HERBICIDE RESISTANCE OF TRANSGENIC PLANTS

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In the name of God the most compassionate and the most merciful

In memory of my brother Hokm Ali

Abstract

A cloned dehalogenase gene, conferring the ability to degrade the herbicide dalapon, was introduced into white clover and tobacco using *Agrobacterium*-mediated transformation. The objectives of this study can be divided into three parts. The first part consists of the evaluation of genetically transformed white clover and tobacco plants for their level of resistance to dalapon, determination of the heritability of the introduced transgene at different stages of growth, and identification of the segregation pattern of the transgene. The second part consists of the study of the quantitative inheritance of the transgene in transgenic tobacco plants. The third part consists of a determination of the number of copies of the transgene integrated into the genome of a transgenic tobacco line, inheritance of the transgene over successive generations and analysis of steady state levels of mRNA of the transgene in leaf tissue. Relationships between the levels of transgene mRNA and the degree of resistance of these plants to dalapon were also assessed.

The resistance of genetically transformed white clover and tobacco plants to dalapon was studied under both *in vitro* and greenhouse conditions using different experimental designs. In the *in vitro* studies, both white clover callus lines and tobacco seedlings showed resistance to high concentrations of dalapon. The level of resistance of the tobacco plants to dalapon was studied under greenhouse conditions using six transgenic lines and one non-transgenic control line. The non-transgenic line was unable to grow at dalapon levels greater than 6.0 kg ha^{-1}, while the majority of the transgenic lines were able to grow at a herbicide level of 48.0 kg ha^{-1}. There were significant differences between the transgenic tobacco lines in their resistance to the dalapon.

The heritability of necrosis, leaf length, leaf width and stem height characters were estimated at various levels of dalapon. The heritability of dalapon resistance for developed transgenic tobacco plants at various levels of dalapon was high. The heritability of dalapon resistance for the characters under study decreased with increasing dalapon levels, with the lowest values of heritability occurring at the highest level of dalapon (48 kg ha^{-1}). The leaf length and leaf width characters had the highest heritabilities, while the necrosis and stem height characters had the lowest heritabilities. The effect of time and the interaction between time and herbicide concentration as environmental factors where lowest for the leaf length

and leaf width characters, while the time effect was highest for the stem height character. The interaction between time and the effects of dalapon were highest for the necrosis character. The heritability of dalapon resistance in transgenic tobacco seedlings grown *in vitro* was significantly lower than in plants, indicating either a low expression of the transgene or a high effect of environmental factors for plants at an early stage of growth. The segregation ratio (resistant:susceptible phenotype) for the transgenic lines was 3:1, and χ^2 test results demonstrated the involvement of single gene inheritance for the lines.

Quantitative inheritance studies of the transgene in tobacco plants using generation means with six generations and 9x9 full diallel mating designs revealed that the additive component of variation was greater than the dominance (hemizygosity) component of variation. The hemizygosity effect was partial and towards the dalapon resistant phenotype. There was significant inter-allelic interaction (epistasis), either between the host plant allele(s) and the dehalogenase transgene or between copies of the transgene. The non-significance of reciprocal effects in the diallel table analysis revealed a lack of maternal or cytoplasmic effects. The analysis of general combining ability and specific combining ability in the diallel table indicated that the majority of transgenic parents had significant general combining ability effect (g.c.a. effects) towards the resistant phenotype, while the non-transgenic parents showed significant g.c.a. effects towards the susceptible phenotype. The progeny derived from crosses between resistant transgenic parents and susceptible, non-transgenic parents showed significant s.c.a. effects towards the resistant phenotype. In contrast, progeny derived from crosses between the susceptible, transgenic parent and non-transgenic parents, as well as progeny derived from crosses between non-transgenic parents showed significant s.c.a. effects towards the susceptible phenotype.

In molecular studies of the copy number of the transgene at different generations of one transgenic tobacco line, the transgenic plants were shown to contain two closely linked copies of the transgene at a single locus, whereas the non-transgenic plants were shown to lack the transgene. It was also shown that the transgene was stably integrated into the plant genome in successive generations and that rearrangement of the integrated transgene did not occur. A dehalogenase-specific mRNA was detected in total RNA extracted from leaves of the transgenic plants. Although all of the transgenic plants contained the same

gene, they showed significant variation in the accumulation of dehalogenase-specific mRNA. In control, non-transgenic plants no dehalogenase-specific mRNA was detected. Although the level of the dehalogenase-specific mRNA in transformed plants varied considerably between the lines, was no significant differences between the individual plants within the lines.

In a two phase selection experiment, some transgenic callus lines exhibited a dissimilarity in expression of the dehalogenase gene and the neomycin phosphotransferase II gene, conferring kanamycin resistance, used in these experiments as a second selectable marker. Some of the genetically transformed cells selected on medium containing kanamycin, when transferred onto medium containing dalapon, did not show resistance to dalapon. Similarly, when transformed cells selected on medium containing dalapon were transferred onto medium containing kanamycin, some of the callus lines did not show resistance to kanamycin. These results show that in some cases selection for one of the transferred genes does not result in expression of the other, non-selected, transferred gene.

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Chapter 1

Introduction

Within the last few years transgenic plants have been developed with traits such as resistance to herbicides, diseases and pests, as well as tolerance to environmental stresses. Furthermore it is expected that in the near future a wide range of crop and pasture plants will be improved by using gene transfer technology to introduce agronomically useful genes into these plants. Once these transgenic plants have been developed, both the expression of the transgene and the phenotypic characteristics of the genetically transformed plants, need to be evaluated.

Herbicide resistance genes for a wide range of herbicides have been recognised, characterised and transferred into a wide range of plants leading to rapid progress in the development of herbicide resistant transgenic plants. Transferring herbicide resistance genes into agronomically important plants is a useful strategy for controlling weeds and increasing agricultural production.

A dehalogenase gene encoding a dehalogenase enzyme, capable of degrading 2,2-dichloropropionic acid, the active ingredient of the herbicide dalapon, has been isolated and characterised. This dehalogenase gene has now been cloned into a plant transformation vector and introduced into both white clover and tobacco. Transgenic plants were resistant to the toxic effects of dalapon.

Once herbicide resistant transgenic plants have been developed, further evaluation is crucial to determine the level of resistance of these plants so that the optimum rate of herbicide can be used in weed control programmes. Heritability of the transgene also has to be studied to establish the stability of expression over successive generations and to determine suitable herbicide levels for subsequent breeding programmes.

Molecular techniques can be used to follow the presence of the transgene in genetically transformed plants as well as to determine the organisation and the inheritance of the transgene over successive generations. Changes in expression of the transgene in plants with different genetic backgrounds (i.e. homozygous and hemizygous genotype), as well as variations in expression of the transgene in plants derived from crosses between transgenic and non-transgenic lines can also be studied using molecular genetic techniques. However, use of traditional plant breeding methods and population genetics is necessary for the breeding of

transgenic plants and their subsequent release as commercial crops. Therefore, to evaluate the effect of a transgene in a population and to estimate those genetic parameters and variations necessary for a breeding programme, there is a need to use appropriate quantitative genetic mating designs, such as generation means, diallel crossing, etc.

After transformation of plant cells, selection for a marker gene is carried out to indirectly select for the particular gene of interest. For example neomycin phosphotransferase II (NPTII) gene is frequently used as a selectable marker in *Agrobacterium*-mediated plant transformation. Imposing a selection pressure on each of the T-DNA genes independently does not necessarily lead to selection of the other gene which is not under selection pressure. It is to be expected that an increase in the selection pressure, through an increase in the selective substrate in the growing medium, would result in cells with a high level of expression of the transgene. In contrast, with no selection pressure, cells with a wide range of resistance would be expected.

In this study, three separate series of experiments are reported. In the first series, the levels of dalapon resistance of transgenic white clover and transgenic tobacco plants, transformed with the dehalogenase gene, were investigated. The levels of resistance of both transgenic white clover and transgenic tobacco plants were studied at an early growth stage (*in vitro*) as well as in fully developed plants (in the greenhouse). The heritability of herbicide resistance was estimated at various levels of herbicide for developed tobacco plants. The segregation pattern of the dehalogenase transgene in transgenic tobacco plants was also evaluated at various levels of herbicide. The results of this series of experiments are presented in Chapter 3.

In the second series of the experiments, the quantitative inheritance of the transgene in developed transgenic plants was studied, using generation means and diallel crossing mating designs. A generation means analysis with six generations was carried out to estimate the additive, dominance, interaction between the transgene and host plant allele(s), as well as broadsense and narrowsense heritabilities. Jinks-Hayman and Griffing full diallel crossing mating designs (9x9), were used to estimate the genetic components of variation, narrowsense and broadsense heritabilities, general and specific combining ability variances as well as general combining ability effects (g.c.a. effects) and specific combining ability

effects (s.c.a. effects). The results of this series of the experiments are presented in Chapter 4.

The third series of experiments mostly involved molecular studies of the dehalogenase transgene. The inheritance and organisation of T-DNA in successive generations derived from a sexually reproduced transgenic line were investigated. The level of dehalogenase-specific mRNA and the relationship between the levels of dehalogenase-specific mRNA in transgenic plants and the level of resistance to the herbicide were also studied. In addition, the effect of selection pressure on the transformed cell lines as well as dual selection for both T-DNA genes were studied in this series of experiments. Results from these experiments are presented in Chapter 5.

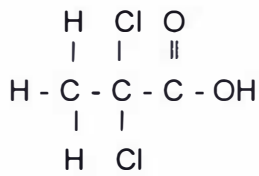
Chapter 2

Literature Review

2.1 Herbicide resistant transgenic plants

2.1.1 2,2-dichloropropionic acid (dalapon)

The herbicide dalapon (2,2-dichloropropionic acid) is classified within the group of chlorinated aliphatic acids. The structural formula of dalapon is given as:



This herbicide is usually used as the sodium salt or as a mixture of sodium and magnesium salts of dalapon. The herbicide can be used in many crops for weed control. The most important use of the chlorinated aliphatic acids, such as dalapon, is as a foliar treatment to control perennial grass weeds in crops and on non-cropped lands (Ashton and Crafts, 1981).

Dalapon itself is a systemic herbicide which controls both annual and perennial grasses. Dalapon may be categorised as a highly selective grass killer, but this selectivity is relative, since at low rates of application it may be used for selective grass control, whereas at higher rates it is a non-selective herbicide. The herbicide is absorbed by both foliage and roots and translocates readily throughout the plant. The recommended rate of usage of dalapon in annual grasses is 2-6 kg ha^{-1} (O'Connor, 1990). Under *in vitro* conditions, the effects of dalapon are altered by growth medium pH. For example, at pH 4.0, maize root growth was inhibited at concentrations of dalapon greater than 10^{-3} M, and dalapon uptake increased sharply at pH 3.0 to 4.0 compared to pH 6.0 to 7.0 (Ingle and Rogers, 1961). These results were taken to indicate that low pH (3.0-4.0) causes a greater uptake of dalapon through the root system. These authors suggest that this increase in dalapon uptake at lower pH by the root system may be related to the increase in hydrogen ions in the media.

Another factor contributing to the effect of the dalapon herbicide is exposure time. In a study of exposure time, at a concentration of $5X10^{-3}$ M dalapon, exposure of up to 8 hours had a linear and maximum inhibitory effect on root

growth, and root elongation ceased after 12 hours exposure (Ingle and Rogers, 1961). Foliar application of dalapon inhibited the growth and the fresh weight yield of oat (*Avena sativa*) plants. By increasing the concentration of dalapon, both fresh weight yield and plant height decreased (Wilkinson, 1962). The patterns of absorption and the distribution of dalapon were studied by Foy (1962). The herbicide was absorbed through the cuticle and the large hypostomatus pores of the leaves, with hypostomatus being the most active routes for herbicide entry. Dalapon was transported out of the green leaves within six hours, but was not significantly transported out ofthe leaves that had previously been depleted of food reserves and left in darkness. It was concluded that dalapon was translocated to regions with high metabolic activity and was associated with the movement of plant food material. Kay (1963) studied the effects of dalapon on three annual clover species. At rates of between 4.5 and 5.5 kg ha^{-1}, applied at different growing stages of the clovers, herbage yields decreased. Different species of the clovers exhibited different levels of resistance to the herbicide and the maturity of the species was delayed from 1 day to 2 weeks in all of the species studied. The plants which were affected severely by the herbicide exhibited a longer delay in maturity.

Translocation of dalapon in plants was traced by applying ^{14}C-labelled dalapon onto the rhizomatous plant, Johnson grass (Hull, 1969). Up to flowering, translocation of dalapon was towards the rhizomes and the pattern of translocation was similar to the translocation pattern of photosynthetic assimilates. During flowering, when the translocation of photosynthetic assimilates towards the rhizomes was limited, translocation of the dalapon from leaves towards the rhizomes was also limited Andersen *et al.* (1962) applied the sodium salt of ^{14}C-labelled dalapon to leaves and roots of sugar beet and yellow foxtail. Autoradiography of the plants indicated that the dalapon entered through the leaves and roots and moved throughout the plants, accumulating to a high level in the younger tissues of both species.

2.1.2 Enzymatic and metabolic effects of dalapon

Hilton *et al.* (1958) studied the interaction between β-alanine and chlorinated aliphatic acids, including dalapon, in yeast (*Saccharomyces cerevisiae*). By

increasing the concentration of dalapon, the growth of the yeast was inhibited. β-alanine offered some protection against the herbicide but was not able to completely reverse the inhibitory effect. These authors suggested that the enzymes involved in pantothenic acid synthesis were possibly important target sites of the herbicidal compounds.

Results of experiments with plants carried out by Hilton *et al.* (1959) showed that dalapon inhibits the pantothenate-synthesis enzyme which is involved in the pantathenic acid biosynthesis pathway of plants. The herbicide inhibited synthesis of pantothenate by competing with pantoate, a precursor of pantothenate, for a site on the pantothenate-synthesis enzyme. The affinity of the herbicide for the enzyme was equal to, or greater than, the affinity of the pantoate. In greenhouse experiments with barley, the interaction between dalapon and pantoate or pantothenate was studied (Ingle and Rogers, 1961). It was concluded that the physiological effect of dalapon was interference with the utilisation of metabolic energy but not interference with respiration nor the production of metabolic energy. Buchanan-Wollaston *et al.* (1992) suggested that dalapon at higher concentrations can cause precipitation of plant proteins, while at lower concentrations the herbicide can cause conformational changes in plant proteins which could affect different metabolic pathways in the plants.

2.1.3 Dehalogenase enzyme

The decomposition of chlorosubstituted aliphatic acids by soil bacteria was reported by Jensen (1957). Organisms which actively degraded 2,2-dichloropropionic acid were isolated and characterised by Hirch and Alexander (1960). The soil organisms responsible were species of the bacteria *Pseudomonas* and *Nocardia* which liberated 90 to 100% of the halogen from dichloropropionate in a period of 2 weeks. Evidence for decomposition was considered to be the production of a chlorine-free component of the herbicide. These microorganisms were capable of growth on media containing several chlorinated propionic acids, including dalapon, as their sole carbon source.

Three strains of *Pseudomonas putida* exhibiting an ability for dichloropropionate decomposition were isolated by MacGregor (1963) from soil treated with 2,2-dichloropropionic acid. The bacteria were able to utilise the

herbicide as a carbon source with high efficiency. Growth of bacteria on dalapon depends on induction of the dehalogenase enzyme. Slater *et al.* (1979) studied 2 strains of *Pseudomonas putida* with different dehalogenase activities. The enzyme activity in one of the strains was 10-40 times greater than other strains. The enzyme was inducible by a range of metabolisable and non-metabolisable chlorinated aliphatic compounds. Liberation of chloride ions from 2,2-dichloropropionic acid was accompanied by the formation of pyruvate. As two chloride ions are released from dalapon and one molecule of pyruvate forms, the conversion of 2,2-dichloropropionic acid to pyruvate may be taken as a measure of dehalogenase enzyme activity (Berry *et al.*, 1979). *Pseudomonas putida* strains with elevated dehalogenase activities and capable of growing on chlorinated aliphatic acids in continuous culture were isolated by Weightman and Slater (1980). In another study, Hardman and Slater (1981) grew sixteen bacterial strains, isolated from soil, capable of growing on different chlorinated propionic acids as a carbon source and identified 5 distinct dehalogenases. While most of the strains had more than one dehalogenase enzyme activity, one strain had just one enzyme activity. Weightman *et al.* (1982) studied *Pseudomonas putida* strain PP3 and found that this organism had multiple dehalogenase enzymes, and that each enzyme was able to dechlorinate certain chlorinated herbicides.

Allison *et al.* (1983) studied fast growing species of *Rhizobium* capable of utilising the 2,2-dichloropropionic acid as a sole carbon and energy source. The organism possessed an inducible dehalogenase enzyme activity. The enzyme activity was inducible by numerous chlorinated aliphatic acids including 2,2-dichloropropionic acid. Beeching *et al.* (1983) constructed two separate plasmids containing the dehalogenase gene from *Pseudomonas putida*. Each plasmid was transferred into a strain of *Pseudomonas putida* lacking the dehalogenase gene and thus herbicide resistant bacteria were produced. Senior *et al.* (1976) isolated organisms from dalapon treated soil and grew them in continuous flow culture containing dalapon. They obtained *Pseudomonas putida* bacteria which had evolved the ability to utilise dalapon as a carbon and energy source via the action of the dehalogenase enzyme. The dehalogenase enzyme was encoded by a large plasmid of 98,800-190,000 daltons in size. Loss of the plasmid resulted in the loss of the ability to utilise or grow on chlorinated aliphatic

acids (Hardman *et al.*, 1986). The dehalogenase gene from *Pseudomonas putida* strain PP3 were isolated and characterised by Thomas *et al.* (1992a, 1992b).

2.1.4 Herbicide resistant transgenic plants

Herbicide resistant plants can be produced via several different mechanisms: 1) reduced herbicide uptake; 2) over-production of the herbicide target site or sites; 3) metabolism, modification or conjugation of the herbicide; 4) mutational alterations in the herbicide target site (Schuls *et al.*, 1990). However, basically two approaches have been used to develop herbicide resistant transgenic plants: 1) modification of the target sites of the herbicide in the plant to render it insensitive to the herbicide or over-production of the unmodified target protein, thus permitting normal metabolism of the plant to continue in spite of the presence of the herbicide; 2) introduction of an enzyme or enzyme system into the plant in order to degrade or detoxify the herbicide before it can act (Botterman and Leemans, 1988).

Several transgenic plants resistant to different herbicides have so far been developed. Glyphosate, a potent broad spectrum, non-selective herbicide which inhibits growth of plants, interferes with biosynthesis of the essential enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. Inhibition of synthesis of this enzyme causes a deficit of aromatic amino acids, an accumulation of shikimate and eventually leads to cellular death (Steinrücken and Amrhein, 1980; Amrhein *et al.*,1980). Comai *et al.* (1985) used a mutant allele of the *aro*A gene from *Salmonella thyphimurium* bacteria encoding EPSP synthase, in which an amino acid substitution of proline to serine caused a decreased affinity for glyphosate through alteration of the herbicide target site without affecting the kinetic efficiency of the enzyme. This mutant *aro*A gene was introduced into the tobacco plant genome using *Agrobacterium*-mediated transformation and the resulting transgenic plants were herbicide-tolerant. The *aro*A gene conferring glyphosate resistant was also successfully transferred to tomato plant, and the resultant transgenic plants, as well as their progeny, exhibited a wide range of tolerance to the herbicide (Fillatti *et al.*, 1987). The isolation of the *aro*A gene from *E.coli* encoding a glyphosate resistant form of EPSP synthase has also been reported (della-Cioppa *et al.*, 1987). Shah *et al.* (1986), isolated a cDNA clone encoding EPSP synthase from a cDNA

library derived from a glyphosate-tolerant, *Petunia hybrida* cell line (MP4-G) that over-produced the enzyme. A chimaeric EPSP synthase gene which would lead to over production of the enzyme was constructed and transferred into susceptible petunia cells. Transformed petunia cells as well as regenerated transgenic plants were tolerant to glyphosate.

The compound 2,4-dichlorophenoxy acetic acid (2,4-D) has an auxin-like hormone activity in plants and at higher concentrations is toxic, especially in dicotyledonous plants. When the *tfd*A gene, responsible for detoxification of 2,4-D, was isolated from the soil bacteria, *Alcaligenes eutrophus*, and cloned into the tobacco plant genome, transgenic tobacco plants showed resistance to the 2,4-D herbicide (Streber and Willmitzer, 1989).

The chloroplast gene *psb*A codes for a photosynthetic quinone-binding membrane protein, this protein being the target for the herbicide atrazine. The quinone-binding membrane protein of atrazine sensitive plants, including *Amaranthus hybridus*, binds atrazine while the quinone-binding membrane protein from an atrazine-resistant biotype, as a result of a single amino acid residue substitution, does not bind the herbicide. Cheung *et al.* (1988) cloned the *psb*A gene from the chloroplast of an atrazine-resistant biotype of *Amaranthus hybridus* and introduced this gene into the tobacco plant genome using *Agrobacterium*-mediated transformation. The transgenic plants were resistant to atrazine herbicide due to the expression of the atrazine-resistant *psb*A gene.

Phosphinothricin (PPT) is a non-selective herbicide which inhibits the activity of glutamine synthetase in plants. De Block *et al.* (1987) isolated a gene from bacteria responsible for detoxifying the herbicide and introduced this gene into tobacco, potato and tomato plants. The resulting genetically transformed plants were able to detoxify the herbicide and showed a high level of herbicide resistance. Lee *et al.* (1988) isolated an acetolactate synthase (ALS) gene, target for sulfonylurea and imidazolinone herbicides, from two lines of tobacco plants that were resistant to each of these herbicides. They also manipulated the genes for enhanced gene expression and reintroduced them into the genome of herbicide-sensitive tobacco plants. The resulting transgenic plants carrying these genes were highly resistant to the herbicide sulfonylurea.

2.1.5 Dalapon resistant transgenic plants

Dalapon resistance has also been manipulated in plants by introducing a gene encoding a detoxifying enzyme. The dehalogenase gene (*Dehl*) from *Pseudomonas putida* bacteria capable of degrading 2,2-dichloropropionic acid has been isolated and characterised (Beeching *et al.*, 1983). The dehalogenase gene (*Dehl*) together with a kanamycin resistance gene, under the control of CaMV 35S and *nos* promoters respectively, were cloned into a plant transformation vector to give plasmid pVW291 (Buchanan-Wollaston *et al.*, 1992). The T-DNA of pVW291, containing both the *Dehl* and kanamycin resistance genes, was transferred into *Nicotiana plumbaginifolia* using *Agrobacterium*-mediated transformation. Subsequent transgenic calli and developed plants were resistant to the toxic effects of dalapon with some individual transgenic plants showing resistance to a level of 110 kg ha^{-1} of dalapon.

2.1.6 Plant transformation methods

2.1.6.1 *Agrobacterium*-mediated DNA transfer to the plant genome

The soil bacterium *Agrobacterium tumefaciens*, harbouring the Ti plasmid, induces crown gall disease in most dicotyledonous plants through the process of transformation of plant cells (see below). The bacterium infects wounded plants and induces genetic changes in these plants whereby the transformed cells of the host plant synthesise carbon and nitrogen compounds that are then utilised by the *Agrobacterium* cells. The Ti plasmids are essential for virulence of the bacteria and they carry genes involved in both plant and bacterial functions. The Ti plasmid is also a natural vector for introducing foreign genes into the genomes of target plants. During *Agrobacterium* infection, only that part of the Ti plasmid between two direct repeats, the T-DNA, is transferred into the plant genome.

The ability of *Agrobacterium* to transfer T-DNA to the plant genome thus offers the prospect of introducing new genes into plants. Because the virulence (*vir*) functions of the Ti plasmid are separate from its oncogenic function, the oncogenic segments of the Ti plasmid within the T-DNA can be deleted without affecting T-DNA transfer. The resulting, non-oncogenic Ti plasmid is called a disarmed Ti plasmid. Foreign DNA can be inserted into the modified T-DNA region

and subsequently transferred from *Agrobacterium* into the plant and integrated into the plant nuclear genome. By using a binary Ti vector system (see Section 2.1.6.1.3), the disarmed T-DNA can be manipulated to carry foreign DNA in a small plasmid which replicates in *E. coli*, while the plasmid containing *vir* genes responsible for DNA transfer to the plant resides and replicates in *Agrobacterium*. The plasmid containing the T-DNA can then be transferred to *Agrobacterium* by using a helper plasmid (Bevan, 1984).

Buchanan-Wollaston *et al.* (1987) developed a plant binary vector system that did not use the T-DNA borders of the Ti plasmid. They constructed a plasmid containing selectable marker genes for both plant (kanamycin) and bacterial (chloramphenicol) use as well as the *mob* and *OriT* functions required for normal bacterial conjugation. This plasmid was then conjugated into *Agrobacterium tumefaciens* harbouring the disarmed Ti plasmid pAL4404. When tobacco leaf discs were infected with this *Agrobacterium tumefaciens*, kanamycin resistant transgenic plants were produced. The experiment showed that through supplying the *vir* function on the disarmed Ti plasmid pAL4404, the second plasmid containing the *mob* and *OriT* sites can be transferred and integrated into the plant genome.

2.1.6.1.1 Ti plasmids

The crown gall disease is a direct result of the transfer of a particular segment of DNA, the T-DNA, from the tumour inducing (Ti) plasmid of *Agrobacterium tumefaciens* to the plant cell where it is integrated into the genome (Zambryski, 1988). Currier and Nester (1976) isolated covalently closed circular Ti plasmid DNA of high molecular weight from *Agrobacterium tumefaciens*. Chilton *et al.* (1978) physically mapped the Ti plasmid pTi-B6-806 by restriction enzyme digestion and determined the segment of the pTi-B6-806 plasmid which incorporates into the plant genome and induces oncogenicity in plants. The size of an octopine-type Ti plasmid, pTi-Ach5 was determined by Ooms *et al.* (1980), using endonuclease digestion, to be 121.3 mega dalton in size. By using the transposable element *Tn 904*, they also obtained several mutants of the plasmid with different characteristics. The precise positions of the T-DNA and *vir* gene on the plasmid were determined by analysing the phenotypes of plants which were infected with the mutants of the plasmid. Ooms *et al.* (1982) analyzed mutants of

the octopine-type Ti plasmid and mapped the Ti plasmid to determine the left border (TL) and right border (TR) regions on the T-DNA and constructed plasmids derived from this plasmid. The widely used pAL4404 plasmid is one of the deletion mutants which contains both the *vir* and *ori* genes. Three types of Ti plasmid; nopaline (Holbrook and Miki, 1985; Vos and Zambryski, 1989), octopine (Holbrook and Miki, 1985; Haaren *et al.*, 1987) and agropine (Czako and Marton, 1986) have been recognised in wild type *Agrobacterium* strains. The analysis of these three types of plasmids indicated that they differ on the basis of homology as well as genetic traits. Moreover, each type of Ti plasmid causes a different tumour morphology and results in the synthesis of a different type of opine. Opines are derivatives of amino acids which can be used as a sole carbon or nitrogen sources by the *Agrobacterium*.

The T-DNA was found to be bounded by essential 25 base pair direct repeats (Zambryski *et al.*, 1982) and these sequences defined the T-DNA borders. The wild-type T-DNA contains 4 genes for tumour induction: *tms* (shooty tumour), *tmr* (rooty tumour), and *tml* (large tumour) which all affect tumour morphology, and *nos* or *ocs* (nopaline or octopine synthase depending on the type of Ti plasmid). Any DNA located between these 2 borders can be efficiently transferred to the plant genome. The T-DNA region transfers as single stranded DNA from the Ti plasmid into the plant with the right border leading; the complementary strand is subsequently synthesised within the plant cell (Stachel *et al.*, 1986). Only the right repeat sequence is essential for the T-DNA transfer process to occur.

2.1.6.1.2 Disarmed Ti plasmids

As discussed above, the wild-type Ti plasmid has the ability to transfer the T-DNA region into the plant genome and this results in gall formation in the host plant. It is possible to delete the T-DNA region between the right and left borders of the Ti plasmid and the resultant plasmid lacks the gall-forming ability. Such a non-oncogenic Ti plasmid is called a disarmed Ti plasmid. It is possible to manipulate the Ti plasmid and thereby insert a foreign DNA segment between the right and left borders of T-DNA, and this foreign DNA is also transferred into the plant genome by *Agrobacterium*. The disarmed Ti plasmid itself, because of its large size, is not suitable for plant genetic transformation (see Section 2.1.6.1.3).

Three genetic components are required for plant cell transformation: the T-DNA, the *vir* genes, and *Agrobacterium* chromosomal genes. The T-DNA and the *vir* region are located on the Ti plasmid. The *vir* region, which provides *trans-acting* factors for T-DNA transfer and contains six essential genes, is about 35 kb outside the T-DNA region. The bacterial chromosomal component consists of at least three genes (Zambryski, 1988).

2.1.6.1.3 Binary vector systems

The binary vector system of transformation is an efficient method of plant genetic transformation based on the distinct functions of the T-DNA and *vir* region. Hoekema *et al.* (1983) used a binary vector system for plant transformation after recognising the distinction between the *vir* and T-DNA regions. They used two different plasmids: pAL4404 containing the *vir* and *ori* regions from the wild type pTi-Ach5 Ti plasmid, and pAL1050 carrying the T-DNA of pTi-Ach5 as well as a wide-range host replicon. The two plasmids were introduced into *Agrobacterium tumefaciens*, and *Agrobacterium* carrying both of these plasmids together had normal tumour-inducing capacity. However *Agrobacterium* carrying only a single plasmid lacked the ability for tumour induction. The experiment indicated that the T-DNA could be located on one plasmid which could be easily manipulated while the virulence genes could be located on a separate plasmid. Furthermore, *E. coli* could be used as a host for the plasmid carrying the T-DNA and this plasmid could be manipulated in *E.coli* and subsequently transferred to *Agrobacterium* carrying the plasmid with the *vir* region. The *Agrobacterium* could then transfer the manipulated T-DNA into the plant genome. Plasmids containing the T-DNA region are termed binary vectors.

A wide range of dicotyledonous plants have been genetically transformed using binary vector systems, for example white clover (White and Greenwood, 1987), tobacco (De Block *et al.*, 1987; Lee *et al.*, 1988), *Amaranthus* (Cheung *et al.*, 1988), tomato (Fillatti *et al.*, 1987).

Though any DNA segment inside the T-DNA borders can be transferred into the genomes of dicotyledonous plants using the *Agrobacterium*-mediated transformation, the transferred gene will only function if promoter and terminator regions of the gene are functional in the plant. In general, the cauliflower mosaic

virus 35S (CaMV 35S) promoter or the nopaline synthase (*nos*) promoter are used in the construction of chimaeric genes. The octopine synthase (*ocs*) and T7 polyadenylation signals are widely used as terminator signals.

Selection of genetically transformed bacteria and plants is crucial in transformation studies. For bacteria, different antibiotic resistance genes are used as selectable markers, while for plants the kanamycin resistance gene is predominantly used as a selectable marker. The reason for using kanamycin resistance for plants is the high susceptibility of many plant cells to this antibiotic.

2.1.6.2 Direct gene transfer methods

The *Agrobacterium*-mediated method of genetic transformation is difficult to use in monocotyledonous plants such as the cereals. However, direct gene transfer methods can be used to transfer a foreign gene into monocotyledonous as well as dicotyledonous plants.

High-velocity microprojectile bombardment (Klein *et al.*, 1987) is a method for direct genetic transformation of plants. A small amount of foreign DNA can be adsorbed onto the surface of tungsten or gold particles of about 1-2 μm, and then introduced into plant cells using high velocity bombardment. Once inside the cell, the foreign DNA becomes integrated into the plant's genome.

Electric shock or electroporation is another efficient direct method for transferring foreign genetic material into the genomes of plant cells and is useful for both dicotyledonous (Bates *et al.,* 1990) and monocotyledonous (Matsuki *et al.*, 1989) plant transformation.

Direct transformation by osmotic shock of plant cells has also been used. Protoplasts derived from *Brassica napus* cells were treated with plasmid DNA containing the hygromycin resistance gene, and subsequently this protoplast/DNA suspension was treated with polyethylene glycol (PEG) to facilitate uptake of the plasmid DNA by the protoplasts (Köhler *et al.,* 1987). Following transformation, the transformed cells could be regenerated to form callus and subsequently to form complete plants. The efficiency of transformation of the *Brassica napus* plants was about 0.07% to 0.1%. Both in electroporation and PEG direct transformation methods, it is necessary to use protoplasts which may be difficult to regenerate into whole plants.

de la Penã *et al.* (1987) devised a method for transferring a foreign gene into rye (*Secale cereale*) by directly injecting plasmid DNA into young floral tillers before meiosis. Seeds from the infected plants were obtained by crossing with ordinary rye plants. Only a low rate of transformed seeds from the transgenic plants were obtained by this method.

2.1.7 Transgene copy number

The number of copies of T-DNA inserted into the plant genome by *Agrobacterium*-mediated transformation varies. Hobbs *et al.* (1990), studying the inter-transformant variability of the copy number of the GUS gene in transgenic tobacco plants, found the copy number of the introduced gene to be between one and several copies per plant genome.

The pea plastocyanin gene was introduced into the tobacco plant genome and the number of copies of the plastocyanin gene in the tobacco genome was determined (Last and Gray, 1990). The copy number of the gene varied between one and two copies per plant genome. The level of expression of the gene in homozygous plants was twice that of the hemizygous plants. Webb *et al.* (1990) genetically transformed roots of three species of legume, *Trifolium repens*, *Trifolium pratense* and *Lotus corniculatus* using *Agrobacterium rhizogenes* and determined copy number of the foreign gene. The genetically transformed *Trifolium repens* roots contained a single copy of the introduced gene, while in *Trifolium pratense* roots, the number of copies varied between one and five, and in *Lotus corniculatus* plants developed from genetically transformed roots copy number varied between two and eight. In another study, Deroles and Gardner (1988b) introduced a kanamycin resistance gene into the genome of petunia using the *Agrobacterium*-mediated technique and analyzed the T-DNA structure in a large number of kanamycin resistant, transgenic plants. In this case, the copy number of the T-DNA varied between one and six copies per plant genome. In addition, the majority of plants with multiple copies of the T-DNA did not show an inheritance pattern other than that of single copy gene inheritance. Lawton *et al.* (1987) introduced the soybean β-conclycinin gene into petunia and analyzed the resultant transgenic plants for the number of copies of the foreign gene. The majority of the transgenic plants contained one to two copies of the transgene, while one plant had ten copies

of the transgene. Brunold *et al.* (1987) introduced a plasmid containing two selectable markers into the tobacco plant genome using a direct gene transformation technique and subsequently selected for antibiotic resistant transformed plants. In this case, the number of copies of the gene varied from one to five per plant genome.

5.1.8 Transgene Copy number determination

A variety of methods have been used to determine the number of copies of a transgene which have become integrated into the plant genome and the organisation of the transgene(s) within the genome. Methods used to determine the number of copies of introduced T-DNA in genetically transformed plants include restriction fragment size variation (David and Tempe, 1987), diluted plasmid (Webb *et al.,* 1990), and comparison of signal intensity (Mouras *et al.,* 1987).

Internal fragments of T-DNA have been labelled with ^{32}P and used as a probe to detect the number of copies of an opine gene in genetically transformed plants (David and Tempe, 1987). In this case the number of bands was used to determine the copy number of the T-DNA. Genomic DNAs of transgenic plants digested with a restriction enzyme and hybridized with labelled probe have also been used to detect the copy number and molecular structure of the end of the T-DNA (Zambryski *et al.*, 1980). This study provided direct evidence that the T-DNA can be integrated into the plant genome and that it can be tandemly repeated within the genome. Sequence analysis of the junction of the T-DNA in some clones indicated several direct repeats as well as inverted repeats. It was postulated that these direct and inverted repeats may be involved in the process of T-DNA transfer from *Agrobacterium* to the plant genome. Deroles and Gardner (1988b) also used restriction fragment size variation to determine the number of copies of an introduced T-DNA in genetically transformed petunia plants. In another study, Hobbs *et al.* (1990) used a 1.9 kb DNA fragment within the T-DNA region as a probe. They determined the copy number of the T-DNA in individual transformants by analysing the occurrence of a unique restriction enzyme site inside the T-DNA. Genomic DNA from transgenic plants was digested with this enzyme and fragments with different sizes with respect to the border fragments between the T-DNA and the plant DNA were generated. Following hybridisation

of the digested plant genomic DNA with the probe, bands with different sizes were detected, each band representing a single copy of the T-DNA. Last and Gray (1990) detected the number of copies of a transgene by digesting plant genomic DNA with *Eco*RI, transferring the digested DNA onto a nylon membrane by Southern blotting, and subsequently hybridizing with a labelled cDNA probe. The autoradiograph of the Southern blot was scanned to determine the number of copies of the transgene which had integrated into the plant genome.

Webb *et al.* (1990) determined the copy number of a transgene in *Trifolium repens* and *Trifolium pratense* roots using a diluted plasmid method. They compared the intensity of the bands of the diluted plasmid with the intensity of plant genomic bands and thus determined the copy number of the gene. Mouras *et al.* (1987) hybridized the chromosomes of transgenic tobacco plants with a ^{3}H-labelled fragment of an introduced gene as a probe. The intensity of the signal increased with increasing amounts of the probe DNA and with increasing exposure time. These results showed that the intensity of the bands depended on the amount of plant DNA, the exposure time, and the specific activity of the radioisotope.

Since the intensity of bands in an autoradiograph varies depending on the amount of target DNA, the exposure time, and the specific activity of the labelled DNA probe, the determination of gene copy number by band intensity is not a highly sensitive method (Mouras *et al.,* 1987). Furthermore, this method does not give sufficient information about the organisation of the transgene(s) within the genome of the host plant. In contrast, the restriction fragment size variation methods which determine copy number and organisation of the transferred gene(s) in the host plant genome by using T-DNA restriction enzyme site information are more informative (Zambryski *et al.,* 1980; David and Tempe, 1987; Hobbs *et al.,* 1990).

2.1.9 Variation in transcription of the transgene

Following transformation and the regeneration of transgenic plants, it is important to study the expression of the transgene. There can be considerable variation in the level of expression from the same foreign gene in different transformed plants. Variability in the levels of mRNA could arise from differences in gene copy number, mRNA stability, and rates of transcription of the transgene

(Lawton *et al.,* 1987). The effect of the position of the transgene in the genome on the level of expression of the transgene has also been reported to be an important factor (Hobbs *et al.,* 1990). Lawton *et al.* (1987) suggest that differences in expression of foreign genes are associated with differences in transcription rather than with differences in translation. In relation to the expression of the transgene in subsequent generations, in some cases there is a similarity in expression of the gene in the different generations (Dean *et al.*, 1988), while in other cases it is possible that expression of the transgene in the progeny is different from that in the parents (Czernilofsky, 1986). Deroles and Gardner (1988b), studying the expression of a kanamycin resistance gene in transgenic petunia plants, found differing levels of expression of the transgene. Low expressing plants were shown by Southern blotting to contain T-DNA in their genomes. In another study, Deroles and Gardner (1988a) reported that the expression of the transgene could be lost despite the presence of the gene in the genome. To determine the interactive effect of T-DNA genes in transformed plants, Deroles and Gardner (1988a) introduced T-DNA containing two copies of the kanamycin resistance gene, under two different promoters, into petunia plants using the *Agrobacterium*-mediated transformation system. Their results indicated that deletions in various parts of the T-DNA, including both the right and left borders, could occur and that a significant ratio of the transformed plants did not express either one or both of the T-DNA genes, due to truncation or deletion within the T-DNA region. Another reason for both low expression and silencing of the transgene within plants is the level of methylation of the introduced gene (Meyer *et al.*, 1992; Hobbs *et al.*, 1993; Meyer and Heidmann, 1994) brought about by the plant methylation system.

An analysis of the expression of a foreign gene in independently transformed plants showed a very high level of variability in expression of this gene (Jones *et al.*, 1985). Variations in locus number and T-DNA copy number per locus both contributed to the variability in the level of foreign gene expression (Dean *et al.,* 1988). The level of the protein coded for by the foreign gene can also vary significantly among individual transformants within the same transformation experiment. Dean *et al.* (1988) also concluded that locus number could contribute to between-transformants variability, since any one T-DNA locus had a finite probability of contributing the same level of expression.

An (1986) tested the activity of both chloramphenicol acetyltransferase

(CAT) and neomycin phosphotransferase II (NPTII) transgenes in forty independent transformed tobacco calli. The level of transcription of the CAT varied two hundred-fold between individual transformants in the study. The level of NPTII activity also varied within the transformants, but this variation was lower than the variation in CAT activity.

The expression of a gene can be quantified by measuring the amount of mRNA transcribed from that gene. An (1986) developed a method for estimating the absolute amount of mRNA by comparing the intensity of mRNA in a test sample to that of an internal standard consisting of homologous copy RNA (cRNA). The relative quantity of mRNA can be calculated by optical density, using densitometer scanning of Northern blot autoradiograms. However, relative signal density is a variable value and one which can be affected by several factors such as the specific activity of the radioisotope, the exposure time for autoradiography and the type of probe being used. Another physical limitation is that X-ray film is sensitive only between optical density of 0.5 to 2.8. Moreover, the signal intensity of X-ray film over time is not linear. An alternative is storage phosphor technology, such as the PhosphorImager (information supplied by Molecular Dynamics, U.S.A.) which has the ability to capture images from both strong and weak signals in a single exposure, and exhibit linearity in quantification of the signals. Moreover, the storage phosphor screen is 10-100 times more sensitive to incident radiation than X-ray film, so images that require a week or more exposure with X-ray film, can be seen overnight following exposure to storage phosphor screen.

With respect to the T-DNA mutagenesis and complementation, a recessive *pale* (*cs*) mutant in *Arabidopsis thaliana* was identified by T-DNA mediated insertional mutagenesis (Koncz *et al.*, 1990). These mutants were confirmed by transformation with T-DNA containing the full length cDNA of the wild type (*cs*) gene. This resulted in transformed plants with a normal green phenotype and the authors concluded that they had achieved positive complementation of the T-DNA induced mutation.

2.1.10 Inheritance of the transgene

In general, a foreign gene introduced into a plant genome is conserved during meiosis and is expressed in the progeny. In most cases, segregation of the

cloned gene can be interpreted within the framework of Mendelian genetics (Budar *et al.*, 1986). Deroles and Gardner (1988b), in analysing the inheritance of a large number of kanamycin resistant transgenic plants, reported that half of the progeny plants showed normal Mendelian inheritance, while in some other progeny plants, this was not so. Transgenes introduced into the plant genome may be present in the progenies and may also be present in F_1 plants resulting from crosses between transgenic and non-transgenic plants (McKing *et al.,* 1987).

The majority of primary transgenic plants resulting from transformation are genetically hemizygous. Homozygous transformed plants can be achieved by selfing of hemizygous plants and screening the progeny. Consequently, the progeny population has a mixed structure consisting of homozygous transgenic, hemizygous transgenic, and plants which lack any copies of the transgene (Koncz *et al.*, 1990).

The integrated T-DNA can be organised as tandem repeats in the plant genomic DNA. In plants where the transgene is arranged in 3-5 tandem copies, the transgene could be inherited as a single Mendelian factor. Transgenic plants and their progenies were analyzed for T-DNA segregation pattern by David and Tempe (1987). The segregant negative progeny plants did not have the T-DNA, while other non-segregant progeny did have the T-DNA fragment within their genomes.

The nitrate reductase (NR) structural genes from tobacco plants were transferred into the genome of nitrate reductase deficient mutants of tobacco to complement their nitrate reductase deficiency (Vaucheret *et al.*, 1989). These transgenic tobacco plants were able to grow on media containing nitrate as the nitrogen source. The inheritance pattern in progeny of these transgenic plants was 3:4 for resistant transgenic progenies and 1:4 for nitrate reductase deficient progeny plants. Moreover, amongst backcross progeny developed through crosses between the transgenic progeny and the nitrate reductase deficient mutant parents, the segregation ratio was 1:1. These results indicated that the NR gene was inherited as a single Mendelian factor.

2.2 Quantitative genetics

2.2.1 Quantitative characters which are controlled with a few genes

Biometrical genetics is generally used for the study of continuously varying characters. Continuous variations in quantitatively inherited characters is mediated by a large number of genes, each having such a small effect that they can only be studied in one set of genes and in terms of the average effect of the genes. Metric or quantitative characters are also referred to as a polygenic system. The phenotypes of individuals in any family or other appropriate group yields at least two biometrical quantities which are used in quantitative analysis, the mean of the distribution as a first degree statistic and the variance and covariance as a second degree statistics (Mather and Jinks, 1977).

As described above for quantitative inheritance studies, continuous metrical characters are referred to as characters which are controlled by a large number of genes or a polygenic system. However this definition is not valid for all characters as it is possible that a quantitative character is controlled by two or three loci, or even a major proportion of a quantitative character could be controlled by a single effective gene. For example, in wheat the inheritance of a quantitative character, heading time, and the number of genes contributing to the inheritance of this character have been studied by Wehrhahn and Allard (1965). A minimum of four genes was involved in inheritance of this character. One of these loci, locus number one, had an effect on the phenotype as large as the other three loci combined and as a result it was concluded that locus number one clearly was of major importance in the inheritance of the heading time character. This is not in agreement with the assumptions of continuous characters in which the characters are controlled by a large number of polygenes with small and similar effects. As a result these assumptions are not valid for all quantitative characters. Furthermore, it is possible that one gene with a major effect is involved in inheritance of a quantitative character. In quantitative studies, any locus which contributes significantly to expression of the phenotype is called an effective factor or major gene, while loci with a minor effects on the expression of the phenotype are known as minor genes. Thoday (1961) studied the relationship between polygenes and the major genes affecting the inheritance of quantitative characters. The major genes had a significant effect on expression of quantitative characters.

Jana (1971) described a biometrical model for analysing quantitative characters resulting from qualitatively acting genes with 2 or 3 loci. Quantitatively inherited characters could be controlled by few loci. Jana (1972a), studying the additive, dominance and other genetical components for several quantitative characters, concluded that these characters were controlled by two or three loci. The net blotch resistance character in barley (*Hordeum vulgare*) was studied quantitatively by Douglas and Gordon (1985). The number of effective factors involved in resistance to the disease was approximately one. They concluded that resistance to net blotch was mainly controlled by a single, partially dominant gene with a major effect. Lee *et al.* (1968) in an inheritance study of the level of gossipol compound, one of the polyphenolic substances produced by pigment glands and stored in cotton seed, reported that this compound was qualitatively controlled by two independent loci.

Additive, dominance and epistasis genetic effects, for simply inherited characters can be estimated using a linear expectation model (Jana, 1972b). This provides an extension of the generation means analysis for several types of loci which can be analyzed by segregation methods. A diallel crossing mating design for a two gene system, was used to estimate the genetical parameters for the level of gossipol in cotton (Lee *et al.,* 1968). The result of this diallel analysis showed that 94% of the total genetic variance was additive, while only 5% was due to dominance and epistasis. Some characters controlled by 2 or 3 genes have been studied quantitatively in different plants (Legg and Collins, 1971; Willson and Lee, 1971; Jana, 1972a; Jana, 1972b).

Hobbs *et al.* (1993) studied the copy number of a *uid*A transgene, responsible for GUS activity, and also the interaction between the copy number of this transgene and the level of GUS activity in transgenic tobacco plants. There was no apparent interaction between alleles at the same or different loci. Extra copies of the transgene integrated into the plant genome acted additively. Hence two copies of the gene produced two-fold the level of GUS activity of one copy, and three copies three-fold the expression. This relationship between extra copies of the transgene and an increasing level of transgene expression was defined as an additive effect. Subsequently these authors categorised the transgenic plants into two groups: plants with a high level of GUS activity (H-type) and plants with a low level of GUS activity (L-type). The levels of GUS activity in a transgenic F_1

population developed from a cross between two parents with high level of GUS activity (H-type) were similar to the H-type parents. Also the levels of GUS activity in a transgenic F_1 population developed from a cross between two parents with low levels of GUS activity (L-type) were similar to the L-type parents. However, the levels of GUS activity in a transgenic F_1 population developed from a cross between a parent with low level of GUS activity (L-type) and a parent with a high level of GUS activity (H-type) were most similar to the L-type parent. Hence, the L-type partially or totally suppressed the expression of the H-type. Hobbs *et al.* (1993) concluded that a low level of GUS activity suppresses the expression of genes conferring a high level of GUS activity. The individual plants in a F_2 population developed from a cross between L-type x H-type parents showed segregation in the level of GUS activity. Some plants were observed with GUS activity levels similar to those of the L-type parent, others with levels similar to the H-type parent and yet other individuals had intermediate levels. However, some individuals of the F_2 population had levels of GUS activity up to twice that of the H-type parent. This significantly high level of GUS activity in some individual plants was postulated to be due to "transgressive segregation" in the F_2 population.

2.2.2 Partitioning the genetic variance

In quantitative studies, measurements are based on phenotype, and the phenotype can be divided into genetic and environmental components. The genetic component can be further subdivided into additive (A), dominance (D) and inter-allelic interaction or epistasis (I). The additive genetic component arises from differences between two homozygotes (i.e. AA and aa) and is defined in Figure 2.1. The deviation of each of the homozygote parents from the mid parent (*m*) is indicated by (*d*). The departure from mid parent is positive for the homozygote parent with the increasing allele (AA) and is negative for the homozygote parent with the decreasing allele (aa) The dominance (intra-allelic interaction) is the departure of the heterozygote (*h*) from the mid parent and it is positive when the heterozygote is similar to the homozygote parent with the increasing allele, and is negative when the heterozygote is similar to that of the homozygote parent with the decreasing allele (Mather and Jinks, 1977; 1982). The above definition can be summarized in Figure 2.1 (from Mather and Jinks, 1977).

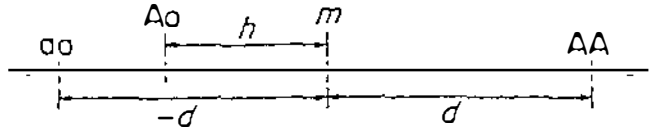


Figure 2.1. The deviation of homozygote and heterozygote genotypes from the mid parent (mid point). The *d*, *-d* and *h* indicate the deviation of the parent with increasing allele (AA), the parent with decreasing allele (aa), and the heterozygote (Aa) from the mid parent (*m*).

The inter-allelic interaction (epistasis) arises from the modification of the additive and dominance effects at one locus, resulting from allelic substitution at the other locus. The variance of these phenotypic and genetical components are as follows (Mather, 1949):

$$\sigma^2_P = \sigma^2_G + \sigma^2_E$$

where, σ^2_P is the phenotypic component of variation, σ^2_G is the genotypic component of variation and σ^2_E is the environmental component of variation. The total genotypic component of variation can be further subdivided into three components:

$$\sigma^2_G = \sigma^2_A + \sigma^2_D + \sigma^2_I$$

where, σ^2_A is variance of additive effect of genes (variance of additive), σ^2_D is variance of intra-allelic interaction effect of gene, i.e. variance of interaction between the (A) allele and the (a) allele (variance of dominance) and σ^2_I is variance of non-allelic effects of genes (variance of non-allelic interaction or

variance of epistasis). Furthermore, the non-allelic interaction can be further sub partitioned (Cockerham, 1954) as follows:

$$\sigma^2_i = \sigma^2_{AA} + \sigma^2_{AD} + \sigma^2_{DD}$$

where, σ^2_{AA} is additive x additive interaction variance, σ^2_{AD} is additive x dominance interaction variance, and σ^2_{DD} is dominance x dominance interaction variance.

2.2.3 Genetical mating designs

For estimating the genetical components as well as the genetical components of variations, the use of appropriate mating designs is required. A mating design is a system of mating to develop particular sets of progeny. Kearsy (1965), explained the random biparental mating (RBIP) design as crosses of parents in pairs to develop the full sib families (there are no half sib families in this design). Comstock and Robinson (1948, 1952), described three kinds of mating designs consisting of design 1, 2 and 3 (North Carolina I, II and III). In design 1, a random samples of a male parent is mated with a number of different female parents. In design 2, the half sib families with a common female parent and a common male parent are produced by systematic crossing of n_1 females with n_2 males to produce n_1n_2 progeny Design 3 has a restricted use because restricted families are used in this design.

The diallel mating design has been extensively used to obtain the genetical components of variation as explained by Jinks and Hayman (1953), Hayman (1954a, 1954b) and Mather and Jinks (1977). The Jinks and Hayman diallel analysis method deals with the differences within a set of inbred lines and their progeny. Generation means analysis is based on different generations such as inbred parents, F_1, F_2 and different backcrosses. The generation means methods provide detailed information on genetical components. The generation means analysis and diallel mating design are discussed in detail in Sections 2.2.4 and 2.2.5 respectively.

2.2.4 Generation means

A generation means mating design for testing inter-allelic interaction was introduced by Mather (1949). Means of six generations of *Nicotiana rustica*, $\bar{P}_1$, $\bar{P}_2$, $\bar{F}_1$, $\bar{F}_2$, $\bar{B}C_1$ and $\bar{B}C_2$ were used by Jinks (1956) to estimate the genotype-environmental interaction and the additive and dominance effects of the gene. Hayman (1958a) described a theory of different generations derived from crosses between two inbred lines and the expectation of the means of the parents and some of the descendant's family and generations. He also introduced a model to estimate additive, dominance and epistasis effects using the results of an analysis of generation means, and for testing the adequacy of the additive-dominance model. In the main, two models are used to analyze the generation means and to estimate the different genetical components: firstly, the three parameter model, which is suitable for estimating the mean, additive and dominance (*m, d, h*) in the absence of epistasis; and secondly the six parameter model consisting of mean, additive, dominance and additive x additive, additive x dominance, dominance x dominance types of epistases (*m,d,h,i,j,l*). Mather and Jinks (1977), used P_1, P_2, F_1, F_2, BC_1 and BC_2 generations in a generation means analysis and described a method for analysing three and six parameter models to estimate mean, additive, dominance and epistases. Bhullar *et al.* (1982) estimated the genotype x environment interaction, epistasis, linkage, additive and dominance for wheat protein as a quantitative character. They crossed high and low protein content cultivars of wheat (P_1 and P_2) to developed the F_1, F_2, BC_1 and BC_2 generations. The generation means mating design with the six generations (P_1, P_2, F_1, F_2, BC_1 and BC_2) was used to estimate the genetical components. The results of the study showed that the mean and additive effect for the protein content were significant and positive, while the dominance effect showed a partial toward the parent with the low protein phenotype.

In cases of inappropriate design of the generation means, a simple three parameter model of additive dominance effects fails to account for the observed variation in the generations. There are three possible causes for this: the presence of genotype x environment interactions, linkage and epistasis (Perkins and Jinks 1970). In an expanded model of generation means analysis in *Nicotiana rustica*, twenty-one generations, P_1, P_2, F_1, F_2, BC_1, BC_2 and fifteen other generations were

used by Perkins and Jinks (1970). They used a scaling test to test the adequacy of the additive-dominance model. Their results indicated linkage and genotype x environment interactions for height and flowering time characters. The scaling test also unambiguously detected the presence of linkage among genes involved in controlling height. In another study, Jinks and Perkins (1970) developed a model using F_2 and backcrosses of *Nicotiana rustica*, to estimate genetical components. The results of this study, however, did not show evidence of a dominance component of variation for height and flowering time. In a more complicated model, Jinks and Perkins (1968) used a twenty-one generation model to study linkage and epistasis in tobacco plants. Results of the study indicated that the simple additive-dominance model was inadequate due to the presence of some form of epistasis.

In a generation mean analysis, Mather and Jinks (1977) used mean phenotype (mid-parent) as mean, *m*. The mean phenotype for each generation was formulated as the departure from the mid-parent *m*. As an example of 6 generations, consisting of P_1, P_2, F_1, F_2, BC_1 and BC_2, components of mean, additive, dominance and epistasis are given in Table 2 1 (from Mather and Jinks, 1982).

However, Hayman (1958a) in a generation mean analysis, used the F_2 mean in the analysis instead of the parent. Hence, the components of mean in Hayman's model is based on the F_2 mean. As an example of 6 generations consisting of P_1, P_2, F_1, F_2, BC_1 and BC_2, as based on Hayman's model, components of mean, additive, dominance and epistasis are given in Table 2.2 (from Hayman, 1958a).

Table 2.1. Components of mean (based on mead-parent mean)

Generations	Mean Phenotype					
	m	*d*	*h*	*i*	*j*	*l*
P_1	1	1	0	1	0	0
P_2	1	-1	0	1	0	0
F_1	1	0	1	0	0	1
F_2	1	0	1/2	0	0	1/2
BC_1	1	1/2	1/2	1/4	1/4	1/4
BC_2	1	-1/2	1/2	1/4	-1/4	1/4

Table 2.2. Components of mean (based on F_2 mean)

Generations	Mean Phenotype					
	m	*d*	*h*	*i*	*j*	*l*
P_1	1	1	1/2	1	1	1
P_2	1	-1	-1/2	1	1	1
F_1	1	0	1/2	0	0	0
F_2	1	0	0	0	0	0
BC_1	1	1/2	0	1/4	0	0
BC_2	1	-1/2	0	1/4	0	0

A statistical model for generation means is proposed as follows (from Mather and Jinks, 1982):

Mean=$m + \alpha d + \beta h + \alpha^2 i + 2\alpha\beta j + \beta^2 l$

m : mean of all generations in a cross

[*d*]: additive effect

[*h*]: dominance effect

[*i*]: interaction between additive effects (additive x additive)

[*j*]: interaction between additive and dominance(additive x dominance)

[*l*]: interaction between dominance effects (dominance x dominance)

α, β, α^2, $2\alpha\beta$, and β^2 are coefficients of the genetic parameters.

2.2.5 Diallel crossing

Hull (1945) was first to mention some aspects of diallel crossing in maize. Jinks and Hayman (1953) introduced the analysis of diallel cross, as a set of possible matings between several genotypes. The genotypes are defined as individuals, clones, lines etc., and if there are "n" of them, there are "n^2" mating combinations. Therefore, the diallel table is an arrangement of a square of n measurements corresponding to offspring with a common parental genotype. The theory of Hayman's diallel crossing (Hayman, 1954a; Hayman, 1954b; Hayman,

1956; Hayman, 1960) is based on the following assumptions:

1. Diploid segregation.
2. No differences between reciprocal crosses.
3. Independent action of non-allelic gene (no epistasis).
4. Homozygous parents.
5. Gene independently distributed between the parents.
6. No multiple allelism.

With respect to assumption 4, Kempthorn (1956) introduced another assumption in the theory of diallel crossing, in which the homozygosity of parents can be ignored if the parent had a similar F (coefficient of inbreeding) value. Assumption 3 does not alter the relative importance of additive and dominance variance (Matzinger *et al.*, 1960; 1966; 1972). Hayman (1954b) and Jinks (1954), introduced a method for analysing a diallel table and for subsequently estimating the genetical components. Hayman (1956) used diallel crossing to estimate dominance, additive and average dominance for characters such as, plant height and flowering time in the *Nicotiana rustica*. He also described mean, additive and dominance components in terms of *m*, *d* and *h*, in the absence of epistasis for the characters. The general combining ability based on Hayman's description is composed of both additive and dominance portions, while the specific combining ability mainly involves dominance and epistasis.

2.2.5.1 Analysis of Jinks-Hayman diallel table

Statistical analysis of the diallel table to estimate the variances, covariances of arrays and genetical components was introduced by Jinks and Hayman (1953), and described in more detail by Mather and Jinks (1977, 1982). Variance of all the offspring of the i'th parent (i.e. i'th complete array) is denoted by V_r. The covariance between the offspring and their recurrent parents are denoted by W_r as follows:

Variance of the array:

$$V_{ri} = (\Sigma_j X_{ij}^2 - X_{i.}^2/m)/m-1 \quad \text{for each array}$$

Where i=1,...,P, and P is the number of parents, j=1,...,m, where m is the progeny number and $X_{i.}$ is the summation across all j observation for each i.

Mean variance across arrays:

$$\overline{V}r = (\Sigma_i V_{ri})/P$$

Variance of array mean:

$$V\overline{x} = (\Sigma_l X_l^2 - X_{.}^2/P)/P-1$$

Where:

X_i is the ith phenotype variate, l=1,..., a, where a is the number of array means, P is the number of parents and $X_{.}$ is the sum of parental array mean.

Variance of parents:

$$V_P = (\Sigma_i X_i^2 - X_{.}^2/P)/P-1$$

Where:

X_i is the ith parent phenotype (leading diagonal of diallel table) and X. is the sum of entries in the leading diagonal (parents).

Covariance of array:

$$Wr_i = (\Sigma_{jk}\ X_{ij}\ Y_{ik} - ((X_{i.} - Y_{i.})/m))/m-1$$

Where:

i and j are defined as before and K=1,...,P,

Mean covariance across the arrays:

$$\overline{W}r = (\Sigma_i Xr_i)/P$$

Mean of the i'th array:

$$r = (\Sigma_j X_{ij})/m$$

Four genetical components D, H_1, H_2, and F, can be estimated (Jinks and Hayman, 1953; Mather and Jinks, 1982) as follows:

$$D = \Sigma 4uvd^2$$
$$H_1 = \Sigma 4uvh^2$$
$$F = \Sigma 8uv(u-v)dh$$
$$H_2 = \Sigma 16u^2v^2h^2$$

Where:

D is twice the additive genetic component of variation in panmictic.

H_1 and H_2 measures dominance components of variation.

F accounts for non-independent contributions of additives (d) and dominance (h) when gene frequencies (u and v) are unequal.

Additive genetic variance and dominance genetic variance can be estimated from D, H_1 and H_2 when it is assumed that the parameters apply to a random mating base population of which the parents used in the diallel are a sample. With the assumption of random mating (u=v=0.5), and absence of epistasis, D and H_2 become D_R and H_R (R denotes for random mating). The additive and dominance components of variations can be estimated from D, H_1 and H_2 (Falconer, 1981) as follows:

$$\sigma^2_A = (1/2)D$$
$$\sigma^2_D = (1/4)H_2$$
$$\sigma^2_O = H_1$$

2.2.5.2 Graphical analysis of Jinks-Hayman diallel

In a diallel crossing with a certain value of H_1/D, the points Vr/Wr are distributed along a straight line of unit slope inside the limiting parabola, $Wr^2 = VrxVp$ (Hayman, 1954b). When the limiting parabola is plotted for an attribute, completely recessive parents correspond to points at the upper ends of the sloping line, and completely dominant parents are laid at the lower part of the sloping line.

Regression of Wr against Vr can also be analyzed for an attribute. The standard error of regression, R-square and slope (β) can be estimated by regression analysis, and tested using the F-test. A simple additive dominance model is indicated when the regression slope does not differ significantly from unity, as determined by a t-test. Generally, appropriate regression equations have higher slope (β) closest to 1, relatively low standard error and high R-square.

2.2.5.3 Griffing diallel

Griffing (1956b), described four diallel crossing techniques depending upon whether either the parental inbreds or the reciprocal F_1s are included or both. Four possible diallel crossing methods were subsequently explained by Griffing as follows:

1. Parents, one set of F_1's and reciprocal F_1's included (all PxP combinations. where P in number of parents).
2. Parents and one set of the F_1's are included but reciprocal F_1's are not ($^1/_2P(P+1)$ combinations).
3. One set of F_1's and reciprocals are included but not parents (P(P-1) combinations).
4. One set of F_1's but neither parents or reciprocal F_1's included ($^1/_2P(P-1)$ combinations).

Each method could be analyzed based on random or fixed model sampling assumptions. Hence, based on the methods or the statistical assumptions, eight different analyses could be carried out using Griffing's diallel method.

Griffing's method of analysis of diallel cross data involve general and specific combining abilities. Combining abilities can be interpreted as additive, dominance and various types of epistasis; therefore the predominant types of genetic variances can be determined.

The genetic variances between the crosses is partitioned into two components: variance of general combining ability (σ^2_{gca}) which contains additive variance and additive x additive type of epistasis, and variance of specific combining ability (σ^2_{sca}) which contains dominance variance as well as all other types of epistasis. The relation between σ^2_{gca} and σ^2_{sca} with genetic components was defined by Griffing (1956b) and Kempthorn (1955) in terms of additive and non-additive variances.

The estimates of genetic components of variations and epistasis are derived from covariance of full-sib and half-sib families as follows:

$$cov_{H.F} = [(1+F)/4]\sigma^2_A+[((1+F)/4)^2]\sigma^2_{AA}+[((1+F)/4)^3]\sigma^2_{AAA}+....$$

$$cov_{F.S} = [(1+F)/2]\sigma^2_A+[((1+F)/2)^2]\sigma^2_D+[((1+F)/2)^2]\sigma^2_{AA}+ [((1+F)/2)^3]\sigma^2_{AD}+[((1+F)/2)^4]\sigma^2_{DD}+....$$

where:

σ^2_A : additive genetic variance of random mating, non-inbred (i.e. panmictic)

σ^2_{AA} : additive x additive type of epistasis

σ^2_{AAA}: additive x additive x additive type of epistasis

σ^2_D : dominance x genetic variance of panmictic

σ^2_{AD} : additive x dominance type of epistasis

σ^2_{DD} : dominance x dominance type of epistasis

The variance components in terms of the covariances between relatives are:

$$\sigma^2_{gca} = cov_{H.S.}$$

$$\sigma^2_{sca} = cov_{F.S.}-2cov_{HS}$$

The broadsense and narrowsense heritabilities can be estimated using the σ^2_{gca} and σ^2_{sca} as follows:

$$h^2_{BS} = (2\sigma^2_{gca}+\sigma^2_{sca})/(2\sigma^2_{gca}+\sigma^2_{sca}+\sigma^2_e)$$

$$h^2_{NS} = 2\sigma^2_{gca}/(2\sigma^2_{gca}+\sigma^2_{sca}+\sigma^2_e)$$

There are several advantages of the combining ability analysis (Griffing, 1956a; 1956b) over the diallel analysis as used by Mather and Jinks (1982), since the combining ability variances estimate a simple and reliable genetic situation and a genetic model in the presence of epistasis. On the other hand, the diallel analysis of Mather and Jinks (1977) in which the absence of epistasis is one of the basic assumptions, is not necessary in Griffing's diallel analysis. In this case, there is no limitation on the number of alleles and the analysis can be used for any number of alleles per locus and any number of loci.

2.2.6 Heritability

The heritability of any character is a measure of the relative importance of heredity. Therefore, heritability may be considered as the usefulness of a character under selection. A character can be hereditary in the sense of being determined by 1) total genotype, and 2) average transmission from the parent to the offspring. The genotype versus environment meaning leads to the first definition of heritability known as descriptive heritability. Descriptive heritability is equal to the ratio of the all of the genotypic variance σ^2_G (additive, dominance and epistasis) to the phenotypic variance σ^2_p and is commonly symbolised by h^2_{BS}. The later meaning leads to the second definition of heritability known as narrowsense heritability and is equal to the ratio of additive genetic variance σ^2_A to the phenotypic variance of individuals in the population, and is commonly symbolised h^2_{NS} (e.g. Nyquist, 1991). In plant breeding, characters with a high narrowsense heritability are of particular interest, since characters with higher narrowsense heritability have higher genetic advance (ΔG) under selection (see Section 2.2.7), and also may be evaluated more readily in field trials. When narrowsense heritability is high, more reliance can be placed on mass selection, and when heritability is low, more emphasis must be placed on progeny, sib or family selection (Nyquist, 1991).

The broadsense heritability is generally of little interest in plant breeding except in clonal or hybrid cultivars. All of the genotypic variance in some population is accounted for not just the additive variance. Example include a population with different genotype structure (mixture of homozygotes and

heterozygotes), asexually propagated species with high heterozygosity (i.e. fruit and forest trees), and apomictically propagated species. To obtain an unbiased broadsense heritability in self-fertilising species, Nyquist (1991) recommended using an F_2 population derived from a cross between parents within a random mating population.

It is possible that by changing the environment, the genotypic and additive effects change. We can assume that, in the presence of genotype x environment interactions, changes in environment could result in changes in phenotype. The environment could be defined as either microenvironment, e.g. temperature, light, level of nutrition etc., or macro-environment, e.g. area (location), growing season, year etc.

In plants, the observed variance is the variance of phenotypic values of individuals within a population. The family heritability in plants is the observed phenotypic variance among family means. In fact, family heritability is the ratio of the among family additive genetic value component to the phenotypic variance among family means. For within family heritability, the observed variance is the phenotypic variance among individuals within the families. If individuals in a plant species can be measured, heritability on an individual basis can be calculated. In some cases e.g. dry mass of some plants, heritability can not be measured on an individual basis.

To estimate heritability, Pederson (1972) evaluated and compared four mating designs, the partial diallel cross, the full diallel cross, and the North Carolina designs I and II. The σ^2_A, σ^2_D and σ^2_W were measured and a true genotype x environment interaction was assumed to be absent. Of these four designs, the partial diallel design was the most preferred, followed by the North Carolina design II, the complete diallel and then the North Carolina design I.

To estimate the heritability and standard error of heritability, Gordon *et al.* (1972) used 2 cross-classified models. The first model consisted of block, environment, genotype, and genotype x environment interaction. The second model consisted of year, site, site x block interaction, block within environment, genotype, genotype x site interaction, genotype x year interaction and genotype x site x year interaction. Gordon *et al.* (1972) also estimated all of the variance components and standard errors of heritabilities. In another study, Gordon (1979) estimated heritability and standard errors of heritabilities for perennial crops. Pesek

and Baker (1971) gave a detailed numerical example for computation of the standard error of a heritability estimate.

Using cross classification designs to estimate the genotypic and environmental components of variations is more common in heritability studies. It is possible to ignore the class classification structure of locations and year, and simply regard those microenvironments as a single criterion of classification. Comstock and Moll (1963) suggested, when the cross classification structure was ignored, that the family x environment mean square underestimates the contribution due to the variance components for family x location and family x year interactions. In contrast the family component is overestimated by a similar amount. In another study, Carmer *et al.* (1989) ignored the environment effect in the cross classification. In some studies, the heritability has been estimated without taking into account either location or time. In such situations, the heritability estimate increases significantly compared to that which includes location and time.

To estimate the heritability through cross-classification designs, Nyquist (1991) explained several designs. The first used as completely randomised design (CRD) as the simplest model with the possibility of year x location combination, while, the second more common experimental design used as randomised complete block (RCB) design. The location, year and replication can all be classified in this (RCB) design. Furthermore, besides the location, year, and replication effects, partitioning the plot to within plot is possible in these designs. By analysing the designs, the heritability can be estimated through dividing the genotypic variance by the phenotypic variance. For perennial species, Nyquist (1991) also explained a split-block design in which the macro environment was assigned as a whole unit.

According to Gordon (1979), the variance components of the effects should be obtained by equating the mean squares to their expected values for estimating the heritability. Negative estimates of the variance components should be reported and/or used to avoid introducing a bias in any expression involving the components, as the conversion of negative estimates to zero can result in a truncated sampling distribution of the variance component.

In a method presented by Warner (1952), heritability was estimated using additive and dominance variance. The estimate was made entirely on the basis of the F_2 and backcrosses. In this method, the estimate of non-heritable variance is

not necessary. The genetic variance of the F_2 population was proposed as (1/2)D+(1/4)H, and the genetic variance of BC_1 and BC_2 was proposed as (1/2)D+(1/2)H. The additive component (D) represents the portion of the variance which can be attributed to the additive effect of the genes, and the dominance component (H) represents the portion of the variance due to deviation from additivity. It is assumed that the environmental component (E) of variation is comparable in all three segregating populations (BC_1, BC_2, F_2). Under this assumption, the variance of the F_2 and BC_1+BC_2 can be estimated as follows:

$$VF_2 = (1/2)D+(1/4)H+E$$
$$VBC_1+VBC_2 = (1/2)D+(1/2)H+2E$$

The total F_2 variance can be determined as a total phenotypic component of variation. To estimate the genotypic component of variation, the F_2 variance is multiplied by 2 and then subtracted from the (BC_1 + BC_2) variance.

The heritability can be estimated as follows:

$$h^2 = [(1/2)D]/VF_2 = [(1/2)D]/[(1/2)D+(1/4)H+E]$$

Warner (1952) estimated the standard error of the heritability as follows:

$$h^2_{NS} = [2(VF_2)-(VBC_1+VBC_2)]/VF_2$$

and the standard error of heritability can be estimated as follows:

$$S.E.(h^2_{NS}) = \{2\{[(VBC_1+VBC_2)^2/dfF_2]+(VBC_1^2/dfBC_1)+(VBC_2^2/dfBC_2)\}/VF_2^2\}^{1/2}$$

In this formula dfF_2, $dfBC_1$ and $dfBC_2$ are degrees of freedom of F_2, BC_1 and BC_2 respectively. The significance of h^2 can be tested by F distribution with n_1 and n_2 degrees of freedom.

where:

$$n_1 = dfF_2, \text{ and,}$$
$$n_2 = (VBC_1+VBC_2)^2/[(VBC_1^2/dfBC_1)+(VBC_2^2/dfBC_2)]$$

Mather and Jinks (1982) estimated the broadsense and narrowsense heritabilities using genetical components which were themselves estimated through Jinks-Hayman diallel cross analysis as follows:

$$h^2_{BS} = [(1/2)D+(1/2)H_1-(1/4)H_2-(1/2)F]/[(1/2)D+(1/2)H_1-(1/4)H_2-(1/2)F+E]$$

$$h^2_{NS} = [(1/2)D+(1/2)H_1-(1/2)H_2-(1/2)F]/[(1/2)D+(1/2)H_1-(1/2)H_2-(1/2)F+E]$$

2.2.7 Genetic advance (ΔG) and number of effective factors

Heritability is commonly used to predict a selection response. Genetic advance or expected gain from selection can be estimated as follows (Nyquist, 1991):

$$\Delta G = K\sigma P h^2$$

where:

ΔG is selection differential in standard units, σP is the phenotypic standard deviation of F_2 population, K is intensity of selection and h^2 is heritability.

The number of genes (effective factors) contributing to the variance of quantitative characters within and between populations are fundamental for the study of mechanisms of heredity and evaluation. A procedure was outlined by Lande (1981) for estimating the minimum number of freely segregating genetic factors contributing to the difference in a quantitative character between two populations that have diverged by artificial or natural selection. The number of effective factors (genes) can be estimated by comparing the phenotypic means and variance in the two populations and in their F_1 and F_2 generations, as well as in backcrosses (BC_1 and BC_2). An estimate of the minimum number of genetic factors was estimated as follows:

$$nE = (\mu P_2-\mu P_1)^2/8\sigma^2 s \leq n$$

where:

$$\sigma^2 s = 2\sigma^2 F_2-\sigma^2 BC_1-\sigma^2 BC_2$$

The number of effective factors (genes) involved on expression of a certain character was estimated by Mather and Jinks (1982) using parents mean, F_1 mean, and H_2 as follows:

$$K = (MF_1\text{-}MP)^2 / [(1/4)H_2]$$

Where MF_1 and Mp are the overall means for the F_1 progeny and the parents respectively.

Chapter 3

Phenotypic expression and heritability of resistance to dalapon

3.1 Introduction

After the transfer of the dehalogenase gene into the plant genome and the regeneration of genetically transformed plants, it is necessary to evaluate the phenotype of the transgenic plants. A study of the phenotypic expressivity of the transgene at various levels of herbicide as well as at various times after herbicide application, is necessary for evaluation of the stability of phenotypic expression in the transformed plants. A study of the heritability of the transgene leads to understanding whether expressivity of the transgene is stable across changing environmental conditions. The objectives of different aspects of this chapter are outlined below.

3.1.1 Phenotypic expression of transgene

The objectives here are to quantify the phenotypic expression of callus grwoth and leaf necrosis following dalapon application to transgenic white clover calli and transgenic white clover plants respectively; to quantify the phenotypic expression of various characters (i.e. height, leaf length, leaf width, necrotic surface of leaves) at various growth stages following dalapon application to transgenic tobacco plants (seedlings and developed plants); level of resistance of transgenic lines to the applied herbicide at various growth stages. Various cross classification designs were carried out to quantify the phenotypic expression of the transgene for various characters and lines.

3.1.2 Heritability of transgene

The objectives here are to estimate heritability of some of the characters under various herbicide levels for the genetically transformed tobacco plants; and to estimate the phenotypic correlations between the characters under study.

The heritability of characters was estimated using various appropriate cross classification designs.

3.1.3 Inheritance of transgene

The objectives here are to determine the segregation pattern of the transgenic tobacco lines during successive generations and at various levels of herbicide separately, as well as pooled over the herbicide levels. The inheritance studies were carried out using both adjusted and unadjusted χ^2 methods.

3.2 Materials and Methods

3.2.1 Herbicide resistance of transgenic white clover

3.2.1.1 Developing the transgenic white clover

Stolon internode segments of white clover, *Trifolium repens* line WR8 (White, 1984) were genetically transformed by Dr. D.W.R. White using *Agrobacterium tumefaciens* strain LBA4404 and the binary vector pAS501. The T-DNA of plasmid pAS501 included two genes which were introduced into the plant genome: a) a dehalogenase gene which is responsible for the degradation of dalapon herbicide by detaching the chlorine ions from the herbicide structure, and b) a neomycin phosphotransferase II (NPTII) gene which is responsible for the detoxification of kanamycin and thus facilitates the growth of transformant cells in media containing kanamycin as a selective agent.

WR8 agar medium (this is a callus growth medium and should not be confused with the WR8 white clover line) supplemented with 100 mg L^{-1} of kanamycin after autoclaving the medium (methods for preparing the WR8 medium and antibiotics stock solutions are given in Appendix 2 and Appendix 3 respectively) was used as callus induction medium, as well as for selection of genetically transformed cells. The calli were transferred aseptically onto the WR8 plates and incubated in a 28°C culture room with continuous white light at an intensity of 10 w m^{-2}. The callus lines were subcultured onto fresh medium every 21 days. Also a non-transgenic callus line was developed by culturing WR8 white clover on WR8 medium without added kanamycin.

Following several rounds of subculturing of calli on WR8 medium, the line numbered 66 showed vigorous growth and had differentiated into green tissue. The green tissue was transferred onto B5+2ip (Appendix 4) shoot induction medium to allow shoots to develop. The newly grown shoots were transferred onto fresh B5+2ip medium every 21 days until 3-4 strong petioles containing trifoliate leaflets had developed. For root induction, shoots containing 3-4 petioles and a piece of attached callus, were transferred into long plastic tubes with tight caps containing B5 (Gamborg *et al.*, 1968) slope agar medium (Appendix 1) without growth regulators. The shoots were transferred to fresh B5 medium every 21 days. After transferring shoots to fresh medium 2-3 times, roots had started to initiate and develop. Approximately six weeks after root initiation, the plantlets were washed

carefully under running tap water to remove agar and were then transferred into pots containing water-saturated soil. The pots were covered with transparent plastic lids for a few days to prevent water stress of the plantlets. The pots were placed in the glasshouse and sub-irrigated.

Growth of the plants in the glasshouse was very slow and adventitious roots from the stolon did not develop. As a result the plants were very weak and, after developing to late vegetative phase, started senescing. To maintain the plant material once senescence had begun, stolons were cut and planted into fresh soil. Each cut stolon contained 5-6 nodes and 1 petiole with a trifoliate leaflet. After 4-5 rounds of clonal propagation, the plants finally started to develop adventitious roots, coinciding with rapid and vigorous growth (Figure 3.1). The plants were grown up to late vegetative stage, approximately 3 months after adventitious root formation.

3.2.1.2 Herbicide resistance of *in vitro*-grown transgenic white clover calli

3.2.1.2.1 White clover callus lines

Three genetically transformed white clover callus lines (see section 3.2.1.1) were randomly chosen from a collection of transformed white clover calli and used in phenotypic expression experiments. These three lines were numbered as line number 1, 2 and 3. The control non-transgenic callus line was developed from internode culture of the WR8 white clover line.

3.2.1.2.2 Experimental layout

A factorial experimental design, with four white clover callus lines as factor (A), and five levels of herbicide as factor (B) was established with 7 blocks in a randomized complete block format. The lines consisted of the 3 transgenic white clover callus lines and one non-transgenic base line (WR8) callus (see section 3.2.1.2.1). The five concentrations of dalapon (Aldrich) used were 0, 100, 200, 300 and 400 mg of active component L^{-1}. The experimental unit was a petri dish containing a single piece (250 mg) of callus.

WR8 agar medium supplemented with the levels of herbicide (0, 100, 200, 300 and 400 mg dalapon L^{-1} added after autoclaving the medium) was used as callus growing medium. Under aseptic conditions, 250 mg callus tissue (initial fresh

weight) was transferred onto each experimental unit (plate). The plates were incubated in a 28°C culture room with continuous white light at an intensity of 10 w m^{-2} for 21 days. The final fresh weight of callus tissue of each of the plates was weighed on an analytical balance and this weight (mg) was recorded. The callus growth-gain was calculated for each experimental unit by subtracting the final fresh weight from the initial fresh weight.

Analysis of data of the callus growth-gain was carried out under a fixed model assumption using the SAS (statistical analysis system) programme. The statistical procedure of analysis was based on the general linear model (Proc. GLM) of the SAS programme. The linear statistical model for the experimental design was:

$$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \epsilon_{ijk}$$

where:

X_{ijk}=single callus observation, μ=population mean, α_i=effect of$_i$'th block, β_j=effect of$_j$'th callus line, γ_k=effect of$_k$'th herbicide level, $(\beta\gamma)_{jk}$=interaction effect between$_j$'th callus line and$_k$'th herbicide level and, ϵ_{ijk}=error term.

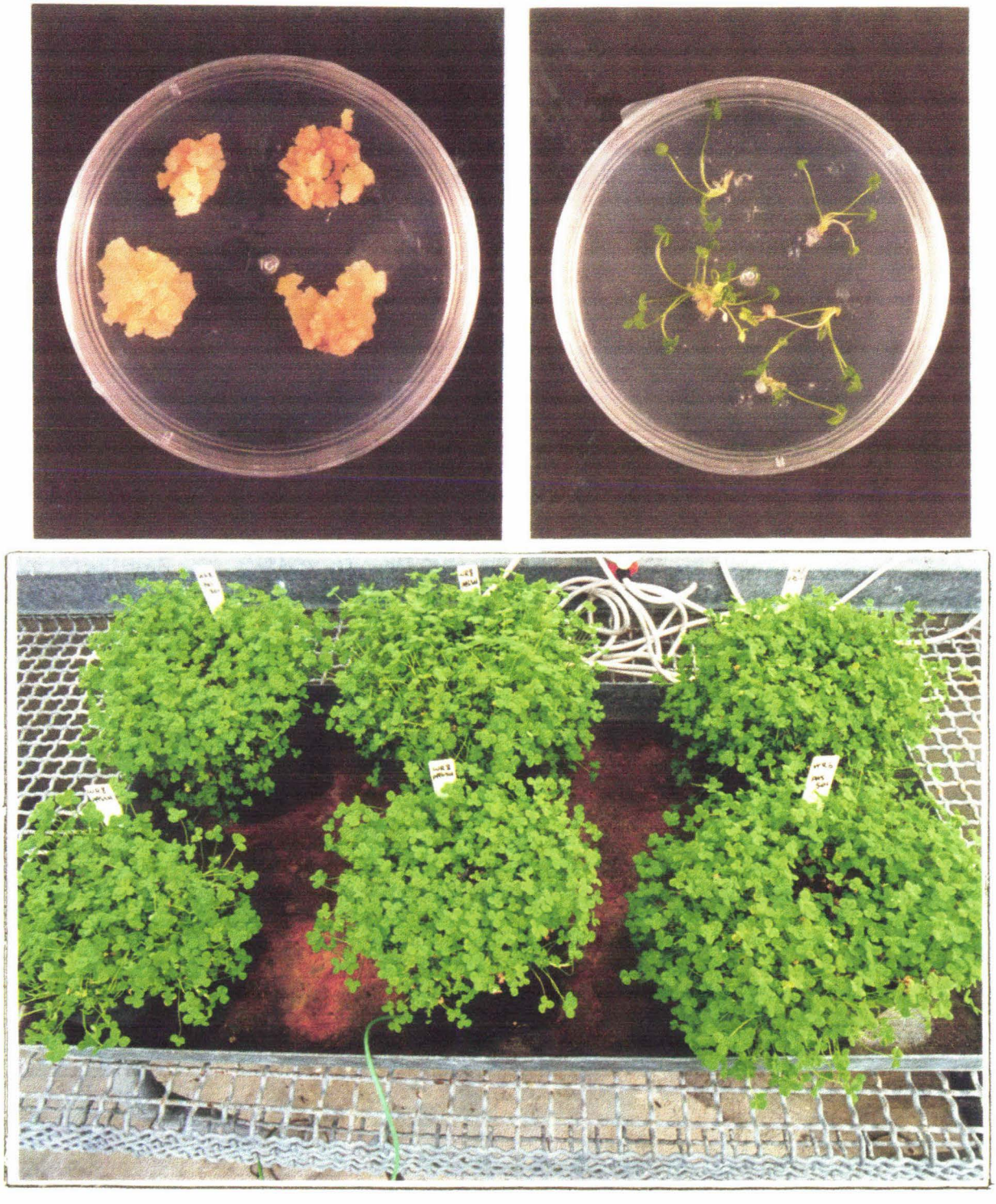


Figure 3.1. Development of dalapon resistant transgenic white clover plants. Genetically transformed calli (top left), shoots (top right) and fully developed transgenic white clover plants (bottom) are shown.

3.2.1.3 Herbicide resistance of transgenic white clover plants

A factorial experimental design with two factors was employed. A non-transgenic white clover line (WR8) and a transgenic line (number 66) were levels of factor (A). Three levels of dalapon (0 (water), 6 and 12 kg active component ha^{-1}), made up factor (B) in the factorial design with two blocks in a randomised complete block format. The plants were sprayed with a swing sprayer with high accuracy. The pressure of the sprayer was held constant at 200 kilo pascal during the herbicide application Pots were sprayed randomly and distributed into each block. Necrosis of each leaflet was estimated after 17 days in 1/8 of each pot (the pots were divided in 8 equal areas and one of the areas randomly selected) and recorded by giving scores from 1 to 10 (low level of necrosis to high level of necrosis). Each score represented approximately a 10% increment in necrotic leaf area. Estimation of necrosis was by eye (Lefkovitch, 1991; Cummins and Aldwinckle, 1983; Bradley, 1953; Finney, 1950). The scores were normalized using normit transformation. The analysis of the data was carried out under a fixed model assumption using the SAS programme. The statistical procedure of analysis was based on the GLM procedure (proc. GLM). The linear statistical model for the experimental design that was used was as follows:

$$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \epsilon_{ijk}$$

where:

X_{ijk}=single plant observation, μ=population mean, α_i=effect of $_i$'th block, β_j=effect of $_j$'th white clover line effect, γ_k=effect of $_k$'th herbicide level, $(\beta\gamma)_{jk}$=interaction between $_j$'th white clover line and $_k$'th herbicide level and ϵ_{ijk}=error term.

3.2.2 Herbicide resistance of transgenic tobacco

3.2.2.1 Tobacco seed material

Tobacco (*Nicotiana tabacum, L.)* line KKD leaf discs were genetically transformed by Dr D.W.R. White using *Agrobacterium tumefaciens* strain LBA4404 containing the disarmed Ti plasmid pAL4404 and the binary vector pAS501 , as described for tobacco transformation (Chapter 5, Section 5.2.9). Several

transformant leaf discs subsequently developed into complete (R_0) plants (see Chapter 4, Section 4.2.1.1 for abbreviations) that went on to flower and produce seeds. Seeds from these selfed plants were harvested separately. From this collection, six transgenic seed lines: 6849-1 (49-1), 6851-1 (51-1), 6851-3 (51-3), 6851-5 (51-5), 6851-14 (51-14) and 6854-18 (54-18) were randomly selected and used as transgenic seed material in all subsequent experiments for this study (greenhouse, *in vitro*, mating designs, and molecular genetic experiments). The non-transgenic base line KKD, was used as a control.

3.2.2.2 Herbicide resistance of *in vitro*-grown tobacco seedlings

Approximately 0.1 g seed from each line was placed onto a piece of clean cloth material, dipped into 95% ethanol for 30s and transferred to 20% bleach containing 3-5 drops of wetting agent (Cetavlon) for 20 min. The seeds were then washed four times with sterile distilled water, distributed on the surface of plates containing $^1/_2$MS medium supplemented with 0.8% Difco agar (Appendix 5), and incubated in a 28°C culture room under continuous light for 8 days at an intensity of 10 w m^{-2} to allow the seedlings (R_1) to develop.

A factorial experimental design consisting of two factors (lines and dalapon levels, see below) and three blocks in a randomized complete block format was established. The six transgenic lines and one non-transgenic line were determined as levels of factor (A), and six concentrations of dalapon (Aldrich) at rates of 0, 100, 200, 300, 400 and 500 mg active component L^{-1} made up the levels of factor (B). Experimental units were single petri dishes, each containing 20 tobacco seedlings. The analysis of the data was carried out under mixed model assumptions in which block and line effects were assumed to be random effects and herbicide to be fixed effect using the SAS programme. The statistical procedure of analysis was based on the GLM procedure (Proc. GLM).

The $^1/_2$MS medium supplemented with 0.8% Difco agar, 3 mM MES [2-(N-Morpholino) Ethanesulfonic Acid] (United States Biochemical Corporation Cat. No.18886), and dalapon (0, 100, 200, 300, 400 or 500 mg L^{-1}, added after autoclaving the medium) was used to achieve the levels of factor B (herbicide levels). Twenty seedlings were transplanted from the growth medium to individual plates containing the different levels of herbicide. After transplantation the plates

were sealed with sealing film and transferred to a 28°C culture room with continuous light at an intensity of 10 w m^{-2}. After 30 days, the total fresh weight (mg) of the 20 seedlings was measured on an analytical balance to give the total fresh weight attribute. The statistical model for the experimental design was:

$$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \epsilon_{ijk}$$

where:

X_{ijk}=single plant observation, μ=population mean, α_i=effect of$_i$'th block, β_j=effect of$_j$'th tobacco line effect, γ_k=effect of$_k$'th herbicide level, $(\beta\gamma)_{jk}$=interaction between$_j$'th tobacco line and$_k$'th herbicide level and ϵ_{ijk}=error term.

Variance components were estimated and used. The expectations of mean squares (EMS) and their standard errors were based on the method of Crump (1946, 1951). The correct F-test was applied using the method of Satterthwaite (Satterthwaite, 1941; 1946). The heritability of the tobacco seedling fresh weight *(in vitro)* was estimated using the variance components estimated by the linear statistical model as discussed above. The genotypic and phenotypic components of variations are as follows:

$$\sigma^2_P = \sigma^2_\alpha + \sigma^2_\beta + \sigma^2_\gamma + \sigma^2_{(\beta\gamma)} + \sigma^2_\epsilon$$

where:

σ^2_P=phenotypic variance, σ^2_α=variance of block, σ^2_β=variance of line (genotypic variance), σ^2_γ=variance of herbicide, $\sigma^2_{(\beta\gamma)}$=variance arisen from interaction of line and herbicide and, σ^2_ϵ=variance of residual (error).

The heritability (h^2) can be estimated as follows:

$$h^2 = \sigma^2_\beta / \sigma^2_P$$

The standard errors of heritabilities were estimated by a procedure based on the method described by Gordon *et al.* (1972).

3.2.2.3 Herbicide resistance of transgenic tobacco plants

These experiments were carried out under greenhouse conditions. The necrosis, stem height, leaf length and leaf width characters were measured over

time during growth after herbicide application. The drymass (g), as a time-excluded character, was measured once at the end of the experiment. The experimental design for the "time" characters was a factorial design consisting of 2 factors, ((A) tobacco lines, (B) herbicide levels), 3 blocks and four times as a split plot. Repeated measurements adjusted were used in analysis of variance (Rowell and Walters, 1976; Gill, 1986). The experimental units were single pots, each containing five plants with an equal distance between each plant.

Six transgenic lines (49-1, 51-1, 51-3, 51-5, 51-14 and 54-18), and one non-transgenic base line KKD, were levels of factor (A). Seven levels of herbicide, 0 (water), 1.5, 3, 6, 12, 24 and 48 kg of the active component ha^{-1} made up the levels of factor (B). Analysis of the data was carried out under a mixed model assumption, in which block and line effects were assumed to be random effects while the other effects were assumed to be fixed effects in the model. The analysis of the data was carried out using the SAS programme. The statistical procedure of analysis was based on the GLM procedure (Proc. GLM).

To develop the tobacco seedings, seeds of each line were surface sterilized (Section 3.2.2.2) and spread onto plates containing $^1/_2$MS + 0.8% (w/v) Difco agar. After 28 days, seedlings (at the 2-3 leaf stage) were transplanted into soil-filled pots in the greenhouse. Five seedlings were transplanted into each pot, which had previously been subirrigated to saturation point, at an equal distance from each other. Following transplantation, the pots containing the seedlings were immediately subirrigated and covered with transparent plastic bags for 3-4 days. These bags, containing a few holes, were used to facilitate a humid micro-environment and to prevent drought shock. The pots were subsequently subirrigated weekly. Twenty-six days after transplantation, leaf height and leaf width measurements were determined for the fourth leaf from the base of the each seedling. This leaf was tagged with a coloured plastic collar for subsequent measurements. Herbicide (Icipon®, ICI) containing 74% (w/w) 2,2-dichloropropionic acid (dalapon) as the active component, was made into a solution and used for spraying the plants. The herbicide for each level was dissolved in distilled water and sprayed onto the plants with a highly accurate swing sprayer. The pressure of the sprayer was maintained at a constant 200 kilo pascal during the herbicide application. Pots were randomly selected for herbicide application and after applying the herbicide, the sprayed pots were randomly distributed inside the blocks

again. The humidity and temperature of the greenhouse were measured using a thermohygrogram. The greenhouse temperature was within the range of 20-30°C while the relative humidity was within the range of 50-85%. The four measurement times were 2, 5, 8 and 11 day after herbicide application.

The following four attributes were measured at each time point:

1. Stem height, measured from soil surface to apical meristem (mm).
2. Necrosis (proportion of the leaf surface covered by necrotic lesions after herbicide application), estimated by eye.
3. Leaf length, measured from leaf tip to axil (mm).
4. Leaf width, measured at the widest part of the lamina (mm).

Total drymass (g) was measured once only, at the end of the experiment. Plants were removed from the pots together with the soil. The roots were washed under running tap water, then each plant was put in a paper bag, and dried at 68°C in a ventilated oven for 7 days. The linear statistical model for the time-included characters was as follows:

$$X_{ijklm} = \mu + \alpha_i + \beta_j + \rho_k + (\beta\rho)_{jk} + \delta_{ijk} + \theta_{(ijk)} + \Gamma_m + (\beta\Gamma)_{jm} + (\rho\Gamma)_{km} + (\beta\rho\Gamma)_{jkm} + \epsilon_{ijklm}$$

where:

X_{ijk}=single plant observation, μ=population mean, α_i=effect of $_i$'th block, β_j=effect of $_j$'th tobacco line, γ_k=effect of $_k$'th herbicide level, $(\beta\gamma)_{jk}$=interaction effect between $_j$'th tobacco line and $_k$'th herbicide level, $(\alpha\beta\rho)_{ijk}$=error (a), θ_l=effect of $_l$'th individual plant, Γ_m=effect of $_m$'th time, $(\beta\Gamma)_{jm}$=interaction effect between $_j$'th line and $_m$'th time, $(\rho\Gamma)_{km}$=interaction effect between $_k$'th herbicide level and $_m$'th time, $(\beta\rho\Gamma)_{jkm}$=interaction effect between $_j$'th line, $_k$'th herbicide level and $_m$'th time and ϵ_{ijklm}= error (b).

For drymass, as a time-excluded character, the following model was used:

$$X_{ijkl} = \mu + \alpha_i + \beta_j + \rho_k + (\beta\rho)_{jk} + \epsilon_{ijkl} + \theta_{(ijkl)}$$

Description of notations are as described above for the time included characters.

3.2.2.4 Methods for estimating the heritability (h^2) of characters

For the time-included characters, F-test, variance components of the effects, heritabilities and standard errors of heritabilities were estimated using the AOVFASBW statistical programme (I.L., Gordon unpublished). The method for estimating the heritabilities and the standard errors of heritabilities were based on the statistical models of Gordon *et al.* (1972).

Phenotypic variance and heritabilities were estimated as follows:

$$\sigma^2_P = \sigma^2_\alpha + \sigma^2_\beta + \sigma^2_{(\alpha\beta)} + \sigma^2_\delta + \sigma^2_\theta + \sigma^2_\tau + \sigma^2_{(\beta\tau)} + \sigma^2_\epsilon$$

where:

σ^2_P=phenotypic variance, σ^2_α=variance of block, σ^2_β=variance of line (genotypic variance of lines), $\sigma^2_{(\alpha\beta)}$=variance of interaction between block and line, σ^2_δ variance or error (a), σ^2_θ=within plot variance (genotypic variance of individual plant), σ^2_τ=variance of time, $\sigma^2_{(\beta\tau)}$=variance of interaction between line and time, σ^2_ϵ=variance of error (b).

There were two genetic variances in the experiment, the variance arising from line (σ^2_β), and the variance arising from individual plants within the line ($\sigma^2_{g(\theta)}$). Since a proportion of variance of individual plants within the line (σ^2_θ) is an environmental variance, the environment-free variance of line ($\sigma^2_{g(\theta)}$) can be estimated as follows:

$$\sigma^2_{g(\theta)} = K\sigma^2_\theta$$

where the K ratio can be estimated using Smith (1936) equations as follows:

$\sigma^2_{e(\theta)} = \sigma^2_\epsilon / t'$ (as plants were each observed over t pooled times)

$\sigma^2_{g(\theta)} = \sigma^2_{(\beta)} + \sigma^2_{e(\theta)}$

$K = \sigma^2_{g(\theta)} / \sigma^2_{(\theta)}$

The environment-free variance of individual plants within the line was estimated by multiplying the variance of individual plants within the line by the K

ratio ($\sigma^2_{g(\theta)} = K\sigma^2_{\theta}$). Subsequently the three types of heritability, population (line), plant (within) and broadsense were estimated as follows:

Population (line)	$h^2 = \sigma^2_{\beta}/\sigma^2_P$
Plant (within)	$h^2 = \sigma^2_{g(\theta)}/\sigma^2_P$
Broadsense	$h^2 = (\sigma^2_{\beta} + \sigma^2_{g(\theta)})/\sigma^2_P$

As the standard error of each of these heritabilities were available, their significances were tested with the t-test.

The phenotypic variance of the drymass character was estimated as follows:

$$\sigma^2_P = \sigma^2_{\alpha} + \sigma^2_{\beta} + \sigma^2_{(\alpha\beta)} + \sigma^2_{\rho} + \sigma^2_{\theta} + \sigma^2_{(\beta\rho)} + \sigma^2_{\epsilon}$$

where:

σ^2_P=phenotypic variance, σ^2_{α}=variance of block, σ^2_{β}=variance of line (genotypic variance of population), $\sigma^2_{(\alpha\beta)}$=variance of interaction between block and line, σ^2_{ρ}=variance of herbicide, σ^2_{θ}=variance of individual plants (genotypic variance of population), $\sigma^2_{(\beta\rho)}$=variance of interaction between line and herbicide, σ^2_{ϵ}=variance of residual (error). The population, plant and broadsense heritabilities for the drymass character (time-excluded) were estimated as described above for the time-included characters.

3.2.3 Inheritance of dalapon resistance

For the 6 transgenic lines 49-1, 51-1, 51-3, 51-5, 51-14 and 54-18, the number of independent functional dehalogenase gene integration events was determined by a segregation analysis of dalapon resistance in progeny plants (R_1) originating from seeds of selfed, tissue culture-derived, transgenic parents (for developed plants grown under greenhouse conditions). The number of resistant and susceptible phenotypes for each line at each levels of dalapon (1.5, 3.0, 6.0, 12.0, 24 and 48.0 kg ha^{-1}) was recorded separately and used for a χ^2 test. Since three blocks consisting of five plants per experimental unit were used for each line, a total of 15 plants was tested for each line at each level of herbicide.

In the χ^2 test, since the degree of freedom was 1, the approximation of the χ^2 distribution was improved and a more exact probability value was obtained from

the χ^2 table by imposing a correction for continuity. This correction is intended to make the actual distribution, as calculated from discrete data, more nearly like the χ^2 distribution based on normal deviation (Steel and Torrie, 1980). The approximation calls for the absolute value of each deviation to be decreased by 0.5 as follows:

$$\text{Adjusted } \chi^2 = \sum \frac{(|\text{observed-expected}| - 0.5)^2}{\text{expected}}$$

Homogeneity of the 3:1 segregation ratios over all levels of herbicide for each line tested using the pooled χ^2 formula (Steel and Torrie, 1980) as follows:

$$\chi^2 = \frac{\sum (n^2_i/n_i) - n^2_j/n_{..}}{P(1-P)}$$

Where: j=1 or 2, P=1/4 or 3/4

In pooled segregation analysis, since three blocks consisting of five plants per experimental unit were used for each line and the results pooled over each level of herbicide, a total of 90 plants was tested for each line. The susceptible (negative) plants within each of the lines were recognized by two different procedures: a) plants which were phenotypically similar to those of the non-transgenic line at each level of herbicide were recorded as susceptible plants to give a phenotypic based identification; b) the level of necrosis of each plant at each level of herbicide was determined based on the level of necrosis of the non-transgenic control line to give a quantitative identification. Individual segregant plants for each of the lines were recognized on the basis of the level of leaf necrosis for each level of herbicide. Thus for 1.5 kg herbicide ha^{-1}, plants with more than 6% of necrosis were recorded as susceptible plants. For 3.0, 6.0, 12.0, 24.0 and 48.0 kg of herbicide ha^{-1} of herbicide, plants with more than 10, 25, 40, 50, and 65% of necrosis respectively were recorded as susceptible plants

(quantitative based identification). In the χ^2 test, since the degree of freedom was 1, a more exact probability value was obtained from the χ^2 table by imposing a correction for continuity as indicated above for the adjusted χ^2.

3.3 Results

3.3.1 Herbicide resistance of transgenic white clover

3.3.1.1 The herbicide resistance of *in vitro*-grown transgenic white clover calli

The analysis of variance of white clover callus growth-gain data is presented in Table 3.1. The significance ($P<0.001$) of the line, as a main effect, indicates that there are significant differences in average callus growth-gain of the lines in the presence of herbicide irrespective of other effects such as the herbicide and block effects. Furthermore, the significance ($P<0.00.1$) of the herbicide effect (another main effect) indicates that the levels of herbicide have significant effects on callus growth-gain irrespective of other effects.

The significant ($P<0.001$) effect of the interaction between line and herbicide levels indicates that as well as the main effects (line and herbicide levels), there are also variation amongst particular line x herbicide combinations which are departure from the main effects. Hence, each line shows a different phenotypic expressivity at the different levels of herbicide.

The mean total callus growth-gain of the white clover callus lines are presented in last column of Table 3.2. The transgenic line number 1 had the highest growth rate followed by line number 2, control and line number 3 .

All herbicide levels also have a significant effect on callus growth-gain such that by increasing the concentration of the herbicide, the rate of callus growth decreased significantly (last row of Table 3.2). Lower levels of the herbicide have less effect on callus growth-gain, while higher levels of the herbicide are more effective in decreasing callus growth-gain. Increasing the concentration of herbicide from 0 to 100 mg L^{-1} or from 100 to 200 mg L^{-1} led to a decrease in the callus growth-gain of between 30-40%, while increasing the concentration from 200 mg L^{-1} to 300 mg L^{-1} led to a decrease of about 50%, and from 300 mg L^{-1} to 400 mg L^{-1} to a decrease of 72%.

Since the interactions between line and herbicide level are significant, comparing the callus fresh weight gain of the white clover lines at various dalapon levels is appropriate. The callus growth-gain of the white clover lines at various dalapon levels are indicated in Table 3.2. At a zero level of herbicide, the non-transgenic control line showed the highest callus growth-gain, while increasing

concentrations of the herbicide led to significant decreases in callus growth-gain with no growth-gain at the 300 mg L^{-1} treatment. However the transgenic lines show only a slow decline in growth rates with increasing concentrations of herbicide.

In contrast with the control line, the transgenic lines continue to grow at a level of 400 mg L^{-1} of the herbicide. There are also differences between transgenic lines, especially at higher levels of the herbicide (Table 3.2). The callus growth-gain of the transgenic line number 3 at 400 mg L-1 of dalapon is 2.5 times that of the transgenic line number 1. However there are no differences in callus growth-gain between lines 1 and 2 for this level of herbicide. An analysis of the data for the interaction between herbicide and line (Table 3.2.), indicates that the control line at the zero herbicide level has a greater callus growth-gain than the transgenic lines. This may possibly reflect differences in age of the callus since the control callus line was freshly grown while the transgenic callus lines were somewhat older. These results show that there are significant differences between the lines before the application of herbicide. However when the herbicide level increases, callus growth-gain of the control plant decreases sharply compared with callus growth-gain of the transgenic lines.

With respect to the phenotypic expressivity of transgene, all of the transgenic lines show significantly higher callus growth-gain (phenotypic expression) than the control non-transgenic line in the presence of herbicide. The phenotypic expression of the lines differ with changes in herbicide concentration, and higher concentrations of herbicide caused a decrease in phenotypic expressivity of lines. The transgenic lines themselves do not show similar phenotypic expression in the presence of herbicide. Thus although an increase in concentration of herbicide caused a decrease in phenotypic expressivity for all of the transgenic lines, line number 3 showed a smaller decrease in phenotypic expressivity compared to the other lines. In relation to the herbicide concentration. The significant differences between the lines before applying the herbicide could be due to somaclonal variation. Somaclonal variation could have resulted from stresses induced by tissue culture, transformation and selecting agent (van Lijsebetten *et al.,* 1991; Errampali *et al.,* 1991).

Regarding the significant differences in phenotypic expressivity of the lines at higher concentrations of herbicide, it can be interpreted that the transferred gene

in the calli of the transgenic lines was responsible for the increased resistance to the herbicide. The herbicide dalapon inhibits the synthesis of β-alanine and pantothenate enzymes (Hilton *et al.*, 1958; 1959) as well as causing precipitation or conformational changes in plant proteins which could affect a large number of different metabolic pathways in the plant (Buchanan-Wollaston *et al.*, 1992).

3.3.1.2 Herbicide resistance of white clover plants

The analysis of variance of white clover leaf surface necrosis area data is presented in Table 3.1.

The total mean leaf necrosis surface of transgenic white clover and non-transgenic white clover lines are 44.35 and 83.58 respectively (last column of Table 3.3). The leaf necrotic surface of the control non-transgenic line is significantly higher than the transgenic line. As a comparison, the leaf necrotic surface of the non-transgenic control line is 1.88 times more than that of the transgenic line.

The level of leaf necrosis at 6.0 and 12.0 kg ha^{-1} of herbicide were 46.92 and 81.01% respectively (last raw of Table 3.3), indicating the significant effect of herbicide on leaf necrotic surface area of the leaves.

The significance differences in leaf necrosis level of each of the white clover lines at various levels of applied herbicide (interaction) indicate the different response of the control non-transgenic and transgenic lines to the applied herbicide at different concentrations (Table 3.3). The non-transgenic white clover line shows a significant increase in leaf necrosis in response to increasing herbicide concentration. When the response of the control non-transgenic line and the transgenic line to the levels of applied herbicide are compared, it is apparent that the leaf necrosis level of the transgenic line at 12 kg ha^{-1} is not statistically different from that of the control line at 6.0 kg ha^{-1} of herbicide (Table 3 3).

Increasing concentrations of the herbicide caused an increase in the level of leaf necrosis of each of the lines as indicated above. This indicates that an increase in concentration of the herbicide led to different phenotypic expressivity of the lines. From a comparison of the levels of leaf necrosis of the control line at 6.0 kg ha^{-1} and the transgenic line at 12.0 kg ha^{-1}, it can be concluded that the non-transgenic white clover line has a natural level of herbicide resistance which enables the plant to tolerate a low level of herbicide (e.g. 6.0 kg ha^{-1}). However,

higher levels of herbicide killed the control plants, indicating that dalapon at these concentrations has an irreversible effect on normal plant metabolism. Thus this level of herbicide (6.0 kg ha^{-1}) can be considered a threshold level for normal clover plants, and transgenic clover plants can tolerate level beyond this threshold and are able to continue growth.

It can also be concluded that phenotypic expressivity in the presence of the herbicide is relative for the transgenic lines, hence at a certain level of herbicide, a transgenic line may be also affected by the herbicide. On the other hand, the transgene facilitated an increase in the resistance threshold in transgenic lines. Based on the results shown above, it is interpreted that the dehalogenase enzyme conferred resistance to the herbicide through degradation in the resistant transgenic plants, as discussed on Section 3.3.1.1. These transgenic plants were thus able to grow in the presence of the herbicide and exhibit a resistant phenotype.

The interaction means comparison (Table 3.3) indicates that the phenotypic expressivity of the white clover lines changed with increasing concentrations of herbicide. Hence, an increase in concentration of herbicide resulted in decrease in phenotypic expressivity of the transgene in white clover line.

Table 3.1. Analysis of variance of white clover callus fresh weight (in vitro) and leaf necrosis surface area of white clover plants

Effect	Mean Square (MS)	
	Callus growth-gain	Leaf necrosis surface
Block	24796.66 **	4745.02 **
Line	40307.39 **	98982.09 **
Herbicide	429635.76 **	697436.52 **
LinexHerbicide	43180.22 **	54004.53 **
error	2083.73	5682.24

** : significant at 1% probability level.

Table 3.2. Callus growth-gain[1] of white clover for lines, herbicide levels and interaction between lines and herbicide levels

Lines	Herbicide (mg L^{-1})					Line Mean[3] ($LSD_{5\%}$=23.4)
	0.00	100	200	300	400	
Cont.	544.67 a[2]	173.67 e	55.00 gh	000.00 i	00.00 i	154.67 C
1	348.67 b	333.83bc	298.00bc	105.00 fg	22.17h i	221.53 A
2	288.33 cd	170.83 e	134.00 ef	82.00 fg	20.00 hi	187.67 B
3	295.00 d	249.17 d	181.67 e	157.50 e	55.00 gh	139.03 D
Hcide. Mean[4] ($LSD_{5\%}$=26.16)	366.17 A'	231.88 B'	167.17 C'	86.13 D'	24.29 E'	175.73 (Grand mean)

[1] Callus growth-gain was calculated by subtracting the initial callus fresh weight from the final callus fresh weight (see Section 3.2.1.2)

[2] Callus growth-gain of different white clover lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).

[3] Callus growth-gain of different lines. Means with the same letter are not significantly different (P=0.05).

[4] Callus growth-gain at different levels of herbicide. Means with the same letter are not significantly different (P=0.05).

Table 3.3. Leaf necrosis surface for white clover lines, herbicide levels and interaction between lines and herbicide levels

Herbicide (kg ha^{-1})	Line		Herbicide Mean[2]
	Transgenic	Control	($LSD_{5\%}$=13.21)
6.0	24.73 c[1]	69.11 b	46.92 B
12.0	63.97 b	98.05 a	81.01 A
Line Mean[3] ($LSD_{5\%}$=15.01)	44.35 B'	83.58 A'	63.79 (Grand mean)

[1] Leaf necrosis surface of different white clover lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Leaf necrosis surface of different levels of herbicide. Means with the same letter are not significantly different (P=0.05).
[3] Leaf necrosis surface at different lines. Means with the same letter are not significantly different (P=0.05).

3.3.2 Herbicide resistance of transgenic tobacco lines

3.3.2.1 The resistance of *in vitro*-grown tobacco seedlings to dalapon

The analysis of variance of tobacco seedling fresh weight data is presented in Table 3.4.

Table 3.4. Analysis of variance of tobacco fresh weight

Effect	Mean Square (MS)
Block	1441.84 ns
Line	3943.93 *
Herbicide	140388.19 **
LinexHerbicide	11634.84 **
error	1678.81

ns, * and ** : not significant, significant at 5% and significant at 1% probability levels respectively.

The total mean fresh weights of the tobacco lines are presented in the last column of Table 3.5. The highest fresh weights were produced by the transgenic lines 51-1 and 54-18, followed by 51-5, 51-3, 49-1, 51-14, while the lowest fresh weight was produced by the non-transgenic line, KKD.

There are also significant ($P<0.01$) differences in mean fresh weight between the herbicide levels (last row of Table 3.4). Although the seedling fresh weight decreased with increasing concentrations of dalapon (Table 3.5), there did not appear to be a direct correlation. For example, increasing the dalapon levels from 0 to 400 mg L^{-1}, had a more or less similar effect on fresh weight reduction (about 30% reduction for each level), while increasing dalapon levels from 400 to 500 mg L^{-1} led to a greater reduction in seedling fresh weight (about 85% reduction).

The interactions between tobacco lines and herbicide levels are significant ($P<0.01$). The fresh weight of each line at different levels of dalapon are presented in Table 3.5. The tobacco lines showed different responses to the different levels of herbicide. The non-transgenic line KKD responded more rapidly to increasing concentrations of herbicide than the transgenic lines. In addition, the fresh weight of KKD decreased more sharply with increasing herbicide concentrations and there

was no measurable seedling fresh weight for the 500 mg L^{-1} treatment. There was also significant variation among the transgenic lines with increasing herbicide concentrations, especially at the highest level of the herbicide. At lower herbicide levels, the differences in ratio of phenotypic expressivity is much lower than that at the highest concentration of the herbicide. There is more than four times the difference between the high resistant and low resistant transgenic plants at the 500 mg L^{-1} treatment for lines 51-1 and 51-14 (Table 3.5). This indicates that phenotypic expressivity of the lines decreases significantly with increase in concentration of applied herbicide.

3.3.2.2 Heritability of fresh weight of *in vitro*-grown tobacco seedlings

The heritability of fresh weight of *in vitro*-grown tobacco seedlings is based on the total fresh weight measurement, there being no individual seedling measurements. Thus an estimate of only the population heritability is used for this character. Furthermore, there is no time component included for this character and only the tobacco lines, herbicide concentrations, blocks and interactions are used as the sources of variation.

The estimate of population heritability of the fresh weight character was 0.106±08026, which was significantly low. For the seedling fresh weight, the estimated heritability is very low, this indicates that phenotypic expressivity of the transgene at the seedling stage is very low. These results also indicate the herbicide is more toxic to seedlings. This could be because either the environmental effects, including herbicide, are more important than genetic effect at the early stages of growth or that expression of the transgene at the seedling stage is not as high as in developed plants (see Section 3.3.2.4).

Table 3.5. Fresh weight of tobacco seedlings for lines, herbicide levels and interaction between lines and herbicide levels

Line	Herbicide (mg L^{-1})						Line Mean[2]
	0	100	200	300	400	500	($LSD_{5\%}$=0.024)
KKD	0.243 abc[1]	0.146 de	0.102 efgh	0.060 hijk	0.033 ijkl	0.000 l	0.097 C
49-1	0.204 bcd	0.219 bc	0.129 efg	0.111 efgh	0.086 ghi	0.024 jkl	0.129 A
51-1	0.253 ab	0.196 cd	0.135 efg	0.103 efgh	0.100 efgh	0.033 ijkl	0.137 A
51-3	0.263 ab	0.201 cd	0.114 efgh	0.099 efgh	0.082 ghij	0.020 chile	0.130 A
51-5	0.229 bc	0.191 cd	0.152 de	0.114 efgh	0.108 efgh	0.009 chile	0.134 A
51-14	0.227 bc	0.146 def	0.110 efgh	0.099 efgh	0.090 fghi	0.007 chile	0.113 A
54-18	0.297 a	0.224 bc	0.133 efg	0.090 fgh	0.069 hij	0.011 chile	0.137 A
Hcide. Mean[3] ($LSD_{5\%}$=0.023)	0.245 A'	0.189 B'	0.125 C'	0.097 D'	0.081 D'	0.015 E'	0.125 (Grand mean)

[1] Seedling fresh weight of different tobacco lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Seedling fresh weight of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Seedling fresh weight at different levels of herbicide. Means with the same letter are not significantly different (P=0.05).

3.3.2.3 Herbicide resistance of tobacco plants

As discussed in Materials and Methods, four time-dependent attributes and one time-independent attribute were studied in the tobacco plants under greenhouse conditions. The time-dependent attributes studied in greenhouse were: the proportion of the leaf surface covered by necrosis (NC), stem height (SH), leaf length (LL) and leaf width (LW). Total drymass (DM) was studied as a time-independent attribute. The response of each plant to herbicide was determined by measuring the attributes in each of the lines as follows:

Approximately two hours after applying herbicide, leaves of the sensitive plants started to wilt and approximately 48 hours later the colour of the necrotic part of the leaves changed from yellow to brown. The analysis of variance of leaf surface necrosis of tobacco plants are presented in Table 3.6.

The significance ($P<0.001$) of the line main effect indicates that there are significant differences in average necrosis surface area of the lines in the presence of herbicide irrespective of other effects such as the herbicide and block effects. The significance ($P<0.001$) of the herbicide effect (another main effect) indicates that the levels of herbicide have significant effects on necrosis surface area irrespective of other effects. The non-significant effect of time indicates that there are no significant differences in average necrosis surface area of all lines at different times after herbicide application irrespective of other effects such as the herbicide and block effects.

The significant ($P<0.001$) effects of interactions indicate that, as well as the main effects, there are inconsistencies apparent amongst various combinations of the main effects.

Table 3.6. Analysis of variance of the necrosis character

Effect	Mean Square (MS)
Block	1087.32 ns
Line	77479.11 **
Herbicide	481957.84 **
LinexHerbicide	7931.59 **
error(a)	1197.39 **
Within plot	830.29 **
Time	738.97 ns
TimexLine	1484.69 **
TimexHerbicide	1251.58 **
TimexLinexHerbicide	289.27 **
error(b)	75.21

ns, * and ** : not significant, significant at 5% and significant at 1% probability levels respectively.

The total mean leaf necrosis surface of each tobacco line is presented in the last column of Table 3.8. Comparing the values for total mean leaf necrosis of the lines, there are no significant differences between the non-transgenic, control tobacco line (KKD), and the transgenic line 51-14. The effect of each of the applied herbicide concentrations on levels of leaf necrosis is presented within Table 3.8. Increase in the concentration of the herbicide led to an increase in the necrotic surface of the leaves (last row of Table 3.8). although interaction effects between line and herbicide levels is significant, the independent comparisons of both the total mean of the lines and the total mean of the herbicide levels only give a general idea about the performance of each main effect without considering the performance of the individual lines at individual levels of herbicide. These combinations, and their departures from main effects, are discussed below.

The significance of this interaction indicates that there are differences between the necrosis surface area of the lines at various levels of herbicide. Differences in the patterns of leaf necrosis for each of the lines across levels of applied herbicide (interaction between herbicide and line) indicate differences in resistance of the tobacco lines (Table 3.8). Similarity in leaf necrosis surface area of the transgenic lines at the lowest level of herbicide (1.5 kg ha^{-1}) indicates similarity in phenotype of the transgenic lines at the lowest level of herbicide. The

non-transgenic line KKD showed a significant increase in leaf necrosis in response to increasing herbicide concentration. Although the transgenic tobacco line 51-14 at herbicide levels of 1.5 and 3.0 kg ha^{-1} showed less necrosis than the control line (higher resistance), at levels higher than 3.0 kg ha^{-1} it exhibited a similar phenotype to that shown by the non-transgenic line. This indicates that 51-14 line exhibited a resistance phenotype up to 3.0 kg ha^{-1} of herbicide, while at concentrations higher than 3.0 kg ha^{-1} exhibited a non-resistant phenotype. Possible reasons for this will be discussed in Chapter 5. There were also significant differences between the other resistant transgenic tobacco lines at herbicide concentrations of more than 1.5 kg ha^{-1}. However at 48 kg of herbicide ha^{-1}, variation amongst the resistant transgenic lines was lower and there were no significant differences between the 51-1, 51-3 and 51-5 lines at this level of herbicide. The transgenic tobacco line 49-1 shows the lowest level of leaf necrosis at the highest level of herbicide. Thus the level of leaf necrosis of this line at 48 kg of herbicide ha^{-1} was less than the level of necrosis of the control non-transgenic line at 6.0 kg of herbicide ha^{-1}.

It appears that while resistance has been conferred by the transgene, its expression varies significantly from line to line. Phenotypic expression of the lines changed with increasing level of herbicide, although there were no differences between the lines at lowest level of herbicide.

The interactions between lines and herbicide levels revealed several changing patterns amongst the lines with increasing herbicide concentration. While all transgenic lines showed a resistant phenotype at the lowest levels of herbicide, the 51-14 line began to respond similar to the susceptible control line with increasing herbicide concentration (more than 3.0 kg of herbicide ha^{-1}), eventually becoming indistinguishable from the control. Another line (54-18), exhibited threshold behaviour, being the most highly resistant up to 6.0 kg of herbicide ha^{-1}, followed by a marked change to susceptibility above this level. Yet another line (49-1), showed a steady middle level of resistance through all herbicide levels, being the most resistant at the highest level. The other transgenic lines were similar to the 49-1 line until the highest level of herbicide where they showed moderate susceptibility. These variations in phenotypic expression probably arise through differential expression of the transgene. This may be as a result of either the expression of the transgene itself or from some interaction with the host

genotype. The quantitative genetics and molecular genetics results in Chapter 4 and Chapter 5 respectively will elaborate on these issues further.

As mentioned above, the interaction effect of the time component and leaf necrosis level indicate that the leaf necrosis level of the non-transgenic control line, KKD, and the transgenic line 51-14, increased after herbicide application over time (Table 3.9). For example, in both lines, the necrosis level at time 1 is lower than that at subsequent times. The increase in necrosis over time indicates that the plants are unable to recover following application of the herbicide. In contrast, the resistant transgenic tobacco lines show a decrease in necrosis level over time following herbicide application. These results indicate that the resistant lines are able to recover following herbicide application.

The analysis of variance of the stem height data for tobacco plants is presented in Table 3.7. All of the effects in this Table, except the block effect, are significant. The total stem height of each of the lines (last column of Table 3.10) indicate significant differences in these values between the lines. The effect of applied herbicide at various concentrations on total stem height is also significant (Table 3.10). As can be seen from Table 3.8 and Table 3.10, there is a similar pattern of response to applied herbicide at various concentrations for both the stem height and leaf necrosis characters. However, in contrast to leaf necrosis, the total stem height increased significantly over time (Table 3.11), indicating that the stem height continued to grow after herbicide application. The stem height increased approximately 2.7 times from the first measurement (time 1) to the last measurement (time 4, Table 3.11).

The stem height of each of the lines at various levels of herbicide (interaction) and changes with time (Tables 3.10 and 3.11) indicates a pattern of response inverse to that of leaf necrosis (Tables 3.8 and 3.9). This is illustrated in Figure 3.2 where the stem height of the transgenic line 54-18 can be compared with that of the control line KKD at 0 and 48 kg of herbicide ha^{-1}, eleven days after herbicide application (time 4). Similarity in stem height of the lines at lowest level of herbicide (1.5 kg ha^{-1}) indicate similarity in phenotype of the lines at the lowest level of herbicide. From the data, it can be interpreted that the herbicide residue remains active in the plant after herbicide application. Thus the resistant plants are able to degrade the herbicide over time and facilitate the growth of these plants.

Table 3.7. Analysis of variance of stem height character

Effect	Mean Square (MS)
Block	8831.53 ns
Line	249509.75 **
Herbicide	284287.63 **
LinexHerbicide	8110.91 *
error(a)	3812.39 **
Within plot	2330.34 **
Time	716980.25 **
TimexLine	16999.21 **
TimexHerbicide	30538.21 **
TimexLinexHerbicide	1077.42 **
error(b)	366.74

ns, * and ** : not significant, significant at 5% and significant at 1% probability levels respectively.

Table 3.8. Leaf necrosis surface area for lines, herbicide levels and interaction between lines and herbicide levels

Line	Herbicide(kg ha^{-1})						Line Mean[2]
	1.5	3.0	6.0	12.0	24.0	48.0	($LSD_{5\%}$=4.74)
KKD	19.67 m[1]	63.57 j	77.43 d	86.06 c	91.67 b	100.00 a	73.06 A
49-1	2.52 r	12.55 o	15.12 no	32.70 l	56.17 h	69.78 e	31.47 C
51-1	0.00 r	13.52 no	30.23 l	59.38 g	62.97 f	85.90 c	42.00 B
51-3	3.08 r	9.12 p	17.03 mn	49.68 i	68.38 fg	86.57 c	38.98 BC
51-5	1.67 r	6.85 pq	31.95 l	36.60 k	69.87 e	86.40 c	38.89 BC
51-14	2.80 r	35 83 k	76.48 d	90.62 b	92.23 b	99.78 a	66.29 A
54-18	1.42 r	2.73 r	4.08 qr	44.75 j	51.45 i	91.98 b	32.74 C
Hcide. Mean[3] ($LSD_{5\%}$=4.74)	4.45 F'	20.60 E'	36.06 D'	57.11 C'	70.39 B'	88.63 A'	46.21 (Grand mean)

[1] Leaf necrosis surface of different tobacco lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).

[2] Leaf necrosis surface of different lines. Means with the same letter are not significantly different (P=0.05).

[3] Leaf necrosis surface at different levels of herbicide. Means with the same letter are not significantly different (P=0.05).

Table 3.9. Leaf necrosis surface for lines, times and interaction between lines and times

Line	Time				Line Mean[2] ($LSD_{5\%}$=4.74)
	1	2	3	4	
KKD	62.76 c[1]	75.11 a	76.46 a	77.54 a	76.06 A
49-1	35.49 hi	33.35 ig	29.82 k	28.29 k	31.47 C
51-1	42.95 d	43.84 d	41.97 e	39.24 fg	42.00 B
51-3	41.44 e	40.34 ef	37.84 gh	36.33 ghi	38.98 BC
51-5	40.81 ef	38.84 fg	38.91 fg	36.98 ghi	38.89 BC
51-14	61.97 c	66.55 bc	67.24 bc	69.25 b	66.26 A
54-18	37.67 gh	33.64 ig	30.70 gk	28.95 k	32.74 C
Time Mean[3] (Not significant)	46.16	47.38	46.13	45.23	46.21 (Grand mean)

[1] Leaf necrosis surface of different tobacco lines at various times (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Leaf necrosis surface of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Leaf necrosis surface at different times. Means are not significant (P=0.05).

Table 3.10. Stem height (mm) for lines, herbicide levels and interaction between lines and herbicide levels

Line	Herbicide (kg ha^{-1})							Line Mean[2]
	0	1.5	3.0	6.0	12.0	24.0	48.0	($LSD_{5\%}$=8.46)
KKD	134.83 ab[1]	59.88 l	39.38 op	28.72 qr	22.20 s	22.60 s	21.40 s	47.00 C
49-1	132.73 ab	96.87 g	95.30 g	83.83 i	88.98 i	55.20 m	45.33 no	85.45 B
51-1	140.87 a	111.23 d	105.10 ef	88.50 i	60.57 l	67.22 k	34.17 pq	86.81 B
51-3	134.05 ab	106.53 ef	104.70 ef	109.60 e	72.27 *j*	61.85 l	43.02 no	90.28 B
51-5	136.78 ab	101.07 fg	108.02 e	88.98 *i*	89.93 hi	49.07 mn	38.17 p	87.42 B
51-14	130.75 ab	58.17 l	43.55 no	38.25 op	23.35 s	26.07 *r*	20.73 s	48.70 C
54-18	142.68 a	119.90 cd	129.98 b	126.48 bc	93.53 hg	86.28 i	46.48 n	106.47 A
Hcide. Mean[3] ($LSD_{5\%}$=4.46)	136.10 A'	93.38 B'	94.43 B'	80.20 C'	64.41 D'	52.62 E'	35.62 F'	78.85 (Grand mean)

[1] Stem height of different tobacco lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Stem height of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Stem height at different levels of herbicide. Means with the same letter are not significantly different (P=0.05).

Table 3.11. Stem height (mm) for lines, different times and interaction between times and lines

Line	Times				Line Mean[2] ($LSD_{5\%}$=8.46)
	1	2	3	4	
KKD	32.59 o[1]	40.22 m	50.43 ij	64.76 i	47.00 C
49-1	45.27 kl	63.53 ij	100.27 f	132.75 b	85.45 B
51-1	48.38 kl	67.93 i	103.72 e	127.20 c	86.81 B
51-3	50.84 k	70.48 h	107.16 d	132.63 bc	90.28 B
51-5	45.43 kl	67.90 ij	110.77 c	127.58 c	87.42 B
51-14	34.14 no	42.51 lm	54.91 j	63.25 ij	48.70 C
54-18	57.14 j	85.39 g	130.45 bc	152.93 a	106.47 A
Time Mean[3] ($LSD_{5\%}$=1.96)	44.83 A'	62.27 B'	93.95 C'	114.44 D'	78.85 (Grand mean)

[1] Stem height of different tobacco lines at various times (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Stem height of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Stem height at different times. Means with the same letter are not significantly different (P=0.05).

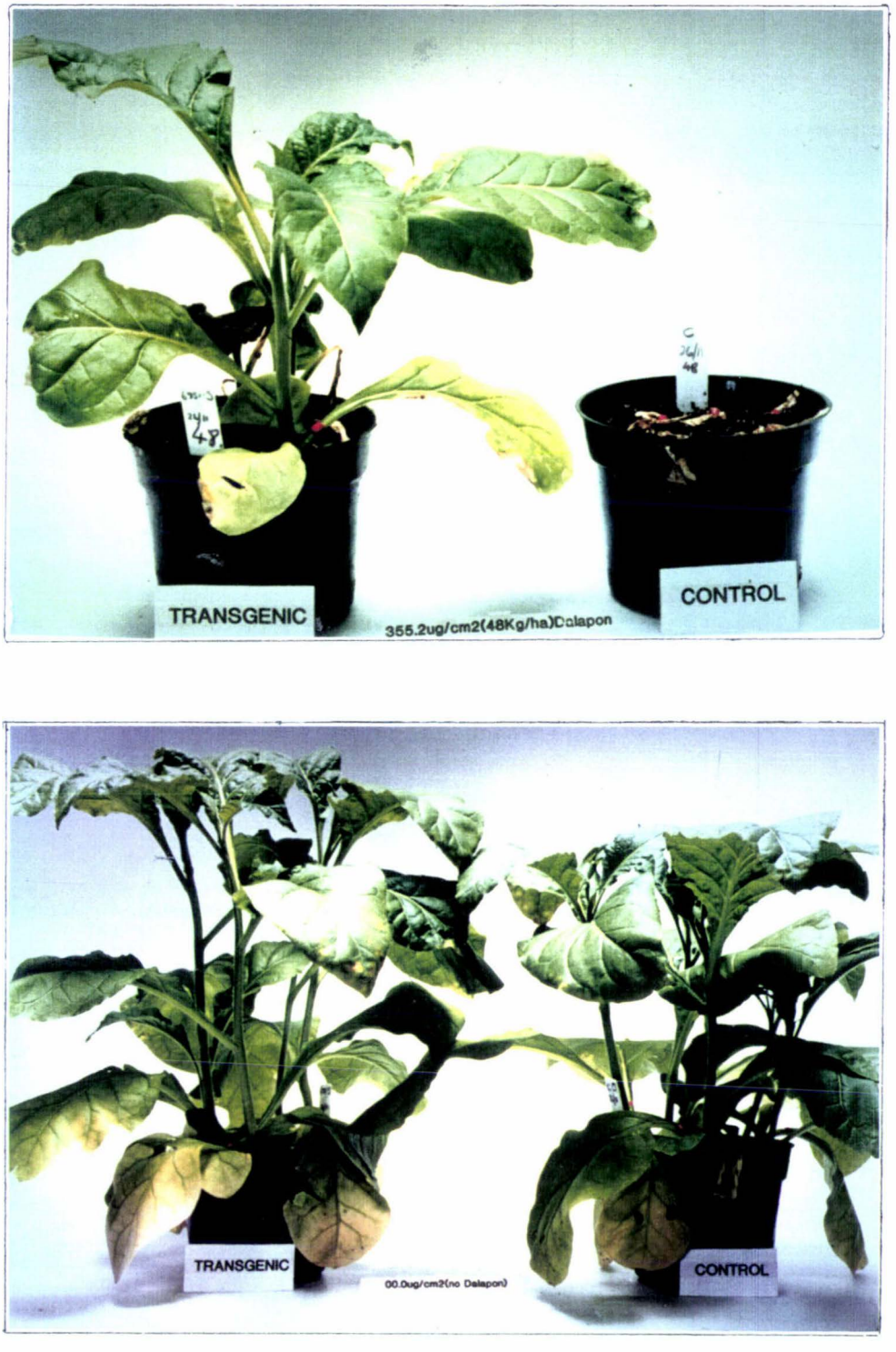


Figure 3.2. Stem height of the transgenic plants, line 54.18 (left) and non-transgenic plants, *Nicotiana.tabacum* line KKD (right) at 48 Kg ha^{-1} (top) and no herbicide application (bottom).

The analysis of variance of leaf length and leaf width characters are presented in Table 3.12.

Table 3.12. Analysis of variance of leaf length and leaf width characters

Effect	Mean Square (MS)	
	LL	LW
Block	14964.29 ns	3293.39 ns
Line	202606.92 **	46389.37 **
Herbicide	35447.82 **	10608.86 **
LinexHerbicide	5371.65 ns	1777.69 ns
error(a)	5770.15 **	1678.81 **
Within plot	2360.65 **	735.08 **
Time	24897.30 **	6574.96 **
TimexLine	235.32 *	128.90 **
TimexHerbicide	1032.09 **	287.57 **
TimexLinexHerbicide	122.09 **	47.76 **
error(b)	65.62	25.08

ns, * and ** : not significant, significant at 5% and significant at 1% probability levels respectively.

The effects of herbicide application on leaf width and leaf length attributes were considerably lower than those on necrosis and stem height, although these two characters showed similar patterns in response to the herbicide. The total leaf length and leaf width of each of the lines as well as interactions between lines and herbicide levels are presented in Tables 3.13 and 3.14 respectively.

Although the interaction effects between herbicide levels and these two characters are significant, the response of these two characters to herbicide applied at various concentrations is very low (Tables 3.13 and 3.14 respectively). This is possibly due to a low growth of the measured leaf. Furthermore, both the differences in leaf length and leaf width between the resistant transgenic lines as well as between the low resistant transgenic line (51-14) and the non-transgenic control line are very low compared with the corresponding values for stem height and necrosis level.

There is a significant increase in both leaf length and leaf width of each of the lines over time (LinexTime interaction, Table 3.15 and 3.16). Although the increases in total leaf length and width over time are significant, these increases

are small compared with the increase and stem height. As an example, leaf length and leaf width increase by only 11% and 8% respectively over time (compare time 1 and time 4, Tables 3.15 and 3.16). In contrast, stem height increases by 269% over the same period (Table 3.10). Furthermore, there are no significant differences between the 0, 1.5, 3.0 and 6.0 kg ha^{-1} levels of herbicide nor between the 12.0, 24.0 and 48.0 kg ha^{-1} levels of herbicide for leaf length.

Table 3.13. Leaf length (mm) for lines, different levels of herbicide and interaction between lines and levels of herbicide

Line	Herbicide (kg ha^{-1})							Line Mean[2] ($LSD_{5\%}$=10.41)
	0	1.5	3.0	6.0	12.0	24.0	48.0	
KKD	136.45 g[1]	113.65 m	111.48 m	96.92 p	87.32 q	89.05 q	80.r	102.28 C
49-1	149.50 c	143.63 d	140.63 ef	130.20 hi	135.45 gh	135.26 gh	122.00 j	136.68 B
51-1	153.98 bc	149.98 c	144.52 d	149.68 c	137.80 fg	148.53 c	115.87 I	143.33 A
51-3	135.65 gh	162.90 a	154.30 b	163.18 a	129.30 i	133.78 h	135.80gh	144.98 A
51-5	142.55 de	136.25 g	139.57 f	136.53 g	142.58 de	123.58 j	120.07 k	134.45 B
51-14	102.03 o	101.17 o	100.53 o	107.00 n	71.00 s	85.56 q	87.10 q	93.48 C
54-18	151.43 c	154.52 b	151.27 c	155.20 b	149.47 c	140.45 ef	137.20 fg	148.54 A
Hcide. Mean[3] ($LSD_{5\%}$=10.41)	138.84 A'	137.44 A'	134.61 A'	141.10 B'	121.85 B'	120.19 B'	114.01 B'	129.39 (Grand mean)

[1] Leaf length of different tobacco lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Leaf length of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Leaf length at different levels of herbicide. Means with the same letter are not significantly different (P=0.05).

Table 3.14. Leaf width for lines, different levels of herbicide and interaction between lines and levels of herbicide

Line	Herbicide (kg ha^{-1})							Line Mean[2]
	0	1.5	3.0	6.0	12.0	24.0	48.0	($LSD_{5\%}$=5.61)
KKD	94.78 de[1]	86.67 hi	77.70 n	65.90 q	65.45 q	62.63 r	60.73 r	73.41 B'
49-1	93.23 e	92.30 ef	92.48 ef	84.18 i	84.01 i	81.68 l	83.70 ik	87.37 A'
51-1	95.45 c	95.35 cd	94.46 cde	95.55 cd	85.87 i	86.07 i	77.00 n	89.96 A'
51-3	87 58 gh	97.13 b	93.65 de	95.03 cd	84.95 i	86.78 hı	88.00 gh	90.45 A'
51-5	87.47 gh	86.67 hı	88.26 g	85.92 f	78.96 mn	82.52 chile	69.42 p	82.75 A'
51-14	69.42 p	65.77 q	69.47 p	71.15 p	53.42 s	65.08 q	63.60 q	64.42 B'
54-18	94.90 cde	97.97 ab	96.55 bc	95.77 c	96.35 bc	88.89 fg	90.80 f	94.46 A'
Hcide. Mean[3] ($LSD_{5\%}$=5.61)	88.98 A'	88.83 A'	87.51 A'	84.78 A'B'	78.43 B'	79.09 B'	76.19 B'	83.26 (Grand mean)

[1] Leaf length of different tobacco lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Leaf length of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Leaf length at different times. Means with the same letter are not significantly different (P=0.05).

Table 3.15. Leaf length (mm) for lines, different times and interaction between lines and times

Line	Time				Line Mean[2]
	1	2	3	4	($LSD_{5\%}$=10.41)
KKD	93.52 lm[1]	100.45 k	105.67 j	109.37 i	102.28 C
49-1	127.06 h	136.24 f	140.14 de	143.24 c	136.68 B
51-1	133.78 g	141.12 cd	147.54 d	150.27 bc	143.33 A
51-3	137.23 f	143.82 c	148.46 cd	150.44 bc	144.98 A
51-5	128.45 h	133.82 g	137.90 ef	137.78 f	134.45 B
51-14	89.06 n	92.15 m	95.71 l	97.15 l	93.48 C
54-18	140.72 d	147.88 d	151.80 ab	153.62 a	148.54 A
Time Mean[3] ($LSD_{5\%}$=0.85)	121.40 D'	127.94 C'	132.47 B'	134.52 A'	129.39 (Grand mean)

[1] Leaf length of different tobacco lines at various times (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Leaf length of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Leaf length at different times. Means with the same letter are not significantly different (P=0.05).

Table 3.16. Leaf width (mm) for lines, different times and interaction between lines and times

Line	Time				Line Mean[2] ($LSD_{5\%}$=5.61)
	1	2	3	4	
KKD	68.01 0[1]	71.14 n	75.57 m	78.91 l	73.41 B
49-1	83.56 jk	87.42 h	89.20 ef	92.30 cd	87.37 A
51-1	85.61 ij	89.13 fg	91.92 cd	93.08 bc	89.96 A
51-3	86.68 hi	90.12 ef	91.83 cd	93.16 b	90.45 A
51-5	71.23 n	84.75 j	87.49 h	87.81 gh	82.75 A
51-14	63.23 q	64.87 p	66.30 o	67.26 o	64.42 B
54-18	91.33 de	93.87 b	95.96 a	96.68 a	94.46 A
Time Mean[3] ($LSD_{5\%}$=0.51)	78.52 D'	83.04 C'	85.47 B'	87.03 A'	83.26 (Grand mean)

[1] Leaf width of different tobacco lines at various times (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Leaf width of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Leaf width at different times. Means with the same letter are not significantly different (P=0.05).

The drymass value was determined after the plants were harvested and oven dried *en mass*. Drymass is a time independent character since only one value was determined for this character, as described in Materials and Methods. The analysis of variance of the drymass data is presented in Table 3.17. There are significant differences ($P<0.001$) between the total drymass of different lines (Table 3.18). The effect of applied herbicide at various concentrations on the drymass of each line is presented in Table 3.18. The drymass values indicate a similar pattern of response to the herbicide to that of stem height, suggesting that there is a correlation between the stem height and drymass characters.

Table 3.17. Analysis of variance of drymass character

Effect	Mean Square (MS)
Block	1.873 ns
Line	37.757 **
Herbicide	87.213 **
LinexHerbicide	9.879 *
error	1.354
Within plot	1.490

ns * and ** : not significant and significant at 5% and significant at 1% probability level respectively.

Table 3.18. Drymass (g) for lines, different levels of herbicide and interaction between lines and levels of herbicide

Line	Herbicide (kg ha^{-1})							Line Mean[2]
	0	1.5	3.0	6.0	12.0	24.0	48.0	($LSD_{5\%}$=0.33)
KKD	4.30 a[1]	2.47 efghi	1.40 lmno	0.81 nopq	0.45 pq	0.30 q	0.29 q	1.43 D
49-1	3.14 bcdef	2.81 defg	2.64 defghi	2.23 ghijkl	2.40 fghij	1.77 ijklm	1.28 mnop	2.32 AB
51-1	2.52 efghij	2.55 efghij	2.25 ghijkl	2.05 hijklm	1.53 klmn	1.64 klmn	0.64 opq	1.88 BC
51-3	3.70 abc	3.30 abcde	2.69 defgh	2.66 defgh	1.72 hjkl	1.45 lmn	0.70 opq	2.32 AB
51-5	3.21 bcdef	2.95 cdefg	3.11 bcdefg	2.06 hijklm	1.96 hijkl	1.29 mno	0.71 opq	2.18 B
51-14	2.26 ghijkl	1.80 ijklm	1.49 lmn	0.76 nop	0.32 q	0.23 q	0.11 q	1.00 E
54-18	3.87 ab	3.41 bcd	3.40 bcd	2.92 cdefg	2.82 defg	2.133 ghijkl	0.75 nopq	2.70 A
Hcide. Mean[3] ($LSD_{5\%}$=0.33)	3.29 A'	2.70 B'	2.43 B'	1.93 C'	1.60 D'	1.26 E'	0.64 F'	1.98 (Grand mean)

[1] Drymass of different tobacco lines at various levels of herbicide (interaction). Means with the same letter are not significantly different (P=0.05).
[2] Drymass of different lines. Means with the same letter are not significantly different (P=0.05).
[3] Drymass at different levels of herbicide. Means with the same letter are not significantly different (P=0.05).

The total phenotypic correlations between the leaf necrosis, stem height, leaf length, leaf width and drymass characters are presented below the leading diagonal of Table 3.19. The partial correlations between these characters are given above the leading diagonal of Table 3.19. The total correlation between leaf necrosis and the other characters is negative, since an increase in herbicide concentration led to an increase in the level of necrosis but a decrease in stem height, leaf length, leaf width and drymass. The total correlation between the leaf length and leaf width characters is high. Since both character were measured on the same leaf, the relationship between these two characters may be the cause of this high correlation. The correlations between leaf necrosis and stem height, leaf necrosis and drymass, and drymass and stem height, irrespective of their signs, are medium. However, the correlations between leaf length and leaf width and the other characters are at the lowest level, indicating a lack of correlation between each of these characters.

The partial phenotypic correlation (Table 3.19), which is a correlation between the characters excluding other characters' effects, decreases significantly for correlations between some of the characters. The partial phenotypic correlation between the leaf length and leaf width characters is high, while the correlations between these two characters and the other characters deceases significantly (approximately 0). The partial correlation for stem height and leaf necrosis is -0.601 and for stem height and drymass it is 0.486, indicating a medium correlation between these two characters.

On the basis of the values of partial phenotypic correlation, the characters can be categorised into three categories: characters with a high partial phenotypic correlation consisted of LL and LW; characters with a high to medium to partial correlation consisted of SH and NC, and Dm and SH; and the other partial correlations between some characters with a zero or very low values (Table 3.19)

Table 3.19. Phenotypic correlations between characters. Total phenotypic correlation (below leading diagonal) and partial phenotypic correlation (above leading diagonal) between different characters for tobacco lines

	NC	SH	LL	LW	DM
NC	**1.000**	-0.601	0.065	0.022	-0.150
SH	-0.828	**1.000**	0.022	0.176	0.486
LL	-0.419	0.576	**1.000**	0.825	0.098
LW	-0.461	0.619	0.890	**1.000**	0.005
DM	-0.721	0.821	0.541	0.561	**1.000**

3.3.2.4 Heritability of different characters in transgenic tobacco plants

The heritability of leaf necrosis at various levels of applied herbicide is presented in Table 3.20. The population heritabilities increase slightly from 1.5 to 3.0 kg of herbicide ha^{-1}, and then decrease with increasing levels of herbicide. The minimum population heritability is at 48.0 kg of herbicide ha^{-1}. In contrast, the plant heritability shows a slight decrease with increasing levels of herbicide, from 1.5 to 6.0 kg ha^{-1}, and then an increase up to 48.0 kg ha^{-1} of herbicide. The broadsense heritability decreases with increasing levels of herbicide. This result indicates that the population heritability and plant heritability show different (inverse) reactions to increased levels of applied herbicide. For example, the population heritability decreases with increasing levels of herbicide, especially at higher levels of the herbicide. In contrast, the plant heritability decreases at lower levels of herbicide and then increases with increasing levels of the herbicide.

These results indicate that phenotypic expression of the character changed across varying concentrations of herbicide. The broadsense heritability values indicate that genotypic capability of the character reduces significantly as the concentration of herbicide increases.

Table 3.20. Population, plant and broadsense heritabilities (h^2) of the necrosis character at various levels of herbicide

Herbicide (kg ha^{-1})	$h^2 \pm$ s.e.		
	Population	Plant	Broadsense
1.5	0.585±0.1456 **	0.284±0.1031 **	0.865±0.0789 **
3.0	0.609±0.1489 **	0.253±0.1001 *	0.861±0.0625 **
6.0	0.552±0.1609 **	0.253±0.0612 **	0.803±0.1211 **
12.0	0.388±0.1511 **	0.392±0.1023 **	0.730±0.0900 **
24.0	0.311±0.1432 **	0.410±0.1245 *	0.721±0.0860 **
48.0	0.041±0.0623 **	0.497±0.0978 **	0.538±0.0917 **
Pooled h^2	0.398±0.1530 **	0.3253±0.1092 **	0.760±0.1900 **

* and ** : significant at 5% and significant at 1% probability level respectively.

The population, plant and broadsense heritabilities of stem height are presented in Table 3.21. The heritability of stem height is considerably lower than that of leaf necrosis, indicating that expression of this character is significantly lower than the necrosis character. The population, plant and broadsense heritabilities of stem height at various levels of herbicide (Table 3.21) indicate a pattern similar to those of leaf necrosis (Table 3.20).

The broadsense heritability shows a low variation at herbicide levels of 3.0 to 24.0 kg ha^{-1}, but at 48.0 kg ha^{-1} the heritability decreases significantly. The low heritability of stem height results from a significant effect of the time component of variation, which increases the phenotypic variance, leading to a decrease in heritability. The heritability estimates also indicate that the population heritability and the plant heritability react in an inverse manner at higher herbicide concentrations. Another important point is the low broadsense heritability at both low and high levels of applied herbicide (Table 3.21), such that the genotypic variation is low. It is suggested that, at lower levels of herbicide, plants show a low genotypic variation compared with the phenotypic variation, since the effect of the herbicide is low and there is least variation between the resistant and susceptible plants. At higher concentrations of herbicide, both resistant and susceptible plants are significantly affected by the herbicide, resulting in genotypic similarity between the susceptible and resistant plants (all resistant and susceptible lines highly affected by herbicide). The broadsense heritability of the stem height character also indicates that the phenotypic expression of this character changed by varying the level of herbicide.

Table 3.21. Population, plant and broadsense heritabilities (h^2) of stem height at various levels of herbicide

Herbicide (kg ha^{-1})	$h^2 \pm$ s.e.		
	Population	Plant	Broadsense
1.5	0 078±0 0600 *	0.054±0 0318 **	0.133±0.0786 **
3.0	0.192±0.1198 *	0.153±0.0612 **	0 345±0.1367 **
6.0	0.203±0.1223 *	0 153±0.0555 **	0.357±0.1129 **
12.0	0.207±0.1245 *	0.154±0 0528 **	0.362±0.1167 **
24.0	0.230±0.1312 *	0.219±0 0650 **	0.449±0.1201 **
48.0	0.078±0.0505 *	0.272±0.0735 **	0.350±0.0819 **
Pooled h^2	0.188±0.1201 *	0.172±0.0635 **	0.398±0.1457 **

* and ** : significant at 5% and significant at 1% probability level respectively.

The population, plant and broadsense heritabilities of leaf length are presented in Table 3.22. The population heritability decreased slightly at 3.0 kg of herbicide ha^{-1}, and then started to increase with increasing levels of the herbicide. The highest population heritability was at both 24.0 and 48.0 kg ha^{-1} of applied herbicide.

The plant heritability leaf increased with increasing levels of applied herbicide up to 6.0 kg ha^{-1}, and then decreased with increasing herbicide levels. The lowest plant heritability observed was at 48.0 kg of herbicide ha^{-1}.

The broadsense heritability increased up to 6.0 kg of herbicide ha^{-1} and then started to decrease with increasing levels of herbicide. However the variation in broadsense heritability was very low, and a consistency in broadsense heritabilities at different concentrations of herbicide was apparent. The heritabilities (population, plant and broadsense) of leaf length were greater than those of both stem height and necrosis.

The heritability estimates indicate that the population heritability and the plant heritability of leaf length, in response to applied herbicide, react in an inverse manner to those of necrosis and stem height. For example, with leaf length the population heritability is higher than that of plant heritability at higher levels of herbicide, while at lower levels of herbicide, plant heritability is higher than that of population heritability.

Table 3.22. Population, plant and broadsense heritabilities (h_2) of leaf length at various levels of herbicide

Herbicide (kg ha^{-1})	$h^2 \pm$ s.e.		
	Population	Plant	Broadsense
1.5	0.263±0.1319 **	0.331±0.0920 **	0.594±0.1234 **
3.0	0.220±0.1067 **	0.448±0.1024 **	0.667±0.1171 **
6.0	0.264±0.1289 **	0.548±0.1290 **	0.812±0.0812 **
12.0	0.370±0.1423 **	0.390±0.1167 **	0.760±0.0910 **
24.0	0.443±0.1556 **	0.328±0.1151 **	0.771±0.1034 **
48.0	0.449±0.1402 **	0.287±0.1034 **	0.736±0.1382 **
Pooled h^2	0.310±0.1293 **	0.339±0.1223 **	0.701±0.1498 **

** : significant at 1% probability level.

The population, plant and broadsense heritabilities of leaf width are presented in Table 3.23. These results indicate a pattern similar to that of leaf length (Table 3.22).

As described in experimental methods, drymass was a time independent character and there were no time measurements for this character. Heritabilities of drymass for population, plant and broadsense are 0.470±0.1821, 0.423±0.1268 and 0.893±0.1608 respectively. As can be seen from these values, the population heritability was slightly higher than the plant heritability. The broadsense heritability was considerably larger, suggesting a greater heritability of this character in response to the herbicide.

Table 3.23. Population, plant and broadsense heritabilities (h^2) of leaf width at various levels of herbicide

Herbicide (kg ha^{-1})	$h^2 \pm$ s.e.		
	Population	Plant	Broadsense
1.5	0.234±0.1201 *	0.324±0.0808 **	0.557±0.1245 **
3.0	0.265±0.1498 *	0.417±0.1219 **	0.782±0.1016 **
6.0	0.192±0.1100 *	0.468±0.1082 **	0.660±0.1004 **
12.0	0.236±0.1189 *	0.531±0.1119 **	0.766±0.1015 **
24.0	0.245±0.1203 *	0.544±0.1155 **	0.789±0.0749 **
48.0	0.140±0.1389 ns	0.591±0.2257 **	0.731±0.2233 **
Pooled h^2	0.212±0.113 *	0.485±0.113 **	0.693±0.143 **

ns, * and ** : not-significant, significant at 5% and significant at 1% probability level respectively.

In summary, the results indicate that changes in heritability occur with changes in the concentration of the herbicide. The population and plant heritabilities exhibit inverse responses to increasing concentrations of the herbicide. The high broadsense heritability for all of the characters suggests that the herbicide resistant phenotype will be expressed in subsequent generations.

3.3.2.5 Inheritance of the dalapon resistance phenotype

In relation to the genetic background of the transgenic plants developed from tissue culture (R_0), it must be noted that such plants in most cases have a hemizygous genetic structure (Mouras *et al.,* 1987). As a result, the plants originating from selfed, hemizygous plants (R_1) are undergoing genetic segregation and the presence of all of the possible genotypes (homozygous, hemizygous and segregant plants lacking the transgene) is possible. After applying herbicide onto transgenic tobacco lines derived from selfed transgenic lines (49-1, 51-1, 51-3, 51-5, 51-14 and 54-18), the segregant plants were recognized phenotypically and quantitatively (see Section 3.2.5).

In phenotypic studies of segregant plants, two phenotypes were identified at low (1.5 and 3.0 kg ha^{-1}) and high rates of applied herbicide as follows:

1. At a low concentration, susceptible (negative) plants were phenotypically similar to the control, non-transgenic control plants. Application of herbicide led to the following symptoms in the leaves of these plants; the leaves became concave with respect to the upper part of the leaf and the colour of the leaf lamina changed to yellow, only the area alongside the leaf ribs remaining green. However, in plants with the resistant phenotype, these symptoms were not observed.
2. At high concentrations of herbicide, in plants with the susceptible phenotype, the leaf laminae became covered with necrotic lesions and the plants quickly died. Resistant plants continued to grow at the same concentration of herbicide. These symptoms for susceptible (negative) phenotypes were similar to those of the non-transgenic control line. Owing to the similarity of the plants with susceptible phenotypes within the selfed transgenic lines to the non-transgenic control line, it was concluded that the susceptible

phenotypes were negative plants which lacked the T-DNA and thus the dehalogenase gene.

As outlined in Materials and Methods (Section 3.2.5) the inheritance studies were carried out based on both the pooled segregation analysis (pooled over all levels of herbicide), and separately at each level of dalapon as discussed below.

3.3.2.5.1 Inheritance of the dalapon resistance phenotype based on pooled analysis

In quantitative studies of segregant plants, individual susceptible plants within each transgenic line were recognized on the basis of the level of leaf necrosis for each level of herbicide and for each of the lines. Thus at 1.5 kg of herbicide ha^{-1}, plants with more than 6% of necrosis were recorded as susceptible plants. For 3.0, 6.0, 12.0, 24.0 and 48.0 kg ha^{-1} of herbicide, plants with more than 10, 25, 40, 50, and 65% of necrosis respectively were recorded as susceptible plants (see Table 3.8 and compare the level of necrosis of control line and transgenic lines). The data summarized in Table 3.24 indicate that these tobacco lines, except 51-14, contain a single segregating T-DNA locus. A chi-square (χ^2) test of the number of locus in the progeny of the transgenic lines is consistent (except for the 51-14 line) with the hypothesis that there is one active locus of the dehalogenase in these plants. Table 3.24 summarises the results from the 6 transgenic tobacco lines. The ratios show the number of susceptible to resistant plants for each line with the 3:1 (resistant:susceptible) expected ratio for each line. The χ^2 results indicate the validity of the 3:1 ratio and the hypothesis of single locus inheritance (except the 51-14 line) for the transgenic lines.

It can be concluded therefore, that in pooled analysis the progeny of a majority of the transgenic lines produce segregation ratios which indicate that the dalapon resistance phenotype is inherited in a Mendelian fashion and that a single locus is involved. It is likely that the low herbicide resistance of the 51-14 line indicates a phenotypic similarity between the susceptible as well the low resistant transgenic genotype within the population.

The χ^2 test result, suggests that the R_1 plants derived from selfed seeds of the original transgenic plants have three genotypic structures as follows:

1. Homozygous transgenic plants (both of the homologous chromosomes contain the T-DNA copy or copies),
2. Hemizygous transgenic plants (only one of the homologous chromosomes containing the T-DNA) and,
3. Homozygous segregant plants (no T-DNA integrated in the genome).

Table 3.24. Progeny analysis of the dalapon resistance phenotype of transgenic tobacco lines based on phenotypic and quantitative studies

Line	df	Segregation ratios (resistance:susceptible)	χ^2 value (adjusted)	Probability
49-1	1	66:24	0.059	0.750<P<0.900ns
51-1	1	69:21	0.059	0.750<P<0.900ns
51-3	1	65:25	0.237	0.500<P<0.750ns
51-5	1	70:20	0.237	0.500<P<0.750ns
51-14	1	26:64	99.615	P<<0.005***
54-18	1	64:26	0.533	0.250<P<0.500ns

ns and *** : not significant and significant at 0.001 probability level respectively.

3.3.2.5.2 Inheritance of the dalapon resistance phenotype based on each level of herbicide

The χ^2 test of homogeneity for 3:1 (resistant:susceptible) ratio over all levels of herbicide for each transgenic line indicates that the samples at each level of herbicide are homogenous (except for 51-14 line, last column of Table 3.15). Thus pooling the samples and testing for 3:1 (resistant:susceptible) ratio over all levels of herbicide is appropriate. However, the heterogeneity of pooled χ^2 for the line 51-14 indicates a heterogeneity of segregation for various levels herbicide. Since the heterogeneity of χ^2 for levels of herbicide observed in 51-14 line, the inheritance of transgene was studied separately for each level of herbicide as follows:

The observed resistant:susceptible ratio for different transgenic lines at various level of herbicide together with the χ^2 test results for validity of 3:1 ratio (resistant:susceptible) is summarized in Table 3.25. The χ^2 test results indicate the validity of the 3:1 (resistant:susceptible) ratio and the hypothesis of single locus inheritance for the transgenic lines at various levels of herbicide (except 51-14 line

at levels of herbicide greater than 3.0 kg ha^{-1}). For the transgenic line 51-14, the 3:1 (resistant:susceptible) ratio and the hypothesis of single locus inheritance is valid only for herbicide levels of 1.5 and 3.0 kg ha^{-1}. At concentrations higher than the 3.0 kg ha^{-1}, the number of the plants with susceptible phenotype increased significantly and at higher concentrations, 51-14 line behaved as the control, non-transgenic plants. It can be interpreted that 3.0 kg herbicide ha^{-1} is a threshold for this line. At concentrations greater than 3.0 kg ha^{-1}, this line has a significant χ^2 value which demonstrates that the 3:1 of resistance to susceptible plants is no longer valid. However, for the other resistant transgenic lines, the threshold is greater than 48.0 kg of herbicide ha^{-1}, since these lines have resistant phenotypes with the expected 3:1 ratio up to highest concentrations of herbicide (Table 3.25).

Table 3.25. Progeny analysis of the dalapon resistance phenotype of transgenic tobacco lines based on phenotypic and quantitative studies for each line at each level of herbicide. Number of resistant:susceptible plants for each line and at each level of herbicide and probability for 3:1 ratio (within bracket) as well as homogeneity test of pooled χ^2 over all herbicide levels (last column)

Line	Herbicide (kg ha^{-1})						Homogeneity test of Pooled χ^2 (df=5)
	1.5	3.0	6.0	12.0	24.0	48.0	
49-1	12:3[1]	10:5	11:4	13:2	11:4	9:6	
	0.022[2]	0.200	0.022	0.555	0.022	1.088	**3.556**
	(0.90<P<0.95ns)[3]	(0.50<P<0.75 ns)	(0.90<P<0.95 ns)	(0.50<P<0.25 ns)	(0.90<P<0.95 ns)	(0.25<P<0.50 ns)	**(0.50<P<0.75 ns)**
51-1	13:2	10:5	11:4	11:4	13:2	11:4	
	0.555	0.200	0.022	0.022	0.555	0.022	**2.667**
	(0.25<P<0.50 ns)	(0.50<P<0.75 ns)	(0.90<P<0.95 ns)	(0.90<P<0.95 ns)	(0.25<P<0.50 ns)	(0.90<P<0.95 ns)	**(P=0.75 ns)**
51-3	10:5	13:2	9:6	12:3	10:5	13:2	
	0.200	0.555	1.088	0.022	0.200	0.555	**5.274**
	(0.50<P<0.75 ns)	(0.25<P<0.50 ns)	(0.25<P<0.50 ns)	(0.90<P<0.95 ns)	(0.50<P<0.75 ns)	(0.50<P<0.25 ns)	**(0.25<P<0.50 ns)**
51-5	12:3	12:3	11:4	13:2	9:6	11:4	
	0.022	0.022	0.022	0.555	1.088	0.022	**3.230**
	(0.90<P<0.95 ns)	(0.90<P<0.95 ns)	(0.90<P<0.95 ns)	(0.25<P<0.50 ns)	(0.25<P<0.50 ns)	(0.90<P<0.95 ns)	**(0.50<P<0.75 ns)**
51-14	10:5	9:6	5:10	2:13	0:15	0:15	
	0.200	1.088	11.755	27.222	41.088	41.088	**34.607**
	(0.50<P<0.75 ns)	(0.25<P<0.50 ns)	(P<0.005 ***)	(P<0.001 ***)	(P<0.001 ***)	(P<0.001 ***)	**(P<0.001 ***)**
54-18	9:6	13:2	11:4	10:5	11:4	10:5	
	1.088	0.555	0.022	0.200	0.022	0.200	**3.319**
	(0.25<P<0.50 ns)	(0.25<P<0.50 ns)	(0.90<P<0.95 ns)	(0.50<P<0.75 ns)	(0.90<P<0.95 ns)	(0.50<P<0.75)	**(0.50<P<0.75 ns)**

[1] : Resistant:susceptible phenotype. [2] : χ^2 values for 3:1 ratio (df=1). [3] : Probabilities based on adjusted χ^2.
ns and ***: not significant and significant at 0.001 probability level respectively.

3.4 Discussion

3.4.1 Transgenic white clover

3.4.1.1 Herbicide resistance of *In vitro*-grown transgenic white clover callus

White clover callus growth-gain decreased with increasing concentrations of dalapon in the growth medium. In response to the herbicide, callus growth-gain of the non-transgenic lines was suppressed sharply compared to that of the transgenic lines. Although the growth of the transgenic lines continued at 400 mg L^{-1} of dalapon, the non-transgenic line was unable to grow at a concentration of herbicide greater than 200 mg L^{-1}. Comparing callus growth-gain of transgenic and non-transgenic lines at different levels of herbicide, it is concluded that the herbicide is also absorbed by callus (undifferentiated) cells. Furthermore, it can be concluded that the dehalogenase gene is expressed in the callus cells and facilitates growth of these cells in the presence of the herbicide through decomposition of the herbicide. These results indicate that the measured phenotype of callus growth-gain for each of the transgenic white clover callus lnes changes according to the level of herbicide in the culture medium. Thus, an increase in concentration of herbicide leads to a decrease in callus growth-gain.

There was some variation between the transgenic lines both in absence and presence of herbicide. The variation between the transgenic lines (Table 3.2) in response to the herbicide is probably a result of variation in the level of dehalogenase gene expression and/or somaclonal variation. The variation between the transgenic lines in the absence of herbicide may be a result of somaclonal variation alone. In relation to the sources of somaclonal variation, the stresses induced by tissue culture, transformation and selection agent may lead to somaclonal variation. The somaclonal variation appears to be triggered by the process of *in vitro* culture (Larkin, 1981; 1984), particularly when cell culture remained over a prolonged period as undifferentiated cells (Walbot and Cullis, 1985). Hence, under these circumstances, chromosomal mutations occur at a very high rate. Moreover, the combination of a few stresses may result in a much more dramatic effect. The frequency of the somaclonal variation in plants such as *Arabidopsis thaliana*, induced by tissue culture effects is very high (van Lijsebetten *et al.,* 1991; Errampali *et al.,* 1991). Using the *Agrobacterium*-mediated transformation method, mutations can be caused by T-DNA insertion into the plant genome (insertional mutation). Furthermore somaclonal variation in some plants

such as maize could be accounted for by mobilization of transposable elements due to the conditions in cell culture (Peschke *et al.*, 1986).

Dalapon inhibits the biosynthesis of pantothenate (Hilton *et al.*, 1959) during plant growth. The herbicide also can cause conformational changes in plant proteins which could affect different metabolic pathways in the plants (Buchanan-Wollaston *et al.*, (1992). The dehalogenase gene encodes an enzyme that breaks down the herbicide dalapon. Transgenic white clover callus lines transformed with the dehalogenase gene are resistant to levels of dalapon in the growth medium of up to 400 mg L^{-1}. The dehalogenase gene therefore could be used as a selectable marker to obtain transgenic white clover calli by the addition of 300-400 mg L^{-1} of dalapon in the callus growing medium.

3.4.1.2 Herbicide resistance of transgenic white clover plants

The responses of the non-transgenic, control white clover and the transgenic white clover lines to the herbicide were different. Although the differences in necrosis levels between the transgenic and non-transgenic white clover lines were significant, the levels of resistance of the transgenic white clover plants were not high. Levels of 6.0 and 12 kg ha^{-1} of applied herbicide led to an increase of 24.73 and 63.79 % in necrosis levels respectively for the transgenic white clover line (Table 3.3). Although the highest level of herbicide resistance of the transgenic white clover line was not determined (due to a limitation in the number of plants), it is suggested that the level of resistance of the transgenic line is not as high as those of the resistant tobacco lines. The effect of the herbicide on white clover took significantly longer to become apparent than that on tobacco plants. The growth of the white clover was also slower than that of the tobacco plants. It is suggested that the growth activity of the plant is correlated with the speed of action of the herbicide, plants with more growth activity being more susceptible than plants with less growth activity. To study the relationships between the physiological activity of the plants and herbicide effects, Andersen *et al.* (1962) applied the sodium salt of ^{14}C-labelled dalapon onto leaves and roots of sugar beet and yellow foxtail and showed by autoradiography that the dalapon entered through the leaves and roots and moved throughout the plants, accumulating to a high level in the younger, actively growing, tissues of both species.

3.4.2 Transgenic tobacco

3.4.2.1 *In vitro*-grown tobacco seedling fresh weight

As indicated in Results, the fresh weight of tobacco lines decreased with increasing concentrations of herbicide in the culture medium. As explained above, the herbicide was added to the culture medium and seedlings were transferred from herbicide-free culture medium into medium containing herbicide. Thus the herbicide must be absorbed through the roots and transferred into the aerial parts of the seedlings where it induces physiological effects on the tobacco seedlings. This suggestion is in agreement with the results of Andersen *et al.* (1962), where the movement of dalapon inside the yellow foxtail and sugar beet plants using ^{14}C-labelled dalapon was followed. Results of the present study also show that dalapon can be absorbed through the roots of tobacco seedlings and subsequently suppresses growth. This growth suppression is possibly induced by the inhibitory effect of dalapon on biosynthesis of pantothenate, as discussed in Section 3.4.1. The response of seedlings of the different tobacco lines to different herbicide concentrations was not the same and there were significant differences between these lines. As a comparison, the non-transgenic line KKD showed the highest susceptibility to the herbicide of the seven lines. At the highest concentration of herbicide (500 mg L^{-1}), this line died soon after transferring the seedlings from dalapon-free growth medium into medium containing the herbicide. In addition, at lower concentrations of the herbicide, significant differences were observed between the non-transgenic line and the transgenic lines. The response of the transgenic lines to the herbicide was not the same and there were significant differences between transgenic lines with respect to their responses to different herbicide concentrations. The evidence from other plants (Andersen *et al.,* 1962) indicates that the herbicide is absorbed equally by both resistant and susceptible seedlings, but the responses of the tobacco seedlings to the absorbed herbicide are significantly different. These results indicate that the activity of the dehalogenase enzyme in the transgenic lines facilitates inactivation of the herbicide following absorbtion through the roots.

With respect to the mode of activity of the dehalogenase enzyme, Slater *et al.* (1979) reported that the enzyme was able to liberate the chlorine ion from 2,2-dichloropropionic acid (dalapon) and the resulting dechlorinated compound was

not toxic. There are several microorganisms which are able to decompose dalapon through an intrinsic dehalogenase enzyme activity (Weightman *et al.*, 1982; Wyndham and Slater, 1986; Berry *et al.*, 1979). In the present study, a significantly high level of herbicide resistance in transgenic lines compared to the non-transgenic, control line, indicates that the dehalogenase gene is functional in the plants. In relation to the dehalogenase gene activity in plants, Buchanan-Wollaston *et al.* (1992) transferred the dehalogenase gene into *Nicotiana plumbaginifolia* using *Agrobacterium*-mediated transformation. These transgenic plants showed resistance to dalapon. It is also suggested that differences amongst the transgenic plants in their responses to the herbicide depend upon the relative levels of dehalogenase gene expression in these plants. This suggestion is in agreement with the results of Hobbs *et al.* (1990; 1993), who reported a considerable variation in the level of expression of a *uid*A transgene within genetically transformed tobacco plants. In the present study, at the highest concentration of the herbicide, fresh weight of the transgenic line number 51-1 at 500 mg of herbicide L^{-1} was three times that of the fresh weight of the low resistant transgenic line 51-14 (Table 3.5).

The fresh weight of different tobacco lines (Table 3.5), as a measured phenotype, indicates that the phenotype of the transgenic lines changes with an increase in the concentration of herbicide in the culture media. At higher levels of herbicide, the transgenic lines showed the low resistant phenotype in response to the herbicide. Furthermore, the transgenic lines showed significantly different phenotypes in response to the herbicide, as indicated by the line and herbicide means, and the significance of interaction between line and herbicide (Table 3.5).

3.4.2.2 Heritability of herbicide resistance in *in vitro*-grown transgenic tobacco seedlings

The heritability of fresh weight of *in vitro*-grown tobacco seedlings was significantly low (0.106). It is suggested that at the early growth stage of the plants, expression of the transgene is low and under the effect of environmental factors. Since low heritability means low genetic variability in seedlings, which indicates that the dalapon resistance is poorly expressed at this stage. The population heritability for the fresh weight character was the lowest heritability compared to that of the

developed tobacco plants. The significant difference between the full heritability of seedling fresh weight and the heritability of developed plants suggests that the herbicide has a greater effect on seedlings than on developed plants. On the other hand, the environmental factors are more effective than the gene effect at the early plant growth stage.

3.4.2.3 Transgenic tobacco plants

Based on the chi-square results, the inheritance of dalapon resistance for a single locus in all transgenic lines (except 51-14 line at high concentrations of herbicide) was observed. Hence, the population structure of the majority of the transgenic lines must be under segregation and therefore three genotype could be included in the plant population of the transgenic lines: homozygous plants containing the T-DNA in both homologous chromosomes plus hemizygous plants containing T-DNA in one of the homologous chromosomes; and susceptible (negative) plants which lack the transgene (about 25% of population).

Approximately two hours after herbicide application onto the canopy of greenhouse-grown tobacco plants, susceptible plants started to wilt, and after forty eight hours (first measurement), the necrotic part of the leaf lamina changed to brown. The severity of necrosis varied from a few small spots to total coverage of the leaf lamina, depending on the herbicide concentration as well as the level of resistance of the plant. Higher levels of necrosis were correlated with a decrease in plant growth. The response of the different tobacco lines to different concentrations of herbicide varied. For example, growth of both the non-transgenic tobacco line KKD and the susceptible transgenic line 51-14 almost ceased at levels greater than 6.0 kg of herbicide ha^{-1}. However, growth of the other resistant transgenic tobacco lines continued up to the highest level of herbicide (48 kg ha^{-1}). There were also significant differences in the levels of leaf necrosis between the transgenic tobacco lines in their responses to herbicide applications at various levels. As an example, the response of the low resistant 51-14 transgenic line was similar to that of the non-transgenic tobacco line KKD which was susceptible to the herbicide. In contrast, there were significant differences between the 51-14 transgenic line and the other resistant transgenic lines (49-1, 51-1, 51-3, 51-5 and 54-18). Furthermore, there were considerable differences within the

resistant-transgenic lines (49-1, 51-1, 51-3, 51-5 and 54-18) in response to the herbicide. It is concluded that the levels of resistance of the transgenic lines differ significantly, and that a high variation within the transgenic lines occured. The results indicate that phenotypic expressivity of the lines changes with varying concentrations of herbicide. The transgenic lines themselves also showed different phenotypic expressivity in response to the concentrations of herbicide.

In discussing the inhibitory effect of dalapon herbicide, O'Connor (1990) reported that dalapon is absorbed by both the foliage and the roots of the plant, it is translocated throughout the plant organs, and subsequently suppresses plant growth. Foy (1962) reported that dalapon can be absorbed by the leaf cuticle and the large hypostomatus pores, and is translocated to the metabolically active regions of the plant where it interferes with the metabolic activity of the plant. In the present study, the herbicide was absorbed readily by the plant leaves and prevented plant growth. The dosage of the herbicide for killing weeds as well as crop plants is approximately 6 kg ha^{-1} (O'Connor, 1990). In the present study, a level of herbicide of 6.0 kg ha^{-1} suppressed the growth of the non-transgenic control plants, and this is in agreement with the recommendations of O'Connor (1990). The ability of the herbicide to kill susceptible plants, especially at higher levels, indicates that dalapon at such concentrations has an irreversible effect on plant metabolism, possibly through interference with the biosynthesis of pantothenate (Hilton *et al.*, 1959) and by inducing conformational changes in plant proteins (Buchanan-Wollaston *et al.*, 1992). It can be inferred that the dehalogenase enzyme, resulting from expression of the transferred dehalogenase gene, facilitated herbicide degradation in the resistant transgenic plants. The degradation of herbicide in resistant transgenic plants enabled them to grow in the presence of herbicide. There are several reports concerning the introduction of herbicide resistance genes into plants and the subsequent development of herbicide resistant transgenic plants. For example dehalogenase (Buchanan-Wollaston *et al.*, 1992) and *tfdA* (Streber and Willmitzer, 1989) genes responsible for detoxifying the herbicides dalapon and 2,4-D respectively, have been introduced into the genome of plants, resulting in transgenic plants which showed high levels of resistance to these herbicides. In the present study, the dehalogenase gene facilitate resistance to dalapon both in tobacco and white clover plants.

When the resistance of transgenic tobacco seedlings (only the seedling roots were exposed to the herbicide in the medium), white clover calli and developed tobacco plants (greenhouse-grown) are considered, it can be concluded that the dehalogenase gene is expressed in seedling roots, in undifferentiated cells (calli), as well as in the leaves of the developed plants. In relation to the mode of action of dalapon, Hilton *et al.* (1958; 1959) and Ingle and Rogers (1961) reported that the herbicide interfered with the enzymatic system of plants and other eukaryotic organisms.

The stem height of the plants decreased significantly with increasing levels of applied herbicide and at a level of 6.0 kg ha^{-1}, either the growth of susceptible plants ceased or the plants died. The results of the present study are in agreement with the results of Wilkinson (1962), where increasing concentrations of dalapon led to a significant decrease in plant height. However the resistant transgenic lines 49-1, 51-1, 51-3, 51-5, and 54-18 all continued to grow, up to the highest level of the herbicide (48.0 kg ha^{-1}) tested. There were significant differences between highly resistant-transgenic plants and low resistant 51-14 transgenic plants. As a comparison, at the highest level of the herbicide (48.0 kg ha^{-1}) the 54-18 transgenic line had a greater stem height than control plants at a herbicide level of 3.0 kg ha^{-1}. This indicates that dalapon affects the metabolic activity of the plants and this suppresses stem height growth, especially in susceptible plants. In relation to the effect of dalapon herbicide on plant metabolism, Ingle and Rogers (1961) reported that dalapon affected the physiological activity of plants through interfering with the metabolic energy of the plants, but not with the respiratory system of the plants. In the present study the results show that low concentrations of dalapon decrease the rate of growth of susceptible plants, while higher concentrations inhibit growth of susceptible plants entirely or cause plant death.

The effects of changes over time were different for leaf necrosis and stem height. The level of necrosis did not change over time and the necrotic surface remained constant after herbicide application. However, the stem height of resistant plants increased significantly over time, while the stem height of non-transgenic plants showed no significant changes over time. It is concluded that during the period of time for which it was studied, the leaf necrotic surface did not recover and the total necrotic surface remained constant. In contrast to that of

necrosis, the stem height of the resistant transgenic lines continued to increase, indicating an ability of transgenic lines to recover following herbicide application.

Changes in leaf length and leaf width of each of the lines were also affected by herbicide application, although the effects were very low compared with the effects on necrosis and stem height. The low variation in leaf length and leaf width in response to the herbicide as well low variation in growth over time are related to the growth activity of these characters. The leaf length and leaf width were measured on the fourth leaf from the base of the plant. At the time of herbicide application, the seedlings were actively growing, hence the upper parts of plants towards the apical meristem quickly dominated over the older lower leaves (the measured leaf). As a result, measured leaves was not actively growing and did not show a high response to the applied herbicide. In contrast, stem growth was very active, especially near the apical meristem. As an example, the leaf length and leaf width showed 12% and 8% increases respectively over time while stem height showed an increase of 262% over the same period of time.

The drymass of the tobacco lines in response to different concentrations of herbicide showed a similar pattern to that of stem height, i.e. a decrease with increasing concentrations of herbicide. In a response similar to that of the other characters, the non-transgenic control line and the susceptible 51-14 transgenic line showed the lowest increase in drymass in response to the herbicide compared to the other resistant transgenic lines. Wilkinson (1962) studied the effect of dalapon on drymass of oat (*Avena sativa*) plants and reported that the drymass of the plants decreased with increasing herbicide concentrations. In another study, Kay (1963) examined the effect of dalapon on herbage yield of three clover species. The herbage yield of the plants decreased significantly at level of dalapon of 4.5-5.0 kg ha^{-1}. In the present study, the non-transgenic line and the low resistant transgenic line 51-14 at a low level of herbicide, showed a reduction in drymass similar to that reported by Kay (1963) and Wilkinson (1962) However for the resistant transgenic lines, the decrease in drymass at each level of herbicide (especially at low levels of herbicide) was very low. As an example, the 49-1 transgenic tobacco line at a level of herbicide of 48.0 kg ha^{-1} produced a similar drymass to the control at a level of 3.0 kg ha^{-1}.

Since the majority of the transgenic lines are highly resistant to the herbicide, it is likely that the dehalogenase gene activity in the resistant transgenic

lines facilitates resistance to the herbicide through synthesis of the dehalogenase enzyme and the subsequent decomposition of the herbicide. In microorganisms, expression of the dehalogenase gene has been reported to result in resistance of organisms to this herbicide (Hirch and Alexander, 1960; MacGregor, 1963; Beeching *et al.*, 1983; Senior *et al.*, 1976; Hardman *et al.*, 1986). It is concluded that the activity of the dehalogenase gene in transformed plants is similar to that in bacteria with respect to the decomposition of dalapon (Buchanan-Wollaston *et al.*, 1992). However, the levels of resistance of the transformed plants varies, suggesting that the level of expression of the transferred gene is different for the different transformed lines. Differences between the genetically transformed plants in their responses to dalapon will be discussed in molecular studies in Chapter 5.

3.4.2.4 Heritability of herbicide resistance in transgenic tobacco plants

The population heritability of the herbicide induced necrosis character increased with increasing herbicide levels from 1.5 to 6.0 kg ha^{-1} and then decreased gradually, reaching a minimum at 48.0 kg of herbicide ha^{-1}. In contrast, the plant heritability initially decreased with increasing levels of herbicide up to 6.0 kg ha^{-1} and then started to increase with increasing concentration of herbicide, with the highest heritability being observed at 48.0 kg ha^{-1}.

The population and plants showed contrasting responses to increasing herbicide concentrations, i.e. an increase in the level of applied herbicide lead to a decrease in population heritability but an increase in plant heritability. As discussed in the previous section, the individual plants of each of the transgenic tobacco lines were a genetical mixture of all the genotypes. As a consequence the response of each of the individual plants to the herbicide lead to a genetic variation between the plants within the experimental units. The population genetical component of variation arose from differences between each of the tobacco lines. The estimation of heritability of characters at each level of herbicide, revealed that the heritabilities of population and plant should be considered separately. Furthermore, the broadsense heritability, which is a summation of the population and plant heritabilities, did not give any detailed information about population heritability or plant heritability as separate heritabilities. Hence, detailed information

could only be achieved by considering the plant and population heritabilities separately.

The population and plant heritabilities for the stem height character indicated that the population heritability initially increased at lower concentrations of the herbicide and then began to decrease with increasing concentrations of herbicide. In contrast, the plant heritability increased with increasing concentrations of the herbicide. The stem height had the lowest heritability of the characters studied, indicating the significant effect of environment on this character.

Heritability of the leaf length character was higher than that of necrosis and stem height. The experimental block as an environmental factor also had least effect on heritability of this character. The population heritability decreased slightly at herbicide level of 3.0 kg ha^{-1} and then began to increase with increasing levels of the herbicide, with the highest estimate of heritability being observed at 48.0 kg ha^{-1}. There was slight increase in plant heritability from 1.5 to kg ha^{-1} and then a decrease with increasing levels of the herbicide.

When the plant and population heritabilities of necrosis and stem height are compared with the population and plant heritability of leaf length, the leaf length character shows an inverse pattern of response to all levels of herbicide to those of necrosis and stem height. For example, in contrast with stem height and necrosis, the population heritability of leaf length increased with increasing the concentrations of herbicide, while the plant heritability decreased with increasing levels of herbicide. It is concluded that each of the characters has a particular population or plant heritability in response to the herbicide and this may vary from one character to another. The heritability of each character also depends on growth activity associated with that character as well as the response of the character to the herbicide. The leaf length character has a higher heritability estimate compared with the necrosis and stem height characters, which indicates a low response of this character to the environmental factors, as discussed above.

The leaf width character had a similar pattern of heritability to that of the leaf length character. Although the variation in heritability estimates for the herbicide levels was more than the leaf length character, the highest population heritability and lowest plant heritability was observed at a herbicide level of 48.0 kg ha^{-1}.

The heritability of the drymass character was similar for both population and plant. It is indicated a low variation in plant and population heritabilities for this character compared with the other characters.

The four characters, leaf necrosis, stem height, leaf length and leaf width, all showed different heritabilities in response to changes in the environmental factors of time, herbicide levels and experimental block. The environmental factor of time was particularly important for stem height, while that of herbicide level was particularly important for leaf necrosis. However these environmental factors had a minimal affect on the heritabilities of the leaf length and leaf width characters. Changes in genotypic variance of both population and plants in response to the herbicide were features of each of the characters. The level of plant and population heritabilities varied for each character, depending on the level of the applied herbicide.

It is suggested that the cross classification designs which includes time and experimental site (equivalent to herbicide in this study), is an appropriate method for estimating the heritabilities, since more environmental effects and their interactions are considered. Nyquist (1991) described several experimental designs for estimating plant heritability, including a completely randomised design model comprising site and year combinations, as well as cross classification design models such as randomised complete block, split plot and split block models. Increasing the complexity of the experimental design led to an increase in the number of environmental effects, interactions between the environmental effects, error terms, and a subsequent decrease in the estimated value of heritability. Instead of using such complex experimental designs, a simple experimental design is sometimes used in heritability studies. For example Carmer *et al.* (1989) used a simple experimental design which ignored site and/or time effects from the cross classification and included the block effect as the only environmental effect, resulting in a heritability estimate considerably higher than the designs which included site and time. Comstock and Moll (1963) suggested that when the cross classification structure is ignored, the genotype-by-environment mean square underestimates the contribution of the variance components for genotype-by-site and genotype-by-year interaction. Furthermore, in genetical designs such as diallel crossing (Hayman, 1958b; Griffing 1956b), generation means (Mather and Jinks, 1977; 1982), and factorial mating designs (Comstock and Robinson, 1952), only the

block effect was considered as the environmental component of variation in estimating the heritability. However, in the present study the main environmental factors of time and block, as well as genotype/environmental interactions and different levels of herbicide were all used for heritability estimates and these are thus likely to be more accurate than the above genetical design methods.

With respect to the recommended level of herbicide for controlling the weeds within transgenic population, the level of resistance of transgenic plants and the level of resistance of the non-transgenic plants should both be considered. The resistance of transgenic tobacco lines to dalapon under greenhouse conditions indicated that at a herbicide level of 6.0 kg ha^{-1}, growth of both the non-transgenic control line and the low resistant transgenic line (51-14) were suppressed significantly. However, the resistant transgenic lines continued to grow up to the highest level of herbicide (48.0 kg ha^{-1}). It should be noted that, although the resistant transgenic lines continued to grow in the presence of the herbicide, the growth rates of these lines decreased with increasing levels of herbicide. The optimum level of the herbicide to control weeds in the population of transgenic plants should be 6.0 kg ha^{-1}, since at this level weeds can be controlled while the transgenic plants are able to grow with the least inhibitory effect of the herbicide. Furthermore, the heritability of the characters under study are also considerably higher at this level of herbicide, indicating that optimum genetic advance in selection programmes can be expected for this level of herbicide.

With respect to the suitable character in herbicide resistance study, it appears that leaf necrosis is a suitable character for evaluating the resistance of plants to the herbicide dalapon, since this character has a high heritability and shows a high response to different levels of herbicide with an easily distinguishable form. This character is also a direct measure of resistance to the herbicide, since in the absence of herbicide the necrosis surface value is zero, while in presence of herbicide, depending on both the herbicide level and herbicide resistance level, the necrosis surface value can vary from 0 to 100%. Furthermore, this character is not significantly affected by time and the necrotic surface remains constant for a period of time after herbicide application. A second suitable character showing a clear response to different levels of herbicide concentrations is stem height. However this second character has the lowest heritability of the characters studied and shows a significant time effect. The two other characters, leaf length and leaf

width, as a result of lack of growth and the least response to the herbicide, are not suitable for the evaluation of dalapon resistance, although the heritability of these characters is high.

Comparing the resistance of the tobacco lines at the seedling stage (*in vitro*) and developed plant stage (in the greenhouse), the developed plants showed higher resistance to the herbicide than did the seedlings. Furthermore the heritability of the seedlings (*in vitro*) was significantly lower than that of the developed plants. It is suggested that evaluation of herbicide resistance at the seedling stage is not as useful as at the developed plant stage.

3.4.2.5 Inheritance of the dalapon resistance phenotype

In pooled analysis (Table 3.24), in the majority of cases, the dehalogenase gene is conserved during meiosis and is expressed in the progeny. In transgenic lines (except the 54-14 line), the segregation of the dehalogenase gene can be interpreted within a framework of Mendelian genetics.

In pooled analysis, only one case was observed where the transmission or the expression of the gene into the progeny was not achieved as expected. This was probably an artifact due to sub optimal expression of the dalapon resistance gene leading to an under estimation of the number of resistant plants. The resistant transgenic lines indicate a good agreement between the expression of the gene and inheritance of herbicide resistance for a single locus. However for the low resistant line (51-14), there is no such agreement.

Further analysis of inheritance using segregation data of each level of herbicide for each line (Table 3.25) showed that all of the transgenic lines, except 51-14, show single locus inheritance for various levels of herbicide. The homogeneity test of the χ^2 (last column of Table 3.25) also confirmed the validity of pooled analysis and involvement of single locus inheritance for transgenic lines (except 51-14). For the transgenic line 51-14, at concentrations higher than the 3.0 kg ha^{-1}, the number of the plants with a susceptible phenotype increased significantly and plants behaved as the control, non-transgenic plants. Hence the 3.0 kg of herbicide ha^{-1} is the threshold for this line. However, for the other resistant transgenic lines, the threshold is greater than the 48.0 kg of herbicide ha^{-1},

since these lines show resistant phenotypes with the expected ratio up to the highest concentration of herbicide (see Table 3.25).

Chapter 4

Quantitative genetics of herbicide resistance

4.1 Introduction

There is little information in the literature about quantitative genetics of transgenic plants. Phenotypic expression of a transgene in a population is continuous not discrete, and so it is important to estimate the additive, dominance, and epistasis effects of the transgene. This is important from the threshold problem in Chapter 3, which make it clear that the methods of quantitative genetics were appropriate for quantitative study of transgene. In addition the quantitative genetics provide information relating to breeding programmes and selection strategies. Three genetical designs, the generation means, the Jinks-Hayman diallel and the Griffing diallel were carried out to study the transgene effect in a plant population. For the generation means analysis, the transgene effect was studied in some detail. In the generation means analysis one transgenic and four successive generations of theses parents were used. For the Hayman-Jinks diallel, although less information on the gene effect was achieved, the number of the parental lines was significantly greater than for the generation means analysis. The Griffing method of diallel analysis gave the least detailed information about the gene action. However the result of the Griffing method would be of benefit for applied plant breeding. The objectives of different aspects of this chapter are outlined below.

4.1.1 Quantitative inheritance of transgene

The objectives here are to estimate basic genetic components such as additive, dominance and degree of dominance of the transgene, presence or absence of maternal effect, as well as the interaction of the transgene with host plant allele(s) (epistasis). A generations means mating design experiment, consisting of six generations, as well as Hayman-Jinks (9x9) full diallel-crossing mating design experiments were carried out to estimate genetic components and epistasis.

4.1.2 Heritability of transgene

The objectives here are to estimate the narrowsense and broadsense heritabilities of the transgene and to estimate the genetic advance (ΔG) for the stem height and leaf necrosis characters of transgenic plants can be useful in predicting the gain from selection. A generation means mating design experiment, consisting of six generations, as well as Jinks-Hayman and Griffing (9x9) full diallel-crossing mating designs data were used to estimate the heritablities and the genetic advance (ΔG) of individual characters.

4.1.3 Combining ability

The objectives here are to estimate the general and specific combining ability variances (σ^2_{gca} and σ^2_{sca}), the general combining ability effects (g.c.a. effects), and the specific combining ability effects (s.c.a. effects) of the transgenic plants in cases of crosses between transgenic plants as well as between transgenic and non-transgenic plants. The Griffing (9x9) full diallel-crossing mating design were carried out to estimate the general combining ability variance (σ^2_{gca}), specific combining ability variance (σ^2_{sca}), general combining ability effects (g.c.a. effects) and specific combining ability effects (s.c.a. effects). These estimates provide useful summaries to assist plant breeders in deciding which method to use.

4.2 Materials and Methods

4.2.1 Plant development for transgenic crossing

4.2.1.1 Homozygous plant development

True breeding (homozygous) and subsequent sexual generations are used for inheritance studies. This Section discusses their preparation. The following abbreviations are used:

TP : Transgenic Parent.

NP : Normal parent (non-transgenic).

TF_1: Transgenic F_1, (either between transgenic and non-transgenic, or between two transgenic).

TF_2: Transgenic F_2.

TBC_1: Transgenic backcross1 (cross between TF_1 and transgenic parent).

TBC_2:Transgenic backcross 2 (cross between TF_1 and non-transgenic KKD parent).

R_0 : Transgenic plants originating from tissue culture

R_1 : Plants developed from selfed R_0 plants

R_2 : Plants developed from selfed R_1 plants

R_3 : Plants developed from selfed R_2 plants

R_1 transgenic seeds, which are produced from selfed R_0 plants originating from tissue culture, mostly have a hemizygous genetic background for the transgene. Subsequent generations segregate for transgenic and non-transgenic genotypes. In genetical studies, it is desirable that parent plants are homozygous. To develop homozygous transgenic plants for the transgene, screening methods were conducted in the greenhouse as follows:

Seeds from the transgenic lines 49-1, 51-1, 51-3, 51-5, 51-14 and 54-18, and the non-transgenic tobacco (*Nicotiana tabacum*) line KKD were surface sterilised and spread on the surface of a ½MS (Appendix 5) plate supplemented with 0.8% Difco agar, as described in Chapter 3, Section 3.2.2. At the 2-leaf stage, 40 R_1 seedlings from each line were transplanted into soil as a single plant in each pot. Twenty-six days after transplanting, at the 4-6 leaf stage, plants were sprayed with 2,2-dichloropropionic acid (dalapon) at a rate of 12 kg active component ha^{-1}.

Ten days after herbicide application, susceptible (negative) plants were discarded since these plants lacked the transgene. The remaining transgenic plants were grown to flowering and their seeds were harvested.

Seeds from the remaining resistant plants (homozygous and hemizygous transgenic plants) were collected and recorded separately and stored in a cool room as reference seeds. A portion of the seeds from single plants was surface sterilised separately and spread on the surface of plates containing $^1/_2$MS solid medium. At the 2 leaf stage, 42 seedlings (R_2) were transplanted into water-saturated soil in 35 x 40 cm trays as 6 x 7 seedlings per column and row. The seedlings were covered with transparent plastic bags containing holes for a few days. The trays were subirrigated weekly until seedlings reached the 5-6 leaf stage. Before herbicide application at the rate of 12 kg active component ha^{-1}, half of each tray was covered with plastic as a control, and the other half (21 seedlings) was sprayed with herbicide. Following herbicide application, dead or susceptible (negative) seedlings in each tray were recorded. Any tray which contained dead plants was recorded as a segregant or hemizygous transgenic plant and was discarded. In contrast, trays with no dead plants were recorded as homozygous plants for the transgene. The homozygous transgenic tobacco seedlings from the trays were transplanted into single pots and allowed to develop to flowering. Seeds were then produced by selfing, collected, and recorded as homozygous transgenic parent seeds for each line. The homozygous transgenic R_3 plants developed from these homozygous seeds were used in quantitative genetic studies as described in the subsequent sections.

4.2.1.2 TF_1, TF_2, TBC_1 and TBC_2 seeds production

The homozygous 51-1 transgenic R_3 parent plants and the non-transgenic KKD line, were crossed and the seeds harvested as TF_1 seed material. The TF_2 seeds were harvested from the selfed TF_1 plants To develop the TBC_1 and TBC_2 plants, the TF_1 plant flowers were emasculated, covered and pollinated with:

1. 51-1 to achieve backcross 1 (TBC_1) seed material or
2. Control non-transgenic KKD line to achieve backcross 2 (TBC_2) seed material which was used for generation means mating design.

4.2.2 Quantitative inheritance studies

4.2.2.1 Generation means

Two homozygous parental tobacco line, the 51-1 transgenic line homozygous for and the control non-transgenic base line, KKD were used to develop the different generations as discussed above (Section 4.2.1.2). The seeds of the different generations, TF_1, TF_2, TBC_1, TBC_2, TP_1 (51-1) and non-transgenic KKD parent NP_2, were sown directly into soil in the greenhouse and subirrigated for seedling growth. Twenty-three days after sowing, five seedlings were transplanted into a single pot in the greenhouse.

A randomised complete block design with six tobacco generations (as mentioned above) and four blocks was established. Experimental units were single pots, each containing 5 single plants. Each of the TF_1, TP_1 (51-1) transgenic parent and the non transgenic parent NP_2 generations consisted of 20 plants. The TF_2 and backcrosses (TBC_1 as well as TBC_2) generations consisted of 160 and 100 plants respectively.

4.2.2.1.1 Attributes

When seedlings were at the 5-6 leaf stage, the pots were sprayed randomly with dalapon at rate of 6 kg ha^{-1}. Four days after herbicide application, the necrotic surface of leaves was recorded by giving scores from 1 to 10 (high level of resistance to low level of resistance). Each score represented approximately a 10% increment in necrotic leaf area. Estimation of necrosis was by eye (Lefkovitch, 1991; Cummins and Aldwinckle, 1983; Bradley, 1953; Finney, 1950). The stem height was measured from the soil surface to the apical meristem (mm).

4.2.2.1.2 Generation means analysis

A generation means analysis (Mather and Jinks, 1977; 1982) was carried out to analyze the generation means data and estimate the genetical components of additive (*d*), dominance (*h)* and mean (*m).* The generation mean analyses were carried out if the weighted analysis of variance was significant for generations. Further genetic components, such inter-allelic interactions (epistases) were

estimated using the following model:

$$Y = m + \alpha d + \beta h + \alpha^2 i + 2\alpha\beta j + \beta^2 l$$

Where:

Y : mean of a generation

m : mean of all generations in a cross

[*d*]: additive effect

[*h*]: dominance effect

[*i*]: interaction between additive effects (additive x additive)

[*j*]: interaction between additive and dominance(additive x dominance)

[*l*]: interaction between dominance effects (dominance x dominance)

α, β, α^2, $2\alpha\beta$, and β^2 are coefficients of the genetic parameters.

For six generations consisting TP_1, NP_2, TF_1, TF_2, TBC_1 and TBC_2, the components of mean are given in Chapter 2, Table 2.1 (from Mather and Jinks, 1982) . The estimates of the parameters were obtained by using a weighted least square analysis of the data, since the number of individuals and variances differed for each generation. Weighted generation means were used by taking weights as the reciprocals of squared standard errors of each mean (Mather and Jinks, 1977; 1982). All of the six generations were tested with two, three, four, five and six parameters to obtain the best model. The models (except the model with six parameters) were tested for adequacy using the chi-square test (scaling test). As mentioned above, since the degree of freedom for a model with six parameters is 0 (6-6=0), a test of this model with the scaling test is not possible. The weighted least square method (Mather and Jinks, 1982) was used to estimate the genetical parameters. The matrix inversion method as a general approach was used (Rowe and Alexander, 1980) to analyze the data as follows:

$$\mathbf{E\,G = O} \quad \text{or} \quad \mathbf{(E'W)E\,G = (E'W)\,O}$$

$$\mathbf{G = E^{-1}\,O} \quad \text{or} \quad \mathbf{G = (E'WE)^{-1}\,E'WO}$$

Where:

O : column vector of generation means

G : vector of genetic parameters to be estimated by least square

E : matrix, depending on the genetic model for six generations in terms of 2, 3, 4, 5, and 6 genetical parameters (Chapter 2, Table 2.1)

W : diagonal of $[1/\sigma^2_{xi}]$ (reciprocal of variance of generations)

E´ : transpose of **E**

$\mathbf{E^{-1}}$: inverse of **E**

Standard errors of the components were obtained as follows:

$$\mathbf{V_G = (E'WE)^{-1}}$$

4.2.2.1.3 Generation variance analysis

The components of variation of the six generations were estimated according to Mather and Jinks (1982) as follows:

$$V_{P1} = E_W$$
$$V_{P2} = E_W$$
$$V_{F1} = E_W$$
$$V_{F2} = (1/2)D+(1/4)H+E_W$$
$$V_{BC1} = (1/2)D+(1/4)H-(1/2)F+E_W$$
$$V_{BC2} = (1/4)D+(1/4)H+(1/2)F+E_W$$

Where:

E_W is the non-heritable component of variation, D is the additive component of variation, H is the dominance component of variation and F is the dependent contribution of d and h over all loci. These four genetical parameters were estimated as follows:

$$E_W = (1/4)(V_{P1}+V_{P2}+2V_{F1})$$
$$D = 4V_{F2}-2(V_{BC1}+V_{BC2})$$
$$H = 4(V_{BC1}+V_{BC2}-V_{F2}-W_E)$$
$$F = V_{BC1}-V_{BC2}$$

Since there are four estimates for the four parameters, we can neither estimate the standard deviation of the D, H, E_W and F nor test the adequacy of the additive dominance model (Mather and Jinks, 1982).

4.2.2.1.4 Heritability

The broadsense and narrowsense heritabilities were estimated using variance of generations, which estimated by generation means analysis. The heritability estimates were based on family variance and genetical components. The method of Warner (1952) was used to estimate the heritability and standard error of the heritability as follows:

$$VTF_2 = V_A+V_D+V_E$$

$$VTBC_1+VTBC_2 = V_A+2V_D+2V_E$$

where:

VTF_2, $VTBC_1$ and $VTBC_2$ are variances of TF_2, TBC_1 and TBC_2 generations respectively. The V_A , V_D , V_E are additive, dominance and environmental components of variation respectively.

The component of variation of the TF_2 generation was determined as a total phenotypic component of variation. To estimate the genotypic component of variation, the component of variation of the TF_2 generation was multiplied by 2 and the result subtracted by (TBC_1 + TBC_2) component of variation as follows:

$$V_A = 2(VTF_2)-(VTBC_1+VTBC_2)$$

Or

$$V_A = 2(V_A+V_D+V_E)-(V_A+2V_D+2V_E)$$

where:

$$V_E = (V_{P1}+V_{P2}+2V_{F1})/4$$ (Mather and Jinks, 1977)

The narrowsense heritability was estimated as follows:

$$h^2_{NS} = V_A/VTF_2 = V_A/(V_A+V_D+V_E)$$

The standard error of narrowsense heritability was estimated as the square root of the following equation:

$$S.E.(h^2_{NS}) = \{2\{[(VTBC_1+VTBC_2)^2/dfTF_2]+(VTBC_1^2/dfTBC_1)+(VTBC_2^2/dfTBC_2)\}/VTF_2^2\}^{1/2}$$

In this formula the $dfTF_2$, $dfTBC_1$ and $dfTBC_2$ are degrees of freedom of TF_2, TBC_1 and TBC_2 respectively. The significance of h^2_{NS} was tested by F distribution with n1 and n2 degrees of freedom.

where:

$n1=dfTF_2$, and,

$n2 = (VTBC_1+VTBC_2)^2/((VTBC_1^2/dfTB_1)+(VTBC_2^2/dfTBC_2))$

The expectation of broadsense heritability can be calculated using different formulae as follows:

$$h^2_{BS} = (V_{F2}-V_E)/V_{F2}$$

where:

$V_E = (V_{P1}+V_{P2}+2V_{F1})/4$ (Mather and Jinks, 1977)

4.2.2.1.5 Method of estimating the number of effective factors

The number of the effective factors (genes) contributing to the quantitative characters was estimated using the procedure outlined by Lande (1981). The method estimates the minimum number of freely segregating genetic factors:

$$nE = (\mu NP_2-\mu TP_1)^2/8\sigma^2 s \leq n$$

where:

$$\sigma^2 s = 2\sigma^2 TF_2-(\sigma^2 TBC_1+\sigma^2 TBC_2)$$

the standard error of gene number (Lande, 1981) is the square-root of the following expression:

$$V(nE) \approx nE^2 = 4\{[(\sigma^2TP_1/NTP_1+\sigma^2NP_2/NNP_2)/(\mu NP_2-\mu TP_1)]+ [Var(\sigma^2_s)/\sigma^4s]\}$$

the $Var(\sigma^2s)$ can be estimated as:

$$Var(\sigma^2s) = 2\sigma^2TF_2/NTF_2+2\sigma^2TF_1/NTF_1$$

4.2.2.3. Diallel crossing programme and TF_1 and F_1 seed production

Six homozygous R_3 transgenic tobacco lines, 49-1, 51-1, 51-3, 51-5, 51-14, 54-18 and three non-transgenic lines, KKD, Kutsaga 51 (K51) and Burleigh 21 (B21) were the parental lines (9 lines in total). All of the possible crosses were made between the parental lines and subsequently 72 (=9(9-1)) lots of TF_1 seed material were harvested. The 81 lots of seed material consisting 72 lots of hybrid seeds (off the leading diagonal of diallel table), and 9 lots of selfed parents seeds (leading diagonal of diallel table) were used as the material for the diallel crossing mating design.

4.2.2.4 Jinks-Hayman diallel crossing design

Seventy-two TF_1 and F_1 (progeny of cross between two non-transgenic parents) seeds (including reciprocals) plus 9 homozygous parents were used as material for a 9x9 full diallel crossing mating design. A randomised complete block design which comprised 81 treatments (lines) with three blocks, was established under greenhouse conditions. The experimental unit was a single pot containing 5 plants with an equal distance between each plant.

4.2.2.4.1 Attributes

Dalapon herbicide at a rate of 6 kg ha^{-1} was sprayed on the plants at the 5-6 leaf stage. Four days after the herbicide application, the leaf necrosis score and the stem height of each seedling were measured as described above.

4.2.2.4.2 Analysis of diallel table

Statistical analysis of the diallel table was carried out using the Jinks and Hayman (1953), and Mather and Jinks (1977, 1982) methods to estimate the variance of arrays as well as covariances between the arrays, as follows:

Variance of i'th array:

$$Vr_i = (\Sigma_j X_{ij}^2 - X_{i.}^2/m)/m-1 \quad \text{for each array}$$

where i=1,...,P, and P is number of parents. j=1,...,m, where m is progeny number and $X_{i.}$, is the summation across all j observation for each i .

Mean variance across arrays:

$$\bar{V}r = (\Sigma_i Vr_i)/P$$

Variance of array means:

$$\bar{W}r = (\Sigma_l X_l - X_{.}^2/P)/P-1$$

Where:

X_l is the l'th phenotype variate, l=1,..., a, where a is number of array mean and $X_{.}$ is the sum of parental array mean.

Variance of parents:

$$V_P = (\Sigma_i X_i^2 - X_{.}^2/P)/P-1$$

where X_i is the ith parent phenotype (leading diagonal of diallel table) and $X_{.}$ is the sum of entries in the leading diagonal (parents).

Covariance of array:

$$Wr_i = (\Sigma_{jk} X_{ij} Y_{ik} - ((X_{i.} - Y_{i.})/m))/m-1$$

Where i and j are defined above and K=1,...,P,

Mean covariance across the array:

$$\bar{\bar{W}}r = (\Sigma_i Wr_i)/P$$

Mean of the ith array :

$$r = (\Sigma_j X_{ij})/m$$

To test the significance of (Wr+Vr) and (Wr-Vr) arrays, the arrays' data were statistically analyzed and tested using the F-test. The significance of (Wr+Vr) indicated the presence of dominance, while non-significance indicated the absence of dominance. Non-significance of (Wr-Vr) indicated that additive-dominance is appropriate and alleles are distributed independently, while significance of the source of the variation indicated inadequacy of the simple additive-dominance model and the presence of non-allelic interaction (Mather and Jinks, 1982).

In a diallel crossing with a certain value of H_1/D (H_1 is the dominance component of variation and D is the additive component of variation), the points Vr/Wr (Vr is variance of arrays and Wr is covariance of arrays in the diallel table) are distributed along a straight line of unit slope inside the limiting parabola, $Wr^2 = VrxVp$, (Hayman, 1954b). The regression of Wr against Vr was analyzed for each attribute and the standard error of regression, R-square and regression line slope (β) were estimated by regression analysis, and tested using the F-test. The limiting parabola was plotted for each attribute using the limiting parabola formula ($Wr^2 = VrxVp$).

4.2.2.4.3 Genetical components

Genetical components were estimated using the method of Jinks and Hayman (1953) and Mather and Jinks (1982). Basic array statistics were averaged over 3 blocks before estimating the components. Since the equations of Mather and Jinks (1882) are for full diallel, in the present study (for half diallel analysis), the half of the TF_1 error mean square was removed from all of the coefficients The genetical components of additive (D), dominance (H_1, H_2) and F were estimated for

each of the attributes as follows:

$$D = V_P - E_P$$

$$H_1 = 4\bar{V}r + V_P - 4\bar{W}r - [((4n-2)/n)E_F + E_P]$$

$$H_2 = 4\bar{V}r + 4V\bar{r} + [(4(n-1)^2/n^2)EF - (4(n-1)/n^2)EF$$

$$F = 2V_P - 4\bar{W}r - [(2(n-2)/n)E_P]$$

where:

V_P is variance of parents, E_P is parents' error mean square, E_F is F_1 error mean square, H_1 and H_2 are dominance components of variation, and F accounts for the non-independent contribution of additive and dominance effects when gene frequencies (*v* and *u*) are unequal (additive-dominance covariance). Since no worthwhile estimates of the errors of these components is available (Mather and Jinks, 1982), standard errors of these genetical components are not estimated.

The expectations of $\bar{V}r$, $\bar{W}r$, $V\bar{r}$, and V_p related with the genetical components, are given as follows:

$$\bar{V}r = (1/4)D + (1/4)H_1 - (1/4)F$$

$$\bar{W}r = (1/2)D - (1/4)F$$

$$V\bar{r} = (1/4)D + (1/4)H_1 - (1/4)H_2 - (1/4)F$$

$$V_p = D$$

where:

D is additive component, H_1 and H_2 are dominance components and F is the covariance of additive and dominance components.

Some further statistics can be estimated from the components of variation as follows:

(H_1/D) : measures the average degree of dominance over all loci. The degree of dominance can be described as: $H_1 < D$ partial dominance, $D = H_1$ full dominance and $H_1 > D$ over dominance.

$(0.5F)/[D(H_1 - H_2)]^{1/2}$: measures the extent to which dominance levels vary from one locus to another. The absolute value varies from 0 to 1, where 1 indicates a constant dominance level over all loci.

$[(4DH_1)^{1/2}+F]/[(4DH_1)^{1/2}-F]$: measures the proportion of dominant to recessive alleles over all parents.

$(\bar{uv})$: is the product of the frequencies of dominant and recessive alleles, which can be estimated as $H_2/(4H_1)$, with a maximum value of 0.25 (0.5x0.5=.25).

$r_{(p,Wr+Vr)}$: is the correlation between Wr+Vr and mean of common parent of array.

4.2.2.4.4 Estimating heritability through Jinks-Hayman diallel

The Mather and Jinks (1982) method was used to estimate the broadsense and narrowsense heritabilities using genetic components as follows.

$$h^2_{BS} = [(1/2)D+(1/2)H_1-(1/4)H_2-(1/2)F]/[(1/2)D+(1/2)H_1-(1/4)H_2-(1/2)F+E]$$

$$h^2_{NS} = [(1/2)D+(1/2)H_1-(1/2)H_2-(1/2)F]/[(1/2)D+(1/2)H_1-(1/2)H_2-(1/2)F+E]$$

where:

E is the pooled error mean-square estimated from analysis of variance of (n(n+1)/2) genotypes.

4.2.2.5 Method for analysis of Griffing diallel

The method of general combining ability (GCA) and specific combining ability (SCA) analysis and estimating the general combining ability effects (g.c.a. effects) and specific combining ability effects (s.c.a. effects) were based on the method 2 (parents and one set of F_1 included but reciprocal F_1's are not), model I and II (fixed and random effect respectively) of Griffing (1956a). The general combining ability is the average performance of a line hybrid combination (e.g. parental performance) while the specific combining ability in certain combinations (hybrid progenies) do better or worse than would be expected on the basis of the average performance of the parental lines involved

For method 2 of diallel analysis (Griffing, 1956b), the following summation notations were used:

$$X_{i.} = \sum_{j} x_{ij} = x_{i1}+\ldots+x_{i9}$$

where $x_{ij} = x_{ji}$, $X_{j.}$ need not be considered (because reciprocal F_1's were not included).

$$X_{..} = \sum_{i \le j} \sum x_{ij} = x_{11}+x_{12}+\ldots+x_{89}+x_{99}$$

where $X_{i.}$ is total performance of i'th line and $X_{..}$ is total performance over all lines.

The analyses of GCA and SCA resulting from partitioning of the line (variety) sum of squares are given in Table 4.1 (from Griffing, 1656b).

Table 4.1. Analysis of variance for Method 2 giving expectation of mean square based on the assumptions of model I (fixed) and model II (random)

Source	df	SS	MS	Expectation of Mean Squares	
				Model I (Fixed)	Model II (Random)
GCA	P-1	S_g	M_g	$\sigma^2+(P+2)(1/(P-1))\sum\sigma_{gi}^2$	$\sigma^2+\sigma_s^2+(p+2)\sigma_g^2$
SCA	P(P-1)/2	S_s	M_s	$\sigma^2+[2/P(P-1)]\sum_i\sum_j\sigma_{sij}^2$	$\sigma^2+\sigma_s^2$
Error	m	S_e	M_e	σ^2	σ^2

where:

$$S_g = [1/(P+2)]\{\sum_{i.}(X_{i.}+x_{ii})^2-(4/P)X_{..}^2\}$$

$$S_s = \sum_{i \le j} \sum x_{ij}^2-[1/(P+2)]\sum_{i}(X_{i.}+x_{ii})^2+[2/(P+1)(P+2)]X_{..}^2$$

The statistical model used for the combining ability analysis of model I (fixed) is as follows:

$$x_{ij} = \mu+g_i+g_j+s_{ij}+(1/bc)\sum_{k}\sum_{l}e_{ijkl}$$

where:

i,j = 1,...,P (number of parents)

k = 1,...,b (number of blocks)

l = 1,...,c (number of within plot observations),

μ is the population mean, g_i (g_j) is the g.c.a. effect, s_{ij} is the s.c.a. effect

such that $s_{ij} = s_{ji}$ and e_{ijkl} is the effect peculiar to $_{ijkl}$'th observation.

The restrictions which were imposed in this analysis were as follows:

$$\Sigma g_i = 0,$$
$$\Sigma s_{ij}+s_{ii} = 0 \text{ (for each i)}$$

In the model I (fixed), $M_e = M_e/bc$. The following F ratios were used to test for g.c.a. and s.c.a. effects. To test the differences among g.c.a. effects:

$$F_{[p(p-1),m]} = M_g/M_e$$

and to test for differences among s.c.a. effects:

$$F_{[p(p-1/2)/2,m]} = M_s/M_e$$

The effects were estimated as follows:

$$\hat{\mu} = [2/(P(P+1)]X_{..}$$
$$\hat{g}_i = [1/(P+2)]\{X_{i.}+x_{i}-(2/P)X_{..}\}$$
$$\hat{s}_{ij} = x_{ij}-[1/(P+2)]\{X_{i.}+x_{ii}+X_{j.}+x_{jj}\}+[2/(p+1)(P+2)]X_{..}$$

Variances of g.c.a. and s.c.a. effects were estimated as follows:

$$Var(\hat{g}_i) = (P-1)/[P(P+2)]\sigma^2$$
$$Var(\hat{s}_{ij}) = (P^2+P+2)/[(P+1)(P+2)]\sigma^2 \quad (i \neq j)$$

The statistical model used for the combining ability analysis for model II (random) is as follows:

$$x_{ij} = \mu+g_i+g_j+s_{ij}+(1/b)\Sigma_k b_k+(1/b)\Sigma_k (bv)_{ijk}+(1/bc)\Sigma\Sigma_{kl} e_{ijkl}$$

where all effects except μ were random variable.

The expectations of combining ability mean squares based on Table 4.1 are as follows:

$$E(M_{e'}) = (1/bc)[\sigma_e^2 + c\sigma_{bv}^2] = \sigma^2$$

The following F ratios were used for testing hypotheses pertaining to the different variance components. To test $\sigma_g^2 = 0$:

$$F_{[P-1),\ P(P-1)/2]} = M_g/M_s$$

and to test $\sigma_s^2 = 0$:

$$F_{[P(P-1)/2,\ m]} = M_s/M_{e'}$$

The variance components for g.c.a and s.c.a. effects were estimated as follows:

$$\sigma_g^2 = [1/(P+2)](M_g - M_s)$$

$$\sigma_s^2 = M_s - M_{e'}$$

4.3 Results

4.3.1 Generation means analysis

A generation means mating design experiment was carried out for the detailed study of the gene effects and inter-allelic interactions (epistasis), as well as for estimating other genetical components. Results of the analysis of a generation means and estimates of components of the mean and components of variations for the necrosis and stem height characters are given in the following Section.

4.3.1.1 Components of means

Analysis of variance of the leaf necrosis and stem height characters shows that there are significant differences in these characters between different generations. The means of leaf necrosis and stem height of the different generations are presented in Table 4.2.

Table 4.2. Mean necrosis and stem height (mm) of different generations

Generation	Necrosis	Stem height
TP_1 (51-1)	0.652 c	59.95 a
NP_2 (KKD)	2.967 a	2.80 c
TF_1	0.763 c	53 70 a
TF_2	1.402 b	35.98 b
TBC_1	0 670 c	53.79 a
TBC_2	1.499 b	27.53 b
LSD 5%	0.440	8.67

Means with the same letter within each column are not significantly different (P=0.05%).

For leaf necrosis, the TP_1 (51-1), TF_1 and TBC_1 generations show the lowest mean necrosis scores (highest resistance) in response to the herbicide. The differences between the TP_1, TF_1 and TBC_1 generations are not significant, indicating phenotypic similarity between the three generations. Furthermore, the results indicate that TF_1 and TBC_1 are phenotypically similar to the transgenic parent TP_1. The highest necrosis is shown by the non-transgenic parent NP_2

(KKD), indicating that this line has the lowest herbicide resistance. The leaf necrosis values for the TF_2 and TBC_2 generations fall between the high and low resistant classes.

There are no significant differences in stem height between the TP_1 (51-1), TF_1 and TBC_1 generations. These generations also have significantly higher stem heights. The phenotypic similarity of TF_1 as well as TBC_1 with the transgenic parent TP_1 with respect to stem height, indicates the dominance of the transgenic parent over the non-transgenic parent. The non-transgenic parent NP_2 (KKD) shows the least mean stem height compared to the other generations. The stem height values for the TF_2 and TBC_2 generations fall between the high and low resistant classes.

The significant differences that are evident between the generations with respect to herbicide resistance show that analysis of the data through generation means and the estimation of the mean and variance components is valid. The result of the analysis of generation means using the six parameters model led to estimates of *m* (mean genotype), *d* (additive), *h* (dominance), *i* (additive x additive epistasis), *j* (additive x dominance epistasis) and *l* (dominance x dominance epistasis) values for leaf necrosis and stem height. These estimated values are presented in Tables 4.3 and 4.4.

For leaf necrosis (Table 4.3), the result of the analysis indicates that all of the estimates for genetical parameters are highly significant. The significance of the genetical parameters indicate an involvement of additive, dominance, as well as additive x additive, additive x dominance and dominance x dominance epistases in controlling the necrosis character. The high significance of the additive effect suggests an important effect of homozygous alleles in the expression of the resistant phenotype. On the other hand, the homozygous transgenic genotypes show a high resistance phenotype in the presence of the herbicide. The significance of the additive effect indicates an important effect of the hemizygous genotype in the expression of the resistance phenotype. Thus, the transgenic homozygous and hemizygous genotypes are clearly important in controlling the herbicide resistance character in this line. The negative estimate of the additive (*d*), indicates the distribution of additive and dominance allele(s) in the transgenic parent which have the effect of decreasing the leaf necrosis. The significance of *i*, *j* and *l* estimates, indicates the involvement of additive x additive, additive x

dominance and dominance x dominance epistases. In relation to the presence of epistasis, it is suggested that there is an interaction between transgene and host tobacco allele(s) in controlling this character.

Because it was not possible to test the six parameter model by the χ^2 test, an additional analysis of the data was carried out using all possible combinations of the above parameters. This made it possible to test for the best model by the joint scaling test (except for the six parameter model as mentioned above). The results of this analysis and the joint scaling test are presented in Table 4.3. The results indicate the inadequacy of a simple additive-dominant model by non-significance of one or more of the parameter(s), and/or the significance of χ^2 of the relevant model as indicated in the table. In conclusion, it is indicated that the results of the six parameter model give the best fit for the data, and indicate the validity of this model for estimating the additive, dominance and epistasis for the leaf necrosis character.

For the stem height character, the analysis of the six parameter model shows that estimates of *m*, *d*, *h*, and *i* only are significant whereas *j* and *l* epistases are not significant (Table 4.4). The results indicate that additive, dominance, as well as additive x additive epistasis are involved in controlling the stem height character. To determine the best model for estimating the genetical components, all of the possible models were tested by means of a joint-scaling test (except for the six parameter model). The results of this test are presented in Table 4.4. The table indicates that the best model to estimate the mean component is the model containing *m*, *d*, *h*, and *i*. The results of all the possible models reveal that for stem height, besides the additive and dominance effects, the additive x additive type of epistasis is involved. Thus it can be interpreted that an interaction between the transgene and host tobacco allele(s) is involved in the expression of the transgene

The additive dominance ratio estimated by mean component is 0.78 for each of the leaf necrosis and stem height characters, indicating a partial dominance of the allele(s) for both characters.

Table 4.3. The estimates of the genetic components of means, based on 2,3,4,5 and 6 parameter models for interaction types, as well as a test of significance for the necrosis character

	Number of parameters in the model				
Component	2	3	3	6	4
$\bar{m}$	1.28±0.03 **	1.04±0.03 *	1.77±0.05 **	3.08±0.35 **	1.84±0.15 **
$[\bar{d}]$	-1.00±0.04 **	-1.11±0.04 *	-1.12±0.04 **	-1.16±0.05 **	-1.12±0.04 **
$[\bar{h}]$	-	-	-1.06±0.08 **	-4.39±0.85 **	-1.15±0.21 **
$[\bar{i}]$	-	0.73±0.06 *	-	-1.27±0.35 **	-0.07±0.16 ns
$[\bar{j}]$	-	-	-	0.66±0.22 **	-
$[\bar{l}]$	-	-	-	s.08±0.52 **	-
χ^2	187.21 **	45.94 **	16.40 **	-	16.17 **

Table 4.3. Continued...

	Number of parameters in the model				
Component	4	4	5	5	5
$\bar{m}$	1.78±0.05 **	1.79±0.05 **	1.81±0.15 **	2.61±0.32 **	1.81±0.05 **
$[\bar{d}]$	-1.14±0.05 **	-1.12±0.04 **	-1.14±0.05 **	-1.09±0.05 **	-1.16±0.05 **
$[\bar{h}]$	-1.09±0.09 **	-1.22±0.18 **	-1.14±0.21 **	-2.98±0.70 **	1.45±0.24 **
$[\bar{i}]$	-	-	-0.04±0.16 ns	-0.83±0.32 **	-
$[\bar{j}]$	0.13±0.17 ns	-	0.12±0.17 ns	-	0.31±0.20 ns
$[\bar{l}]$	-	0.19±0.20 ns	-	1.13±0.41 **	0.40±0.25 ns
χ^2	15.81 **	15.53 **	15.74 **	8.68 **	13.12 **

ns, (*), * and ** : not significant, significant at 10%, %5% and 1% probability level respectively.

Table 4.4. The estimates of the genetic components of means, based on 2,3,4,5 and 6 parameter models for interaction types, as well as a test of significance for the stem height character

	Number of parameters in the model				
Component	2	3	3	6	4
$\bar{m}$	37.42±0.61 **	45.70±1.00 **	30.46±0.83 **	12.93±9.66 *	19.45±3.74 **
$[\bar{d}]$	32.39±0.75 **	28.09±0.85 **	27.88±0.83 **	28.60±0.91 **	28.48±0.85 **
$[\bar{h}]$	-	-	21.14±1.71 **	51.71±23.29 *	34.45±4.73 **
$[\bar{i}]$	-	-14.80±1.42 **	-	18.42±9.62 *	11.86±3.92 **
$[\bar{j}]$	-	-	-	-4.66±5.99 ns	-
$[\bar{l}]$	-	-	-	-0.94±1.42 ns	-
χ^2	162.26 **	54.09 **	10.06 **		0.93 ns

Table 4.4. Continued...

	Number of parameters in the model				
Component	4	4	5	5	5
$\bar{m}$	30.66±0.80 **	31.29±0.89 **	19.76±3.78 **	14.55±9.43 ns	31.35±0.91 **
$[\bar{d}]$	28.18±0.89 **	28.50±0.88 **	28.66±0.91 **	28.38±0.87 **	28.60±0.91 **
$[\bar{h}]$	21.07±1.72 **	8.08±5.49 ns	34.20±4.75 **	46.85±0.22 **	8.40±5.57 ns
$[\bar{i}]$	-	-	11.68±3.94 **	16.66±9.35 *	-
$[\bar{j}]$	-4.66±5.71 ns	-	-3.32±5.73 ns	-	-1.96±5.82 ns
$[\bar{l}]$	-	14.34±5.72 **	-	-7.70±13.62ns	13.96±5.83 **
χ^2	9.40 **	3.78 ns	0.59 ns	0.61 ns	3.67 *

ns, (*), * and ** : not significant, significant at 10%, %5% and 1% probability level respectively.

4.3.1.2 Components of variation

The genotypic structure of each of the six generations (TP_1, TF_1, TF_2, TBC_1, TBC_2 and NP_2) are as follows:

Since tobacco is a selfed pollinating plant, it can be assumed that the non-transgenic parent line (NP_2) has a homozygous genotypic structure due to long term selfing. The 51-1 transgenic parent is also homozygous for the transgene following three rounds of selfing and selection of the resistant phenotype (see Section 4.2.1.1). The TF_1 transgenic progeny has a hemizygous genotypic structure, and the TF_2 plants have all three genotypes including homozygous and hemizygous for transgene as well as susceptible genotype (lack of transgene). The TBC_1 plants have a homozygous and hemizygous genotypic structure for the transgene, and the TBC_2 plants have a hemizygous genotype for the transgene and the susceptible genotype. Therefore, except the parents and the TF_1 generations, the other generations (TF_2, TBC_1 and TBC_2) are under segregation.

Hence genetical variation in either the parents or the hemizygous TF_1 plants should not be present. The variation for each of these three genotypes (TF_1, TP_1 and NP_2) must therefore be related to variations caused by the environment. In relation to the source of the variation among the TF_2 and backcrosses (TBC_1 and TBC_2), these generations contain both genetical and environmental components of variation. The estimates of variance for each of the generations for the leaf necrosis and stem height characters is presented in Table 4.5. Of all the generations, the TF_2 and TBC_2 generations have the highest estimate of variance values. It is indicated that these two generations undergo genetical segregation and that the presence of susceptible (negative) plants in the population of the two generations is the reason for the high level of variance. The low variance in the TBC_1 generation is related to the dominance (hemizygosity effect) of the transgene and this accounts for the similar pattern of herbicide resistance shown by both homozygous and hemizygous genotypes.

Estimates of additive variance components (D), dominance variance components (H) and F (additive dominance covariance), non-heritable variance components (E_w), dominance ratio ($(H/D)^{1/2}$) and $F/(DxH)^{1/2}$ ratio for the leaf necrosis and stem height characters are presented in Table 4.6.

Table 4.5. The variance of necrosis and stem height for different generations

Generation	Necrosis	Stem height
TP_1 (51-1)	0.054	55.62
NP_2 (KKD)	0.152	11.25
TF_1	0.115	47.90
TF_2	0.837	599.61
TBC_1	0.175	236.70
TBC_2	0.904	575.86

Table 4.6. Estimates of D, H, F E_w and ratios for necrosis and stem height

Component	Necrosis	Stem height
	Estimated value	Estimated value
D	1.390	1588.90
H	0.132	689.17
F	-0.829	339.16
E_w	0.109	40.67
$(H/D)^{1/2}$	0.308	0.66
$F/(DxH)^{1/2}$	-1.935	0.32

For necrosis, the additive component of variation (1.390) is more than 10-fold higher than the dominance component of variation (0.132), indicating that the effect of the additive allele(s) is greater than the effect of the dominance allele(s). The high negative value of the additive dominance covariance (-0.829), indicates that the distribution of dominance alleles is responsible for the low necrosis (high herbicide resistance) in the transgenic parent.

The degree of dominance of necrosis is 0.308 and this indicates a partial dominance of herbicide resistance gene(s) in the relevant plants. The dominance ratio is in agreement with the relatively high level of dominance suggested by the analysis of mean (Section 4.3.1.1). The $F/(DxH)^{1/2}$ ratio is -1.935, which is in contrast with the assumption that the highest value of the ratio must be unity (Mather and Jinks, 1982). The overestimate of this ratio is probably due to inter-allelic interaction effects.

For stem height, the additive component of variation is more than 2-fold greater than the dominance variance component, suggesting a greater effect of the additive allele(s) for this character as well. The positive value of F indicates the presence of a dominant allele(s) for higher stem height in the transgenic parent. The value of the degree of dominance, $(H/D)^{1/2}$, is 0.66 which agrees with the relatively high level of dominance suggested by the analysis of mean (Section 4.3.1.1). This ratio indicates a partial dominance of the allele(s) for this character. The $F/(DxH)^{1/2}$ ratio of 0.32 indicates a variation, both in sign and magnitude, of dominance (h) in the different alleles. The values for the narrowsense and broadsense heritabilities of leaf necrosis, as estimated by the Mather and Jinks (1982) method, are 0.83 and 0.87 respectively, while those for stem height are 0.93 and 0.67 respectively. There is a considerable difference between the narrowsense and broadsense heritabilities of stem height, suggesting the presence of non-additive components of variation (dominance and epistasis) which have a significant effect on the stem height character.

The number of effective factors (genes) involved in the expression of the necrosis and stem height characters are 0.72 and 0.73 respectively, suggesting that resistance to the herbicide is controlled by one effective factor. However the number of effective factors for both of the characters are probably underestimated, since these values are both less than unity.

The values of the genetic advance for the necrosis and stem height characters, where 1% of the population with the lowest necrosis and the highest stem height were selected, are 2.01 (towards decreasing necrosis)

and 42.02 mm (towards increased stem height) respectively. These values indicate a significant response of the population to selection for herbicide resistance.

4.3.2 Jinks-Hayman diallel crossing

The quantitative inheritance of herbicide resistance was estimated using leaf necrosis and stem height characters, measured on tobacco seedlings. The parents were three inbred non-transgenic lines, KKD, Kutsaga 51 (K51), Burleigh 21 (B21), and six transgenic lines (49-1, 51-1, 51-3, 51-5, 51-14 and 54-18) as described above. The analysis of the data set using the Jinks and Hayman (1953) method led to an estimate of the additive and dominance effects, and an indication of the presence of epistatic effects of the transgene with the host plant allele(s).

An analysis of variance for the leaf necrosis and stem height characters of the full diallel table are presented in Table 4.7. The significance of the parental variance suggests an additive genetic variation for both attributes. The significance of female x male or genic interaction (i.e. hybrids), indicates differences among the 81 progeny of the crosses in the full diallel table can not be entirely accounted for in terms of additive-dominance variation, and the presence of non-allelic variation is possible (Mather and Jinks, 1982).

The sums of squares for hybrids (interaction) can be partitioned further into reciprocal and genic interactions (Table 4.7). Non significance of the reciprocal mean square against the error mean square (Mather and Jinks, 1977) indicated that there was no maternal or cytoplasmic effect for both of these characters. In cases where there is no evidence for the presence of maternal or cytoplasmic effects (no evidence of differences between the progenies of reciprocal crosses), it is possible to combine the reciprocal progenies together and then analyze the data (Mather and Jinks, 1977; 1982).

Table 4.7. Analysis of variance of parents and reciprocals for necrosis and stem height characters

Source of variation	df	Mean Square (MS)	
		Necrosis	Stem height
Block	2	0.762 **	6890.91 **
Female	8	6.022 **	5264.79 **
Male	8	5.338 **	5447.96 **
FemalexMale	64	0.330 **	376.72 **
Genic interaction	28	0.733 **	841.36 **
Error	160	0.057	102.61
Reciprocal	36	0.018 ns	15.34 ns

ns and ** : not-significant and significant at 1% probability level respectively.

Since there were no differences between the reciprocal crosses, an analysis of variance was carried out, combining the reciprocal crosses as well as the within plot observations. The analysis of variance of the diallel table separately for the parents, TF_1s and pooled (parents together with TF_1s), for the leaf necrosis and stem height characters are presented in Tables 4.8 and 4.9 respectively. The significant differences between the genotypes indicates that the analyses of the diallel crosses data and the estimates of the genetical components and the components of variation are valid.

Table 4.8. Analysis of variance of parent, TF_1 and pooled (parents and TF_1s) for necrosis

Source of variation	Parent		TF_1		Pooled	
	df	MS	df	MS	df	MS
Block	2	0.021 ns	2	0.383 **	2	0.357 **
Genotype	8	2.768 **	35	1.241 **	44	1.508 **
Error	16	0.022	70	0.051	88	0.046

ns and ** : not significant, significant at 1% probability level respectively.

Table 4.9. Analysis of variance of parents, TF_1s and pooled (parents and TF.s) for stem height

Source of variation	Parent		TF_1		Pooled	
	df	MS	df	MS	df	MS
Block	2	129.4 *	2	3452.7 **	2	3322.5 **
Genotype	8	2692.9 **	35	1224.6 **	44	1475.9 **
Error	16	381.5	70	105.5	88	94.1

ns, * and ** : not significant, significant at 5% and 1% probability levels respectively.

The analyses for mean leaf necrosis and stem height of diallel crosses for parents are presented in Table 4.10. There are significant differences in the levels of leaf necrosis as well as in the stem height between the parental lines. The mean level of leaf necrosis and stem height of F_1 and TF_1 progeny (hybrids) resulting from crosses between the relevant parental lines are presented in Tables 4.11 and 4.12 respectively. For leaf necrosis, the 49-1 transgenic parent shows the lowest leaf necrosis (high resistance) of the all parental lines (Table 4.11). In contrast, the non-transgenic lines show the highest necrosis (lowest resistance) of the parental lines. The F_1 progeny resulting from crosses between non-transgenic parents show high susceptibility (high leaf necrosis) to the herbicide compared to that of the other TF_1 progeny resulting from crosses between transgenic parents. However, in most of the crosses, the TF_1 progenies resulting from a cross between resistant transgenic and susceptible non-transgenic parents show a high level of resistance to the herbicide (lowest leaf necrosis). Furthermore, the susceptible transgenic parent, 51-14, and TF_1 progenies derived from a cross between this line and non-transgenic parents, show significantly low resistance to the herbicide (Table 4.11).

For stem height, the KKD, K51, B21 and 51-14 parents (Table 4.12) show the lowest values (lowest resistance). In contrast, the other transgenic parental lines, especially the 49-1 parent, show the highest stem height of the parental lines. The F_1 progenies derived from crosses between K51xB21, KKDxK51, B21xKKD non-transgenic lines and the TF_1 progeny

resulting from crosses between KKDx51-14, 51-14xK51, 51-14xB21 and 51-5xB21 show the lowest stem height (lowest resistance) of all the crosses (Table 4.12). The TF_1 progenies derived from crosses between non-transgenic and transgenic parents (except for the 51-14 line), show stem heights similar to those of the resistant transgenic parents. However, in crosses involving the 51-14 transgenic parent and non-transgenic parents, the TF_1 progeny have low stem heights, while for crosses involving the 51-14 parent and resistant transgenic parents, the TF_1 progenies have stem heights similar to those of resistant parents. This indicates that the expression of the low resistant phenotype in 51-14 line is suppressed by the resistant transgenic lines.

In one of the crosses (51-5xB21), it is evident that the transgene expression in the transgenic parent (51-5) is suppressed by the susceptible non-transgenic parent (B21). This could be due to effects of background allele(s) of the non-transgenic B21 parent. However, in crosses between the 51-5 parent and the other transgenic or non-transgenic lines, the hybrid progenies have a resistance phenotype similar to the 51-5 transgenic parent. Hence this indicates that only on some occasions could the expression of the transgene be suppressed by the host background genome. This is type of inter-allelic interaction (epistasis) between host plant allele(s) and the transgene. The statistical analysis results also indicate the involvement of epistasis for herbicide resistance (see below).

Table 4.10. Mean necrosis and stem height of tobacco parental lines of diallel table

Line	Character	
	Necrosis score	Stem height
KKD	2.89 a	5.11 d
K51	2.40 ab	9.50 cd
B21	2.18 ab	12.54 cd
49-1	0.13 d	90.54 a
51-1	0.89 c	53.13 b
51-3	0.68 c	52.49 b
51-5	0.89 c	57.70 b
51-14	2.03 ab	9.68 cd
54-18	0.73 c	60.33 d
$LSD_{5\%}$	0.35	5.6

Means with the same letter within the columns are not significantly different.

Table 4.11. Mean necrosis of different crosses of diallel table

	KKD	K51	B21	49-1	51-1	51-3	51-5	51-14
K51	2.78 ab							
B21	2.54 bc	2.45 bcd						
49-1	1.28 fgh	0.68 nopqr	0.65 opqr					
51-1	1.01 hijklmn	1.1 hijk	1.26 fghi	0.30 st				
51-3	1.60 f	1.09 hijk	1.05 hijklm	0.34 rst	0.83 klmnop			
51-5	1.24 ghij	1.03 hijklm	2.34 cde	0.44 qrst	0.99 hijklmn	1.00 hijklmn		
51-14	2.16 de	2.23 cde	2.05 e	0.92 hijklmn	1.08 hijkl	1.16 ghijk	1.49 fg	
54-18	1.06 hijkl	1.12 hijk	1.05 hijklm	0.51 pqrs	1.06 hijkl	0.50 pqrs	0.71 mnopq	1.12 hijk

Means with the same letter are not significantly different.

Table 4.12. Mean stem height of different crosses of diallel table

	KKD	K51	B21	49-1	51-1	51-3	51-5	51-14
K51	5.1 k							
B21	4.9 k	9.2 k						
49-1	55.0 defghij	69.8 bcd	81.4 ab					
51-1	46.5 ghij	54.4 defghij	48.7 efghij	69.5 bcd				
51-3	40.6 ghij	53.8 defghij	55.0 defghij	70.8 bcd	45.0 ghij			
51-5	40.8 ghij	56.3 defghij	16.2 k	76.2 abc	53.9 defghij	52.4 defghij		
51-14	9.8 k	14.2 k	19.5 k	58.6 cdefgh	45.0 ghij	45.6 ghij	48.9 efghij	
54-18	39.7 hij	36.6 j	38.1 ij	67.9 bcde	55.7 defghij	47.7 fghij	59.7 cdefg	54.6 defghij

Means with the same letter are not significantly different.

4.3.2.1 Basic array statistics

The basic array statistics, mean variance of arrays ($\bar{V}r$), variance of parents (VP), covariance of arrays ($\bar{W}r$), variance of arrays mean ($V\bar{r}$) and covariance of arrays/variance of arrays (Wr/Vr) regression equation, as well as the F-test for regression for both characters are presented in Table 4.13. The regression was significant as shown by F significant, large coefficient of variation (R^2), and ($\hat{\beta}$) being significantly greater than zero. Since the regression slope ($\hat{\beta}$) was significantly different from zero, it was therefore appropriate to conduct a t-test on the departure of the $\hat{\beta}$ from unity (Mather and Jinks, 1982).

The Wr/Vr regression plot for necrosis and stem height, including R^2 and regression slope ($\hat{\beta}$) are presented in Figure 4.1. The R^2 and regression slope estimates are 0.93 ± 0.9 and 0.85 ± 0.9 respectively for leaf necrosis and 0.93 ± 0.9 and 0.88 ± 0.9 respectively for stem height. The genetical component of variation and other statistics for both of the characters are presented in Table 4.13. The analysis of variance for necrosis indicates that there are significant differences between (Wr+Vr) parental arrays, while the non-significance of (Wr-Vr) between parental arrays indicates homogeneity of (Wr-Vr) arrays. The homogeneity of (Wr-Vr) between the parental arrays reveals that the data may be adequately accounted for by an additive-dominance model for the necrosis character. The heterogeneity of (Wr+Vr) suggests the presence of dominance allele(s). The non-significance of the t-test indicates that the regression slope ($\hat{\beta}$) does not significantly deviate from unity. The significance of (Wr+Vr) and $\hat{\beta}=1$ suggest the presence of dominance for the necrosis character. In the present study there is good agreement between the regression analysis and (Wr-Vr), the supporting the adequacy of the additive dominance model for the necrosis character. Furthermore, the result suggests an absence of inter-allelic interaction for this character.

For stem height, the analysis of variance indicates significant differences between both (Wr+Vr) and (Wr-Vr) parental arrays (Table 4.13). The heterogeneity of (Wr+Vr) indicates the presence of non-additive variation (dominance) for this character, while the significance of (Wr-Vr) suggests the presence of inter-allelic interaction and/or correlated gene distribution for the stem height character. The result of the parental array analysis indicates that the presence of inter-allelic interaction (epitasis) between the transgene and the host plant genome is possible. Thus, there is a possibility of interaction between the transgene and host tobacco allele(s) which are involved in plant stem height. A high level of additive allele effects were indicated by the D estimates in both characters. Relatively high values of D for necrosis (0.86) and stem height (789.46), indicate the presence as well as the importance of the additive component of variation for both characters (Table 4.13).

The estimates of H_1 and H_2 (dominance) for the leaf necrosis character are 0.20 and 0.31 respectively, and for the stem height character are 49.15 and 153.23 respectively (Table 4.13), indicating the presence of dominant allele(s). The significant of dominance generally indicates the resistance of hemizygous transgenic genotypes (TF_1s) to the herbicide. The estimate of the additive dominance ratio, $(H_1/D)^{1/2}$, is 0.41 and 0.25 for necrosis and stem height respectively, which indicates partial dominance for both of these characters. As a comparison between these characters, the effect of the dominance allele(s) for the necrosis character is greater than that of the stem height character. It is suggested that the inequality of H_1 and H_2 for both characters is derived from an unequal frequency of dominant and recessive alleles in the parents. Furthermore, the $\tilde{uv}$ is 0.40 and 0.78 for leaf necrosis and stem height respectively, indicating unequal allele frequency in the relevant loci for both characters. The positive values of F for both characters shows that there are more dominant allele(s) in the parental lines than recessive alleles, irrespective of whether they have an increased or decreased effect on the character (Mather and Jinks, 1982). There is no estimate for $0.5F/[D(H_1-H_2)]^{1/2}$ because the estimate of H_2 is

bigger than that of H_1, leading to a negative result inside the parenthesis (square root of a negative value is impossible). It is suggested that this could result from an overestimate of the H_2 component or an underestimate of the H_1 component.

Correlations between the common parent mean and (Wr+Vr) for the leaf necrosis and stem height characters are highly significant (0.91 and -0.85), with positive and negative values respectively. The positive value as well as the significance of the correlation coefficient for the necrosis character indicates that the direction of dominance is towards the parents with a low necrosis phenotype (resistant phenotype). Thus the parents with low necrosis show dominance over those with high necrosis (low resistance). The positive value and significance of the correlation coefficient for the stem height character suggests that the allele(s) which lead to greater stem height in the presence of the herbicide are dominant. Furthermore, the distribution of dominant to recessive allele(s) is correlated with a common parent phenotype for both of the characters.

The broadsense heritability and narrowsense heritability values are 0.91 and 0.74 for leaf necrosis and 0.80 and 0.72 for stem height respectively. The heritability of these characters indicates that the genetical component of variation makes up the major proportion of the component of variation for both of the characters. The narrowsense heritabilities are lower than the broadsense heritabilities for both characters, indicating the importance of the effect of the dominant allele(s) (H_1 and H_2) on the heritability of each of the characters. Although the narrowsense heritability of the characters is similar, the broadsense heritability for leaf necrosis is higher than that of stem height, suggesting that the effects of the dominant allele(s) are greater for necrosis than those for the stem height character.

In a graphical analysis, the parental arrays can be compared with respect to the dominance allele(s) by plotting the covariance of parental arrays (Wr) against the variance of parental arrays (Vr), as illustrated in Figure 4.1 for the necrosis and stem height characters. Any (Wr/Vr) point close to the origin corresponds to parental arrays with the greatest

proportion of dominant alleles. With regarding to the high value of R^2 for both necrosis and stem height (0.93), it is interpreted that epistasis, correlated gene distribution and/or environmental effects do not interfere with dominance.

Table 4.13. Array statistics and components of variation for necrosis score

Statistics	Character	
	Necrosis	Stem height
Regression equation	Wr=0.17+0 85Vr	Wr=127.88+0.88Vr
F-test	90 03**	88.38**
V_p	0.88	813.30
$\bar{V}r$	0.30	300.41
$\bar{W}r$	0.43	329.22
$V\bar{r}$	0.18	176.60
t-test	ns	ns
Wr+Vr	**	**
Wr-Vr	ns	**
D	0.86	789.46
H_1	0.20	49.15
H_2	0.31	153.23
F	0.30	20.82
$(H_1/D)^{1/2}$	0.41	0.25
$0.5F/[D(H_1xH_2)]^{1/2}$	-	-
$\bar{u}\bar{v}$	0.40	0.78
Dom./rec.	1.08	1 11
$r_{(Pr,Wr+Vr)}$	0.91	-0.85
h^2_{BS}	0.91	0.80
h^2_{NS}	0.74	0.72
K(Effective factor)	0.5	0.70

ns and ** : not significant and significant at 1% probability level respectively.

In relation to Figure 4.1 (necrosis), it is concluded that the 51-1 (5) transgenic parent had the highest proportion of dominant allele(s), while the non-transgenic parents, KKD (1), B21 (2), and K51 (3) have the lowest proportion of dominant alleles compared to the other parental arrays. The other transgenic arrays, except 51-14 (8), were scattered around the origin which indicates the presence of a high proportion of dominant allele(s) in these transgenic parents. The results indicate that dominance is an important genetical component of variation for the character. The result of the analysis of (Wr+Vr), (Wr-Vr) and H_1 components also indicate that dominance is the major genetical component, as discussed above.

A graphical analysis for stem height (Figure 4.1) indicates that the non-transgenic parents K51 (2), B21 (3) and KKD (1) have the highest recessive allele(s), while the transgenic lines, 51-1 (5), 51-3 (6) 49-1 (4) and 54-18 (9) show the greatest proportion of dominant allele(s) between the parental lines. The 51-5 (7), 51-14 (8) and KKD (1) parental lines showing no dominance, hence it is suggested that additive alleles are predominant for these lines.

The estimate of the number of effective factors (genes) involved in resistance to herbicide is 0.5 and 0.7 for necrosis and stem height respectively. It is suggested that for the genetic control of herbicide resistance, one gene has a major effect. With regard to the estimated values of the effective factors (both less than one), it is suggested that these values are possibly underestimated.

In summary, resistance to the herbicide (low leaf necrosis as well as high stem height) is controlled by both additive (homozygous) and dominance (hemizygous) alleles. Thus both the homozygous and the hemizygous transgenic genotypes show significantly high levels of resistance to the herbicide. However, the hemizygous transgenic phenotypes show slightly more resistance to the herbicide than the transgenic hemizygous genotypes (partial dominance). For the stem height character, besides the additive and dominance effect, inter-allelic interactions (epistasis) between

the transgene and host tobacco allele(s) and/or correlated genes distribution are possible. However, there is no evidence for the involvement of inter-allelic interaction for the necrosis character. For both of these characters, the additive component of variation is more important than the dominance component of variation and the dominance is partial and towards low necrosis as well as high stem height. The distribution of dominant allele(s) is more frequent in transgenic parents while the distribution of recessive alleles(s) is observed in non-transgenic parents.

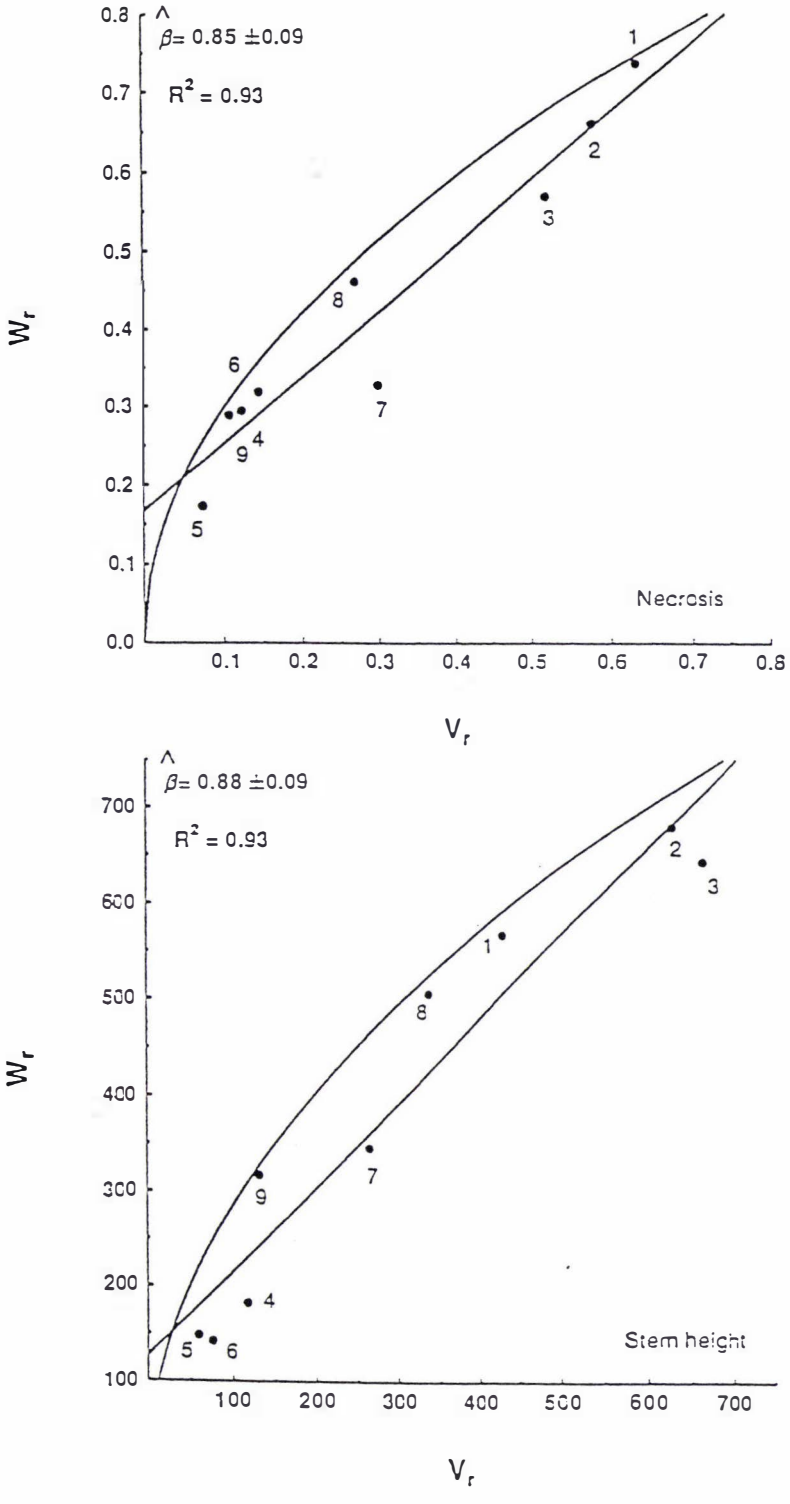


Figure 4.1. The Wr/Vr graph of necrosis and stem height of tobacco lines. Nine tobacco lies: KKD (1), B21 (2), K51 (3), 49-1 (4), 51-1 (5), 51-3 (6), 51-5 (7), 51-14 (8) and 54-18 (9) in response to the herbicide. The straight line is a regression line and the curvilinear line is a limiting parabola (Wr^2 = VrxVp).

4.3.3 Griffing diallel

The analysis of the diallel data by the Griffing method (Griffing, 1956b) was conducted to estimate the general and specific combining ability effects, as well as the variances of general and specific combining abilities. Although the Griffing method for analysing the data gives less information on gene action, the information obtained by the method is very useful for applied plant breeding, since the method gives a useful result for the selection of the best parental line(s) as well as the best progeny derived from a cross between the parental lines.

The analysis of variance of general and specific combining ability (GCA and SCA) for model I and model II assumptions are given in Tables 4.14 and 4.15 for leaf necrosis and stem height respectively. The analysis of variance revealed that both the variances of general combining ability and the specific combining ability for both models were highly significant for both characters. The significance of the variance of general combining ability revealed the presence of additive genetic variance, with a possibility of epistasis (Griffing, 1956b). The high significance of the specific combining ability variance indicated the presence of dominance variance, with the possible presence of additive x dominance and dominance x dominance epistasis. These general and specific combining ability variance components are presented in Tables 4.14 and 4.15 for necrosis and stem height respectively. For both of the characters, the general combining ability variance is significantly greater than the specific combining ability variance, indicating the preponderance effect of additive allele(s). As we are dealing here with the homozygosity or hemizygosity effect for the transgene, the dominance effect can be referred to as the hemizygosity effect. Hence, the variance of specific combining ability is probably best interpreted as an indication of a hemizygosity variance and an epistasis effect.

Table 4.14. Analysis of variance of GCA and SCA effects for the necrosis character

Source of variation	Model I		Model II		
	df	MS	df	MS	Variance Component±s.e.
GCA	8	2.311 **	8	2.030 **	0.201±0.105
SCA	36	0.101 **	36	0.101**	0.072±0.024
Error	1080	0.006	88	0.03	

** : significant at 1% probability level.

Table 4.15. Analysis of variance of GCA and SCA effects for the stem height character

Source of variation	Model I		Model II		
	df	MS	df	MS	Variance Component±s.e.
GCA	8	2185.500**	8	2.030 **	188.339±99.424
SCA	36	115.397**	36	0.101**	56.066±28.633
Error	1080	4.361	88	0.031	

** : significant at 1% probability level.

With respect to the relationship between the genetic components of the variations, Griffing (1956b) suggested that the total component of genetic variation amongst the progenies of the cross between two homozygous parents is equal to twice the GCA variance component plus the SCA variance component. On the other hand, the GCA variance component is equal to twice the additive (additive x additive is possible) variance component, while the SCA variance corresponds to the dominance (epistasis possible) component of variation. Total genotypical component of variations

with respect to variance of general combining ability ($2\sigma^2_g$) and variance of specific combining ability (σ^2_s) can be defined by the following formula: $\sigma^2_G = 2\sigma^2_g + \sigma^2_s$. On the other hand, the total genetic variance (σ^2_G) is equal to the additive genetic variance ($2\sigma^2_g$) plus the dominance genetic variance (σ^2_s). The additive dominance ratio is a good indicator of progeny performance in selection programmes and can be calculated using the following formula:

$$2\sigma^2_g/(2\sigma^2_g+\sigma^2_s)$$

This ratio in the present study is 0.848 and 0.913 for necrosis and stem height respectively (Table 4.16), suggesting that the variance of specific combining ability (σ^2_s) or dominance component of variation is of little importance compared to that of the additive component of variation (σ^2_g).

The broadsense and narrowsense heritability estimates are 0.999 and 0.979 for leaf necrosis and 0.982 and 0.962 for stem height respectively (Table 4.16). There is little difference between the narrowsense and broadsense heritabilities for both of these characters. It is suggested that the similarity between the broadsense and narrowsense heritability estimates is related to the considerably lower σ^2_s component compared to that of the σ^2_g component. An analysis of the diallel data using the Griffing method led to a significant underestimate of the dominance component of variation (σ^2_s) and an overestimate of the additive component of variation ($2\sigma^2_g$).

Table 4.16. Additive dominance ratio, broadsense heritability and narrowsense heritability for necrosis and stem height

	Necrosis	Stem height
Ratio	0.848	0.913
h^2_{BS}	0.999	0.987
h^2_{NS}	0.979	0.962

The estimates of general combining ability and specific combining ability effects (g.c.a. and s.c.a. effects, see Section 4.2.2.5), together with the significance test for leaf necrosis and stem height characters are presented in Tables 4.17 and 4.18 respectively. The estimates of g.c.a. effects are given on the leading diagonal of the Tables, while the s.c.a. effects are given below the leading diagonal of the Tables. For the leaf necrosis character (Table 4.17), the occurrence of genotypes with negative and significant values indicates a significant g.c.a. effect with respect to resistance to herbicide (low necrosis). The KKD, B21, K51 and 51-14 parents contributed genes to their progenies conferring susceptibility to the herbicide (significant positive g.c.a. estimate). In contrast the lines, 49-1, 54-18, 51-3, 51-1 and 51-5 contributed genes conferring resistance, as indicated by significant negative g.c.a. estimated values. There was considerable variation for this general combining ability. The line 49-1 shows the greatest g.c.a. with a negative estimated value (-0.663), showing the best general combining ability of all of the parental lines.

The s.c.a. estimated values for progenies derived from crosses between the three non-transgenic parents (KKD, K51 and B21) all are positive and significant, indicating transmission of susceptibility to the herbicide form non-transgenic parents to their hybrid progenies derived from crosses between these parents. However significant and negative s.c.a. estimates for hybrid progenies derived from crosses between transgenic parents (except 51-14 parent) and the non-transgenic parents, indicate herbicide resistance for these transgenic hybrid progenies (except 51-5xB21). Significantly negative s.c.a. estimated values for hybrid progenies derived from crosses between transgenic parents, indicate that these hybrid progenies exhibit herbicide resistance similar to the transgenic parents.

Table 4.17. Estimates of g.c.a. effects (leading diagonal) and s.c.a. effects (below diagonal) for necrosis character

	KKD	K51	B21	49-1	51-1	51-3	51-5	51-14	54-18
KKD	**0.617 ****								
K51	0.479 **	**0.419 ****							
B21	0.189 **	0.302 **	**0.463 ****						
49-1	0.055 ns	-0.347 **	-0.415 **	**-0.663 ****					
51-1	-0.575 **	-0.294 **	-0.172 **	0.006 ns	**-0.295 ****				
51-3	0.059 ns	-0.257 **	-0.340 **	0.074 ns	-0.202 **	**-0.339 ****			
51-5	-0.588 **	-0.497 **	0.772 **	-0.005 ns	-0.176 **	-0.233 **	**-0.1597 ****		
51-14	-0.045 ns	0.214 **	-0.010 ns	-0.008 ns	-0.220 **	-0.099 ns	0.054 ns	**0.328 ****	
54-18	-0.454 **	-0.195 **	-0.307 **	-0.276 **	-0.455 **	-0.055 ns	-0.030 ns	-0.105 *	**-0.369 ****

ns, * and ** : not significant, significant at 5% and 1% probability level respectively.
Standard error of general combining ability = (0.016)
Standard error of specific combining ability = (0.051)

The estimates of g.c.a and s.c.a effects for stem height (Table 4.18) indicate that all parental lines (except for 51-14) show significant and positive g.c.a. estimates. The non-transgenic lines KKD, B21, and, K51 show significant negative g.c.a. estimated values, indicating a significant g.c.a. estimate in the direction of low stem height (susceptible). The 49-1 parent shows the highest positive g.c.a. estimates of the parents (highly resistant) while the 51-14 parent shows non-significant g.c.a. estimates.

The s.c.a. estimates for crosses between the non-transgenic parents are significant with negative values, while most of the TF_1 progenies derived from crosses between resistant transgenic and non-transgenic parents are significant with positive values, indicating the dominance of the herbicide resistance phenotype in these crosses. There is considerable variation in the s.c.a. estimates between the TF_1 progenies derived from crosses between the transgenic parents.

Tables 4.17 and 4.18 indicate that transgenic parents with the resistance phenotype are dominant over those with the susceptible phenotype, irrespective of whether they are transgenic or non-transgenic parents. However, an exception is the progeny derived from the 51-5xB21 cross which have a high susceptibility to the herbicide, although the 51-5 transgenic parent is a resistant line. It is suggested that an interaction between the transgene and host plant allele(s) (epistasis) leads to either a suppression or a decrease in expression of the transgene in these progenies.

With respect to the interactions between the resistant and susceptible lines, when the 51-14 low resistant transgenic parent was crossed with those of the resistant transgenic parents, all of the hybrid progenies exhibited a resistant phenotype similar to the resistant parent. This indicates that resistant parents are dominant over the low resistant parent. The s.c.a. estimate of the progenies derived from crosses between transgenic lines and non-transgenic lines in the majority of cases are significant with negative values, indicating that the production of highly resistant hybrid progenies

derived from crosses between transgenic and non-transgenic parents is possible.

Table 4.18. Estimates of g.c.a. effects (leading diagonal) and s.c.a. effects (below diagonal) for stem height character

	KKD	K51	B21	49-1	51-1	51-3	51-5	51-14	54-18
KKD	**-17.71 ****								
K51	-10.19 **	**-11.72 ****							
B21	-8.55 **	-10.21 **	**-13.59 ****						
49-1	2.24 ns	11.11 **	24.55 **	**25.69 ****					
51-1	12.44 **	14.38 **	10.49 **	7.96 **	**7.02 ****				
51-3	7.36 **	14.60 **	17.60 **	5.88 *	13.01 **	**6.20 ****			
51-5	7.19 **	16.70 **	-21.53 **	-0.84 ns	4.44 ns	5.12 *	**6.59 ****		
51-14	-7.08 **	-8.69 **	-1.47 ns	-1.65 ns	3.35 *	4.80 *	7.73 **	**0.19 ns**	
54-18	4.96 *	4.16 ns	0.82 ns	10.27 **	3.77 ns	10.96 **	0.62 ns	12.30 **	**7.73 ****

ns, * and ** : not significant, significant at 5% and 1% probability levels respectively.
Standard error of general combining ability = (0.016)
Standard error of specific combining ability = (0.051)

4.4 Discussion

4.4.1 Generation means

4.4.1.1 Components of means

Knowledge of the type of gene action involved in the expression of a particular character is useful for choosing breeding procedures to be used for improving that character. The additive and dominance components can be defined as a homozygous and hemizygous allele effect in the present study. The significance of additive alleles, indicates the importance of homozygous alleles in homozygous plants in expression of the resistance character, while the significance of dominance in present study indicates the significance of hemizygosity in expression of the resistance phenotype. The significance of both additive and dominance effects in the present study indicates that both homozygous genotypes and hemizygous genotype exhibit a herbicide resistance phenotype. Where the additive gene action signifies that standard selection procedures would be effective in improving the character, the dominance gene action signifies the importance of production of hybrids.

The main propose of the genetical designs are to estimate the genetic parameters and provide basic information regarding the gene effects in a plant population, such information being necessary for plant breeding. The analysis of generation means provides a basic and useful information about gene action as well as inter-allelic interactions (epistases). Through generation means analysis, it is possible to provide detailed information on additive, dominance, and all types of epistasis including additive x additive, additive x dominance and dominance x dominance (Mather and Jinks, 1977; 1982)

In the present study although the dominance (hemizygosity) gene effect is smaller than the additive (homozygosity) gene effect, the dominance is a highly significant factor in herbicide resistance. In fact the performance of TF_1 (Table 4.2) deviates significantly from mid-parent and is closer to the transgenic parent, indicating almost full dominance for herbicide resistance. Therefore, the hemizygous TF_1 plants show a herbicide resistant phenotype similar to the homozygous transgenic parent.

The differences between mean leaf necrosis and stem height of the transgenic parent (TP_1), TF_1 and TBC_1 are not significant, indicating a similarity in

phenotype for these three genotypes. The similarity of TF_1 and TBC_1 to the transgenic parent (TP_1) indicates a similarity between hemizygous and homozygous genotypes which facilitates the resistance of the TF_1 and TBC_1 progeny to the herbicide (giving low leaf necrosis and high stem height values). The leaf necrosis of the non-transgenic parent is significantly higher, and its stem height lower, than that of the other genotypes, reinforcing the distribution of the herbicide resistant allele(s) in the transgenic parental line.

In the present study, the results of means analysis indicate the presence of additive, dominance and inter-allelic interactions (epistases) for both characters. Therefore, the full model with six parameters is more reliable. Furthermore, in the scale test, all of the six generations are not considered together in the estimate. The result of the generation means analysis indicate the involvement of additive [$\bar{d}$], dominance [$\bar{h}$], and three types of epistasis (additive x additive [$\bar{i}$], additive x dominance [$\bar{j}$], dominance x dominance [$\bar{l}$]) for leaf necrosis, as well as additive, dominance and additive x additive epistasis for stem height. It should be noted that in the present study, the dominance effect is actually the hemizygosity effect as discussed above. For leaf necrosis, the significance of additive [$\bar{d}$], and dominance [$\bar{h}$] indicates that the distribution of additive and dominance allele(s) in the parents, as well as the interaction between the transgene(s) and host tobacco allele(s) in the form of additive x additive [$\bar{i}$], additive x dominance [$\bar{j}$] and dominance x dominance [$\bar{l}$], is possible. However for stem height, additive [$\bar{d}$], dominance [$\bar{h}$], and only the additive x additive [$\bar{i}$] type of epistasis is significant.

In relation to epistasis, it is concluded that in the host plant genome, the allele(s) involved in controlling a particular character have a special type of interaction with the transgene as discussed above. In general, the more important epistases detected by the six parameter model were of additive x additive, additive x dominance, dominance x dominance for necrosis and additive x additive for stem height. The non significance of other types of epistasis for stem height may be due to cancelling positive and negative effects from different loci.

For leaf necrosis, the negative values of additive [$\bar{d}$] and dominance [$\bar{h}$],

suggest a distribution of additive and dominant allele(s) in the transgenic parents with a low leaf necrosis. Although the dominance gene effect is smaller than the additive gene effect, the effect of dominance is highly significant and indicates that dominance is a significant factor in herbicide resistance. The degree of dominance as estimated by the departure of TF_1 and the parents mean from the mid-parent, is 0.95. In fact the perfonmance of TF_1 (Table 4.2) deviates significantly from the mid-parent and is closer to the transgenic parent, indicating almost full dominance for herbicide resistance. In view of the significant additive genetic variance for both of the characters, it is concluded that the additive effect contributes significantly to the inheritance of the herbicide resistance trait.

The importance of a fixable gene effect in the form of an additive effect for herbicide resistance indicates the potential for improvement in these characters through selection. Any selection system that utilizes additive variance should be effective. Among these systems, mass selection, pure line selection and other procedures which can lead to the selection of resistant plants would be a suitable procedure.

4.4.1.2 Components of variation

The additive component of variation is much higher than the dominance component of variation for both the leaf necrosis and the stem height characters, suggesting the greater importance of the additive component compared to the dominance component, as mentioned above. The dominance ratio, as estimated using the component of variation, is lower than the degree of dominance estimated by the parent and TF_1 mean for both characters.

The $(H/D)^{1/2}$ indicated partial dominance for resistance to the herbicide for both of the characters, which is in agreement with that estimated by mean analysis. However the dominance ratio estimated here for $(H/D)^{1/2}$ is lower than that estimated by mean analysis. The difference between the two dominance ratio estimates are probably due to the magnitudes of single additive alleles (h's)) and single dominance alleles (d's), where dominance ratio $(H/D)^{1/2}$ provides an estimate of the average dominance of genes. The additive dominance estimates indicate that the additive component of variation is an important component of variation and that dominance for the characters was partial. The $(H_1/D)^{1/2}$ were 0.41 and 0.25 for

leaf necrosis and stem height respectively, indicating partial dominance for resistance to the herbicide. For leaf necrosis, the high negative value of F (covariance of additive dominance effects), indicates that the allele(s) controlling this character are distributed in the transgenic parent. The high positive value of F for stem height (high stem height) indicates that the allele(s) affecting stem height are also distributed in the transgenic parent.

In theory the estimate of the $F/(DxH)^{1/2}$ ratio should reach a maximum of unity if all of the [h] values have a similar sign. Otherwise, where some h values are negative and some positive, the ratio is expected to be less than unity or $F<(DxH)^{1/2}$. Thus the ratio is expected to vary between zero and one. In the present study, the ratio is greater than unity for the necrosis character, in contrast with the expected theoretical value. The overestimate of the F value is probably caused by the presence of inter-allelic interactions (epistasis), correlated genes, and/or a different effect of environment on successive generations. However the ratio was positive and less than unity for the stem height character (0.32), suggesting that there are variations in sign of the h values. Thus some of the alleles show negative dominance, while the others show positive dominance.

The estimates of broadsense heritabilities for both of the characters are significantly high. This high broadsense heritability indicated that in the expression of the herbicide resistant phenotype, the genotypic part is much greater than the non-genotypic (non-heritable) part. The broadsense heritability of the characters is considerably higher than the narrowsense heritability. And this indicated that the dominance (hemizygosity) effect is significant in the expression of the herbicide resistance character. In addition, high values of heritability indicate the expression of resistance to the herbicide in subsequent generations. In relation to the high heritability of the characters, it is suggested that this results from a high genetic component of variation, since there is a wide range of variations between the genotypes of the parents. For a plant breeding point of view, the higher estimates of the narrowsense heritability indicate that the improvement of plants towards herbicide resistance is expected to be rapid with a high efficiency.

The genetic advance value predicts what gain from selection can be achieved in each cycle of selection. This depends largely on the heritability of the character, phenotypic variance, and the proportion of the population being selected. The estimates of the genetic advance are 2.01 and 42.02 for leaf necrosis and

stem height respectively. These values indicate that for necrosis, at one cycle of selection, a plants with reduction of 2.01 in their leaf necrosis score and an increase of 42.02 cm in their stem height can be achieved. The considerably higher genetic advance for herbicide resistance indicates a greater response of the successive generations for selecting herbicide resistant plants. Furthermore, the high value of the genetic advance indicates a distribution of additive allele(s) in the transgenic parent which thereby facilitates a high response to selection in subsequent generations. The estimate of effective factors was less than one (minimum number of effective factors) for both the necrosis and stem height characters. It is suggested that the number of effective factors (genes) is underestimated due to presence of epistasis (Mather and Jinks, 1977).

4.4.2 Jinks-Hayman diallel crossing

The mean analysis for the stem height and leaf necrosis characters, indicated that the KKD, K51, B21 and 51-14 parents (Table 4.10) showed susceptibility to the herbicide. In contrast, the other transgenic parents, especially the 49-1 parent, showed the highest resistance of all parents.

The mean analysis of the stem height and necrosis characters, for the F_1 and TF_1 progenies derived from different parental lines indicated a remarkable variation in resistance to the herbicide. The F_1 hybrid progenies derived from crosses between the non-transgenic lines were susceptible to the herbicide (Tables 4.11 and 4.12). The TF_1 hybrid progenies derived from crosses between the non-transgenic and the transgenic parents (except for the 51-14 line), had stem height similar to those of the resistant transgenic parents. In crosses involving the 51-14 transgenic parent and non-transgenic parents, the TF_1 hybrid progenies were susceptible to the herbicide, while for crosses involving the 51-14 parent and the resistant transgenic parents, the TF_1 hybrid progenies were resistant the herbicide similar to those of the resistant parents. This indicates that the low resistant genotypes were suppressed by the resistant genotypes.

For (51-5 x B21) TF_1 hybrid progeny, it is evident that the transgene in the transgenic parent (51-5) is suppressed by the susceptible non-transgenic parent (B21). However, in crosses between the 51-5 parent and the other either transgenic or non-transgenic parents, the hybrid progenies show herbicide resistance similar

to the 51-5 transgenic parent. This indicates that only in one of the crosses of 51-5 parent is the expression of the transgene suppressed by the host background genome by means of epistasis. The interaction effect between the allele(s) (epistasis) will be discussed in Section 4.4.4.

The analysis of the diallel data sets for both leaf necrosis and stem height indicate that estimates of additive components of variation (D) are greater than the estimate of dominance components of variation (H_1 and H_2). In other words, the additivity was of major importance for the low necrosis and high stem height in the presence of the herbicide. This conclusion is supported by the significance of (Wr+Vr) between arrays for both characters.

The homogeneity of the (Wr-Vr) arrays suggests the absence of epistasis (Mather and Jinks, 1982). In the present study, the estimate of the (Wr-Vr) values is not significant for the leaf necrosis character, suggesting the absence of an epistasis (inter-allelic interaction). However, the estimate is significant for the stem height character and suggests the presence of an inter-allelic interaction. Hayman (1954b) stated that in an analysis of diallel crossing data, the (Wr-Vr) should be in agreement with the regression analysis. The presence of epistasis can be shown by the significance of (Wr-Vr) or by the significant deviation of the Wr/Vr regression coefficient ($\hat{\beta}$) from unity. In the present study, the result of the analysis of (Wr-Vr) arrays (non-significant) is in agreement with the estimate of the regression coefficient ($\hat{\beta}=1$) which does not significantly deviate from unity for leaf necrosis. However, there is disagreement between the (Wr-Vr) analysis and the regression result for the stem height character. For this character, (Wr-Vr) is significant and indicates the presence of a inter-allelic interaction, while estimates of the regression coefficient ($\hat{\beta}$) do not show deviation from unity. This disagreement between these two results indicated the presence of epistasis between the transgene and host plant allele(s) (inter-allelic interaction) for the stem height character (Mather and Jinks, 1982). However, Matzinger *et al.* (1960, 1966, 1972), in relation to the assumption of absence of inter-allelic interaction (epistasis) in diallel analysis, suggests that the failure of this assumption does not alter the relative importance of additive and dominance variances.

All types of epistases can be estimated for TF_1, TF_2 and backcrosses using the generation mean analysis (Mather and Jinks, 1977; 1982; Hayman, 1958b), as

discussed above. From a generation means analysis it is possible to provide detailed information on additive, dominance and all kinds of inter-allelic interactions (additive x additive, additive x dominance additive x additive).

The products of frequency of dominance and recessive alleles ($\bar{uv}$) for necrosis and stem height are 0.40 and 0.78 respectively, suggesting an unequal distribution of the transgene in the parents. Differences in parents with respect to the presence of the transgene only in transgenic parents is suggested by a deviation of the estimate from 0.25 (u=v=0.5 and uv=0.25). This deviation from 0.25 is due to an inequality in the number of transgenic parents which have the transgene in their genomes and non-transgenic parents which lack the transgene. The values of F (0.30 and 20.81 for leaf necrosis and stem height respectively) as well as dominance/recessive ratio values (1.08 and 1.11 for necrosis and stem height respectively) indicated that the dominant alleles are more frequent for both of the characters.

In a graphical analysis (Figure 4.1), a similar distribution of the dominance and recessive alleles was found for both the necrosis and stem height characters. All of the transgenic parents (except 51-14) showed a higher frequency of dominant alleles compared with the non-transgenic parents. The 51-14 line showed almost no dominance. The graphical results are in agreement with the results obtained from F and dominance/recessive estimates, mentioned above. In addition, the distribution of dominance alleles in transgenic parents as well as the presence of dominance, predominantly in transgenic lines, is in agreement with the analysis of variance of (Wr+Vr). Graphical analyses do provide some information on the degree of dominance and the presence of the inter-allelic interactions (Mather and Jinks, 1977; 1982). However this approach failed to provide either an estimate of the proportion of dominant to recessive alleles in the parents or the consistency of the distribution of the dominance across the loci.

The correlation coefficient of common parent (Pr) and (Wr+Vr) was significant and positive for the necrosis character and significant and negative for the stem height character. The significant coefficient suggests a directional dominance for these characters. For necrosis, the negative coefficient suggests that phenotypes with low necrosis are dominant over phenotypes with high necrosis. However for the stem height character, phenotypes with the greater stem height are dominant over the phenotypes with the low stem height in the presence

of the herbicide. A similar result was obtained from the distribution of parents on the Wr/Wr graph.

Heritability estimates were high for both characters in this part of the study. There were small differences between narrowsense and broadsense heritabilities, especially for stem height. The narrowsense heritabilities of the characters were similar, while the broadsense heritability of stem height was greater than that of leaf necrosis. The higher broadsense heritability of stem height indicates a greater non-additive component of variations in stem hight than in leaf necrosis. The relatively high narrowsense heritability for these characters indicates that the additive component of variation is more important than the other components of variation for the characters. The narrowsense heritability is more important and appropriate for self pollinated crops (e.g. tobacco plants). The high narrowsense heritability suggests that appropriate progress could be made in a herbicide resistance selection programme.

4.4.3 Griffing diallel crossing

The general and specific combining ability variances for both the leaf necrosis and stem height characters are significant, suggesting the presence of both additive and dominance components of variation. The dominance variance for both characters was very small compared with the additive variance. It should be noted that Griffing's method of diallel cross analysis is a flexible method. The combining ability of the characters provide a simple account of the genetic situation and the genetic model on which the analysis is not based on absence of epistasis. Furthermore, the analysis may be used for any number of alleles per locus and any number of loci. It should be noted that the results of the present study reveals that data analyzed using Griffing's model shows lower dominant (lower σ^2_s) than the Jinks and Hayman (1953) model. With regard to the low σ^2_s estimate by the Griffing method, Hayman (1956) suggested that the general combining ability variance was composed of both additive and dominance portions while the specific combining ability variance mainly involved dominance. Hence, it is interpreted that the general combining ability variance is composed of both additive and dominance components of variation.

The Griffing method of analysis of diallel cross data is involved in the

estimate of general and specific combining variances. The variances of combining abilities are genetically defined as additive, dominance and various kinds of inter-allelic interactions (epistasis). The total genetic variation among the progeny of the cross between two homozygous parents is equal to twice the σ^2_g component plus the σ^2_s components of variation (Griffing, 1956b). On the other hand the general combining ability component of variation (σ^2_g) is equal to half of the additive (additive x additive is possible) component of variation while the specific combining ability component of variation (σ^2_s) is equal to the dominance (inter-allelic interaction is possible) component of variation.

The estimated values of g.c.a. effects for leaf necrosis (Table 4.17) are significant and negative for transgenic parents, while for non-transgenic parents the estimates are statistically significant and positive. It is interpreted that the transgenic parents show a significant g.c.a. estimated values toward the low leaf necrosis, while the non-transgenic parents show a significant g.c.a. estimated values towards the high necrosis (low resistance to herbicide). Although all of the transgenic lines show significant g.c.a. estimated values, considerable differences within the transgenic lines can be observed. The estimated values of g.c.a. effects for stem height (Table 4.18) give positive values (higher stem height) for transgenic lines, while the non-transgenic lines give negative g.c.a. estimated values (susceptible to herbicide).

The estimated values of s.c.a. effects for leaf necrosis and stem height (below diagonal of Tables 4.17 and 4.18 respectively) show that crosses between the non-transgenic parents are positive for leaf necrosis and negative for stem height values (susceptible). In contrast, crosses between resistant transgenic parents and non-transgenic parents show a negative s.c.a. value for necrosis and a positive value for stem height, suggesting the presence of dominance allele(s) in the transgenic parents, as discussed above. However, in some crosses involving the transgenic parents, estimated values of the s.c.a. are not similar to the g.c.a. of the resistant transgenic parent, suggesting the dominance of susceptible phenotypes in some crosses (e.g. in 51-5 x B21 cross).

With respect to the transmission of herbicide resistance from parents to the progenies for various crosses, the hybrid F_1 and TF_1 progenies can be divided into three groups as follows:

1. Crosses between the three non-transgenic parents (KKD, K51 and B21).

The F_1 hybrid progenies derived from crosses between these parents were all susceptible to the herbicide.

2. Crosses between the three non-transgenic parents (KKD, K51 and B21) and the resistant transgenic parents (49-1, 51-1, 51-3, 51-5 and 54-18). The TF_1 hybrid progenies (except 51-5 x B21) were resistant to the herbicide.
3. Crosses between the transgenic parents (49-1, 51-1, 51-3, 51-5, 51-14 and 54-18). The TF_1 hybrid progenies were resistant to the herbicide similar to those of the resistant transgenic parents.

Although the non-transgenic lines and their progenies resulting from crosses between them did not show resistance to the herbicide, when crossed with the transgenic parents (except 51-14 parent), the TF_1 progenies were resistant to the herbicide. It can be interpreted that the transgene expressed efficiently in these two types of hybrids progenies. Since the low resistance transgenic line (51-14) suppressed by the resistant parents, the resistant lines are dominant either over the non-transgenic or the low resistance transgenic lines. As an exception, the TF_1 progeny of the (51-5xB21) cross indicates that expression of the transgene is suppressed by the host background genome as discussed above.

The comparison of g.c.a. values of individual lines for leaf necrosis and stem height (Tables, 4.17 and 4.18) shows that 49-1, 51-1, 54-18, 51-3, 51-5 transgenic lines have negative g.c.a. effects for leaf necrosis and positive g.c.a. effects for stem height. These lines exhibit the best general combining ability and could be used in crossing programmes with the aim of producing of herbicide resistant, hybrid plants. It is suggested that information on the g.c.a. and s.c.a. effects on transgenic tobacco may be of interest for understanding the transgene effect as well as for the production of transgenic hybrids and for selecting high performance transgenic parents and hybrid progenies.

Of particular importance to the plant breeder with the aim of breeding herbicide resistant plants is the predominance of additive gene action for the stem height and leaf necrosis characters. This suggests that improvement of these characters can be achieved through the breeding procedures and may result in superior pure-line varieties. The presence of a significant dominance gene action (hemizygosity effect) is also important from a plant breeding point of view, since it may be possible to obtain resistant hybrid plants through choosing an appropriate

transgenic parent and a non-transgenic cultivar for producing the TF_1 hybrid.

The minor differences between the narrowsense and broadsense heritabilities possibly resulted from the involvement of both additive and dominance components of variation in the general combining ability variance (σ^2_g) which increased the general combining ability variance component compared to the specific combining ability variance component, as discussed above.

From the genetical information perspective, this analysis is superficial compared with those of the generation means and Hayman-Jinks diallel methods. However, because this analysis is not restricted by the assumptions required for the other methods, it is possible to use this method more generally. It is interesting that in this case, all methods agree for the genetic effects of the transgene. Thus the general combining ability and specific combining ability analysis give the essential information on gene effect as well as the other methods do.

4.4.4 Inter-allelic interaction (epistasis)

All three mating designs including generation means analysis, Jinks-Hayman diallel analysis and Griffing diallel analysis indicated the involvement of inter-allelic interactions (epistases) for the transgene. Two types of inter-allelic interaction (epistasis) can be accounted for the present study: the allelic interaction between the transgenes in transgenic lines (e.g. between the 51-14 and the other transgenic lines) and the inter-allelic interaction between the plant background genome and the transgene (e.g. between 51-5 and B21 lines).

The evident case of the interactions between the transgene with a high and low level of resistance observed for the low resistant 51-14 transgenic line and the other resistant transgenic lines (Tables 4.11, 4.12, 4.17 and 4.18). The TF_1 progenies derived from crosses between the non-transgenic lines and low resistant transgenic line, 51-14, were susceptible to the herbicide. The progenies derived from crosses between the resistant transgenic lines were resistant to the herbicide. However, in crosses between the low resistant transgenic line, 51-14, and the resistant transgenic lines, the TF_1 hybrid progenies were resistant to the herbicide, similar to those of the resistant transgenic parents.

With respect to the interaction between the transgenes, Hobbs *et al.* (1993) crossed two groups of transgenic tobacco plants with high and low levels of GUS

activity. The transgenic F_1 progenies developed from a cross between two parents with low level of GUS activity (L-type), had a low level of GUS activity. Similarly, the transgenic F_1 progenies developed from a cross between two parents with high level of GUS activity (H-type) had a high level of GUS activity (H-type). However, the level of GUS activity in transgenic F_1 progenies developed from crosses between parents with a low level of GUS activity (L-type) and a high level of GUS activity (H-type), totally or partially suppressed by the L-type gene. Therefore, they concluded that a low level of GUS activity suppresses the expression of genes conferring a high level of GUS activity.

In the present study, the progenies developed from either crosses between two susceptible parents or two resistant parents were similar to those of reported by Hobbs *et al.* (1993) as discussed above with an exception. In contrast to that of the Hobbs *et al.* (1993) results, in the present study in crosses between the herbicide resistant transgenic lines (49-1, 51-1, 51-3, 51-5 and 54-18) and the low resistant transgenic line (51-14), the TF_1 hybrid progenies showed resistance to the herbicide similar to those of the resistant parents. In the other words, in the present study in crosses between the low resistant and resistant transgenic lines, the resistance gene shows dominance over the susceptible gene, which is in contrast with that of reported by Hobbs *et al*. (1993). This may have resulted from either using a different gene or different tobacco line in their studies.

The apparent case of the interaction between the transgene and the plant genome is in the TF_1 progeny derived from cross between the 51-5 and B21 lines (Tables 4.11, 4.12, 4.17 and 4.18). These hybrid progenies exhibit a susceptible phenotype similar to that of the non-transgenic B21 parent. However the crosses between 51-5 and the other transgenic lines indicate a resistance phenotype similar to that of the 51-5 transgenic parent or the other resistant transgenic parents (in cases of crosses with resistant parents). It must be noted that the cross between the two lines was made reciprocally (B21x51-5 and 51-5 x B21), hence in one set of crosses the 51-5 line was a female parent. There is therefore no possibility of incorrect emasculation or other crossing error. The generation means, Jinks-Hayman diallel and Griffing diallel analyses show the presence of epistasis with the transgene. The results of mean analysis (Table 4.11 and 4.12) as well as g.c.a and s.c.a. effect (Table 17 and 4.18) clearly show the background allele(s) of the non-transgenic parents have an effect on the phenotypic expression of the transgene.

For the (51-5xB21) cross, there is a significant interaction between the transgene and the background allele(s). This can be defined as an epistasis effect of the background genotype on phenotypic expressivity of the transgene.

In summary, the generation means data indicate that the herbicide resistance phenotype is expressed in different generations of the transgene. The expression of gene remains active in the sexually developed progeny derived from the transgenic line (51-1). Therefore it can be expect that the phenotypic expressivity of the transgene conserved in different generations and that the progeny derived from cross between the transgenic and non-transgenic line will remains active. Therefore where this transgenic line is released as a cultivar,this cultivar will exhibit a resistant phenotype in subsequent generations. However, there is a significant epistasis effect evident for the transgene which may be due to an interaction of the transgene with the allele(s) of the background genotype. Thus the effect of the background genotype mask the expression of the transgene in the transgenic plants. This epistasis could result either from interaction between a certain or interaction between different copies the transgene while more than one copy of transgene is present in the transgenic plants. In relation to the first possibility, the Jinks-Hayman and Griffing diallel were set up with a wide range of transgenic and non-transgenic parents to identify wether these effects resulted from background genotype or occurred for all of the crosses. The significance of the additive and dominance effects derived from these experiments indicates that the homozygous transgenic plants exhibit a resistant phenotype during the subsequent generations.

From a plants breeding point of view, since tobacco is a self-pollinating plant, the transgenic homozygous genotype can be selected and introduced as a transgenic pure line. This genotype can subsequently be used as a resistant cultivar for a long period of time (e.g. up to 7-10 years). The dominance effect indicates that if the homozygous transgenic line is crossed with a non-transgenic line, the resultant hemizygous progeny will exhibit a resistant phenotype similar to that of transgenic parent (because the dominance effect is almost full). However, although this hemizygous phenotype is similar to that of the transgenic parent, it can not be recommended as a cultivar for long term use, because this genotype is under segregation and in the next generation, due to segregation, all possible

genotypes could be obtained. However the transgene could be introduced to the agronomically important cultivars through backcrossing and selection.

In applying these results to cross-pollinated plants with a high a level of heterozygosity in their background alleles, the homozygous plants for the transgene allele(s) can be developed through either selfing or half sib mating (in case of self incompatibility) and selection of good performance, resistant, transgenic plants. The resultant selected transgenic plants will be homozygous for the transgene but may not necessarily be homozygous for the other background alleles. To study the genetical parameters of the cross-pollinated plants, genetical designs such as factorial designs (Comstock and Robinson, 1952) can be used to estimate the genetical parameters. Where additive and full dominance occurred in cross pollinated plants, the phenotypic expression of the resistant phenotype in both homozygous and hemizygous plants is to be expected. However if the hemizygous plants exhibit the susceptible phenotype, partial dominance, no-dominance or epistasis between the transgene and the host plant genome are all possible.

The Jinks-Hayman and Griffing diallel analyses show that the phenotypic expressivity of the transgene varies significantly within the transgenic parental lines as well as within the hybrid progeny plants. This variation shows that after transformation, a selection strategy may be used to select a resistant transgenic line(s). As mentioned above, the most suitable strategy is a selection based on resistant homozygous transgenic line rather than of the development of hybrid plants (no over dominance). Since the heritability of the characters under study were high, the gain from selection of superior transgenic lines may also be high. Using a selection strategy such as pure line selection requires only a short time to improve and evaluate the selected transgenic line(s).

Chapter 5

Molecular genetics of herbicide resistance

5.1 Introduction

The main objective of the plant molecular studies was to follow the expression and inheritance of the dehalogenase transgene in successive, sexually reproduced generations. The binary vector plasmid pAS501 was developed and transformed into *E.coli* by BioTechnica International (Boston, U.S.A). The T-DNA of the plasmid pAS501 includes two genes: a) a dehalogenase gene which is responsible for the degradation of dalapon herbicide by removing the chlorine ions from the herbicide, and b) a neomycin phosphotransferase II (NPTII) gene for the detoxification of kanamycin, which allows growth of transformed plant cells on media containing kanamycin as a selective agent. Plasmid pAS501 had previously been transferred into *Agrobacterium tumefaciens* strain LBA4404 (see Chapter3, Section 3.2.2.1). Different generations derived from one homozygous transgenic tobacco line were used in T-DNA inheritance and organisation studies. The relationships between the levels of dehalogenase-specific mRNA and the levels of resistance to dalapon were studied in different transgenic lines which showed various levels of resistance to the herbicide. The objectives of different aspects of this chapter are outlined below.

5.1.1 T-DNA inheritance and organisation

The T-DNA inheritance and organisation in one homozygous transgenic tobacco line 51-1 (R_3), as well as the inheritance and organisation of the T-DNA in different generations including transgenic TF_1, backcrosses (TBC_1 and TBC_2) and TF_2 plants developed from a cross between this homozygous transgenic line and the non-transgenic control line, KKD were all analyzed by Southern hybridisation.

5.1.2 levels of the dehalogenase gene mRNA

Dehalogenase transgene specific mRNA levels in transgenic tobacco plants which exhibit different levels of resistance to the herbicide dalapon were studied to

determine the relationships between the dehalogenase-specific mRNA levels and levels of resistance to the herbicide. Total plant RNA was isolated and the dehalogenase-specific mRNA was detected and compared using a RNA dilution dot-blot technique.

5.1.3 Variability in the level of expression of T-DNA genes

The objectives of this part of the study was to determine the relationship between the expression of the two selectable T-DNA genes (NPTII and dehalogenase) in tobacco calli transformed with T-DNA, under *in vitro* conditions. The resistance threshold for genetically transformed tobacco callus lines in the presence of dalapon herbicide as well as the reasons for some of the transgenic lines selected for kanamycin resistance not showing resistance to dalapon are also investigated. A two phase selection (dual selection) experiment was carried out to check the expression levels of the T-DNA genes in response to kanamycin and dalapon.

5.2 Materials and Methods

5.2.1 Isolation of plasmid pAS501 from *Agrobacterium*

Agrobacterium tumefaciens, strain LBA4404, containing modified Ti plasmid pAL4404 (Ooms *et al.*, 1982) was used in all plant transformation experiments. Plasmid pAS501 contains a tetracycline resistance gene as a bacterial selectable marker outside the T-DNA borders and kanamycin resistance and dehalogenase genes within the T-DNA.

The binary vector plasmid pAS501 had previously been transferred into the *Agrobacterium*. To isolate plasmid DNA, *Agrobacterium* containing pAS501 was grown in TY medium (White and Greenwood, 1987). *Agrobacterium* containing plasmid pAS501 was streaked on the surface of TY medium (appendix 6) supplemented with 10 mg L^{-1} tetracycline antibiotic (TY + Tc^{10}), and plates were incubated at 28°C for 2 days. Bacteria were subsequently streaked on a fresh TY + Tc^{10} plate to produce single colonies.

5.2.1.1 A modified STET method of plasmid DNA isolation

The STET method used for the isolation of plasmid DNA from *Agrobacterium* were based on that given in Sambrook *et al.* (1989) with some modifications. Materials used for isolation of plasmid DNA from *Agrobacterium* are presented in Appendix 7. The method used was as follows:

1. A number of colonies of *Agrobacterium tumefaciens* strain LBA4404 containing pAS501 was harvested from the plate (see above), inoculated into 10 ml of TY+ Tc^{10} liquid medium and incubated at 28°C on a gyratory shaker at 100 rpm until late exponential log phase growth had occured.
2. Cells were collected by centrifugation at 3500 rpm for 5 minutes in a Sorvall SS-34 rotor. The supernatant was removed and the pellet was resuspended in 100 μl of TY liquid medium.
3. STET buffer (700 μl) was added, mixed carefully and the bacterial suspension was transferred to a 1.5 ml microfuge tube.
4. After lysozyme (20 μl of 10 mg L^{-1} solution) was added, the suspension was mixed immediately and incubated at room temperature for 10 minutes.

5. The suspension was placed in a boiling water bath for 2 minutes and then immediately spun in a microfuge for 10 minutes.
6. Either the supernatant was transferred to a fresh tube, or the pellet was removed using a toothpick. DNase-free RNase (13 μl of 10 mg ml^{-1} solution) was added, the tube was briefly vortexed and then incubated at 37°C for 10 minutes.
7. Phenol and chloroform (150 μl of each) were added, the tube was vortexed for 15 second, spun for 5 minutes in a microfuge and the aqueous phase was transferred to a fresh microfuge tube; this step was then repeated.
8. An equal volume of cold (-20°C) isopropanol was added to the aqueous phase and the tube was vortexed and placed at -80°C for 15 min.
9. DNA was pelleted by centrifugation in a microfuge for 15 minutes. The tubes were drained and dried under vacuum.
10. The DNA pellet was dissolved in 100 μl of 100 mM NaCl.
11. The DNA was reprecipitated by adding 300 μl of cold (-20°C) absolute ethanol and incubating at -80°C for 5 minutes.
12. Tubes were spun for 10 minutes in a microfuge, the tube was drained, and then washed with 1 ml of 75% ethanol followed by centrifugation in a microfuge for 5 minutes.
13. The pellet was dried under vacuum, redissolved in 40 μl sterile H_20 and stored at 4°C.

5.2.1.2 Analysis of isolated plasmid DNA

The isolated plasmid DNA was diluted 250 times (4 μl ml^{-1}) in sH_20. The A 260 of the diluted DNA was determined in a spectrophotometer using sterile H_2O as a blank. The concentration of plasmid DNA was calculated using the following formula:

DNA (μg ml^{-1}) = Absorbency (260) x 50 x dilution factor.

5.2.1.3 Horizontal mini gel electrophoresis

Materials for agarose gel electrophoresis preparation are given in Appendix 8. For plasmid DNA analysis, a 0.7% (w/v) agarose horizontal slab mini gel (96 x

67 x 3.9 mm) was used. Agarose was dissolved in 1xTAE buffer by heating in a microwave oven. Before pouring the gel, 0.1 volume of a 10 mg L^{-1} ethidium bromide was added to the gel. Electrophoresis was usually carried out at 75 mA. The gel was photographed after electrophoresis using U.V. light emittance.

5.2.1.4 Determination of molecular weight of DNA samples

A *Hind*III+*Hind*III/*Eco*RI digest of Lambda DNA was used as a standard to determine the molecular size of sample DNA fragments. Following electrophoresis, the relative mobilities of DNA fragments were measured on the gel. The molecular size of DNA fragments were determined on a semi logarithmic paper by plotting the relative mobility as a linear scale on the "X" axis versus the $\log_{10}$ of the molecular weight on the "Y" axis (Sanger *et al.*, 1982).

5.2.1.5 Competent cell preparation and Transformation of *E.coli* with plasmid pAS501

5.2.1.5.1 Preparing *E.coli* strain DH5α competent cells

1. LB broth (10 ml) (Appendix 9) was inoculated with *E. coli* strain DH5α and incubated overnight at 37°C on a gyratory shaker at 200 rpm.
2. The stationary phase culture was diluted $^1/_{100}$ into 40 ml of fresh LB broth and incubated at 37°C on a gyratory shaker at 200 rpm until the cells reached an OD_{600} of 0.5.
3. The cells were collected by centrifugation at 3500 rpm for 5 minutes in a Sorvall SS-34 rotor at 4°C.
4. The supernatant was discarded and the cells were resuspended in 10 ml of cold 60 mM $CaCl_2$ at 4°C.
5. Another 10 ml of cold 60 mM $CaCl_2$ was added and the cells were left on ice for 30 minutes.
6. The cell suspension was centrifugated at 3500 rpm for 5 minutes in a Sorvall SS-34 rotor at 4°C to pellet the cells and the supernatant was discarded.
7. The cells were resuspended in 4 ml of cold 60 Mm $CaCl_2$.

   For storing the remaining competent cells (step 7), 50% glycerol was added

to give a final concentration of 15%. Aliquots of 300 µl were distributed in microfuge tubes, frozen in liquid nitrogen, and stored at -80°C.

5.2.1.5.2 Transformation of *E.coli* with plasmid pAS501

1. To 300 µl of competent cells, 20 ng of plasmid pAS501 DNA was added and placed on ice for 1 hour.
2. The cells were heat shocked at 42°C for 2.5 minutes and then 700 µl of LB broth was added and the cells were incubated at 37°C for 2 hours.
3. Aliquots of 1 µl, 10 µl and 100 µl were spread on LB plates supplemented with 10 mg L^{-1} tetracycline and the plates were incubated at 37°C overnight.

5.2.2 Restriction enzyme site mapping of plasmid pAS501

E. coli strain DH5α containing pAS501 was inoculated into LB + 10 µg ml^{-1} tetracycline broth medium and was then incubated at 37°C on a gyratory shaker at 200 rpm overnight. Plasmid was isolated by the STET method as described in Section 5.2.1.2.

5.2.2.1 Conditions for restriction enzymes digestion

5.2.2.1.1 Single digests

The following Boehringer Mannheim incubation buffers were used for different restriction enzymes: buffer B for *Eco*RV, *Hind*III and *Bam*HI; buffer A for *Sac*II; buffer M for *Bgl*II and *Sph*I; buffer H for *Eco*RI and *Pst*I incubation. Incubation temperature for all the above enzymes was 37°C. For *Sma*I restriction enzyme, buffer A was used with incubation at 25°C. Incubation times were for 1-2 hours.

5.2.2.1.2 Double digests

1. *Eco*RI+*Sma*I: DNA was digested with *Sma*I at 25°C with buffer A for 1 hour, and digestion was checked by running a sample on an agarose gel. The digest was precipitated with two volumes of absolute ethanol, pelleted, dried

and resuspended in sH_2O. The sample was then digested with *Eco*RV using buffer B at 37°C for 1 hour.

2. *Eco*RI+*Sac*II: DNA was digested with *Sac*II in buffer A, then digested with *Eco*RI in buffer H as described above. Incubation temperature was 37°C.
3. *Eco*RV+*Bgl*II: The sample DNA was first digested with *Eco*RV in buffer B and then with *Bgl*II in buffer M at 37°C.
4. *Eco*RV+*Pst*I: DNA was digested with *Eco*RV in buffer B, then digested with *Pst*I in buffer H.

5.2.2.2 Restriction mapping of plasmid pAS501

There were no *Eco*RI or *Hind*III restriction sites in the plasmid pAS501 (Appendix 16, Figure A.1). Single or double digests were set up with *Bgl*II, *Sac*II, *Sma*I, *Pst*I, and *Eco*RV according to the protocol outlined above. After digestion, the samples were run on an agarose mini-gel (Section 5.2.1.3) along with the lambda *Hind*III+*Eco*RI/*Hind*III standard. The restriction fragment sizes for pAS501 digested with the enzymes *Eco*RV, *Sac*II, *Bgl*II *Pst*I and *Sma*I are presented in Appendix 16, Figures A.1 and A.2.

When the plasmid pAS501 was digested with *Bgl*II, two fragments of 19.6 kbp and 5.4 kbp were produced. There was only one known *Bgl*II site outside the T-DNA and upstream of the right border of T-DNA. Hence it was concluded that the other site, by considering the size of the T-DNA region and the distance between the right border and the known site of *Bgl*II, must be located inside the NPTII gene. When the plasmid pAS501 was digested with *Eco*RV, at least four fragments with sizes of approximately 14.9 kbp, 5 kbp, 4.1 kbp and 1 kbp were produced. A double digest of the plasmid with *Eco*RV and *Bgl*II (Appendix 16, Figure A.2) produced five fragments: 17.4 kbp large fragment, 3.5 kbp, 2.6 kbp, 2x1.5 kbp and 1 kbp

When the plasmid was digested with *Sac*II, DNA fragments of approximately 1.78 kbp, 3.9 kbp, and 4.22 kbp were produced. Double digests with *Eco*RV and *Sac*II produced fragments of approximately 14.7 kbp, 4.2 kbp, 2.6 kbp, two of 1.75 kbp, and 1 kbp. It can be concluded that at least two *Eco*RV sites are located within the small *Sac*II fragments.

When the plasmid was cut with *Sma*I (Appendix 16, Figure A.1), five

fragments of approximately 17.4 kbp, 4 kbp, 1.7 kbp, 1.1 kbp, and 780 bp were produced. Furthermore, a *Sma*I/*Sac*II double digest (Appendix 16, Figure A.3) cut the 4 kbp *Sma*I fragment to produce 2.25 and 1.75 kbp fragments. The enzyme *Pst*I cut the plasmid (Appendix 16, Figure 3.A) to produce fragments of approximately 22.85, 1.25 kbp and two small size fragments of approximately 500 and 350 bp which were not visible on this gel. All of the *Pst*I fragments are located between the T-DNA borders since there are no *Pst*I sites outside the T-DNA region.

5.2.3 Making a dehalogenase gene probe

The 3.9 kbp *Sac*II fragment was isolated from plasmid pAS501 and subcloned into the *Sac*II site of plasmid vector pSK+ as described in the following Section.

5.2.3.1 Isolation of DNA fragments using a modified DEAE method

The method used for the isolation of DNA fragments was based on that of Dretzen *et al.* (1981) with some modifications.

A *Sac*II digest of plasmid pAS501 was run on a 0.7% gel until the fragments were separated, as visualised under U.V. light. Using a razor, a vertical slit was cut below the band of interest and a piece of DEAE paper was fitted into the slit. The gel was squeezed firmly against the paper and electrophoresis was resumed until the DNA had run onto the membrane as indicated under U.V. light. The paper was removed from the slit, washed three times in gel running buffer, dried on a piece of clean towel paper to remove the buffer from the membrane, placed in the bottom of a microfuge tube and 100 μl of DEAE buffer (Appendix 10) was added. The tubes were incubated at 65°C for 30 minutes. The membrane to was then transferred to another tube containing 50 μl of DEAE buffer and incubated at 65°C for a further 20 minutes. The paper was removed from the solution and liquid from both tubes was combined. To recover the DNA, $MgCl_2$ (1.5 μl of 1M) was added to the supernatant followed by 2 volumes of 100% ethanol and incubated at -80°C for 15 minutes. The DNA was pelleted and resuspended in 15 μl sH_20. The fragment was subcloned into *Sac*II digested pSK+ plasmid vector using the following DNA ligation method:

reaction mixture:

1.	5 x ligase buffer (BRL)	4	µl
2.	T4 DNA ligase (1u μl^{-1} ; BRL)	2	µl
3.	Vector pSK+ 60 ng μl^{-1}	1	µl
4.	DNA fragment 60 ng μl^{-1}	3	µl
5.	sH_2O	10	µl
	total volume	20	µl

The reaction mixture was incubated at 4 °C overnight to ligate the fragment.

5.2.3.2 *E.coli* transformation and selection for transformed cells

After autoclaving LB medium, 100 µl of each of 100 mg L^{-1} ampicillin (filter sterilised), 1 mg L^{-1} IPTG (isopropyl β-D-thiogalactoside, sigma No. I6758) dissolved in water, and 1 mg L^{-1} of X-gal (5-bromo-4-chloro=3indolyl-β-D-galactoside, Sigma No. B4252) dissolved in dimethyl formamide, were added to the media before pouring into the plates.

E.coli transformation was carried out by adding 20 ng of ligated vector to 300 µl of competent cells (Section 5.2.1.5.2). The tube containing cells were placed on ice for 1 hour, heat shocked at 42°C for 2.5 minutes and then transferred to ice for approximately 10 minutes. To the tube containing cells, 700 µl of LB broth was added and incubated at 37°C for 2 hours. Aliquots of 1 µl , 10 µl and 100 µl of transformed cells were spread on the surface of LB+Ampicillin+IPTG+X-gal plates (see above) which were then incubated overnight at 37°C.

Transformed white colonies were inoculated into 10 ml of LB medium supplemented with 100 mg L^{-1} ampicillin and incubated at 37°C with shaking. Plasmid were isolated using the modified STET method described in Section 5.2.1.2. The plasmid containing the correct dehalogenase gene fragment was designated pSDH.

5.2.3.3 Polymerase chain reaction (PCR) amplification of dehalogenase gene

To check the presence of the dehalogenase gene within the *Sac*II fragment of plasmid pAS501, the PCR technique was used. The size and orientation of the

dehalogenase gene in plasmid pSDH was determined by PCR using different primers as described below:

PCR reaction mixtures:

(a) E3* and universal primers.

1.	pSDH plasmid DNA (1ng μl^{-1})	1	μl
2.	10x Taq polymerase buffer (Promega)	10	μl
3.	Taq DNA polymerase enzyme (5u μl^{-1}, Promega)	0.5	μl
4.	E3 primer (20 pmol)	1	μl
5.	Universal primer (20 pmol)	1	μl
6.	10 mM dNTP	10	μl
7.	sH_2O	76.5	μl
	total vol.	100	μl

* The E3 primer is a 24 mer (5′-GATCCTGACGTAAGGGATGACGCA-3′) which is homologous to a sequence within the CaMV 35S gene promoter upstream of the dehalogenase gene.

(b) universal and reverse primers;

1.	pSDH plasmid DNA (1ng μl^{-1})	1	μl
2.	10x Taq polymerase buffer (Promega)	10	μl
3.	Taq DNA polymerase (5u μl^{-1}, Promega)	0.5	μl
4.	E3 primer (20 pmol)	1	μl
5.	Revers primer (20 pmol)	1	μl
6.	10 mM dNTP	10	μl
7.	sH_2O	76.5	μl
	total vol.	100	μl

The reaction mixtures were incubated in a DNA Thermal Cycler 480 (Perkin Elmer) using 30 cycles of 1 minute at 92°C for denaturation, 1 minute at 50°C for annealing the primers to homologous sequences of single stranded plasmid DNA and 1 minute at 72°C for polymerisation of new DNA strands. The resultant fragment is approximately 1.5 kb as indicated in Appendix 16, Figure A.3.

5.2.3.4 Preparation of a dehalogenase gene probe

Plasmid pSDH was digested with *SacII* and *PstI* restriction enzymes and a 730 bp fragment was isolated and subcloned into the *SacII*+*PstI* sites of the plasmid vector pSK+. The resultant plasmid was designated pDH7.

5.2.3.5 A method of making [α-^{32}P]-dCTP-labelled probe

The method used for making a labelled probe DNA was based on that of Feinberg and Vogelstein (1983; 1984) with some modifications.

1. Approximately 20 ng of 730 bp DNA fragment was dissolved in 21 μl of sH_2O. The DNA was denatured by heating for 3 minutes in a boiling water bath and then immediately chilled on ice.
2. Labelling reaction mixture

denatured DNA (from step 1)	21	μl
10 mM dATP	1	μl
10 mM dGTP	1	μl
10 mM dTTP	1	μl
2.5x random primers solution(BRL)	20	μl
5 μl (approximately 50 μCi) [α-^{32}P] dCTP (Amersham)	5	μl
Klenow fragment (40 u μl^{-1}; BRL)	1	μl
final volume (adjust with sH_2O)	50	μl

3. The reaction was mixed briefly and incubated at 37-40°C for 10 minutes.

5.2.3.5.1 Determining quality of labelled probe DNA

1. A 1 μl aliquot of the reaction was spotted onto TLC (thin layer chromatography) plate.
2. The bottom edge of the TLC plate was placed in phosphate buffer for 10-15 minutes.
3. The thin layer chromatography (TLC) plate was exposed to X-ray film for 5 minutes.
4. The X-ray film was developed, fixed and checked for incorporation of nucleotides into newly synthesized DNA.

5.2.3.5.2 Purification of labelled probe DNA

Labelled probe can be purified by a number of different methods e.g. using Sephadex® G-50 or other columns, or ethanol precipitation of DNA. In this study, due to better results, the ethanol precipitation method was used as follows:

1. To each sample, 150 µl of cold (-20°C) absolute ethanol was added.
2. The samples were placed into a lead-container and incubated at -20°C for 2 hours.
3. The samples were spun at 15,000 rpm for 15 minutes.
4. The supernatants were carefully discarded and the DNA tubes dried and resuspended in 100 µl of sH_20.

5.2.4 Plant DNA isolation

The method used for the plant DNA isolation was based on that of Ellis *et al.* (1984) with some modifications.

Buffers and solutions used for the plant DNA isolation are outlined in Appendix 11. The actual method is outlined as follows:

1. Approximately 10 g of fresh tissue was collected from tobacco leaves and frozen with liquid nitrogen.
2. The frozen tissue was grounded with a cold pestle and mortar to a fine powder.
3. The samples were allowed to warm slightly before adding 10 ml of extraction buffer per 10 g of leaf tissue and thorough mixing .
4. To give a final volume of 0.2% w/v of SDS, 0.1 ml of 20% SDS was added and mixed by grinding.
5. To each sample, 15 ml of chloroform:*iso*-amylalcohol (24:1) was added and thoroughly mixed.
6. The mixture was transferred to a 30 ml Corex tube and centrifugated for 10 minutes at 4000 rpm in a Sorvall SS-34 rotor at 4°C.
7. The top aqueous phase was transferred to a fresh tube.
8. Two volumes of cold (-20°C) absolute ethanol was added to the aqueous phase and placed at -20°C for at least 3 hours. A thick floating DNA formed

on the surface was carefully transferred to a microfuge tube, which was centrifugated briefly, and then the supernatant was discarded.

9. The DNA was dried under vacuum.
10. sH_20 (700 μl) was added to the DNA which was then left for 2-3 hours at either room temperature or 4°C to allow the DNA to dissolve.
11. To the DNA solution, 0.5 volume of phenol was added, mixed and centrifuged in a microfuge for 5 minutes.
12. To the aqueous layer, two volumes of cold (-20°C) absolute ethanol was added and centrifuged for 15 minutes in a microfuge.
13. The tubes were drained carefully, 1 ml of 70% ethanol was added, and the tubes were centrifuged for 5 minutes. The tubes were again drained and then dried under vacuum.
14. The DNA was redissolved in 100 μl sH_20 and the DNA concentration was determined.

5.2.5 Typical method of plant DNA restriction enzyme digestion

1.	Plant genomic DNA (1 $\mu g\ \mu l^{-1}$)	30	μl
2.	Restriction enzyme (10 u μl^{-1})	15	μl
3.	10x restriction Buffer	25	μl
4.	sH_20	180	μl
	final volume	250	μl

the mixture was incubated at 37°C for 3 hours.

5.2.6 Plant RNA isolation using guanidine isothiocyanate

The method used for plant RNA isolation was based on that of MacDonald *et al.* (1987) with some modifications Buffers and solutions used for plant RNA isolation are outlined in Appendix 12. The actual method is outlined as follows:

1. Approximately 2 g of plant tissue was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. A pre-chilled spatula was used to scrape the powder into a 25 ml beaker containing 7 ml guanidine isothiocyanate buffer and a magnetic stirring bar.

2. The mixture was stirred vigorously for 2 minutes then poured into a 15 ml Corex tube and centrifugated for 10 minutes at 10000 rpm in a Sorvall SS-34 rotor at 4°C.
3. The mortar and pestle were cleaned between each sample by wiping out with 50% ethanol.
4. To precipitate the RNA, 0.0025 volume of glacial acetic acid and 0.5 volume of cold (-20°C) absolute ethanol was added to the mixture.
5. The suspension was mixed by gentle inversion, and then capped and left at -20°C for 3 hours to allow the RNA to precipitate.
6. The samples were centrifugated at 3000 rpm in a Sorval SS-34 rotor at 4°C for 15 minutes.
7. The supernatant was discarded and 1 ml urea buffer (plus extra if required) was added to the pellet.
8. The suspension was poured into a 12 ml plastic tube and 1 ml equilibrated phenol was added.
9. The tube was capped, shaken for 2 minutes, and then 1 ml chloroform:*iso*-amylalcohol (24:1) was added and the tubes were shaken for a further 2 minutes.
10. The samples were centrifuged at 3000 rpm in a Sorvall SS-34 rotor for 15 minutes.
11. The aqueous layer and interface were transferred to a fresh 12 ml plastic tube and 3.5 ml equilibrated phenol was added.
12. the tubes were capped, shacken for 2 minutes and centrifugated at 3000 rpm in a Sorvall SS-34 rotor for 15 minutes.
13. When there was a thick protein layer or when the aqueous phase was gelatinous, a third phenol extraction was carried out.
14. The aqueous layer was carefully transferred to a 15 ml siliconised Corex tube.
15. The RNA was precipitated by adding 0.1 volume of 20% sodium acetate and 2.5 volume of cold (-20°C) absolute ethanol and incubation at -20°C overnight.
16. The tubes were centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 15 minutes.
17. The pellet was resuspended initially in 1 ml DEPC treated water (although

additional H_2O was sometimes necessary), and then 2 volume of 4M sodium acetate was added and the tubes were incubated for 3 hours at 4°C.

18. The tubes were centrifuged at 10,000 rpm in a Sorvall SS-34 rotor for 15 minutes.
19. The pellet was resuspended in 1 ml DEPC water and 2 volume of 4.5 M sodium acetate was added.
20. The RNA was again precipitated at 4°C for 3 hours, and spun at 3,000 rpm for 15 minutes.
21. The pellet was resuspended in 400 μl DEPC treated water and then transferred to siliconised microfuge tubes.
22. Sodium acetate (40 μl of 20%) and 1 ml cold (-20°C) absolute ethanol was added and the tubes was stored at -20°C until required.

The RNA samples were prepared and run on a large formamide/formaldehyde gel. The materials for preparing the RNA samples and formamide/formaldehyde gel are outlined in Appendix 13.

5.2.7 Southern blotting

The buffers and solutions used for Southern blotting are outlined in Appendix 14. The Southern blotting methods used for plant DNA are described in the following Sections.

5.2.7.1 Running the DNA samples on gel

1. To prepare the agarose gel, 1.75 g of agarose (BRL) was dissolved in 250 ml of 1xTAE buffer by heating in a microwave.
2. Ethidium bromide (25 ml, 10 mg ml^{-1}) was added to the gel and mixed before pouring the gel in a gel tray (20 x 20 cm).
3. The gel was allowed to cool for 30-40 minutes and then covered with 2250 ml of 1xTAE buffer.
5. Restriction enzyme digested plant genomic DNA (30 μg)was loaded in each well and the gel was run at 30 mA overnight.
6. Following electrophoresis, the agarose gel was placed in 0.25 M HCl for

about 20 minutes for depurination of DNA.

7. The gel was rinsed in distilled water and placed in denaturation buffer for 30 minutes at room temperature with shaking.
8. The gel was rinsed in distilled water and placed in neutralising solution for 15 minutes at room temperature with shaking; this step was repeated.

5.2.7.2 Capillary blotting

1. A container was filled with 20xSSC and flat glass plate was placed as a bridge on top of the container made from three sheets of 3 MM paper was placed over the glass plate with its end submerged in the 20xSSC. The wick was saturated with 20xSSC.
2. The gel was placed on the wick and surrounded with plastic film and air bubbles were removed from beneath the gel.
3. A sheet of positively charged Hybond-N^+ nylon membrane was cut to the exact size of the gel, pre-soaked with 20xSSC, and placed on top of the gel.
4. Three pieces of 3 MM paper were cut to the exact size of the gel, pre-wetted with 20xSSC, and placed on top of the nylon membrane. A pack of paper towels was placed on top of the 3 MM papers. A weight of approximately 0.5 kg was placed on top and the assemble was left overnight for DNA transfer from the gel onto the membrane to occur.

5.2.7.3 Alkali fixation of DNA to the membrane

Following blotting but before removing the membrane from the gel, the membrane was marked with a pencil to allow later identification of lanes. The membrane was placed between 2-3 layers of 3 MM paper pre-wetted with 0.4 M NaOH and left for 20 minutes to fix the DNA to the membrane. The membrane was removed and washed briefly in 5xSSPE for up to 1 minute. To store the membrane for later use, the membrane was sealed in plastic film and stored at 4°C.

5.2.7.4 Hybridisation of probed DNA to membrane

The hybridisation of membrane was carried out in two separate steps as follows:

1. Prehybridisation: 50 µl of herring sperm DNA (10 mg ml^{-1}) was heated in a boiling water bath for 3 minutes to denature the DNA. The DNA was immediately chilled in ice and was then added to the prehybridisation solution. The membrane was placed in a plastic bag and then prehybridisation solution (Appendix 14) was poured into the bag. The bag was sealed and incubated at 65°C with shaking for 1 hour.
2. Hybridisation: the labelled probe DNA was denatured by heating in a boiling water bath for 3 minutes and it was then immediately chilled on ice. The denatured probe was added to the prehybridisation solution (step 1) and incubated with the membrane for a further 12-15 hours at 65°C with shaking.

After hybridisation, the membrane was washed twice in 500 ml of 2xSSPE, 0.1% (w/v) SDS at room temperature for 15 minutes The membrane was then washed in a 500 ml of 1xSSPE, 0 1% SDS at 65°C with shaking for 15 minutes. The filter was removed and sealed in plastic film and then exposed to X-ray film at -80°C for 1 week.

5.2.8 RNA dot blotting technique

1. The concentration of RNA samples was determined and aliquots containing 20 µg of RNA were removed. The RNA was precipitated with ethanol and dried as described above (Section 5.2.6).
2. The RNA samples were resuspended in 6 µl of DEPC treated H_2O and to this was added 3 volumes of the following solution:

formamide	500	µl
10 x MOPS buffer	100	µl
formaldehyde	162	µl

3. The samples were incubated at 65°C for 5 minutes and then immediately

chilled on ice.

4. A Bio-Dot (BioRad) dot blotting apparatus with a 12x8 well format was used to load the samples onto Hybond-N+ membrane. The membrane was cut to the exact size of 9 x 12 cm. The apparatus was assembled and connected to a vacuum pump.
5. Each RNA sample (24 μl) was divided in two 12 μl samples.
6. To the first well, 6 μl of the sample containing 10μg of RNA was loaded, and to the second well 3 μl (5 μg). Formamide/formaldehyde/MOPS solution (3 μl) (step 2) was added to the remaining 3 μl RNA sample to achieve a 1:1 dilution and 3 μl of this was added to the 3'rd well. This sequential dilution continued up to well number 8. Thus 8 sequential dilutions from 1 to 1/128 were achieved for each RNA sample in each lane.
7. The pump was disconnected, the apparatus was dismantled and the membrane was placed between 2-3 layers of 3 MM paper pre-wetted with 0.05 M NaOH for 5 minutes.
8. The remaining 12 μl of each sample (from step 6) was loaded onto a second membrane in the same manner.
9. One of the membranes was hybridised with the labelled 730 DNA fragment from within the dehalogenase gene and the other membrane (as a control) was hybridised with labelled 18S and 25S rDNA fragments isolated from plasmid pBG35 (Ellis *et al.,* 1983; Goldsbrough and Cullis, 1981) digested with *Kpn*I, *Bam*HI, *Hind*III and *Eco*RI restriction enzymes.

5.2.8.1 Dot blot Hybridisation

The method for prehybridisation and hybridisation were similar to those of described above for Southern blot hybridisation. The exposure time to X-ray film was approximately 24 hours for the membrane hybridized with the labelled 730 bp fragment and 2 hours for the membrane hybridized with the labelled rDNA fragments.

5.2.9 Tobacco leaf transformation method

The method used for tobacco leaf transformation was based on that of Rogers *et al.* (1986) with some modifications. The solutions and materials are given in Appendix 15. The actual transformation method is outlined as follows:

1. The KKD tobacco line seeds were surface sterilised and spread on the surface of a plate containing 1/2 MS medium (Appendix 5) solidified with 0.8% Difco agar. Plates were incubated in a culture room at 28°C for 1 week.
2. Two seedlings were transplanted into a single 230 ml transparent polystyrene container (Lifetech, New Zealand) containing 35 ml of 1/2 MS medium supplemented with 0.8% Difco agar, and this was incubated in a culture room at 28°C for a further 3-8 weeks.
3. Fully expanded leaves were cut into leaf discs with a sterile cork borer (6 mm diameter).
4. *Agrobacterium tumefaciens* containing plasmid pAS501 was inoculated into 10 ml of TY medium supplemented with 10 mg L^{-1} tetracycline and incubated at 28°C with shaking for ~30 hours.
5. The *Agrobacterium* was spun down at 3500 rpm for 5 minutes in a Sorvall SS-34 rotor and resuspended in 1 ml of 10 mM $MgSO_4$.
6. Approximately 25 leaf discs were transferred into a sterile 20 ml bottle containing 1 ml of the *Agrobacterium* suspension.
7. The bottle was shaken and the leaf discs were then blotted onto sterile filter paper and transferred onto the surface of NicI plates (25 leaf discs on each plate).
8. The plates were incubated in a culture room at 28 °C for 4 days.
9. The leaf discs were transferred to NicII and NicII/kanamycin-free plus dalapon media to select for genetically transformed cells.
10. The transformed cells were grown to callus and then to shoots in NicII and NicII/kanamycin-free plus dalapon media.
11. The differentiated calli and shoots from the NicII medium were transferred to NicII/kanamycin-free plus dalapon medium whereas the calli grown in the NicII/kanamycin-free plus dalapon medium were transferred to NicII medium

so that selection for both of the T-DNA genes (dehalogenase and kanamycin) could be achieved.

12. Shoots containing a few small leaves together with a small section of callus were removed and transferred to NicIII or NicIII/kanamycin-free plus dalapon plates.

It should be noted that, the shoots from the NicII plates were transferred to NicIII plates and the shoots from the NicII/kanamycin-free plus dalapon plates were transferred to NicIII/kanamycin-free plus dalapon plates to allow roots to develop.

5.3 Results

5.3.1 T-DNA organisation in tobacco line 51-1 and subsequent generations

The binary vector plasmid pAS501 (Appendix 17, Figure A.5) has been developed by insertion of both a dehalogenase gene and a neomycin phosphotransferase II (NPTII) gene, together with left border (LB) and right border (RB) regions of T-DNA, into the *Eco*RI site of plasmid pRK290 (Ditta *et al.,* 1985; Appendix 17, Figure A.6).

In order to obtain a fragment of DNA from within the dehalogenase gene to use as a probe, the plasmid pAS501 was mapped by restriction enzyme digestion as described in Materials and Methods (5.2.2). A restriction enzyme site map of plasmid pAS501 is presented in Figure 5.1. It should be noted that restriction site positions indicated on the map are approximations. The size of the plasmid is approximately 25 kb and is similar to the size of the T-DNA plus plasmid pRK290. There are no *Hind*III or *Eco*RI cleavage sites in the plasmid DNA (Appendix 16, Figure A.1). However, there are several restriction enzyme sites within the T-DNA region, which allowed the cloning of a dehalogenase gene fragment.

The DNA fragment between the *Sac*II site downstream of the dehalogenase gene and the *Pst*I site within the gene was isolated and ligated into the multiple cloning site of the plasmid vector pSK+. The resulting plasmid, pSDH, was digested with both *Pst*I and *Sac*II to isolate a 730 bp fragment from within the dehalogenase gene (Appendix 16, Figure A.4) . This fragment was used as a DNA probe in RNA and DNA hybridisation studies.

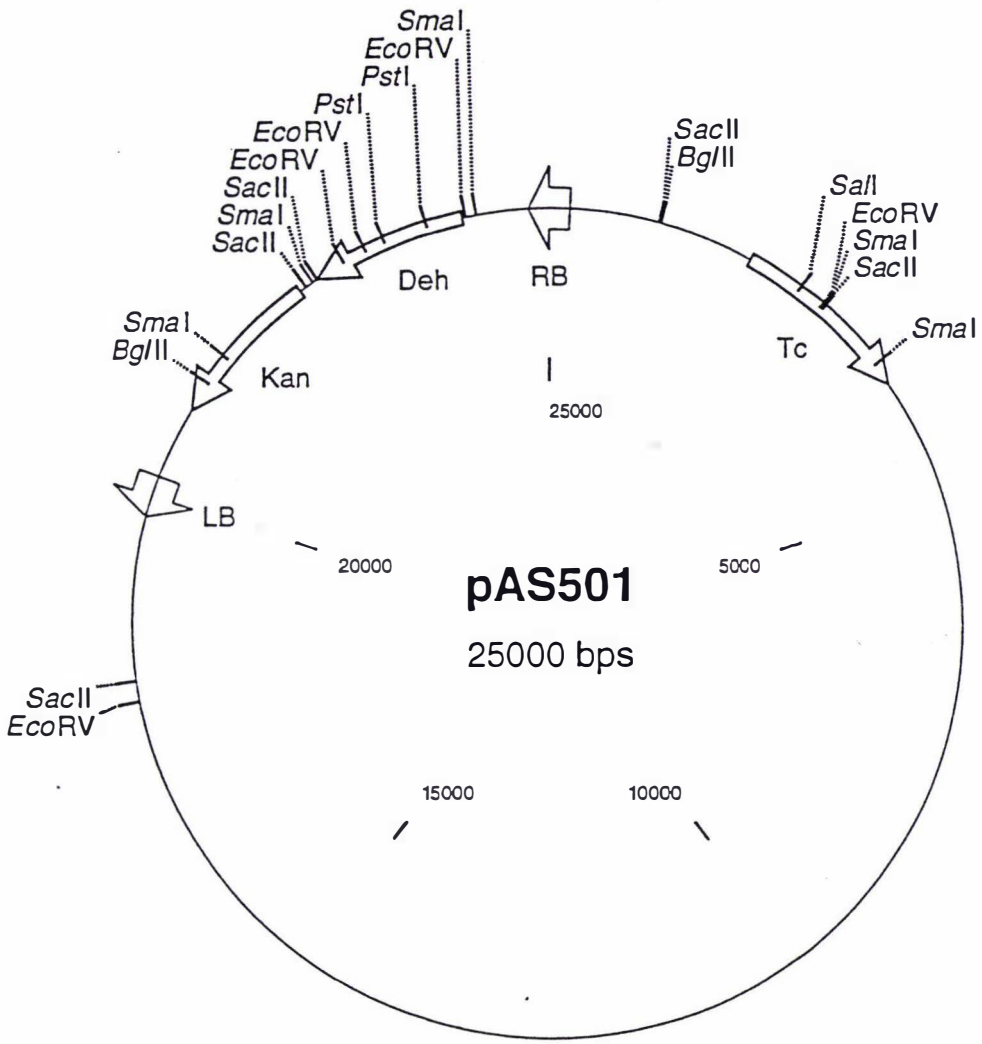


Figure 5.1. A restriction enzyme map of plasmid pAS501. Positions of restriction enzyme cleavage sites were determined by analysis of fragments resulting from single and double digests of the plasmid with the various restriction enzymes. The known *Bgl*II and *Sma*I enzyme sites, outside the T-DNA borders of plasmid pRK290 (Ditta *et al.*, 1985), were key points for determining the position of other enzyme sites.

The plant material used to study T-DNA organisation were; 51-1 homozygous transgenic parental line, the KKD a non-transgenic parental line, as well as TF_1 (51-1 x KKD), TBC_1 (TF_1 x 51-1), TBC_2 (TF_1 x KKD) and TF_2 (selfed TF_1) progeny plants. Detailed information about these lines is given in Chapter 4, Sections 4.2.1.2 and 4.2.2.1. From each of the parents and TF_1 lines, four plants were randomly selected and used in the T-DNA organisation studies. Since segregation of the T-DNA in the TBC_1 population did not occur, 15 plants were randomly selected. Fifteen plants were selected from the TBC_2 line, consisting of 9 resistant and 6 susceptible (negative) plants. However, due to a higher segregation frequency in the TF_2plants, 19 plants consisting of 12 resistant and 7 susceptible plants were selected for the T-DNA organisation studies. Isolated plant DNA was digested separately with either *Eco*RI or *Bgl*II restriction enzymes. As discussed above, although there is no *Eco*RI recognition sites on the plasmid, there is a single *Bgl*II recognition site within the T-DNA region. Therefore the *Bgl*II enzyme was used to obtain further information about copy number and the organisation of T-DNA integrated into plant DNA, as well as for confirmation of the result from the *Eco*RI digestion. From each generation (except the non-transgenic line) one plant was selected and its DNA was digested with *Bgl*II alone and with *Bgl*II/*Eco*RI together.

The number of copies of the dehalogenase gene integrated into plant genomic DNA can be estimated from the number of bands detected by Southern hybridisation that represent T-DNA flanked by plant genomic DNA. Since there are no *Eco*RI restriction site within the T-DNA region, the size of the fragments produced by *Eco*RI digestion depend upon the *Eco*RI sites within the flanking plant genomic DNA. The Southern blot result for DNA from the 51-1, KKD and TF_1 plants digested with *Eco*RI is presented in Figure 5.3. The stem heights of the plants are presented in Table 5.1. There are two copies of the dehalogenase gene for 51-1 and TF_1 plants, while the non-transgenic parent, KKD lacks the dehalogenase gene, therefore there are no sequences in control non-transgenic plants with homology to the dehalogenase gene. The size of the hybridizing fragments are approximately 7.2 and 5.2 kbp, indicating that two copies of the T-DNA are located within the genome of the 51-1 and TF_1 plants. Furthermore, since the size of both fragments are less than 10 kbp (expected for two copies of the T-DNA repeated in tandem), the T-DNAs are not likely to be tandem repeats.

The larger fragment indicates that the flanking region of the T-DNA is about 2.2 kbp and that the flanking *Eco*RI sites are located some distance from the T-DNA, while the smaller fragment indicates that the flanking *Eco*RI sites are located near the right and left borders of the T-DNA within the plant genome. The effect of the herbicide on both parents, as well as TF_1 plants, is presented in Figure 5.2A.

The Southern blot result for DNA from TBC_1 plants digested with *Eco*RI is presented in Figure 5.4. All of the TBC_1 plants show two similar sized bands hybridising to the dehalogenase probe. The Southern blot result is in agreement with result for resistance to the herbicide as shown by generation mean (Chapter4, Section 4.3.1). There are no susceptible (negative) plants in the TBC_1 generation (Figure 5.2B) and all of the plants have a similar herbicide resistant phenotype. The Southern blot results are also in agreement with the inheritance pattern of the transgene in the TBC_1 generation. Hence the resistance phenotype is correlated with the integration of T-DNA into the plant genome. The stem heights of these TBC_1 plants used as a resistance symptom and measured after the herbicide application, as well as the presence or lack of the T-DNA are presented in Table 5.2.

The stem heights and of the TBC_2 plants are presented in Table 5.3. For the TBC_2 generation, the Southern blot result of an *Eco*RI digest shows that the susceptible (negative) plants lack the transgene in their genome, (Figure 5.5.), whereas TBC_2 plants with a resistance phenotype carry two copies of the dehalogenase gene. Hence the result of the Southern analysis of the TBC_2 plants is in agreement with the segregation pattern and phenotype of the plants. The result also reveals that the TBC_2 population is undergoing genotypic segregation. By comparing the TBC_1 and TBC_2 Southern blot results, it can be concluded that the hemizygous plants have both a similar herbicide resistance phenotype and T-DNA organisation as the homozygous transgenic plants, while the susceptible (negative) plants have a susceptible phenotype and lack integrated T-DNA. The phenotype of the susceptible (negative) plants is similar to the non-transgenic control plants. Furthermore, the Southern blot analysis also shows the absence of T-DNA in the genomes of susceptible (negative) plants. This result indicates that there is a direct correlation between the presence of T-DNA and the herbicide resistance phenotype in transgenic plants.

The stem height and the presence or lack of the T-DNA for each of the TF_2

plants is presented in Table 5.4. In the TF_2 generation, the susceptible (negative) plants lack T-DNA (Figure 5.6), while the resistant transgenic plants have two copies of the T-DNA integrated into their genomes.

To obtain more information on the organisation of the T-DNA in the transgenic plants, genomic DNA of the 51-1 parent (lane 1, Figure 5.5), the TBC_1 generation (lane 6, Figure 5.4), the TBC_2 generation (lane 2, Figure 5.5) and the TF_2 generation (lane 18, Figure 5.6) was digested with *Bgl*II and *Eco*RI/*Bgl*II and probed with the 730 bp dehalogenase gene fragment. The Southern blot result of the *Bgl*II digests of DNA for these plants is presented in Figure 5.7. The Southern blot result of the single *Bgl*II digest (lanes 1, 2, 3, and 4) indicates that two copies of the T-DNA are integrated into the genomes of the different plants. The organisation of the T-DNAs in the genomes of each of these plants is the same. The results for the double digest of the plant DNAs with *Bgl*II/*Eco*RI (lanes 5, 6, 7, and 8) are similar to the single enzyme digest results. It is suggested that the recognition sites for both *Bgl*II and *Eco*RI are close to each other within the plant's genome and adjacent to the integration sites for the T-DNA.

Regarding the organisation of the T-DNAs in the host plant genome, it is possible that the two T-DNA copies are integrated in one chromosome with only a short distance between them. The reasons for this suggestion are given as follows:

1. In the case of integration of the T-DNAs in two separate chromosomes, the hemizygous TF_1 plants (i.e TF_1 plants in generation mean) would be expected to have a single band in the Southern analysis. This is because, in the hemizygous TF_1 plants, only one of the homologous chromosomes would be inherited from a transgenic parent, while the other homologous chromosome would be inherited from the non-transgenic control parent. From the similarity of the bands of the two genotypes, it is concluded that both copies are integrated into the same chromosome.
2. The chi-square results (see Chapter 3 Section 3.3.2.5) indicate single locus inheritance in the resistant transgenic 51-1 line.

Therefore, from the chi-square and Southern blot results, it can be concluded that two copies of the dehalogenase are located in one chromosome close to each other (linked genes) and act as one locus. Three possible arrangements of the integrated T-DNAs are illustrated in Figure 5.8.

Figure 52. Effect of dalapon on different generations of tobacco. The transgenic parent 51-1, non-transgenic parental plants *Nicotiana tabacum* (line KKD) and (51-1 x KKD)TF_1, plants (A). The non-transgenic plants are highly susceptible to the herbicide, while the transgenic parental plants as well as the TF_1 plants are resistant to the herbicide. The (51-1 x TF_1) backcross (TBC_1) are resistant to the herbicide (B). Segregant susceptible plants were not observed in the TBC_1 plants. By comparing the transgenic parent plants and the non-transgenic control plants the TBC_1, plants is concluded that the transgene is dominant in the transgenic parent.

Table 5.1. Presence of the dehalogenase gene and stem height of parents and TF_1 plants

Line	Plant number	T-DNA	Stem height (mm)
51-1	1	+	77
51-1	2	+	75
51-1	3	+	69
51-1	4	+	65
KKD	5	-	12
KKD	6	-	15
KKD	7	-	20
KKD	8	-	18
TF_1	9	+	79
TF_1	10	+	66
TF_1	11	+	75
TF_1	12	+	68

Table 5.2. Presence of the dehalogenase gene and stem height of TBC_1 plants

Plant number	T-DNA	Stem height (mm)
1	+	70
2	+	80
3	+	62
4	+	65
5	+	60
6	+	60
7	+	55
8	+	55
9	+	62
10	+	67
11	+	80
12	+	80
13	+	69
14	+	65
15	+	65

Table 5.3. Presence of dehalogenase and stem height of TBC_2 plants

Plant number	T-DNA	Stem height (mm)
1	+	60
2	+	65
3	-	20
4	-	12
5	+	55
6	+	62
7	-	11
8	-	5
9	+	58
10	+	65
11	-	10
12	-	12
13	+	62
14	+	50
15	+	60

Table 5.4. Presence of the dehalogenase gene and stem height of TF_2 plants

Plant number	T-DNA	Stem height (mm)
1	+	82
2	+	52
3	+	70
4	+	70
5	+	52
6	+	50
7	-	20
8	-	20
9	-	15
10	-	11
11	-	10
12	-	20
13	-	20
14	+	75
15	+	80
16	+	55
17	+	72
18	+	50
19	+	85

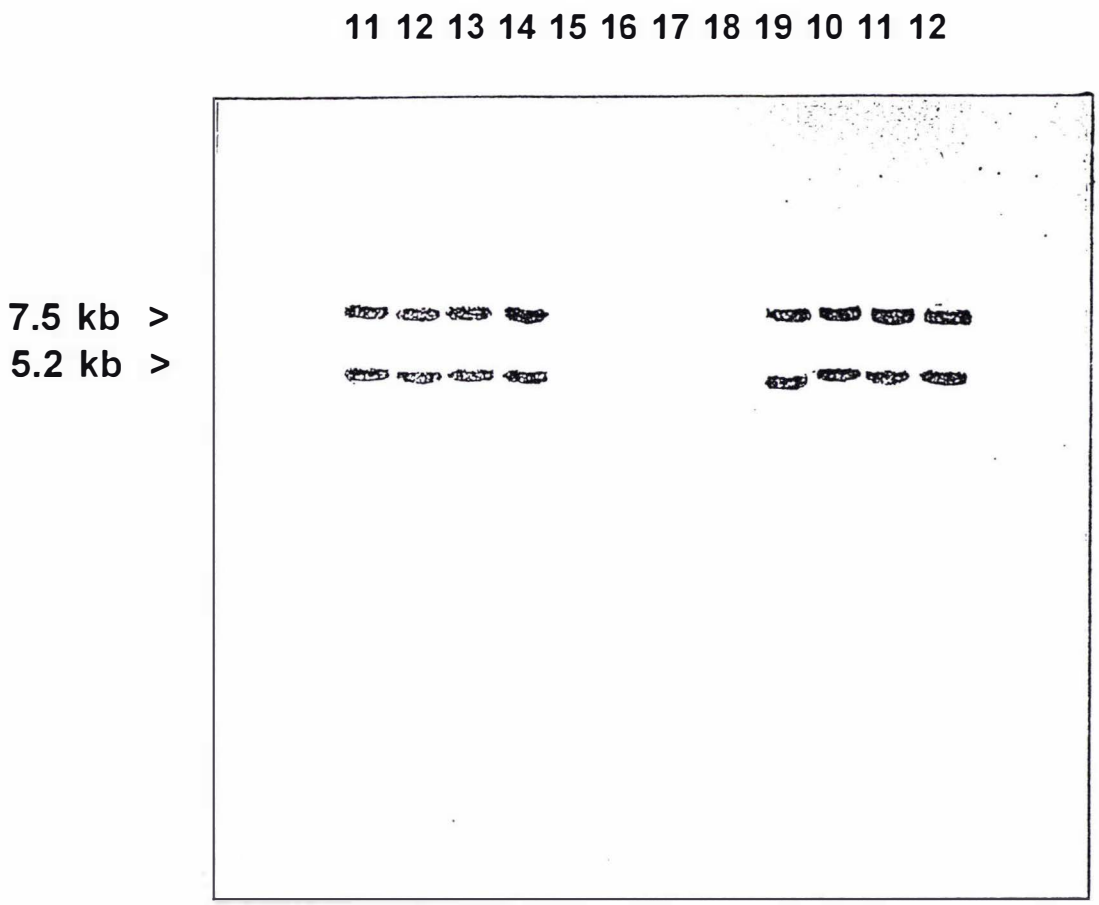


Figure 5.3. Southern blot of parent and TF_1 plants. The Southern blot result of transgenic 51-1 parent (lanes 1, 2, 3 and 4), non-transgenic parent KKD (lanes 5, 6, 7 and 8) and (51-1 x KKD)TF_1 plants (lanes 9, 10, 11 and 12). Genomic DNA of each of the plants was digested with *Eco*RI and hybridized with the labelled 730 bp dehalogenase gene fragment.

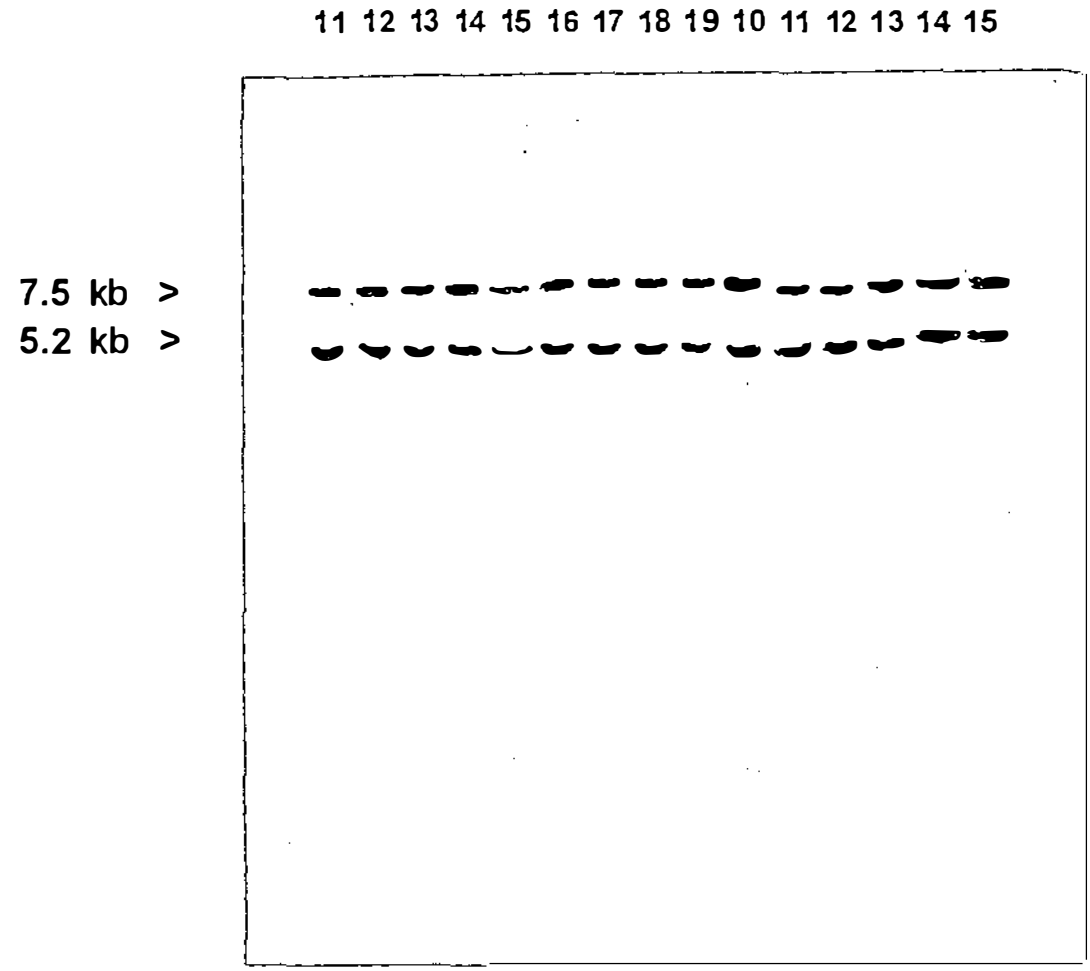


Figure 5.4. Southern blot result of TBC_1 plants. Genomic DNA of each of the plants was digested with *Eco*RI and hybridized with the labelled 730 bp dehalogenase gene fragment. All of the plants show the two fragments of 7.2 and 5.2 kb.

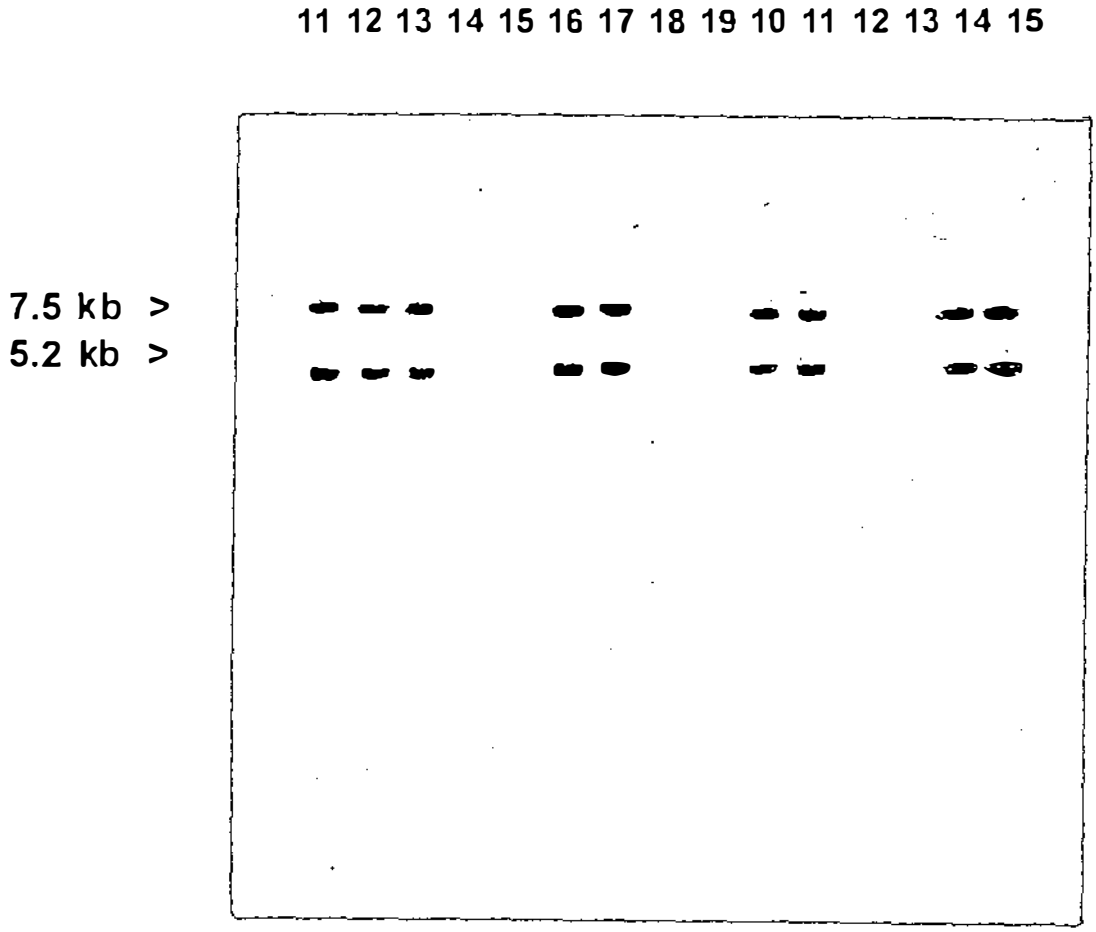


Figure 5.5. Southern blot result of TBC_2 plants. Genomic DNA of each of the plants was digested with *Eco*RI and hybridized with the labelled 730 bp dehalogenase gene fragment.

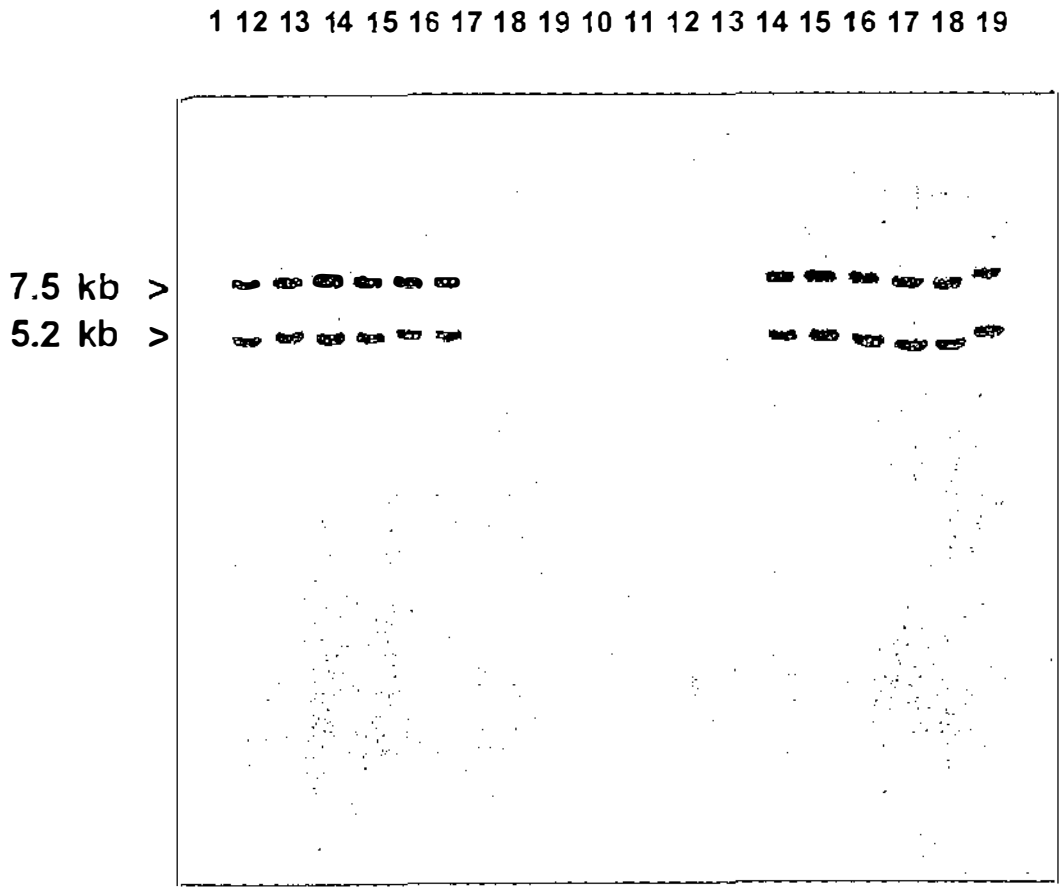


Figure 5.6. Southern blot result of TF_2 plants. Genomic DNA of each of the plants was digested with *Eco*RI and hybridized with the labelled 730 bp dehalogenase gene fragment.

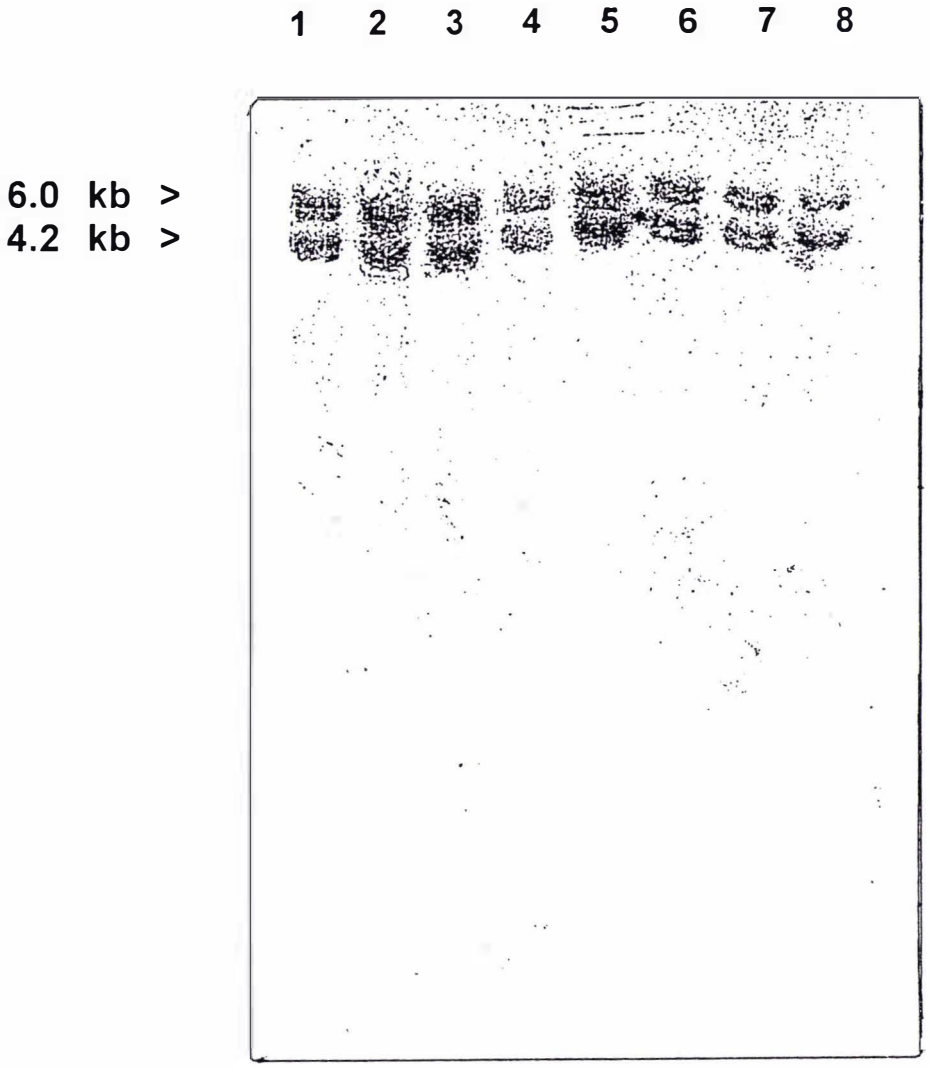
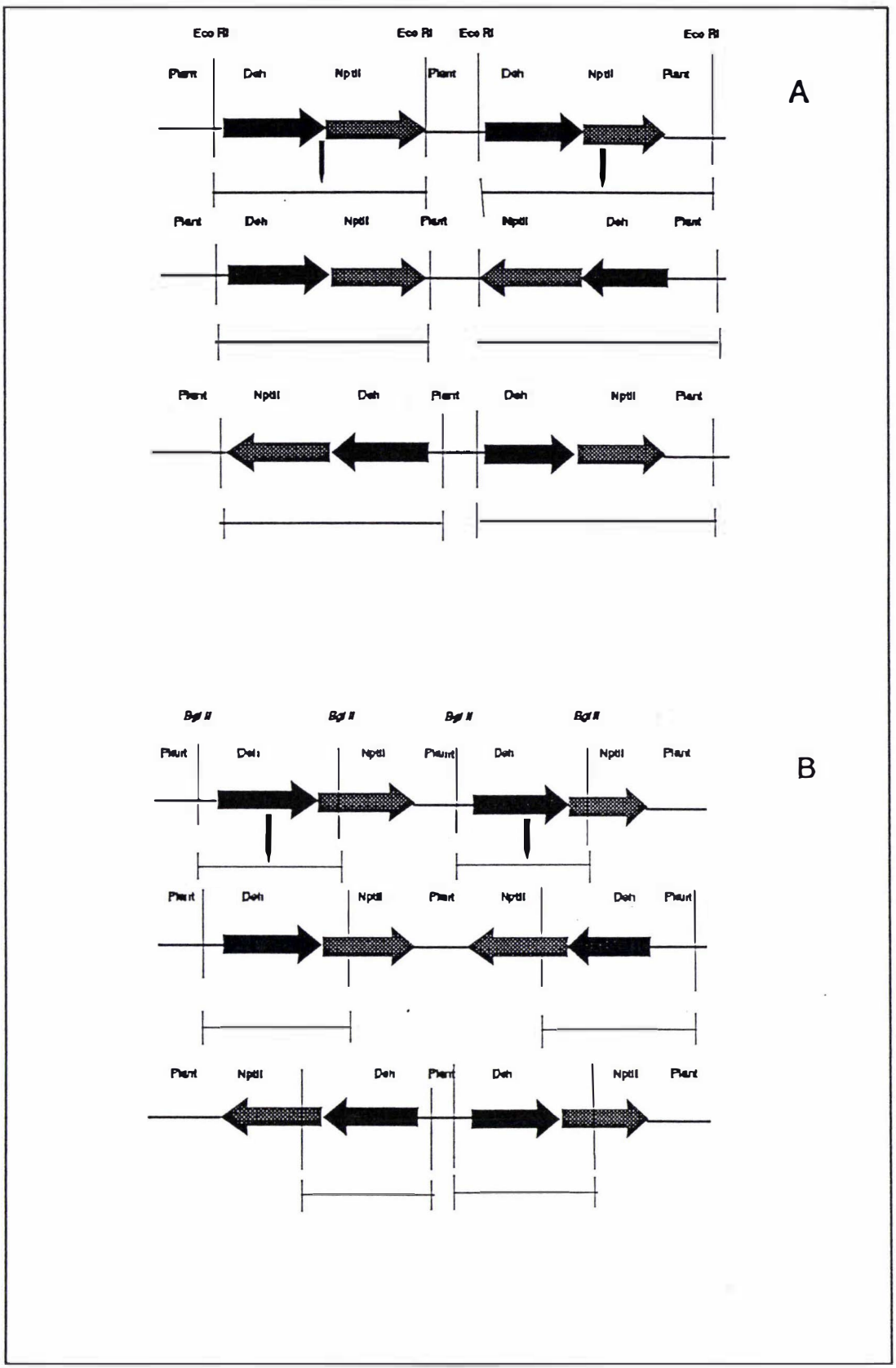


Figure 5.7. Southern blot of *Bgl*II and *Eco*RI/*Bgl*II digests of DNA extracted from transgenic plants. The transgenic plant DNA was digested with *Eco*RI/*Bgl*II (lanes 1, 2, 3, and 4) and *Bgl*II (lanes 5, 6, 7, and 8) and hybridized with the 730 bp labelled dehalogenase gene fragment. Lanes 1 and 5 are the parental line 51-1 (equivalent to lane number 1 in Figure 5.3), lanes 2 and 6 are the TBC_1 plants (equivalent to lane number 4 in Figure 5.4), lanes 3 and 7 are the TBC_2 plants (equivalent to lane number 2 in Figure 5.5) and, lanes 4 and 8 are TF_2 plants (equivalent to lane number 18 in Figure 5.6).

Figure 5.8. Proposed model for T-DNA organisation in 51-1 transgenic line. To determine the organisation of the T-DNA, plant genomic DNA was digested with *Eco*RI (A) and *Bgl*II (B) and hybridized with labelled 730 bp fragment of dehalogenase gene..


Eco RI
Eco RI
Eco RI
Eco RI
Plant
Deh
NptII
Plant
Deh
NptII
Plant
A
Plant
Deh
NptII
Plant
NptII
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Bgl II
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Deh
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Deh
NptII
Plant

5.3.2 Dehalogenase-specific mRNA level

The diallel crossing results indicate that there are significant differences between the transgenic and non-transgenic parents as well as between the TF_1 progeny, in their responses to the herbicide (see Chapter 4, Sections 4.3.2 and 4.3.3). To check the relationship between the level of resistance of the transgenic plants to the herbicide and the level of steady state dehalogenase-specific mRNA, the following plant lines with different herbicide resistant phenotypes were used.

1. The 51-1 homozygous transgenic parent, highly resistant to herbicide.
2. Non-transgenic line KKD, highly susceptible to herbicide.
3. (51-1 x KKD)TF_1 plants, highly resistant to herbicide.
4. (B21 x 51-1)TF_1 plants, with low resistance to herbicide .

Six single plants were randomly selected in each line and total RNA was isolated from these plants. The integrity of RNA was confirmed by separation on a formamide/formaldehyde gel.

The non-transgenic plants were used as control plants to check that the probe did not hybridise to mRNA other than the transgene dehalogenase-specific mRNA. To compare the signal intensity between single plants of each line, the following internal comparison was used; the line with the highest signal intensity at the lowest amount of RNA (0.16 μg) was given a score of 1 (e.g. lane 16 Figure 5.11). The signal intensities of the other lines were then compared with this line and a score was determined. For example, in Figure 5.11, lane number 19 at 10 μg (2xdilution) has a similar intensity to that of the lane 16 at 0.16 μg (128x dilution) and therefore the score of this plant is $1/64 \approx 0.16$ (see Table 5.5)

The total RNA isolated from each plant was quantified spectrophotometrically and 40 μg was denatured, divided into two equal sub samples and loaded onto a positively charged nylon membrane in 2x sequential dilutions (see Materials and Methods Sections 5.2.8 and 5.2.8.1). One of the membranes was hybridized with the labelled 730 bp dehalogenase gene fragment to check the level of dehalogenase-specific mRNA. To check that there was an equal amount of total RNA loaded onto each well of the membrane, the another membrane was hybridized with labelled 18S and 25S rDNA fragments

isolated from plasmid pBG35 (Ellis *et al.,* 1983; Goldsbrough and Cullis, 1981) digested with *Kpn*I, *Bam*HI, *Hind*III and *Eco*RI restriction enzymes (see Material and Methods, Section 5.2.8.1). A map of plasmid pBG35 is given in Figure 5.9.

Following hybridisation, the membrane probed with the dehalogenase gene fragment was exposed for 24 hours while the membrane probed with the rDNA probe was exposed for only 2 hours. Although the dot blot result for the rDNA-probed membrane indicates that there is some variation in the amount of RNA loaded onto the membrane for each sample, this variation is small and can not account for the large variation seen for the membrane probed with the dehalogenase gene. Figure 5.11 indicates that there are significant differences in steady state dehalogenase-specific mRNA levels between the different lines.

None of the six non-transgenic KKD plants (Figure 5.11, lanes 1, 2, 3, 13, 14 and 15) show a signal on the membrane when hybridized separately with the labelled dehalogenase probe for all of the RNA loadings. By comparing the two membranes hybridized with the labelled rDNA probe and the dehalogenase probes, it can be concluded that the control plants do not have RNA homologous to the dehalogenase gene. In contrast, all of the transgenic lines have dehalogenase-specific mRNA, although there are significant differences in steady state dehalogenase-specific mRNA levels for the dehalogenase gene between the plants. It should be noted that the dot blot result of the non-transgenic plants is in agreement with the Southern blot results where no homologous DNA sequences were detected in control plants. The variation in steady state dehalogenase-specific mRNA level of (51-1 x KKD) TF_1 plants (Lanes 10, 11, 12, 22, 23 and 24, Figure 5.11) and 51-1 transgenic parent plants (lanes 4, 5, 6, 16, 17 and 18, Figure 5.11) is very low compared with susceptible (51-5 x B21) TF_1 plants (Lanes 7, 8, 9, 19, 20 and 21, Figure 5.11). The dot blot result indicates that the susceptible plants, either control or low resistant (51-5 x B21)TF_1 transgenic plants, have a total lack of or only a very low level of the dehalogenase-specific mRNA. The level of resistance of the plants to the herbicide is presented in a diallel crossing analysis in Chapter 4, Tables 4.11, 4.12, 4.17 and 4.18 and discussed in Sections 4.4.3 and 4.4.4. By comparing the dot blot results and responses of the plants to the herbicide, it is concluded that there is a correlation between the level of resistance to the herbicide and the level of dehalogenase-specific mRNA. The RNA dot blot results also indicate that within the transformants, the molecular-based differences

are related to the level of steady state dehalogenase-specific mRNA of the transgene. An internal comparison of the dot blot result (Table 5.5) indicates that there is significant difference in dehalogenase-specific mRNA levels between the resistant and susceptible transgenic plants.

An analysis of variance of the intensity of those signals on the RNA dot blot which hybridized with the dehalogenase probe is presented in Table 5.6. There are significant ($P>0.001$) differences in signal intensity between the lines. However the non-significance of replications indicated a similarity between the intensity of the plants within each of the lines.

The mean signal intensity of the each of the lines is presented in Table 5.7. The intensity of signal of the 51-1 transgenic parent and the resistant (51-1 x KKD)TF_1 hybrid progeny are not significantly different. However there are significant differences between these two lines and the susceptible (51-5 x B21)TF_1 hybrid progeny. The intensity of the signal of the (51-5 x B21)TF_1 hybrid progeny is significantly low.

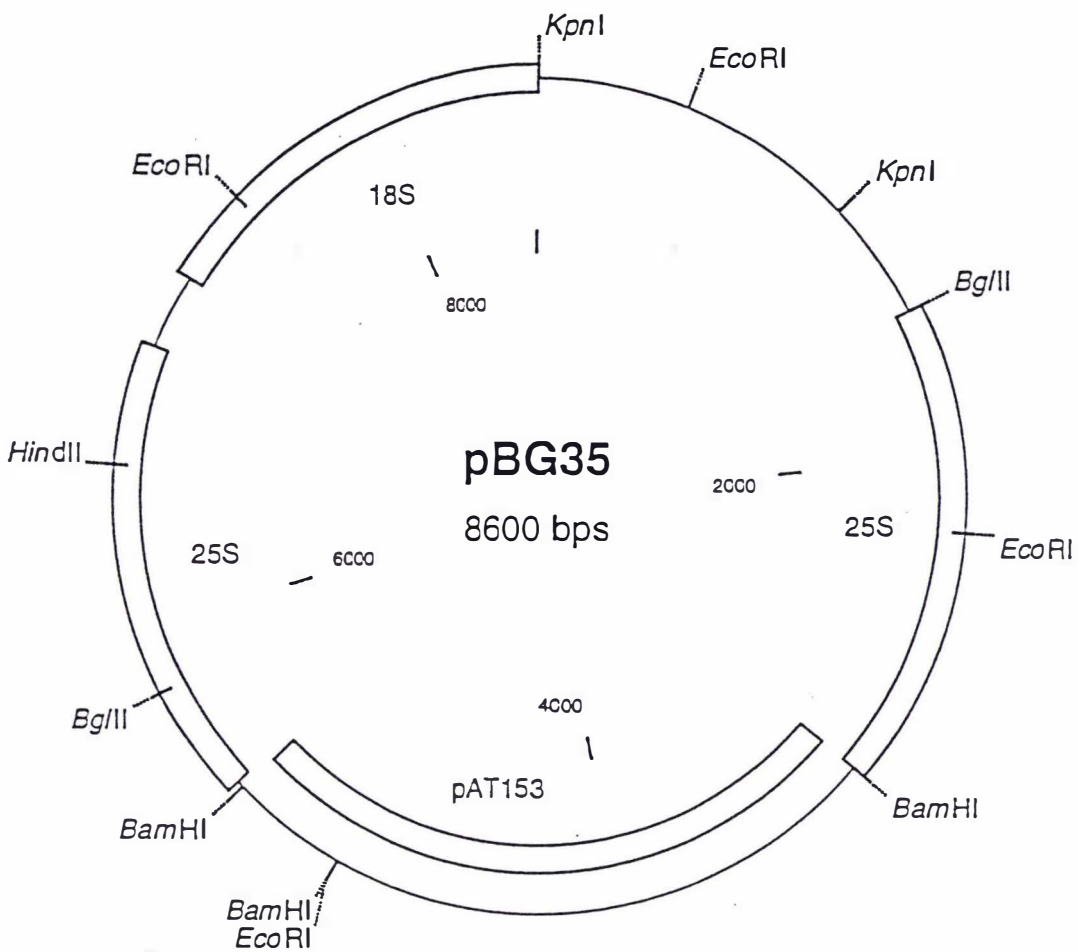


Figure 5.9. Restriction enzyme map of plasmid pBG35. This plasmid contains two copies of the 25S and one copy of the 18S rDNA. The plasmid was digested with *Eco*RI, *Hind*III, *Bam*HI and *Kpn*I restriction enzymes and the resulting fragments were used as DNA probes in RNA studies.

Table 5.5. Internal comparison of signal intensity between samples in the dot blot experiment

Line	Plant number	Relative signal intensity
KKD	1	0
KKD	2	0
KKD	3	0
KKD	13	0
KKD	14	0
KKD	15	0
51-1	4	0.25
51-1	5	0.25
51-1	6	0.50
51-1	16	1.00
51-1	17	0.25
51-1	18	0.50
TF_1(S) *	7	0.016
TF_1(s) *	8	0.016
TF_1(s) *	9	0.032
TF_1(s) *	19	0.016
TF_1(s) *	20	0.016
TF_1(s) *	21	0.016
TF_1(r)**	10	0.25
TF_1(r)**	11	1.00
TF_1(r)**	12	0.125
TF_1(r)**	22	1.00
TF_1(r)**	22	0.125
TF_1(r)**	24	1.00

* (51-5 x B21) susceptible TF_1
** (51-1 x KKD) resistant TF_1

Table 5.6. Analysis of variance of the RNA dot blot signal of lines probed with the dehalogenase gene

Effect	MS
Replication	0.1035 ns
Line	0.5392 **
Error	0.0641

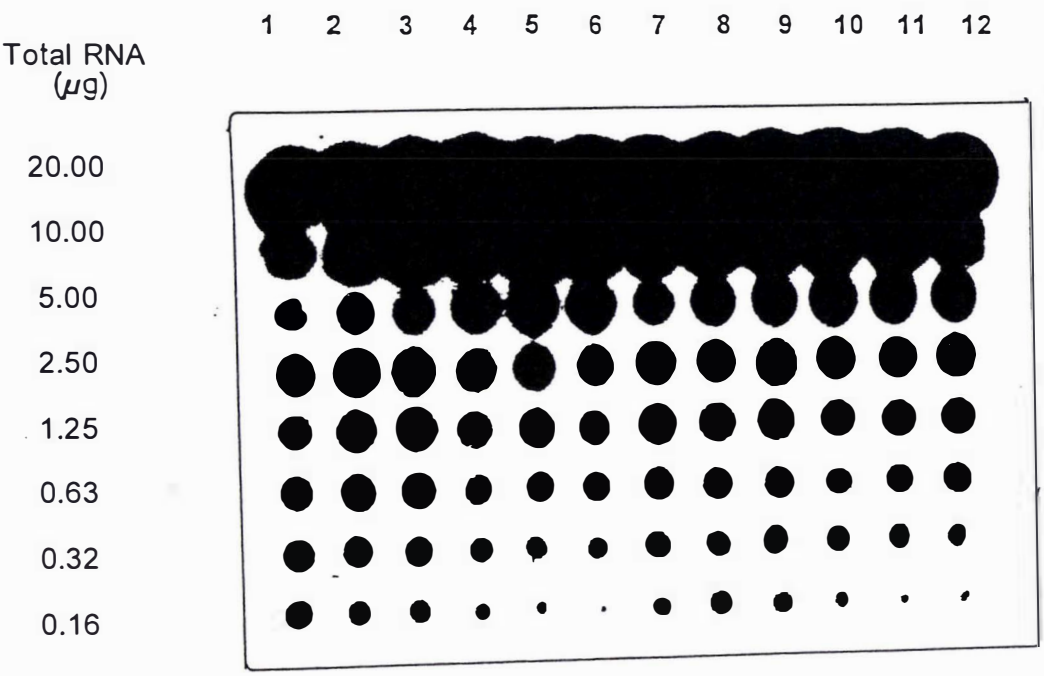
ns and **: non-significant and significant and 1% probability level respectively.

Table 5.7. Mean intensity of signal of RNA dot blot probed with the dehalogenase gene for different transgenic lines

Line	Mean
51-1 parent	0.583 a[1]
(51-1 X KKD)TF_1	0.458 a
(51-5 x B21)TF_1	0.019 b

[1] Means with the same letter within the column are significantly different (P=0.05%).

Figure 5.10. Dot blot of RNAs from susceptible and resistant tobacco lines probed with a rRNA gene probe. Total RNA was isolated from six individual plants from each of the following lines: non-transgenic parent, KKD (lanes 1, 2, 3, 13, 14 and 15); transgenic parent 51-1, (lanes 4, 5, 6, 16, 17 and 18); (51-5 x B21)TF_1 (lanes 7, 8, 9, 19, 20 and 21) and (51-1 x KKD)TF_1 (lanes 10, 11, 12, 22, 23 and 24). RNA was isolated from fully expanded leaves, transferred to positively charged nylon membrane, and probed with ^{32}P radiolabelled 18S and 25S rDNA fragments isolated from plasmid pBG35 digested with *Kpn*I, *Bam*HI, *Hind*III and *Eco*RI restriction enzymes (Figure 5.9). Autoradiography was for 2 hours.


1 2 3 4 5 6 7 8 9 10 11 12
Total RNA (μg)
20.00
10.00
5.00
2.50
1.25
0.63
0.32
0.16

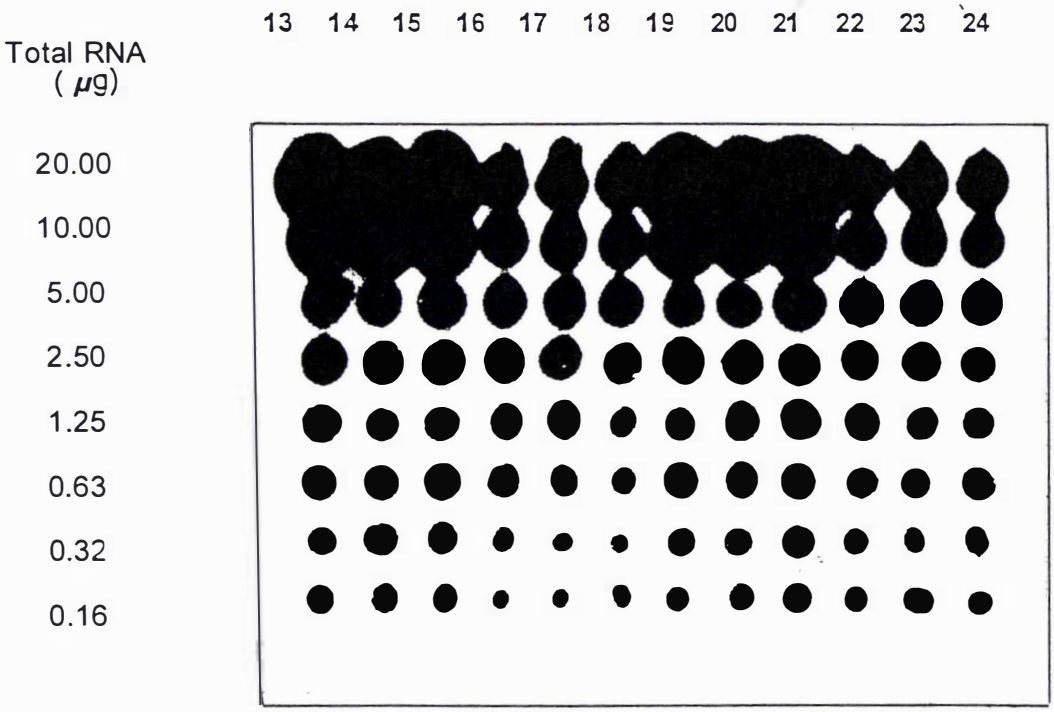
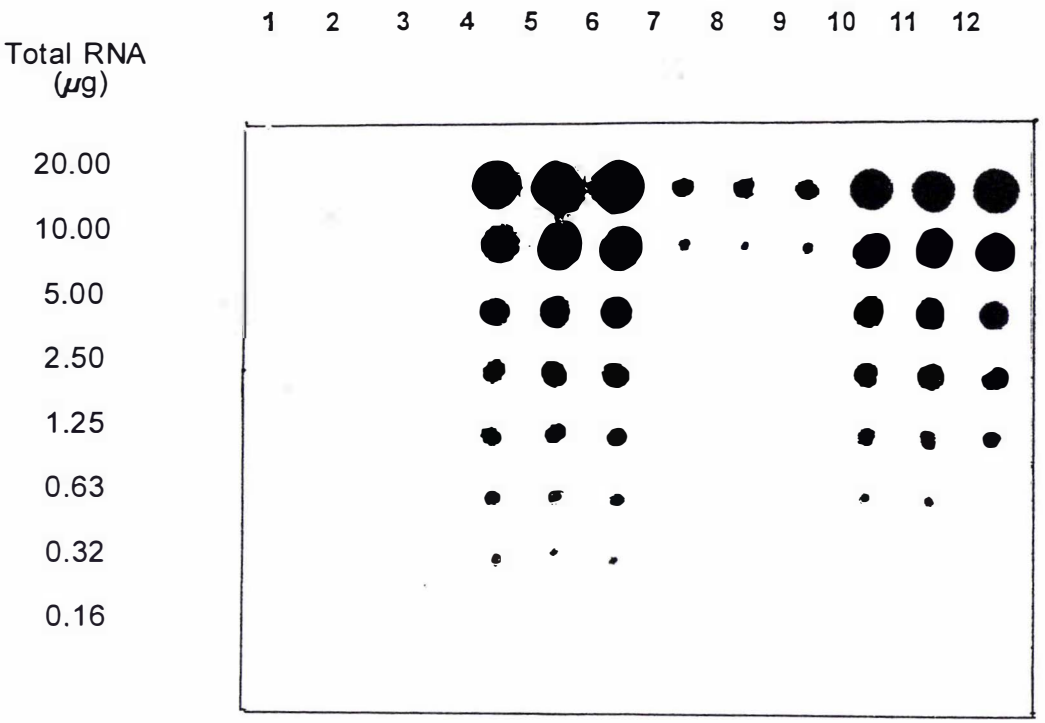
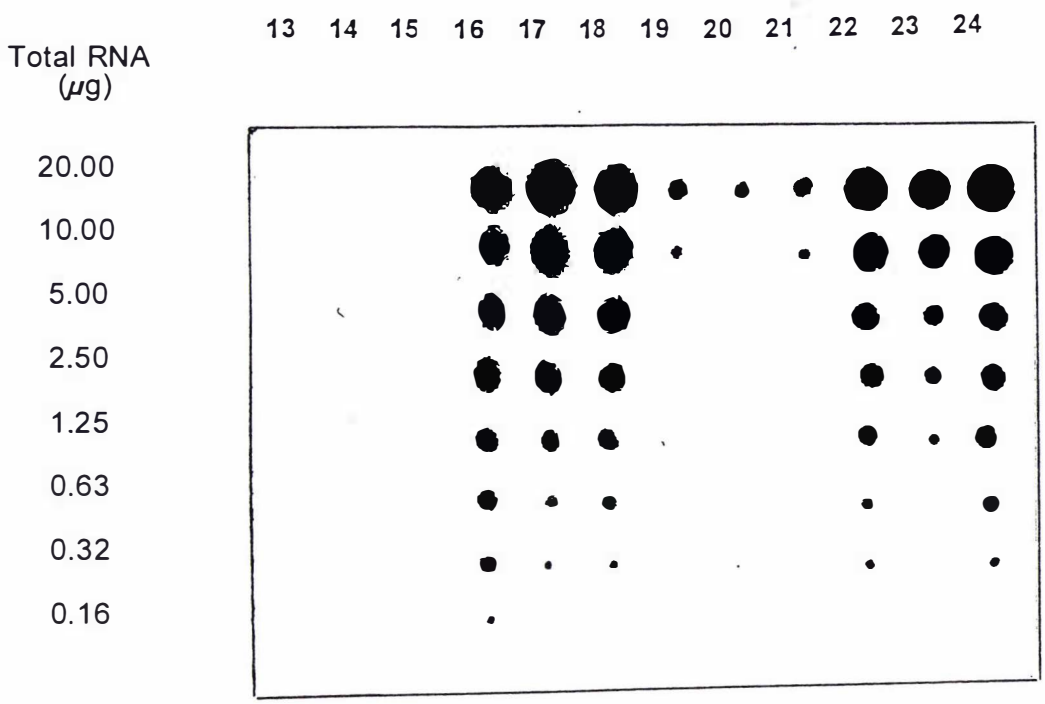

13 14 15 16 17 18 19 20 21 22 23 24
Total RNA (μg)
20.00
10.00
5.00
2.50
1.25
0.63
0.32
0.16

Figure 5.11. Dot blot of RNAs from susceptible and resistant tobacco lines probed with the dehalogenase gene. Total RNA was isolated from six individual plants from each of the following lines: non-transgenic parent, KKD (lanes 1, 2, 3, 13, 14 and 15); transgenic parent 51-1, (lanes 4, 5, 6, 16, 17 and 18); (51-5 x B21)TF_1 (lanes 7, 8, 9, 19, 20 and 21) and (51-1 x KKD)TF_1 (lanes 10, 11, 12, 22, 23 and 24). RNA was probed with a ^{32}P-labelled the 730 bp DNA fragment of the dehalogenase gene, and exposed to X-ray film for 24 hours. The autoradiography does not show dehalogenase-specific mRNA activity in control plants. However, there is dehalogenase-specific mRNA in the transgenic parental line, (51-1 x KKD)TF_1 and (51-5 x B21)TF_1 progeny plants


1 2 3 4 5 6 7 8 9 10 11 12
Total RNA (μg)
20.00
10.00
5.00
2.50
1.25
0.63
0.32
0.16


13 14 15 16 17 18 19 20 21 22 23 24
Total RNA (μg)
20.00
10.00
5.00
2.50
1.25
0.63
0.32
0.16

5.3.3 Two phase (dual) selection of genetically transformed cells

The tobacco leaf disc transformation method utilised a binary vector system, plasmid pAS501 and *Agrobacterium tumefaciens* strain LBA4404, according to the protocol outlined in Materials and Methods (Section 5.2.9). After co-cultivation of tobacco leaf discs with *Agrobacterium,* approximately twenty five tobacco leaf discs were placed onto each plate containing Nicl medium (no selection agent) to allow infection of *Agrobacterium* in the wounded tissues. The plates were incubated in a culture room for 4 days and the leaf discs were subsequently transferred onto medium containing 300 mg L^{-1} kanamycin or medium supplemented with 100, 200, 300 and 400 mg L^{-1} of dalapon herbicide to select for kanamycin resistant as well as dalapon resistant transformed cells respectively. The control non-transformed leaf discs were unable to grow in the medium containing kanamycin or on 300 or 400 mg L^{-1} of dalapon. The non-transformed leaf discs turned yellow a few hours after being transferred onto the medium containing 400 mg L^{-1} of dalapon herbicide. After shoot formation, green transformed calli from medium containing kanamycin were transferred onto medium supplemented with 100, 200, 300 or 400 mg L^{-1} of dalapon. When calli from the medium containing kanamycin were transferred onto medium containing dalapon, growth of some of the calli declined or ceased altogether and some of them died. Growth of the calli on the second selective media is shown in Figure 5.12 The result of the selection of transformed as well as control, non-transformed calli in different selective media is presented in Table 5.8. In the second phase of the selection (calli transferred from media contain kanamycin onto the media contain dalapon) the number of dead plants on 400 mg L^{-1} dalapon was significantly higher than on 200 and 300 mg L^{-1} dalapon. This indicates that by increasing the concentration of selective agent in the growth medium, the transformed cells with low levels of expression of the transgene may fail to grow.

The selection results for each of the T-DNA genes (NPTII and dehalogenase) can only be correlated with the particular gene under selection and cannot necessarily be correlated with the other T-DNA gene. To obtain high levels of expression for each specific gene, that gene must be selected by using the appropriate selection agent. For example, selection for expression of the NPTII gene can not be expected to result in the simultaneous selection for expression of

the dehalogenase gene via indirect selection. It is suggested that direct selection for a gene is more appropriate than indirect selection.

The results indicate that dalapon can be used as a selective agent in tobacco plant transformation at concentrations of 300 and 400 mg L^{-1}. However concentrations lower than the 300 mg L^{-1} are not recommendable due to resistance of all or some of the control tobacco leaf discs.

Table 5.8. Selection of transgenic tobacco cells against two selective agents. Response of the genetically transformed and non-transformed tobacco cells to kanamycin and dalapon are indicated in the upper part and lower part of the Table respectively.

Line	First selective agent	Number of calli	Second selective agent	Dead	Alive	% of dead callus
	Kn^{300} *	80	$Dlpn^{100}$	-	80	0
	Kn^{300}	80	$Dlpn^{200}$	4	76	5.00
	Kn^{300}	80	$Dlpn^{300}$	7	73	8.75
Transgenic	Kn^{300}	80	$Dlpn^{400}$	15	65	18.75
	$Dlpn^{100}$	80	Kn^{300}	80	-	100.00
	$Dlpn^{200}$	80	Kn^{300}	35	45	43.75
	$Dlpn^{300}$	80	Kn^{300}	16	64	20.00
	$Dlpn^{400}$	80	Kn^{300}	10	70	12.50
	-	80	$Dlpn^{100}$	-	80	0
	-	80	$Dlpn^{200}$	25	65	31.25
Control	-	80	$Dlpn^{300}$	80	-	100.00
	-	80	$Dlpn^{400}$	80	-	100.00
	-	80	Kn^{300}	80	-	100.00

* mg L^{-1} of selective agent in culture media. the Kn and Dlpn are abbreviations for kanamycin and dalapon respectively.

Figure 5.12. Resistance of tobacco calli against both kanamycin and dalapon. Tobacco leaf discs transformed with T-DNA containing both a dehalogenase and a NPTII gene were transferred onto medium containing kanamycin to select for cells expressing the NPTII gene. Then kanamycin resistant calli were transferred onto growth medium supplemented with 400 mg dalapon l^{-1} to select for expression of the dehalogenase gene. Some of the differentiated calli lines when transferred onto the medium containing dalapon turned yellow after a few hours and subsequently died, while other lines continued to grow.

5.4 Discussion

5.4.1 Transgene copy number

In the present study, the T-DNA organisation in 51-1 homozygous transgenic parent, (51-1 x KKD)TF_1, (TF_1 x 51-1)TBC_1, (TF1 x KKD)TBC_2 and TF_2 (selfed TF_1) plants from the generation means mating design experiment (see Chapter 4) was determined using information based on restriction enzyme sites within the T-DNA region. This is a preferred method of determining the copy number and organization of T-DNA integrated in the plant genome (Hobbs *et al.*, 1990; David and Tempe, 1987; Zambryski *et al.*, 1980).

As a result of the transformation process, single or multiple copies of the T-DNA may become integrated into plant nuclear DNA. The actual number of copies of the T-DNA can be determined by using restriction site information coupled with Southern blotting. Hence different fragment sizes in a Southern blot analysis indicate a variation in the size of the plant DNA junction with the integrated T-DNA, while multiple bands indicate multiple copies of the T-DNA within the plant genome. In the present study, Southern hybridisation of *Eco*RI and *Bgl*II digests of DNA derived from 51-1 transgenic plants show that these plants contain two copies of the transgene, whereas the non-transgenic plants do not contain the transgene. This result also shows that the homozygous transgenic parental plant 51-1 line, as well as the TF_1 hemizygous plants, contain two copies of the transgene. The chi-square test result (Chapter 3, Section 3.3.2.5) indicates that a single locus inheritance hypothesis can be accepted and that a single gene controls resistance in the 51-1 transgenic line. Since Mendelian inheritance for the gene is observed, it is assumed that the two T-DNA copies are inserted at a single locus. Since neither tandem repeats nor head to head or tail to tail inverted repeats of T-DNA were detected, (the results indicate that there must be a piece (e.g. filler DNA, see below) of plant DNA located between two copies of the T-DNA), and the size of the fragments are less than 10 kb (equivalent to two copies of the T-DNA), several conclusions can be drawn from the Southern analysis:

(i) All of the plants that exhibited the dalapon resistance contain the T-DNA while the plants lacking the T-DNA show no evidence of the dalapon resistance phenotype, as seen in the susceptible plants of TBC_2 and TF_2, as well as the non-transgenic control parent.

(ii) The transgenic plants show inheritance of one dalapon gene in phenotypic studies (Table 3.25), whlie two copies of the T-DNA determined by Southern analysis, suggesting that two copies of the T-DNA are integrated into the same chromosome at one locus with only a small distance between them i.e. they are linked genes separated by a piece of plant DNA or by filler DNA. The formation of filler DNA due to illegitimate recombination has been reported both in mammalian (Roth *et al.,* 1991) and higher plants (Wessler *et al.,* 1990). Formation of filler DNA during T-DNA integration can be caused by polymerase slipping and template switching during DNA repair synthesis (Gheysen *et al.,* 1991). For T-DNA copies in the present study, It could be interpreted that the filler DNA contains both *Eco*RI and *Bgl*II recognition sites located between the two copies of the T-DNA.

(iii) All of the plants with two copies of the T-DNA transmitted their dalapon-resistant phenotype to their progeny as a single locus.

Several possibilities regarding the insertion of T-DNA in the plant genome have been suggested. For the present study, three possible arrangement of the T-DNA genes in the plant DNA are illustrated in Figure 5.8. This Figure indicates that the T-DNAs can be integrated in various orientations with respect to each other. For example, they can occur in the form of a direct tandem repeat or in the form of an inverted repeat and in either case can occur as either tandem or interspersed repeats. In this context, there are several reports indicating that tandem repeats of the T-DNA in the form of either direct or indirect repeats is possible (Jorgensen *et al.,* 1987; Spielman and Simpson, 1986). It is suggested that the insertion of two copies of the T-DNA in one cell may be caused by transformation of the same plant cell by two *Agrobacterium.* It is also possible that the T-DNA disrupts the integrity of the target plant DNA and thus may result in errors in replication and repair at the insertion site, leading to the formation of direct and indirect T-DNA repeats. In relation to T-DNA copy number, Zambryski (1988) suggested that multiple copies of the T-DNA in the plant's genome was either the result of replication and repair of the T-DNA during insertion into the plant DNA or the result of replication and ligation of the T-DNA in a random fashion prior to integration.

The Southern blot results also indicate that except for the susceptible (negative) plants, integration of the T-DNA into the plant DNA is stable in

successive generations of the 51-1 transgenic line (TF_1, TF_2, TBC_1, and TBC_2) and that further rearrangement of the T-DNA does not occur.

5.4.2 Transcription of the transgene

A dehalogenase-specific mRNA was detected in dot blots of total RNA extracted from leaves of the transgenic plants. The transgenic lines showed at least a significant variation in the accumulation of the dehalogenase-specific mRNA in dot blot experiments. In control, non-transgenic plants, the dot blot results showed that there was no dehalogenase-specific mRNA transcription for these plants, demonstrating that there was no transcription from endogenous gene(s), homologous to the chimaeric dehalogenase gene, in the tobacco plants. Transcription of the dehalogenase gene of transformed plants varied considerably between the 51-1 and the TF_1 lines, although the variation was not significant between the individual plants within the lines. Analysis of the expression of the dehalogenase gene demonstrates that there is a substantial variation between independent transformants in the levels of expression as discussed below.

The phenotype of the (51-1 x KKD)TF_1 and (51-5 x B21)TF_1 plants with respect to their resistance to herbicide is presented in Tables 4.11, 4.12, 4.17 and 4.18. The resistance/susceptibility of the different tobacco lines, as well as progenies derived from crosses between transgenic lines, and between transgenic and no-transgenic lines, to the herbicide are presented in Chapter 4, Tables 4.11, 4.12, 4.17 and 4.18. There was significant variation in herbicide resistance for the progenies derived from crosses between the two different transgenic lines and from crosses between the transgenic and non-transgenic lines. The dot blot experiment was conducted to compare the levels of dehalogenase-specific mRNA in lines, and to establish any correlation between the herbicide resistance phenotype and the level of dehalogenase-specific mRNA in these plants.

The internal quantitative comparison of dehalogenase-specific mRNA (Figure 5.11) showed significant difference between the transgenic lines. This variability between different plants can be attributed to differences in the amount of dehalogenase-specific mRNA in each plant. There was good agreement between the levels of transgene dehalogenase-specific mRNA and the levels of resistance of the corresponding transgenic plant to herbicide. For example, highly resistant

plants from 51-1 and (51-1 x KKD)TF_1 progeny lines showed a high level of steady state dehalogenase-specific mRNA. In contrast, the most susceptible (51-5 x B21)TF_1 progeny which showed the lowest resistance to herbicide, also had a low level of the steady state dehalogenase-specific mRNA.

The variation in steady state dehalogenase-specific mRNA level between the transformed lines was considerably higher than that of the plants within each transformed line. For example, as discussed above, the (51-5 x B21)TF_1 plants showed significantly lower dehalogenase-specific mRNA than (51-1 x KKD)TF_1 plants. However, variation in dehalogenase-specific mRNA within each of the (51-5 x B21)TF_1, (51-1 x KKD)TF_1, as well as the 51-1 parental line was very low (six plants for each line were used in dot blotting). The variation between the 51-1 parent and the (51-1 x KKD)TF_1 progeny plants was also very low. The statistical analysis of the signal intensity of the lines (Table 5.6) also indicates significant differences between the lines (i.e. between 51-1, (51-5 x B21)TF_1, (51-1 x KKD)TF_1 and the non-transgenic control line). However variation within single plants of each line is not significant. It can be concluded that the variation within single plants of each transformed line is not significant, while variation between the lines is significant.

The mean phenotype comparisons (Tables 4.11 and 4.12) and s.c.a. effects comparisons (Table 4.17 and 4.18) for the (51-5 x B21)TF_1 cross showed that these plants exhibit a susceptible phenotype similar to that of the non-transgenic B21 parent plants. The mRNA studies of the dehalogenase gene, show that the level of dehalogenase-specific mRNA is very low for this cross. These results suggest that the expression of the transgene is lowered due to crossing with the non-transgenic line. Such gene silencing in plants can be caused by several factors. Coordinated inactivation (co-suppression) of two genes in plants and epistasis, where one gene is suppressed by another gene have been reported for transgenes (Fujiwara and Beachy, 1993; Hobbs *et al.,* 1993; Matzke and Matzke, 1991; Meyer *et al.,* 1992). Inactivation of, or lowering the expression of a gene(s) by means of epistasis has been studied widely in plants. For example, some of the genes that confer resistance to stem rust in tetraploid wheats are suppressed in hexaploid wheat, probably due to a suppressor gene located on chromosome 7D of the hexaploid wheat (Dyck, 1987). This is also the mechanism for suppression of the rye ribosomal RNA genes in wheat (Flavell, 1989). It is likely that the

expression of the transgene in the (51-5 x B21)TF_1 cross is suppressed by means of an epistasis effect of the background gene(s).

Another reason for both low expression and silencing of the transgene within plants is the level of methylation of the introduced gene brought about by the plant methylation system. Meyer *et al.* (1992) found that silencing the maize *A1* gene was correlated with the methylation. Rathore *et al.* (1993), gave various possible explanations for transgene silencing including methylation of promoters, interference of RNA strands with the transcription process, and degradation of mRNA. Prols and Meyer (1992), studying the inactivation of T-DNA genes in three transgenic plants, found that the inactivation of both T-DNA genes in one of the lines was associated with integration into a region of highly repetitive DNA, while the integration sites of the other two lines were essentially unique. The integration regions of the two latter lines, both of which expressed the foreign genes at characteristically different levels, showed a distinct methylation pattern that was stably conserved for these regions. The characteristic methylation pattern of the two integration regions was also imposed on the border region of the integrated fragments. They suggested that the region of plant DNA adjacent to the T-DNA may have been responsible for the differences in the level of gene expression by means of methylation. In another study, unusual gene interactions between two foreign genes were studied by Matzke and Matzke (1990). The expression of one of the foreign genes, which encoded kanamycin resistance, was suppressed in some, but not all, of the transformed plants after the introduction of the second gene, which encoded hygromycin resistance. In this case, the reversible cytosine methylation of the promoters of the T-DNA genes was shown to correlate with the activation/inactivation cycle of the genes. Variability in expression could also be a consequence of position effects of adjacent genomic DNA (Hepburn, 1983) which could influence the level of mRNA in the plant, which in turn could arise from differences in either mRNA stability, or rates of transcription of the foreign gene.

A high variation in the transcription of a transgene in genetically transformed plants has been reported for numerous transgenic plants. An (1986) also quantified the expression of a transgene within transformants and reported a variation of approximately two hundred-fold in the levels of transcription between transformants. In the present study, variation in the level of transcription of the transgene was also high and the level of dehalogenase-specific mRNA of the transgene was associated

with the level of dehalogenase-specific mRNA of the transgene in transgenic plants. For the present study, it is suggested that the variability in transgene expression must reflect differential rates of steady state dehalogenase-specific mRNA. It is concluded that differences in expression of the transgene are associated with differences in the level of dehalogenase-specific mRNA of the transgene. It is also suggested that the differences between the hybrid progeny and the transgenic parents may be caused by an epistasis effect, as discussed above.

5.4.3 Two phase selection

The 51-14 transgenic line exhibits a very low herbicide resistance compared to the other resistant lines and at level of more than 6.0 kg ha^{-1} this line did not differ from the control line. Therefore this experiment was carried out to investigate the effect of direct and indirect selection on expression of the dalapon resistance transgene.

Plant transformants produced using the *Agrobacterium*-mediated technique can vary widely in the level of expression of the introduced gene. The NPTII and dehalogenase genes were transferred into the plant cells using the *Agrobacterium*-mediated system and hence it was expected that the transformed cells would show resistance to both kanamycin and dalapon. In some callus lines there was a dissimilarity in expression of the dehalogenase gene and the NPTII gene in transgenic callus lines. Some of the genetically transformed cells selected on medium containing kanamycin, when transferred onto medium containing dalapon, did not show resistance to dalapon. Similarly, when transformed cells selected on medium containing dalapon were transferred onto medium containing kanamycin, some of the lines did not show resistance to kanamycin. Therefore it is concluded that selection for one of the T-DNA genes under selection pressure in some cases did not give a corresponding result for the other, non-selected T-DNA gene.

As discussed above, there can be considerable variation in the levels of expression of a foreign gene in transformed plants. The differences in level of expression of two marker genes under the control of one type of promoter (*nos*) was reported by An (1986). There was a considerable variation in the level of expression. However, in the present study the dehalogenase and NPTII genes were under two different promoters (CaMV 35 and *nos*) with two distinct

polyadenylation signals (see Appendix 17, Figure A.5). It can be expected that by increasing the selection pressure through an increase in the selective substrate in the growing medium, cells with high expression of the transgene will be achieved. In contrast, under no selection pressure, cells with a wide range of resistance would be expected. It is concluded that imposing a selection pressure on each of the genes independently will not give a similar result for the other gene not under selection pressure. As shown in Table 5.8 for the selection of transformed cells in this study, the number of dead calli increases with increasing concentrations of herbicide used in the selection media. Therefore increases in the level of herbicide caused an elimination of the calli with low transgene expression. An (1986), studying the level of CAT (chloramphenicol acetyl transferase) and NPTII gene expression in transformed tobacco callus lines, found that the level of CAT expression varied 200-fold between the individual transformants. The NPTII activity of the transformed callus lines also varied although the variation was not as significant as that of CAT activity. There was not a good correlation between the levels of CAT gene and NPTII gene expression. An (1986) suggested that the differences were partially due to the selection pressure on gene expression since the transformed tissues were maintained on a medium containing kanamycin but lacking chloramphenicol.

Another possible cause for the suboptimal, or the complete lack of, expression of the introduced T-DNA genes could be partial deletions of the T-DNA region during integration of the T-DNA into the plant DNA. To check the interactive effect of T-DNA genes in transformed plants, Deroles and Gardner (1988a) introduced T-DNA containing two copies of the kanamycin resistance gene, under two different promoters, into petunia plants using *Agrobacterium*-mediated transformation. Their results indicated that deletions in various parts of the T-DNA, including both the right and left borders, were possible and that a significant proportion of the transformed plants did not express either one or both of the T-DNA genes, due to truncation and deletion in the T-DNA region. As discussed above, it is also possible that methylation of either the T-DNA gene(s) itself or the promoter region of the gene may be brought about by the plant methylation system.

Chapter 6

General discussion

6.1 level of resistance of transgenic lines to dalapon

Expression of the dehalogenase gene in transgenic tobacco lines led to the ability of these lines to grow at high concentrations of dalapon herbicide. Following the application of herbicide in greenhouse experiments, the transgenic lines showed significant variation in their responses to various levels of the herbicide. The rate of dalapon used to control grass weeds is 6.0 kg of herbicide ha^{-1} and at this rate most pasture and crop plants are killed by the herbicide as well (O'Connor, 1990). The level of resistance to the herbicide varies significantly between the transgenic tobacco lines and hence the lines can be classified into three classes: lines with a high level of resistance to the herbicide (e.g. 49-1, 51-1, 54-18); lines with a medium resistance to the herbicide (e.g. 51-3, 51-5); and a line with low resistance to the herbicide (51-14). Such variation has also been reported in other plant transformation studies (Lawton *et al.,* 1987; Hobbs *et al.,* 1990; Hobbs *et al.,* 1993). By comparing the levels of resistance of control and transgenic lines, it can be concluded that expression of the dehalogenase gene in the resistant transgenic lines conferred resistance to the herbicide, but with variation in expression. However at early growth stages (*in vitro*), seedlings of the same transgenic tobacco lines showed lower resistance to the herbicide than plants in the greenhouse. At the seedling stage, growth of the control and the resistant transgenic plants stopped at levels of dalapon in the media of approximately 300 and 500 mg L^{-1} respectively. The range of resistance of the transgenic tobacco lines at the seedling stage is considerably lower than that of developed plants. Since the herbicide was applied to the roots of the seedlings via growth medium, and to the leaves of the developed plants grown in soil by spraying, it is possible that the differences in resistance of the seedlings and developed plants reflects differences in the level of uptake of the herbicide through roots and leaves and /or differences in growth medium.

With respect to the differences in growth of the white clover callus as well as seedling fresh weight, leaf length and leaf width of the tobacco lines, some differences in the characteristics are evident within the individual transgenic lines in the absence of herbicide application (Tables 3.2, 3.5, 3.13 and 3.14). These differences may be due to somaclonal variation (Larkin, 1981; 1984). Somaclonal

variation is due to stress-induced mutations arising from tissue culture (Walbot and Cullis, 1985). The frequency of somaclonal variation in plants such as *Arabidopsis thaliana* induced by tissue culture effects is high (van Lijsebetten *et al.*, 1991), and may be as high as 5% (Errampalli *et al.*, 1991).

With regard to the effect of the plant species on expression of a transgene, the transgenic white clover callus (*in vitro*) and mature white clover plants (greenhouse-grown) showed lower resistance to the herbicide than mature, resistant, transgenic, tobacco lines. However, it should be noted that there was only one transgenic white clover line in the greenhouse experiments and as a consequence the results are not as informative as those from the several lines of transgenic tobaccos. By comparing the levels of resistance of the transgenic tobacco lines with those of the white clover transgenic line in both *in vitro* and in greenhouse experiments, it is suggested that resistance to the herbicide may be influenced by the plant species into which the transgene is introduced.

In order to determine the correlation between resistance of the transgenic tobacco lines to herbicide at the early growth stage (seedlings grown *in vitro*) and for fully developed plants (grown in the greenhouse) these two stages of growth were compared using rank correlation (Steel and Torrie, 1980). The value for correlation is 0.91 and is highly significant, indicating a strong correlation between herbicide resistance of seedlings and herbicide resistance of fully developed plants of the same line.

Herbicide at the highest level significantly suppressed growth of the resistant transgenic lines. The recovery or suppression of plants after application of the highest level of herbicide can be considered as a factor in determining the resistance or susceptibility of a certain line to the herbicide. Considering the significant effect of the time component in the greenhouse experiments, it is concluded that the resistant transgenic tobacco plants had the ability to recover following the application of high levels of the herbicide.

In contrast to the tobacco plants, differentiation of the transgenic white clover calli to green calli took a very long time. Following the differentiation of calli to shoots, formation of roots from shoots was also very slow. Hence regeneration of transgenic white clover plants was a slow process compared to regeneration of tobacco plants. Furthermore, the white clover line WR8 is not agronomically useful. However a method for regenerating shoot meristems directly from cotyledons of

white clover seedlings has recently been developed (White and Voisey, 1994). Subsequently, two important cultivars of white clover (Grasslands Huia and Grassland Tahora) were genetically transformed using *Agrobacterium*-mediated transformation and regenerated using this method (Voisey *et al.*, 1994).

Considering the significant effect of dominance and the lower additive/dominance ratio of the transgene as shown by quantitative studies (Chapter 4), transfer of the transgene from a transgenic line with good regeneration potential into cultivar(s) with agronomically superior characters indirectly by backcrossing should be possible.

6.2 Inheritance of the transgene based on phenotypic and molecular studies

The result of the chi-square test of the greenhouse experiments indicates the involvement of single locus inheritance for the transgenic lines (Table 3.25). However, Southern hybridisation of *EcoR*I and *EcoR*I/*Bgl*II digests of DNA derived from 51-1 transgenic plants (Figure 5.7) show that these plants contain two copies of the T-DNA adjacent to each other (i.e. are closely linked genes). This result also shows that the homozygous, transgenic, parental 51-1 line, as well as the TF_1 hemizygous plants, contain two copies of the T-DNA. The chi-square test result (Table 3.25) indicates that a single locus inheritance hypothesis is valid for the 51-1 line and that a single gene controls resistance in this transgenic line. Since sexual segregation for a single locus was observed for the 51-1 line, it is assumed that the two T-DNA copies were inserted at a single locus in the transgenic plants. Furthermore, from the Southern analysis it is concluded that the T-DNA is inherited in subsequent, sexually reproduced generations of the 51-1 line. All of the plants that showed the dalapon resistance contain the dehalogenase gene. In contrast, the plants lacking the T-DNA showed no evidence of the dalapon resistance phenotype, as seen in the susceptible (negative) plants TBC_2 and TF_2, as well as the non-transgenic control parent.

All of the plants with two copies of the T-DNA transmitted their dalapon-resistant phenotype to their progeny as a single gene as indicated by the chi-square test (Table 3.25). As regards the inheritance of the transgene from a molecular point of view, the Southern blot results also indicate that the transgene is inherited by the subsequent, sexually-derived progeny. There is good agreement

between the molecular studies of gene inheritance and the high value of heritability estimated by means of the phenotypic analysis.

6.3 Heritability of herbicide resistance

The values for heritability estimated from the greenhouse experiments show medium to high heritability for the majority of the characters at various levels of herbicide. The population and plant heritabilities show opposite responses to increasing levels of the herbicide. Thus on the one hand, an increase in concentration of the herbicide caused a decrease in population heritability and an increase in plant heritability for necrosis and stem height, while on the other hand it caused an increase in population heritability and a decrease in plant heritability for leaf length and leaf width. Each character has a special population or plant heritability in response to increasing levels of herbicide and it may vary between the characters. The heritability of each character also depends on growth activity and response to the herbicide. Leaf length and leaf width has a higher heritability estimate compared to that of necrosis and stem height, which indicates a low response of these characters to environmental factors.

Comparing the necrosis heritabilities of plant, population and broadsense, at various levels of herbicide, the plant heritability shows the highest value at 48.0 kg dalapon ha^{-1} (Table 3 20), while the population (line) heritability value is highest at 3.0 kg ha^{-1} and is lowest at 48.0 kg ha^{-1}. From a plant breeding point of view, the plant heritability is important for selecting resistant single plants. In the present case, it seems that selection of resistant plants should be based on the single plant selections, since selections based on population only consider the best lines and ignore the single plants within the line. The broadsense heritability is of interest from a biological point of view. Since the heritability of the necrosis character for the plants in this study is highest at the 48.0 kg ha^{-1} level, it is suggested that the best genetic advance (ΔG) will be gained by selecting the resistant single plants at this level of herbicide.

The values for heritabilities of necrosis and stem height characters, estimated through the mating designs (Chapter 4), are higher than those of the experimental design carried out in the greenhouse (Chapter 5) with tobacco plants. Of the heritabilities which are estimated by mating designs (generation means and

diallel crossing), only the block effect is considered to be an environmental component of variation. Hence, the low block component of variation leads to a decrease in the phenotypic component of variation and subsequently an increase in the estimated value of the heritability. However the experimental designs, carried out in the greenhouse, involved more environmental components of variation besides the block effect, such as the time effect, interaction of time with the other effects, and the interaction of the block effect with the other effects. These extra components of variation led to an increase in the environmental components of variation and subsequently to a decrease in heritability. There are also significant differences between the heritability values estimated by different mating designs. For example, the heritability estimated by the Griffing method was significantly higher than that of the other methods. It is suggested that the heritabilities estimated by the mating designs, in which only the block effect is considered as an environmental factor, leads to an overestimate of heritability compared to that of the experimental designs. Furthermore, the heritability values estimated through the greenhouse experiments are more reliable than the values estimated by mating designs.

The transgenic tobacco plants at the seedling stage (*in vitro*) showed the lowest heritability (0.1) comparing to that of the developed tobacco plants. It is concluded that the heritability changes, depending on the stage of growth of the plants, and that at the early growth stage the environmental factors are more significant than the transgene effect. As mentioned above, the heritability of herbicide resistance at the seedling stage is very low, suggesting that selection for herbicide resistance at an early stage of development is not as efficient as at the fully developed stage. The low heritability of herbicide resistance at an early stage can be caused by either a high proportion of environmental components of variation or a low genotypic components of variation. With regard to the experimental result, it is suggested that both low genetical variation and high environmental effects were the cause of the low heritability for herbicide resistance at the early growth stage.

6.4 Expression of the transgene

The transgenic lines show significant differences in their level of expression of the dehalogenase gene, as shown in the dot blot experiment (Figure 5.11).

Although all of the transgenic plants contained the same dehalogenase gene, the steady state levels of dehalogenase-specific mRNA detected in a dot blot showed significant differences between the different transgenic lines. The dot blot result also indicates a correlation between herbicide resistance and the level of expression of the dehalogenase gene. For example, the highly resistant plants derived from the 51-1 parental line and the (51-1 x KKD)TF_1 progeny line show high levels of dehalogenase specific-mRNA. In contrast, the most susceptible (51-5 x B21)TF_1 progeny had a low level of dehalogenase-specific mRNA of the transgene. The variation in level of dehalogenase specific-mRNA between lines is considerably higher than the variation within lines. For example, the variation in dehalogenase-specific mRNA levels within each of the (51-5 x B21)TF_1, (51-1 x KKD)TF_1 and 51-1 parental lines is very low (six plants for each line in the dot blot). The variation between the 51-1 parent and (51-1 x KKD)TF_1 progeny plants was also very low. This suggests that in the susceptible (51-5 x B21)TF_1 plants, the level of dehalogenase gene expression is lowered by an effect of the control B21 parent.

In relation to the inter-allelic interaction (epistasis), the generation means, Jinks-Hayman diallel and Griffing diallel show the presence of inter-allelic interaction with the transgene. The results of the mean analysis (Tables 4.11 and 4.12) and s.c.a. effects (Tables 4.17 and 4.18) clearly show the effect of the background allele(s) of the non-transgenic parent on the expression of the transgene in the (51-5 x B21)TF_1 hybrid progeny. However the crosses between 51-5 and the other transgenic and non-transgenic lines indicate a resistance phenotype similar to that of the 51-5 transgenic parent and the other resistant transgenic parents (in cases of crosses with resistant parents). Inactivation of, or lowering of, the expression of a gene(s) by means of inter-allelic interaction has been studied widely in normal plants. Examples are; stem rust resistance in tetraploid wheats is suppressed in hexaploid wheat, probably due to a suppressor gene (Dyck, 1987), and suppression of rye ribosomal RNA genes in wheat (Flavell, 1989).

This interaction in the present study could be responsible for the decrease in the level of expression of the transgene, possibly through methylation of the transgene by the host plant methylation system. As reported by Hobbs *et al.*, (1993), methylation of a transgene by the host plant methylation system is one of

the reasons for either a reduction in, or a lack of transgene expression in transgenic plants. In the present study, as mentioned above, the 51-14 transgenic parent and (51-5 x B21)TF_1 hybrid did not show a high resistance to the herbicide compared with the other resistant parental lines or the progeny hybrids. Determining the relationship between methylation and level of expression of the transgene would be a good subject for a future study. Such a study could resolve the question as to whether the epistasis effect directly results from methylation of allele(s) or whether mechanisms other than methylation cause the epistasis effect.

6.5 Quantitative genetics studies

With regard to the quantitative inheritance studies of the transgene, the herbicide resistance studies of the transgenic tobacco lines under greenhouse conditions (Chapter 3) showed a significantly wide range of variation between the transgenic lines. There was also some overlap between the level of resistance of the different lines for each of the measured characters. These variations between the lines, as well as the overlap between the different lines, indicate a continuity for the characters which are measured following application of herbicide to the tobacco plants. This continuity revealed that the characters can be studied using the quantitative genetic approaches (Mather and Jinks, 1977). Furthermore, there are several quantitative characters that have been shown to be controlled by one or few loci (Jana, 1971; Wehrhahn and Allard, 1965; Jana, 1972a; Douglas and Gordon, 1985).

Resistance to the herbicide occurred in the subsequent generations of the transgenic line(s) with a similar pattern to that of the parent plants. For example, in generation means mating design, the homozygous transgenic parent (51-1) as well as other generations (TF_1, TF_2, TBC_1 and TBC_2) showed significantly higher resistance to the herbicide than the control non-transgenic line, although there were significant differences between these generations. It is suggested that these differences between the generations are related to different segregation patterns in the generations. In addition, in diallel crossing mating designs, the resistance pattern of the selfed, homozygous, transgenic plants (Tables 4.11 and 4.12) were similar to the pattern of resistance of plants in the greenhouse experiments. It is concluded that the dehalogenase gene is expressed in the subsequent generations

derived by sexual reproduction and these plants show a pattern of expression similar to that of the original, tissue culture-derived, transgenic plants. The similarity of TF_1 and TBC_1 to the transgenic parent (KKD) indicates the presence of dominance allele(s) in the transgenic parent which facilitates herbicide resistance of these generations.

Further genetical analysis revealed the presence of additive, dominance and epistasis effects as well as a high additive/dominance ratio for the transgene. The Jinks-Hayman diallel cross analysis also reveals the presence of additive, dominance and epistasis with a high additive/dominance ratio for the transgene. However, analysis of the diallel cross data set by the Griffing method results in a high estimated value for the additive component of variation ($2\sigma^2_g$) and trivial estimated value for the dominance allele effect (σ^2_s). It is suggested that this model overestimates the additive effects and underestimates the dominance (hemizygosity) effect. In comparing the three mating designs, the generation mean mating design gives more detailed information about allelic effects, presence or absence of epistasis, type of epistasis, and variances of generations. While the Jinks-Hayman diallel method only provides information about the absence or presence of epistasis and about the type of epistasis, more parental lines can be used and the result is based on a variety of lines. Although analysis of the diallel data set by Griffing method gives the least information on the gene effect, it does give useful information about g.c.a. and s.c.a. estimated values which can be used in applied plant breeding for selecting the best parent as well as the best crosses between the parental lines.

The plants originating from selfed hemizygous plants are under genetic segregation and the presence of all of the possible genotypes (homozygous, hemizygous and susceptible (negative) plants lacking the transgene) is possible. Ordinarily, the dominance effect involves intra-allelic interaction between recessive and dominance alleles of the same gene in a heterozygous genotype. However, in the hemizygous transgenic parental genotypes there is only a single version of the allele(s) present and there is no recessive allele(s) against the transgene on the host chromosome. Thus only one of the homologous chromosomes contains the transgene. The dominance effect as used here refers to the hemizygosity effect of the transgene. The generation mean mating results indicate the presence of partial dominance for the transgene with a high additive/dominance ratio. The dot

blot result indicates a similar amount of dehalogenase-specific mRNA in 51-1 homozygous and hemizygous (51-1 x KKD)TF_1 plants, which is in agreement with the result of the generation means mating design. The dominance effect could therefore be interpreted as the level of expression of the transgene in the hemizygous transgenic plants.

The analysis of variance for combining ability shows that both general combining and specific combining ability variances are highly significant for both of the necrosis and stem height characters. The general combining ability variance is significantly higher than the specific combining ability variance with a high general combining ability/specific combining ability variance ratio. The higher magnitude of the general combining ability variance compared with the specific combining ability variance indicates the predominance of an additive (fixable) gene effect in the genetic control of herbicide resistance among the parents used in the diallel cross. The significant specific combining ability variance suggests a considerable amount of non-additive (hemizygosity or dominance) variance among the parents used for the diallel crossing.

Of particular importance to the plant breeder with the aim of breeding herbicide resistant plants is the predominance of additive gene action for stem height and necrosis characters. This suggests that improvement of these characters can be achieved through the breeding procedures and may result in superior pure-line varieties. The presence of a significant dominance gene action (hemizygosity effect) is also important from a plant breeding point of view, since it may be possible to obtain resistant hybrid plants through choosing an appropriate transgenic parent and a non-transgenic cultivar for producing the TF_1 hybrid.

The comparison of g.c.a effects of individual lines for necrosis and stem height (Tables, 4.17 and 4.18) shows that 49-1, 51-1, 54-18, 51-3, 51-5 transgenic lines have negative g.c.a effects for necrosis and positive g.c.a effects for stem height. These lines indicate the best general combining ability and could be used in crossing programmes with the aim of production of herbicide resistant hybrid plants. In breeding programs where the aim is to improve a certain character, the plant breeder needs to predict the result of the selection programme for the character under selection. The selection gain or genetic advance (ΔG) values depend mainly on the narrowsense heritability, the phenotypic variance of the character and the proportion of the population of plants which are selected at each

selection cycle. In the present study, the high estimated value of the genetic advance for the stem height and necrosis characters suggests that selection for herbicide resistant plants should be highly successful. Thus if the plant breeder were to set up a selection method for selecting highly resistant plants within the population of F_2 transgenic plants, plants with a significantly higher resistance phenotype would be expected for the next generation.

The number of effective factors (genes) estimated through the various methods of quantitative genetics is less than one, in contrast with the result achieved by Southern blot. This disagreement can be explained as follows: a) the underestimate arising from the quantitative studies may be the result of the presence of epistasis effects. b) in quantitative studies, estimates of the number of genes are based on hypotheses and theory, and thus the estimated value can show a high variation. It can be concluded that the quantitative approach for estimating the number of genes is not as reliable as the Southern blot method.

With regard to the epistasis effects, the genetical analyses (Chapter 4), indicated the involvement of inter-allelic interactions (epistases) for the transgene. Two types of inter-allelic interaction (epistasis) can be accounted for in the present study: the allelic interaction between the plant background genome and the transgene (e.g. between 51-5 and B21 lines as discussed above), and the allelic interaction between the transgenes in transgenic lines (e.g. between the 51-14 and the other transgenic lines).

Epistasis in the form of interactions between transgenes with high and low levels of resistance were observed for the 51-14 transgenic line and the other resistant transgenic lines (Tables 4.11, 4.12, 4.17 and 4.18). The TF_1 progenies derived from crosses between the non-transgenic lines and the susceptible transgenic line, 51-14, were susceptible to the herbicide, whereas the progenies derived from crosses between the resistant transgenic lines were resistant to the herbicide. These results are similar to those reported by Hobbs *et al.* (1993) for the GUS gene in transgenic tobacco plants. However, in crosses between the susceptible transgenic line, 51-14, and the resistant transgenic lines, the TF_1 hybrid progenies showed a similar level of resistance to the herbicide to those of the resistant transgenic parents. This indicates dominance of the herbicide resistant phenotype over the herbicide susceptible phenotype in crosses between the transgenic plants.

6.6 *in vitro* dual selection

The levels of resistance of the transgenic lines to herbicide are significantly different. The 51-14 low resistant transgenic line has the lowest resistance to the herbicide of all of the transgenic lines. It is suggested that the susceptibility of the 51-14 transgenic line results from direct selection for the kanamycin gene and only an indirect selection for the dehalogenase gene. This could have been caused by selection of leaf discs of transformed tobacco on medium containing kanamycin but not dalapon, with a consequence that cells were not under selection pressure for the dehalogenase gene. The low level of expression of the dehalogenase gene in this susceptible line may have been caused by either a deletion of part of the dehalogenase gene within the T-DNA region during integration of the T-DNA into the plant DNA, or methylation of the dehalogenase gene. To select for transformed cells with a high level of gene expression, it is suggested that, where feasible, direct selection for the transgene is more reliable than indirect selection.

From the selection results for the NPTII and dehalogenase genes it can be concluded that selection for a particular gene of T-DNA can only be correlated with the particular gene under selection and cannot necessarily be correlated with the other T-DNA gene. To obtain high levels of expression for each specific gene, that gene must be selected for using the appropriate selection agent. Thus, selection for expression of the NPTII gene cannot be expected to result in the simultaneous selection for expression of the dehalogenase gene.

From the results it can be concluded that dalapon at 300 and 400 mg L^{-1} can be used as a selective agent in plant transformation.

6.7 Possible further studies

The quantitative studies of the transgene reported here were carried out using transgenic tobacco. The quantitative inheritance of the transgene in white clover needs to be determined using a wide range of white clover lines.

Since only one line of transgenic white clover was used in the present study, the determination the range of resistance to the herbicide was impossible. Resistance experiments with a wide range of transgenic white clover lines need to

be carried out. And, the number of T-DNA copies as well as the levels of expression of the transgene in these lines need to be determined.

As discussed in Chapter 4 and 5, transgene scilencing can be attributed to methylation pattern in the plant DNA.. There is some evidence of correlation between either a lack of expression or low expression of the transferred genes in the transformed plants and methylation of theses genes in the literature. Transferred DNA integrated into a hypermethylated chromosomal region could become methylated, due to the spread of the methylation pattern from adjacent regions of plant genomic DNA. However integration of transferred DNA into the unmethylated region could leave the transferred DNA hypomethylated and transcriptionally active (Pröles and Meyer 1992). There were significant differences between the tobacco lines in their levels of resistance to the herbicide. This high variation could have resulted from factors such as methylation, and/or copy number of the transgene. An analysis of the DNA methylation pattern for transferred DNA regions and adjacent plant genomic DNA for the tobacco lines in this study with medium and high levels of resistance to the herbicide (51-5, 51-3, 51-1, 54-18 and 49-1) and the line with the lowest resistance to the herbicide (51-14) as well as the (51-5xB21)TF_1 hybrid could reveal relationships between methylation patterns and levels of transcription for these lines. For such methylation studies, different restriction enzymes such as *Hpa*II, *Msp*I, *Hha*I, *Bst*UI (Meyer and Heidmann, 1994), *Ava*I and *pst*I (Hobbs *et al,* 1993) can be used.

Transgene scilencing can also be attribute to the interactions between the transgene and background allele(s) of plants. it should be possible to self the (51-5xB21)TF_1 plants to achieve all possible genotypes, including homozygous transgenic, hemizygous transgenic and negative (lacking the transgene), and evaluate these crosses for phenotypic similarities in the progeny and the 51-5 parent. If the phenotypic similarities in some of the progeny of the selfed (51-5xB21)TF_1 plants (TF_2) and 51-5 homozygous parental plants are observed, it can be concluded that the effect is due to homologous chromosomes derived from the B21 parent.

Since the T-DNA inheritance and organisation was studied only for the line 51-1, and the successive generations of this transgenic tobacco line, it could be useful to study the copy number, inheritance and organisation of T-DNA for transgenic tobacco lines 49-1, 51-3, 51-5, 54-18 and especially for 51-14 which

has the lowest level of resistance to the herbicide. This study could reveal relationships between the copy number and organisation of T-DNA and the level of resistance to the herbicide.

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Appendices

Appendix 1. B5 medium

Stock solutions:

A.	Micronutrients (store in freezer)	g/100 ml
	$MnSO_4.4H_2O$	1.320
	H_3BO_3	0.300
	$ZnSO_4$	0.200
	$Na_2MoO_4.2H_2O$	0.025
	$CuSO_4.5H_2O$	0.0025
	$CoCl_2.6H_2O$	0.0025
B.	Vitamins (store in freezer)	g/100 ml
	Nicotinic acid	0.100
	Thiamine.HCl	1.000
	Pyridoxine.HCl	0.100
C.	Calcium chloride (store in refrigerator)	g/100 ml
	$CaCl_2.2H_2O$	15.0
D.	Potassium iodide (store in Amber bottle in refrigerator)	g/100 ml
	KI	0.075

Preparation of B5 medium

Ingredient	Amount L^{-1}
$NaH_2PO_4.2H_2O$	0.170 g
KNO_3	0.134 g
$MgSO_4.7H_2O$	0.250 g
Ferric EDTA	0.040 g
myo-Inositol	0.100 g
Sucrose	20 00 g
B5 stock solution A (micronutrient)	1.000 ml
B5 stock solution B (vitamins)	1.000 ml
B5 stock solution C ($CaCl_2.2H_2O$)	1.000 ml
B5 stock solution D (KI)	1.000 ml

Final pH adjusted to 5.5 with 0.2N KOH or 0.2N HCl. To prepare agar plates add 0.8% Difco agar. Autoclave at 121°C for 15 minutes.

Appendix 2. WR8 medium

Ingredients	Amount L^{-1}
$NaH_2P0_4.2H_20$	0.170 g
$KN0_3$	2.500 g
$(NH_4)_2S0_4$	0.134 g
$MgS0_4.7H_20$	0.040 g
myo-Inositol	0.100 g
Sucrose	20.00 g
B5 stock solution A.	1.000 ml
B5 stock solution B.	1.000 ml
B5 stock solution C.	1.000 ml
B5 stock solution D.	1.000 ml
2,4-D	0.500 mg
Kinetin	0.500 mg
Picloram	0.050 mg

Adjust pH to 5.5

Autoclave at 121°C for 15 minutes.

Preparing growth regulators:

2,4-D: Dissolve 5 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) in 200-500 µl of absolute ethanol, heat slightly, then gradually dilute to 10 ml with sH_2O.

Kinetin: Dissolve 5 mg Kinetin in small amount of 0.5N HCl by heating slightly then dilute to 10 ml with sH_2O. Wrap with aluminium foil, store in dark.

Picloram: Prepare as 2,4-D using 0.05 mg ml^{-1} of picloram.

Appendix 3. Antibiotic stock solutions

Kanamycin	100 mg ml^{-1} in H_20
Ampicillin	100 mg ml^{-1} in H_20
Tetracycline	5 mg ml^{-1} in methanol
Cefataximine	100 mg ml^{-1} in H_20

Appendix 4. B5+2ip medium

Add 300 µl (0.5 mg L^{-1}) of 6-[r,r-dimethylallylamino]purine (2ip)* to B5 medium before autoclaving.

Adjust pH to 5.5

*To preparing 2ip solution: Dissolve 0.5 mg 2ip in 1 ml of 0.5N HCl.

Appendix 5. MS medium

Stock solutions:

Stock	Constituents	Concentration g/200 ml	Volume stock solution in final medium mg L^{-1}	Final concentration in medium mg L^{-1}
A.	NH_4NO_3	16.500	20	1650.0
B.	KNO_3	19.000	20	1900.0
C.	H_3BO_4	0.248	5	6.2
	KH_2PO_4	6.800		170.0
	KI	0.033		0.830
	$Na_2MoO_4.2H_2O$	0.010		0.250
	$CoCl_2.2H_2O$	0.001		0.025
D.	$CaCl_22H_2O$	17.600	5	440.0
E.	$MgSO_4.7H_2O$	14.800	5	370.0
	$MnSO_4.4H_2O$	0.892		22.30
	$ZnSO_4.7H_2O$	0.342		8.60
	$CuSO_4.5H_2O$	0.001		0.025
F.	Ferric EDTA	2.600	6	40.0
G.	Thiamine.HCl	0.004	5	0.10
	Nicotinic acid	0.020		0.50
	Pyridoxine.HCl	0.020		0.50
	Glycine	0.080		2.00

* Stock solutions should be stored in the refrigerator (should be discarded after 6 weeks).

Preparation of MS medium

Ingredient	MS medium Amount L^{-1}	1/2MS medium Amount L^{-1}
myo-inositol	0.100 g	0.050 g
sucrose	30.00 g	15.0 g
MS stock A	20 0 ml	10.0 ml
MS stock B	20.0 ml	10.0 ml
MS stock C	5.0 ml	2.5 ml
MS stock D	5.0 ml	2.5 ml
MS stock E	5.0 ml	2.5 ml
MS stock F	5.0 ml	2.5 ml
MS stock G	5.0 ml	2.5 ml

Adjust final pH to 5.8 with 0.2N KOH or 0.2N HCl. To prepare the solid agar, add 0.8% (w/v) Difco agar before autoclaving. Autoclave at 121°C for 15 minutes.

Appendix 6. TY medium

Ingredient	g L^{-1}
Difco bactotryptone	5.0
Difco yeast extract	3.0
$CaCl_2.2H_20$	0.87

to prepare solid agar, add 15 g L^{-1} of agar (Davis) before autoclaving.

Appendix 7. Plasmid DNA isolation; modified STET method

STET buffer:

Tris-HCl pH8.0	500	mM
EDTA	50	mM
Triton X-100	5%	(v/v)
Sucrose	8%	(w/v)

Lysozyme (sigma L-6876) 10 mg L^{-1} in sH_2O.
(Prepare lysozyme immediately before use)
Isopropanol
Ethanol 75% and 100%
Chloroform:*iso*-amylalcohol 24:1
Phenol equilibrated with STET buffer (without triton)
100 mM NaCl
DNase free RNase (Sigma R-2638) 10 mg ml^{-1}

Prepared RNAase as below (Sambrook *el al.*, 1989):

Dissolve 10 mg of RNAase in 10 mM Tris-HCl (pH 7.5), heat to 100°C for 15 minutes, allow to cool slowly to room temperature.

Appendix 8. Horizontal gel electrophoresis

1xTAE electrophoresis buffer:

Tris	4.84	g L^{-1}
Acetic acid	1.14	ml L^{-1}
0.5M EDTA pH 8	2.00	ml L^{-1}

To prepare agarose gels, dissolve agarose (BRL 5510 UB) in 1xTAE buffer by heating into the microwave oven. Add 1/1000 vol.(v/v) of 10% (w/v) ethidium bromide before pouring the gel. Cover the gel with 1xTAE buffer contain 1/1000 vol.(v/v) of 10% (w/v) ethidium bromide.

Appendix 9. LB Medium

Ingredient	Amount g L^{-1}
Difco bactotryptone	10.0
Difco yeast extract	5.0
NaCl	10.0

Final pH adjusted to 7.5 with 0.2N NaOH. To prepare solid agar medium, add 1.5 g L^{-1} agar (Devis) before autoclaving.

Appendix 10. Material for DNA fragment isolation from gel

DEAE solution:

1M Tris-HCl (pH 7 9)	0.4	ml
0.5 M EDTA (pH 8)	0.8	ml
5 M NaCl	l8	ml
sH_20	31.8	ml
Total vol.	40	ml

(Filter sterilise the solution)
DEAE paper.

Appendix 11. Material for plant DNA extraction

Extraction buffer 1:
- 3 x SSC
- 0.1 M EDTA pH8
- 0.1 M sodium diethyldithiothiocarbamate
- 1/100 vol. of 20% (w/v) SDS (sodium dodecylsulfate)

Ethanol (absolute)
Equilibrated phenol

Appendix 12. Material for plant RNA isolation

DEPC treated water:

Add 1 ml of diethylpyrocarbonate (DEPC) per litre of water in the fume hood, shake well to dissolve.
Incubate at 37°C overnight.
Autoclave at 121°C for 15 minutes.
All solutions must be made up with DEPC treated water.

Guanidine isothiocyanate:

Guanidine isothiocyanate	42.7	g
0.25 M sodium citrate	10	ml
10% N-Lauryl sarcosine	5	ml
β-mercapto-ethanol	700	μl
Adjust final volume to 100 ml with sH_20		

Do not autoclave, wrap the bottle in tinfoil and store at 4°C or room temperature.

Urea buffer:

Urea	42	g
1 M Tris-HCl pH 8.0	5	ml
0.5 M EDTA pH 8.0	20	μl
10% (w/v) SDS	1	ml
Adjust final volume to 100 ml with sH_20		
N.B. Do not autoclave, prepare fresh before use.		

Sodium acetate buffers:

1) 20% sodium acetate buffer:

Sodium acetate $3H_20$	20	g
Adjust pH to 5.5 with glacial acetic acid.		
Adjust final volume to 100 ml with sH_20.		
Autoclave at 121°C for 15 minutes.		
Store at 4°C.		

2) 4.5 M acetate buffer:

Sodium acetate $3H_20$	61.2	g
Adjust pH to 6.0 with glacial acetic acid.		
Adjust volume to 100 ml with sH_20.		
Autoclave at 121°C for 15 minutes.		
Store at room temperature.		

Equilibrated phenol:

AR phenol	500	g
Hydroxy-quinoline	0.6	g
0.1M Tris-HCl pH 8.5	500	ml

Shake well, then continue to shake periodically for 1-2 hours before finally allowing phenol to settle. Decant off buffer and overlay with fresh buffer. Repeat this equilibration twice more. All of the glassware extraction equipments must be baked at 180°C for 4 hours and plastic wares must be autoclaved at 121°C for 45 minutes.

Materials and buffers for formaldehyde gel (large):
(DEPC treated water must be used in all solutions)

10xMOPS/EDTA buffer:

1) 3-(N-morpholino)propane-sulphonic acid (MOPS)	52.3	g
2) EDTA pH 7.5	1.9	g

Adjust pH to 7.5 with 10N NaOH
Adjust final volume to 250 ml
Added 250 ml MOPS to 250 ml EDTA and obtained final correct concentrations.
(Wrapped on aluminium foil and stored at room temperature)

Buffer A:

10x MOPS/EDTA buffer	300	µl
sH_2O	700	µl

Deionized formamide:

AR formamide	50	ml
Mixed-bed ion exchange resin	5	g

(e.g. BDH Amberlite - MB1)
Stirred slowly for 30 min at room temperature.
Filtered twice through Whatman No. 1 filter paper.
Store as 1 ml aliquots at -20°C.

Formamide/formaldehyde.

AR formaldehyde	89	µl
Deionized formamide	250	µl

Gel loading buffer:

Sucrose	400	mg
Buffer A (288 µl H_2O/32 µl xMOPS/EDTA)	320	µl
Mix to dissolve then add:		
Xylene cyanol	5	mg
Bromophenol blue	5	mg
Mix to dissolve then add:		
AR formaldehyde	178	µl
Deionized formamide	500	µl

Store as 100 µl aliquots at -20°C
Before use thawed and added 12.5 µl ethidium bromide (10 mg ml^{-1}).

Gel running buffer (= 1x MOPS/EDTA):

MOPS/EDTA buffer	225	ml
Water	2025	ml

Appendix 13. RNA sample preparation

A) Determined concentration of RNA sample and removed volume containing 30 µg RNA. Precipitated RNA over 3-4 hours with ethanol at -20°C.

B) Prepare following mixture

RNA (as dry pellet)	30	µg
Buffer A	5.5	µl
Resuspend RNA and added:		
Formamide/formaldehyde	12	µl
Heated at 70°C for 10 minutes and quenched on ice and added:		
Gel loading buffer	4.5	µl

Pouring formaldehyde gel:

Agarose	2.5	g
10xMOPS/EDTA buffer	2.5	ml
sH_20	180	ml

Mixed in a 500 ml flask and dissolved in microwave, stirred when it was cooling and added:

AR formaldehyde	45	ml

Mixed and poured into gel tray which already have been wiped out with ethanol.

Allowed to set the gel for 30-40 minutes.

Covered the gel with 2250 ml of running buffer, loaded the samples and ran overnight at 25V for 14 hours.

Appendix 14. Materials for Southern blotting

Hybond-N+ nylon membrane (Amersham)
20xSSC solution (Sambrook *et al.*, 1988):

NaCl	3.0	M
Sodium citrate	0.3	M

Alkali fixation buffer:
- 0.4 M NaOH for DNA
- 0.05 M NaOH for RNA

Denaturing solution:

NaCl	1.5	M
NaOH	0.5	M

Neutralising solution:

NaCl	1.5	M
Tris-HCl pH 7.2	0.5	M
EDTA	0.001	M

Material for hybridization of membrane for Southern blotting:

20xSSPE
SDS 10% (w/v)
100xDenhardt's solution:
- Bovine serum albumin (BSA) 2% (w/v)
- Ficole™ 2% (w/v)
- Polyvinylpyrolidone (PVP) 2% (w/v)

Hering sperm DNA (sonicated) 10 mg ml^{-1}

Made prehybridisation solution as:

20xSSpE	6.25	ml
100x Denhardt's solution	1.25	ml
10% SDS	1.25	ml
sH_20	16.25	ml
Final volume	25.00	ml

Appendix 15. Materials for tobacco leaf transformation

Nicl medium:

To MS medium without solution G (vitamins) add following solutions)

B5 vitamins	1 ml	L^{-1}
BAP* (Benzyl amino purine)	1 μg	ml^{-1}
NAA** (Naphtalene acetic acid)	0.1 μg	ml^{-1}

*BAP prepared as a 1 mg ml^{-1} stock in 0.1N NaOH.
**NAA prepared as 0.5 mg ml^{-1} by dissolving 0.5 mg NAA in 50 μl absolute ethanol then make up to 1 ml with sH_20

NicII medium:

NicI medium supplemented with 100 μg ml^{-1} Cefotaximine and 300 μg ml^{-1} kanamycin

NicIII medium:

NicII medium without BAP and NAA.

NicII/kanamycin-free plus dalapon:

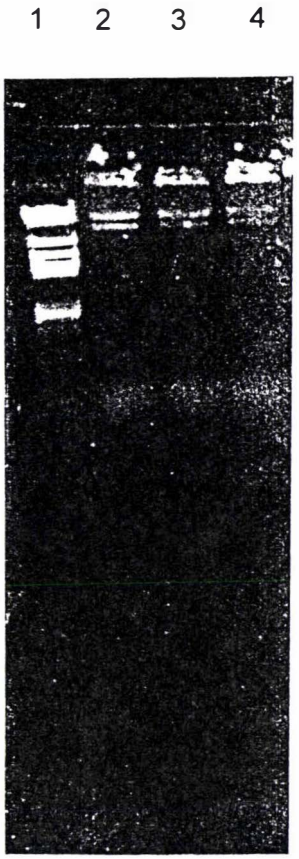
NicII medium without kanamycin but supplemented with, 0.00, 100, 200, or 400 μg ml^{-1} dalapon

NicIII/kanamycin-free plus dalapon:

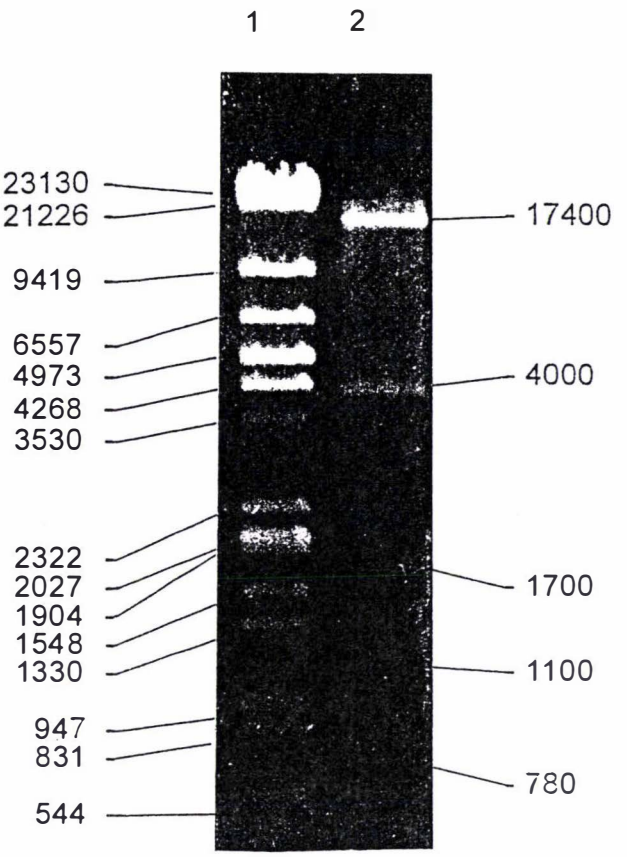
NicIII medium without kanamycin but supplemented with, 0.00, 100, 300 or 400 μg ml^{-1} of dalapon.

Appendix 16. DNA electrophoresis results used for the plasmid mapping

Figure A.1. Restriction enzyme digests of plasmid pAS501 (*Eco*RI, *Hind*III, *Sma*I, *Bgl*II, *Sac*II *Eco*RV). (A) lane 1: λ *Hind*III*Eco*RI+*Hind*III standard, lane 2: undigested pAS501, lane 3: pAS501 digested with *Eco*RI, lane 4: pAS501 digest with *Hind*III. (B) lane 1: λ *Hind*III*Eco*RI+*Hind*III standard, lane 2: pAS501 digest with *Sma*I. (C) lane 1: λ *Hind*III*Eco*RI+*Hind*III standard, lane 2: pAS501 digest with *Bgl*II, and (D) lane 1: pAS501 digest with *Sac*II, lane 2: pAS501 digest with *Eco*RV, lane 3: λ *Hind*III*Eco*RI+*Hind*III standard.


1 2 3 4

A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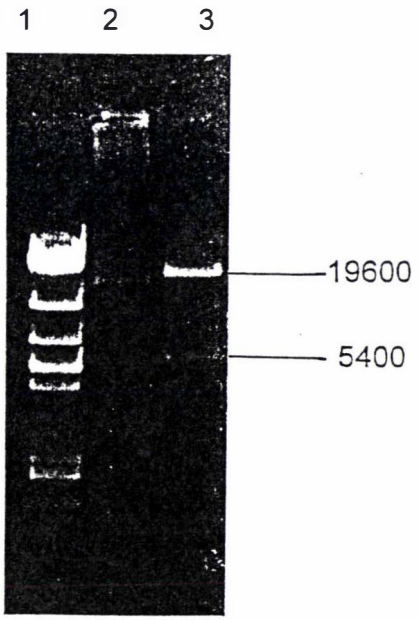
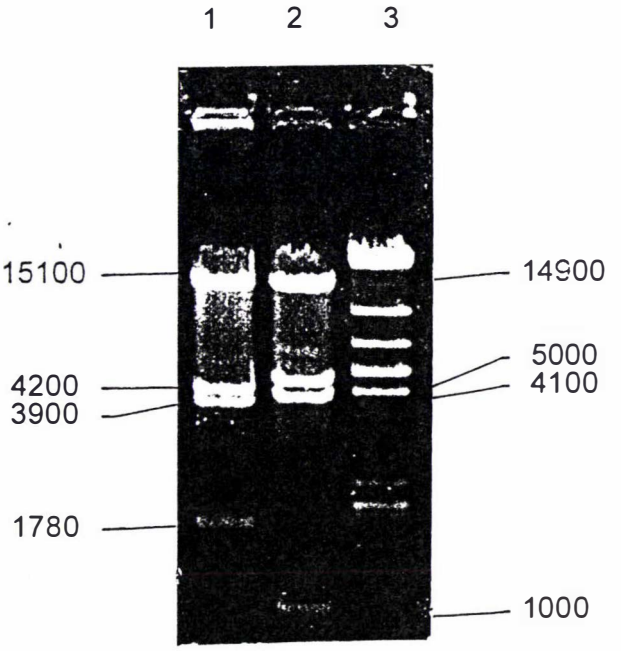
23130
21226
9419
6557
4973
4268
3530
2322
2027
1904
1548
1330
947
831
544
17400
4000
1700
1100
780

B

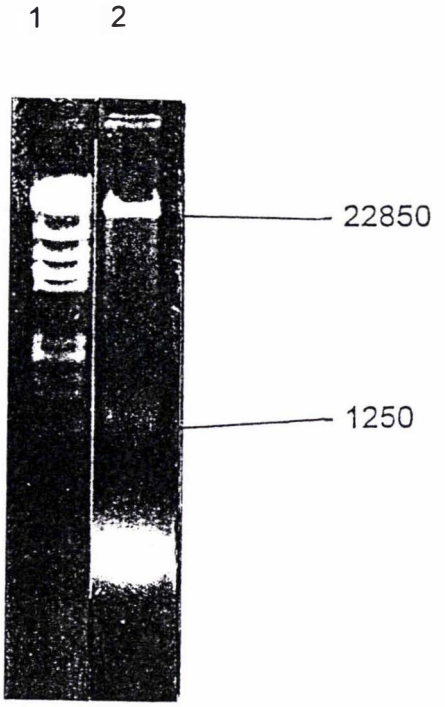

1 2 3
19600
5400

C

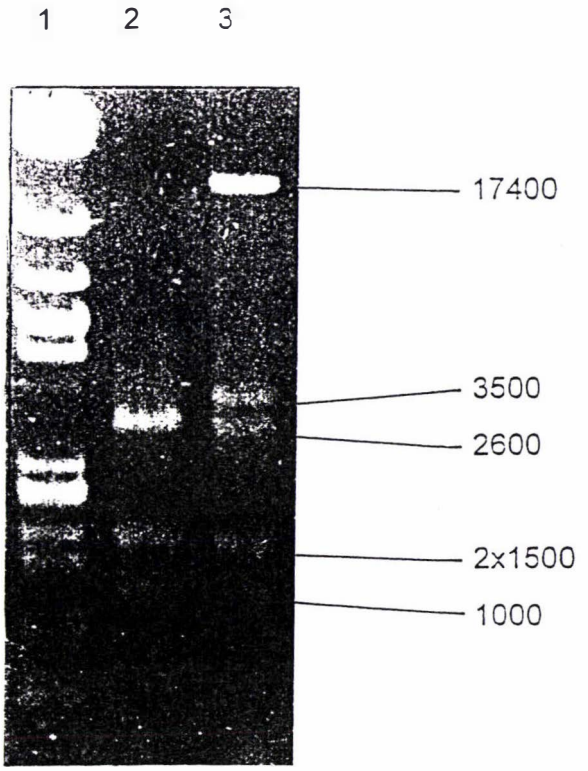

1 2 3
15100
4200
3900
1780
14900
5000
4100
1000

D

Figure A.2. Restriction enzyme digest of plasmid pAS501 (*Pst*I, *Eco*RI/*Bgl*II). (A) lanes 1: λ *Hind*III*Eco*RI+*Hind*III standard, lane 2: pAS501 digest with *Pst*I, (B) lane 1: λ *Hind*III*Eco*RI+*Hind*III standard, lane 2: pAS501 digest with *Eco*RI/*Bgl*II.



A



B

Figure A.3. Restriction enzyme digest of plasmid pAS501(*Sac*II/*Eco*RI, *Sma*I/*Sac*II) and PCR product of the dehalogenase gene. (A) lanes 1: pAS501 digested with *Sac*II/*Eco*RI, lanes 2: λ *Hind*III*Eco*RI+*Hind*III standard, (B) lane 1: pAS501 digested with *Sma*I/*Sac*II, lane 2: λ *Hind*III*Eco*RI+*Hind*III standard, and (C) lanes 1: λ *Hind*III*Eco*RI+*Hind*III standard, lane 2: PCR product of the dehalogenase gene .

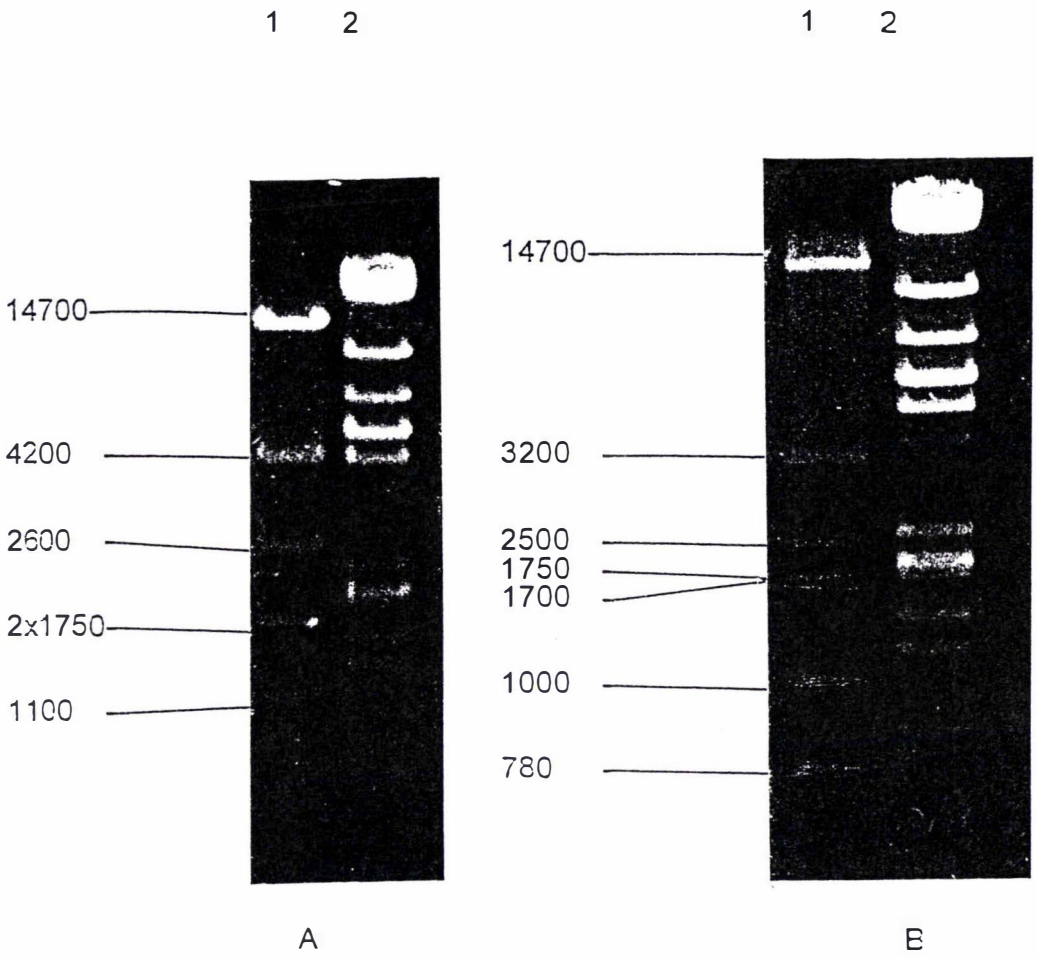
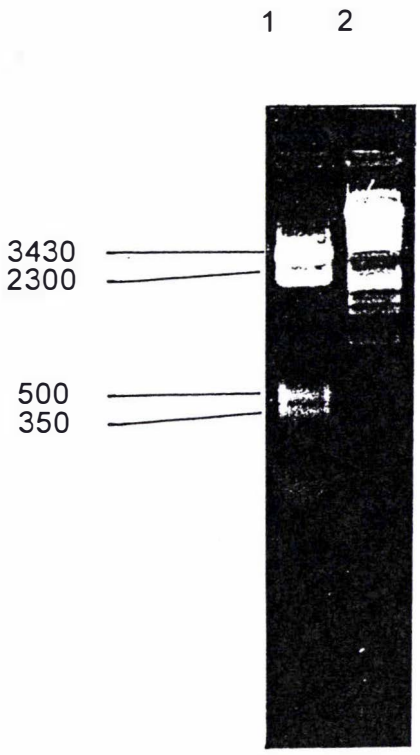
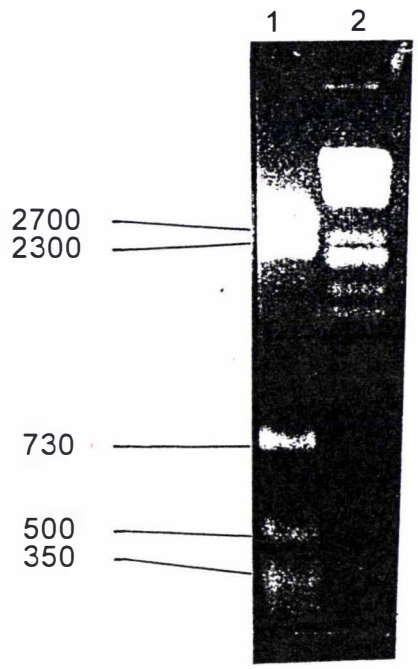

1 2
14700
4200
2600
2x1750
1100
A
1 2
14700
3200
2500
1750
1700
1000
780
B
1 2
1550
nonspecific
C

Figure A.4. Restriction enzyme digest of plasmid pSDH. (A) lane 1: pAS501 digested with *Pst*I lane 2: λ *Hind*III*Eco*RI+*Hind*III standard, and (B) lane 1: pAS501 digested with *Pst*I/*Sac*I, lane 2: λ *Hind*III*Eco*RI+*Hind*III standard.



A



C

Appendix 17. Maps of plasmids pAS501 and pRK290.

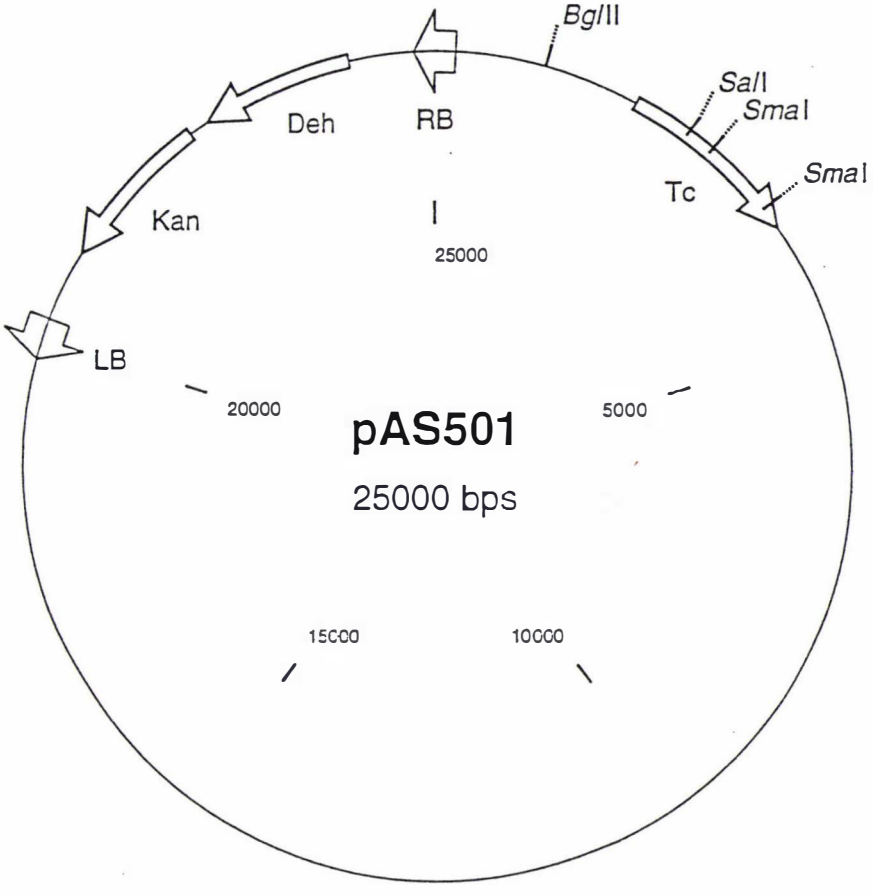


Figure A.5. Binary vector plasmid pAS501. The pAS501 binary T-DNA vector for plant transformation has been constructed by insertion of a T-DNA region containing right border (RB) and left border (LB) sequences into the *Eco*RI site of the pRK290 vector. The T-DNA region contains a dehalogenase gene (Deh) and a kanamycin resistance (Kan) gene. The arrows show the location and direction of transcribed regions of these genes.

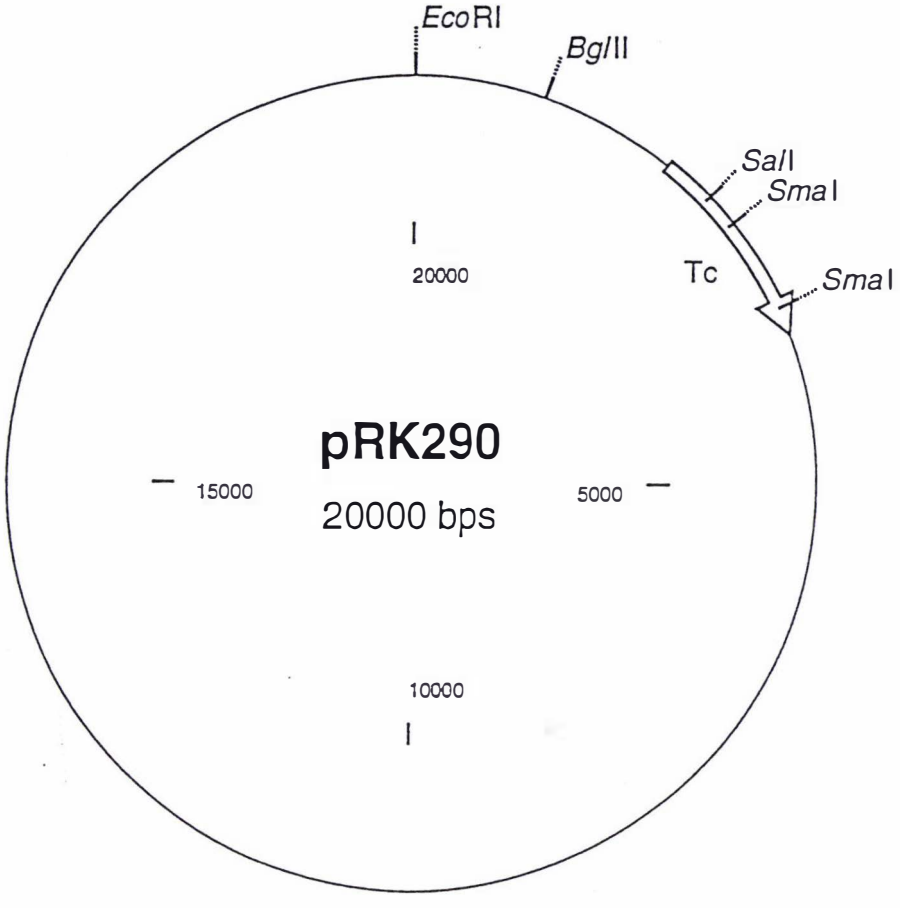


Figure A.6. The pRK290 broad host range plasmid vector. Restriction sites are shown with coordinates (bp) measured anticlockwise from the *Eco*RI site. The *Eco*RI and *Bgl*II sites, outside the tetracycline gene are available for cloning foreign DNA fragments. The *Sma*I sites are located inside the tetracycline gene. The arrow shows the direction and minimum extent of transcription of the tetracycline gene.