Bushiness in micropropagated *Zantedeschia:* An investigation at the environmental, physiological and molecular levels

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Abstract

The commercial *Zantedeschia* industry faced a potentially serious problem with the cultivar Florex Gold periodically producing 'bushy' plants with several short non-flowering shoots and squat tubers with multiple nodes. These plants, derived from tissue culture, resulted in a reduced financial return to the *Zantedeschia* industry.

A number of potentially causative factors were investigated in this project but no single factor emerged as the cause of the bushy syndrome. The selection of tubers with multiple eyes for initiation into tissue culture, or tubers themselves recently derived from tissue culture, may be a contributing factor to bushiness. A variable growing environment (screen house), shortened storage time (11 weeks) and plant position were also shown to accentuate individual bushy characteristics.

Endogenous cytokinin concentration and profile, determined by high performance liquid chromatography and radioimmunoassay, in tubers at three stages in the *Zantedeschia* life cycle were similar between bushy and non-bushy plants and cannot explain the bushy phenotype. Micropropagation of Florex Gold on Murashige and Skoog medium supplemented with low cytokinin concentration (4.4 μM 6-benzylaminopurine, 6-BAP) did not produce bushy plants or tubers. The use of elevated cytokinin during micropropagation did not produce plants with a range of bushy symptoms, although elevated thidiazuron (13.6 μM), but not 6-BAP, was shown to affect flower spathe length and plant shoot number. Florex Gold, when compared to a range of non-bushy *Zantedeschia* cultivars, displayed greater sensitivity to cytokinin during micropropagation assessed by a root length bioassay. Increased sensitivity to 6-BAP, during micropropagation may indicate a predisposition to bushiness.

Differences in the DNA methylation profile of bushy and non-bushy plants were found in DNA using DNA Amplified Fragment Length Polymorphism. However, the isolated sequences were not similar to any gene(s) currently listed in public databases. Differential gene expression levels, determined by cDNA Amplified Fragment Length Polymorphism, found 60S and 18S ribosomal RNA down-regulated in bushy Florex Gold. These two genes may play a role in the expression of the bushy phenotype.

Changes in DNA methylation and differential gene expression patterns and the results from the micropropagation experiments, provide evidence that *Zantedeschia* Florex Gold tubers selected for commercial micropropagation should be deep and apically dominant with few nodes and buds, and not themselves recently derived from tissue culture.

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
^{3}H-iPATA	^{3}H-isopentenyladenosine-trialcohol
^{3}H-ZRTA	^{3}H-zeatin riboside-trialcohol
5mdC	5-methyl-deoxycytidine
6-BAP	6-benzylaminopurine
^{14}C-AMP	^{14}C-adenosine-5'-monophosphate
ADP	adenosine 5'-diphosphate
AFLP	amplified fragment length polymorphism
AMP	adenosine 5'-monophosphate
AtIPT1 to 9	*Arabidopsis thaliana* isopentenyltransferases 1 to 9
ATP	adenosine 5'-triphosphate
B	amount of ^{3}H-ZRTA or ^{3}H-iPATA bound to the antibody in the presence of ZR or iPA
B_0	amount of ^{3}H-ZRTA or ^{3}H-iPATA bound to the antibody in the absence of ZR or iPA
BLAST	basic local alignment search tool
BV	bed volume
C_{18}	octadecyl silica
cDNA	complementary DNA
*cis*ZOG1	*cis* zeatin *O*-glucosyltransferase gene
CKO	cytokinin oxidase gene
ckx1	cytokinin oxidase gene
CPM	counts per minute
CPPU	*N*-phenyl-*N'*-(2-chloro-4-pyridyl)urea
CRE1	cytokinin response 1 gene
CTAB	cetyltrimethylammonium bromide
cZ	*cis* zeatin
cZ9G	cis zeatin 9 glucoside
cZNT	*cis* zeatin nucleotide
cZOG	*cis* zeatin-*O*-glucoside
cZR	*cis* zeatin riboside
cZROG	*cis* zeatin riboside-*O*-glucoside

d	day
DDRT-PCR	differential display reverse transcription PCR
DE52	DEAE cellulose 52
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
df	degrees of freedom
DPU	diphenylurea
DZ	dihydrozeatin
DZ3G	dihydrozeatin-3-glucoside
DZ7G	dihydrozeatin-7-glucoside
DZ9A	dihydrozeatin-9-alanine (dihydrolupinic acid)
DZ9G	dihydrozeatin-9-glucoside
DZMP	dihydrozeatin riboside-5'-monophosphate
DZNT	dihydrozeatin nucleotide
DZOG	dihydrozeatin-*O*-glucoside
DZR	dihydrozeatin riboside
DZROG	dihydrozeatin riboside-*O*-glucoside
EDTA	ethylenediaminetetraacetate
EST	expressed sequence tag
EXO I	exonuclease I
FGC	Fast Growing Callus
FW	fresh weight
GA_3	gibberellic acid
HPLC	high performance liquid chromatography
IBA	indole-3-butyric acid
iP	isopentenyladenine
iP7G	isopentenyladenine-7-glucoside
iP9G	isopentenyladenine-9-glucoside
iPA	isopentenyladenosine
iPDP	isopentenyladenosine-5'-diphosphate
iPMP	isopentenyladenosine-5'-monophosphate
iPNT	isopentenyladenine nucleotide
ipt	isopentenyltransferase gene
iPTP	isopentenyladenosine-5'-triphosphate
LSD	least significant difference

MET1	DNA methytransferase gene
MPC	Magnetic Particle Concentrator
mRNA	messenger RNA
mT	*meta* topolin
NAA	naphthalene acetic acid
NCC	Nodular Compact Callus
OAcDZR	*O*-acetyldihydrozeatin
OAcZR	*O*-acetylzeatin
oT	*ortho*-topolin
oTR	*ortho*-topolin riboside
OXDZ	*O*-xylosyldihyrozeatin
OXZ	*O*-xylosylzeatin
PCR	polymerase chain reaction
PVPP	polyvinylpolypyrrolidone
RAPD	random amplified polymorphic DNA
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SAP	shrimk alkaline phosphatase
SE	standard error of the mean
SSC	sodium chloride and sodium citrate buffer
T1	tuber one year from tissue culture
T2	tuber two years from tissue culture
TDZ	thidiazuron
TEA	triethylamine
TEMED	NNN'N'-tetramethylethylenediamine
TIF	Technology for Industry Fellowship
tRNA	transfer RNA
tZ	*trans* zeatin
tZR	*trans* zeatin riboside
UV	ultra violet
v/v	volume/volume
Z	zeatin
Z7G	zeatin-7-glucoside

Z9A	zeatin-9-alanine (lupinic acid)
Z9G	zeatin-9-glucoside
ZDP	zeatin riboside-5' diphosphate
ZMP	zeatin riboside-5' monophosphate
ZNT	zeatin nucleotide
ZOG	zeatin-*O*-glucoside
ZOG1	zeatin *O*-glucosyltransferase
ZOX1	zeatin *O*-xylosyltransferase
ZPT	zeatin riboside 5' triphosphate
ZR	zeatin riboside
ZROG	zeatin riboside-*O*-glucoside

Chapter One: Introduction

1.1 Overview

Zantedeschia are New Zealand's number two flower export earner after orchids. *Zantedeschia* export cut flower sales totalled $8.7 million in 2001 (Statistics New Zealand, 2002). Export tuber sales of all tuberous crops, including *Zantedeschia*, totalled $15.7 million in the same year (Statistics New Zealand, 2002). The main export destinations of *Zantedeschia* are Europe (especially The Netherlands and Germany), Japan and the United States of America (Statistics New Zealand, 2002). Exports of cut flowers have increased almost seven times over the last 13 years, from $1.1 million in 1989 to $8.7 million in 2001. New Zealand *Zantedeschia* export cut flower sales reached $10 million in 1998. New Zealand only supplies 0.5% of the world cut flower market and there is, therefore, considerable potential for growth (Statistics New Zealand, 2002). In New Zealand approximately 2.5 million *Zantedeschia* plants are produced for the local market from tissue culture per year. New Zealand *Zantedeschia* growers also source tissue culture plants from India, Vietnam and Tonga (S. Simpson, personal communication, 2002).

The *Zantedeschia* industry in New Zealand faces a potentially serious problem, with some previously high quality cultivars periodically producing weak plants, often with several non-flowering shoots, resulting in a reduced financial return. The problem is found in plants derived from tissue culture and is referred to as the 'bushy' syndrome. Bushy *Zantedeschia* plants have tubers with multiple nodes and many eyes (buds). These produce short multiple shoots, and flowers with reduced stem length. Cultivars reported to be susceptible to bushiness include Florex Gold, Mango and Hot Shot. In the mid 1990s, bushiness was observed in The Netherlands, in plants derived from overseas tissue culture laboratories. In 1998, bushy plants were reported in tuber material originally derived from New Zealand tissue culture plants. If tubers of certain cultivars continue to produce bushy plants with multiple shoots, there will be a significant impact on tuber sales. A large percentage of New Zealand *Zantedeschia* tubers are exported to the world market and an understanding of the cause of the syndrome is vital to New Zealand producers.

This research was funded by a Technology for Industry Fellowship (TIF) from Technology New Zealand in conjunction with Multiflora Laboratories Limited. Multiflora is one of the largest plant tissue culture laboratories in New Zealand and *Zantedeschia* is one of the major crops micropropagated at Multiflora. New Zealand *Zantedeschia* growers and Dutch importers also offered cooperation and material for research, in an effort to understand the bushy syndrome. Consequently, the majority of this research addressed the bushy syndrome from a commercial tissue culture perspective.

1.1.1 The genus *Zantedeschia*

1.1.1.1 Botanical classification and morphology

Zantedeschia are still popularly, although confusingly, known as arum lilies or calla lilies, both *Arum* L. and *Calla* L. being distinct genera in the Araceae (Singh, 1996). The reason for the analogue *Arum* is the similarity in the leaves and that of *Calla* is that *Zantedeschia* was initially classified as *Calla* by Linnaeus (Singh, 1996). The genus *Zantedeschia*, which is in the family Araceae, is divided into two distinct sections. Section I contains only one species, *Z. aethiopica*, which is evergreen. Section II contains five species, *Z. albomaculata*, *Z. elliottiana*, *Z. jucunda*, *Z. pentlandii* and *Z. rehmannii*, all of which are winter dormant (Funnell, 1994). Extensive breeding of *Zantedeschia* species in Section II has produced numerous cultivars of varying spathe colours and sizes (Singh, 1996). Flowers range in colour from yellow, through orange to deep red and pink (Funnell et al., 1988). Exact parentage is unknown due to extensive interbreeding, although the yellow flowered cultivars prone to the bushy syndrome, such as Florex Gold, Mango and Hot Shot, are thought to have a strong *Z. elliottiana* background with some *Z. pentlandii*. However, non-bushy yellow flowering cultivars such as Best Gold also have a *Z. elliottiana* background (S. Simpson, personal communication, 1999).

The *Zantedeschia* flower is botanically an inflorescence consisting of a spadix, bearing female flowers at the base and male flowers above, which are surrounded by a modified leaf, the spathe (Corr and Widmer, 1987; Funnell et al., 1988). Leaf form is cultivar dependent. Leaf shape varies from lanceolate to ovate through to triangular and hastate. Leaf maculation or flecking can also be present. Maculation is cultivar dependent and is

also a sign of plant maturity when present. *Zantedeschia* have a storage organ, typically with numerous buds, which survives during the winter (Funnell, 1994). The buds (eyes) on the *Zantedeschia* storage organ have a range of flowering potentials. Dominant buds are physically swollen and encircled by a number of smaller axillary buds arranged spirally on the tuber. Some of these smaller axillary buds are physically swollen. Both the dominant buds and physically swollen axillary buds are readily induced to flower (Funnell and Warrington, 1994). *Zantedeschia* storage organ shape is a central organ, with additional organs or nodes growing off the side. These may grow to the same size as the central organ. Botanically whether the storage organ is a corm, tuber or rhizome, has not been resolved (Funnell, 1994). The term tuber will be used to describe the storage organ of *Zantedeschia* in this thesis.

Two cultivars were investigated during this research: the first cultivar, Florex Gold, is susceptible to bushiness, and the second cultivar, Best Gold, has not shown signs of bushiness. Florex Gold has deep yellow flowers and triangular leaves marked with translucent maculation. It grows to 70 cm tall, and has on average one flower per 25g (40 mm) tuber (A. Wearmouth, personal communication, 2000). Best Gold is a golden yellow flower variety with triangular leaves with translucent maculation. Best Gold grows true to type from seed and was not micropropagated prior to 1999. Best Gold grows to 80 cm tall and has two flowers per 25g (40 mm) tuber (A. Wearmouth, personal communication, 2000).

1.1.1.2 Commercial life cycle of *Zantedeschia*

The *Zantedeschia* are indigenous to southern and eastern Africa (Funnell, 1994). In their natural habitat, *Zantedeschia* in Section II exhibit a seasonal cycle of growth. Vegetative growth and flowering occurs during the late spring through to summer and leaves die down in winter. Plants require a period of 'rest' before growth begins the next season (Funnell, 1994). The commercial life cycle of *Zantedeschia* in New Zealand begins with the planting of seed or tissue culture plantlets in the spring (Figure 1.1). The plant will normally have one shoot above the ground. At the end of the growing season vegetative growth senesces, leaving 10 to 30 g tubers, which are lifted in early winter. Tubers produced at the end of the first growing season after micropropagation are referred to as T1 tubers (tubers one year from tissue culture). The tubers are cool stored at constant temperature (6 to 10°C) or held at ambient

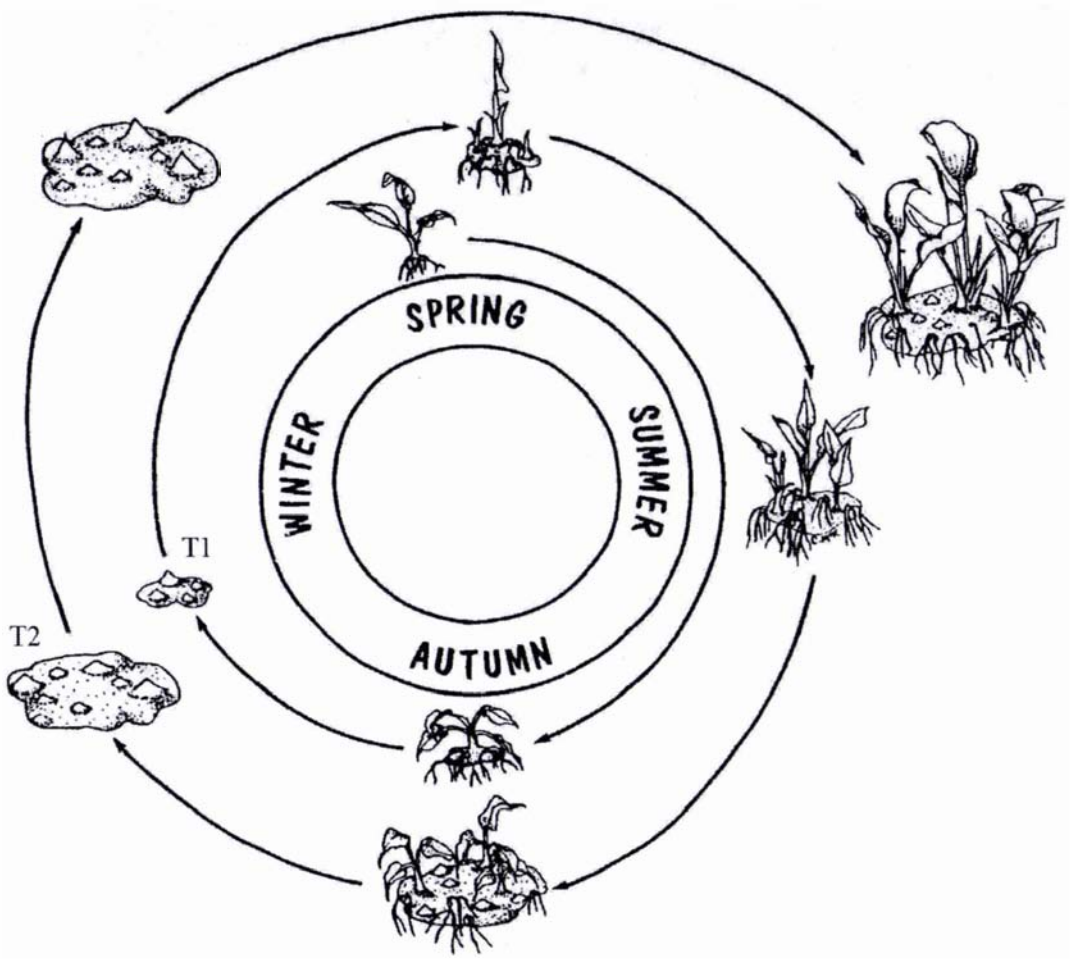


Figure 1.1: Life cycle of *Zantedeschia* from tissue culture plantlet to T1 tuber, first flowering and T2 tuber (adapted from Funnell and Warrington, 1994). A T1 tuber is a tuber one year from tissue culture. A T2 tuber is a tuber two years from tissue culture. A T1 tuber, typically heavier than 25g, produces one to two flowers with gibberellic acid (GA_3) treatment.

temperature (3 to 15°C). Many T1 tubers produced commercially in New Zealand are exported to the Northern Hemisphere, to be grown for flower production. These tubers are grown on (forced), in the Northern Hemisphere summer, in what would have been the normal winter dormant period in New Zealand (Funnell et al., 1988; Funnell and Warrington, 1994).

In the second growing season T1 tubers are replanted and the dominant buds on the tuber produce primary shoots. Above ground each of these primary shoots typically present two to three protective sheath leaves followed by two expanded leaves below the flower (Funnell and Warrington, 1994). A large tuber, typically heavier than 25 g, produces one to two flowers with gibberellic acid (GA_3) treatment. The time from planting to flowering takes between 60 and 140 days, depending on growing conditions. In New Zealand, flowering occurs between November and late January (Funnell et al., 1988; Funnell and Warrington, 1994). Tubers produced at the end of the second growing season after micropropagation are referred to as T2 tubers (tubers two years from tissue culture) (Figure 1.1). Tissue culture plants and *Zantedeschia* plants for autumn or winter flower production are typically grown under protected greenhouse cultivation.

1.1.2 Bushy syndrome in Florex Gold

1.1.2.1 First reports of bushiness in New Zealand Florex Gold

A major buyer of New Zealand tubers in The Netherlands reported the bushy syndrome in the *Zantedeschia* cultivar Florex Gold, in 1998. The Florex Gold tubers were from New Zealand. Multiflora tissue culture laboratory produced the tissue culture plantlets, which were grown for one season in New Zealand. The tubers were then exported to The Netherlands in early 1998 as T1 tubers. The tubers were planted in the northern hemisphere spring for flower production. They produced a large number of short shoots with a limited number of flowers, or no flowers at all, instead of producing one or two long stemmed flowers. Bushiness is usually first reported during the second growing season. This syndrome had been seen previously in micropropagated *Zantedeschia* material produced by Indian and Dutch tissue culture laboratories (S. Simpson, personal communication, 1999). Over the 1998/1999 season in New Zealand, bushy plants were observed on several properties with plants produced by a number of different tissue

culture laboratories and growers. Reports also came from other countries about bushy *Zantedeschia* plants.

1.1.2.2 Suggestions for the bushy syndrome

Many suggestions have been made as to why the *Zantedeschia* cultivar Florex Gold developed bushy characteristics. Most of these were based on industry 'gut feeling' and anecdotal information. There was a wide spread assumption amongst *Zantedeschia* growers that the bushy plants were induced by a high level of the plant hormone, cytokinin, in the culture medium. It was thought that the cytokinin caused disturbed metabolism two years (and many cell division cycles) after the tissue culture plants were removed from the media. During commercial micropropagation it had also been observed that the yellow flowered *Zantedeschia* cultivars, including Florex Gold, were more responsive to cytokinins. Yellow flowered cultivars micropropagated on media containing the same cytokinin concentration, have an increased multiplication rate, in comparison to pink or red cultivars (S. Simpson, personal communication, 1999). As a consequence, media containing lowered cytokinin concentration were routinely used during the multiplication of the yellow flowered *Zantedeschia* cultivars at Multiflora Laboratories. Anecdotal information, therefore, suggested that both changes in cytokinin concentration and profile two years after tissue culture, and sensitivity to cytokinin during micropropagation, were playing a role in the expression of the bushy phenotype.

Further suggestions for the cause of the bushy syndrome included the parentage of Florex Gold and/or the Florex Gold genotype. It was suggested that *Z. elliottiana*, one of the possible parents of Florex Gold, was somehow contributing to the bushiness, as the cultivars first expressing the bushy syndrome, Florex Gold, Hot Shot and Mango, may all have *Z. elliottiana* parentage. It was also suggested that the cultivar itself had a predisposition to bushiness. This was because plants with the bushiest symptoms were first seen in Florex Gold.

Two practices of New Zealand *Zantedeschia* tuber growers were also questioned regarding their role in the expression of the bushy syndrome. The practice of New Zealand tuber growers selecting new tissue culture lines from T1 or T2 tubers may have enhanced juvenile characteristics and bushiness in *Zantedeschia* Florex Gold. This

practice was in an effort to produce *Zantedeschia* tubers free from virus. However, there are reports of gradual physiological change during micropropagation leading to rejuvenation (Webster and Jones, 1989; Marks, 1991). Such rejuvenation may have occurred in tissue cultured *Zantedeschia* from previously micropropagated T1 or T2 tubers. Rejuvenation of micropropagated *Zantedeschia* plants may have caused the reduction in leaf maculation, the reduced percentage of leaves with mature triangular leaf shape and the reduced percentage of flowers observed in bushy plants.

New Zealand tuber growers had also been supplying multiple eyed tubers for initiation into tissue culture, as there was the perception that tubers with many eyes were superior, as they produced more flowering shoots. However, a large number of shoots may not necessarily have equated to a large number of strong, tall flower stems.

The reduced light and lower temperature growing conditions for *Zantedeschia* in The Netherlands, compared with those in New Zealand, were also questioned because of the large differences in these conditions between the two countries. The bushy condition was first seen after an unusually cool, shady summer in The Netherlands. In The Netherlands, optimal temperature regimes of 19 to 22°C during sprouting, 15 to 18°C after sprouting, and 18 to 24°C (day) and 13 to 16°C (night) during flowering are recommended by one *Zantedeschia* grower. The growing conditions used by other *Zantedeschia* growers in The Netherlands are unknown. These temperatures, however, are lower than those recommended in the New Zealand Calla Council Growers' Handbook (Clemens, 1994; Funnell and Warrington, 1994). Low temperature (<12°C) and low light conditions may result in reduced flower yields and stem length (A. Wearmouth, personal communication, 1999).

The reduced length and variable temperature of storage was also suggested as a possible factor contributing to the abnormal growth phenotype of Florex Gold, because tubers were sent to The Netherlands from New Zealand and stored for different lengths of time and at different temperatures. Forcing was also implicated because Dutch tuber growers were buying New Zealand tubers and forcing them to grow and flower during their natural rest period. Forcing tubers may be disturbing their life cycle and has been suggested as a cause of bushiness.

Included in the numerous possible causes of bushiness were also that Dutch tuber growers had been buying smaller grade/size tubers and trying to flower these. The smaller sized tubers may not be able to support flower growth. Additional variation due to plants growing at the edge of the tray and edge of the bench was also suggested to contribute to the expression of the bushy syndrome.

1.1.2.3 New Zealand commercial *Zantedeschia* growing conditions and bushiness

Extensive research investigating optimal commercial *Zantedeschia* growing conditions has been conducted including Clemens (1994) and references therein, and others (Dennis et al., 1994; Funnell and Warrington, 1994; Clemens et al., 1998; Funnell and Heins, 1998; Wright, 1998; Chen et al., 2000; Wright and Burge, 2000). The New Zealand Calla Council Growers' Handbook has recommended guidelines for growing *Zantedeschia* in New Zealand (Clemens, 1994).

The optimal commercial growing conditions include a minimum temperature for tuber and plant growth of 5 to 6°C, and a mean daily temperature of 21 to 26°C. Plants grown under average daily temperatures encountered in the field during the New Zealand summer (16 to 19°C) will attain only approximately 20% of the final tuber weight of those grown under the optimal temperature near 25°C. Total plant height and flower stem length also increases with increasing growing temperature. The number of days from planting until flowering is also reduced with increasing growing temperatures. Tuber growth is thought to be independent of day length. Light levels are important for tuber and plant growth, with light and temperature interacting to determine tuber size at the end of a growing season. Under summer conditions, 50% shading can result in greater final tuber size than if plants are grown in full summer light. Flower stem length is also increased under reduced light levels (Funnell and Warrington, 1994; Funnell et al., 1987b; Corr and Widmer, 1990). Funnell and Warrington (1994) also recommend the use of a slow release fertiliser (19N-2.6P-10K). Rates of 2 g L^{-1} and 4 g L^{-1}, have provided optimal flowering in peat and bark-based growing mediums respectively (Funnell and Warrington, 1994). Storage regimes of 10 weeks at 8°C or four weeks at 15°C are recommended to prevent damage during transport and increase the number of flowering buds. Storage at higher constant temperatures (22 to 25°C) reduces the number of buds developing into primary shoots, due to the rapid establishment of apical dominance (Corr and Widmer, 1988; Funnell

and MacKay, 1988; Funnell et al., 1988; Funnell and Warrington, 1994). As bushiness is a recent syndrome, it is not known whether these 'optimal' growing conditions alter the expression of the bushy phenotype. However, some of the practises (conditions outlined in Section 1.1.2.2) have been at variance to the recommended optimal growing conditions.

1.2 Cytokinins and bushiness

Various reports have implicated cytokinins in both the induction and expression of tissue culture syndromes comparable to bushiness in *Zantedeschia* (Anderson et al., 1982; Besse et al., 1992; Topoonyanont et al., 1999; Topoonyanont and Debergh, 2001; Eeuwens et al., 2002). When the TIF contract was being developed, it was considered that the cytokinins could be implicated in the expression of the bushy syndrome as the syndrome reflects characteristics ascribed to cytokinins such as reduction in apical dominance (Mok, 1994). Transgenic plants with elevated cytokinin levels caused by over expression of the isopentenyltransferase (*ipt*) gene (cytokinin synthase gene) from *Agrobacterium tumefaciens* had reduced stature, the release of apical dominance, changes in vascular development, and in some cases, an inhibition of root growth (Smart et al., 1991; Hobbie et al., 1994; McKenzie et al., 1998). An understanding of cytokinin biosynthesis, perception, and metabolism is therefore important when assessing a possible role for cytokinins in the expression of the bushy syndrome.

1.2.1 Cytokinin structure

Cytokinins, compounds first recognised by their ability to induce cell division in certain plant tissue cultures, are now known to act in combination with other phytohormones to evoke a diversity of responses. These include release of buds from apical dominance, bud and shoot development, cell division, organ formation, cell and organ enhancement, retardation of chlorophyll breakdown, chloroplast development, dormancy breaking, delay of senescence, leaf expansion and preferential translocation of nutrients and organic substances to cytokinin treated tissues (Brzobohatý et al., 1994; Mok, 1994; Arteca, 1996; Suttle, 2001). Cytokinins are highest in meristematic regions and areas of continued growth potential including roots, young leaves, developing fruits and seeds (Arteca, 1996). There are three forms of cytokinins: the natural isoprenoid and aromatic cytokinins and the synthetic diphenylureas (DPU's). The natural cytokinins are adenine

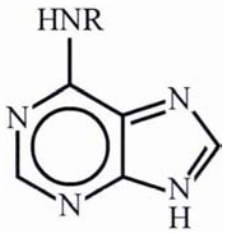
derivatives, and can be classified by the configuration of the N^6-side chain as isoprenoid, usually with an unsaturated isoprenoid side chain, or aromatic, with ring substitutions at the N^6-position (Figure 1.2) (Mok and Mok, 2001). Three origins of cytokinins have been suggested for the biosynthesis of cytokinins in plants: de novo synthesis, the transfer RNA (tRNA) pathway and de novo synthesis by plant associated microorganisms (Holland, 1997; Ashby, 2000; Kakimoto, 2001; Mok and Mok, 2001; Takei et al., 2001).

1.2.2 Cytokinin biosynthesis

There are two major groups of cytokinin found in plants. These are the "free" cytokinins, extractable from plant tissue in aqueous alcoholic solution and the "bound" cytokinin, released after alkaline hydrolysis and associated with the tRNA. Cytokinin biosynthesis involves the production of both of these groups. The biosynthetic pathway leading to aromatic cytokinins is entirely unknown (Mok and Mok, 2001).

1.2.2.1 tRNA pathway

It has been speculated that the tRNA pathway is a possible source of cytokinins as it involves the breakdown of nucleic acids, in particular tRNAs that contain cytokinins adjacent to a specific anticodon (Letham and Palni, 1983; Prinsen et al., 1997). Dimethylallyl pyrophosphate (DMAPP) together with the isopentenyladenosine (iPA) residue at the site adjacent to the anticodon is catalysed by tRNA isopentenyltransferase (EC 2.5.1.8) to give isopentenylated tRNA (Takei et al., 2001). The isopentenyl side chain can then possibly be hydroxylated to the *cis* configuration or a *cis*-hydroxylated side chain is directly transferred to the adenine moiety. McGaw and Burch (1995) suggest, however, that there is strong evidence that free cytokinin activity is not mediated via tRNA (McGaw and Burch, 1995). An argument against tRNA being a source of free cytokinin is the fact that *cis* zeatin (cZ) is the major cytokinin in tRNA. However, isomerisation of cZ to *trans*-zeatin (Z or tZ) by *cis-trans* isomerases can occur (Bassil et al., 1993; Mok and Mok, 2001). Another objection is that tRNA turnover occurs in all tissues, while cytokinin production is localised e.g. in root tips and shoot meristems. Therefore, if tRNAs were a major source of cytokinins, regulatory mechanisms must operate at the level of the metabolism of the released cZ (Mok and Mok, 2001).



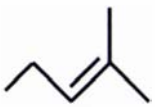
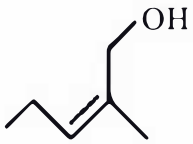
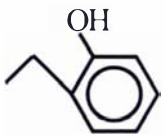
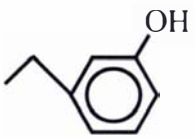
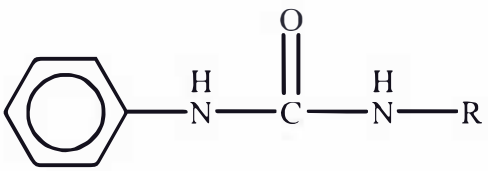
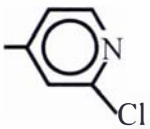
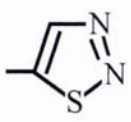
R=	Name; abbreviation
	isopentenyladenine; iP
	*trans*-zeatin; Z or tZ
	*cis*-zeatin; cZ
	dihydrozeatin; DZ
	6-benzylaminopurine; 6-BAP
	6-(*O*-hydroxybenzlamino)-purine, *ortho*-topolin (oT)
	6-(3-hydroxybenylamino)-purine, *meta*-topolin (mT)
	
	*N*-phenyl-*N*′-(2-chloro-4-pyridyl)urea; CPPU
	*N*-phenyl-*N*′-(1,2,3-thidiazol-5-yl)urea, thidiazuron; TDZ

Figure 1.2: Structures of natural adenine and synthetic phenylurea cytokinins (Mok and Mok, 2001).

1.2.2.2 De novo synthesis by plant-associated microorganisms

Up to 2001, little progress was made in determining the mechanism and rate of biosynthesis of free cytokinins, due to the extremely low levels of the endogenous cytokinins in plant tissues and the central role of the most likely precursors in cellular metabolism (Letham and Palni, 1983). Plant pathogens such as *A. tumefaciens*, which causes crown gall disease, contain the *ipt* cytokinin biosynthetic gene on the transferred DNA (T-DNA) of the Ti plasmid. The T-DNA is integrated into the genome of host plant cells after infection (Jameson, 2000). As numerous microorganisms have been shown to secrete cytokinins (Jameson, 2000), Holland (1997) suggested that plants did not synthesise their own cytokinins, but merely metabolised those produced for them by plant-associated microbes.

1.2.2.3 De novo biosynthetic pathway

The rate-limiting step in cytokinin biosynthesis in plants, modelled on the *Agrobacterium ipt* gene, was thought to be the addition of a 5-carbon chain to the N^6 of adenosine 5'-monophosphate (AMP). Up to 2001, despite extensive studies to identify cytokinin biosynthetic genes in plants, none was explicitly identified and characterised. Takei et al. (2001) and Kakimoto (2001) were the first to identify cytokinin biosynthetic isopentenyltransferases in a higher plant at the molecular level. Takei et al. (2001) and Kakimoto (2001) searched the *Arabidopsis thaliana* genome sequences for proteins that could code for isopentenyltransferases and identified nine putative genes for isopentenyltransferases (AtIPT1 and AtIPT3 to AtIPT9). AtIPT2 is a putative tRNA *ipt*. Takei et al. (2001) showed that the purified recombinant AtIPT1 exhibited DMAPP:AMP isopentenyltransferase activity but could use all three nucleotides (AMP, adenosine 5'-diphosphate [ADP] and adenosine 5'-triphosphate [ATP]). Kakimoto (2001) subjected AtIPT4 to detailed analysis and demonstrated cytokinin biosynthesis in plants by over expression of AtIPT4, and that cytokinins are at least in part, synthesised from ATP and ADP in plants, in preference to AMP.

In the de novo biosynthetic pathway in plants, the isopentenyl group is transferred from DMAPP to the N^6 of ATP or ADP, resulting in the production of isopentenyl-adenosine-5'-triphosphate (iPTP)/isopentenyl-adenosine-5'-diphosphate (iPDP). This reaction is thought to be catalysed by DMAPP:ATP/ADP isopentenyltransferases

(Kakimoto, 2001). It is proposed that one of the methyl groups of iPTP and iPDP are hydroxylated to produce *trans*-zeatin-riboside 5' triphosphate (ZPT) and *trans*-zeatin-riboside-5' diphosphate (ZDP), respectively, followed by dephosphorylation.

Recently, the presence of another de novo biosynthetic pathway was demonstrated in *A. thaliana*, although the reactions that constitute this pathway have not been identified yet. In this pathway *trans*-zeatin-riboside-5'-monophosphate (ZMP) is synthesised independently of isopentenyladenosine-5'-monophosphate (iPMP) (Åstot et al., 2000). Kakimoto (2001) propose if one of the methyl groups of iPTP and iPDP were hydroxylated to produce ZPT and ZDP, respectively, followed by dephosphorylation, the ATP/ADP-derived pathway outlined above could be the iPMP-independent pathway reported by Åstot et al. (2000).

1.2.3 Cytokinin metabolism

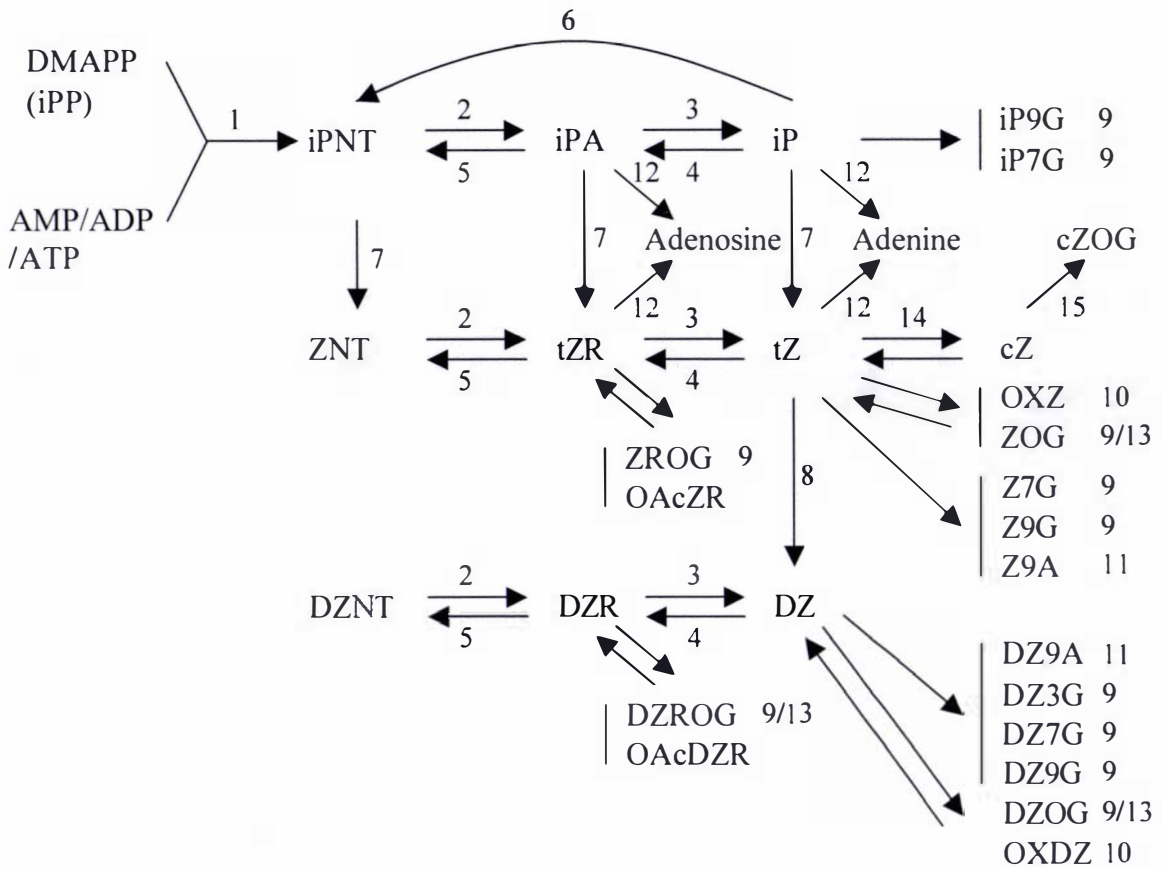
After the hydroxylation of the iPMP side chain, the cytokinins are subjected to a complexity of metabolic transformations in the plant cell with the consequent generation of aglycones, glucosides, ribosides, nucleotides, amino acid conjugates, reduction and oxidation products (Figure 1.3). These metabolic events can be categorised under four broad headings, namely: conjugation, hydrolysis, reduction and oxidation (McGaw and Burch, 1995).

1.2.3.1 Conjugation

Ribosides and nucleotides: The cytokinin ribosides (e.g. *trans*-zeatin riboside [ZR or tZR], dihydrozeatin riboside [DZR] and iPA) and their 5′, mono-, di- and tri-phosphates are probably the most abundant naturally occurring cytokinins (McGaw and Burch, 1995). Ribosides have a ribose sugar attached to the 9 position of the purine ring. Cytokinin ribosides are the predominant forms in cytokinin translocation via xylem from roots to shoots in higher plants (Letham and Palni, 1983). Nucleotides are the first formed cytokinins and may also be the predominant metabolites formed from free cytokinins, when cytokinins are supplied exogenously. Nucleotides are formed when the ribose sugar moiety of the ribose is esterified with phosphoric acid. The nucleotides are subsequently rapidly metabolised to other cytokinin forms (Jameson, 1994; McGaw

and Burch, 1995). Nucleotides are believed to be formed to facilitate uptake across the cellular membranes (Brzobohatý et al., 1994).

Glucosides and xylosides: The naturally occurring cytokinins also undergo N- and *O*-glucosylation. Cytokinin N-glucosides are formed when a glucose sugar molecule is attached to the 3, 7 or 9 position of the purine ring (Brzobohatý et al., 1994; McGaw and Burch, 1995; Mok and Mok, 2001). *O*-glucosylation occurs when the glucose sugar molecule is attached on the N^6-substituted side chain (Brzobohatý et al., 1994). N-glucosyl conjugation is considered to be important in the regulation of the levels of cytokinin. The 7- and 9- glucosides are biologically inactive and are extremely stable in the tissues in which they are formed (Brzobohatý et al., 1994; McGaw and Burch, 1995). They may serve as deactivation or detoxification forms formed when exogenous cytokinin levels are so high as to be toxic. In contrast, the *O*-glucosides appear to be candidates for cytokinin storage forms rather than as a means of inactivating cytokinins. The *O*-glucosides are catalysed to active cytokinins by *β*-glucosidases (McGaw and Burch, 1995; Mok and Mok, 2001). While cytokinin glucosides appear to be ubiquitous cytokinin conjugates, cytokinin-*O*-xylosides seem to be specific for *Phaseolus vulgaris* (Martin et al., 1993). Two enzymes catalysing the formation of the *O*-glycosyl derivatives of Z have been characterised and the genes identified. *O*-glucosyltransferase (Martin et al., 1999b) and *O*-xylosyltransferase (Martin et al., 1999a) occur in seeds of lima bean (*Phaseolus lunatus*) and bean (*Phaseolus vulgaris*) respectively. The ZOG1 gene (zeatin *O*-glucosyltransferase) was isolated first from *P. lunatis* (Martin et al., 1999b). Based on the ZOG1 sequence, the ZOX1 gene (zeatin *O*-xylosyltransferase) was cloned from *P. vulgaris* (Martin et al., 1999a). Zeatin-*O*-xylosyltransferase enzyme is associated with the nucleus as well as the cytoplasm, and may be involved in the nuclear-cytoplasmic transport of cytokinins and related molecules or, possibly, with chromatin of rapidly dividing cells (Martin et al., 1993).



Enzyme		Gene
1.	Isopentenyltransferase	AtIPT1, AtIPT4
2.	5' nucleotidase / non-specific phosphatases	
3.	adenine nucleotidase	
4.	adenine phosphorylase	
5.	adenine kinase	
6.	adenine phosphoribosyltransferase	
7.	microsomal mixed function oxidases	
8.	zeatin reductase	
9.	*O*-glucosyltransferase	*ZOG1*
10.	*O*-xylosyltransferase	*ZOX1*
11.	*β*-(-9-cytokinin alanine)synthase	
12.	cytokinin oxidase	*ckx1, CKO*
13.	*β*-glucosidase	*Zm-p60.1 (glu1)*
14.	zeatin *cis-trans* isomerase	
15.	*cis* zeatin *O*-glucosyltransferase	*cisZOG1*

Figure 1.3: A schematic representation of the biosynthesis and metabolism of cytokinins (adapted from Jameson, 1994). The first steps of the de novo biosynthetic pathway are shown and are catalysed by isopentenyltransferases AtIPT1 and AtIPT4. AtIPT4 uses ATP and ADP in preference to AMP. Where no enzyme number is listed, the conversion is known to occur but no enzyme or gene is isolated.

Amino acids: Conjugation of the cytokinins may also occur with the alanine at the 9 position of the purine nucleus to yield lupinic acid (Z9A) and dihydrolupinic acid (DZ9A) (Jameson, 1994; McGaw and Burch, 1995). The role of these conjugates is probably similar to that of the N-glucosyl cytokinins. They are biologically inactive and extremely stable compounds. As with glucosylation, the formation of amino acid conjugates is a common response of plant tissues to xenobiotic material and presumably, by rendering them more water soluble, facilitates their deposition in the vacuole (McGaw and Burch, 1995).

1.2.3.2 Hydrolysis

Hydrolysis of the cytokinin ribosides and nucleotides is a major form of metabolism, which occurs when cytokinins are externally supplied. The cytokinin ribosides and nucleotides undergo hydrolysis to their corresponding free bases and ribosides. The *O*-glucosides have also been shown to be readily hydrolysed by enzymes such as β-glucosidase, but not the N-glucoside or N-alanyl conjugates, which are extremely stable (McGaw and Burch, 1995).

1.2.3.3 Reduction

The reduction of the cytokinin side chain leads to the production of the dihydroderivatives (e.g. dihydrozeatin [DZ] and DZR). Reduction confers cytokinin stability and resistance to degradation from cytokinin oxidase (Jameson, 1994; McGaw and Burch, 1995).

1.2.3.4 Oxidation

Cytokinins are degraded by cytokinin oxidases which oxidatively cleave the N^6-isoprenoid side chain of cytokinin free bases and ribosides to yield adenine and adenosine, respectively (Houba-Hérin et al., 1999; Mok and Mok, 2001). Side chain cleavage leads to the irreversible loss of cytokinin activity and may be important in the regulation of cytokinin activity (Jameson, 1994; McGaw and Burch, 1995). The isolation of cytokinin oxidase genes by Houba-Hérin et al. (1999) and Morris et al. (1999), represented the first characterisation of a plant gene strictly involved in cytokinin metabolism (Morris et al., 1999). Houba-Hérin et al. (1999) purified and

cloned a maize cytokinin oxidase (CKO) and showed transient expression of the enzyme in moss protoplasts, therefore allowing the functionality of the recombinant enzyme to be demonstrated. Morris et al. (1999) purified to homogeneity the major cytokinin oxidase in immature maize kernels and expressed the gene (*ckx1)* in *Pichia.* The cytokinin oxidase gene caused secretion of active glycosylated cytokinin oxidase in *Pichia.* The cytokinin oxidase enzyme is a flavoprotein containing covalently bound flavin adenine dinucleotide, but no detectable heavy metals (Bilyeu et al., 2001).

1.2.4 *Cis* isomers

Zeatin-type cytokinins naturally occur in either the *cis*- or *trans*-isomeric forms. It has long been thought that only the tZ derivatives function as plant hormones. The *cis*-derivatives were regarded as unimportant, biologically inactive plant constituents (Tay et al., 1986; Suttle and Banowetz, 2000). Occasional reports describing the presence of *cis*-type cytokinins in various plant tissues have appeared over the years. *Cis*-zeatin and its riboside have been reported for a number of plant species, including sweet potato tubers (Hashizumi et al., 1982), potato tubers (Nicander et al., 1995; Suttle and Banowetz, 2000), rice shoots and roots (Takagi et al., 1985; Izumi et al., 1988), hop fruit (Watanabe et al., 1981), male flower buds of *Mercurialis* (Durand and Durand, 1994), chickpea (Emery et al., 1998) and xylem sap of wheat and oats (Parker et al., 1989). It was suggested that in many of these studies, the *cis*-cytokinins identified were artefacts arising from tRNA degradation during tissue disruption and sample preparation (Kaminek, 1982; Tay et al., 1986). Recently, however, reports have appeared describing the isolation of *cis*-cytokinins from various plant sources where an artefactual genesis of these compounds was highly unlikely. Using extraction conditions specifically designed to minimise RNA degradation, *cis*-cytokinins (and particularly *cis* zeatin riboside [cZR]) were found to be the predominant cytokinins in developing chick pea seeds (Emery et al., 1998) and young lupin fruit (Atkins et al., 1998). Therefore, it is clear that specific plant tissues do contain significant amounts of cZ derivatives, although the physiological significance of these Z isomers in plant development remains unknown (Suttle and Banowetz, 2000). The occurrence of cZ as a minor cytokinin constituent could be explained by its origin from tRNA, but its presence as the major component in some plants or plant organs may signify a direct pathway of biosynthesis (Mok and Mok, 2001). The isopentenyl side chain can possibly be hydroxylated to the *cis* configuration or a *cis*-hydroxylated side chain is

directly transferred to the adenine moiety (Mok and Mok, 2001). A *cis* zeatin-specific *O*-glucosyltransferase gene (*cis*ZOG1) was isolated from maize (Martin et al., 2001). The maize enzyme recognises as substrates cZ and UDP-glucose but not cZR, tZ or *trans* zeatin riboside (tZR). This finding indicates the existence of *cis*-specific regulatory elements in plants. A recently identified cytokinin receptor did not respond to cZ, suggesting that the *cis* cytokinin isomers are biologically inactive (Section 1.2.7).

1.2.5 Aromatic cytokinins

The aromatic cytokinins can occur naturally (Mok and Mok, 2001). The structures of the aromatic cytokinins suggest considerably different biosynthetic pathways from that of Z and related isoprenoid cytokinins (Strnad, 1997). Aromatic cytokinin metabolism can be classified under four main headings analogous to isporenoid cytokinins: interconversion, hydroxylation, conjugation, and oxidative degradation (Strnad, 1997). Hydroxylated forms of 6-benzylaminopurine (6-BAP), *meta*-topolin (mT) and *ortho*-topolin (oT), occur naturally with the accompanying nucleosides, nucleotides and *O*-glucosides plus N-glucosides and 9-alanyl conjugates (Strnad, 1997; Mok and Mok, 2001). Two aromatic cytokinins were isolated from *Z. aethiopica* immature fruits and identified as 6-(*o*-hydroxybenzylamino)-purine or oT (Chaves das Neves and Pais, 1980b) and 6-(*o*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine or *ortho*-topolin riboside (oTR) (Chaves das Neves and Pais, 1980a). It was suggested that these cytokinins were involved in inhibition of spathe senescence and regreeening.

1.2.6 Diphenylurea cytokinins

DPU-type cytokinins are synthetic cytokinin-like compounds, some of which are extremely active e.g. thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-5-yl)urea) and CPPU (*N*-phenyl-*N'*-(2-chloro-4-pyridyl)urea) (Mok and Mok, 2001). DPU's are used in tissue culture, as they are active at relatively low concentrations (Karanov et al., 1990). Several modes of action have been postulated for DPU-type cytokinins. These include inhibition of cytokinin oxidase activity (Jones and Schreiber, 1997; Laloue and Fox, 1989), direct interaction with cytokinin receptors (Section 1.2.7) (Haberer and Kieber, 2002) and feedback inhibition on endogenous cytokinin biosynthesis (Lewis et al., 1996a).

1.2.7 Cytokinin perception

The action of cytokinins at the molecular and the whole plant level is still largely unknown (Mok and Mok, 2001). A cytokinin receptor has been recently identified. Inoue et al. (2001) identified *CRE1* (*cytokinin response 1*) as a histidine kinase cytokinin receptor from *Arabidopsis*. Mutations in the *CRE1* gene caused a cytokinin-insensitive phenotype in *Arabidopsis*. The phenotype displayed reduced sensitivity to the cytokinin, kinetin, and the auxin, naphthalene acetic acid (NAA), in tissue culture. The mutant was also less responsive to other cytokinins, including tZ, iPA, 6-BAP and TDZ (Inoue et al., 2001). The expression of *CRE1* conferred a cytokinin responsive phenotype in a mutant yeast, lacking endogenous hybrid kinases (Inoue et al., 2001; Lohrmann and Harter, 2002). The phenotype responded to tZ, iPA, 6-BAP and TDZ. The phenotype did not, however, respond to the less active cytokinin cZ (Inoue et al., 2001; Haberer and Kieber, 2002). In addition to receptor genes such as *CRE1*, proposed cytokinin targets include cell cycle genes and genes affecting shoot meristems formation. As cytokinins stimulate cell division and formation of meristematic tissues, a link between these events and cytokinin action is expected (Mok and Mok, 2001). Mok and Mok (2001) suggested that the study of action/perception will need to address the apparently indistinguishable biological effects of adenine (natural) and phenylurea (synthetic) cytokinins. However, this may now be resolved as TDZ binds to the *CRE1* receptor and initiates the cytokinin signal transduction chain at least in the mutant yeast system.

1.3 Somaclonal variation and bushiness

1.3.1 Somaclonal variation

In plants regenerated from tissue culture, numerous kinds of genetic variation have been observed, such as changes in DNA sequence (point mutations, activation of transposons), changes in chromosome structure (duplications, translocations) and in chromosome number (leading to polyploidy or aneuploidy) (De Klerk, 1990). In addition to genetic variation, epigenetic or non-genetic variation occurs frequently (Meins, 1983; De Klerk, 1990; Smulders et al., 1995). Epigenetic variation, in contrast to genetic variation, is not transmitted during meiosis and is reversible during the life of the plant (Meins, 1983; De Klerk, 1990). Epigenetic variation is observed in plants

originating from both adventitious and from pre-existing apical/axillary meristems. Genetic variation, in contrast, only occurs in plants regenerated from adventitious meristems. Epigenetic variation is predictable, whereas genetic variation is not, i.e. the same conditions will usually result in the same epigenetic variation. Epigenetic variation is a physiological response and is, therefore, in one direction (De Klerk, 1990). Genetic variation is supposed to be entirely random with reference to functional or adaptive value. However, genetic variation will, as a rule, be in the direction of decreased vigour and yield (De Klerk, 1990). Larkin and Scowcroft (1981) define all variation in plants from cell and callus cultures as somaclonal variation.

Somaclonal variation constitutes a fundamental problem in commercial micropropagation (De Klerk, 1990; Arnholdt-Schmitt et al., 1995). There have been reports that somaclonal variation has limited the expansion of the tissue culture technology in banana (Peraza-Echeverria et al., 2001) and oil palm (Besse et al., 1992; Jones et al., 1995; Rival et al., 1997; Jones, 1998; Rival et al., 1998a; Rival et al., 1998b; Jaligot et al., 2000; Rival et al., 2000; Matthes et al., 2001; Eeuwens et al., 2002). Somaclonal variation may arise from pre-existing or induced variation (Skirvin et al., 1994). Somaclonal variation is thought to be affected by biological (genotype, explant type), medium (plant growth regulators) and physical (duration of culture) factors (De Klerk, 1990; Skirvin et al., 1994). Insight into the process(es) leading to this variation is still limited (Smulders et al., 1995). Growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-BAP have been implicated in the induction of somaclonal variation, and it is suggested that the somaclonal variation rate increases as the overall concentrations of the growth regulators rise, but their direct relationship to this phenomenon is still debated (Skirvin et al., 1994). Arnholdt-Schmitt et al. (1995) suggests that cytokinin activity can be effective in genome modification only if the cultured material is dynamically changing with respect to molecular differentiation. Somaclonal variation may be the underlying mechanism responsible for the bushy syndrome.

A well known example of somaclonal variation is the capacity of plant tissue to acquire the ability to grow in culture in the absence of phytohormones (habituation) (Meins and Lutz, 1980; Meins et al., 1980; Meins, 1983; Meins et al., 1983; Jäger et al., 1997). Habituation is a stable heritable loss (Jäger et al., 1997). Jackson and Lyndon (1988) suggest that cells with different states of habituation, and different competences to

habituate, may exist in the intact plant, and that the degree of habituation may change as the cells develop. Many suggestions have been put forward as to why plant cell cultures become habituated including a) increased biosynthesis of the growth substance, or b) decrease in the rate of degradation or c) altered sensitivity of the cells to the growth substance (Jackson and Lyndon, 1990) or d) a possible activation and expression of genes that bypass the requirement of specific growth factors (Bisbis et al., 1998) or e) interaction of some or all of these (Jackson and Lyndon, 1990). Habituation for a growth substance may be induced by low concentrations of the same or a different growth substance. The difference in concentrations required to induce or reverse the process might be more than ten-fold (Jackson and Lyndon, 1990).

1.3.2 Bushiness and comparable syndromes in other micropropagated species

Even a decade ago Capellades Queralt et al. (1991) suggested that for more and more micropropagated ornamental plants, problems of bushy growth were arising. This aberration did not occur directly after tissue culture plants were planted out, but during the successive stage of development to marketable plants. Capellades Queralt et al. (1991) suggested that this problem was likely to be due to an inappropriate cytokinin treatment, caused by doses which were too high, or too many subcultures on a proliferation medium. Comparable or similar syndromes to bushiness in micropropagated plants reported in the literature are detailed in Table 1.1.

Gerbera jamesonii: Bushiness has been reported in micropropagated *Gerbera*. The abnormality was characterised by numerous leaves, a limited numbers of flowers that had short peduncles and were smaller in size, delayed flowering and small laminae (Topoonyanont et al., 1999). Topoonyanont et al., (1999) suggested that high levels of cytokinin in the *in vitro* process induced the abnormality. They also suggested that it was cultivar specific and may have been a temporary change in plant behaviour and morphological characteristics. Further research by Topoonyanont and Debergh (2001) found the abnormality was enhanced by high kinetin concentration and the selection of the main axis as the source of inocula for micropropagation, compared to adventitious or axillary shoots. They also found the addition of the fungicide imazalil to the propagation medium overcame bushiness, but in the case of extreme bushiness, only partial changes occurred.

Table 1.1: Bushiness or comparable syndromes seen in other micropropagated species.

Syndrome	Plant	Reference
Bushiness	*Gerbera jamesonii*	Topoonyanont et al., 1999, Topoonyanont and Debergh 2001
'Mantled' flowering	Oil palm (*Elaeis guineensis* Jacq)	Besse et al., 1992, Jones et al., 1995, Rival et al., 1997, Jones 1998, Rival et al., 1998a and 1998b, Jaligot et al., 2000, Rival et al., 2000, Matthes et al., 2001 Eeuwens et al., 2002
Multi-apexing	Strawberry (*Fragaria ananassa*)	Anderson et al., 1982
Tissue-proliferation	Rhododendron 'Montego'	Mercure et al., 1998

Elaeis guineensis Jacq (oil palm): The "mantled" syndrome has been reported in 5% of oil palms derived from somatic embryos. The "mantled" phenotype describes an apparent feminisation of male parts in flowers of both sexes. This variation may result in partial or complete flower sterility, which directly affects oil production, depending on the severity of the abnormality (Rival et al., 2000). "Mantled" oil palm varies in its occurrence and intensity between different clonal lines, between palms of the same clonal line and between different flowers of the same individual variant palm. Reversions to the normal phenotype have been found to occur, leading to a complete recovery of the normal phenotype for 100% of the slightly "mantled" individuals, and for 50% of the severely "mantled" ones after nine years in the field (Rival et al., 1998b; Jaligot et al., 2000; Rival et al., 2000).

There was a widespread assumption that the abnormal flowering was induced by some component of the tissue culture process and this was in some way associated with disturbed cytokinin metabolism (Jones, 1998). There were two different callus types described during the micropropagation of oil palm: soft and friable callus named Fast Growing Callus (FGC) and Nodular Compact Callus (NCC). The NCC regenerated into plants with fewer than 5 % abnormal inflorescences, while 100 % of the plants regenerated from FGC had abnormal inflorescences (Besse et al., 1992). There were no significant amounts of cytokinins detected in FGC, whereas they accumulated in NCC. Besse et al. (1992) suggested that there appeared to be a close relationship between the ability of FGC to regenerate plants with abnormal inflorescences and low levels of cytokinins. These results led Besse et al. (1992) to hypothesise that the malformation of oil palm inflorescences was a physiological disorder associated with tissue disorganisation and abnormally low endogenous cytokinin levels. Jones et al. (1995) analysed cytokinin content in tissue cultures and regenerant plants of cell lines producing both normal and abnormal flowers. They compared these to zygotic embryos, seedlings and immature inflorescences. In all cases, except one, between-clone differences were greater than any normal/abnormal differences. Jones (1998) studied cytokinin metabolism in abnormal and normal lines using radiolabelled 6-BAP and isopentenyladenine (iP). Jones (1998) found cytokinin uptake was faster in normal clones compared with abnormal oil palm clones during the 6-BAP and iP feeding experiments. He hypothesised that there may be a difference in cell permeability to, or transport of, cytokinins.

Eeuwens et al. (2002) investigated culture conditions of embryoid multiplication and found that, in general, reducing the transfer interval to two or four weeks from eight weeks, and/or using high concentrations of the cytokinin kinetin (0.25 mg L^{-1}) and low concentrations of the auxin NAA (0 or 0.1 mg L^{-1}) resulted in a high incidence of mantled flowering.

Flow cytometric analysis performed on the different callus types did not reveal any variation in their nuclear DNA content, thus eliminating the possibility of an alteration of the ploidy level as the source of somaclonal variation (Rival et al., 1997; Rival et al., 1998b). Random amplified polymorphic DNA (RAPD) analysis failed to show any significant banding pattern changes between the mother palm and its clonal offspring, or between true to type and variant phenotypes (Rival et al., 1998a; Rival et al., 1998b). On the basis of observed characteristic features of the "mantled" somaclonal variants (spatial heterogeneity, temporal instability, absence of any detectable defect in DNA organisation), an epigenetic alteration of genome expression was then proposed as a hypothesis to explain the origin of the variant plants (Jaligot et al., 2000).

Jaligot et al. (2000) demonstrated a correlation between DNA hypomethylation (Section 1.3.3) and the "mantled" somaclonal variation in oil palm in both calli and leaves using two techniques: high performance liquid chromatography (HPLC) quantification of relative amounts of 5-methyl-deoxycytidine (5mdC) and an *Sss*I-methylase accepting assay, based on the enzymatic saturation of CG sites with labelled methyl groups. The trend towards hypomethylation detected by HPLC, was subtler in leaves than in calli. Jaligot et al. (2000) found that in leaves of regenerated palms the global DNA methylation rates, determined by HPLC, were variable in intensity from one genotype to another. The methylation rate data did not always allow discrimination between normal regenerants of one clonal group and abnormal regenerants of another. DNA methylation levels were found to be lower in seed-derived palms from various different origins than in abnormal regenerants taken as a whole.

Despite the importance of the decrease in methylation rate, it is likely that very few of the corresponding cytosines have a relevant biological role in the onset or in the maintenance of the studied somaclonal variation (Rival et al., 2000). Rival et al. (2000) then aimed to target more precisely the sequences with decreased methylation which could account for the "mantled" phenotype. This was done using methylation-sensitive

RFLP and amplified fragment length polymorphism (AFLP) studies involving isoschizomeric restriction enzyme pairs showing differential sensitivity to the methylation of deoxycytidine residues (e.g. *Msp* I [from *Moraxella* species] and *Hpa* II [from *Haemophilus. parainfluenzae*]). Rival et al. (2000) found changes in DNA methylation, at CCGG sites, using methylation sensitive AFLP. The changes correlated to the "mantled" abnormality were detectable amongst a limited number of samples and within a very restrained portion of methylatable sites.

Fragaria ananassa (Strawberry): Anderson et al. (1982) reported a multi-apexing abnormality in strawberry. The abnormal strawberry plants had an excessive number of small-branched crowns. When plants of this type were split and the individual crowns planted separately, each grew into a single crown. On plants not split, one crown in the cluster eventually became dominant. Leaves on multi-apexed plants had shorter more slender petioles and smaller laminae than those on normal plants, giving the multi-apexed plants a stunted, bushy appearance. Anderson et al. (1982) observed large differences in susceptibility of cultivars to multi-apexing. They found that, in the presence of indole-3-butyric acid (IBA), higher 6-BAP concentrations increased the frequency of abnormal plants. Multi-apexing appeared to be related to the duration of culture in media containing higher concentrations of 6-BAP. They also found that the addition of gibberellin decreased the incidence of affected plants.

Rhododendron 'Montego': Tissue-proliferation was reported in *Rhododendron* by Mercure et al. (1998). This tissue culture syndrome was described as crown tumours that produced shoots that were smaller than normal, with short internodes and a whorled arrangement of small leaves. The shoots proliferated rapidly without the presence of cytokinin in the tissue culture medium (Mercure et al., 1998). Tissue-proliferation was seen during tissue culture but sometimes it was not seen for up to one to three years after initial propagation from tissue culture material.

1.3.3 DNA methylation in plants

Variation in DNA methylation has been proposed as a mechanism that may explain the wide range of changes that can occur after tissue culture propagation, although other mechanisms cannot be excluded (Kaeppler and Phillips, 1993a; Smulders et al., 1995). Alterations in methylation pattern can occur in cultured plant cells, with a general

tendency towards demethylation (hypomethylation) of cytosine residues (Finnegan et al., 1993). Altered patterns of DNA methylation may result from imbalances between the activity of the enzymes involved in maintenance methylation and DNA replication and cell division, or could be related to other events that occur in cultured cells, such as chromosome breakage and repair (Peschke et al., 1991; Finnegan et al., 1993). The hormone concentration of tissue culture can also affect the level of DNA methylation in cultured cells (Finnegan et al., 1993). These alterations in methylation pattern could affect the expression of specific genes, including transposable elements, and/or could affect chromatin structure on a more global level. Changes in DNA methylation could be related to the late replication of heterochromatin, which in turn results in chromosome breakage events. In addition, deamination of 5-methyl cytosine is known to ultimately result in a base change from a cytosine to a thymine. This could be another mechanism of genetic change (Kaeppler and Phillips, 1993a; Kaeppler et al., 2000).

DNA methylation is a reversible modification of the DNA of chromosomes in plant cells, in which a methyl group is added to either the cyclic carbon in C5-methylcytosine, or to non-cyclic nitrogen residues of adenine (N6-adenine) or cytosine (N4-cytosine) (Finnegan and Kovac, 2000). Cytosine-5-methylation is the most common DNA modification in eukaryotes (Finnegan and Kovac, 2000). In plants up to 32% of cytosine residues are modified (Brown, 1989). The methyl groups are added by specific enzymes, the DNA methylases or DNA methyltransferases, after cytosine residues are incorporated into DNA chains (Doerfler, 1983; Watson et al., 1987). There are two types of DNA methylases whose actions are distinguished by the state of the methylated DNA. To modify DNA at a new position requires the action of de novo methylase, which presumably recognises DNA by virtue of a specific sequence (Lewin, 2000). To modify hemimethylated, or newly replicated, DNA with methyl groups only at specific sites on the parental strand, requires the action of maintenance methylase. Maintenance methylation of the new strand restores the original pattern of methylation (Finnegan et al., 1993; Kaeppler and Phillips, 1993a; Lewin, 2000). The methyl groups are removed by demethylase enzymes (Lewin, 2000). Plant genes encoding plant DNA methyltransferases have been isolated by similarity to their mammalian counterparts (Finnegan and Dennis, 1993). Plants have at least three families of methyltransferases which differ in protein structure and function (Finnegan and Kovac, 2000; Finnegan et al., 2000).

The addition of methyl groups to cytosine residues can occur at any cytosine residue but predominantly occurs in cytosines in the strand-symmetrical sequences CpG and CpNpG especially CpApG and CpTpG (Kaeppler and Phillips, 1993a; Finnegan et al., 1998). Methylcytosine is not randomly distributed throughout the nuclear genome but is concentrated in repeated sequences (Finnegan et al., 1998). In plants, DNA methylation is mainly restricted to the nuclear genome, suggesting that the smaller chloroplast and mitochondrial genomes do not require this additional level of gene control (Finnegan et al., 1998).

There is increasing evidence that DNA methylation directly inhibits gene expression in eukaryotes (Holliday, 1987; Kaeppler and Phillips, 1993b; Xiong et al., 1999). Methylation generally represses transcription either directly, by blocking the binding of transcription factors, or indirectly, as shown in mammalian systems, through proteins that bind methylated DNA resulting in deacetylation of nearby histones and decreased transcription (Cedar, 1988; Finnegan et al., 2000).

DNA methylation mutants provide experimental systems to directly address the role of cytosine methylation in development of plants (Vongs et al., 1993; Kakutani, 1997). To date, two kinds of DNA hypomethylation mutants in *A. thaliana* have been isolated; *ddm1* (*decrease in DNA methylation*) mutants (Vongs et al., 1993; Kakutani, 1997; Kakutani et al., 1999), and transgenic plants expressing the DNA methyltransferase gene (MET1) in antisense orientation (Finnegan and Dennis, 1993; Finnegan et al., 1996; Ronemus et al., 1996). Some of these *A. thaliana* mutants show a variety of developmental abnormalities, strongly suggesting the DNA methylation is important for various processes of plant development (Finnegan et al., 1996; Ronemus et al., 1996; Kakutani, 1997; Kakutani et al., 1999). Some plants that have substantially reduced levels of DNA methylation, display a number of phenotypic abnormalities (Finnegan et al., 1998). These include loss of apical dominance, reduced stature, altered leaf size and shape, reduced root length, homeotic transformation of floral organs, and reduced fertility (Finnegan et al., 1996; Ronemus et al., 1996; Kakutani, 1997; Finnegan et al., 1998). Phenotypes were variable between plants of the same family and plants with the lowest methylation levels were most severely affected; the abnormal phenotype became more severe in successive generations of progeny from self-pollinated plants (Finnegan et al., 1996).

1.3.3.1 DNA methylation and tissue culture

Variation in DNA methylation patterns in tissue culture regenerants has been described in rice (Xiong et al., 1999), maize (Kaeppler and Phillips, 1993b), potato (Harding, 1994), tomato (Smulders et al., 1995), carrot (Arnholdt-Schmitt et al., 1991; Arnholdt-Schmitt et al., 1995), oil palm (Rival et al., 2000; Matthes et al., 2001) and banana (Peraza-Echeverria et al., 2001). In most studies, however, the identity of the affected DNA is unknown, making a direct correlation between methylation and specific phenotypic changes difficult (Matthes et al., 2001). It has been considered that demethylation drugs could be helpful in stimulating differential gene activity in tissue cultures (Holliday, 1987; Arnholdt-Schmitt et al., 1991). Methylation can be reduced by treatment with 5-azacytidine and 5-azadeoxycytidine. The drug 5-azacytidine is incorporated into DNA during replication or repair and cannot be methylated, because the 5' position is blocked (Holliday, 1987; Lewin, 2000).

Since the process of regeneration during tissue culture bypasses the normal fertilisation and development events of seed grown plants it is possible that the normal epigenetic program is not established in regenerated plants (Kaeppler et al., 2000). Plants may not undergo the cycle of general demethylation followed by remethylation, observed in the early stages of mammalian embryo development (Finnegan et al., 1996). Developmentally programmed plant epigenetic patterns and resetting of those patterns, therefore, may be quite important in somaclonal variation. Those patterns may be variable within the explant source, and resetting of patterns outside the normal process of meiosis and fertilisation may be imprecise (Kaeppler et al., 2000). The details of the resetting mechanisms in plants remains to be determined (Finnegan et al., 2000).

Smulder et al. (1995) suggested that longer periods of tissue culture induced more variation in DNA methylation among regenerant tomato shoots. Brown (1989) found that although plants may be phenotypically normal after tissue culture, this did not preclude the possibility that, at least in terms of gene methylation, such plants may be disturbed and exhibit a range of methylation changes deviant from controls. The converse is also true, that even though plants may be phenotypically abnormal, methylation status for a particular gene may be identical with controls.

1.3.3.2 Methods for studying DNA methylation

In parallel with the interest in the functional role of DNA methylation, there has been a series of developments in the methods used for detecting DNA methylation (Xiong et al., 1999). The methods include the HPLC estimation of global DNA methylation rates as used in oil palm (Jaligot et al., 2000), and the *SssI*-methylase accepting method, based on the enzymatic saturation of CG sites with labelled methyl groups, also used in oil palm (Jaligot et al., 2000). A third method is described as the bisulphite treatment method, in which unmethylated cytosine residues are converted to uracil, whereas, methylated cytosine residues remain unchanged. The DNA is then amplified by polymerase chain reaction (PCR) and sequenced. Uracil is replaced by thymine during PCR amplification. The remaining cytosine residues in the sequence obtained correspond to a methylated cytosine in the starting DNA (Frommer et al., 1992; Finnegan et al., 1993). Another method is the modification of the AFLP technique, a method originally developed by Vos et al. (1995). In this technique, DNA is digested with the restriction enzyme *Eco* RI, and one of the isoschizomers *Hpa* II and *Msp* I, whose ability to cleave at the sequence CCGG is affected by the methylation state (Reyna-López et al., 1997; Xiong et al., 1999; Peraza-Echeverria et al., 2001). The fragments are ligated to appropriate adapters and amplified with primers that anneal to the complementary sequences in the adapters. After PCR the DNA is separated by polyacrylamide gel electrophoresis. Comparison of patterns reveal differences indicative of DNA fragments whose methylation state did or did not change (Reyna-López et al., 1997; Xiong et al., 1999; Peraza-Echeverria et al., 2001).

Peraza-Echeverria et al. (2001) demonstrated the usefulness of the modified AFLP technique for detecting DNA methylation differences in banana and indicated that DNA methylation differences were associated with micropropagation. Rival et al. (2000) and Matthes et al. (2001) investigated differences in DNA methylation levels between normal and mantled micropropagated oil palm, using the modified AFLP technique. Matthes et al. (2001) suggested that the method was an effective way of detecting variation in tissue culture-derived plants. The modified AFLP technique was used in this research to examine differences in the DNA methylation profile between bushy and non-bushy Florex Gold.

1.3.4 Differential gene expression in plants

A significant limitation of investigating differences in DNA methylation patterns between bushy and non-bushy plants using genomic DNA AFLP, is that differences in methylation profile are often in highly repetitive non-coding regions of the DNA (Matthes et al., 2001). Higher organisms contain about 100,000 different genes, of which only a small fraction, perhaps 15%, are expressed in any individual cell (Liang and Pardee, 1992). As detailed in Section 1.3.3, cytosine methylation has been shown to have a high negative correlation with gene expression (Holliday, 1987). Direct comparison of gene expression levels between bushy and non-bushy plants, however, may give some insight into the underlying biological process(es) (Liang and Pardee, 1992). Differences in gene expression are examined by comparing messenger RNA populations. Differences in gene expression need to be examined during identical stages of development to ensure the changes seen are physiologically relevant. A number of methods exist for comparing messenger RNA populations, and are discussed below.

1.3.4.1 Methods for studying differential gene expression

The methods for studying differential gene expression include the first *in vitro* technique for the determination of transcript patterns. This was differential display reverse transcription PCR (DDRT-PCR) developed by Liang and Pardee (1992). DDRT-PCR employs a reverse transcriptase (RT) reaction on mRNA primed by a set of oligonucleotide primers anchored to the polyadenylate tail of a subset of mRNAs. The resulting cDNA is then amplified with the same anchored oligo(dT) primer and a short arbitrary primer that anneals at different positions relative to the first primer. Labelled dATP is introduced in the reaction and the labelled products are separated on a DNA sequencing gel and visualised by autoradiography (Liang and Pardee, 1992). The DDRT-PCR technique permits recovery and cloning of the corresponding cDNAs (Liang and Pardee, 1992). In DDRT-PCR, there is no accurate relationship between signal strength and the initial concentration of the corresponding mRNA, which precludes quantitative measurement of transcript concentration (Kuhn, 2001).

Other techniques that compare mRNA populations and differential gene expression include cDNA AFLP (Bachem et al., 1996), high density membrane filters (Desprez et

al., 1998; Kuhn, 2001) and cDNA microarrays (Schena et al., 1995). The cDNA AFLP technique uses the standard AFLP protocol on a cDNA template. In cDNA AFLP, double-stranded cDNA is digested with two restriction enzymes, or an anchored oligo(dT) primer and a restriction enzyme and adapters of arbitrary sequence are ligated to the fragments. Subsequently, the fragments are amplified by PCR using the adapter sequence plus one or more selective bases at the 3' end, and the results are displayed on DNA sequencing gels (Bachem et al., 1996).

The cDNA membrane array (macroarray) technique uses nylon membranes on which double-stranded DNA fragments are deposited at indexed locations with high speed arraying machines. The basic principle of acquiring gene expression data using membrane arrays consists of hybridisation of an unknown sample to the ordered set of immobilised molecules on the array surface. This produces a specific hybridisation pattern that can be read and compared to the pattern produced by a different sample to a given standard (Desprez et al., 1998; Kuhn, 2001).

The array elements of cDNA microarrays are single-stranded DNAs. These arrays are produced by robotically depositing denatured target DNAs, e.g. PCR-amplified inserts from cDNA, at defined locations onto microscope slides. The probes from two independent samples are prepared and labelled with fluorescent nucleotides. Both probes are mixed together and allowed to hybridise to the same microarray under a coverslip. After washing off the unbound probe, the fluorescent DNA molecules that have hybridised to DNA fragments on the microarray are excited by light and the fluorescence signal associated with each element of the microarray is read by an array scanner and compared to the other sample (Schena et al., 1995; Kuhn, 2001).

Microarrays and macroarrays can only be used to analyse the expression of cloned genes (Durrant et al., 2000). Microarray and macroarray facilities were not available during this thesis; therefore the cDNA AFLP technique was chosen to investigate differences in gene expression between bushy and non-bushy plants. The cDNA AFLP technique was also an extension of the already used genomic DNA AFLP technique. The cDNA AFLP method has previously yielded important differentially expressed genes from eukaryotes (Dellagi et al., 2000).

1.4 Aims of project

The objective of this TIF funded project was to develop a broad understanding of the variables associated with the bushy syndrome in *Zantedeschia* Florex Gold and to make recommendations to the current commercial production protocol for Florex Gold, in order to minimise bushiness and its associated symptoms of poor cut flower production. More specifically, the aims of the project were to examine the variables anecdotally associated with the bushy syndrome.

A key aim of the project was to determine if particular environmental conditions affected the bushy syndrome by assessing light and temperature growing environments, growing position within the greenhouse, tuber storage length and temperature.

In order to address the anecdotal suggestions that cytokinins play a role in the expression of the bushy syndrome, a further aim of the project was to determine the endogenous cytokinin concentration and profile in tubers of different degrees of bushiness, at three stages in the *Zantedeschia* life cycle

Various aspects of the micropropagation protocol had been proposed as the cause of bushiness. Another aim of the project was to determine the effect of the micropropagation environment on the development of the bushy phenotype by examining explant age, the number of tuber explant eyes, the effect of different concentrations of the cytokinins 6-BAP and TDZ, and the sensitivity of different cultivar explants to cytokinins.

An additional aim of the project was to determine if bushiness was due to somaclonal variation by investigating DNA methylation profiles and levels of gene expression in bushy and non-bushy plants in order to investigate whether there was a molecular basis to the bushy syndrome.

Chapter Two: The effect of genotype and environment on the development and/or expression of the bushy syndrome in *Zantedeschia*

2.1 Introduction

The experiments outlined in this chapter were designed to obtain a broad understanding of the characteristics associated with the bushy phenotype in *Zantedeschia* Florex Gold. Bushy plants are described by the industry as short plants with multiple shoots, few or no flowers, and squat tubers with multiple nodes and multiple eyes (buds). Experiments were designed to test several aspects of commercial production that were considered might influence the expression of the bushy syndrome in *Zantedeschia*.

Anecdotal evidence led to the formulation of the hypotheses that either the Florex Gold genotype or the parentage of Florex Gold were factors causing or contributing to the bushy syndrome. These two hypotheses were investigated as extreme cases of bushiness were mostly seen in the cultivar Florex Gold. In addition, the probable parent of Florex Gold, the yellow species *Z. elliottiana*, is also the probable parent of the two other cultivars expressing the bushy syndrome. The exact parentage of Florex Gold, Hot Shot and Mango (the other two cultivars expressing the bushy syndrome) is unknown.

The bushy syndrome has only been reported since the mid 1990s', and the problem may, therefore, have been caused by the selection of particular tubers for micropropagation. Two hypotheses were proposed: firstly, that the bushy syndrome is a direct response to the use of tubers for micropropagation that were themselves recently from tissue culture (T2 tubers), and, secondly, that if tubers with too many eyes or buds were used to initiate tissue cultures, then plants with a large number of weak non-flowering shoots might arise.

Cytokinins were implicated in the bushy syndrome as they have previously been linked to the induction and expression of tissue culture syndromes comparable to bushiness in mantled oil palm (Besse et al., 1992; Eeuwens et al., 2002), bushy *Gerbera* (Topoonyanont et al., 1999; Topoonyanont and Debergh, 2001), and multi-apexing strawberry (Anderson et al., 1982). This research, therefore, investigated the hypothesis

that elevated cytokinin during micropropagation, led to cytokinin carry-over and bushiness in progeny.

Differences in *Zantedeschia* growing and tuber storage conditions were also suggested as likely causes of the bushy syndrome, as the growing and storage environments may vary considerably between growers and countries and may have influenced plant/tuber performance and therefore bushiness. Experiments were designed to test factors such as the effect of reduced light and lower temperature on the development of bushy characteristics. Additional factors included lower storage temperature and shorter storage times, forcing tubers to grow during their natural rest period, and growing plants at the edge of the tray and edge of the bench.

Data from some of the experiments described in this chapter has been published D'Arth et al. (2002).

2.2 Materials and Methods

2.2.1 Tuber selections for Experiments One, Two, Three and Four

The tubers investigated in Experiments One to Four came from two cultivars, Best Gold and Florex Gold.

Best Gold selections: The Best Gold T2 tubers were raised from seed and grown at Pukekaroro Exotics, Northland, New Zealand. The tubers were lifted on the 17 March 1999. The selections were:

few-eyed	three tubers all with few eyes.
multi-eyed	one tuber with multiple eyes.

Florex Gold selections:

control Florex Gold	one tuber, originally derived from a tissue culture plant, but for the past 12 years it had been grown in the home garden of *Zantedeschia* breeder, D. Harrison in Palmerston North, New Zealand.
bushy	six T2 tubers selected from tubers that displayed bushy symptoms. Although no selection was made of specific bushy plants while the tubers were in the ground, the cured tubers were later selected for their squat/flat, knobbly appearance, with many eyes.
few-eyed	four tubers with few eyes.
multi-eyed	three tubers with multiple eyes.
	T. Reed, a commercial *Zantedeschia* grower in Auckland, New Zealand, grew both the few-eyed and multi-eyed Florex Gold selections. The tubers were lifted on the 20 January 1999.
Dutch	six tubers. This selection was supplied by J. Kapityen (The Netherlands) and was selected from a group of tubers from plants that displayed bushy symptoms during the Dutch growing season. The plants were originally propagated by tissue culture and grown to the T1 tuber stage in New Zealand. They were then exported to The

Netherlands in 1998 by A. Wearmouth (Pukekaroro Exotics, Northland). The bushiest tuber/plant was Dutch tuber F.

Tuber selections for detailed experimental analysis: The few and multi-eyed Florex Gold tuber selections, and the Dutch Florex Gold tuber selection were used in Experiment One and concurrently in Experiments Two, Three and Four.

2.2.2 Tissue culture

Tuber eyes (>4 mm) were excised from mother tubers together with a wedge of tissue (3 to 5 mm) under the eye. These were soaked in 1 g L^{-1} of the fungicide Thiram (Nufarm NZ Limited, Manurewa, New Zealand) for 4 to 5 h then surface-sterilised for 20 min in a 0.3% sodium hypochlorite solution, prior to initiating into culture. This sterilising procedure was repeated on d 2 and 3. Culture vessels comprised 98 mm diameter, clear plastic tissue culture tubs with snap on lids (Vertex Pacific Ltd, New Zealand). Cultures were maintained at 22 ± 2°C with a 16 h photoperiod provided by cool, white fluorescent tubes (Philips, Australia) with a photon flux density of 7 μmol m^{-2} s^{-1}.

For Experiments One to Four tissue culture plants were micropropagated on one of two media series, the standard media series (Experiment One) or the alternative media series (Experiment Two) (Table 2.1).

Experiment One. Standard media series: Tissue culture material from all selections was cultured on a series of standard tissue culture media, based on a modification of Cohen (1981). Cohen used MS media (Murashige and Skoog, 1962), supplemented with the cytokinin 6-BAP. Each tuber was cultured separately through the tissue culture media series, with each step on media supplemented with a decreasing concentration of 6-BAP. The details are the property of Multiflora Laboratories Limited and are covered by intellectual property rights. Tissue was sub-cultured onto fresh medium every three to four weeks.

Table 2.1: Experimental design outlining the treatments of the different Best Gold and Florex Gold selections during the first two growing seasons of the *Zantedeschia* life cycle. Tissue culture plants were initiated from tubers, the number of tubers initiated per selection is indicated in brackets. Ticks indicate the procedures applied to each selection.

			Selection						
			Best Gold		Florex Gold				
Procedure	Stage in life cycle	Experiment number	Few-eyed (3)	Multi-eyed (1)	Control (1)	Bushy (6)	Few-eyed (4)	Multi-eyed (3)	Dutch (6)
Standard tissue culture MS media	Tissue culture	One	✓	✓	✓	✓	✓	✓	✓
Elevated cytokinin MS media		Two					✓	✓	✓
Standard growing conditions	First growing season	One	✓	✓	✓	✓	✓	✓	✓
Environmental factorial experiment		Three					✓	✓	✓
Standard storage conditions	Tuber storage	One	✓	✓	✓	✓	✓	✓	✓
Storage factorial experiment		Four					✓	✓	✓
Standard growing conditions	Second growing season	One	✓	✓	✓	✓	✓	✓	✓
Environmental factorial experiment		Three					✓	✓	✓

Experiment Two. Alternative micropropagation media series: Few and multi-eyed and Dutch Florex Gold Selections were concurrently cultured on six alternative media series. Shoots were proliferated on tissue culture media supplemented with three elevated levels of 6-BAP (13.3 μM, 26.6 μM and 44.4 μM) and three levels of TDZ (4.5 μM, 9.1 μM and 13.6 μM) for four weeks. Each tuber was cultured separately through the tissue culture media series. After four weeks on the media supplemented with elevated cytokinin, tissue was sub-cultured onto fresh medium every three to four weeks, onto media containing an equivalent concentration of 6-BAP as used in the standard media series.

Tissue culture plantlets were produced for exflasking at the end of four subcultures. The plantlets were rinsed in water to remove any medium, and wrapped in wet paper towels to prevent the plantlets drying out before planting.

2.2.3 First growing season

2.2.3.1 Experimental design

Each treatment was made up of 200 plants from tissue culture, planted in four trays, each containing 50 plants (5 x 10 grid pattern). Plantlets from several tubers were needed to provide the 200 plants per treatment. However, each tray contained the same number of plants from a particular tuber. If a treatment was made up of three tubers, 17, 17 and 16 plants respectively were planted into each tray. If a treatment was made up of four tubers, 13, 13, 12 and 12 plants respectively were planted into each tray. If a treatment was made up of six tubers, 9, 9, 8, 8, 8 and 8 plants respectively were planted into each tray. The number of plants from each tuber was allocated randomly. Plantlets derived from each tuber were kept separate during tissue culture and planting. Trays were blocked into four random blocks within two greenhouses. Trays within each block were randomised.

2.2.3.2 Growing environment

Tissue culture plants were grown in either the standard growing environment (Experiment One) or concurrently in those environments outlined in the environmental factorial experiment (Experiment Three).

Experiment One. Standard growing environment: Zantedeschia plantlets produced by tissue culture were planted out in 58 x 40 x 18 cm polystyrene trays (Barnes Plastics, New Zealand) in pumice:peat (50:50 [v/v]) potting mix (Appendix A). Tissue culture plantlets were covered with Evolution cloth (Vege-Gro Supplies, Auckland, New Zealand) for the first 6 to 11 d, during the hardening off period from culture to plastic greenhouse conditions. Plants were hand watered as required every 1 to 2 d for 4 weeks. The plants were then watered using a T-Tape (T-systems Int. Inc, California, USA) drip irrigation system with three rows of drip tape, across the width of the tray. Plants were watered daily for 15 min from November to December 1999, 18 min from January to March 2000 and 6 min from mid-March 2000, at a rate of 250 mL per tray, per min.

Plants were grown in two identically controlled greenhouses. The greenhouses were screened internally with 50% shading (Aluminet, Polysack-Plastic Industries, Israel). Light levels within the greenhouse averaged 750 μmol m^{-2} s^{-1} at midday, in December and January, on a clear day. A minimum air temperature of 15°C was maintained in the houses. Cooling was set to start at 21°C using vents and either one or two fans. An evaporative cooling system operated if the air temperature reached 27°C.

Experiment Three. Environmental factorial experiment: Few, multi-eyed and Dutch Florex Gold Selections were also grown at two light levels: 750 μmol m^{-2} s^{-1} (50% shading) and 300 μmol m^{-2} s^{-1} (80% shading), and two temperature environments: a heated greenhouse as previously described, and an unheated screen house, in a 2 x 2 factorial (Figure 2.1). The plants in the screen house were watered as outlined in Experiment One above. Overhead plastic in the screen house, prevented rain from watering the plants (Figure 2.1).

2.2.4 Harvesting T1 plants and tuber storage

2.2.4.1 Harvesting

Water was turned off after 17 weeks (26th April, 2000), the foliage dried down and was subsequently removed. The tubers were tipped out from the trays and removed by hand. T1 tubers were weighed and scored for the number of nodes as seen underneath the

A

B

C

D

Figure 2.1: Environmental factorial experiment: Plants derived from tissue culture grown in heated greenhouse and 750 μmol m^{-2} s^{-1} (A), heated greenhouse and 300 μmol m^{-2} s^{-1} (B), unheated screen house and 750 μmol m^{-2} s^{-1} (C), and unheated screen house and 300 μmol m^{-2} s^{-1} (D). Photos are of seven week old tissue culture plantlets.

tuber and the number of tuber eyes (physically swollen axillary buds). Tubers from each tray were bagged into groups containing tubers from each individual mother tuber and then bagged into one bag for each tray. The tubers in the net bags were stored in plastic mesh crates.

2.2.4.2 Tuber storage

Experiment One. Standard tuber storage conditions: Tubers were stored at 9 ± 1°C, in the dark, for 11 weeks.

Experiment Four. Tuber storage factorial experiment: Tubers were stored at two temperatures, 9 ± 1°C (standard), or 15 ± 1°C and for two time periods, 11 (standard) or 21 weeks, in a 2 x 2 factorial arrangement. All tubers were stored in the dark.

2.2.5 Second growing season

2.2.5.1 Experimental design

T1 tubers were replanted in the same tray as the previous growing season. Tubers from each individual mother tuber were randomly selected from the appropriate bag. Not all the tubers were grown in the second growing season. Each tuber was weighed and scored for the number of tuber nodes as seen from underneath the tuber and the number of eyes. The tubers were placed in a plastic mesh tray in order of planting and dipped for 1 min in GA_3 (Grocel GA, Nufarm NZ Limited, Manurewa, New Zealand [100 mg L^{-1}]). The tubers were surface dried for 30 to 60 min before planting. Tubers from the alternative storage conditions of 9°C and 15°C stored for 21 weeks were dipped in GA_3 (100 mg L^{-1}), Benlate (0.5 g L^{-1}), Kocide (1.5 g L^{-1}) and Thiram (1.5 g L^{-1}).

Each treatment comprised 72 tubers, planted in four trays, each containing 18 tubers (3 x 6 grid pattern). Depending on the number of tubers derived from an original mother tuber, tubers from several original mother tubers were needed to provide the 72 tubers per treatment. However, each tray contained the same number of tubers from the original mother tubers. If a treatment was made up of three original mother tubers, 6 tubers of each were planted per tray. If a treatment was made up of four mother tubers, 5, 5, 4 and 4 tubers of each were planted in each tray. If a treatment was made up of six

mother tubers, 3 tubers of each were planted in each tray. The number of plants from each tuber was allocated randomly at the beginning of the experiment and remained consistent in each tray. Trays were blocked into four random blocks within one greenhouse. Trays within each block were randomised.

2.2.5.2 Growing environment

Tubers were planted out in 58 x 40 x 18 cm polystyrene trays in pumice:peat potting mix (Appendix A). Trays were covered with 2 cm of sawdust. A T-Tape drip irrigation system was used, with two rows of drip tape per tray across the length of the tray. Plants were watered once a day for 15 minutes from August to October 2000, and 7 minutes from November 2000 to March 2001, at a rate of 250 mL per tray per min.

Experiment One. Standard growing environment: Plants were grown in one controlled greenhouse (identical to the one used in the first growing season – Section 2.2.3.2). Light levels within the greenhouse averaged 750 μmol $m^{-2}s^{-1}$ at midday, on a fine day, in December and January.

Experiment Three. Environmental factorial experiment: Few and multi-eyed and Dutch Florex Gold selections were returned to the same growing environment as for the first growing season (Section 2.2.3.2).

2.2.6 Harvesting T2 plants

Irrigation was discontinued after 33 weeks (1st April, 2001) to induce foliar senescence and the foliage was subsequently removed. The T2 tubers were harvested as described in Section 2.2.4.1.

2.2.7 Stock plants

2.2.7.1 Stock plant: Year one

Mother tubers that were the source of all *in vitro* initiated lines were planted and grown to determine plant morphology. The tubers were planted 2 cm below the medium surface, in 20 cm diameter pots, with pumice:peat potting mix (Appendix A). Saucers

were placed under each pot. The saucers were painted with Spin Out (Griffin Corporation, USA), a biocide and growth retardant that contains cupric hydroxide. This prevented roots growing out of the bottom of the pots and coming into contact with wet surfaces that may contain pathogens such as *Erwinia carotovora* subsp. *carotovora*. The tubers were grown in a greenhouse (Section 2.2.3.2). Irrigation was discontinued before harvest to induce foliar senescence.

2.2.7.2 Stock plants: Year two

One tuber from each stock plant was replanted at the same time as the T2 tubers (Section 2.2.5.2) in the same greenhouse. Tubers were weighed and scored for the number of nodes and number of eyes. Tubers were dipped in GA_3 as detailed in Section 2.2.5.1. Tubers were planted out in 58 x 40 x 18 cm polystyrene trays in the same conditions as described in Section 2.2.5.2.

2.2.8 Assessments

Year one assessments: After foliage senescence and tuber harvest, the weight of the tuber and the number of nodes and eyes were recorded.

Year two assessments: The number of primary shoots growing from each tuber was recorded for one block of *Zantedeschia* tubers grown in the greenhouse. Flowers were harvested as in commercial practise using a twisting, pulling motion. Flowers were harvested as soon as the spathe was fully unfurled and the spadix yellow in colour. Flower weight, flower stem length, spathe length, flower quality and colour and the number of days from planting until flowering, were recorded for each tuber.

Flowers were given a quality score, on a five-point scale as detailed below:

1 non-complete flower spathe
1.5 non-complete flower spathe but some spathe overlap
2 some flower spathe distortion
2.5 minimal distortion and
3 no distortion.

Flowers with a score of 2 and above were considered of desirable quality.

Flower colour was determined by comparing the inside of the flower spathe with The Royal Horticultural Society London Colour Chart (Royal Horticultural Society (Great Britain), 1990). Most flowers scored 12A, 12B, 12C, 13A, 13B and 13C. Flowers with desirable colour scored 12A, 12B and 12C. The 12A to 12C scores were chosen as desirable as they were golden yellow. The 13A to 13C scores were more lemon yellow. T. Reed (personal communication, 2001) suggested that Florex Gold flowers from bushy plants were more lemon yellow. The desirable colour scores were the same for both Best Gold and Florex Gold.

The tuber weight and the number of tuber nodes and eyes were recorded after foliage senescence and tuber harvest.

2.2.9 Spray regime

Zantedeschia plants were routinely sprayed each week. The sprays used varied from week to week using the insecticides Diazinon 50W (1 g L^{-1}), Dimilin 25W (0.5 g L^{-1}), Lannate L (1.2 mL L^{-1}) or Mavrik flo (0.3 mL L^{-1}). The fungicide used was Rovral WP (1 g L^{-1}).

2.2.10 Treatment for *Erwinia carotovora* subsp. *carotovora*

When a plant became infected with *Erwinia carotovora* subsp. *carotovora*, the plant including the tuber and the surrounding medium was removed. This was replaced with new sterile medium and covered with sawdust. The remaining plants within the tray were each treated with a 100 to 200 mL solution of Benlate (0.5g L^{-1}) and Kocide DF (1.5g L^{-1}). These trays were monitored and treated weekly.

To prevent the spread of *Erwinia* infection within the greenhouse, the entire *Zantedeschia* crop was treated every four to six weeks. Plants were sprayed with Benlate (0.5g L^{-1}) and Kocide DF (1.5g L^{-1}), at a rate of 1 L per tray.

2.2.11 Data analysis

T1 and T2 tuber data including tuber weight, the number of tuber nodes, and the number of tuber eyes, as well as flower harvest data including flower weight, stem and spathe

length and the number of days from planting until flowering were all analysed by the residual maximum likelihood technique for analysing mixed models (Genstat 5 Release 4.1 Reference Manual Supplement, 1998). Where necessary, transformations were carried out to normalise the data. The means were separated by the least significant differences (LSD) test. The sources of variation caused by blocking, the cultivar/selection, the original source tuber and the location of the individual tuber within the tray were identified and accounted for in the analysis. Additional variation due to plants growing at the edge of the tray and the edge of the bench, being shorter in height, than those in the centre, was also accounted for. To do this plants were given a numerical value for their position in relation to the edge of the tray and edge of the bench. Plants were allocated number one for the first or outer row, number two for the second row, number three for the third row and number four for the fourth and remaining rows. Plant and tuber mortality due to *Erwinia* infection during the second growing season was also accounted for in the statistical analysis. The mean and standard error were calculated (as a percentage of flower number) for desirable flower quality (score 2, 2.5 and 3), desirable flower colour (12A, 12B and 12C), tubers ≥25 g the flowered and the number of flowers per flowering plant.

2.3 Results

2.3.1 Examination of bushy and control Florex Gold plants

The bushiest plants in this research grew from Dutch F tubers. Both Dutch F and control Florex Gold are shown in Figure 2.2. The Dutch F mother tuber had multiple nodes with many eyes and the tuber was shallow in depth. The number of eyes of Dutch tuber F increased from 22 to 183 in one growing season. In contrast the control Florex Gold mother tuber was deep with one dominant eye (not seen in photo as removed for micropropagation) and seven swollen axillary eyes. The control Florex Gold tuber grew from one to three apically dominant tubers in one growing season. The Dutch F tuber grew into a mass of smaller tubers. The control Florex Gold plant had 2 primary shoots, while the Dutch F plant had 22. The control Florex Gold has mature leaves with leaf maculation and a petiole length of 56 cm. The Dutch F plants in contrast had leaves without the characteristic triangular shape and without leaf maculation and petiole length was only 22 cm. The control Florex Gold had two flowers, while Dutch mother tuber F produced no flowers.

2.3.2 Experiment One – The role of the genotype on the expression of individual characteristics of the bushy syndrome

In Experiment One the Best Gold and Florex Gold selections were grown in standard conditions (Table 2.1). The bushy characteristics of each selection were examined (Section 2.3.2.1). Each selection was represented by a number of tubers. As these tubers each had an individual response, these were also analysed (Section 2.3.2.2). For statistical purposes, the tuber responses were compared within selections. A total of 4006 T1 tubers, 822 flowers and 566 T2 tubers were examined. The chi-square statistics are detailed in Table 2.2.

A

22 eyes

B

8 eyes

Petiole Length – 22 cm

Petiole Length – 56 cm

183 eyes

16 eyes

Figure 2.2: Bushy and non-bushy Florex Gold mother plants. Dutch F (A) and control Florex Gold (B). Dutch tuber eyes increased from 22 to 183 eyes and control Florex Gold tuber eyes increased from 8 to 16 in one growing season. Dutch petiole length was 22 cm and control Florex Gold petiole length was 56 cm. Both Dutch F and control Florex Gold were grown in identical sized pots.

Table 2.2: Chi-square probability values of the effect of genotype on individual bushy characteristics: T1 tuber weight, T1 tuber nodes, T1 tuber eyes, flower weight, flower stem length, spathe length, number of days from planting until flowering, proportional increase in T2 tuber weight, proportional increase in T2 tuber nodes and proportional increase in T2 tuber eyes of Best Gold and Florex Gold selections and tubers. The degrees of freedom are listed (df).

	df	T1 Tuber Data			Flower Data				T2 Tuber Data		
		Weight	Nodes	Eyes	Weight	Flower stem length	Spathe length	Number of days from planting until flowering	Proportional increase in weight	Proportional increase in nodes	Proportional increase in eyes
Selection	6	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.247$	$P = 0.021$	$P = 0.016$
Tuber	7	$P < 0.001$	$P < 0.001$	$P < 0.001$	-	-	-	-	$P = 0.016$	$P = 0.045$	$P = 0.165$

2.3.2.1 The effect of genotype on the expression of individual characteristics of the bushy syndrome

T1 tuber results: Analysis of T1 tuber data indicated significant differences between selections in T1 tuber weight ($P < 0.001$), the number of tuber nodes ($P < 0.001$) and the number of tuber eyes ($P < 0.001$) (Figure 2.3). The multi-eyed Best Gold tubers had significantly heavier tubers in comparison with bushy and Dutch Florex Gold tubers. The control Florex Gold had significantly fewer tuber nodes and eyes than every other tuber selection. Multi-eyed Florex Gold tubers had significantly more nodes than all other selections except the Dutch selection.

Flower results: Significant differences were seen between selections for flower weight ($P < 0.001$) and flower stem length ($P < 0.001$) (Figure 2.3). The flowers of the bushy, few-eyed, multi-eyed and Dutch Florex Gold selections were all significantly lighter and shorter than flowers of Best Gold and control Florex Gold. Flower spathe lengths were also significantly different between selections ($P < 0.001$) (Figure 2.3). Flower spathes from the bushy, few-eyed, multi-eyed and Dutch Florex Gold selections and the Best Gold few-eyed selection were significantly shorter than the control Florex Gold and the multi-eyed Best Gold selection. The number of days from planting until flowering also varied between selections ($P < 0.001$) (Figure 2.3). Both the multi-eyed Best Gold and the control Florex Gold took longer to flower than all the other selections.

The percentage of flowers of desirable quality is presented in Figure 2.4. The Best Gold few and multi-eyed and control Florex Gold selections had consistently high quality flowers (95.5%, 87.5% and 95.0% respectively). The percentage of flowers with desirable colour varied considerably between cultivars and selections (Figure 2.4). Photographs of flower shape and colour of one example of each selection are shown in Figure 2.5. The Best Gold few-eyed selection had 73.6% of flowers with desirable flower colour compared with the Best Gold multi-eyed selection, which had only 37.5%. The control Florex Gold selection had 36.3% of flowers with desirable flower colour and the Dutch selection had 69.4%. Few-eyed Best Gold and control Florex Gold had the greatest percentage of tubers at least 25 g that flowered, 50.0% and 48.1% respectively. Dutch Florex Gold and multi-eyed Best Gold had the least number of tubers 25 g or heavier that flowered 35.3% and 38.8% respectively.

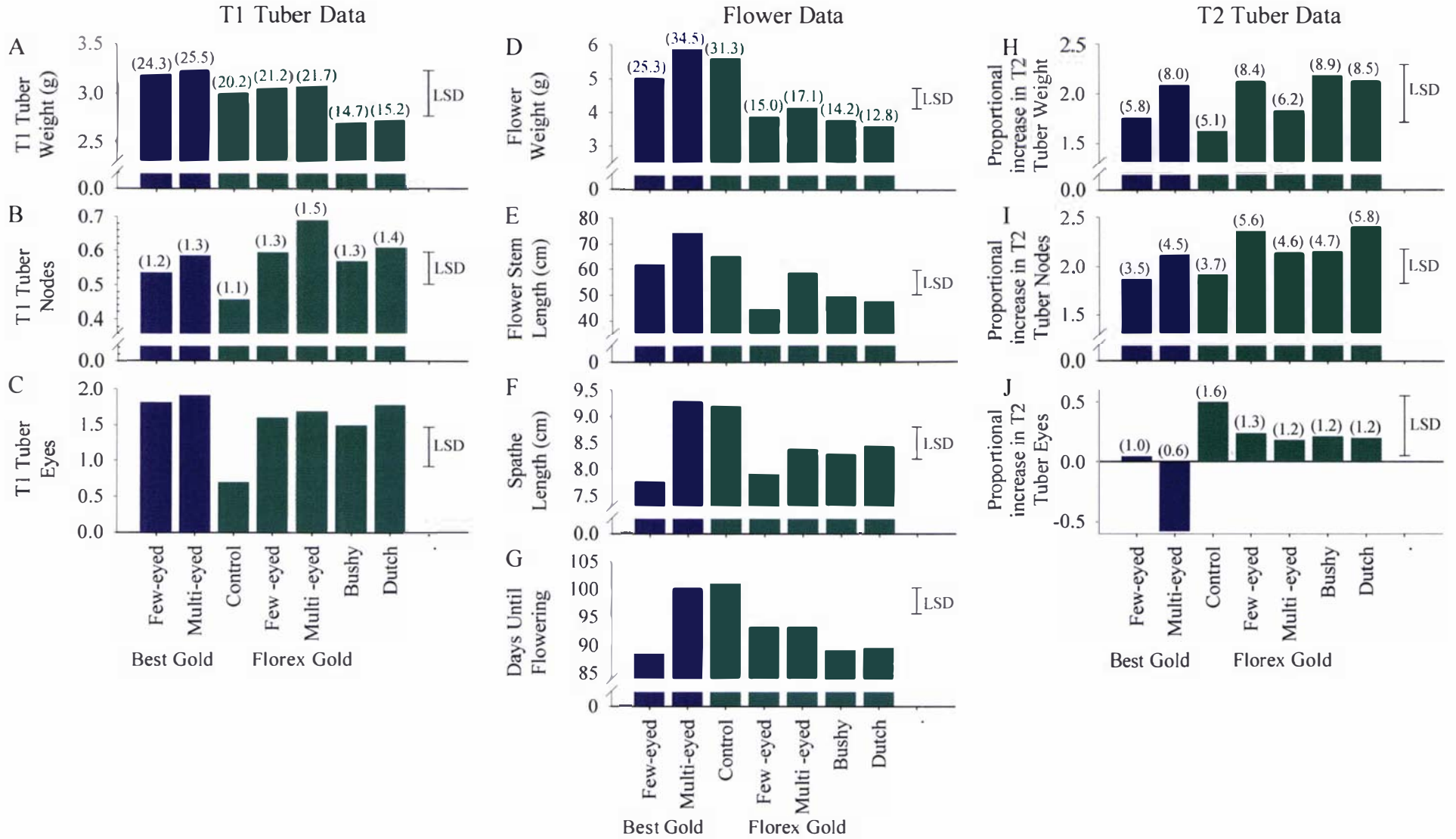


Figure 2.3: The effect of genotype on individual bushy characteristics: T1 tuber weight (A), T1 tuber nodes (B), T1 tuber eyes (C), flower weight (D), flower stem length (E), spathe length (F), number of days from planting until flowering (G), proportional increase in T2 tuber weight (H), proportional increase in T2 tuber nodes (I) and proportional increase in T2 tuber eyes (J) of Best Gold and Florex Gold Selections. Results are presented as either transformed data, with back transformed means in brackets, or untransformed data. Error bars indicate LSD value. Best Gold selections shown in blue; Florex Gold selections shown in green.

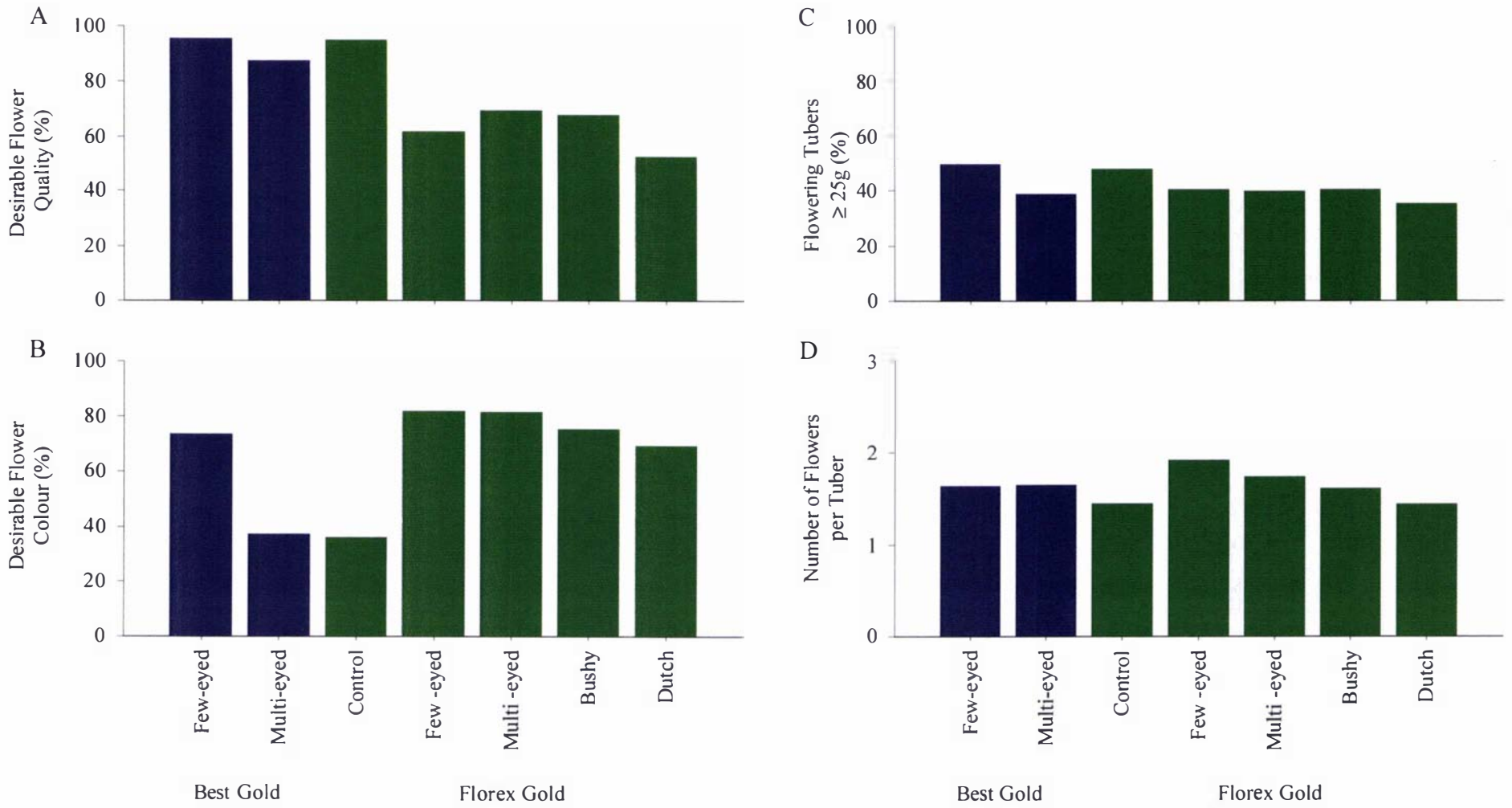


Figure 2.4: The effect of genotype on individual bushy characteristics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C) and the number of flowers per flowering plant (D), in Best Gold and Florex Gold Selections. Best Gold selections shown in blue; Florex Gold selections shown in green.

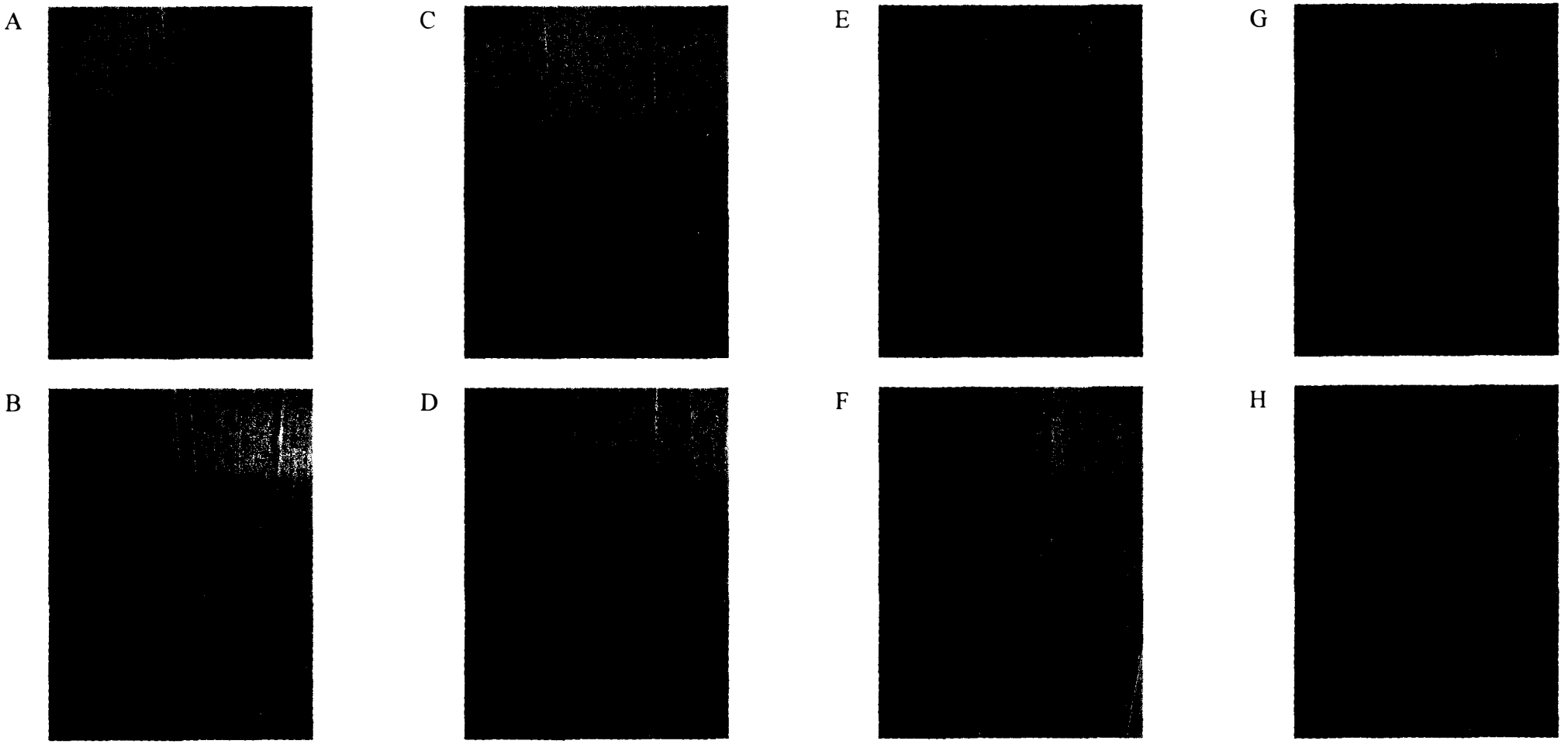


Figure 2.5: Flower colour and shape: control Florex Gold (A), few-eyed Florex Gold (B), multi-eyed Florex Gold (C), bushy (D), Dutch (E) and Best Gold (F), all grown in Experiment One. Multi-eyed micropropagated on MS media supplemented with 44.4 μM 6-BAP (G), and multi-eyed micropropagated on MS media supplemented with 26.6 μM 6-BAP, grown in Experiment Two.

Few-eyed Florex Gold had the most flowers per flowering tuber (1.9), while the control and Dutch Florex Gold had the least (1.5 and 1.4 respectively).

Shoot number: To be considered bushy, the number of primary shoots needs to be five or greater. Control Florex Gold had only 33.3% of tubers with at least five shoots, while bushy had 84.3%, multi-eyed 94.0% and Dutch 100% (Figure 2.6).

T2 tuber results: No significant differences were seen between selections in the proportional increase in T2 tuber weight ($P = 0.247$) (Figure 2.3). There were significant differences between selections in the proportional increase in the number of T2 tuber nodes ($P = 0.021$) and in the proportional increase in the number of T2 tuber eyes ($P = 0.016$) (Figure 2.3). The Best Gold few-eyed and control Florex Gold selections had a significantly lower increase in the number of T2 tuber nodes than the few-eyed and the Dutch Florex Gold tubers. The Best Gold multi-eyed tubers had a significantly lower proportional increase in the number of T2 tuber eyes than all other tuber selections.

2.3.2.2 The variation of individual tubers of each genotype on the expression of individual characteristics of the bushy syndrome

T1 tuber results: In assessing individual tuber weight, node and eye number, significant differences were seen between tubers in a selection in T1 tuber weight ($P < 0.001$), in the number of T1 tuber nodes ($P < 0.001$) and the number of T1 tuber eyes ($P < 0.001$) (Figure 2.7). Plants from the Dutch tuber F had significantly lighter tubers, and fewer tuber nodes and eyes than all other Dutch tubers. The Florex Gold few-eyed tuber D and Best Gold few-eyed tuber A had significantly more tuber nodes and bushy H significantly more tuber eyes than other tubers in the same selection.

Flower results: Plants from Dutch tuber F did not flower. Plants derived from the control Florex Gold tuber and multi-eyed Best Gold tuber C had flowers that were heavier, with longer stem and spathes than most other plants. These tubers also took longer than 100 days to flower (Figure 2.8). The few-eyed Florex Gold tuber C had flowers with longer spathes, which took more than 100 days to flower (Figure 2.8).

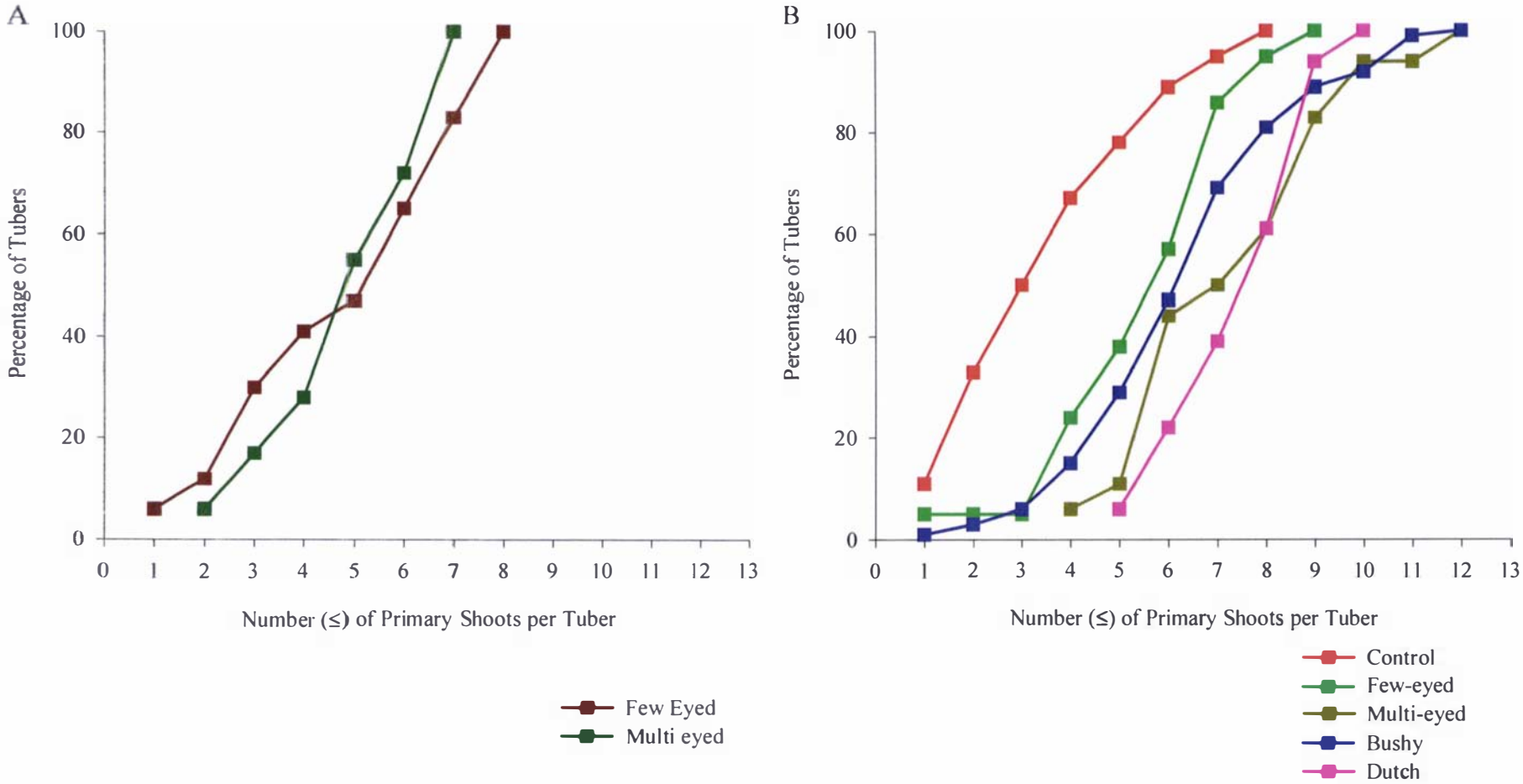


Figure 2.6: The effect of genotype on individual bushy characteristics: the number (≤) of shoots per tuber. Results from Best Gold plants are presented in A. Results from Florex Gold plants are presented in B.

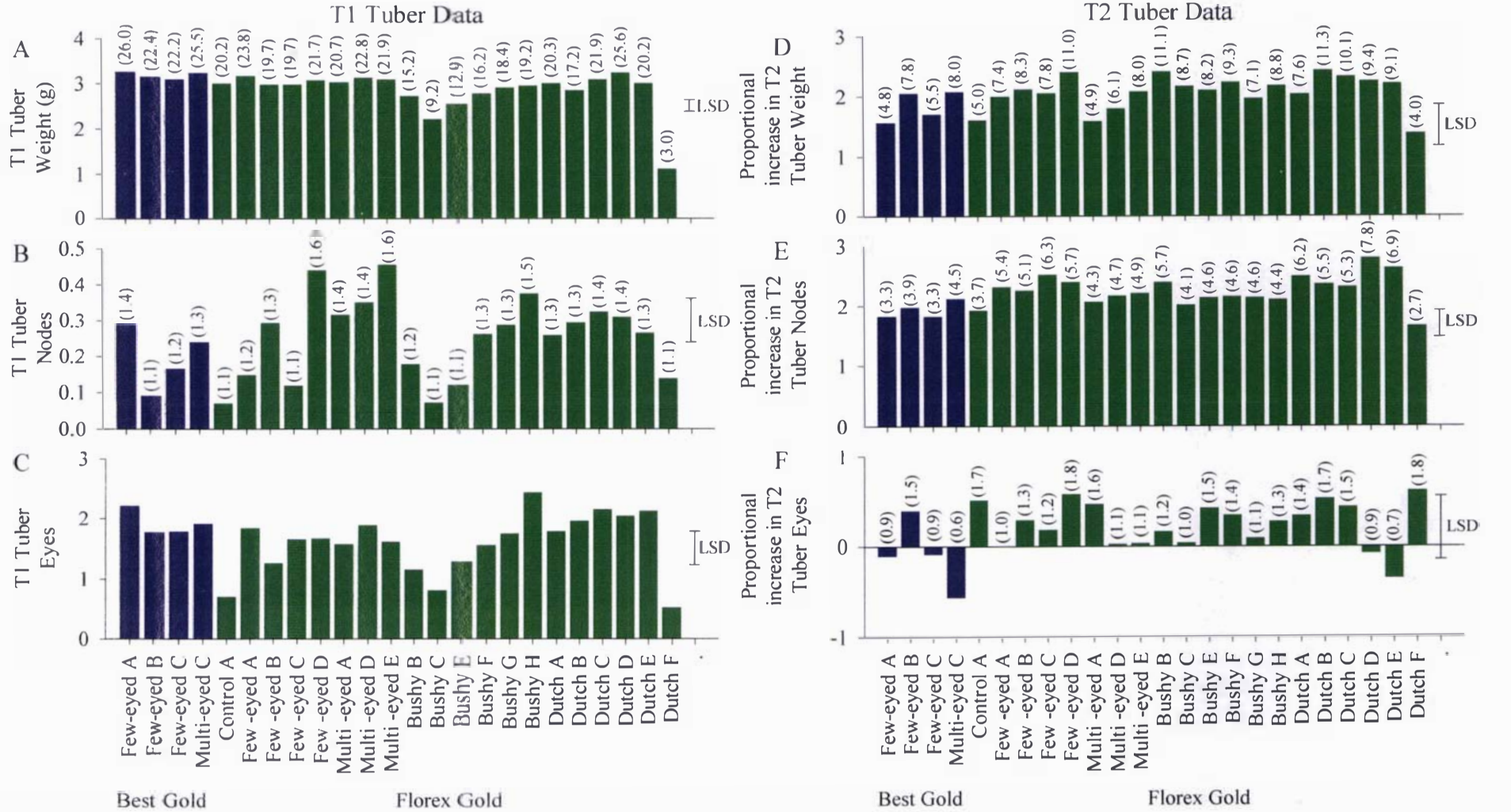


Figure 2.7: The response of each tuber on individual bushy characteristics: T1 tuber weight (A), T1 tuber nodes (B), T1 tuber eyes (C), proportional increase in T2 tuber weight (D), proportional increase in T2 tuber nodes (E) and proportional increase in T2 tuber eyes (F) of individual Best Gold and Florex Gold tuber selections. Results are presented as either transformed data, with back transformed means in brackets, or untransformed data. Error bars indicate LSD value. Best Gold shown in blue and Florex Gold selctions shown in green.

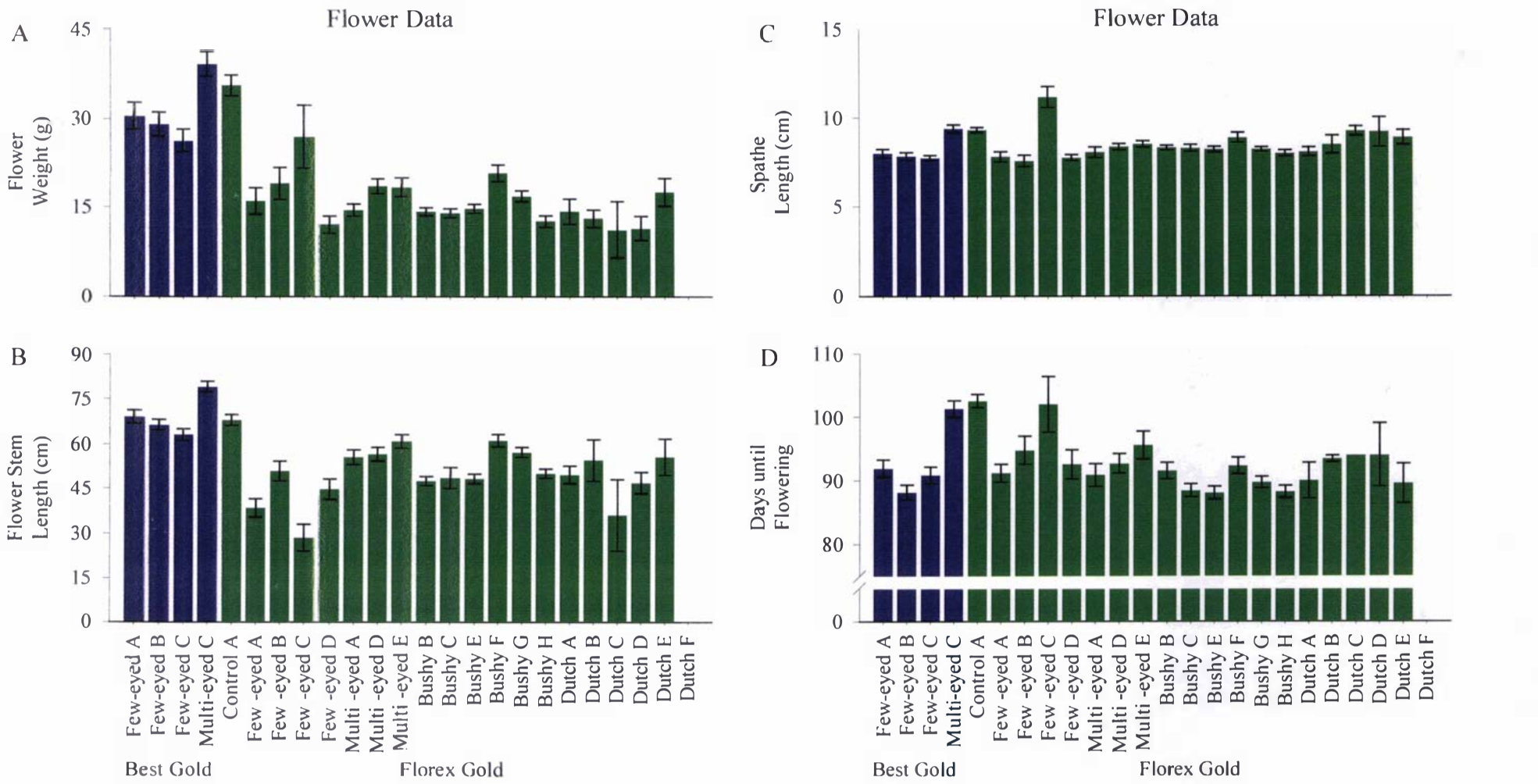


Figure 2.8: The response of each tuber on individual bushy characteristics: flower weight (A), flower stem length (B), spathe length (C), number of days from planting until flowering (D). Error bars indicate standard error. Best Gold selections shown in blue. Florex Gold selections shown in green.

Best Gold few and multi-eyed flowers from individual tubers were consistently of high quality, between 87.5% and 94.9% (Figure 2.9). At least 95% of control Florex Gold flowers were of high quality. The Dutch tuber E had 85.7% of flowers with high quality. The percentage of tubers producing flowers with desirable colour was variable within each selection. The highest percentage of flowers of desirable colour were from plants of Dutch B and D, bushy G and H, few-eyed A, B and D and multi-eyed Florex Gold A and E. Dutch A and C, few-eyed A and D and multi-eyed Florex Gold D, produced the most flowers. The most tubers of at least 25 g that flowered were Dutch D, bushy C and few-eyed Florex Gold B (Figure 2.9).

Shoot number: Best Gold had between 50.0% and 72.3% of tubers with at least five primary shoots (Figure 2.10). Few-eyed Florex Gold had between 25.0% and 100% of tubers and multi-eyed had between 83.3% and 100% of tubers with at least five primary shoots. Bushy had between 55.5% and 100% of tubers with at least five shoots. All Dutch Florex Gold tubers produced at least eight primary shoots (Figure 2.10).

T2 tuber results: The proportional increases in individual T2 tuber weight were significantly different between tubers ($P = 0.016$) (Figure 2.7). Dutch F had a significantly smaller proportional increase in T1 to T2 tuber weight than all other Dutch tubers except Dutch A. Dutch F had only increased 4.0 times from T1 to T2 tuber weight in comparison with the other Dutch tubers that had increased 7.6 to 11.3 times in tuber size. There was a significant difference between tubers in a selection for the proportional increase in the number of T2 tuber nodes ($P = 0.045$) (Figure 2.7). Dutch F had a significantly lower proportional increase in the number of T2 tuber nodes in comparison to other Dutch tubers. Dutch selection D had a significantly greater proportional increase in the number of nodes in comparison to Dutch A, B, C and F. There was no significant difference between tubers within a selection on the proportional increase in the number of T2 tuber eyes ($P = 0.165$) (Figure 2.7).

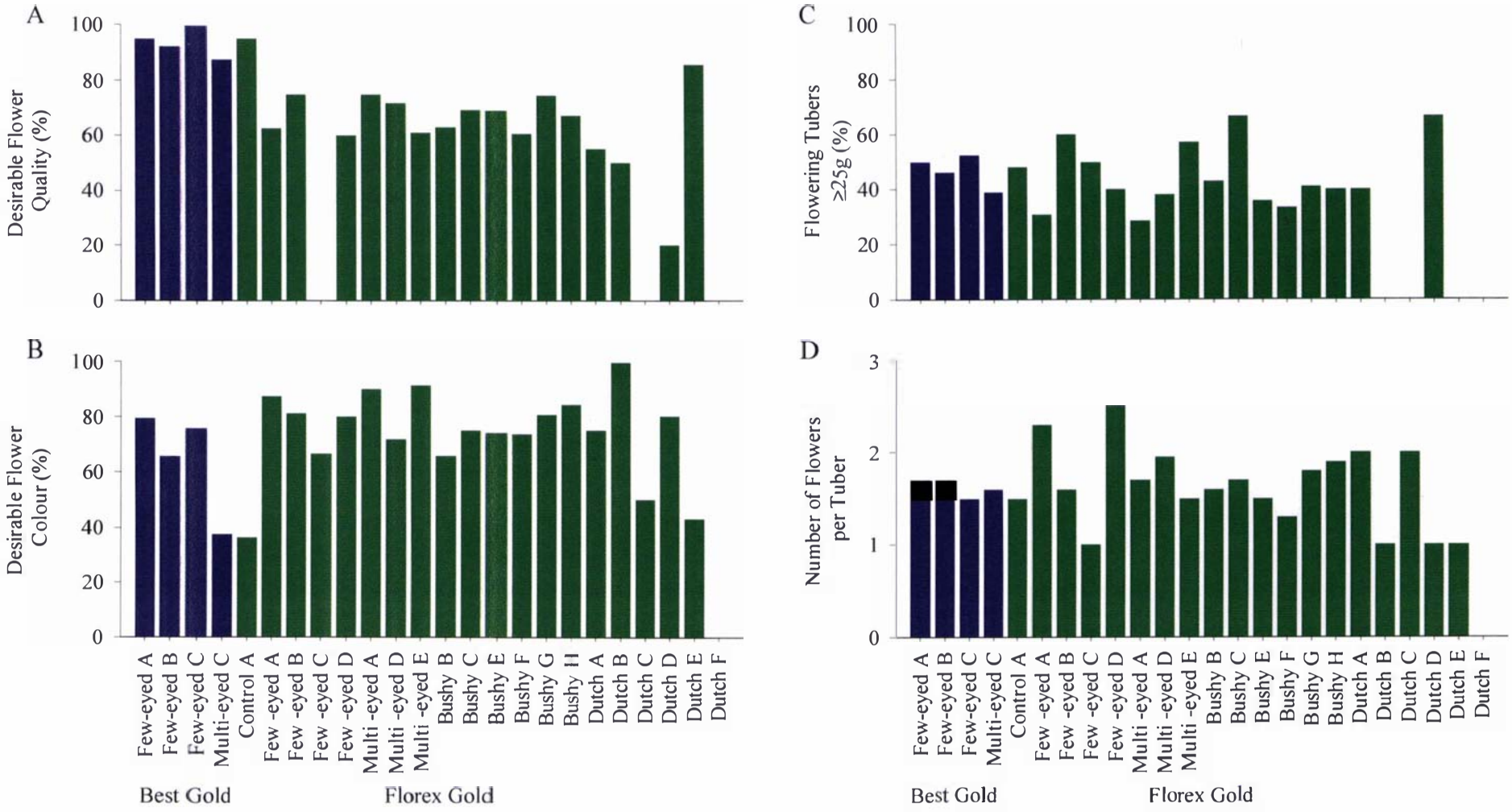


Figure 2.9: The response of each tuber on individual bushy characteristics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C), and the number of flowers per flowering plant (D), in Best Gold and Florex Gold selections. Best Gold selections shown in blue; Florex Gold selections shown in green.

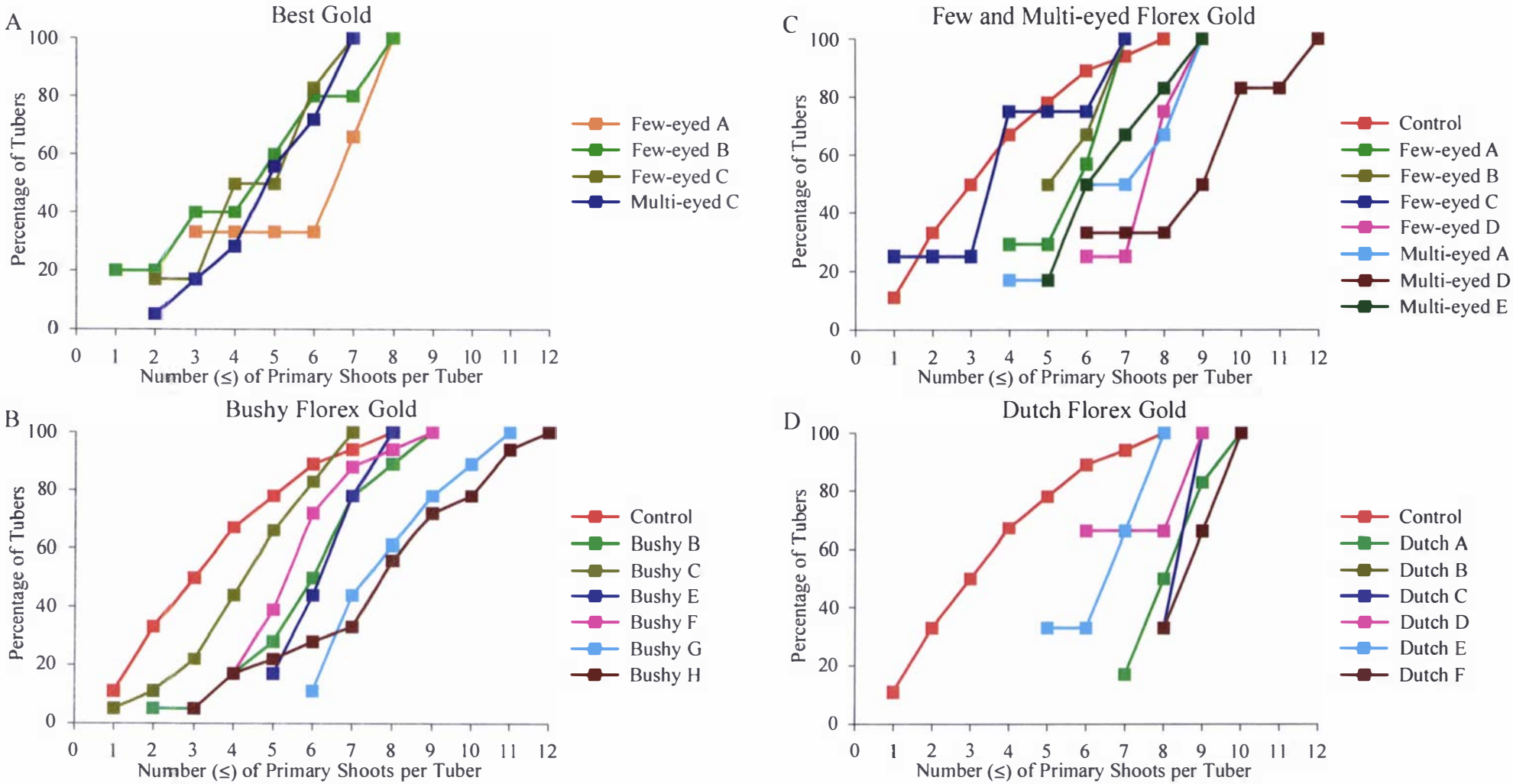


Figure 2.10: The response of each tuber on individual bushy characteristics: the number (≤) of shoots per tuber. Results from individual Best Gold plants are presented in A. Results from individual bushy Florex Gold plants are presented in B. Results from individual few-eyed and multi-eyed Florex Gold plants are presented in C. Results from individual Dutch bushy Florex Gold plants are presented in D. The control Florex Gold plant is presented in B, C and D.

2.3.3 Experiment Two – The influence of elevated cytokinin (6-BAP and TDZ) during micropropagation on the development of the bushy syndrome

The effect of elevated cytokinin on individual bushy characteristics was examined in Experiment Two: 5669 T1 tubers, 1186 flowers and 1058 T2 tubers were harvested and analysed. The chi-square statistics are presented in Table 2.3. Experiment Two examined the response of individual selections to each elevated cytokinin concentration (Section 2.3.3.1) and the effect of elevated cytokinin (Section 2.3.3.2).

2.3.3.1 The response of individual selections to each cytokinin concentration and the development of the bushy syndrome

T1 tuber results: The analysis of T1 tuber data indicated significant differences between plants micropropagated on the various MS media on subsequent tuber weight ($P = 0.002$), the number of tuber nodes ($P < 0.001$) and the number of tuber eyes ($P = 0.036$) (Figure 2.11). Few-eyed tubers from plants micropropagated on MS media supplemented with low cytokinin levels (13.3 μM 6-BAP) were significantly lighter than all other few-eyed tubers. Plants from Dutch tubers micropropagated in the presence of 4.4 μM 6-BAP (standard MS media) had significantly fewer nodes than those grown MS media supplemented with 13.3 and 26.6 μM 6-BAP (elevated cytokinin levels) and all TDZ concentrations. Plants from Dutch tubers micropropagated on all 6-BAP containing media had significantly fewer nodes than those Dutch plants grown on media supplemented with 9.1 and 13.6 μM TDZ. The few-eyed and multi-eyed plants micropropagated in the presence of 9.1 μM TDZ had significantly more nodes at the end of the first growing season than those plants micropropagated on media supplemented with standard and low 6-BAP levels. Both few-eyed and multi-eyed tubers micropropagated on MS media supplemented with low 6-BAP levels had significantly fewer eyes than those grown on elevated 6-BAP and TDZ levels. The Dutch tubers micropropagated on MS media containing low TDZ levels had significantly fewer T1 tuber eyes than those grown on elevated TDZ levels (Figure 2.11).

Table 2.3: Chi-square probability values of the effect of micropropagation media containing elevated concentrations of 6-BAP and TDZ on individual bushy characteristics: T1 tuber weight, T1 tuber nodes, T1 tuber eyes, flower weight, flower stem length, spathe length, number of days from planting until flowering, proportional increase in T2 tuber weight, proportional increase in T2 tuber nodes and proportional increase in T2 tuber eyes of few and multi-eyed and Dutch Florex Gold selections. The degrees of freedom are listed (df).

	df	T1 Tuber Data			Flower Data				T2 Tuber Data		
		Weight	Nodes	Eyes	Weight	Flower stem length	Spathe length	Number of days from planting until flowering	Proportional increase in weight	Proportional increase in nodes	Proportional increase in eyes
Selection	2	$P < 0.001$	$P < 0.001$	$P = 0.026$	$P = 0.001$	$P = 0.263$	$P = 0.823$	$P = 0.019$	$P = 0.231$	$P = 0.409$	$P = 0.407$
Cytokinin treatment	6	$P < 0.001$	$P < 0.001$	$P = 0.001$	$P = 0.180$	$P = 0.919$	$P = 0.116$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.050$
Selection and cytokinin interaction	12	$P = 0.002$	$P < 0.001$	$P = 0.036$	$P = 0.019$	$P = 0.004$	$P = 0.291$	$P = 0.552$	$P = 0.057$	$P = 0.012$	$P = 0.023$

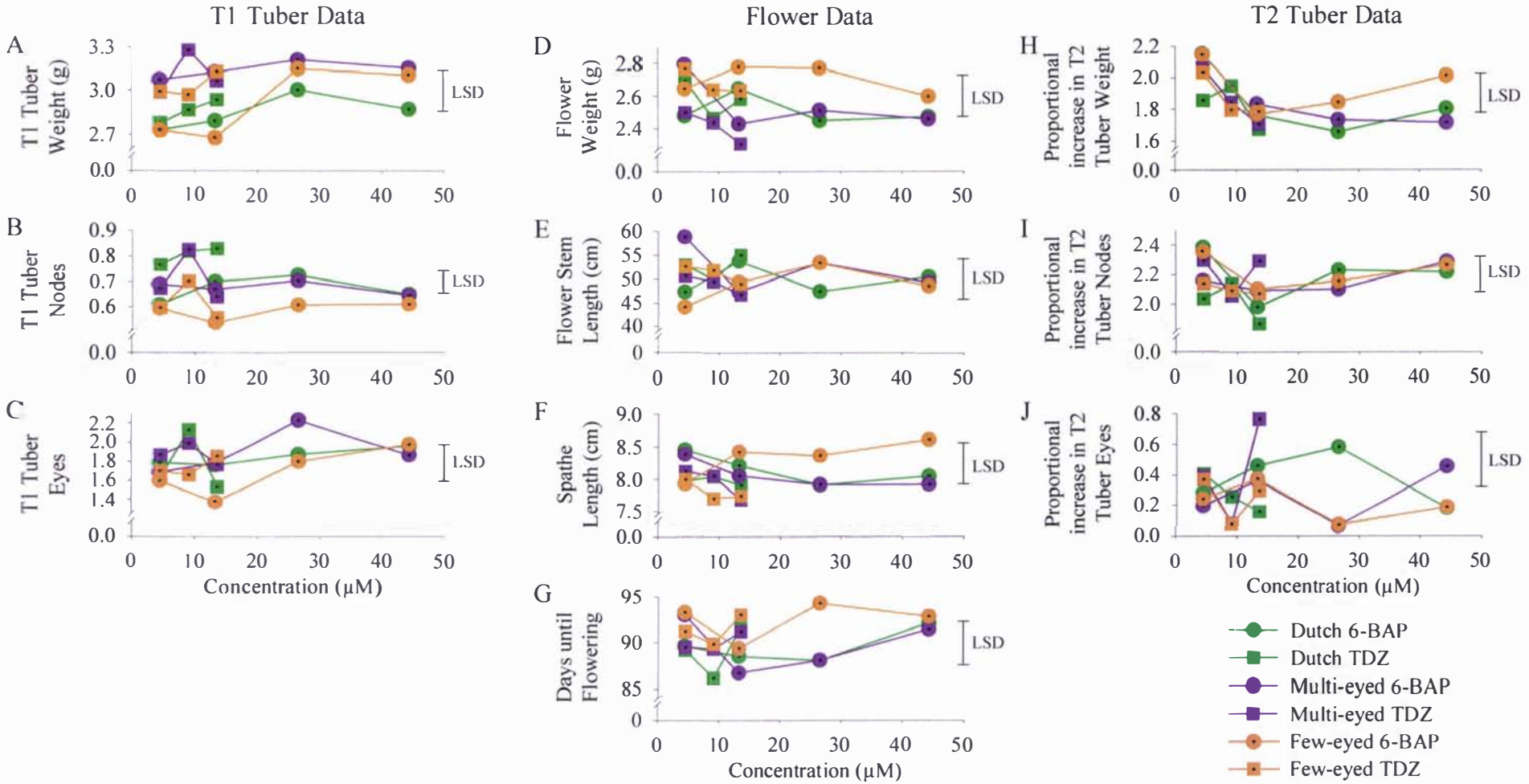


Figure 2.11: The effect of micropropagation media containing different concentrations of 6-BAP and TDZ on individual bushy characteristics: T1 tuber weight (A), T1 tuber nodes (B), T1 tuber eyes (C), flower weight (D), flower stem length (E), spathe length (F), number of days from planting until flowering (G), proportional increase in T2 tuber weight (H), proportional increase in T2 tuber nodes (I) and proportional increase in T2 tuber eyes (J) of few and multi-eyed and Dutch Florex Gold selections. Results are presented as either transformed data, with back transformed means in Table 2.4, or untransformed data. Error bars indicate average LSD value.

Table 2.4: Back transformed means of T1 tuber weight, T1 tuber nodes, flower weight (g), proportional increase in T2 tuber weight, proportional increase in T2 tuber nodes and proportional increase in T2 tuber eyes of few-eyed, multi-eyed and Dutch Florex Gold selections micropropagated on MS media containing elevated cytokinin levels.

A

T1 tuber weight (g)			
	Dutch	Multi-eyed	Few-eyed
4.4 µM 6-BAP	16.74	21.69	21.16
13.3 µM 6-BAP	16.37	22.72	14.68
26.6 µM 6-BAP	20.38	25.12	23.37
44.4 µM 6-BAP	17.96	23.48	22.03
4.5 µM TDZ	16.13	19.80	20.02
9.1 µM TDZ	18.96	26.77	19.61
13.6 µM TDZ	19.07	21.52	22.98

B

T1 tuber nodes			
	Dutch	Multi-eyed	Few-eyed
4.4 µM 6-BAP	1.346	1.493	1.311
13.3 µM 6-BAP	1.513	1.455	1.214
26.6 µM 6-BAP	1.568	1.512	1.336
44.4 µM 6-BAP	1.419	1.410	1.334
4.5 µM TDZ	1.653	1.466	1.315
9.1 µM TDZ	1.783	1.770	1.520
13.6 µM TDZ	1.794	1.398	1.247

D

Flower weight (g)			
	Dutch	Multi-eyed	Few-eyed
4.4 µM 6-BAP	11.93	16.36	14.10
13.3 µM 6-BAP	14.11	11.34	16.07
26.6 µM 6-BAP	11.57	12.32	15.96
44.4 µM 6-BAP	11.82	11.67	13.42
4.5 µM TDZ	14.81	12.17	15.88
9.1 µM TDZ	11.72	11.43	14.00
13.6 µM TDZ	13.24	10.02	13.93

H

Proportional increase in T2 tuber weight			
	Dutch	Multi-eyed	Few-eyed
4.4 µM 6-BAP	8.64	6.42	8.56
13.3 µM 6-BAP	5.65	6.30	5.86
26.6 µM 6-BAP	5.26	5.65	6.33
44.4 µM 6-BAP	6.09	5.55	7.48
4.5 µM TDZ	6.40	7.86	7.62
9.1 µM TDZ	7.02	6.32	6.03
13.6 µM TDZ	5.33	5.50	5.97

I

Proportional increase in T2 tuber nodes			
	Dutch	Multi-eyed	Few-eyed
4.4 µM 6-BAP	5.68	4.67	5.55
13.3 µM 6-BAP	3.91	4.38	4.42
26.6 µM 6-BAP	5.38	4.42	4.64
44.4 µM 6-BAP	4.91	5.23	5.13
4.5 µM TDZ	4.15	5.28	4.58
9.1 µM TDZ	4.57	4.23	4.37
13.6 µM TDZ	3.48	5.25	4.29

J

Proportional increase in T2 tuber eyes			
	Dutch	Multi-eyed	Few-eyed
4.4 µM 6-BAP	1.32	1.22	1.28
13.3 µM 6-BAP	1.58	1.44	1.45
26.6 µM 6-BAP	1.79	1.07	1.07
44.4 µM 6-BAP	1.20	1.58	1.21
4.5 µM TDZ	1.50	1.48	1.45
9.1 µM TDZ	1.29	1.08	1.08
13.6 µM TDZ	1.17	2.15	1.34

Flower results: There was a significant effect between selections micropropagated on the various MS media on flower weight ($P = 0.019$) and flower stem length ($P = 0.004$). There was no effect on spathe length ($P = 0.291$) or the number of days from planting until flowering ($P = 0.552$) (Figure 2.11). Dutch plants micropropagated in the presence of 4.4 or 26.6 µM 6-BAP had significantly lighter flowers than few and multi-eyed plants micropropagated on the same media respectively. Multi-eyed plants micropropagated on MS media supplemented with 13.3 µM 6-BAP, 4.5 or 9.1 µM TDZ had significantly lighter flowers than few-eyed plants micropropagated on the same media. Few-eyed plants micropropagated in the presence of 4.4 µM 6-BAP had significantly shorter stems than multi-eyed plants also micropropagated in the presence of 4.4 µM 6-BAP and few-eyed plants micropropagated in the presence of 26.6 µM 6-BAP.

Desirable flower colour and quality results were variable (Figure 2.12). Dutch plants micropropagated on media supplemented with 26.6 µM 6-BAP, 4.5 or 9.1 µM TDZ and multi-eyed plants micropropagated on media supplemented with 13.3 µM 6-BAP had the highest flower quality (70.9%, 73.9%, 75.6% and 71.1% respectively). Dutch plants micropropagated in the presence of 13.3 µM 6-BAP and few-eyed plants micropropagated in the presence of 13.3 µM 6-BAP, 4.5 or 9.1 µM TDZ had the highest desirable colour. Dutch plants micropropagated in the presence of 26.6 µM 6-BAP and multi-eyed plants micropropagated in the presence of 13.6 µM TDZ had 42.5% of tubers at least 25 g that flowered. Dutch plants micropropagated on media supplemented with 9.1 or 13.6 µM TDZ and multi-eyed plants micropropagated on media supplemented with 9.1 µM TDZ or 13.3 µM 6-BAP all produced more than two flowers per flowering tuber (Figure 2.12).

Shoot results: Dutch, few-eyed and multi-eyed selections grown on the TDZ containing MS media series, had an increasing percentage of plants with at least five primary shoots as the TDZ concentration increased (Figure 2.13). The tubers of all selections had at least five shoots when grown on media supplemented with 13.6 µM TDZ. The 6-BAP media series did not show a similar interaction, and the percentage of tubers with five or more shoots varied on the different concentrations of 6-BAP containing MS media.

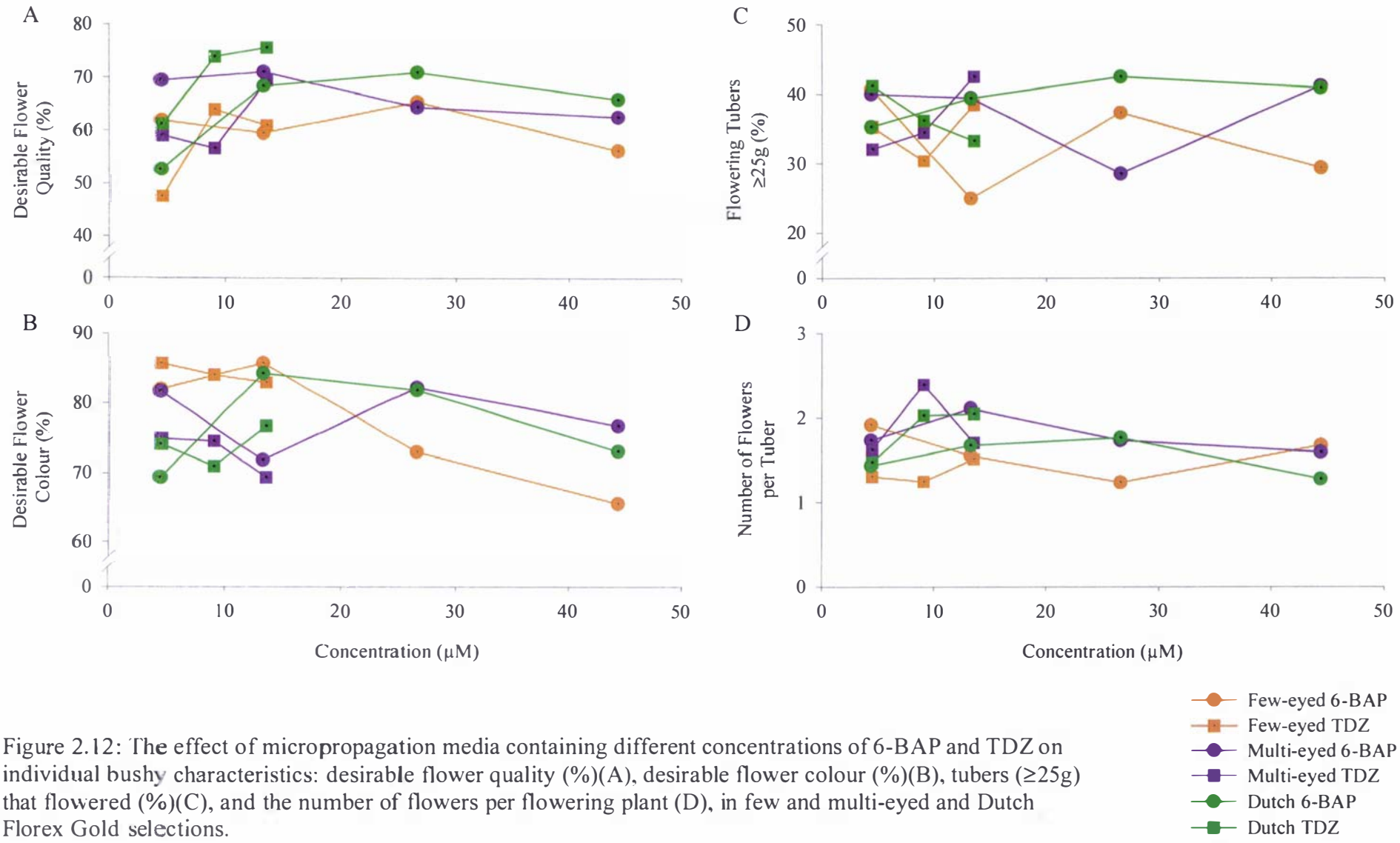


Figure 2.12: The effect of micropropagation media containing different concentrations of 6-BAP and TDZ on individual bushy characteristics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C), and the number of flowers per flowering plant (D), in few and multi-eyed and Dutch Florex Gold selections.

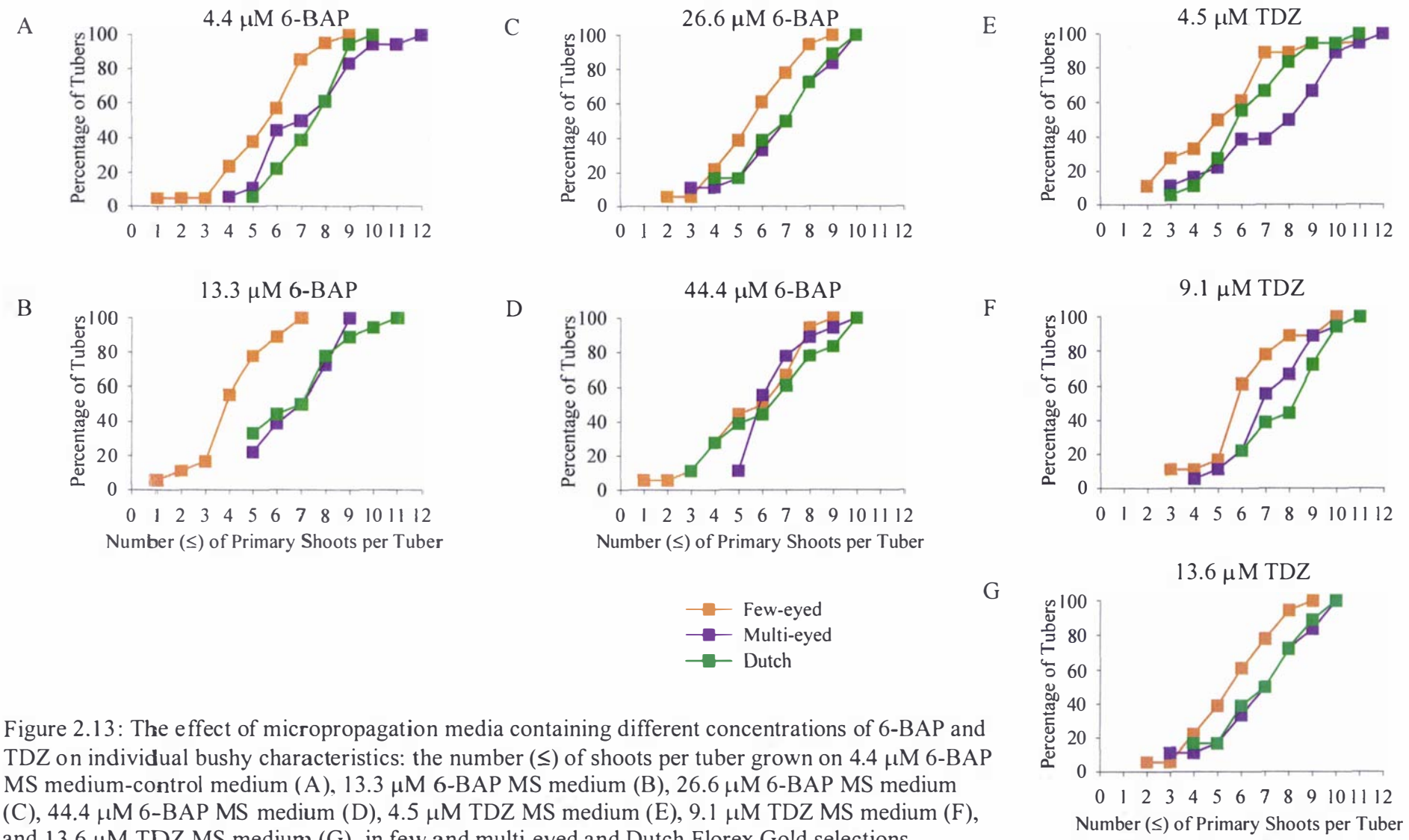


Figure 2.13: The effect of micropropagation media containing different concentrations of 6-BAP and TDZ on individual bushy characteristics: the number (≤) of shoots per tuber grown on 4.4 µM 6-BAP MS medium-control medium (A), 13.3 µM 6-BAP MS medium (B), 26.6 µM 6-BAP MS medium (C), 44.4 µM 6-BAP MS medium (D), 4.5 µM TDZ MS medium (E), 9.1 µM TDZ MS medium (F), and 13.6 µM TDZ MS medium (G), in few and multi-eyed and Dutch Florex Gold selections.

T2 tuber results: Analysis of T2 tuber data showed that there was a significant effect of MS media supplemented with elevated cytokinin on the proportional increase in T2 tuber nodes ($P = 0.012$) and the proportional increase in T2 tuber eyes ($P = 0.023$) (Figure 2.11). Dutch T2 tubers from plants micropropagated in the presence of 13.6 μM TDZ had proportionally fewer nodes than Dutch T2 tubers from plants micropropagated in the presence of 4.4, 26.6 or 44.4 μM 6-BAP or 9.1 μM TDZ and multi-eyed T2 tubers from plants micropropagated on identical media. Dutch T2 tubers from plants micropropagated in the presence of 4.5 μM TDZ had proportionally fewer nodes than multi-eyed T2 tubers from plants micropropagated on identical media. Few-eyed T2 tubers from plants micropropagated on MS media supplemented with 4.4 μM 6-BAP had proportionally more nodes than few-eyed T2 tubers from plants micropropagated on media supplemented with 13.3 μM 6-BAP or 13.6 μM TDZ. Multi-eyed T2 tubers from plants micropropagated in the presence of 13.6 μM TDZ (elevated cytokinin levels) had significantly more eyes than Dutch and few-eyed T2 tubers from plants micropropagated on identical media. It also had proportionally more eyes than multi-eyed T2 tubers from plants micropropagated on all other media except 44.4 μM 6-BAP. Dutch T2 tubers from plants micropropagated on media supplemented with 26.6 μM 6-BAP has proportionally more T2 tuber eyes than few and multi-eyed T2 tubers from plants micropropagated on identical media. There was no significant proportional increase in the T2 tuber weight ($P = 0.057$).

2.3.3.2 The effect of elevated cytokinin MS media on the expression of individual characteristics of the bushy syndrome

T1 tuber results: Examination of tuber data showed significant differences between selections micropropagated on the various media for T1 tuber weight ($P < 0.001$), the number of tuber nodes ($P < 0.001$) and on the number of tuber eyes ($P = 0.001$) (Figure 2.14). Tubers from plants grown on standard and low cytokinin containing MS media were significantly lighter with fewer eyes than those grown on media supplemented with elevated cytokinin levels. Plants micropropagated on standard media (4.4 μM 6-BAP) had significantly fewer tuber nodes than those grown on media supplemented with elevated cytokinin levels.

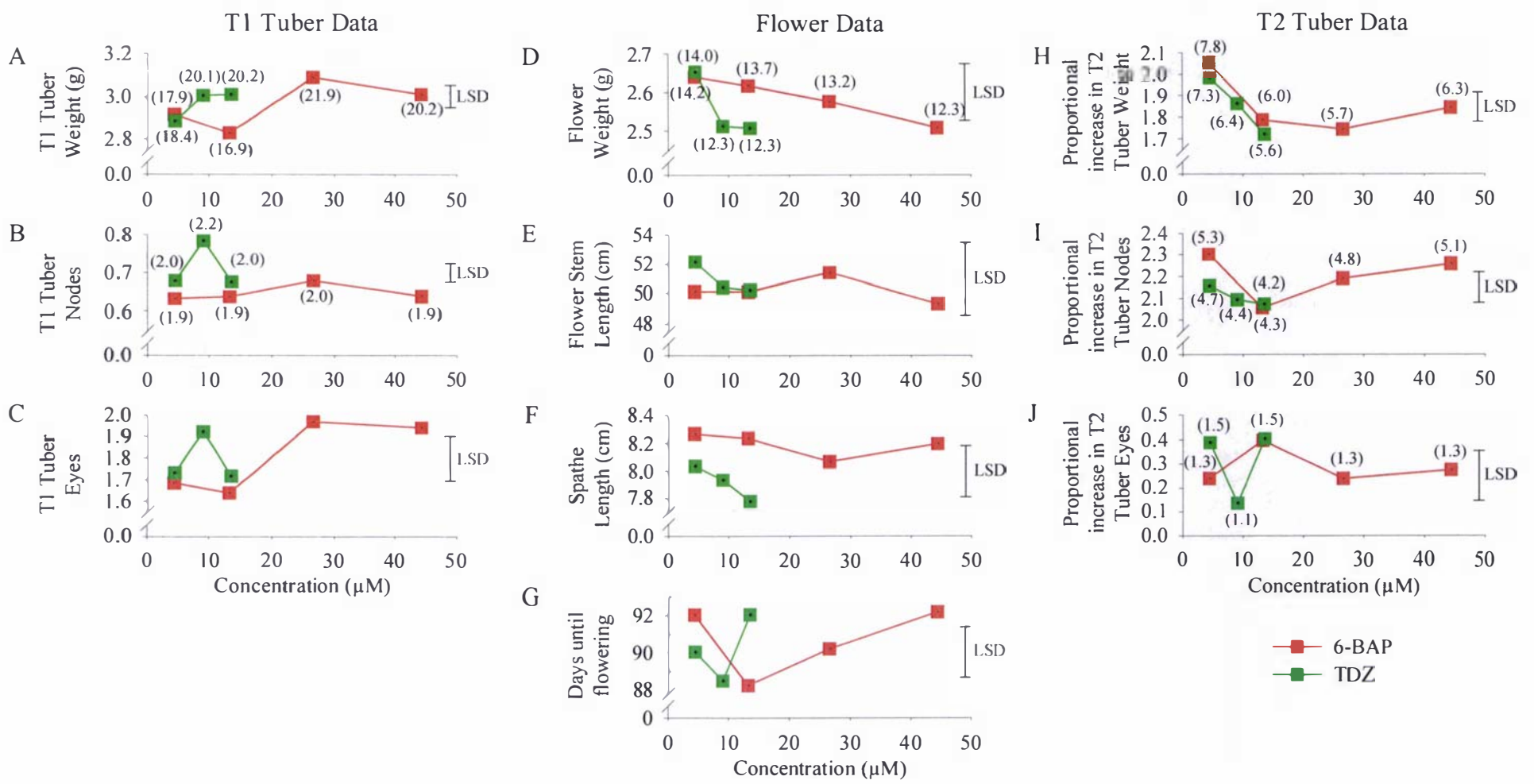


Figure 2.14: The effect of micropropagation media containing different concentrations of 6-BAP and TDZ on individual bushy characteristics: T1 tuber weight (A), T1 tuber nodes (B), T1 tuber eyes (C), flower weight (D), flower stem length (E), spathe length (F), number of days from planting until flowering (G), proportional increase in T2 tuber weight (H), proportional increase in T2 tuber nodes (I) and proportional increase in T2 tuber eyes (J). Data from few and multi-eyed and Dutch Florex Gold have been pooled. Results are presented as either transformed data, with back transformed means in brackets, or untransformed data. Error bars indicate LSD.

Flower results: Micropropagating tissue culture plants on media containing an elevated concentration of 6-BAP or TDZ had little effect on subsequent flower weight ($P = 0.180$) and flower stem length ($P = 0.919$) (Figure 2.14). There was some evidence of an effect on spathe length ($P = 0.116$). Flowers from plants micropropagated on media supplemented with 13.6 μM TDZ, had shorter flower spathes than plants micropropagated on media supplemented with 4.4, 13.3 or 44.4 μM 6-BAP. The number of days from planting until flowering varied between selections micropropagated on various media ($P < 0.001$) (Figure 2.14). Plants micropropagated in the presence of 13.3 μM 6-BAP or 9.1 μM TDZ media flowered in significantly shorter time than those plants tissue cultured on media supplemented with 4.4 or 44.4 μM 6-BAP or 13.6 μM TDZ.

Desirable flower quality and colour and the percentage of flowering tubers at least 25 g was fairly consistent between plants micropropagated on all 6-BAP and TDZ containing MS media (Figure 2.15). Photographs of the multi-eyed selection micropropagated in the presence of 44.4 and 26.6 μM 6-BAP, showing comparable colour to multi-eyed plants micropropagated on standard media, are shown in Figure 2.5. Plants micropropagated on media supplemented with 44.4 μM 6-BAP or 4.5 μM TDZ produced the least flowers with desirable quality (62.0% and 57.3% respectively). Plants micropropagated on media supplemented with 44.4 μM 6-BAP produced the least flowers with desirable colour (72.9%), and produced amongst the least number of flowers per flowering plant (1.5). Plants micropropagated on media supplemented with 4.4 μM 6-BAP (standard media) had the greatest percentage of tubers at least 25 g that flowered (39.2%) (Figure 2.15).

Shoot results: Plants grown on control MS medium (4.4 μM 6-BAP) had 89.5% of tubers with at least five primary shoots (Figure 2.16). Plants grown on all other 6-BAP containing media had fewer tubers with at least five primary shoots. Plants grown on media supplemented with 9.1 and 13.6 μM TDZ had an increased percentage of primary shoots at least five, 92.6% and 100% respectively (Figure 2.16).

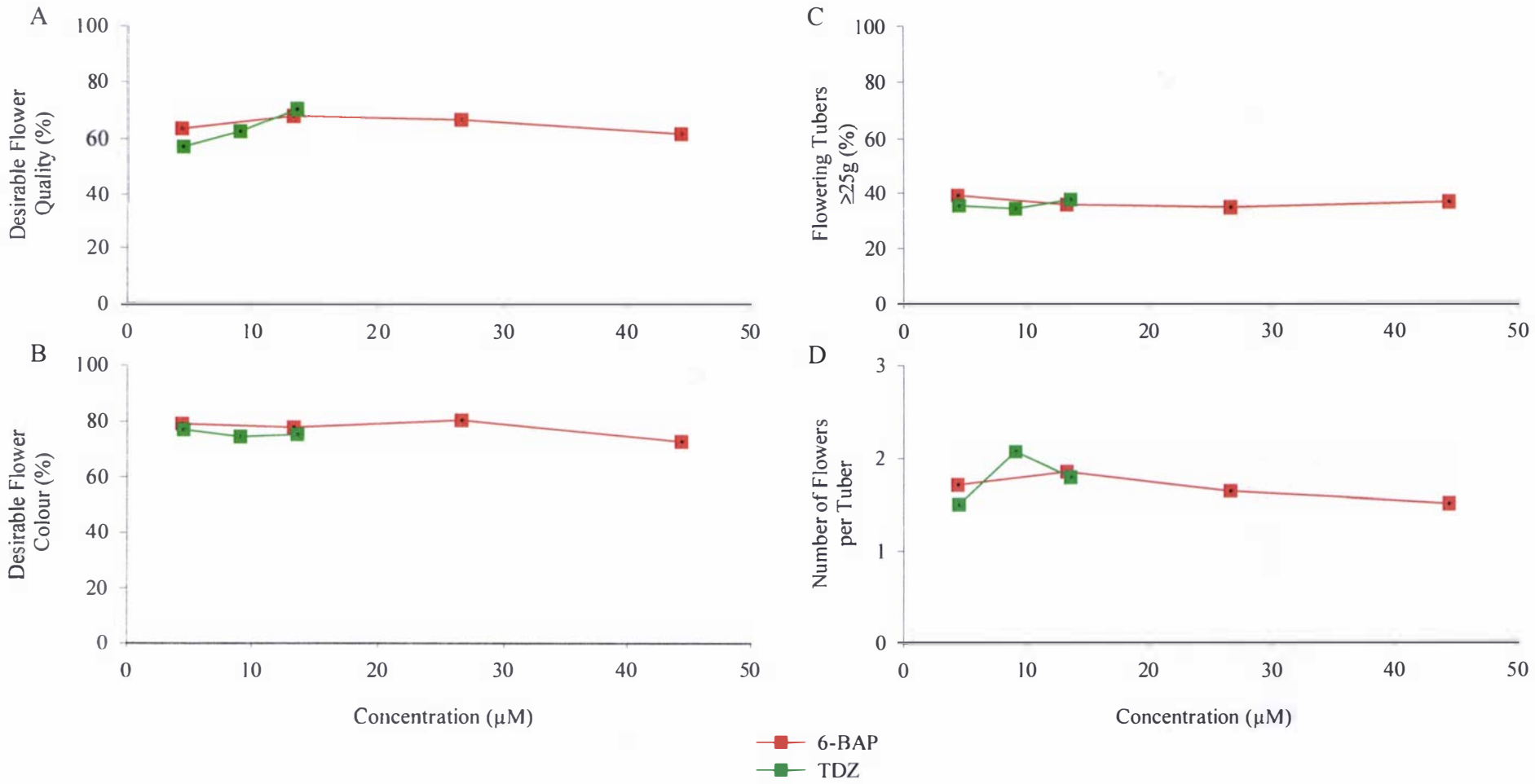


Figure 2.15: The effect of micropropagation media containing different concentrations of 6-BAP and TDZ on individual bushy characteristics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C), and the number of flowers per flowering plant (D). Data from few and multi-eyed and Dutch Florex Gold plants have been pooled.

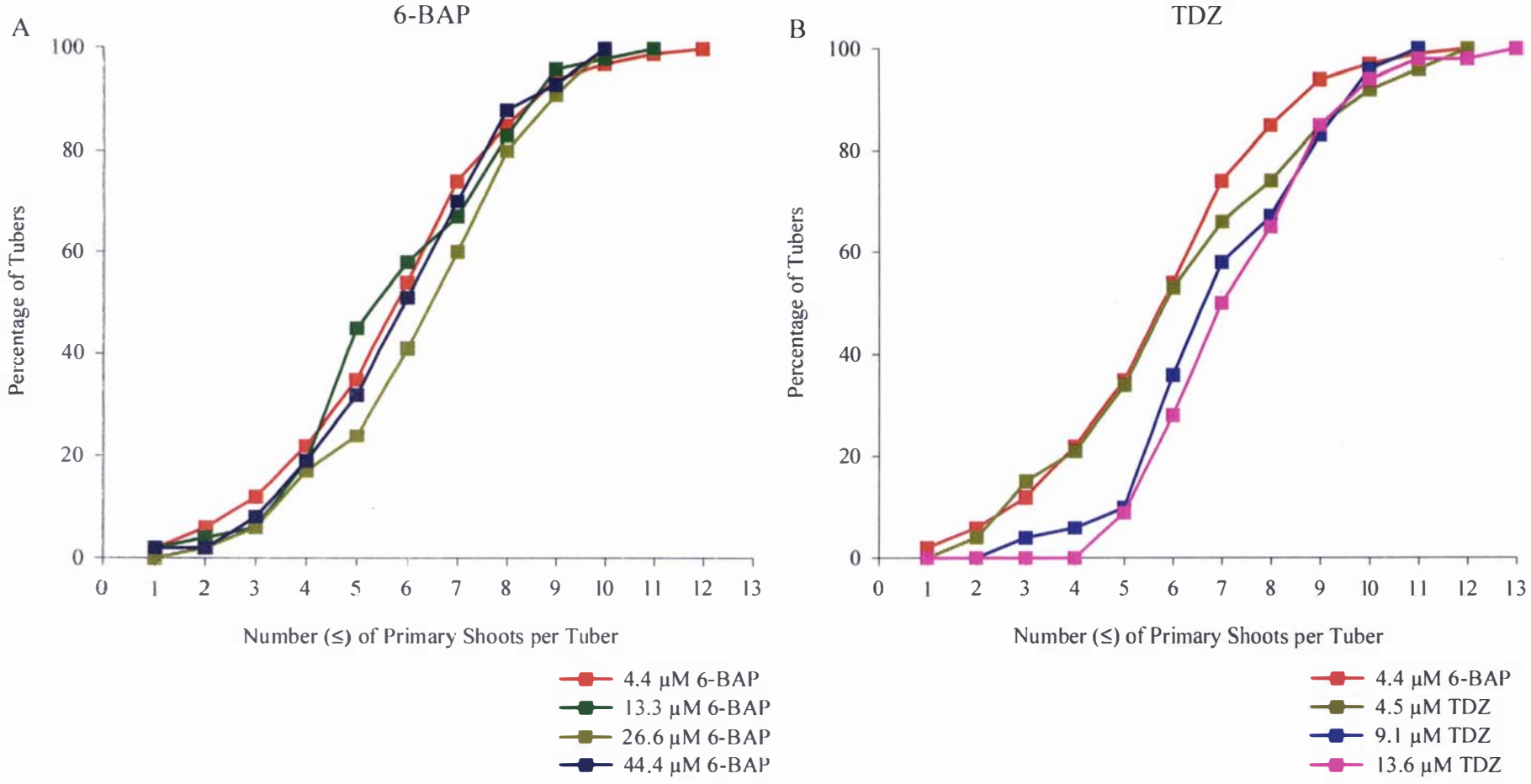


Figure 2.16: The effect of microprogation media containing different concentrations of 6-BAP and TDZ on individual bushy characteristics: the number (≤) of primary shoots per tuber. Results from plants grown on 4.4, 13.3, 26.6 and 44.4 µM 6-BAP are presented in graph A. Results from plants grown 4.5, 9.1 and 13.6 µM TDZ, and 4.4 µM 6-BAP (control) are presented in graph B. Data from few and multi-eyed and Dutch Florex Gold selections were pooled.

T2 tuber results: Significant differences were found between plants micropropagated on the various media in the proportional increase in the T2 tuber weight ($P < 0.001$), the proportion increase in the number of T2 tuber nodes ($P < 0.001$) and the proportional increase in the number of T2 tuber eyes ($P = 0.050$) (Figure 2.14). Plants micropropagated on low cytokinin containing MS media (4.4 μM 6-BAP) and media supplemented with 4.5 μM TDZ had a proportionally greater increase in T2 tuber weight than those micropropagated on elevated cytokinin containing MS media. Plants micropropagated on 4.4 μM or 44.4 μM 6-BAP (the lowest and highest cytokinin levels investigated) had proportionally more nodes than those plants micropropagated on media supplemented with 13.3 μM 6-BAP, 9.1 or 13.6 μM TDZ. Plants micropropagated on media supplemented with 13.3 μM 6-BAP, 4.5 or 13.6 μM TDZ had proportionally more T2 tuber eyes than those plants micropropagated in the presence of 9.1 μM TDZ.

2.3.4 Experiment Three – The influence of temperature and light levels on the development of the bushy syndrome

Experiment Three investigated the effect of different light and temperature growing environments on the expression of the bushy syndrome. Two light environments were chosen, 50% shading (750 μmol m^{-2} s^{-1}) and 80% shading (300 μmol m^{-2} s^{-1}), and two temperature environments, heated greenhouse and unheated screen house, in a 2 x 2 factorial (Table 2.1). A total of 4028 T1 tubers, 386 flowers and 815 T2 tubers were analysed in Experiment Three. The chi-square statistics are summarised in Table 2.5. The effect of each temperature and light factorial level, on each selection (Section 2.3.4.1) and the effect of each temperature and light factorial level on individual bushy characteristics (Section 2.3.4.2) is discussed.

2.3.4.1 The response of individual selections to each temperature and light factorial level and the development of the bushy syndrome

T1 tuber results: There was no evidence to suggest a selection treatment interaction effect on tuber weight ($P = 0.998$), the number of tuber nodes ($P = 0.482$), or the number of tuber eyes ($P = 0.263$) (Figure 2.17).

Flower results: There was not a significant interaction between treatment and selection on flower weight ($P = 0.415$), spathe length ($P = 0.563$) or the number of days until flowering ($P = 0.061$) (Figure 2.17). There was a significant interaction between treatment and selection ($P = 0.027$) on flower stem length. The Dutch, few and multi-eyed flowers grown in the greenhouse at 300 μmol m^{-2} s^{-1} were generally taller than flowers grown at all other factorial levels. The Dutch and the few-eyed flowers grown in the greenhouse at 750 μmol m^{-2} s^{-1}, were significantly shorter than the multi-eyed flowers grown in the same environment.

Table 2.5: Chi-square probability values of the effect of growing temperature (heated greenhouse and unheated screen house) and light levels (300 and 750 μmol m^{-2} s^{-1})(2x2 factorial) on individual bushy characteristics: T1 tuber weight, T1 tuber nodes, T1 tuber eyes, flower weight, flower stem length, spathe length, number of days from planting until flowering, proportional increase in T2 tuber weight, proportional increase in T2 tuber nodes and proportional increase in T2 tuber eyes of few and multi-eyed and Dutch Florex Gold selections. The degrees of freedom are listed (df).

	df	T1 Tuber Data			Flower Data				T2 Tuber Data		
		Weight	Nodes	Eyes	Weight	Flower stem length	Spathe length	Number of days from planting until flowering	Proportional increase in weight	Proportional increase in nodes	Proportional increase in eyes
Selection	2	$P = 0.002$	$P < 0.001$	$P = 0.188$	$P = 0.172$	$P < 0.001$	$P = 0.034$	$P = 0.560$	$P = 0.178$	$P = 0.051$	$P = 0.654$
Temperature and light treatments	3	$P = 0.031$	$P < 0.001$	$P = 0.250$	$P < 0.001$	$P < 0.001$	$P = 0.233$	$P < 0.001$	$P = 0.666$	$P = 0.036$	$P = 0.485$
Selection and temperature and light interaction	6	$P = 0.998$	$P = 0.482$	$P = 0.263$	$P = 0.415$	$P = 0.027$	$P = 0.563$	$P = 0.061$	$P = 0.774$	$P = 0.795$	$P = 0.764$

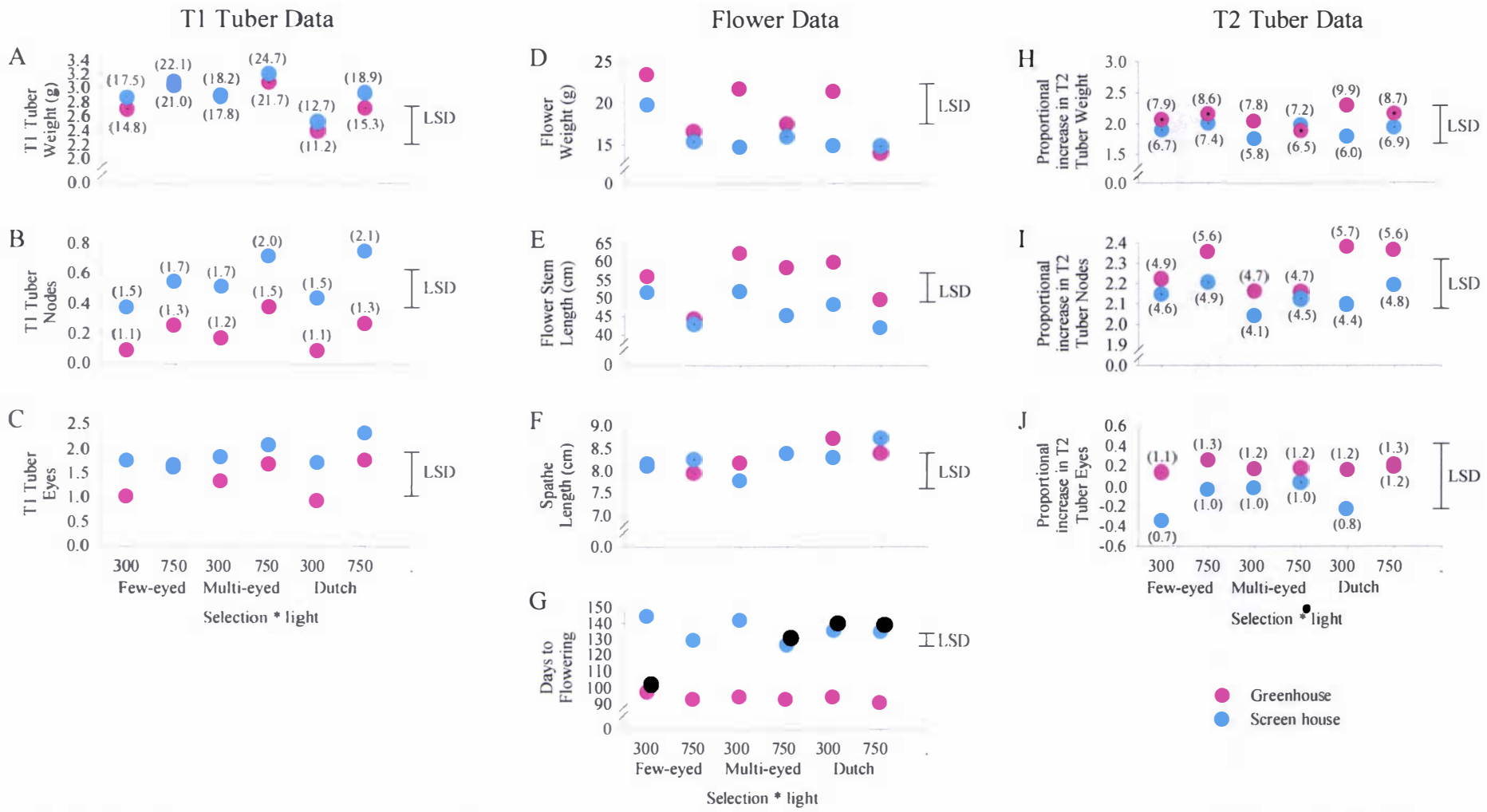


Figure 2.17: The effect of growing temperature (heated greenhouse and unheated screen house) and light levels (300 and 750 µmol m^{-2} s^{-1})(2x2 factorial) on individual bushy characteristics: T1 tuber weight (A), T1 tuber nodes (B), T1 tuber eyes (C), flower weight (D), flower stem length (E), spathe length (F), number of days from planting until flowering (G), proportional increase in T2 tuber weight (H), proportional increase in T2 tuber nodes (I) and proportional increase in T2 tuber eyes (J). Data is presented for few and multi-eyed and Dutch Florex Gold Selections. Results are presented as either transformed data with back transformed data in brackets, or untransformed data. Error bars indicate average LSD value.

There was variation in the percentage of flowers with desirable flower quality or desirable colour (Figure 2.18). The two highest scores for percentage of desirable quality were Dutch flowers grown in the screen house at 300 μmol m^{-2} s^{-1} (91.7%) and multi-eyed flowers grown in the greenhouse at 300 μmol m^{-2} s^{-1} (79.3%). The three highest scores for desirable colour were multi-eyed flowers (98.2%), and few-eyed flowers (86.7%) and Dutch flowers (83.3%), all grown in a screen house at 750 μmol m^2 s^{-1}. The two highest percentages of tubers at least 25 g that flowered were Dutch tubers grown in a screen house at 750 μmol m^{-2} s^{-1} (61.5%) and few-eyed tubers grown in a screen house at 300 μmol m^{-2} s^{-1} (60%) (Figure 2.18).

T2 tuber results: There was no evidence to suggest a significant treatment selection effect on the proportional increase in T2 tuber weight ($P = 0.774$), the proportional increase in T2 tuber nodes ($P = 0.795$) and the proportional increase in T2 tuber eyes ($P = 0.764$) (Figure 2.17).

2.3.4.2 The effect of each temperature and light factorial level in the expression of individual characteristics of the bushy syndrome

T1 tuber results: Analysis of T1 tuber data showed there were significant interactions between light and temperature on T1 tuber weight ($P = 0.031$) and the number of tuber nodes ($P < 0.001$) (Figure 2.19). There was no evidence to suggest a difference in the number of tuber eyes ($P = 0.250$) (Figure 2.19). Tissue culture plants grown in the unheated screen house at 750 μmol m^{-2} s^{-1}, produced heavier tubers with more nodes than those grown at 300 μmol m^{-2} s^{-1} in both the greenhouse and the screen house.

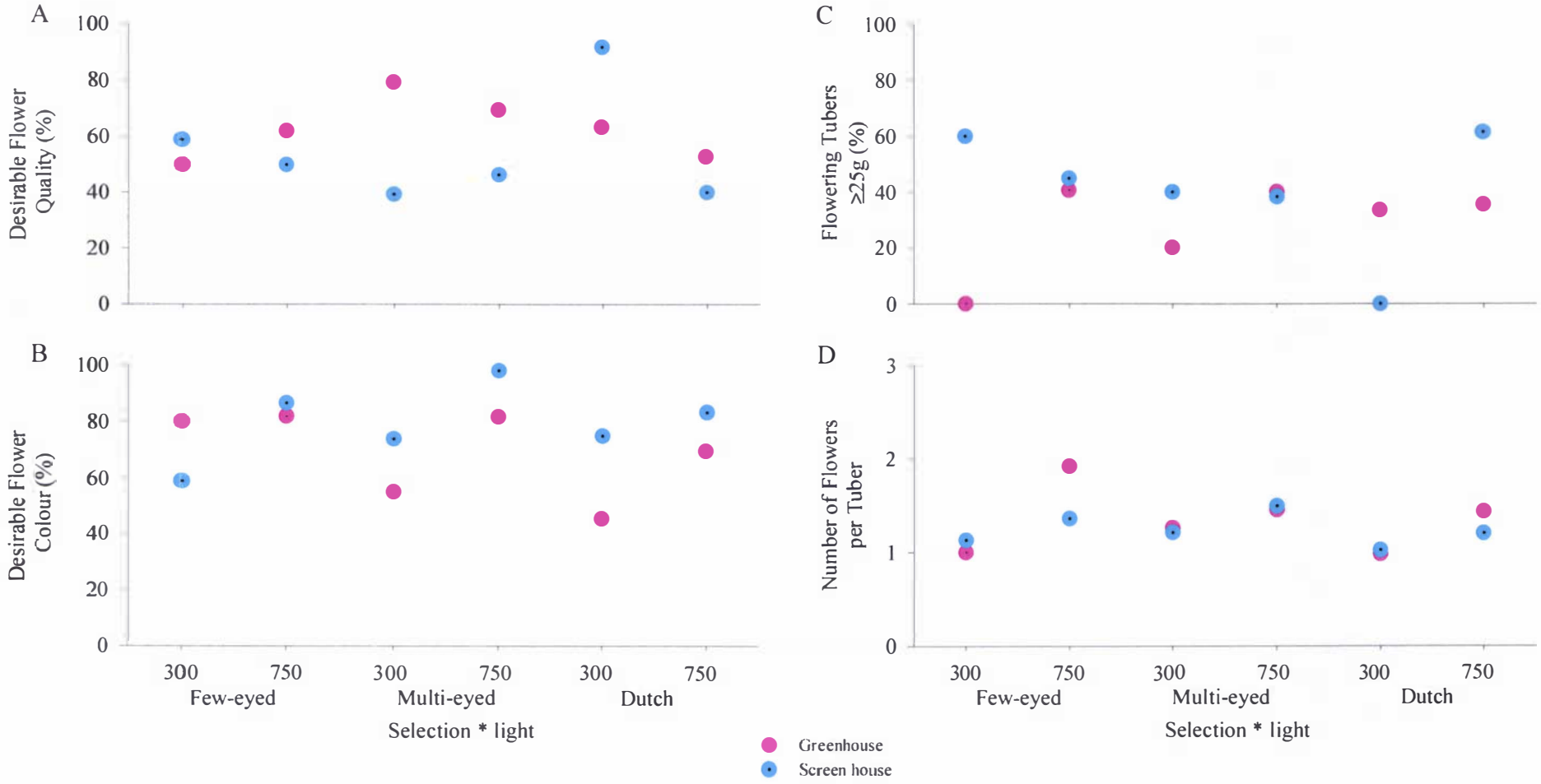


Figure 2.18: The effect of growing temperature (heated greenhouse and unheated screen house) and light levels (300 and 750 μmol m^{-2} s^{-1})(2x2 factorial) on individual bushy characteristics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C) and the number of flowers per flowering plant (D), in few and multi-eyed and Dutch Florex Gold selections.

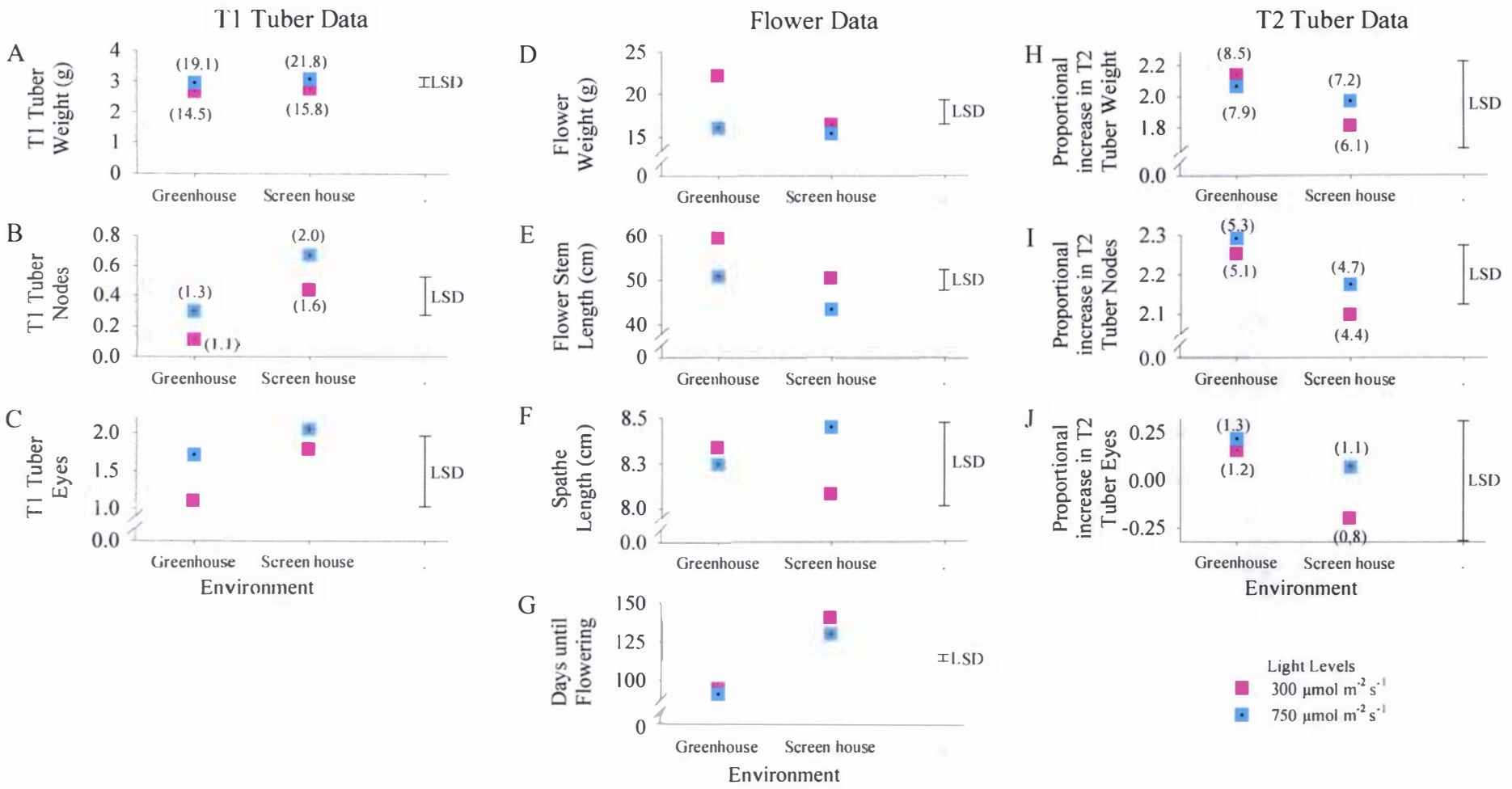


Figure 2.19: The effect of growing temperature (heated greenhouse and unheated screen house) and light levels (300 and 750 µmol m^{-2} s^{-1})(2x2 factorial) on individual bushy characteristics: T1 tuber weight (A), T1 tuber nodes (B), T1 tuber eyes (C), flower weight (D), flower stem length (E), spathe length (F), number of days from planting until flowering (G), proportional increase in T2 tuber weight (H), proportional increase in T2 tuber nodes (I) and proportional increase in T2 tuber eyes (J). Results from few and multi-eyed and Dutch Florex Gold selections have been pooled. Results are presented as either transformed data, with transformed means in brackets. or as untransformed data. Error bars indicate LSD.

Flower results: There was a significant difference between plants grown in different environments in flower weight ($P < 0.001$) (Figure 2.19). Flowers from all plants grown in the unheated screen house, as well as those plants grown the greenhouse at 750 μmol m^{-2} s^{-1}, were all significantly lighter than those grown in the greenhouse at 300 μmol m^{-2} s^{-1}. There was a significant treatment effect on flower stem length ($P < 0.001$) (Figure 2.19). Plants grown in the screen house at 750 μmol m^{-2} s^{-1}, were significantly shorter than flowers from plants grown in all other environments. Flowers from plants grown in the screen house at 300 μmol m^{-2} s^{-1} as well as those grown in the greenhouse at 750 μmol m^{-2} s^{-1}, were significantly shorter than those grown in the greenhouse at 300 μmol m^{-2} s^{-1}. There was no evidence for a treatment effect on spathe length ($P = 0.233$) (Figure 2.19). There was a significant treatment effect on the number of days from planting until flowering ($P < 0.001$) (Figure 2.19). All plants grown in the greenhouse flowered in shorter time than those grown in a screen house. Plants grown in the screen house at 750 μmol m^{-2} s^{-1}, flowered in shorter time than those grown at 300 μmol m^{-2} s^{-1}.

The flowers with highest quality, were grown in the heated greenhouse (63.7% and 66.0%, 750 and 300 μmol m^{-2} s^{-1} respectively) (Figure 2.20). The most flowers with desired colour was grown in the 750 μmol m^{-2} s^{-1} light environments (91.4% and 79.0%, screen house and greenhouse respectively) (Figure 2.20). The percentage of tubers at least 25 g that flowered was slightly higher in the unheated screen house (41.7% and 44.8%) (Figure 2.20). The most flowers per flowering tuber were 1.7 in the greenhouse at 750 μmol m^{-2} s^{-1} (Figure 2.20).

T2 tuber results: There was no evidence to suggest a treatment effect on the proportional increase in the T2 tuber weight ($P = 0.666$), or the proportional increase in the number of T2 tuber eyes ($P = 0.485$) (Figure 2.19). There was evidence to suggest a significant effect on the proportional increase in the number of T2 tuber nodes ($P = 0.036$). The number of T2 tuber nodes decreased when plants were grown in an unheated screen house at 300 μmol m^{-2} s^{-1}, compared to those plants grown in the greenhouse.

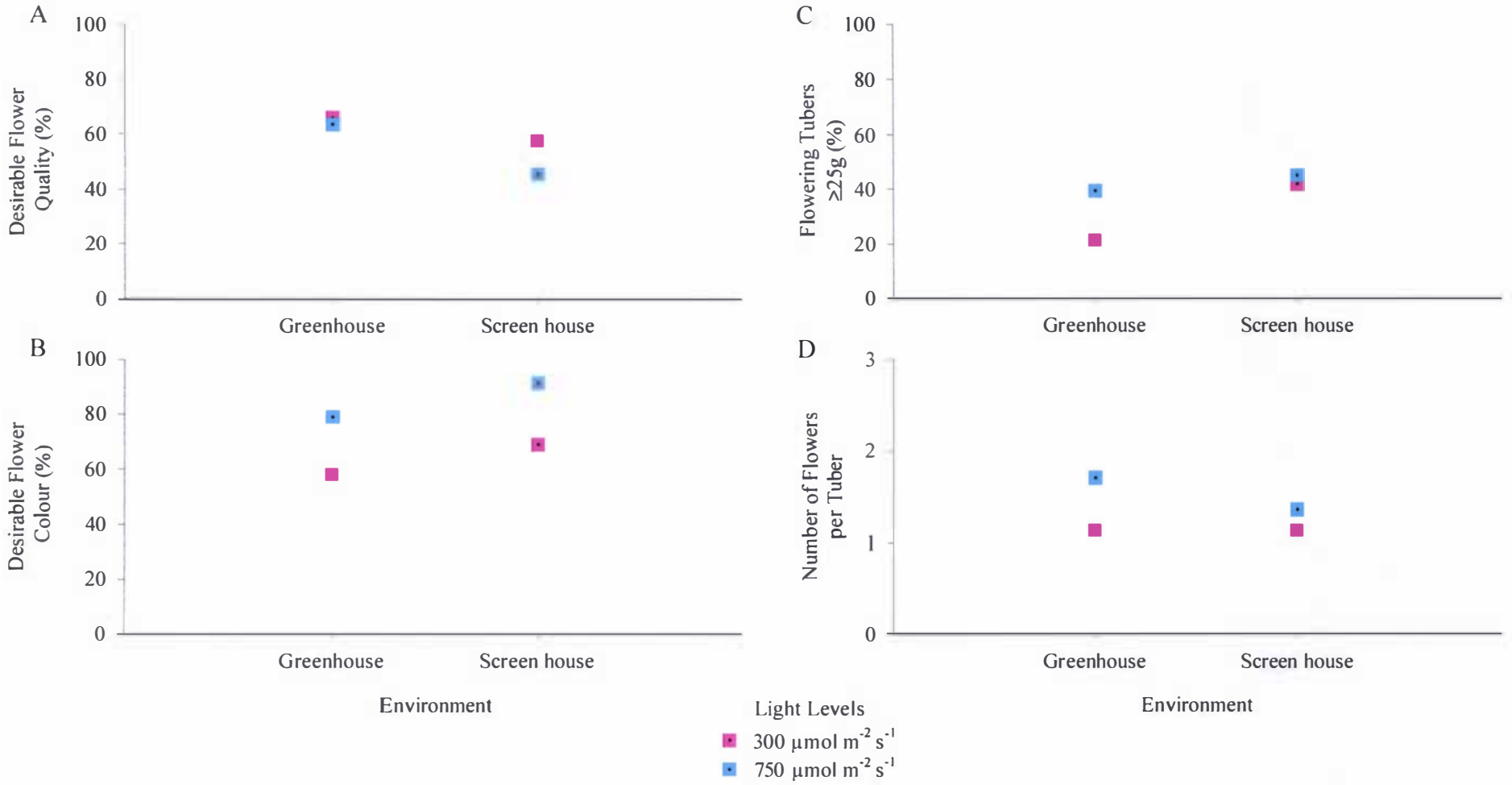


Figure 2.20: The effect of growing temperature (heated greenhouse and unheated screen house) and light levels (300 and 750 µmol m^{-2} s^{-1})(2x2 factorial) on individual bushy characterisitics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C), and the number of flowers per flowering plant (D). Data from few and multi-eyed and Dutch Florex Gold selections have been pooled.

2.3.5 Experiment Four – The influence of the storage environment on the development of the bushy syndrome

Experiment Four examined the effect of different tuber storage environments on the expression of the bushy syndrome. Two temperatures were investigated, 9°C and 15°C, and two time periods, 11 or 21 weeks, in a 2 x 2 factorial. During Experiment Four 416 flowers and 624 T2 tubers were harvested and analysed. The chi-square statistics are summarised in Table 2.6. The effect of each storage temperature and duration factorial level on each selection (Section 2.3.5.1) and the effect of each storage temperature and duration factorial level on individual bushy characteristics (Section 2.3.5.2) are outlined.

2.3.5.1 The response of individual selections to each storage temperature and duration factorial level and the development of the bushy syndrome

Flower results: Assessment of flower data indicated a significant treatment selection effect on flower weight ($P < 0.001$), stem length ($P = 0.001$) and the number of days between planting and flowering ($P = 0.033$) (Figure 2.21). There was no treatment selection effect on spathe length ($P = 0.274$). Few-eyed and Dutch flowers from T1 tubers stored for 21 weeks at 15°C were heavier, than few-eyed and Dutch flower from tubers stored in other environments (Figure 2.21). Flowers from few-eyed T1 tubers stored for 21 weeks at 15°C, and multi-eyed and Dutch tubers stored for 21 weeks at both 15°C and 9°C had significantly longer stems than the same selection stored for 11 weeks. Multi-eyed tubers stored for 21 weeks and few-eyed tubers stored for 21 weeks at 15°C flowered in significantly shorter time than those stored for 11 weeks. Dutch T1 tubers stored for 21 weeks at 9°C flowered in significantly shorter time than those stored for 11 weeks, and 21 weeks at 15°C. Multi and few-eyed T1 tubers stored for 21 weeks at 15°C flowered in significantly shorter time than Dutch T1 tubers stored in the same environment. Multi-eyed T1 tubers stored for 21 weeks at 9°C flowered in significantly shorter time than few-eyed T1 tubers stored in the same environment (Figure 2.21).

Table 2.6: Chi-square probability values of the effect of storage temperature (9 or 15 °C) and duration (11 or 21 weeks)(2x2 factorial) on individual bushy characteristics: T1 tuber weight, T1 tuber nodes, T1 tuber eyes, flower weight, flower stem length, spathe length, number of days from planting until flowering, proportional increase in T2 tuber weight, proportional increase in T2 tuber nodes and proportional increase in T2 tuber eyes of few and multi-eyed and Dutch Florex Gold selections. The degrees of freedom are listed (df).

	df	Flower Data				T2 Tuber Data		
		Weight	Flower stem length	Spathe length	Number of days from planting until flowering	Proportional increase in weight	Proportional increase in nodes	Proportional increase in eyes
Selection	2	$P < 0.001$	$P = 0.001$	$P = 0.581$	$P = 0.020$	$P = 0.444$	$P = 0.638$	$P = 0.190$
Storage treatments	3	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.053$
Selection and storage interaction	6	$P < 0.001$	$P = 0.001$	$P = 0.274$	$P = 0.033$	$P = 1.000$	$P = 0.050$	$P = 0.163$

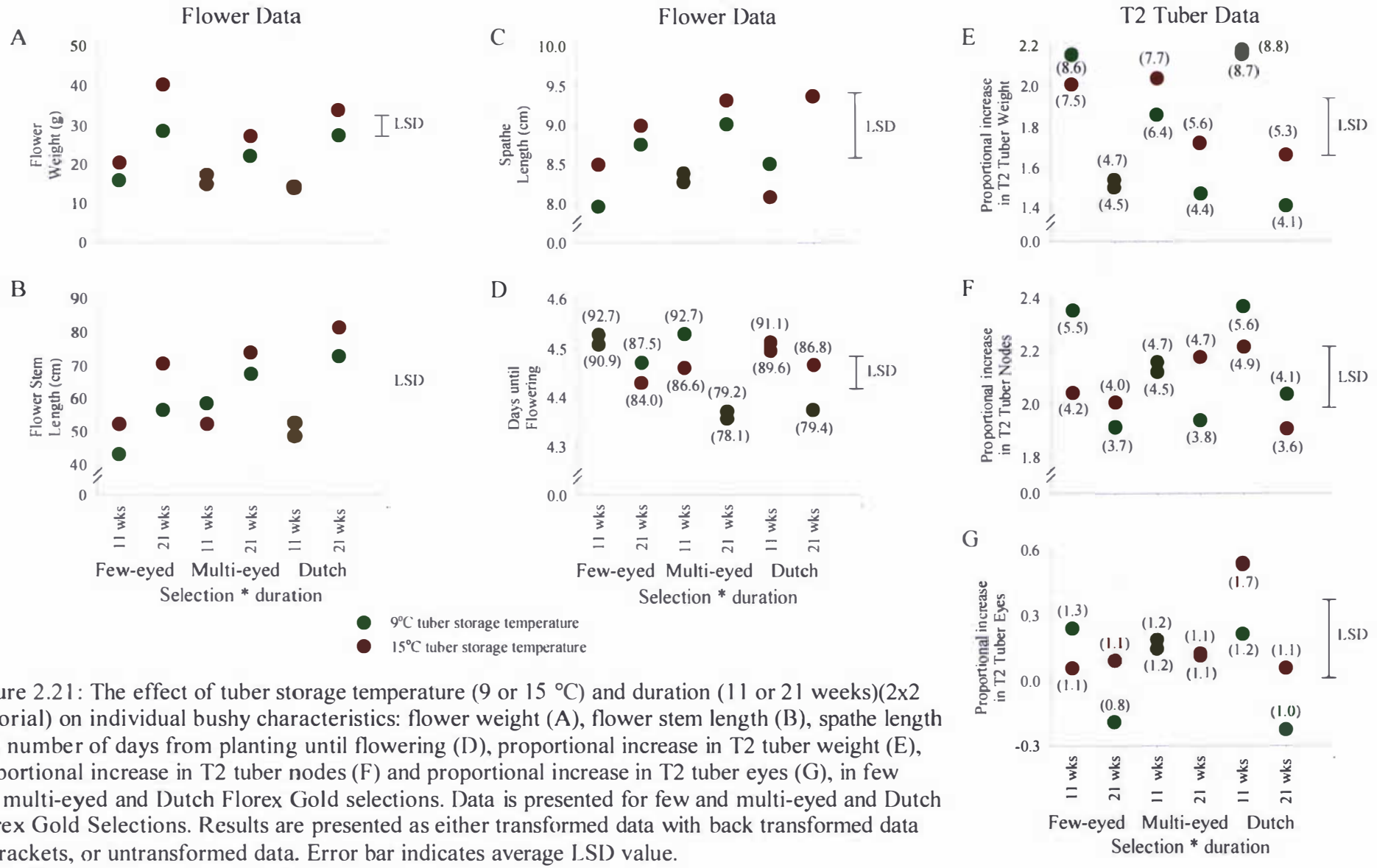


Figure 2.21: The effect of tuber storage temperature (9 or 15 °C) and duration (11 or 21 weeks)(2x2 factorial) on individual bushy characteristics: flower weight (A), flower stem length (B), spathe length (C), number of days from planting until flowering (D), proportional increase in T2 tuber weight (E), proportional increase in T2 tuber nodes (F) and proportional increase in T2 tuber eyes (G), in few and multi-eyed and Dutch Florex Gold selections. Data is presented for few and multi-eyed and Dutch Florex Gold Selections. Results are presented as either transformed data with back transformed data in brackets, or untransformed data. Error bar indicates average LSD value.

Flowers from Dutch T1 tubers stored for 11 weeks at 15°C, and multi-eyed T1 tubers stored at 9°C for 11 and 21 weeks had the highest quality (71.9%, 69.5% and 72% respectively) (Figure 2.22). Dutch T1 tubers stored for 11 weeks at 15°C and few and multi-eyed tubers stored for 21 weeks at 9°C had the highest percentage of desirable coloured flowers (87.5%, 100% and 88.0% respectively). Dutch T1 tubers stored for 21 weeks at 15°C had the lowest number of flowers with desirable colour (38.5%). Dutch T1 tuber stored for 21 weeks at 9°C and multi-eyed T1 tubers stored for 21 weeks at 9°C and 15°C all had the highest percentage of tubers at least 25 g that flowered (approximately 50%). Few-eyed T1 tubers stored for 11 weeks at 9°C and multi-eyed tubers stored for 11 weeks at 9°C and 15°C had the most of flowers per flowering tuber (1.9, 1.7 and 1.7 respectively) (Figure 2.22).

T2 tuber results: There was evidence to suggest a significant treatment selection effect on the proportional increase in T2 tuber nodes ($P = 0.050$) (Figure 2.21). There was no evidence to suggest a significant treatment selection effect on the proportional increase in T2 tuber weight ($P = 0.100$) or T2 tuber eyes ($P = 0.163$). Dutch tubers stored at 15°C for 21 weeks had significantly fewer nodes than Dutch tubers stored for 11 weeks. Dutch tubers stored at 15°C for 21 weeks had significantly fewer nodes than multi-eyed tubers stored in the same environment. Few-eyed tubers stored for 21 weeks at 9 °C had fewer nodes than few-eyed tubers stored for 11 weeks at 9°C. Multi-eyed tubers stored for 21 weeks at 9°C had fewer nodes than multi-eyed tubers stored for 21 weeks at 15°C.

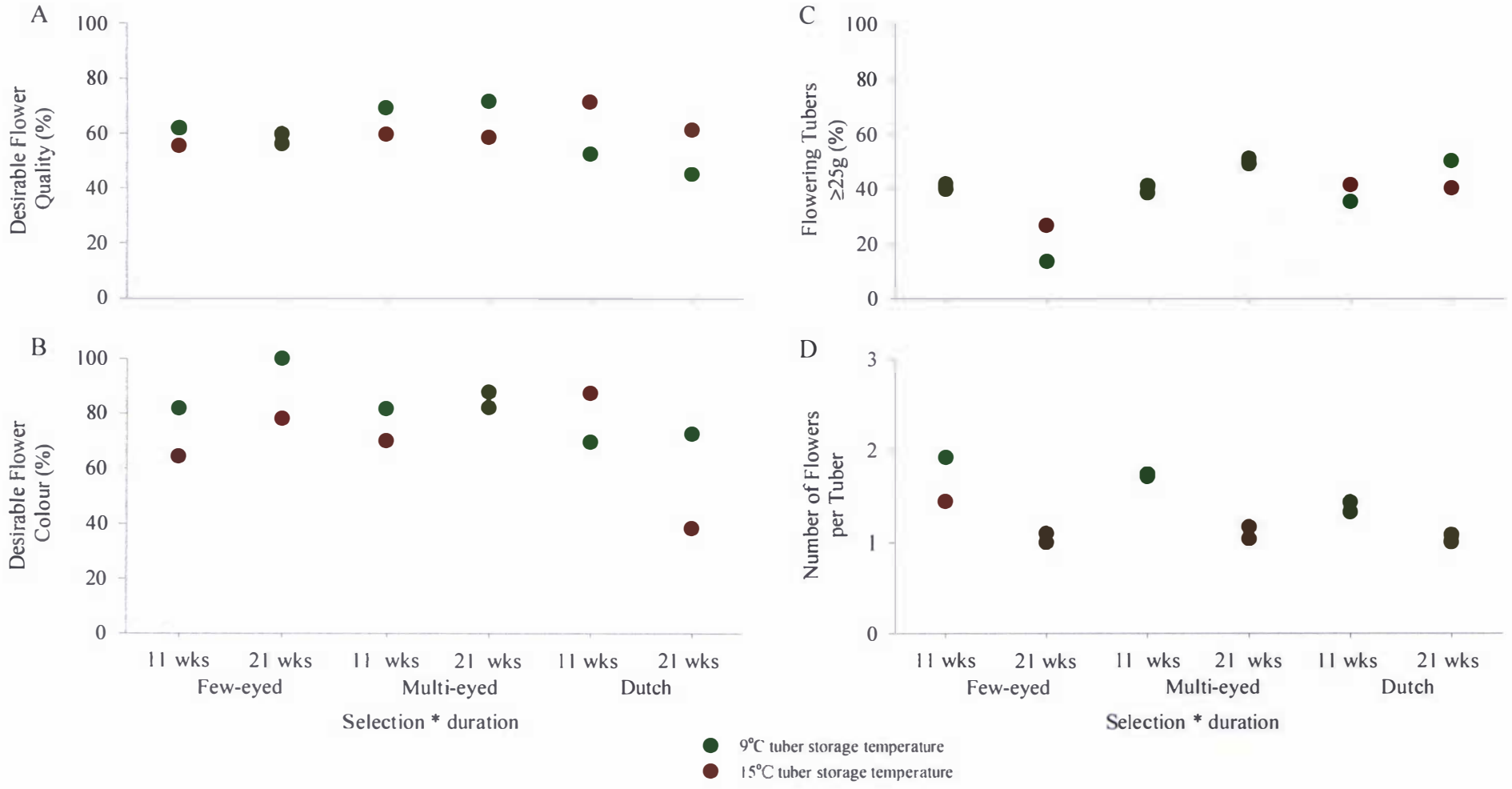


Figure 2.22: The effect of tuber storage temperature (9 or 15 °C) and duration (11 or 21 weeks)(2x2 factorial) on individual bushy characteristics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C) and the number of flowers per flowering plant (D), in few and multi-eyed and Dutch Florex Gold selections.

2.3.5.2 The effect of each storage temperature and duration factorial level on the expression of individual characteristics of the bushy syndrome

Flower results: The analysis of T1 tuber data indicated that there was a significant storage treatment effect on flower weight ($P < 0.001$), flower stem length ($P < 0.001$) and spathe length ($P < 0.001$) (Figure 2.23). Flowers from T1 tubers stored for 21 weeks at both 9°C and 15°C were significantly heavier, taller and had longer spathes than those tubers stored for 11 weeks. Flowers from tubers stored for 21 weeks at 15°C were heavier and taller than from those tubers stored at 9°C for 21 weeks. There was a significant storage treatment effect on the number of days from planting until flowering ($P < 0.001$) (Figure 2.23). T1 tubers stored for 21 weeks flowered in fewer days than those stored for 11 weeks.

The percentage of flowers with desirable flower quality and colour and the percentage of tubers at least 25 g that flowered were fairly similar between storage factorial treatments (Figure 2.24). Those tubers stored at 9°C had the most flowers with desirable colour 85.4% (21 weeks) and 79.2% (11 weeks). Tubers stored for 11 weeks produced a greater number of flowers per flowering tuber, 1.7 (9°C) and 1.5 (15°C), than those tubers stored for 21 weeks (Figure 2.24).

T2 tuber results: There was a significant storage treatment effect on the proportional increase in T2 tuber weight ($P < 0.001$) (Figure 2.23). The weight of tubers stored for 11 weeks were proportionally more than those stored for 21 weeks. The proportional increase in T2 tubers stored for 21 weeks at 9°C was significantly lower than all other treatments. There was a significant treatment effect on the proportional increase in the number of T2 tuber nodes ($P < 0.001$) (Figure 2.23). T1 tubers stored for 21 weeks had a significantly lower increase in nodes than those stored for 11 weeks at 9°C. T1 tubers stored for 21 weeks at 9°C had a significantly lower increase in the number of nodes than those stored for 11 weeks at both 9°C and 15°C. T1 tubers stored for 11 weeks at 15°C had proportionally fewer nodes than those stored for 11 weeks at 9°C. There was some evidence of a treatment effect on the proportional increase in the number of T2 tuber eyes ($P = 0.053$) (Figure 2.23). T2 tubers stored for 21 weeks at 9°C had a proportionally lower increase in the number of eyes than T2 tubers stored for 11 weeks at both temperatures.

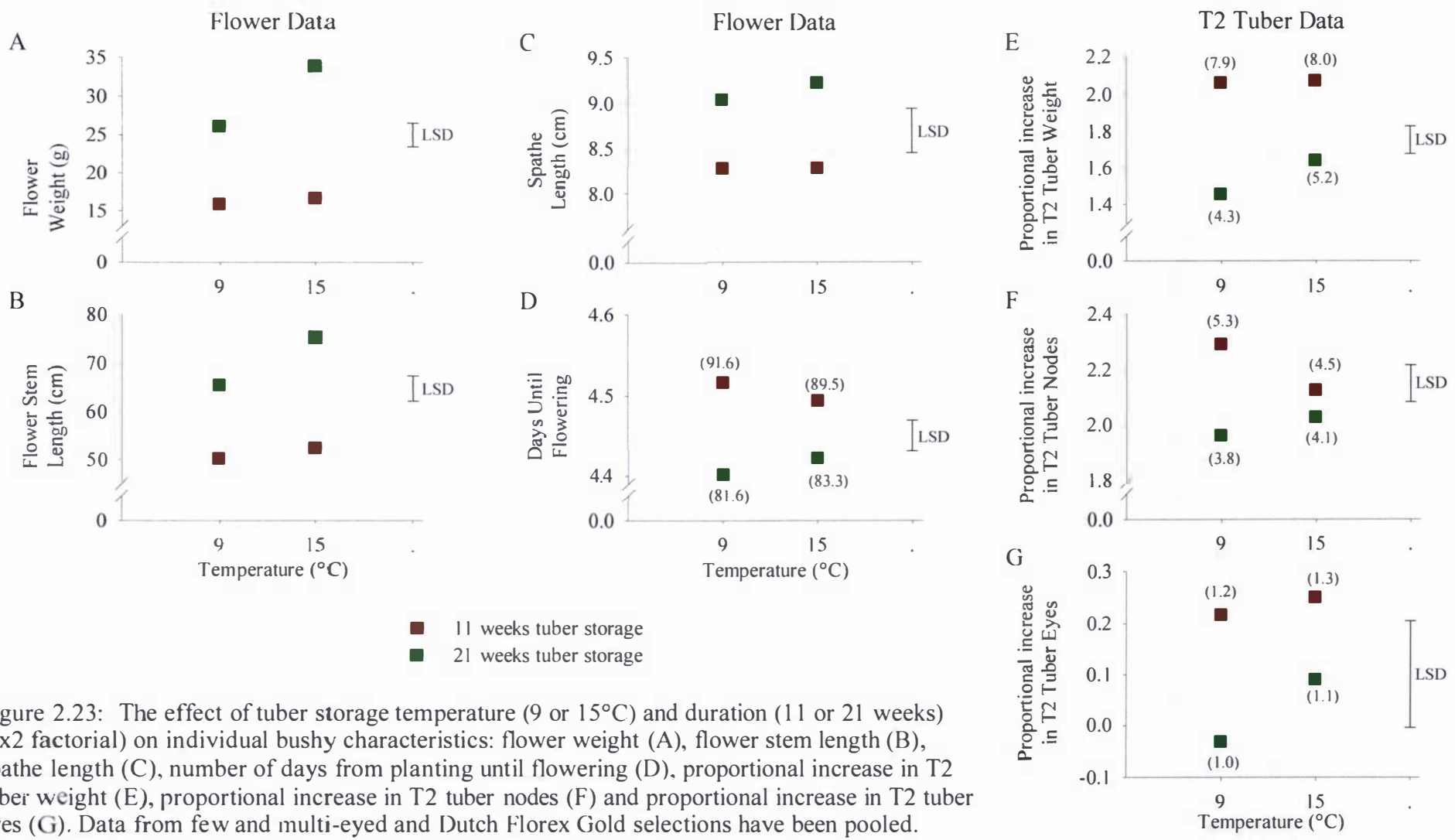


Figure 2.23: The effect of tuber storage temperature (9 or 15°C) and duration (11 or 21 weeks) (2x2 factorial) on individual bushy characteristics: flower weight (A), flower stem length (B), spathe length (C), number of days from planting until flowering (D), proportional increase in T2 tuber weight (E), proportional increase in T2 tuber nodes (F) and proportional increase in T2 tuber eyes (G). Data from few and multi-eyed and Dutch Florex Gold selections have been pooled. Results are presented as either transformed data, with back transformed means in brackets, or untransformed data. Error bars indicate LSD value.

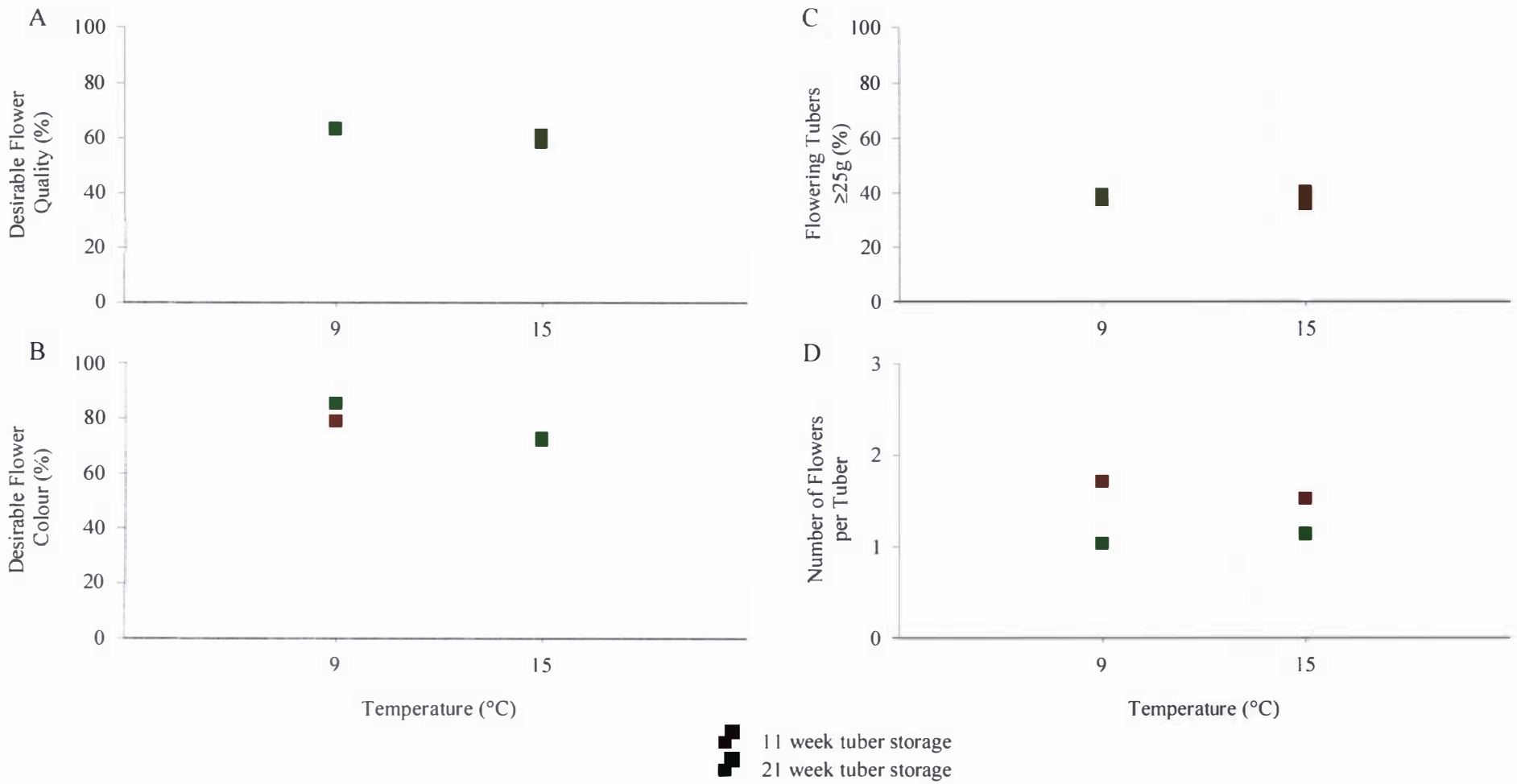


Figure 2.24: The effect of tuber storage temperature (9 or 15°C) and duration (11 or 21 weeks)(2x2 factorial) on individual bushy characteristics: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C), and the number of flowers per flowering plant (D). Data from few and multi-eyed and Dutch Florex Gold selections were pooled.

2.3.6 The expression of individual characteristics of the bushy syndrome in original mother genotypes

Individual characteristics of the bushy syndrome were examined in original mother selections (Selection 2.3.6.1) and individual mother tubers (Section 2.3.6.2) and this information helped to develop the bushy criteria. The chi-square statistics are listed in Table 2.7. The plants in Experiment One to Four were micropropagated from these original mother tubers.

2.3.6.1 The expression of individual characteristics of the bushy syndrome in original mother selections

Flower results: There was a significant difference between selections in flower weight ($P < 0.001$) (Figure 2.25). The control Florex Gold had significantly heavier flowers than all other mother selections. Best Gold had significantly heavier flowers than the Dutch, few-eyed and multi-eyed Florex Gold selections. There were no significant differences in the flower stem length between different mother selections ($P = 0.232$). Differences in spathe length were found between different selections ($P < 0.001$). The spathes of the control Florex Gold were significantly longer than the Dutch and the few-eyed Florex Gold selections. There was no evidence to suggest a difference in the number of days from planting until flowering between selections ($P = 0.108$) (Figure 2.25).

Table 2.7: Chi-square probability values of the individual bushy characteristics of Best Gold and Florex Gold mother selections: flower weight, flower stem length, spathe length and number of days from planting until flowering. The degrees of freedom are listed (df).

	df	Flower Data			
		Weight	Flower stem length	Spathe length	Number of days from planting until flowering
Selection	3	$P < 0.001$	$P = 0.232$	$P < 0.001$	$P = 0.108$
Tuber	-	-	-	-	-

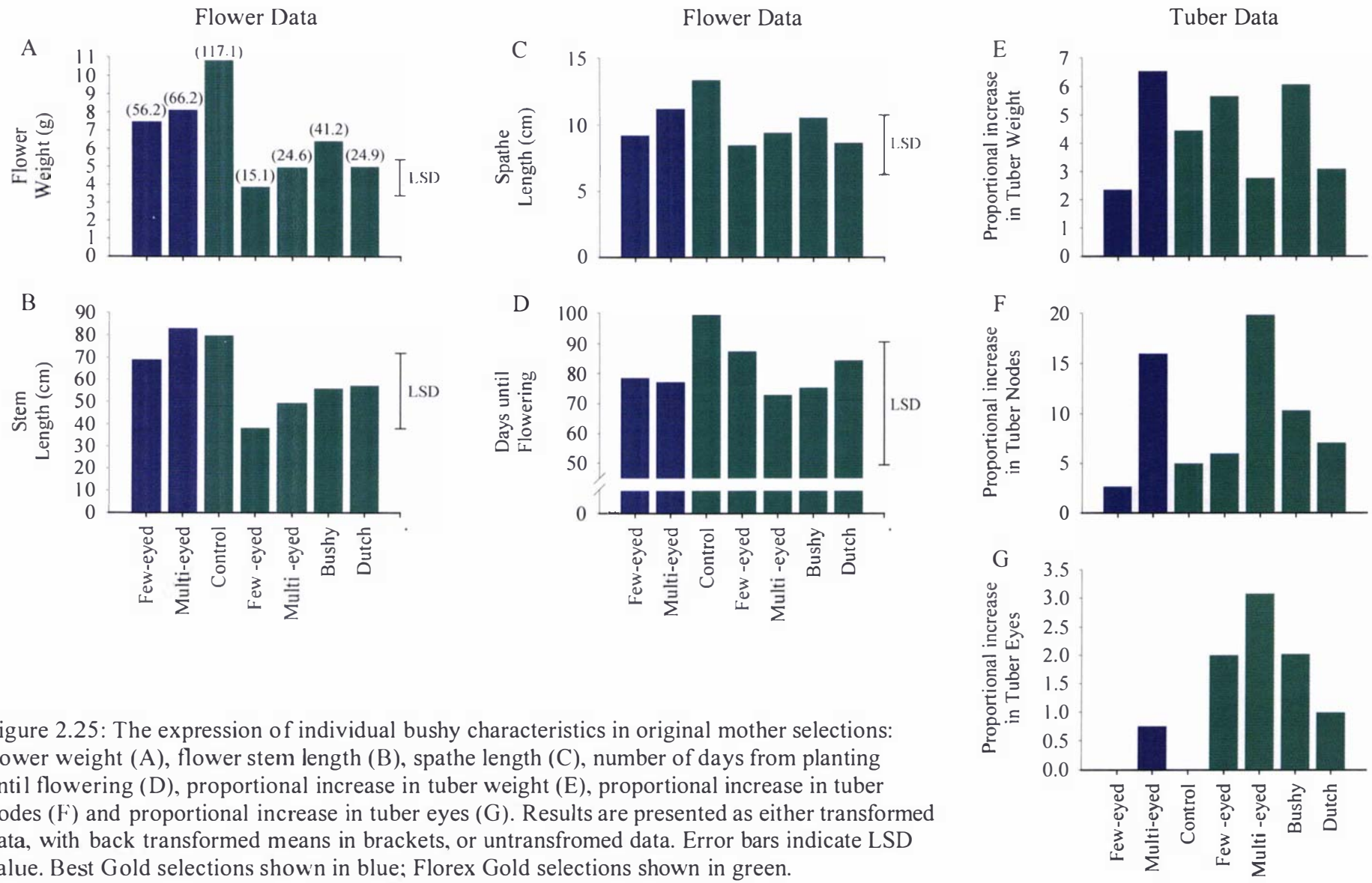


Figure 2.25: The expression of individual bushy characteristics in original mother selections: flower weight (A), flower stem length (B), spathe length (C), number of days from planting until flowering (D), proportional increase in tuber weight (E), proportional increase in tuber nodes (F) and proportional increase in tuber eyes (G). Results are presented as either transformed data, with back transformed means in brackets, or untransfromed data. Error bars indicate LSD value. Best Gold selections shown in blue; Florex Gold selections shown in green.

Plants from the Best Gold multi-eyed selections consistently produced high quality flowers (100%) (Figure 2.26). Flowers from the bushy Florex Gold were also of a high quality (83.3%). Only approximately half of flowers produced from the Dutch, few-eyed Best Gold and the few-eyed and multi-eyed Florex Gold selections were of high quality (63.6%, 50.0%, 50.0% and 50.0% respectively). The control Florex Gold selection produced the least high quality flowers (28.6%). Flowers from the few-eyed Florex Gold selection were consistently of the desired colour (100%). Only approximately half the flowers produced from the Dutch, control, bushy and multi-eyed Florex Gold were of the desired colour (63.6%, 57.1%, 66.7% and 66.7% respectively). Both Best Gold selections had a low percentage of the desired flower colour (few-eyed 33.3%, multi-eyed 25.0%). Most selections had 100% of tubers at least 25 g that flowered, with the exception of Dutch and bushy, which both had 83.3% of tubers, and multi-eyed which had 50.0%. The Florex Gold few-eyed, bushy, Dutch and Best Gold few-eyed produced between 1 to 2.2 flowers per tuber. The control Florex Gold produced 3.5, Best Gold multi-eyed 4 and Florex Gold multi-eyed 6 flowers per tuber (Figure 2.26).

Shoot results: Control Florex Gold had 100% of tubers with two or less primary shoots (Figure 2.27). Best Gold few-eyed had 66% of tubers with four or less primary shoots. All other mother selections had tubers with at least five primary shoots (Figure 2.27).

Tuber results: Best Gold multi-eyed tubers and bushy Florex Gold tubers had the greatest proportional increase in T2 tuber weights (Figure 2.25). Best Gold few-eyed and control Florex Gold tubers had the smallest proportional increase in T2 tuber nodes and eyes.

2.3.6.2 The expression of individual characteristics of the bushy syndrome in each original mother tuber

Flower results: Control Florex Gold, Best Gold few-eyed tuber C and Best Gold multi-eyed tuber B had the highest flower weight. Control Florex Gold and Best Gold few-eyed tuber A and B and Best Gold multi-eyed tuber C had the longest stems (Figure 2.28). Control Florex Gold and Best Gold multi-eyed tuber C and bushy tuber F and G had the longest spathes.

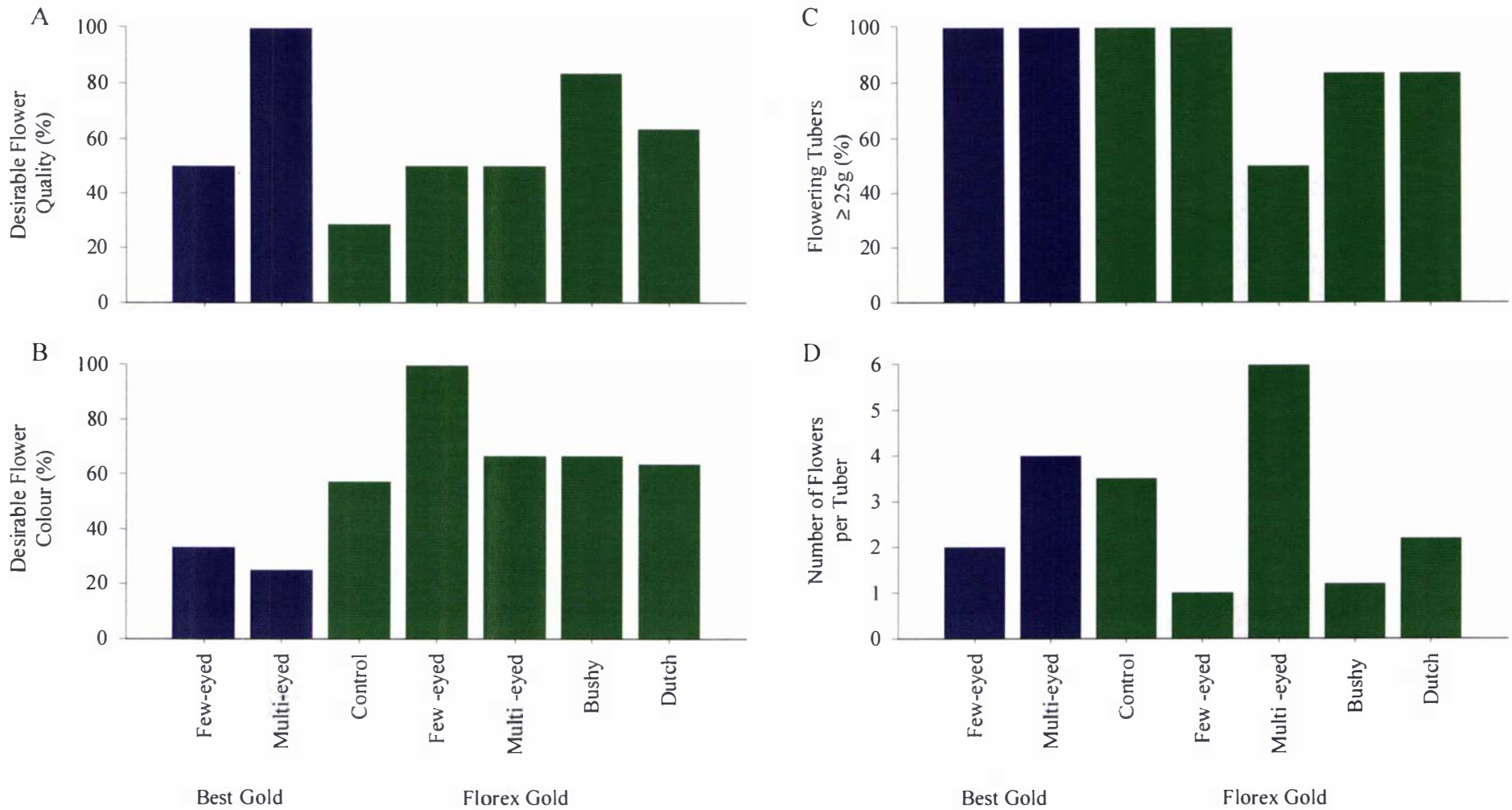


Figure 2.26: The expression of individual bushy characteristics in original mother selections: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C) and the number of flowers per flowering plant (D). Best Gold selections shown in blue. Florex Gold selections shown in green.

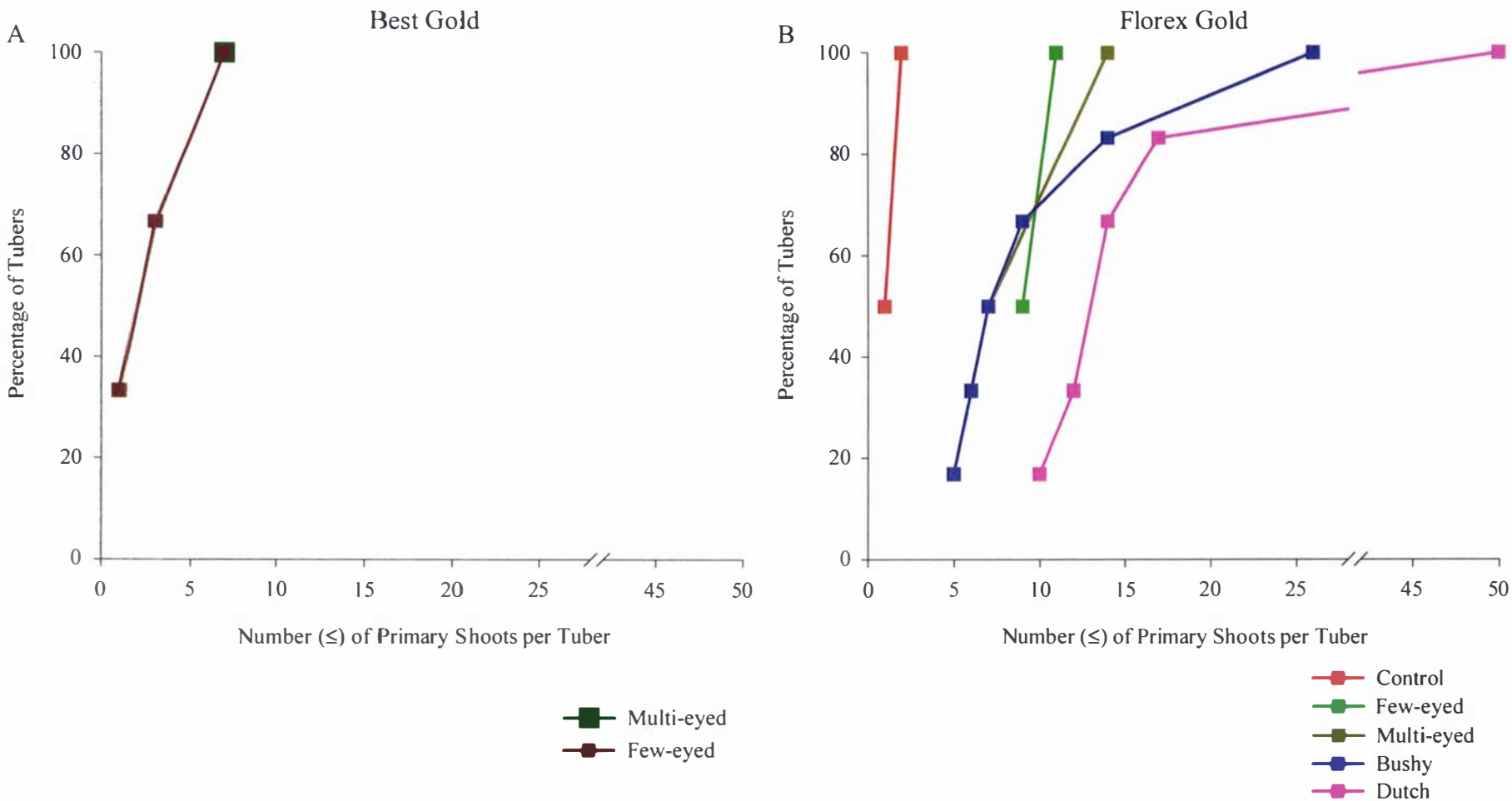


Figure 2.27: The expression of individual bushy characteristics in original mother selections: the number (≤) of shoots per tuber. Results from Best Gold plants are presented in A. Results from Florex Gold plants are presented in B.

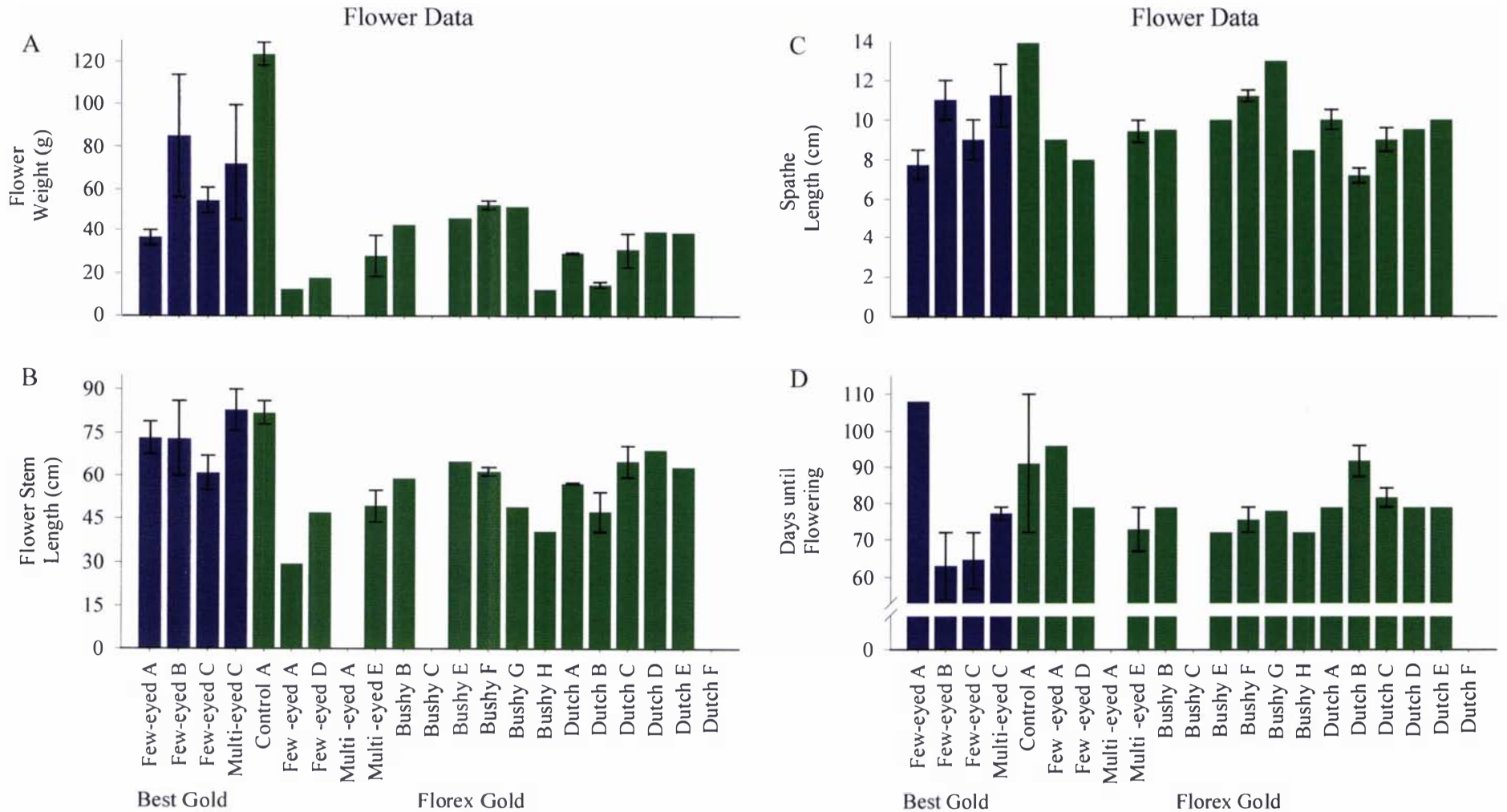


Figure 2.28: The response of each tuber on individual bushy characteristics in original mother selections: flower weight (A), flower stem length (B), spathe length (C), number of days from planting until flowering (D). Error bars when present indicate standard error, where no error bar is present, only one flower was produced. Best Gold selections shown in blue. Florex Gold selections shown in green.

Bushy Florex Gold tubers B, E, H and few-eyed tuber D produced the highest quality and desirable coloured flowers (100%) (Figure 2.29). The tubers of Dutch tuber F, bushy Florex Gold C and multi-eyed A were less than 25 g and did not produce any flowers.

Shoot results: With the exception of Best Gold few-eyed tubers A and B and control Florex Gold, all tubers had at least five primary shoots (Figure 2.30). Dutch tubers A and D had 16 primary shoots, Dutch tuber B had 17, and Dutch tuber F had 50 primary shoots. Bushy Florex Gold tuber H had 26 primary shoots.

Tuber results: Bushy tubers C and H and few-eyed tuber D had the greatest proportional increase in T2 tuber weight (Figure 2.31). Best Gold few-eyed tubers A and C had the lowest proportional increase in T2 tuber nodes. All the Best Gold tuber and control Florex Gold had the lowest proportional increase in T2 tuber eyes.

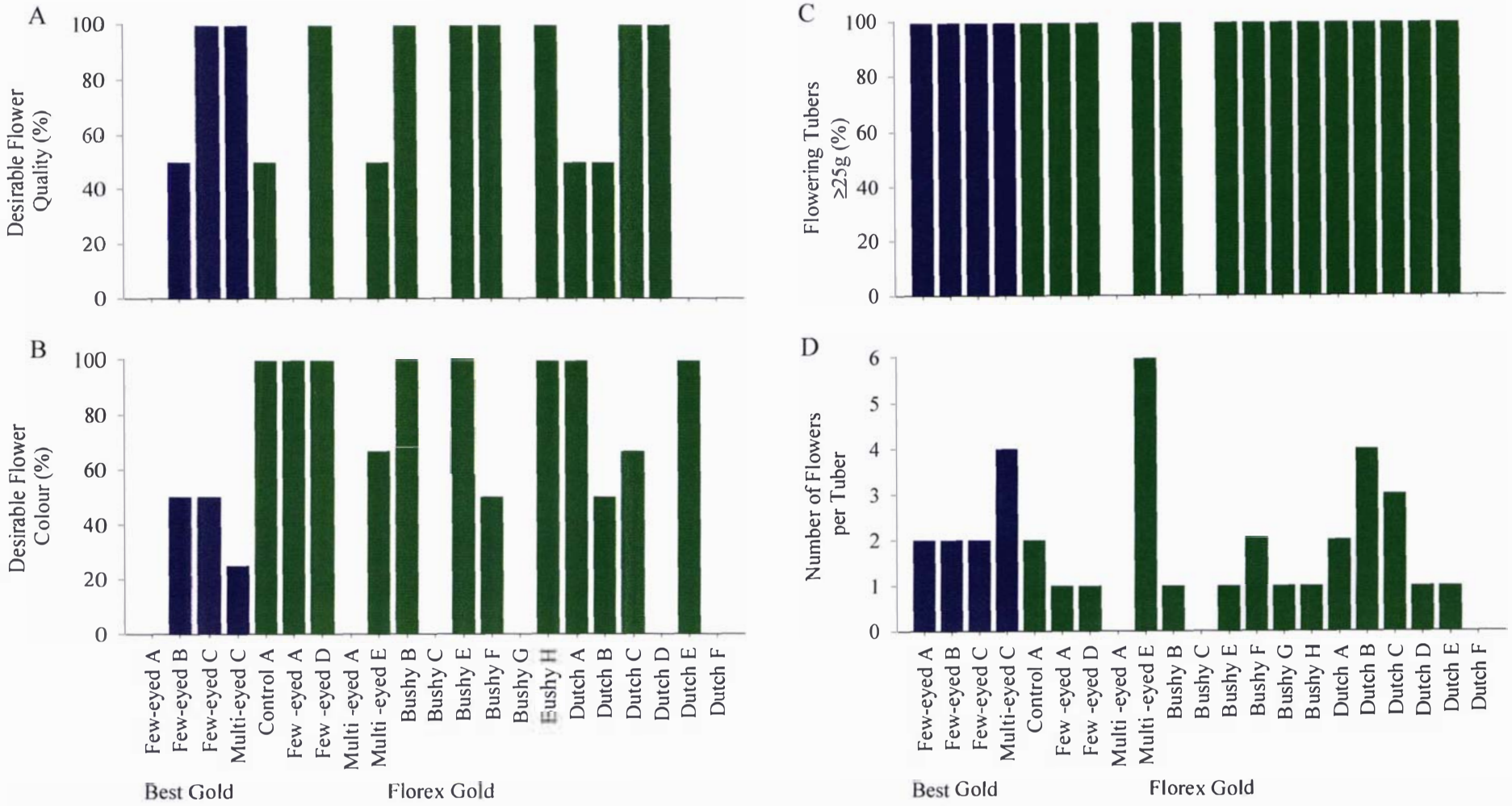


Figure 2.29: The response of each tuber on individual bushy characteristics in original mother selections: desirable flower quality (%)(A), desirable flower colour (%)(B), tubers (≥25g) that flowered (%)(C), and the number of flowers per flowering plant (D). Best Gold selections shown in blue. Florex Gold selections shown in green.

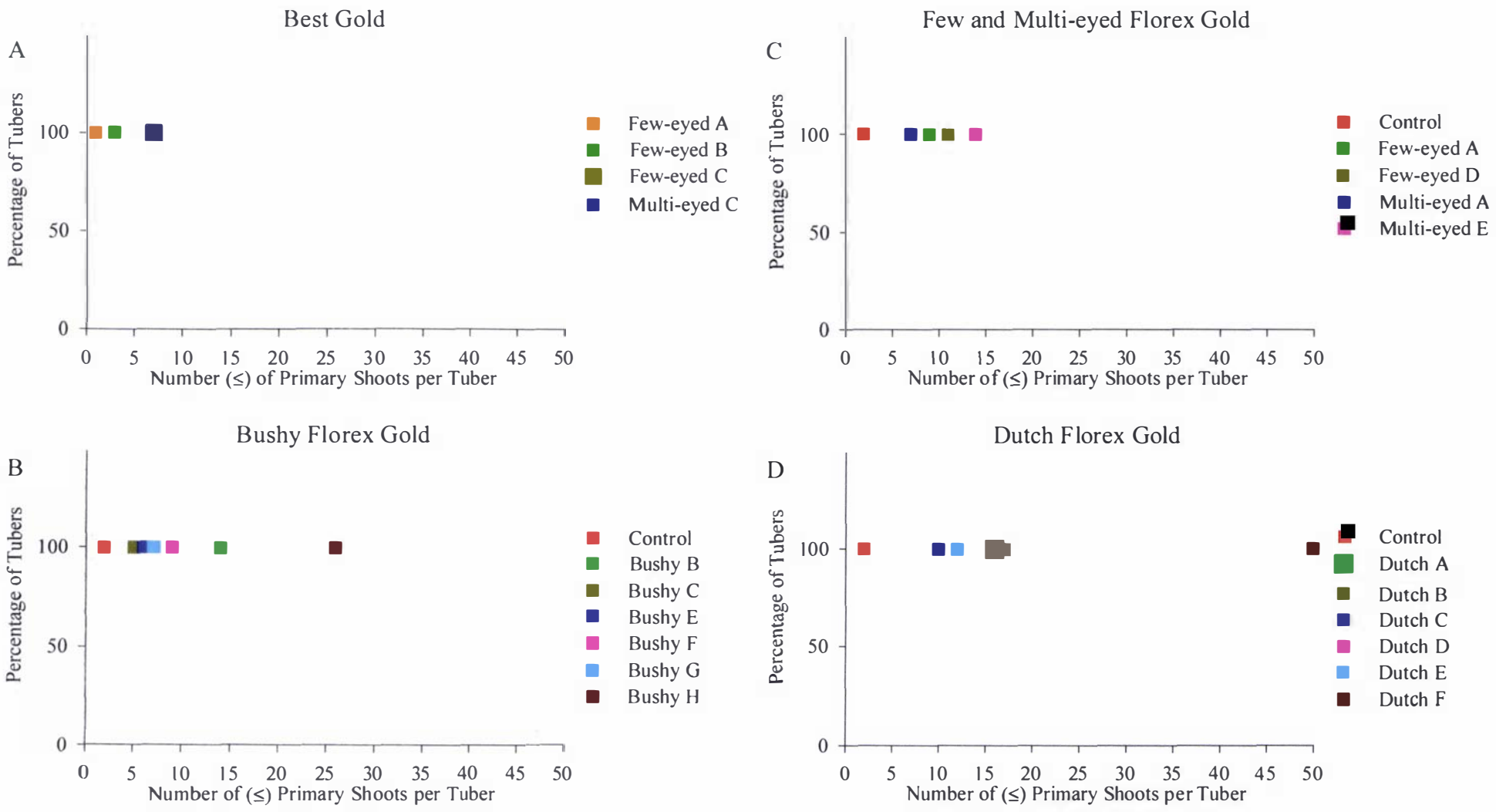


Figure 2.30: The response of each tuber on individual bushy characteristics in original mother selections: the number (≤) of primary shoots per tuber. Results from individual Best Gold plants are presented in A. Results from individual bushy Florex Gold plants are presented in B. Results from individual few-eyed and multi-eyed Florex Gold plants are presented in C. Results from individual Dutch bushy Florex Gold plants are presented in D. The control Florex Gold plant is presented in B, C and D.

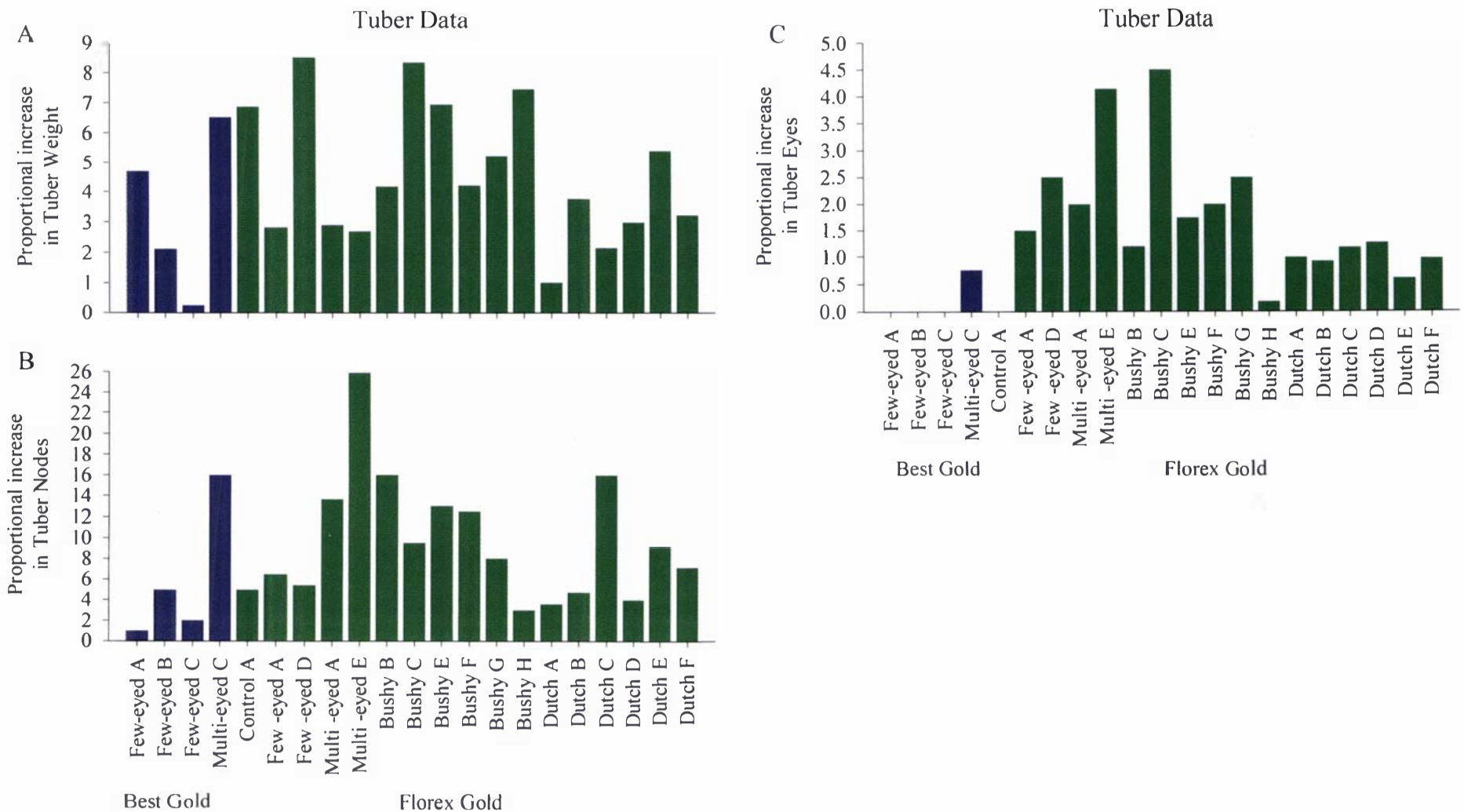


Figure 2.31: The response of each tuber on individual bushy characteristics in original mother selections: proportional increase in tuber weight (A), proportional increase in tuber nodes (B) and proportional increase in tuber eyes (C). Best Gold selections shown in blue. Florex Gold selections shown in green.

2.3.7 Edge Effect

The influence of plant location within the greenhouse on flowering is shown in Figure 2.32. Edge effect data is examined in Experiment One (Section 2.3.7.1), Experiment Two (Section 2.3.7.2), Experiment Three (Section 2.3.7.3) and Experiment Four (Section 2.3.7.4). The chi-square statistics are summarised in Table 2.8.

2.3.7.1 Experiment One – The role of the genotype on the expression of individual characteristics of the bushy syndrome

Analysis of flower data indicated that there was a significant edge effect on flower weight ($P < 0.001$) (Figure 2.33). Flowers from plants grown in position 4 were significantly heavier than those grown in position 1. There was a significant edge effect on flower stem length ($P < 0.001$). Flowers from plants grown in position 1 were significantly shorter than flowers from plants grown in position 2, 3 and 4. Flowers from plants grown in position 2 were significantly shorter than those grown in position 4. There was no evidence to suggest an edge effect on spathe length ($P = 0.510$) and the number of days from planting until flowering ($P = 0.281$) (Figure 2.33).

2.3.7.2 Experiment Two – The influence of elevated cytokinin (6-BAP and TDZ) on the expression of individual characteristics of the bushy syndrome

Assessment of flower data indicated that there was a significant edge effect ($P < 0.001$) on flower weight (Figure 2.33). The mean flower weight of those plants grown in position 1, 2 and 3 were all significantly lighter than those flowers grown in position 4. There was a significant edge effect ($P < 0.001$) on flower stem length. The flower stem length of those plants grown in position 1 and 2 was significantly shorter than those flowers grown in position 4. There was no evidence of an edge effect on flower spathe length due to edge effect ($P = 0.096$), or on the number of days to flowering ($P = 0.128$) (Figure 2.33).

Figure 2.32: Flower height in relation to plant position. Plants were allocated number one for the first or outer row, number two for the second row, number three for the third row and number four for the fourth and remaining rows.

Table 2.8: Chi-square probability values of individual bushy characteristics: flower weight, flower stem length, spathe length and number of days from planting until flowering, in relation to plant position. Plants were allocated number one for the first or outer row, number two for the second row, number three for the third row and number four for the fourth and remaining rows. The degrees of freedom are listed (df).

	df	Flower Data			
		Weight	Flower stem length	Spathe length	Number of days from planting until flowering
Experiment One	3	$P < 0.001$	$P < 0.001$	$P = 0.510$	$P = 0.281$
Experiment Two	3	$P < 0.001$	$P < 0.001$	$P = 0.096$	$P = 0.128$
Experiment Three	3	$P = 0.156$	$P < 0.001$	$P = 0.269$	$P = 0.987$
Experiment Four	3	$P = 0.067$	$P < 0.001$	$P = 0.631$	$P = 0.736$

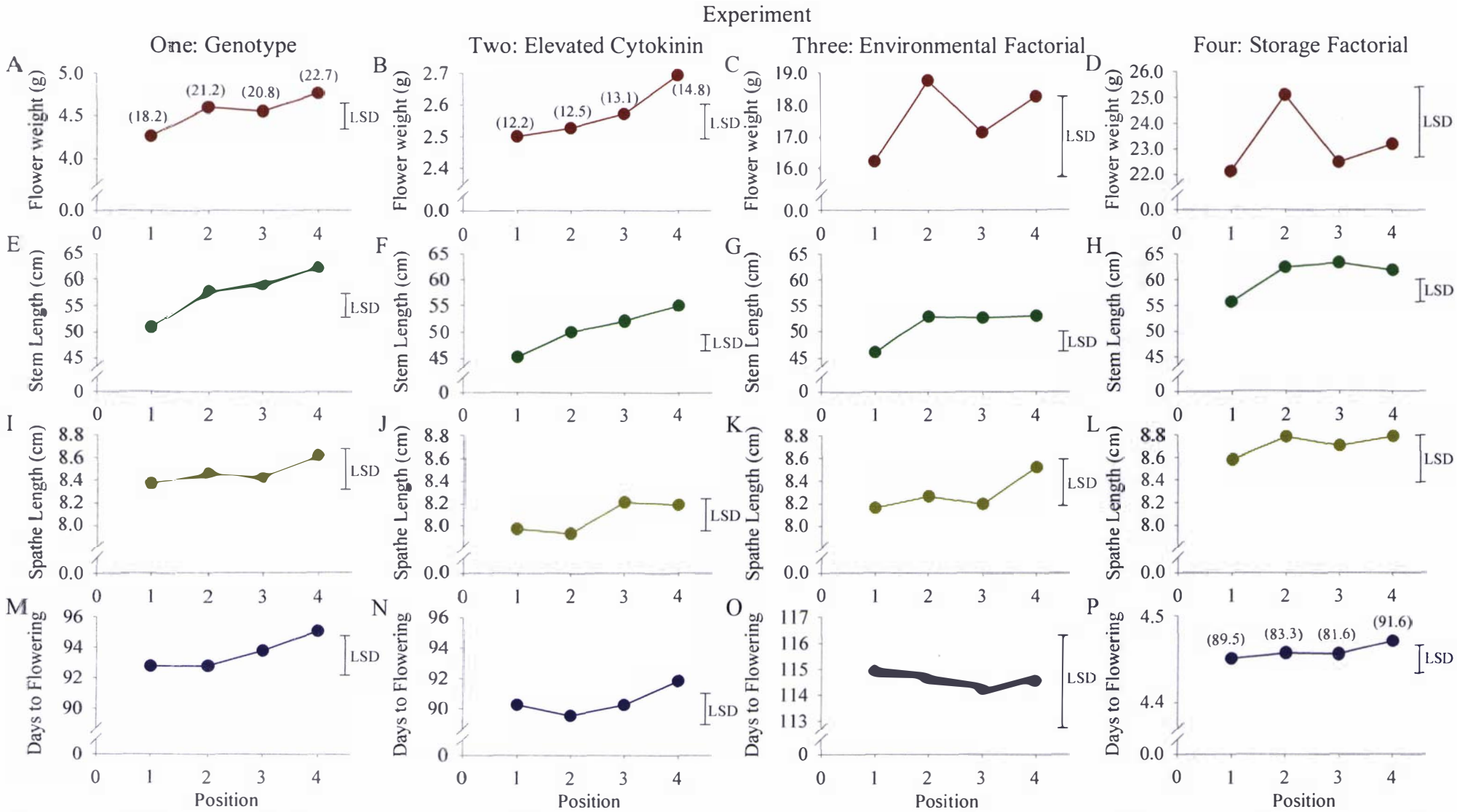


Figure 2.33: Flower weight (A), flower stem length (B), spathe length (C), and the number of days from planting until flowering (D), in relation to plant position. Plants were allocated number one for the first or outer row, number two for the second row, number three for the third row and number four for the fourth and remaining rows. Results from the Experiment One is presented in A, E, I and M, Experiment Two is presented in B, F, J and N, Experiment Three is presented in C, G, K and O and Experiment Four is presented in D, H, L and P. Results are presented as either transformed data, with back transformed means in brackets, or untransformed data. Error bars indicate LSD value.

2.3.7.3 Experiment Three – The influence of temperature and light levels on the expression of individual characteristics of the bushy syndrome

Examination of flower data showed a significant edge effect on stem length ($P < 0.001$) (Figure 2.33). Flowers from those plants grown in position 1 were significantly shorter than flowers from plants in position 2, 3 and 4. There was no evidence of an edge effect on flower weight ($P = 0.156$), spathe length ($P = 0.269$) or the number of days from planting until flowering ($P = 0.987$) (Figure 2.33).

2.3.7.4 Experiment Four – The influence of the storage environment on the expression of individual characteristics of the bushy syndrome

There was a significant edge effect ($P < 0.001$) on flower stem length. The plants grown in position 1 were significantly shorter than those grown in position 2, 3 and 4. There was no evidence of an edge effect on flower weight ($P = 0.067$), spathe length ($P = 0.631$) or the number of days from planting until flowering ($P = 0.736$) (Figure 2.33).

2.4 Discussion

2.4.1 Development of bushy criteria

The criteria against which individual plants were assessed for "bushiness" were developed from the individual characteristics displayed by the original mother selections and tubers (Figures 2.25 to 2.31). The morphology of Florex Gold was highly variable. Some plants were very apically dominant with a small number of very tall petioles and exemplified the non-bushy phenotype (Figure 2.2). At the other extreme, was the typical bushy plant exemplified by plants from Dutch tuber F, which had multiple shoots, with reduced stem length and no flowers. Dutch F leaves had less leaf maculation and leaves were not the characteristic mature triangular shape (Figure 2.2). Dutch tuber F was squat, with multiple nodes and many eyes (Figures 2.2 and 2.28).

Information from a growers' survey helped to determine which criteria were important to commercial *Zantedeschia* growers. These included the number of primary shoots which would need to be five or greater for the plant to be considered bushy (T. Reed, personal communication, 2001). Previous *Zantedeschia* research outlined the expected performance of non-bushy tubers, including *Z. elliottiana* one of the likely parents of Florex Gold. A non-bushy flowering sized *Z. elliottiana* tuber would produce one or two shoots, approximately 60 cm tall, of which one shoot would produce one, or sometimes two, flowers (Funnell et al., 1988; Funnell and Warrington, 1994). One-year-old *Zantedeschia* tubers, at least 25 g, have been induced to flower following a dip in GA_3 (Funnell et al., 1988; Funnell and Warrington, 1994). The approximate stem length of these flowers would be 40 to 50 cm (Appendix A). A symptom of bushy plants would, therefore, be non-flowering GA_3-treated tubers at least 25 g. Flower stem including spathe length below 40 cm could also be considered a bushy symptom, although *Zantedeschia* flower length 30 to 40 cm is considered the smallest or posy flower grade (Appendix A). Consequently, a flower stem length less than 30 cm would be considered a bushy symptom (T. Reed, personal communication, 2001).

Examination of mother tuber selection, and individual tuber selections determined that bushy symptoms could persist for more than one growing season (Figures 2.25 to 2.31). Dutch bushy tuber F displayed bushy symptoms for one season prior to this research

and then for two growing seasons, during which bushiness was investigated and showed no reduction in the severity of symptoms. However, investigation of bushy symptoms in the mother tubers found plants from tubers with less severe bushy symptoms became non-bushy e.g. the New Zealand bushy tubers (Figures 2.25 to 2.31). Bushy symptoms were also passed from mother tuber to progeny after micropropagation e.g. Dutch tuber F (Figures 2.7 to 2.10). It was not tested if bushy symptoms were passed to seed progeny, as this research addressed bushiness from a commercial micropropagation perspective.

Subtle changes in flower colour and reduced flower quality were also associated with bushiness. Bushy H, the bushiest of the New Zealand bushy selection, had 100% of flowers with desirable flower quality and colour (Figure 2.29). Reduced flower quality and colour was not evident in the bushy selections (Figures 2.26 and 2.29). Control Florex Gold had a similar or a smaller percentage of flowers with desirable colour in comparison with the few-eyed, multi-eyed, bushy and Dutch mother selections (Figure 2.26). Consequently, subtle changes in flower colour and quality cannot be attributed to the bushy syndrome.

Assessment of data from a principal component analysis (data not shown) found that one or even a small number of bushy characteristics was not able to describe bushy plants. Consequently, bushiness was considered a syndrome and a range of symptoms needed to be displayed before a plant was considered "bushy". A plant displaying the full bushy syndrome would have a squat multi-node tuber, with many eyes, multiple primary shoots (≥5) that had reduced stem length and no flowers, or flowers with stem length less than 30 cm.

2.4.2 Examination of the bushy syndrome

Several hypotheses were considered during this research including the role of *Z. elliottiana/Z. pentlandii* parentage, the Florex Gold genotype, micropropagation from multiple-eyed or T2 tubers, and elevated cytokinin concentration during micropropagation. Reduced light and lower temperature growing conditions and lower temperature and shorter storage time including forcing were also considered. The *Z. elliottiana* parentage and Florex Gold genotype hypotheses were investigated, as anecdotal evidence suggested a role in the expression of the bushy syndrome. The role

of micropropagation of multiple-eyed or T2 tubers and the use of elevated cytokinin levels were investigated to develop an in depth understanding of the possible role micropropagation played in the expression of the bushy syndrome. Different growing and storage conditions and forcing were examined, as bushiness was most severe in The Netherlands, where *Zantedeschia* tubers were stored and forced/grown in different conditions. These differences in growing and storage conditions were also analysed to provide an appreciation of their effect on *Zantedeschia* morphology.

These hypotheses were tested with four main experiments over two growing seasons (Table 2.1). It would have been ideal to run the experiments sequentially, but time constraints meant the experiments had to run concurrently. The whole experiment was set up in two greenhouses and one screen house. This was to accommodate the four different growing light and temperature growing conditions.

2.4.3 Other possible hypotheses

Numerous other suggestions as to the cause of the bushy syndrome have been made, some of these were discarded at the outset because of information available in the literature that eliminated them as a cause of the bushy syndrome. Such suggestions included the exposure of tubers to some factor that removed apical dominance. This could include the decapitation of the main shoot, which would lead to the emergence, and development of axillary buds as main shoots. Decapitation would remove the source of the inhibiting influence of auxin, thereby enabling the outgrowth of lateral meristems. However, the apical bud of *Zantedeschia* is well protected, being enclosed in several layers of leaf sheaths. Also, the axillary buds surrounding the apical meristem located at the leaf base take over the apical control, thereby restoring the dominance (Ngamau, 2001). Bushiness was observed in hundreds of plants in the same crop and it is unlikely that apical dominance was concurrently lost in so many tubers at the same time.

Exposure to ethylene during storage and transportation was proposed as the reason for the reduction in flower number or length. This suggestion was discarded as *Zantedeschia* is unaffected by exposure of tubers to ethylene (Funnell and MacKay, 1988; Funnell and Warrington, 1994).

Poor mineral nutrition had also been put forward as a cause of the bushy syndrome. However, the effects of growing *Zantedeschia* plants in low nutrient conditions has already been reported (Funnell et al., 1987a; Clemens et al., 1998). Plants grown in low nutrient conditions tended to flower earlier and were shorter than plants grown under higher levels of nutrition (Funnell et al., 1987a). Clemens et al. (1998) found more advanced shoot development and shortened time to flower in *Z. albomaculata* cv. Starlights in low nitrogen conditions. Tubers from plants grown on low nitrogen supply produced over 70% more bloom weight than those grown on high nitrogen. Also almost 100% more flowers developed from tubers grown under low nitrogen, high phosphorus conditions than those given the high nitrogen, high phosphorus treatment (Clemens et al., 1998). As low nitrogen levels increase flower weight and number, a role in the expression of the bushy syndrome is unlikely.

Chen et al. (2000) reported that tuber size in *Zantedeschia* was influenced by growing environment. Plants grown in soil beds, regardless of plantlet size, tended to produce larger tubers than did those grown in pots (Chen et al., 2000). The variation in tuber size also depended on both the size of mother plantlets and the micropropagation method (Chen et al., 2000). Bushy and non-bushy plants were seen growing next to one another in the same growing conditions, and were produced by the same micropropagation method, so it seems unlikely these individual factors influence the bushy syndrome.

Various reports discuss the effects of GA_3 in *Zantedeschia*, including enhanced flower production and an increased number of emerged shoots (Corr and Widmer, 1987; Funnell and MacKay, 1987; Tjia, 1987; Funnell et al., 1988; Corr and Widmer, 1991; Dennis et al., 1994; Clemens et al., 1998; Ngamau, 2001). Ngamau (2001) found the application of GA_3 enhanced both the emergence and the outgrowth of the side shoots, leading to earlier emergence and to an increased number of visible side shoots and developed shoots. The mean and total height of side shoots and developed shoots was also positively influenced. However, the complete emergence of all axillary buds as side shoots could not be attained. The outgrowth of buds terminated or slackened before the unfolding of leaves or shortly thereafter in many cases. This may be due to the apical dominance being re-imposed before the complete outgrowth of the buds (Ngamau, 2001). Corr and Widmer (1987) found flower height was unaffected by GA_3 application in *Z. elliottiana* and *Z. rehmannii*. The GA_3 treatment did not significantly

affect date of first flower, flower stem length or leaf length. Corr and Widmer (1991) found that GA_3 treatment of *Z. elliottiana* and *Z. rehmannii* did not significantly affect date of emergence, number of leaves, number of shoots, or number of leaves per shoot. Therefore, it seems unlikely that GA_3 application plays a role in the expression of bushy symptoms.

2.4.4 Investigated hypotheses

2.4.4.1 *Zantedeschia* cultivars with *Z. elliottiana* parentage may predispose plants to bushiness

Bushiness was first reported in the *Zantedeschia* cultivars Florex Gold, Hot Shot and Mango. The parents of these cultivars are likely to be *Z. elliottiana* and *Z. pentlandii*. However, it is thought these cultivars have more *Z. elliottiana* background than *Z. pentlandii*. It was, therefore, suggested that the *Z. elliottiana* parentage, was somehow predisposing cultivars to bushiness. However, bushiness is not seen in all cultivars with a *Z. elliottiana* background. The yellow cultivar Best Gold has a *Z. elliottiana* background and has shown no signs of bushy symptoms to date. The Best Gold selections investigated during this research had significantly heavier T1 tubers and flowers that were heavier and taller (non-bushy symptoms), in comparison with bushy and Dutch Florex Gold selections (Figures 2.3, 2.4 and 2.5). Therefore, a *Z. elliottiana* background is not singularly responsible for the bushy syndrome. However, because the plants with a range of bushy symptoms had a *Z. elliottiana* background, *Zantedeschia* cultivars with *Z. elliottiana* parentage may have a predisposition to bushiness.

2.4.4.2 Florex Gold genotype may predispose plants to bushiness

A genotype response was proposed as the cause of the bushy syndrome, as bushy symptoms were very severe in some plants of the Florex Gold cultivar or genotype. The plants grown from the Dutch Florex Gold tuber F were the bushiest plants with a range of bushy symptoms. These symptoms included significantly lighter T1 tuber weight, no flowers (Experiment One), or very few flowers (Experiment Two to Four), 100% of plants with primary shoot number at least five and a significantly smaller proportional increase in T1 to T2 tuber weight in comparison to all other Dutch tubers

(Figures 2.7, 2.8 and 2.10). Plants derived from the bushy tuber H also showed signs of bushiness, although not a full range of symptoms. Plants derived from the Bushy tuber H had significantly more T1 tuber eyes, low flower weight, short stem length and 83.3% of tubers with primary shoot number at least five in comparison to other bushy tubers (Figures 2.7 to 2.10). The control Florex Gold plants/tubers showed no signs of bushy symptoms. The control Florex Gold had fewer T1 tuber nodes and T1 tuber eyes, proportionally fewer T2 tuber nodes, the heaviest flowers, longest flower stems and longest spathes compared to all other Florex Gold selections (Figures 2.7 to 2.10).

Plants with a range of bushy symptoms were not present in all Florex Gold tuber selections, therefore bushiness is not singularly caused by the Florex Gold genotype. However, because plants with a range of bushy symptoms were present in some Florex Gold tuber selections, including Dutch F and bushy H, the Florex Gold genotype cannot be ruled out as a predisposing factor to bushiness.

2.4.4.3 Florex Gold plants micropropagated from T2 tubers may be predisposed to bushiness

The bushy syndrome has only been reported since the mid 1990s. Consequently, the problem may have been caused by the selection of clones or individual tubers that were put into tissue culture for micropropagation. The control Florex Gold tuber showed no signs of bushy symptoms, and had not been micropropagated for 12 years. The few-eyed, multi-eyed, bushy and Dutch Florex Gold selections showed a range of bushy symptoms and were micropropagated two years previous (Figures 2.3 to 2.10). Until recently it was common to initiate new micropropagation lines from T1 or T2 tubers. This was to reduce the risk of cucumber mosaic cucumovirus and dasheen mosaic potyvirus infection in the new micropropagation lines (Zettler and Hartman, 1987; Chen et al., 2000). The first plants displaying bushy symptoms in New Zealand were most likely initiated from T1 or T2 tubers. However, bushy symptoms were not seen in all selections micropropagated from T2 material. Therefore, micropropagation of T2 tubers is not singularly responsible for bushy plants but may be a predisposing factor to bushiness.

2.4.4.4 Florex Gold plants micropropagated from tubers with multiple eyes may be predisposed to bushiness

Within the *Zantedeschia* industry there was the perception that tubers with many eyes were superior as they produced more flowering shoots. However, clones with too many eyes (buds) may have been selected for tissue culture and these may subsequently have produced a large number of weak non-flowering shoots. Plants from multi-eyed tubers had more nodes and shoots, but a similar number of flowers compared with the apically dominant control Florex Gold. The flowers produced by the multi-eyed tubers were, however, lighter and shorter (Figures 2.3 to 2.10). Plants with a range of bushy symptoms were not found in all selections micropropagated from tubers with multiple nodes and eyes. Therefore, the micropropagation of plants from tubers with multiple eyes is not singularly responsible for bushy plants. However, because plants with a range of bushy symptoms came from tubers with multiple eyes, the selection of tubers with multiple eyes may be a predisposing factor to bushiness.

2.4.4.5 Elevated concentrations of the cytokinin, 6-BAP, during tissue culture did not induce bushiness in progeny

Elevated cytokinin concentrations during micropropagation had been proposed as the cause of the bushy syndrome. Moreover, various reports have implicated cytokinins in both the induction and expression of other tissue culture syndromes. This includes bushiness in *Gerbera* (Topoonyanont et al., 1999), mantled oil palm (Jones, 1998; Eeuwens et al., 2002), and multi-apexing in strawberry (Anderson et al., 1982). The control cytokinin level (4.4 μM 6-BAP), which is used routinely during commercial micropropagation, did not promote bushy plants. Elevated 6-BAP levels during micropropagation did not produce plants with a range of bushy symptoms. MS media containing elevated levels of 6-BAP had little effect on flower weight, flower stem length, the number of days from planting until flowering, desirable flower quality, desirable flower colour, the percentage of flowering tubers at least 25 g and the proportional increase in the number of T2 tuber nodes and T2 tuber eyes (Figures 2.11 to 2.16).

Elevated TDZ levels were examined, as TDZ, a DPU cytokinin, is extremely active at relatively low concentrations. If elevated cytokinin concentrations during

micropropagation were responsible for the bushy syndrome, it would be expected that the effect would be more pronounced with the use of TDZ. Recent studies on the cytokinin receptor *CRE1* have also shown that TDZ was able to bind to the receptor and act as a natural cytokinin (Inoue et al., 2001) (Section 1.2.7). A carry-over effect of TDZ from tissue culture was evident in only two measured bushy characteristics. Spathe length was reduced and primary shoot number of at least five was increased when plants were micropropagated in the presence of 13.6 μM TDZ (Figures 2.14 and 2.16). Consequently, it is recommended that TDZ should be avoided during micropropagation for those cultivars prone to the bushy syndrome.

2.4.4.6 Variation in light and/or temperature growing conditions did not promote bushy plants but influenced individual bushy symptoms

Bushy Florex Gold, especially those with severe symptoms of the syndrome, was first reported in The Netherlands in 1998. The difference in growing conditions, especially irradiance level and temperature, between The Netherlands, where bushiness was most severe and New Zealand, lead to the hypothesis that low light and low temperature environmental conditions contributed to bushiness. The light (50 and 80% shading) and temperature (greenhouse and screen house) growing conditions investigated during this thesis did not produce plants with a range of bushy symptoms (Figures 2.17 to 2.20). Previous research, focused on optimising *Zantedeschia* growing conditions, supports these findings. Funnell et al. (1987b) found the percentage of plants flowering (symptom of bushiness), in the *Zantedeschia* cultivars Red Emperor and Pink Petticoat, was not influenced by irradiance level or growing environment temperature. Corr and Widmer (1990) found that there was no significant effect of reduced irradiance on date of first flowering, total number of flowers and spathe length in *Z. elliottiana* and *Z. rehmannii*. Corr and Widmer (1990) also found there was no significant effect of air or medium temperature on any characteristic of *Z. elliottiana* growth or on the number of flowers produced by *Z. elliottiana* and *Z. rehmannii*.

However, some light and temperature factorial combinations did influence the appearance of individual bushy characteristics. Therefore, care needs to be taken when selecting light and temperature growing environments, for plants predisposed to the bushy syndrome. Some responses such as increases in flower stem length also have commercial significance in regard to likely increased sale value (Funnell et al., 1987b).

Flowers from plants grown in the greenhouse at 300 μmol m^{-2} s^{-1} (80% shade) were significantly taller than those in all other growing environments, including plants grown in the screen house at 300 μmol m^{-2} s^{-1} (Figures 2.17 and 2.19). Therefore, increased shade level and growing temperature increased flower stem elongation. Previous research in *Zantedeschia* support these findings. Corr and Widmer (1990) also found that the flower stem length of *Z. elliottiana* and *Z. rehmannii* grown in reduced irradiance (170 and 550 μmol m^{-2} s^{-1}) were longer than on plants under full available irradiance (1220 μmol m^{-2} s^{-1}). *Z. rehmannii* plants grown at the higher medium (20 and 25°C) or air temperatures (20°C) were taller with longer flower stem length than those grown at lower temperatures (15°C air and ambient medium temperature). Funnell et al. (1987b) found that the flower stem length was increased by increased shade level. Increasing the shade level from 0 to 72% resulted in excess of 100% increase in flower stem length in both the *Zantedeschia* cultivars Red Emperor and Pink Petticoat. The optimal level of shading varied between cultivars, 82% shading for Red Emperor and 67% shading for Pink Petticoat (Funnell et al., 1987b). Funnell et al. (1987b) also found the total plant height was increased by the interaction of increased shade and temperature level.

The greatest number of flowers per flowering tuber was 1.7 in the greenhouse in the highest light environment (750 μmol m^{-2} s^{-1}[50% shade]) (Figures 2.18 and 2.20). Armitage (1991) also found a decline in yield or the number of flowers per plant in *Zantedeschia* cultivars Majestic Pink, Pink Perfection, Pacific Pink and Black Magic as shade increased, but yield was similar for ambient and 55% shade.

Flowers from plants grown in the greenhouse at 300 μmol m^{-2} s^{-1} had fewer flowers with desirable colour, than those in all other growing environments (Figures 2.18 and 2.20). Funnell et al. (1987b) also noted variation in the development of floral pigment of *Zantedeschia* grown in greenhouses compared to unprotected growing environments. Flowers grown in the unprotected environment showed superior colour development to those grown under protected cultivation.

The number of days from planting until flowering was increased for those plants grown in the screen house due to lower air temperatures (Figure 2.19). Corr and Widmer (1990) also found that *Z. rehmannii*, but not *Z. elliottiana*, grown at lower medium or air temperatures, flowered later than those grown at higher temperatures. Conversely,

Funnell et al. (1987b) found only 2 to 6% of the variation in time to first flowering could be accounted for by shade level and growing environment temperature in *Zantedeschia* cultivars Pink Petticoat and Red Emperor.

Tissue culture plants grown in an unheated screen house at 750 μmol m^{-2} s^{-1} produced heavier T1 tubers with more nodes (Figure 2.19). Funnell and Warrington (1994), in contrast, found that *Zantedeschia* plants grown under average daily temperatures encountered in the field during the New Zealand summer (16 to 19°C) will only attain approximately 20% of the final tuber weight of those grown under the optimal temperature near 25°C.

The growing conditions need to be optimised for each step in the *Zantedeschia* life cycle. In the variable temperature environment (screen house) at 750 μmol m^{-2} s^{-1}, tissue culture plants produced the heaviest T1 tubers with the most nodes. In the second growing season, tubers grown in a controlled temperature environment such as a greenhouse under low irradiance levels (300 μmol m^{-2} s^{-1}), produced flowers of increased weight and stem length but this also reduced the percentage of flowers with desired colour. Commercial *Zantedeschia* growers, therefore, need to balance increased stem and flower weight with desired colour.

2.4.4.7 The temperate and duration of storage did not promote bushy plants but influenced individual bushy symptoms

The reduced length and variable temperature of storage were investigated as tubers first expressing the bushy syndrome were exported to The Netherlands and stored for different lengths of time and at different temperatures compared to the current practice in New Zealand. These conditions may have disturbed the *Zantedeschia* life cycle and influenced the expression of the bushy syndrome. *Zantedeschia* tubers were also 'forced' to grow and flower in The Netherlands, in what would have been their natural rest period (winter) in New Zealand. Therefore, forcing and a greatly reduced dormancy period may have caused bushy symptoms. However, the duration of storage (11 or 21 weeks) and temperature conditions (9 or 15 °C) investigated did not promote plants with a range of bushy symptoms (Figures 2.21 to 2.24). Some storage factorial combinations did influence individual bushy characteristics. Consequently, care needs

to be taken when selecting the tuber storage environment for those cultivars predisposed to the bushy syndrome.

T1 tubers stored for 21 weeks compared to 11 weeks had flowers of increased weight, stem and spathe length (Figure 2.21 and 2.23), but a reduced number of flowers per tuber that flowered (Figure 2.22 and 2.24). Although flowers had increased weight, the diversion of reserves to the apex reduced T2 tuber weights after harvest. These results have horticultural significance for commercial *Zantedeschia* growers. The commercial *Zantedeschia* grower needs to select for increased flower weight, stem and spathe length *or* increased T2 tuber weight. The shorter storage period of 11 weeks, increased the expression of individual bushy symptoms including reduced flower weight, stem and spathe length. The percentage of tubers at least 25 g that flowered, however, was very similar between factorial treatments (Figures 2.22 and 2.24).

Tubers stored for 21 weeks had a lesser number of flowers per tuber that flowered, than those stored for 11 weeks (Figures 2.22 and 2.24). Storage for 21 weeks allowed the establishment of apical dominance, resulting in a reduced proportion of flower buds emerging. Storage temperature had little effect on the number of flowers, per flowering tuber. Funnell and MacKay (1988), however, found increased storage temperature (25°C compared with 8°C), instead of longer storage duration, reduced the proportion of buds emerging.

T1 tubers stored for 11 weeks flowered in 90 days, compared to 82 days for those stored for 21 weeks. A higher temperature for those tubers stored for 11 weeks, decreased the time to flowering. Plants flowered after 89.5 days when stored at 15°C, but flowered after 91.6 days when stored at 9°C (Figure 2.23). Funnell and MacKay (1988) also found that increasing both the temperature at which tubers were stored and the duration of storage reduced the time to flowering. Funnell and Warrington (1994), reported a similar reduction in time to flower by storage at warmer temperatures (12 to 25°C) and/or with increasing duration of storage (4 to 10 weeks). The earlier flowering results from shoot growth and subsequent flower formation occurring during the storage period.

2.4.4.8 Edge effect

Additional variation affecting individual bushy symptoms, due to plants growing at the edge of the tray and the edge of the bench being shorter in height than those in the centre, was investigated (Figure 2.33). Flowers from plants grown in position four (centre) had increased weight and flower length compared with plants grown at the edge, which was probably due to stem elongation as a consequence of shading. The edge effect did not significantly reduce spathe length or reduce the number of days from planting until flowering. Even though the plants grown at the edge were shorter in height, they did not have an increased number of shoots and leaves, and the morphological characteristics of those plants were not bushy. This edge effect was accounted for during statistical analysis, so plants with fewer leaves and shoots, but shorter flowers due to their growing position, were not considered bushy.

2.4.5 Possible improved criteria for non-flowering plants

The criteria against which individual plants were assessed for "bushiness" focused on tuber, flower and shoot number characteristics displayed by the original mother selections. In retrospect, for those plants that did not flower, it would have been beneficial to have additional criteria including the average leaf stem length, to give an indication of overall plant height in relation to the bushy syndrome. Extra measurements investigating juvenility and rejuvenation including the percentage of leaves per plant without leaf maculation and the percentage of leaves without the characteristic mature triangular leaf shape would also have been beneficial.

2.4.6 Conclusions and recommendations

Cultivars with *Z. elliottiana* parentage, the Florex Gold genotype, and micropropagation from T2 tubers with multiple eyes are common factors within all plants displaying bushy symptoms. However, these factors are also common to many non-bushy plants. Consequently, it is suggested that these criteria be considered as factors that may predispose a plant to bushiness and, as such, should be avoided as far as practical. It is recommended that after selection of plants with desirable characteristics for commercial flower production, including long flower stem and spathe length and desirable flower colour and shape, the tubers for micropropagation should be deep, apically dominant

tubers, with few eyes and nodes. Mature tubers should be chosen for micropropagation, especially not tubers themselves recently from tissue culture. The cytokinin in the micropropagation media should be keep to the standard concentration (4.4 μM 6-BAP) and TDZ should be avoided. Growing and storage conditions and edge effect have been shown to accentuate some bushy characteristics. Therefore, careful selection of the growing and storage environment is recommended for those cultivars predisposed to bushiness.

Chapter Three: Investigation of endogenous cytokinins at three stages in the *Zantedeschia* life cycle, in tubers of different degrees of bushiness

3.1 Introduction

Cytokinins were implicated in the expression of the bushy syndrome in Florex Gold because of the well-known effect of cytokinins on the release of apical dominance and promotion of shoot formation (Mok, 1994). Cytokinins were also implicated in the expression of the bushy syndrome, as reports have indicated a close relationship of cytokinins with the induction and expression of comparable tissue culture syndromes (Anderson et al., 1982; Besse et al., 1992; Topoonyanont et al., 1999; Topoonyanont and Debergh, 2001; Eeuwens et al., 2002).

Cytokinin may influence the expression of bushiness in two ways: bushy plants may contain higher concentrations or a different profile of cytokinins and/or they may exhibit a differential sensitivity to cytokinin. Sensitivity to cytokinin during micropropagation was assessed by a root length bioassay, comparing Florex Gold with a range of non-bushy *Zantedeschia* cultivars. Data from this bioassay has been published (D'Arth et al., 2002). A root length bioassay has been used to assess sensitivity to cytokinin in recent work focused on identification of cytokinin receptors (Plakidou-Dymock et al., 1998; Inoue et al., 2001).

The relationship between endogenous cytokinin profile, concentration, and bushiness was examined by analysing tubers at three points in the *Zantedeschia* life cycle. Endogenous cytokinins were analysed in sprouting mother tubers, before bud material was initiated into tissue culture. Endogenous cytokinins were also examined in both dormant and sprouting T1 tubers (or progeny), after tissue culture plants were grown for one season and formed tubers. Bushy symptoms are observed during the second growing season. If cytokinins play a role in the expression of bushy symptoms, differences in cytokinin profile, especially high storage cytokinin concentrations, or differences in the total concentration of biologically active cytokinins might be observed in these tubers.

Extensive research investigating the role of endogenous cytokinins in the life cycle of potato tubers especially dormancy termination has been carried out (Turnbull and Hanke, 1985a, 1985b; Sukhova et al., 1993; Suttle, 1998; Suttle and Banowetz, 2000; Suttle, 2000, 2001). Cytokinin concentration and profile detected in *Zantedeschia* can therefore be compared to cytokinins found in potato, to determine if the cytokinins are similar to the those found in potato during loss of dormancy.

3.2 Materials and methods

3.2.1 Cytokinin sensitivity experiment

The cultivars Florex Gold, Treasure, Sunglow, Chianti and Butterscotch were investigated for sensitivity to cytokinin. Shoot cultures were grown on MS media (Murashige and Skoog, 1962), supplemented with 0, 1.3, 2.6, 4.4 or 13.3 μM 6-BAP (tissue culture initiation and culture conditions are detailed in Section 2.2.2). After three, four-week subcultures, on identical media, cytokinin sensitivity was assessed by measuring the length of the longest root of each tissue culture shoot or cluster of shoots. Thirty roots of each variety were assessed for each 6-BAP concentration. *Zantedeschia* root length data was analysed by analysis of variance (ANOVA), or the Kruskal – Wallis test when the differences in variance between the treatments were too great.

3.2.2 Endogenous cytokinin extraction, separation and analysis

Tuber explants comprising tuber eyes (vegetative buds) and surrounding basal tissue were analysed for endogenous cytokinins. Best Gold tubers were compared to control, few-eyed, bushy, and Dutch Florex Gold tubers. The endogenous cytokinins of both tuber cultivars were compared as sprouting mother tubers and both endodormant and sprouting T1 tubers. Two replicates were investigated at each stage.

Cytokinin extraction, separation and analysis were carried out according to the method described by Jameson et al. (2000). The entire procedure is outlined in Figure 3.1. Unless specified otherwise, all materials were analar grade or its equivalent, and all solvents of HPLC grade purity. MilliQ rated water was used. All extraction and purification procedures were carried out in polypropylene containers or silanised glassware to minimise losses of cytokinin. Silanised glassware was prepared by rinsing glassware with dimethyldichlorosilane (2% [v/v] in 1,1,1-trichloroethane). After drying, the glassware was washed with methanol and finally with MilliQ water.

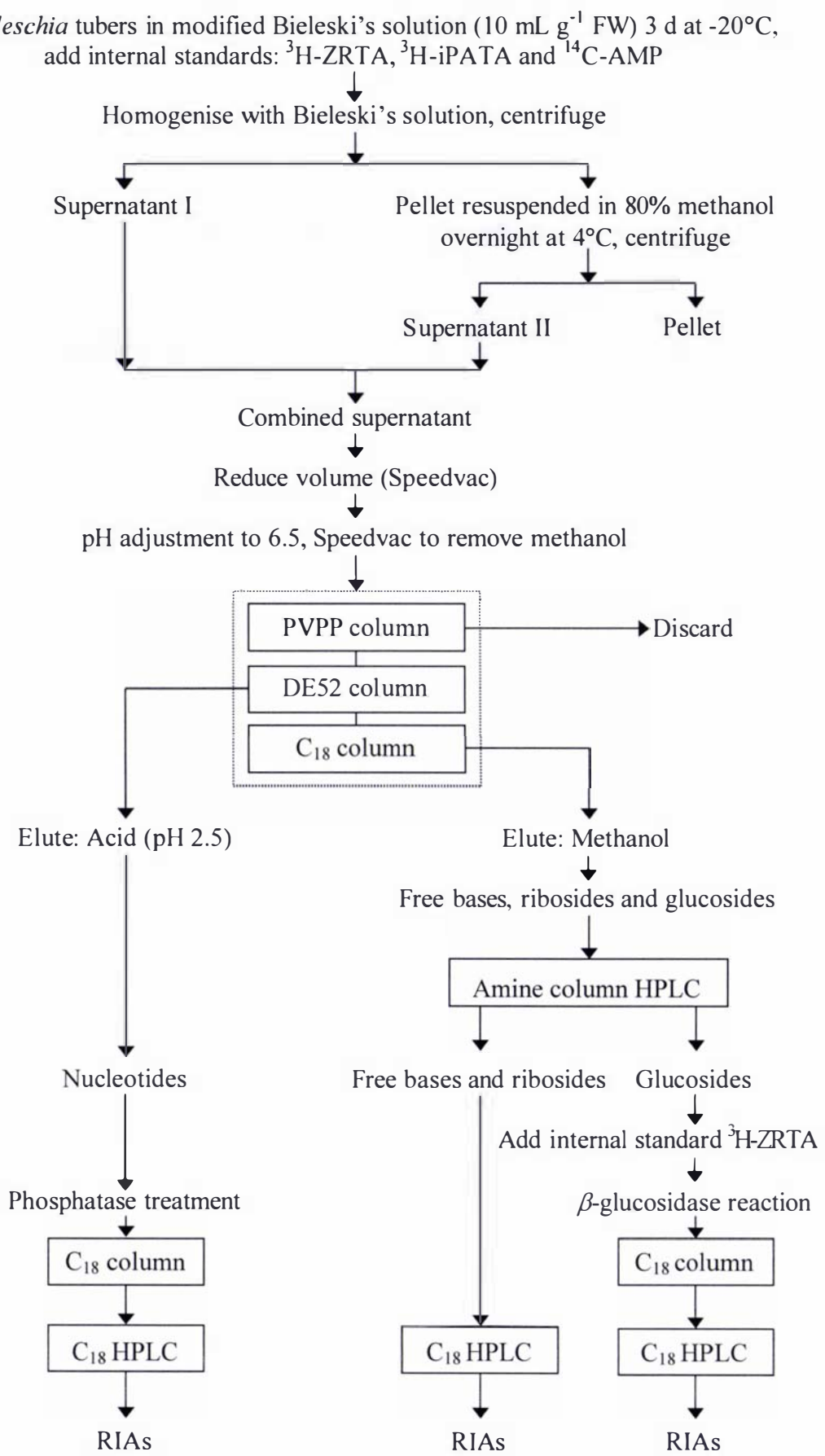


Figure 3.1: Procedures used for extraction, purification, separation and quantification of individual cytokinins in *Zantedeschia* tubers, ▭ indicates column used, ▭ indicates columns are linked.

Between 5 to 10 g of tuber material was placed in modified Bieleski's solution (methanol:H_2O:acetic acid, 70:30:3 [v/v/v]) (10 mL g^{-1} FW), pre-chilled to -20°C. The tuber material remained in the Bieleski's solution for more than 3 d at -20°C to inactivate enzymes. The tuber material was homogenised in a mortar and pestle (previously washed three times in 80% [v/v] methanol). The homogenate was collected. The mortar and pestle was rinsed twice with 80% (v/v) methanol to reduce the losses of the cytokinins, and the rinses were combined with the homogenate.

Approximately 30,000 CPM of each of the internal standards ^{3}H-zeatin riboside-trialcohol (^{3}H-ZRTA), ^{3}H-isopentenyladenosine-trialcohol (^{3}H-iPATA) and ^{14}C-adenosine-5′-monophosphate (^{14}C-AMP) were added to the homogenate, to estimate any losses of cytokinin during the extraction and purification procedures. The internal standards were added after homogenisation, for safety reasons. More correctly, they should have been added during the initial extraction. The internal standards ^{3}H-ZRTA and ^{3}H-iPATA were synthesised according to the protocol in MacDonald et al. (1981), and the ^{14}C-AMP was purchased from Amersham Life Sciences (USA).

The homogenate was centrifuged at 13,000g for 30 min at 4°C. The supernatant was decanted and stored at 4°C. The pellet was resuspended in 80% (v/v) methanol (5 mL g^{-1} FW) and left overnight at 4°C. It was then centrifuged at 13,000g for 30 min at 4°C. The second supernatant was decanted and combined with the first. The combined supernatants were then reduced in vacuo (Savant SpeedVac) to 2 mL. The pH of the sample was adjusted to 6.5 with 1M NaOH. The NaOH was added slowly and mixed quickly on ice to prevent degradation of nucleotides due to high temperature. The sample was further reduced in vacuo to a volume of 2 mL. Samples were loaded onto a pre-conditioned column complex as outlined in Section 3.2.2.1.

3.2.2.1 Column chromatography complex

The cytokinins were purified and separated through a column complex. The complex consisted of three columns individually packed and linked in series. The first column was a polyvinylpolypyrrolidone (PVPP) column to remove phenolics and other impurities. The second column was a DEAE cellulose 52 (DE52, Whatman) anion exchange cellulose column used to collect the cytokinin nucleotides and to remove

impurities. The third column in the series was an octadecyl silica (C_{18}) column, which was used to collect cytokinin free bases, ribosides and glucosides.

PVPP pre-conditioning: The PVPP column was prepared by slurrying PVPP powder in 10 mM ammonium acetate buffer (pH 6.5). Buffer was decanted and fresh buffer added until the pH of the solution stabilised at 6.5. Sodium azide, 0.1% (w/v), was added for storage. The slurry was packed into a 30 mL syringe barrel, which had two discs of number four filter paper (Whatman) placed in the bottom to hold the PVPP in place. The slurry was packed to a final bed volume (BV) of 5 to 10 mL.

DE52 pre-conditioning: The DE52 column was prepared by adding 40 mM ammonium acetate buffer to the DE52 powder. The slurry was allowed to settle. The fines were decanted off and fresh buffer added. The buffer was replaced until the pH of the solution equilibrated at 6.5. Finally, the DE52 was equilibrated in 10 mM ammonium acetate buffer (pH 6.5). Sodium azide, 0.1% (w/v), was added for storage. The DE52 column was packed under gravity, into a 30 mL syringe barrel, allowing 2 mL BV g^{-1} FW. The syringe barrel contained two filter paper discs to prevent the loss of the DE52. The column was washed with 3 BV of 10 mM ammonium acetate buffer (pH 6.5).

C_{18} pre-conditioning: The C_{18} powder (C_{18} Bondesil; Analytichem International) was packed into a 4 or 8 mL syringe barrel, with a pre-fitted filter (Alltech), with 0.4 mL BV C_{18} powder g^{-1} FW. The column was preconditioned with 20 BV of methanol, followed by 20 BV of 10 mM ammonium acetate (pH 6.5).

After each individual column was packed and conditioned, the PVPP, DE52 and C_{18} columns were connected in series. Columns were connected with methanol-washed bungs and syringe needles and care was taken not to disturb the column packing. Multiple sets of the column complexes were set up to process more than one sample at a time. The column series was preconditioned with the equivalent of 1 BV DE52, of 10 mM ammonium acetate buffer (pH 6.5). The pH-adjusted sample (2 to 5 mL) was added directly onto the PVPP column. The complex was eluted with the equivalent of 3 BV DE52 of 10 mM ammonium acetate buffer (pH 6.5). The columns were then disconnected and the PVPP column discarded.

3.2.2.2 Nucleotide recovery and alkaline phosphatase reaction

The DE52 column was eluted with 2.5 BV of 1 N acetic acid (pH 2.5) in order to recover the cytokinin nucleotides. The eluate was stored at -20°C. The sample was then reduced in vacuo to a small volume (ca. 500 μL). Five mL of phosphatase reaction solution was added to the cytokinin nucleotides. The alkaline phosphatase solution consisted of 8.3U of alkaline phosphatase (Sigma EC3.1.3.1), in 5 mL of 0.1 M ethanolamine (pH 9.5) and 0.1 mL 40 mM MgCl. The alkaline phosphatase treatment was carried out at 37°C for 12 h with shaking (80 rpm; Sanyo Gallenkamp PLC orbital incubator 10C400.XX2.C). After the alkaline phosphatase treatment the reaction solution was loaded onto a second preconditioned C_{18} column (minimum 3 mL BV) (Section 3.2.2.1). The column was washed with 3 BV water. The sample was eluted with 3 BV 80% (v/v) methanol to recover the dephosphorylated cytokinins. The sample was stored at -20°C. The eluate was evaporated to near dryness and dissolved in 50 μL 25% (v/v) methanol prior to C_{18} HPLC. Alkaline phosphatase hydrolised the cytokinin nucleotides to their respective ribosides, which allowed the cytokinins to be quantified by radioimmunoassay (RIA).

3.2.2.3 Free base, riboside and glucoside recovery

The C_{18} column was flushed with 3 BV of 80% (v/v) methanol to elute the cytokinin free bases, ribosides and glucosides. The sample was stored at -20°C. The sample was evaporated to near dryness and taken up in 50 μl of 50% (v/v) acetonitrile prior to normal phase HPLC (Section 3.2.2.5).

3.2.2.4 HPLC

For both normal and reverse phase HPLC, a Waters 600 Multisolvent controller, U6K injector port, Waters 490E Programmable multiwavelength UV detector set at 269 nm was used to separate individual cytokinins. HPLC grade solvents were used, diluted with MilliQ water and filtered through a 0.45 μM nylon membrane filter (millipore). To prevent problems with air bubbles, all solvents were sparged for at least 10 min at 100 mL min^{-1} helium prior to use and followed with a 30 mL min^{-1} helium sparging during the entire HPLC run. When HPLC was carried out, the injection loop was

washed with 100 μL methanol five times after each injection. Finally the loop was washed five times with the solvent identical to the initial gradient before each sample or standard injection. Data generated by the detector was collected and processed on an IBM compatible computer using Delta software (v.4.06 Digital Solutions Ltd, Australia).

3.2.2.5 Normal phase HPLC

An amine column (Alphasil 5NH_2 5 μm, 250 mm x 4.6 mm, HPLC Technology) was used to achieve bulk separation of cytokinin glucosides (excluding isopentenyladenine-9-glucoside [iP9G]) from all cytokinin free bases and ribosides (and iP9G) according to a gradient protocol developed by Lewis et al. (1996b) and shown in Table 3.1.

The cytokinin free base and riboside bulk fraction, including iP9G, was collected over the period of 1 to13 minutes. The cytokinin glucoside bulk fraction was collected from 13 to 22 minutes. The separation of glucosides from the other cytokinins was verified by running cytokinin standards on the HPLC at the beginning and end of every four to eight sample HPLC runs. Example retention times are shown in Figure 3.2. The cytokinin standard solution used for normal phase HPLC included zeatin (Z), DZ, ZR, DZR, iP, iPA (all obtained from Sigma, USA), and iP9G, zeatin-*O*-glucoside (ZOG), dihydrozeatin-*O*-glucoside (DZOG), zeatin riboside-*O*-glucoside (ZROG), dihydrozeatin riboside-*O*-glucoside (DZROG), zeatin-9-glucoside (Z9G) and dihydrozeatin-9-glucoside (DZ9G) (all obtained from Apex Chemicals, England). Between 10 to 50 ng of the individual cytokinins was used per 50 μL injection. Two blank solvent runs followed to clean the column prior to sample application. The fractions collected following the second buffer injection were subjected to *β*-glucosidase treatment, C_{18} HPLC and RIA to check for contamination of standard cytokinins. The column was washed and stored in H_20:acetonitrile (50:50 [v/v]).

Table 3.1: Gradient conditions for normal phase (NH_2) HPLC. Solvent A was MilliQ water and solvent B acetonitrile.

Minute	Flow rate mL min^{-1}	%A	%B	Curve
Initial	1.0	10	90	*
6	1.0	10	90	6
7	1.0	20	80	6
16	1.0	30	70	6
17	1.0	50	50	6
27	1.0	50	50	6
28	1.0	10	90	6

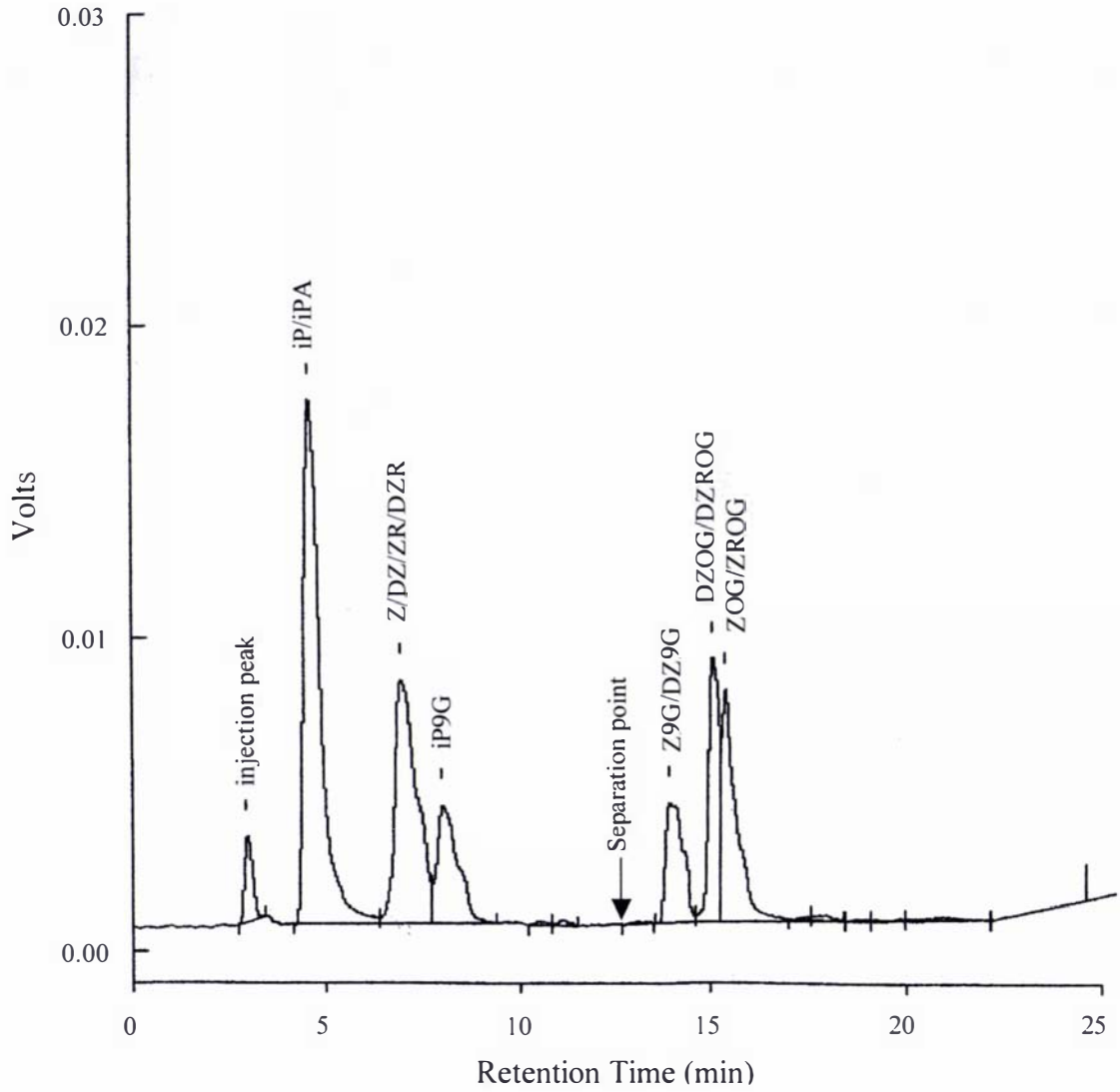


Figure 3.2: Normal phase HPLC separation of cytokinin free bases, ribosides and glucosides. A clear cut was made to separate glucosides (excluding iP9G) from free bases and ribosides at 13 min. Column: Alphasil $5NH_2$, 5 µM, 250 x 4.6 mm. Detection absorbance at 269 nm. Details of the elution gradient are given in Table 3.1.

3.2.2.6 Addition of internal standard and β-glucosidase reaction in glucoside fraction

Approximately 30,000 CPM of the internal standard ^{3}H-ZRTA was added to the glucoside fraction, to estimate any losses of the cytokinin during the last steps of the analysis. The fraction was reduced under vacuum to near dryness (ca. 500 μL). The fraction was dissolved in 5 mL, 50 mM sodium acetate buffer, pH 5.4. An additional 5 mL of β-glucosidase reaction solution was added to the fraction. The β-glucosidase reaction solution consisted of 8U of β-glucosidase (from sweet almonds, Boehringer Mannheim) in 5 mL 50 mM sodium acetate buffer, pH 5.4. The β-glucosidase reaction was carried out at 25°C for 22 h with shaking (80 rpm; Sanyo orbital incubator). β-glucosidase removes the glucosyl group from cytokinin glucosides with a β-glucoside bond linkage. The aglycones can then be detected by anti-cytokinin antibodies. The fraction was run through a second C_{18} column as described for nucleotide recovery (Section 3.2.2.2). The free base and riboside bulk fraction, containing iP9G, and the glucoside bulk fraction were then evaporated to near dryness and taken up in 50 μL of 25% methanol in preparation for C_{18} HPLC.

3.2.2.7 Reverse phase HPLC

Separation of individual cytokinins in each bulk fraction (cytokinin free bases/ribosides, glucosides and nucleotides) was achieved on an octadecyl silica C_{18} column (Beckman Ultrasphere 5 μm, 250 x 4.6 mm) with a 40 mM triethylamine (TEA)/acetic acid buffer (pH 3.35; referred to subsequently as HPLC buffer)/methanol/acetonitrile gradient. The HPLC gradient is that described in Lewis et al. (1996b). See Table 3.2 for gradient conditions.

Table 3.2: Gradient conditions for reverse phase (C_{18}) HPLC. Solvent A was HPLC buffer (40 mM acetic acid, pH 3.35 with TEA), solvent B was methanol:HPLC buffer (80:20 [v/v]) and solvent C was acetonitrile/HPLC buffer (80:20 [v/v]).

Minute	Flow rate (mL min^{-1})	%A	%B	%C	Curve
Initial	1.0	75	25	0	*
1.0	1.0	75	25	0	6
5.0	1.0	72	28	0	5
9.5	1.0	61	39	0	6
10.0	0.8	61	39	0	6
15.0	0.8	60	40	0	7
15.5	1.0	60	40	0	6
20.0	1.0	59	41	0	7
21.0	1.0	75	0	25	6
22.0	1.0	75	0	25	6
26.5	1.0	71	0	29	7
31.5	1.0	70	0	30	7
37.5	1.0	0	100	0	6
47.5	1.0	0	100	0	6
48.5	1.0	75	25	0	6

The samples were eluted with mobile phase gradient. The flow rate was 1 mL min^{-1}, except for min 10 to 15 where it dropped to 0.8 mL min^{-1}. Fractions were collected from the HPLC every 30 s into 1.5 mL centrifuge tubes, for the entire 40 min gradient during each sample run. A cytokinin standard which contained about 20 ng each of Z9G, DZ9G, Z, DZ, cZ, ZR, DZR, cZR, iP9G, iP and iPA was run prior to the first sample application and after every four to eight sample runs to enable retention times of compounds exhibiting cross-reactivity in the RIA, to be compared with retention times of authentic standards. Example retention times are shown in Figure 3.3. Two injections of buffer always followed the cytokinin standard run, to prevent any cross contamination between the standards and samples and the fractions collected following the second buffer injection were subjected to RIA for contamination check.

3.2.2.8 RIA

The cytokinins were quantified using RIA. All tips used to aliquot radioactivity, antibody or RIA solutions were pre-soaked for 5 min. Aliquots (250 to 350 μL) of the fractions from C_{18} HPLC were dried. Fractions collected during C_{18} HPLC from 0 to 25.5 minutes were assayed with monoclonal antibody clone 16 (which cross-reacted with the hydroxylated cytokinins including Z9G and DZ9G). ^{3}H-ZRTA was used as the competitor. Fractions eluting from 26 to 40 min were assayed with monoclonal antibody clone 12 (which cross-reacted with the non-hydroxylated cytokinins including iP9G). ^{3}H-iPATA was used as the competitor. The monoclonal antibodies were a gift from Professor Roy Morris. Protocols for the preparation of monoclonal antibodies can be found in MacDonald and Morris (1985) and Banowetz (1994).

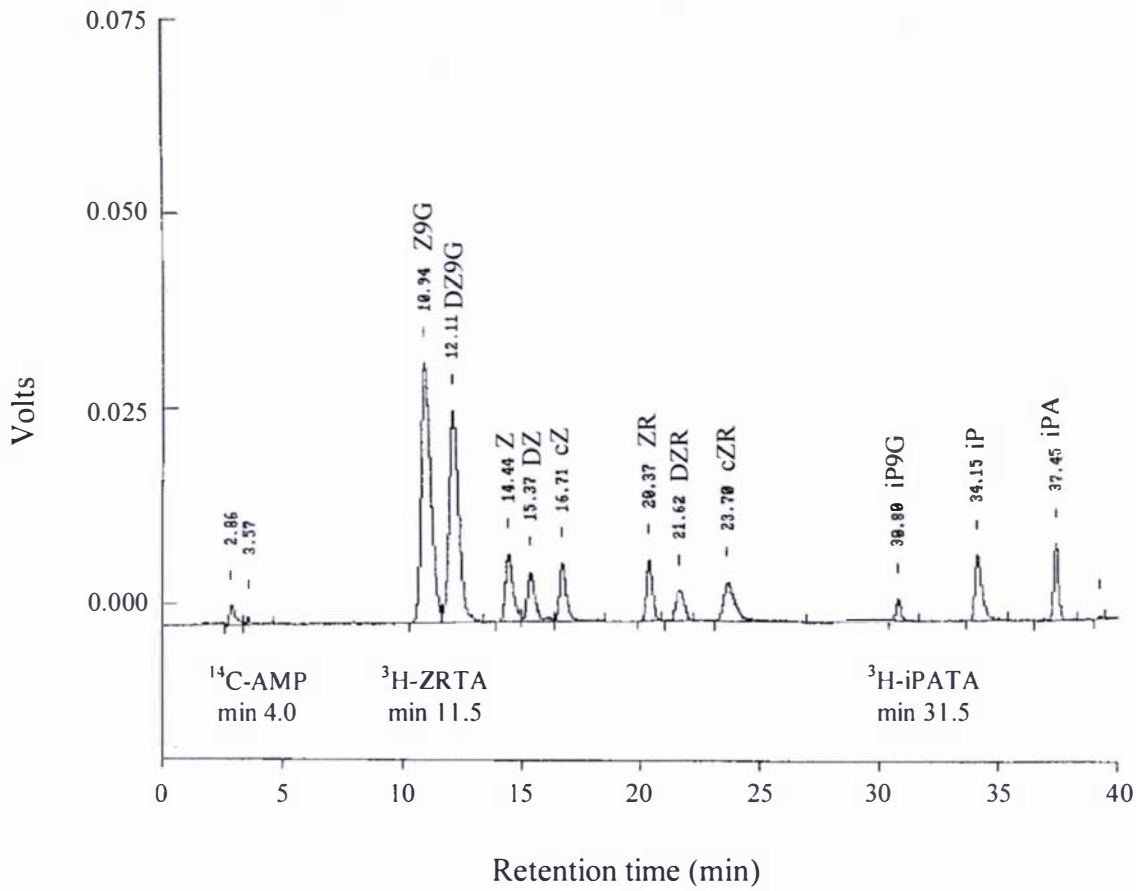


Figure 3.3: Reverse phase HPLC separation of cytokinin standards. Column: 250 x 4.6 mm ocadecyl silica C_{18} (Altex). Detection absorbance at 269 nm. Mobile phase: 40 mM HPLC buffer (pH 3.35)/methanol/acetonitrile (Section 3.2.2.8). Details of the elution gradient are given in Table 3.2.

The fractions were dissolved in 450 μL of the buffer/antigen/antibody assay solution (50 mM sodium phosphate buffer with 0.14 M sodium chloride [NaCl] (pH 7.2), 0.1% (w/v) gelatine (Difco), 0.01% (w/v) ovalbumin (Sigma Grade V); ^{3}H-ZRTA or ^{3}H-iPATA with the radioactive strength adjusted to 5,000 CPMs per 450 μL of reaction solution and anti-cytokinin antibody providing *ca.* 2,500 CPMs binding (B_0) per 450 μL reaction solution. Prior to the addition of 450 μL of buffer to the sample tubes, the B_0 was always checked. The B_0 is the radioactivity (CPM) bound to the antibody present in the 450 μL RIA buffer and which is precipitated by ammonium sulphate in the absence of other cytokinins. The ideal is to have approximately half the added radioactivity bound to the antibody in the absence of other cytokinins. After the addition of the RIA buffer the samples were kept at room temperature with occasional stirring, for 2 to 4 h. Six hundred μL 90% (v/v) saturated ammonium sulphate (pH 7.0) was added and mixed well. After 15 min the tubes were centrifuged at 20,000g for 1 min. The supernatants were then aspirated. The antigen was released by the addition of 50 μL methanol. The released radioactivity was quantified using liquid scintillation counting (Wallac, Milton-Keynes, England) after addition of 1 mL scintillation cocktail (Optiphase HiSafe 2, Wallac).

Standard curves were constructed for each batch of RIAs. A series of dilutions, prepared in triplicate, of ZR or iPA between 0 and 100 pmols were prepared (Appendix B). The minimum detection limit was ca. 1.0 to 2.0 pmol ZR or iPA. Subsequently, the tubes were dried using the Speedvac, and the RIA reaction solution added as described above for samples. A regression line and R^2 value was calculated according to the linear region of each standard curve. The basic form of the calculation equation was:

$$y = ax + b.$$

'y' = B/B_0. ('B' = the amount of ^{3}H-ZRTA or ^{3}H-iPATA bound to the antibody in the presence of ZR or iPA. 'B_0' = the amount of ^{3}H-ZRTA or ^{3}H-iPATA bound to the antibody in the absence of ZR or iPA.).

'x' = log pmol of ZR or iPA.

'a' = the slope of the straight line the equation represents.

'b' = the intercept of the line on the y axis.

The amount of cytokinin present in each fraction was also corrected for percentage cross reactivity with clone 16 or clone 12 (Appendix B).

3.2.2.9 Cross reactivity tests

Cross reactivity for oT, oTR, cZ and cZR were carried out according to the method described by Lewis (1994). The oT and oTR were a gift from Dr Karel Doležal. A series of dilutions was prepared for each of the cytokinin standards oT and oTR between 0 and 1000 pmols and cZ and cZR, between 0 and 100 pmols (Appendix B). The tubes were prepared in triplicate, and the experiment was repeated twice. The prepared tubes were dried using a Speedvac, and the RIA reaction solution added as described in Section 3.2.2.8 for samples. A regression line and R^2 value were calculated for the linear region of the standards. An equation was calculated as discussed in Section 3.2.2.8. Logit transformations of the B/B_0 curves for the cZ, cZR, oT and oTR cytokinins (logit = $\ln[(B/B_0)/(1-B/B_0)]$) were calculated and plotted against the log of the number of pmol of unlabelled competitor (cZ, cZR, oT or oTR) present in the experiment (Trione et al., 1985). The percentage cross reactivity was calculated as the inverse of the molar concentration of the cytokinin standard $B/B_0 = 0.5$, relative to the cytokinin standard (ZR for clone 16, or iPA for clone 12) required to produced the same effect x100 (Badenoch-Jones et al., 1984). The 7-glucosides of both Z and iP were not immuno-reactive with the monoclonal antibodies used in this research. Therefore, their presence was not further investigated.

3.3 Results

3.3.1 Cytokinin sensitivity experiment

In the presence of 6-BAP, root length was significantly different between cultivars ($P < 0.001$). Florex Gold grown on MS media in the absence of 6-BAP had roots that were 91 ± 6 mm long. Root length was reduced by 25% (69 ± 4 mm) when grown in the presence of 4.4 μM 6-BAP (Figure 3.4). In comparison on the same media, the root lengths of Butterscotch and Chianti increased by 44% and 6% respectively, and the root length of Treasure and Sunglow was reduced by 5% and 9% respectively. At 13.3 μM 6-BAP, the root length of Florex Gold was reduced by 94% (5.5 ± 1.6 mm) compared to cytokinin-free media. In comparison the root length of other cultivars had reduced between 32% and 82% (Figure 3.4).

3.3.2 Cross reactivity

The cross reactivities and linearised logit/log plots of the cytokinin standards oT and oTR to clone 16 and clone 12 monoclonal antibodies are presented in Figure 3.5 and 3.6. The cytokinins oT and oTR had very low affinity to both clone 16 and clone 12 antibodies. The percentage cross-reactivity was calculated as follows: (#pmol of unlabelled ZR required for $B/B_0 = 0.5$)/(#pmol of competitor required for $B/B_0 = 0.5$) x 100. The percentage of cross reactivity of oT and oTR to either clone 16 or 12 could not be determined because there was no linear range of antibody competitor reaction at $B/B_0 = 0.5$ (or logit $B/B_0 = 0$). To detect oT and oTR with clone 16 antibody (ZR equivalents), a minimum of 1,787,525.5 and 48,721.3 pmol g^{-1} FW respectively would need to be present in the tissue investigated. To detect oT and oTR with clone 12 antibody (iPA equivalents), a minimum of 1,004.5 and 282.1 pmol g^{-1} FW would need to be present in the tissue sample investigated. These values were calculated at 1 pmol from the equation of oT and oTR for the pmol values 1 to 1000 pmol (clone 12 oT equation $y = -0.051x + 0.9768$, oTR equation $y = -0.0757x + 1.0092$).

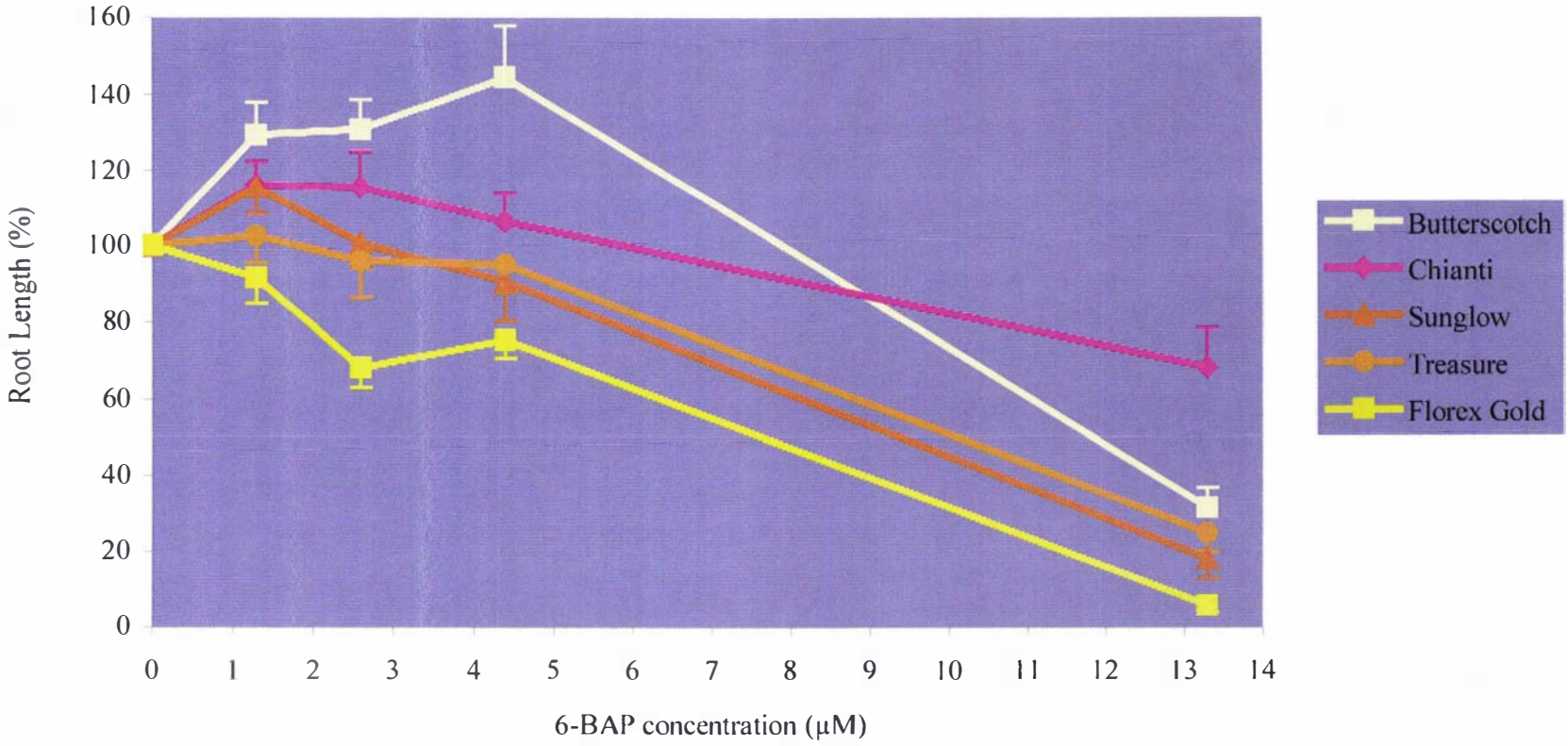


Figure 3.4: Root length, as a percentage of the root length at 0 µM 6-BAP, of the longest root per plant of five *Zantedeschia* cultivars micropropagated on MS media containing 0, 1.3, 2.6, 4.4 or 13.3 µM 6-BAP. Cultivars were grown for three, four-week subcultures on identical media. Data are an average of 30 roots per cultivar; bars indicate SE.

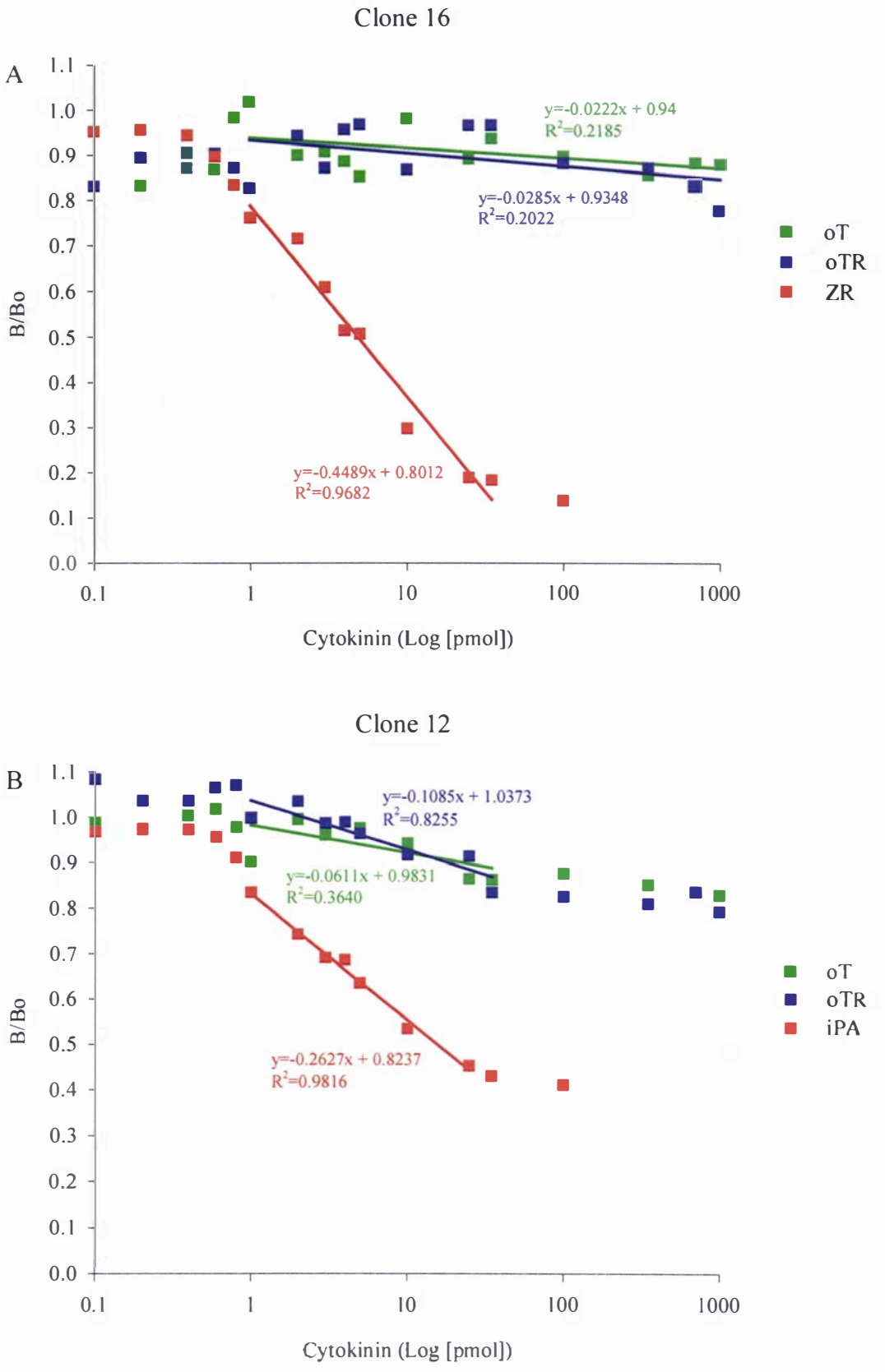
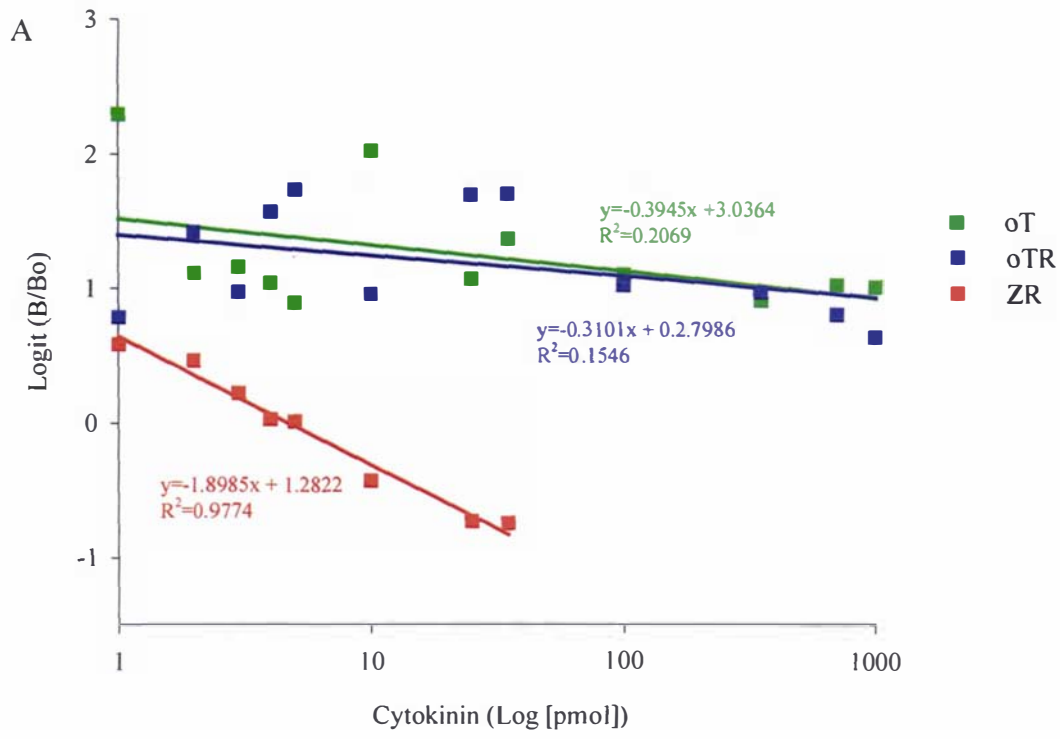


Figure 3.5: Cross reactivity of oT and oTR with the monoclonal antibody clone 16 (A) (anti-ZR antibody) and the monoclonal antibody clone 12 (B) (anti-iPA antibody), were determined by radioimmunoassay. The regression equations and R^2 values were derived from the linear part of the curve. B=binding of ^{3}H-ZRTA or ^{3}H-iPATA in the presence of any labelled ZR or iPA. Bo=binding of ^{3}H-ZRTA or ^{3}H-iPATA in the absence of any labelled ZR or iPA. The x axis has a log scale.

Clone 16



Clone 12

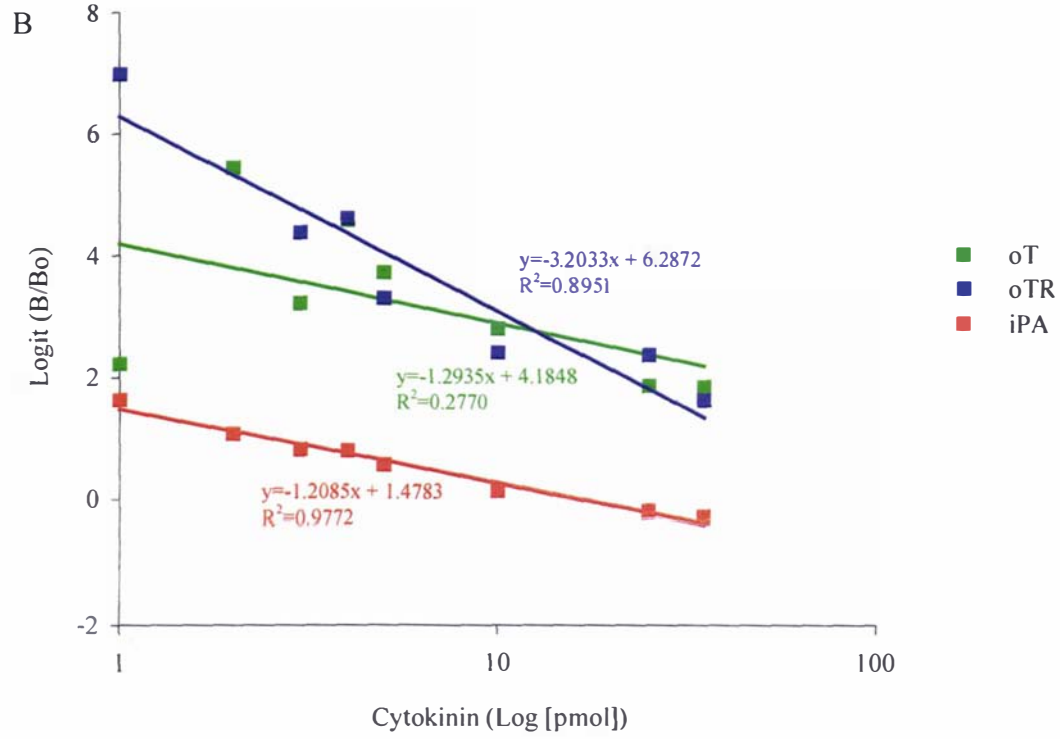


Figure 3.6: Linearized logit/log plots of oT and oTR, with the monoclonal antibody clone16 (A) (anti-ZR antibody) and the monoclonal antibody clone 12 (B) (anti-iPA antibody), were determined by radioimmunoassay. Logit B/Bo = ln [(B/Bo)/1-(B/Bo)]. The regression equations and R^2 values were derived from the linear part of the curve. B=binding of ^{3}H-ZRTA or ^{3}H-iPATA in the presence of any labelled ZR or iPA. Bo=binding of ^{3}H-ZRTA or ^{3}H-iPATA in the absence of any labelled ZR or iPA. The x axis has a log scale.

The cross reactivities and linearised logit/log plots of the cytokinins cZ and cZR to the clone 16 monoclonal antibody are presented in Figure 3.7 and 3.8. The cytokinins cZ and cZR had very low affinity to the clone 16 antibody. The percentage of cross reactivity of cZ and cZR to the clone 16 antibody could not be determined because there was no linear range of antibody competitor reaction at $B/B_0 = 0.5$ (or logit $B/B_0 = 0$). To detect cZ and cZR with clone 16 antibody (ZR equivalents), a minimum of 17.0 and 11.7 pmol g^{-1} FW respectively would need to be present in the tissue investigated. These values were calculated from the equation of the linear range of the standard curves at 2 pmol.

The second blank run of each day of both the amine and C_{18} HPLC collections were subjected to RIA to ensure that there was no contamination in the experimental samples. The second blank of the amine HPLC collection was subjected to all the following extraction and purification processes including C_{18} HPLC before RIA. Contamination was investigated in the fractions corresponding to the cytokinin standards. Cross-reactivity was no greater than 1 pmol in any blank runs.

A critical factor that emerged was the RIA results were improved for the both the dormant and sprouting T1 tubers, compared to the mother sprouting tubers, as newly synthesised ^{3}H-ZRTA and ^{3}H-iPATA was used. As a consequence, the standard curves of ZR and iPA had steeper slopes, and therefore cytokinin concentration could be more accurately determined. The RIA baseline was also lower, and it was easier to determine the presence of cytokinins with low concentrations. The control Florex Gold sprouting mother tubers were processed later with the dormant T1 tubers and therefore these tubers also had improved RIA results, in contrast to the other sprouting mother tubers.

In dormant and sprouting T1 tubers and control Florex Gold sprouting mother tubers the percentage recovery for the internal standards was between 20 to 78% for ^{3}H-ZRTA, 15 to 68% for ^{3}H-iPATA and 10 to 36% ^{14}C-AMP. In sprouting mother tubers (except control Florex Gold tubers) the percentage recovery for the internal standards was between 12 and 28% for ^{3}H-ZRTA. ^{3}H-iPATA and ^{14}C-AMP was not recovered in sprouting mother nucleotide fractions.

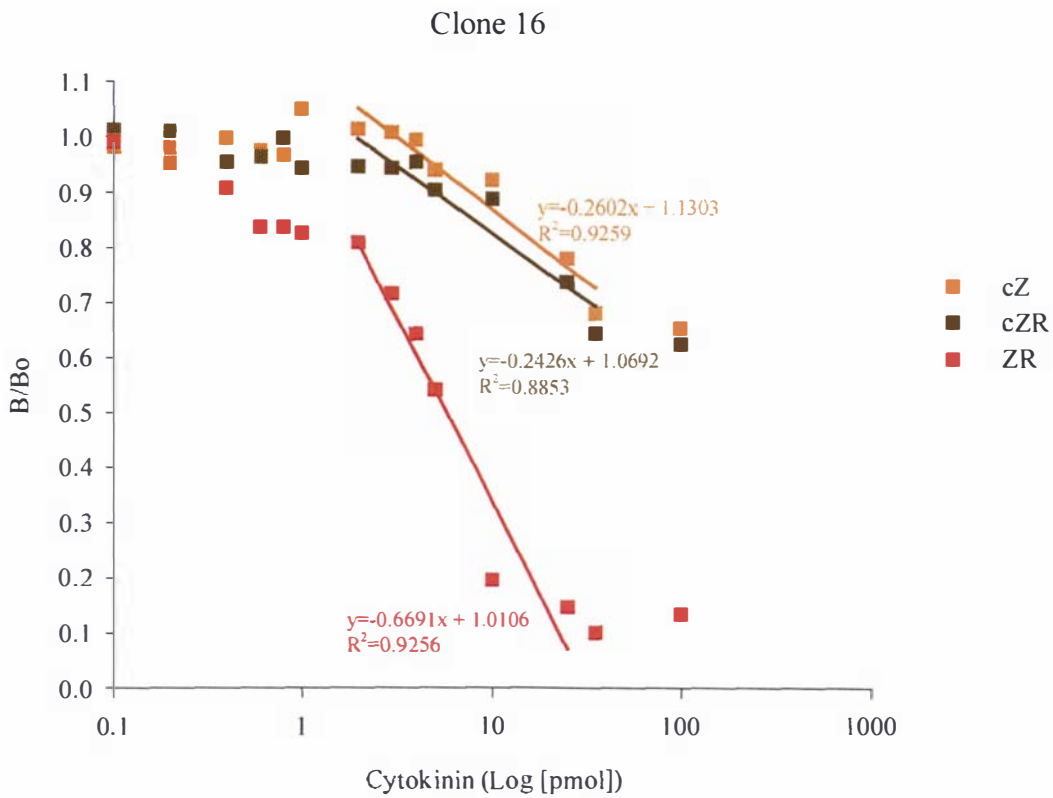


Figure 3.7: Standard curve of ZR, cZ and cZR with the monoclonal antibody clone 16 (anti-ZR antibody), as determined by radioimmunoassay. The regression equations and R^2 values were derived from the linear part of the curve. B=binding of ^{3}H-ZRTA to the antibody in the presence of ZR, cZ or cZR. Bo=binding of ^{3}H-ZRTA in the absence of ZR, cZ or cZR. The x axis has a log scale.

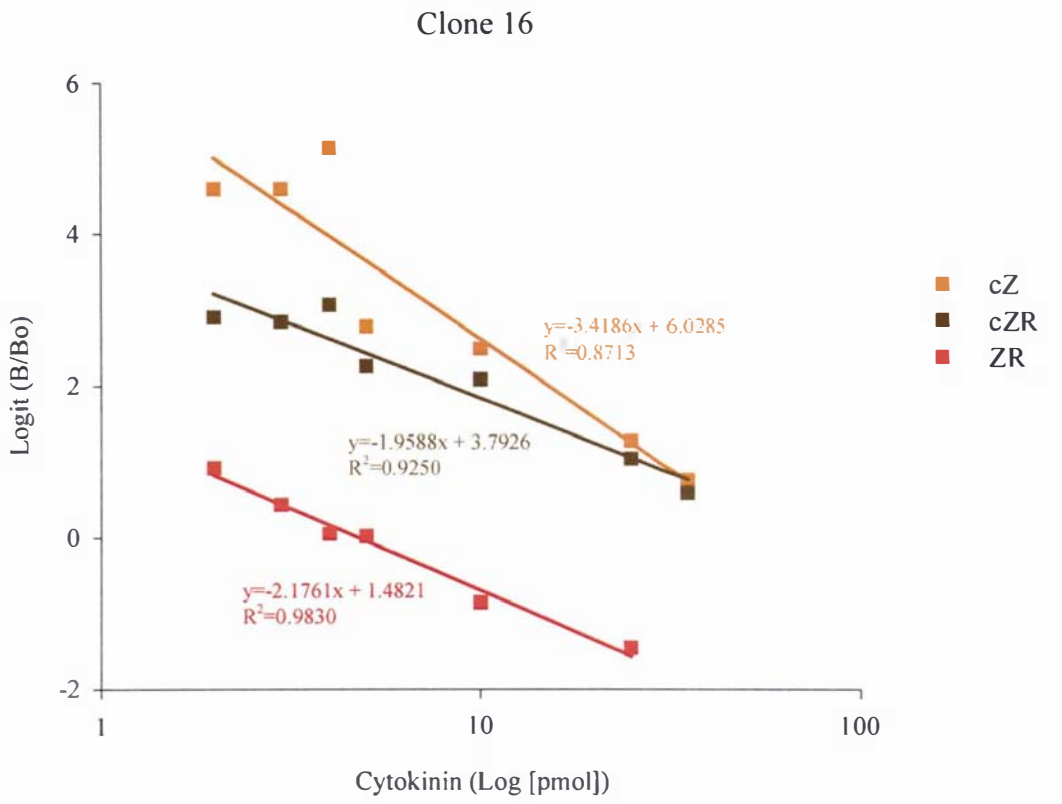


Figure 3.8: Linearized logit/log plot of ZR, cZ and cZR to the monoclonal antibody clone 16 (anti-ZR antibody), as determined by radioimmunoassay. Logit B/Bo = ln[(B/Bo)/1-(B/Bo)]. The regression equations and R^2 values were derived from the linear part of the curve. B=binding of ^{3}H-ZRTA in the presence of ZR, cZ or cZR . Bo=binding of ^{3}H-ZRTA in the absence of ZR, cZ or cZR. The x axis has a log scale.

3.3.3 Endogenous cytokinins in the tuber eyes of *Zantedeschia*

The free base and riboside, nucleotide and glucoside RIA profiles, of one replicate of sprouting T1 Dutch bushy Florex Gold, are presented in Figure 3.9. The nucleotides were measured as their ribosides following treatment with alkaline phosphatase. The glucosides were measured as their aglycones following treatment with β-glucosidase. Fractions 0 to 25 were assayed with the anti-ZR antibody clone 16. Fractions 25.5 to 39.5 were assayed with the anti iPA antibody clone 12. The results shown in Figure 3.9 were not corrected for losses or cross reactivity.

Nucleotide fractions from tuber samples at all three steps in the life cycle had relatively high background during RIA (see Figure 3.9), in comparison with free base and glucoside fractions completed during the same RIA experiment. This made the minimum detection limit higher for the nucleotides, at around 3 to 4 pmol. The minimum detection limit for free bases and ribosides, and glucosides was 1 to 2 pmol. It was also more difficult to determine if nucleotide cytokinins were present above background levels.

On the basis of cross-reactivity with anti-cytokinin antibodies, and by comparison of the retention times between standard cytokinin HPLC peaks and sample RIA peaks, it was determined that the free base and riboside cytokinins present in the tuber eyes and surrounding basal tissue of *Zantedeschia* were DZ, DZR, Z, ZR, cZ, cZR, iP and iPA. Individual cytokinins varied between tuber samples (Figure 3.10). The *cis*-cytokinins are presented as ZR equivalents, as the *cis*-cytokinins had low affinity to the antibodies used and percent cross reactivity could not be determined. All other cytokinins were quantified on the basis of the cross-reactivity of cytokinin standards with either clone 12 or clone 16.

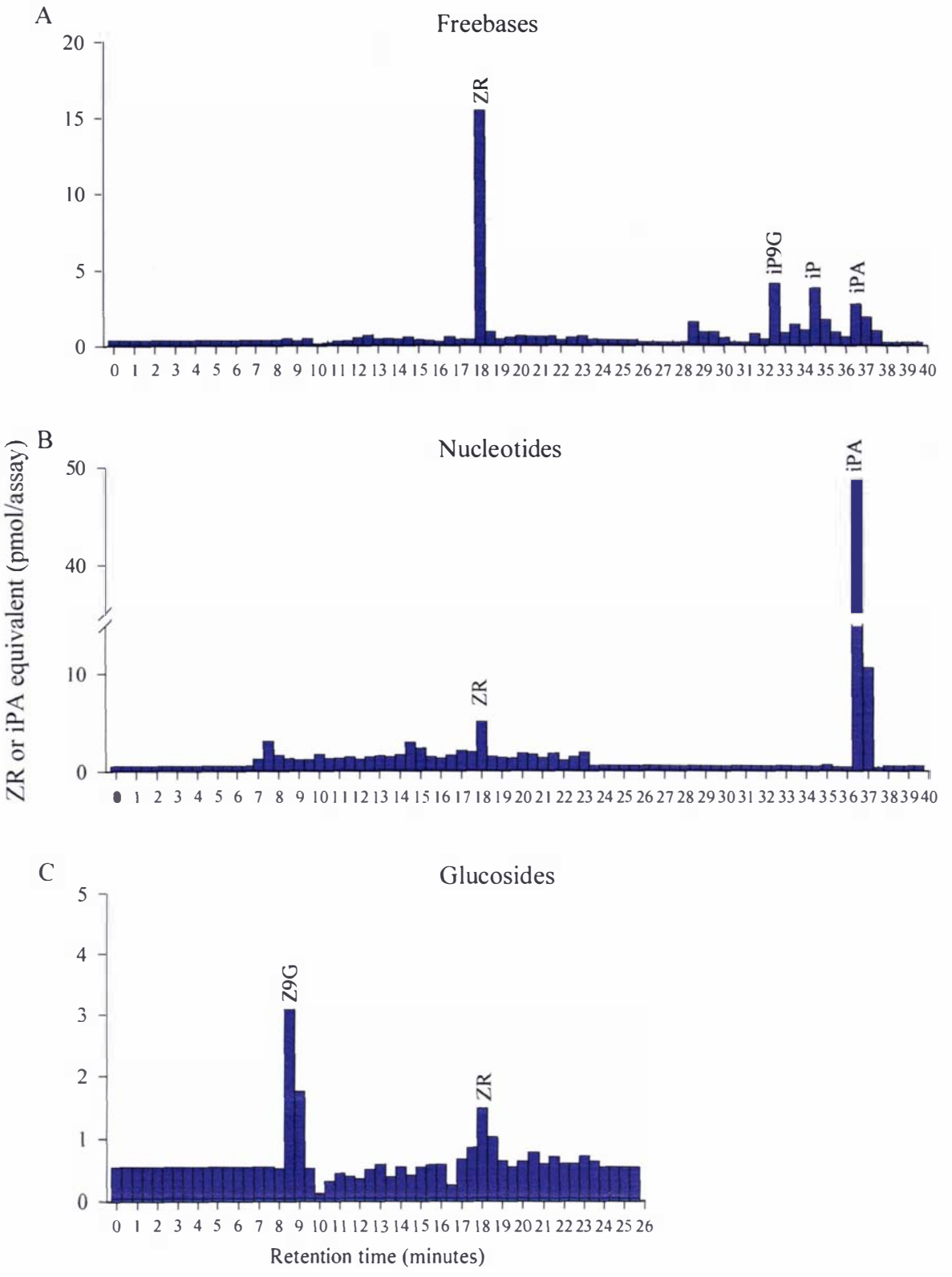


Figure 3.9: Cytokinins present in one replicate of sprouting T1 Dutch Florex Gold. (A) Free bases and ribosides. (B) Nucleotides, measured as their ribosides following treatment with alkaline phosphatase. (C) Glucosides, measured as their aglycones following treatment with β-glucosidase. Purified plant extracts were fractionated by HPLC and individual 0.5 mL fractions were assayed by RIA. Fractions 0 to 25 were assayed with clone 16 antiboby and fractions 25.5 to 39.5 with clone 12 antibody. No cytokinins were present in the glucoside fractions 25.5 to 39.5. Peaks are labelled with the cytokinin standard of the equivalent retention time. Data shown are not corrected for losses or cross reactivity.

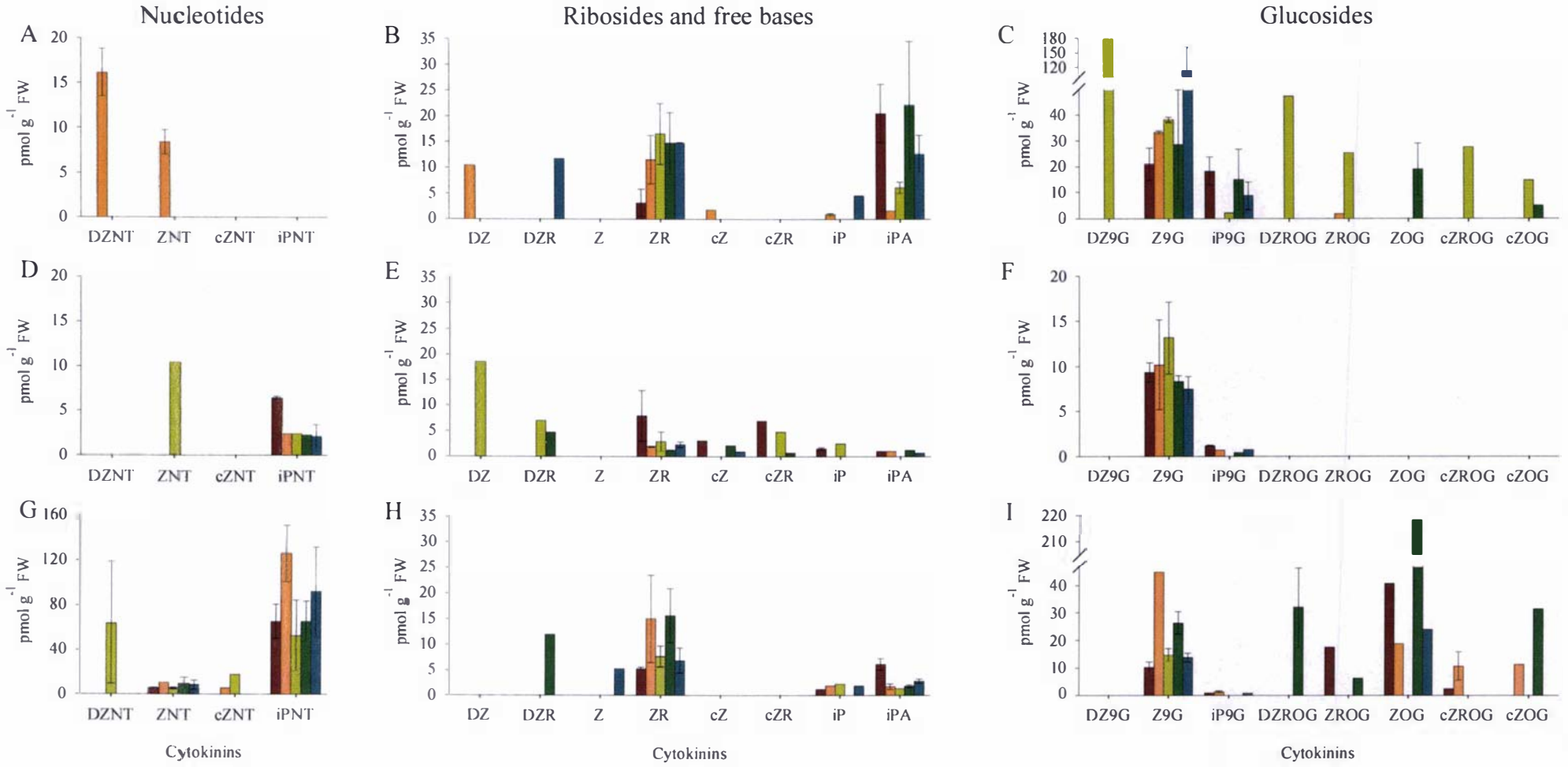


Figure 3.10: The endogenous cytokinin levels in tubers of Best Gold, and the Florex Gold tuber selections - control, few-eyed, bushy and Dutch. The cytokinin content was investigated in sprouting mother tubers (A, B and C), dormant T1 tubers (D, E and F) and sprouting T1 tubers (G, H and I). Nucleotide cytokinins are presented in A, D and G, ribosides and free bases are presented in B, E and H, and glucosides are presented in C, F and I. The cis cytokinins are presented as ZR equivalents. All other cytokinins were quantified taking into account losses and cross reactivity, and presented as pmol g^{-1} FW. Trace cytokinins are not presented. Values presented are means +/- SE (n=2); if no SE is presented, cytokinin was detected in only one sample.

Cytokinin nucleotides were dephosphorylated (Section 3.2.2.2) prior to reverse phase HPLC, so that the dephosphorylated forms could then be separated away from polar contaminants and cross react with the cytokinin antibodies. Their identities could then be determined by comparison with the retention times of the standard cytokinins. As four of the RIA peaks were identical to the retention times of ZR, DZR, cZR and iPA, it could be tentatively concluded that ZNT (zeatin nucleotide), DZNT (dihydrozeatin nucleotide), cZNT (*cis* zeatin nucleotide) and iPNT (isopentenyladenine nucleotide) were present in some of the tuber samples (Figure 3.10).

The glucoside fractions were treated with β-glucosidase (Section 3.2.2.6). The removal of the glucosyl group enabled the *O*-glucosides to cross react with the clone 16 antibody and their identities to be determined. The 9-glucosides cross react with the clone 16 antibody without treatment. By comparison with the retention times of the standard cytokinins, it was determined by RIA that DZ9G, Z9G, iP9G, DZROG, ZROG, ZOG, *cis*-zeatin riboside-*O*-glucoside (cZROG) and *cis*-zeatin-*O*-glucoside (cZOG) were the cytokinins present in some tuber samples (Figure 3.10).

The total cytokinin concentration of Best Gold compared with all Florex Gold samples is presented in Figure 3.11. The total cytokinin concentration of both Dutch and New Zealand bushy Florex Gold, compared with control Florex Gold is presented in Figure 3.12. The cytokinin concentrations are grouped by ribosides and free bases, nucleotides, 9-glucosides, *O*-glucosides, *cis* ribosides and free bases, *cis* nucleotides and *cis* *O*-glucosides. The cytokinin concentrations are presented at the three stages of the *Zantedeschia* life cycle examined. Trace cytokinins were not included in the sum of cytokinin concentrations.

The total cytokinin concentration was highest in sprouting *Zantedeschia* tuber eyes and lowest in endodormant *Zantedeschia* tuber eyes. The sprouting *Zantedeschia* tubers had more nucleotide cytokinins. Nucleotides may have been lost in sprouting mother tubers, excluding control Florex Gold tubers, which were processed at a later date. The internal standard ^{14}C-AMP was not recovered in the sprouting mother tuber nucleotide fractions. The Best Gold sprouting tuber samples had a lower total cytokinin concentration than Florex Gold. No measurable differences in the total cytokinin levels were seen between bushy and control Florex Gold tuber samples.

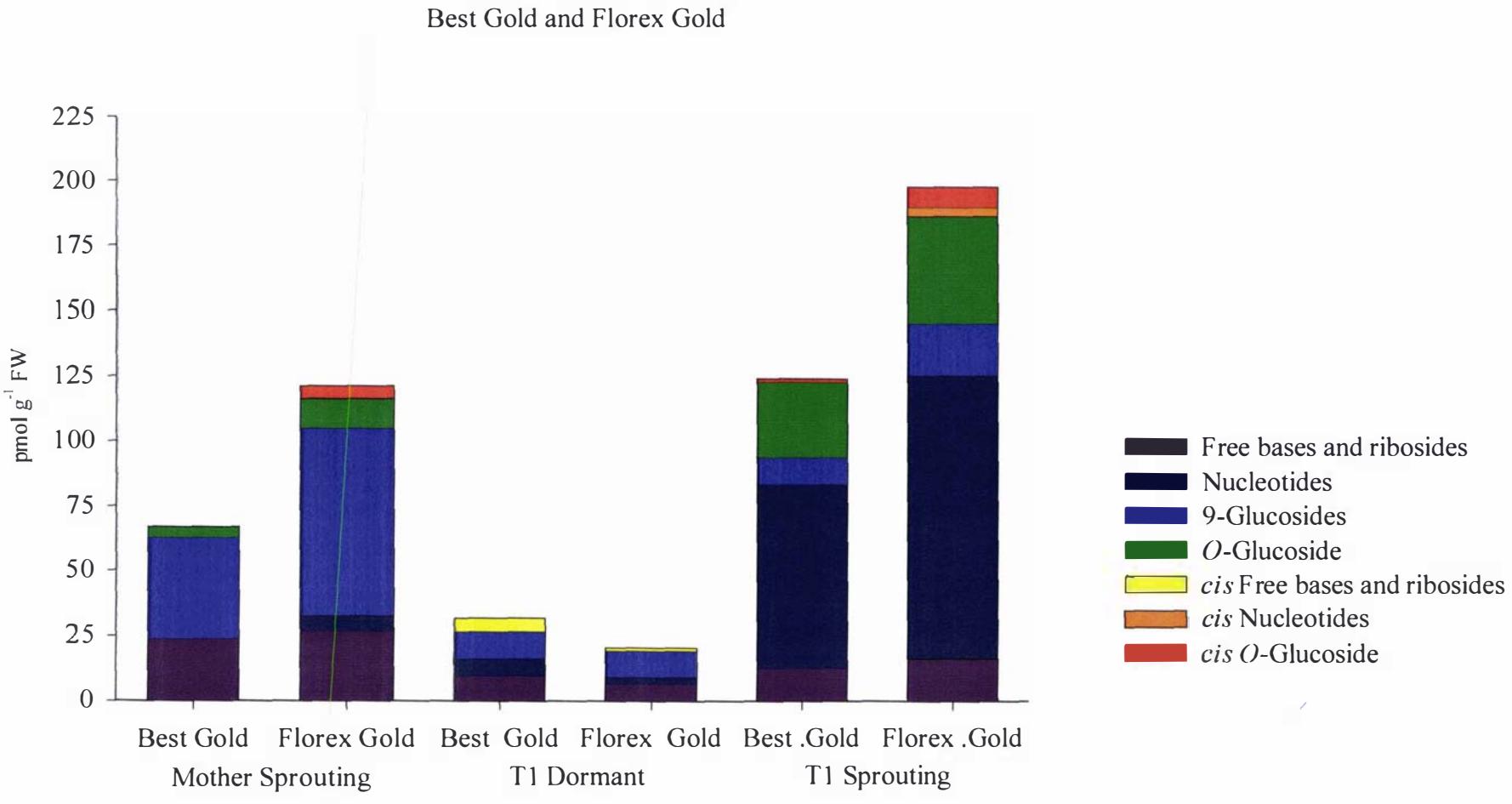


Figure 3.11: The total cytokinin concentrations of Best Gold and all Florex Gold samples. The total cytokinin concentrations are grouped by free bases and ribosides, nucleotides, 9-glucosides, *O*-glucosides, *cis*-free bases and ribosides, *cis*-nucleotides and *cis*-*O*-glucosides. The *cis* cytokinins are presented as ZR equivalents. All other cytokinins were quantified taking into account losses and cross reactivivty, and presented as pmol g^{-1} FW. Trace cytokinins were not included in the sum of cytokinin concentration. The cytokinin concentrations are presented at three stages of the *Zantedeschia* life cycle.

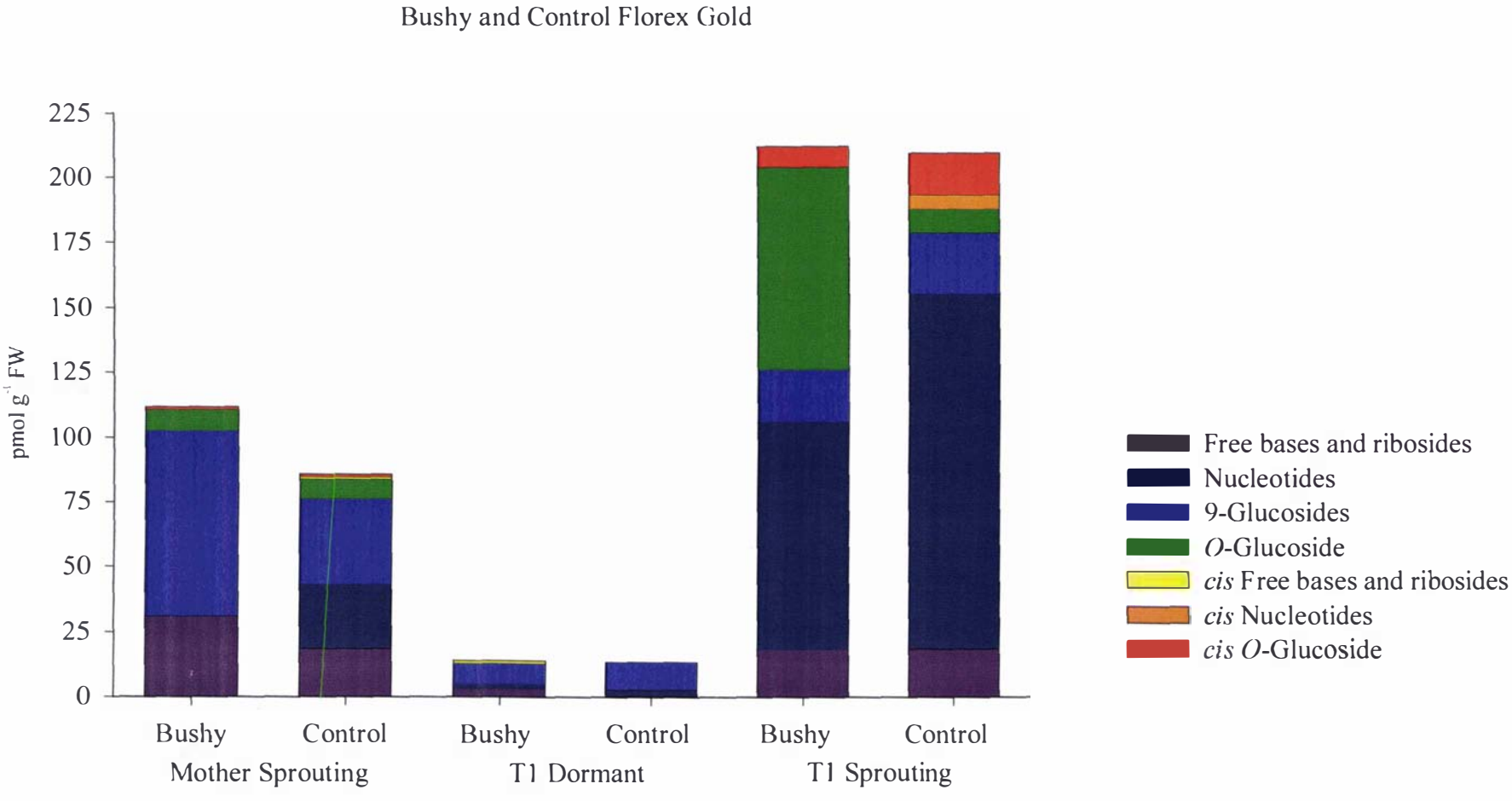


Figure 3.12: The total cytokinin concentrations of Dutch and New Zealand bushy, and control Florex Gold samples. The total cytokinin concentrations are grouped by free bases and ribosides, nucleotides, 9-glucosides, *O*-glucosides, *cis*-free bases and ribosides, *cis*-nucleotides and *cis*-*O*-glucosides. The *cis* cytokinins are presented as ZR equivalents. All other cytokinins were quantified taking into account losses and cross reactivivty and presented as pmol g^{-1} FW. Trace cytokinins are not included in the sum of cytokinin concentration. The cytokinin concentrations are presented at three stages of the *Zantedeschia* life cycle.

3.4 Discussion

3.4.1 *Zantedeschia* Florex Gold was more sensitive to 6-BAP during micropropagation which may predispose it to bushiness

A root length bioassay has been used to assess sensitivity to cytokinin in recent work focused on identification of cytokinin receptors (Plakidou-Dymock et al., 1998; Inoue et al., 2001). A reduction in root length was interpreted by both Plakidou-Dymock et al. (1998) and Inoue et al. (2001) as indicative of increased sensitivity to cytokinin. This bioassay was utilised to assess whether *Zantedeschia* cultivars exhibited differences in sensitivity to the cytokinin 6-BAP, which is routinely used in commercial micropropagation. In this study, the *Zantedeschia* cultivar Florex Gold displayed the greatest sensitivity to cytokinin with reduced root length even at a concentration that promoted root growth in two other cultivars (Figure 3.4). Florex Gold showed an enhanced sensitivity towards the plant growth regulator 6-BAP relative to other cultivars during micropropagation, which may in some way predispose this cultivar to development of the bushy syndrome. This result also validates the use by some tissue culture laboratories of reduced cytokinin during micropropagation of Florex Gold.

3.4.2 Endogenous cytokinins in *Zantedeschia*

Using HPLC and RIA, endogenous cytokinin concentration was investigated in sprouting mother tubers and both dormant and sprouting T1 *Zantedeschia* tubers. Following fractionation by HPLC, a total of 20 cytokinins were detected. These were DZNT, ZNT, cZNT and iPNT nucleotides, DZ, DZR, Z, ZR, cZ, cZR, iP and iPA free bases and ribosides and DZ9G, Z9G, iP9G, DZROG, ZROG, ZOG, cZROG and cZOG glucosides (Figure 3.10).

3.4.2.1 Nucleotides

The T1 sprouting *Zantedeschia* tubers had on average 40-fold more nucleotides than dormant *Zantedeschia* tubers (Figure 3.11 and 3.12). Nucleotides are considered to be the first formed cytokinins (Takei et al., 2001), which are subsequently rapidly metabolised to other cytokinin forms (Suttle, 1998). The cytokinin quantification procedure was optimised for the dormant and sprouting T1 tubers, as well as the control

Florex Gold mothers, which were processed at a later date. The optimised RIA allowed the successful detection of nucleotides. Nucleotides were not detected in the sprouting mother tubers, although they may have been present.

3.4.2.2 Free bases and ribosides

The levels of the cytokinins Z, ZR, iP and iPA in *Zantedeschia* were very similar to those detected in potato tubers. The biologically active cytokinin ZR was highest in sprouting *Zantedeschia* tubers, at levels of 3.2 to 16.5 pmol g^{-1} FW. Z was detected in one replicate of sprouting T1 Dutch bushy tubers at 5.3 pmol g^{-1} FW. Endogenous levels of ZR and Z in potato were between 3 and 14 pmol g^{-1} FW, and between 8 and 16 pmol g^{-1} FW, respectively (Suttle, 1998). The cytokinins iP and iPA were detected in *Zantedeschia* at concentrations of 4.5 pmol g^{-1} FW and 1.3 to 22.0 pmol g^{-1} FW respectively. The cytokinins iP and iPA were, however, not detected in some sprouting and dormant *Zantedeschia* tuber eye samples. The endogenous levels of iP + iPA in potato were between 8 and 29 pmol g^{-1} FW (Suttle, 1998).

DZ and DZR were detected in some dormant and sprouting Best Gold and Florex Gold tubers (10.4 to 18.6 pmol g^{-1} FW). DZ-type cytokinins have previously been reported in jasmonic acid-treated potato plantlets by Dermastia et al. (1994). DZ, DZR and DZ9G were detected at 19 ± 5 ng g^{-1} FW, 81 ± 2 ng g^{-1} FW and 139 ± 25 ng g^{-1} FW respectively (Dermastia et al., 1994). The occurrence of DZ-type cytokinins has not been previously reported in potato tubers (Suttle, 1998, 2001).

3.4.2.3 9-glucoside cytokinins

The 9-glucoside cytokinins found in sprouting *Zantedeschia* appear to be stable metabolites in the tissues in which they are formed. They are substrates for cytokinin oxidase, but it is believed that they are generally protected from this enzyme by compartmentalisation in cells. They are regarded as detoxification or deactivation products (Jameson, 1994). The cytokinin iP9G was found in generally low concentrations in *Zantedeschia* (0.8 to 18.3 pmol g^{-1} FW). In contrast, iP9G was the most abundant cytokinin in potato tubers (Suttle, 1998). In potato, iP9G comprised anywhere from 50 to 90% of the total cytokinin detected. In potato tubers stored continuously at 3°C for 174 days, iP9G levels were 357.5 pmol g^{-1} FW (Suttle, 1998).

In *Zantedeschia* Z9G was detected in most sprouting mother and T1 tuber samples. In sprouting mother and T1 tubers Z9G concentrations varied between 10.2 and 112.2 pmol g^{-1} FW. In dormant T1 tubers Z9G concentrations varied between 7.5 and 13.2 pmol g^{-1} FW. Z9G was not detected in potato extracts (Suttle, 1998).

3.4.2.4 *Cis*-cytokinin isomers

The cross reactivity of the cytokinins cZ and cZR with clone 16 antibody was investigated (Figure 3.9). As the cZ and cZR B/B_0 curves were not parallel with the ZR standard curve, an accurate estimation of the percent cross reactivity with the antibodies could not be determined. The data relating to the cZ and cZR are therefore presented as ZR-equivalents. The minimum detectable levels of cZ and cZR were 17.0 and 11.7 pmol g^{-1} FW respectively.

Cis-cytokinin forms were detected in some *Zantedeschia* tuber samples, although not consistently. In both dormant and sprouting *Zantedeschia* tubers, endogenous levels of cZ varied between 0.8 to 3 pmol ZR equivalents g^{-1} FW and in dormant tubers cZR levels varied between 0.7 to 6.9 pmol ZR equivalents g^{-1} FW. Endogenous levels of cZ and cZR in potato were between 24 and 102 pmol g^{-1} FW and 4 and 10 pmol g^{-1} FW, respectively (Suttle and Banowetz, 2000).

No attempt was made to determine the endogenous levels of cZ9G in *Zantedeschia* tubers. It would be expected to elute after DZ9G and before Z during the reverse phase HPLC separation (Figure 3.3). Endogenous cZ9G was identified in potato tuber sprouts (Nicander et al., 1995). The tuber sprout extracts contained 10 times as much cZ9G as tZ9G, even though cZ was present at a much lower concentration that tZ (Nicander et al., 1995).

Endogenous cZOG and cZROG were detected in some sprouting Best Gold and Florex Gold tubers (4.9 to 31.2 pmol ZR equivalent g^{-1} FW, and 2.4 to 27.5 pmol ZR equivalent g^{-1} FW, respectively). The cZOG cytokinin has been previously detected at the anthesis growth stage in rice (38 pmol g^{-1} FW) (Takagi et al., 1989), but has not been reported in other tubers. The cZROG cytokinin was detected in rice grains. The highest levels were detected in the milk growth stage at 187.2 pmol g^{-1} FW (Takagi et al., 1989). Considerable amounts of cZROG have been tentatively identified in roots

(ca. 30 pmol g^{-1} FW) and meristematic leaves (ca. 20 pmol g^{-1} FW) of stinging nettle (*Urtica dioica* L.) (Wagner and Beck, 1993).

The *cis*-cytokinins detected in *Zantedeschia* tubers during dormancy and sprouting may be from tRNA turnover in the storage tissue, which results in uncontrolled release of *cis*-cytokinins in all tissues. If these *cis*-cytokinins from tRNA contribute to the major source of cytokinins, regulatory mechanisms must operate at the level of metabolism of the released *cis*-cytokinins (Mok and Mok, 2001). There is evidence of *cis*-specific regulatory elements in plants, with the isolation of a maize *cis*-zeatin-specific *O*-glucosyltransferase gene (Martin et al., 2001). The recently identified cytokinin receptor *CRE1* did not, however, respond to cZ, suggesting that the *cis*-cytokinin isomers are biologically inactive (Inoue et al., 2001).

3.4.2.5 Aromatic cytokinins

The cytokinins oT and oTR, which have previously been reported in *Zantedeschia aethiopica* during spathe regreening (Chaves das Neves and Pais, 1980a, 1980b), were unable to be investigated in this research, as the monoclonal antibodies (clone 16 and clone 12), cross reacted with them with very low affinity. The cytokinins oT and oTR would have to have been present at a minimum of 1.8 μmol g^{-1} FW and 49 nmol g^{-1} FW respectively, to be detected by clone 16 and 1 nmol g^{-1} FW and 282 pmol g^{-1} FW respectively to be detected by clone 12. Most of these concentrations are biologically improbable. Due to time constraints, the determination of oT and oTR cytokinin concentrations was not pursued with purchased antibodies.

Naturally occurring 6-BAP, oT and 6-(3-hydroxybenylamino)purine or meta-topolin (mT), their ribosides and 9-glucosides were reported in a wide variety of oil palm tissues (Jones et al., 1996). They were identified using specific antibodies to these groups of compounds in a HPLC/enzyme-linked immunosorbent assay. The ribosides were present at the highest levels, followed by the free base cytokinins and the 9-glucosides were present at the lowest concentrations. The aromatic cytokinins were at very low concentrations in the vegetative crown tissues and in the youngest inflorescence analysed (leaf axil 6) but were found in older inflorescences, embryos, embryoids, and shoot tissues of seedlings and ramets. Without sample replication, no conclusions could be reached on the quantitative differences between tissue types (Jones

et al., 1996). The aromatic cytokinin concentrations were not compared in normal and abnormal mantled oil palm tissue types.

3.4.3 Differences in cytokinins seen between tuber replications

Some of the variation seen between replicates of the same *Zantedeschia* selection may be a consequence of sampling tuber eyes from only a small number of tubers for each analysis, since individual tubers may vary in relative maturity. For example, control Florex Gold ZR levels in sprouting tubers were 23.5 pmol g^{-1} FW in one tuber replicate and 6.6 pmol g^{-1} FW in another. This is a 3.5 fold difference in concentration. Additional variation may be because some of the sprouting *Zantedeschia* mother tubers were harvested up to two months apart (Section 2.2.1), although T1 sprouting tubers were harvested at the same time. The bushy tubers, sourced from the Netherlands, were grown and harvested in the opposite growing season to New Zealand. So their stage of dormancy/maturity may have been different to the New Zealand grown tubers. Individual tubers from potato plants have been suggested to vary in maturity by as much as four weeks (Turnbull and Hanke, 1985b) . The detection of *cis*-cytokinin isomers in only some tuber samples may be due to the low cross reactivity of these cytokinins with the clone 16 antibody. They may be present in other tuber samples, but at levels below detection.

3.4.4 Cytokinins and bushiness

In attempting to discern a role for endogenous cytokinins in the expression of the bushy syndrome, one of the following criteria should be met: either a difference in the sum of biologically active cytokinins should be seen between bushy and non-bushy plants or increased sensitivity might be evidenced by the increased concentration of storage cytokinin forms. Suttle (1998) stated that a three-fold increase in endogenous bioactive cytokinin is needed for the increase to be physiologically significant in potato tubers. Further, Suttle (1998) stated that changes in total bioactive cytokinin content may be of more relevance than changes in individual cytokinin concentration. This is because most cytokinins (with the exception of N-glucoside conjugates) can be readily converted to bioactive forms.

No measurable differences in the total cytokinin levels were seen between bushy and control Florex Gold T1 dormant and sprouting tuber samples (Figure 3.12). This suggests that the sum of biologically active cytokinins is not directly responsible for the expression of the bushy syndrome. An eight-fold higher concentration of *O*-glucosides (storage cytokinins) was found in the bushy compared to the control Florex Gold sprouting T1 tubers (Figure 3.12). However, the higher concentration of *O*-glucosides was detected in New Zealand bushy tubers and not found in the Dutch bushy Florex Gold tubers (Figure 3.10). Therefore, the high levels of *O*-glucosides do not seem to account for the expression of the bushy syndrome.

The role of cytokinins in the expression of a comparable syndrome to bushiness has been investigated in micropropagated oil palm. Besse et al. (1992) investigated the cytokinin content in callus that gave rise to both normal and mantled oil palm. They were unable to detect any cytokinin in FGC (100% abnormal regenerated plants), whereas they did detect cytokinin in NCC (<5% abnormal regenerated plants). They hypothesised that the mantled phenotype involving the malformation of oil palm inflorescences was a physiological syndrome associated with tissue disorganisation and abnormally low endogenous cytokinin levels. Jones et al. (1995) did not agree and suggested that the detection of differences in cytokinin concentration between FGC and NCC was not evidence of the involvement of cytokinins in subsequent floral abnormalities. Rather it demonstrated the expected difference between a tissue containing large, vacuolated non-dividing cells and one with predominantly small, highly cytoplasmic meristematic cells.

Jones et al. (1995) examincd tissue cultures and regenerant plants, including inflorescences, from cell lines producing palms with normal and abnormal flowers and compared with zygotic embryos and seedlings. Jones et al. (1995) found, in most oil palm plant tissues, that the differences in cytokinin levels between clones was greater than any differences between normal and abnormal tissues. An exception was the comparison of two isogenic lines of one clone, one line of which had normal flowering palms and the other abnormal. In this case, significantly higher concentrations of ZR and Z9G were found in the normal line compared to the abnormal line (Jones et al., 1995). Jones et al. (1995) concluded that the concentration of cytokinin per se, was not the sole cause of the mantled flowering abnormality in oil palm. This is in agreement with that found in *Zantedeschia*.

Sprouting Best Gold tubers had less total cytokinin concentration than Florex Gold tubers, with the difference being predominantly in the nucleotides (Figure 3.11). This suggests that there are differences in the endogenous cytokinin profile between these two yellow flowering cultivars. The higher endogenous cytokinin level in the Florex Gold genotype may in some way predispose the Florex Gold genotype to the bushy syndrome.

3.4.5 Endogenous cytokinins in the *Zantedeschia* life cycle

Suttle (1998) detected eight cytokinins in endodormant potato tuber apical buds. These were: ZRMP, ZOG, Z, ZR, iPMP, iP9G, iP and iPA. Suttle and Banowetz (2000) reported the presence of cZ and cZR during potato tuber dormancy. All ten cytokinins detected in potato were also detected in *Zantedeschia* tubers (Section 3.4.2). Ten additional cytokinins were also detected in *Zantedeschia* (Section 3.4.2). These included three additional *cis*-cytokinins cZNT, cZROG and cZOG, the cytokinins ZROG and Z9G and the DZ-type cytokinins (DZNT, DZ, DZR, DZ9G and DZROG). The occurrence of Z9G and the DZ-type cytokinins has not been previously reported in potato tubers (Suttle, 1998, 2001) (Sections 3.4.2.2 and 3.4.2.3).

The most abundant cytokinins found in the sprouting *Zantedeschia* T1 tubers were the nucleotides and *O*-glucosides, followed by the 9-glucosides and free bases and ribosides (iPNT>ZOG>Z9G>ZR>iPA) (Figure 3.10, 3.11 and 3.12). The loss of potato tuber endodormancy and the onset of sprouting were preceded by significant increases in the endogenous levels of Z, ZR, iPMP and iP9G. Increases in the cytokinins ZR, iP9G and iP + iPA were also observed in ecodormant potato tubers prevented from sprouting by storage temperature (Suttle, 1998).

The total cytokinin concentration was 11-fold higher in sprouting *Zantedeschia* tubers than dormant tubers (Figures 3.11 and 3.12). In potato, the total content of cytokinins increased by over seven-fold during postharvest storage and this increase was shown to be the result of de novo synthesis (Suttle, 1998). Endogenous levels of the most abundant cytokinin found in potato, iP9G, rose over 11-fold during storage, regardless of temperature. In potato, iP9G was the most abundant cytokinin, comprising anywhere from 50 to 90% of the total cytokinins detected (Suttle, 1998). In contrast to potato tubers, Z9G was the 9-glucoside cytokinin found in highest concentration in

Zantedeschia tubers. Z9G was 2.5-fold higher in sprouting than dormant *Zantedeschia* tubers. In both *Zantedeschia* and potato, the 9-glucosides were elevated. The reason for the elevated Z9G in *Zantedeschia*, in contrast to iP9G in potato is unknown.

A previous study by Turnbull and Hanke (1985b) found that during potato tuber growth, ZR was the predominant cytokinin detected in all tissues. The total cytokinin concentration fell dramatically after harvest, largely as a consequence of the disappearance of ZR. During storage the levels of cytokinin in the storage tissue remained relatively constant, but increased in the tuber buds. In the buds of tubers stored at 2°C there was a 20 to 50-fold increase in total cytokinin over six weeks, coinciding with the natural break of innate dormancy. At 10°C the rise in the level of bud cytokinins was slower, correlating with the longer duration of innate dormancy (Turnbull and Hanke, 1985b). The RIA used by Turnbull and Hanke (1985b) was specific only for the Z-type cytokinins, any cytokinin with DZ or iP-type side chains could not be detected, so that direct comparison with Suttle's research (1998) and the cytokinins detected in *Zantedeschia* is difficult.

Endogenous levels of iP- and Z-type cytokinins increased in potato tubers prior to the onset of sprout growth (Turnbull and Hanke, 1985b; Suttle, 1998). Further, injection of exogenous cytokinins resulted in premature tuber sprouting (Turnbull and Hanke, 1985a; Suttle, 1998). These observations are consistent with a role for endogenous cytokinins in the process of dormancy termination in potato (Suttle and Banowetz, 2000). Further research may also indicate a role of iP- and Z-type cytokinins in the termination of tuber endodormancy in *Zantedeschia.* Other reports have also hypothesised a role for cytokinins in dormancy breaking in *Gladiolus grandiflorus* (Ginzburg, 1973), onion bulbs (Thomas, 1981) and in buds of certain temperate zone perennials (Powell, 1987). However, these reports did not investigate the endogenous cytokinin concentration and profile during dormancy breaking.

Chapter Four: Molecular studies investigating DNA methylation and differential gene expression

4.1 Introduction

The aim of this research was to develop an understanding of the variables associated with the bushy syndrome. This included analysing the DNA methylation profile and differential gene expression between bushy and non-bushy plants and tubers. DNA methylation and its role in the regulation of gene expression has been hypothesised to be an underlying mechanism in somaclonal variation in other micropropagated species (Arnholdt-Schmitt et al., 1991; Kaeppler and Phillips, 1993b; Harding, 1994; Arnholdt-Schmitt et al., 1995; Smulders et al., 1995; Xiong et al., 1999; Peraza-Echeverria et al., 2001). DNA methylation has also been hypothesised to play a role in the mantled phenotype of micropropagated oil palm, a syndrome comparable to bushiness (Rival et al., 2000; Matthes et al., 2001). As the DNA AFLP technique has proved highly efficient for large scale detection of cytosine methylation (Xiong et al., 1999), this technique was chosen to investigate the role of DNA methylation in the expression of the bushy syndrome. Differences in DNA methylation patterns were examined using a methylation sensitive restriction enzyme *Hpa* II. *Hpa* II recognises the sequence C/CGG but does not cleave ^{m}CCGG and C^{m}CGG (Xiong et al., 1999; Matthes et al., 2001; Peraza-Echeverria et al., 2001).

To investigate expressed genes and their possible role(s) in the expression of the bushy syndrome, a RNA fingerprinting technique, cDNA AFLP, was used to analyse differential gene expression. The cDNA AFLP technique was chosen as it has previously yielded important differentially expressed genes from eukaryotes (Dellagi et al., 2000).

4.2 Materials and Methods

4.2.1 Genomic DNA AFLP

4.2.1.1 DNA extraction and quantification

Zantedeschia leaves of similar age were harvested from plants of identical age, in their second growing season after tissue culture (T2). Leaves were harvested from Best Gold plants grown from tubers B and C; control Florex Gold; few-eyed plants grown from tubers A and B; bushy plants grown from tubers B and F; and Dutch plants grown from tubers E and F (for a description see 2.2.1). The *Zantedeschia* leaves were harvested into liquid nitrogen and each sample was ground in liquid nitrogen in a 1.5 mL centrifuge tube with a grinding tool. The grinding tool was prepared by heating in a glass Pasteur pipette in a Bunsen flame, close to where the pipette narrowed, until the two sections separated. The thicker section of the pipette was again passed through the flame to create a small bulb, which was used to grind the tissue. One tool was prepared for each sample processed.

An aliquot of 600 µL of CTAB extraction buffer (2% [w/v] hexadecyltrimethylammonium bromide [CTAB], 1% [w/v] polyvinyl-pyrrolidone, 1.4 M NaCl, 100 mM Tris [hydroxymethyl] aminomethane hydrochloride [Tris HCl] [pH 8.0] and 20 mM ethylenediaminetetra-acetic acid disodium salt [EDTA]) was added to the ground tissue. The sample was then incubated at 65°C for 30 to 60 min. Following incubation, 600 µL of chloroform was added to the supernatant and the tubes were shaken vigorously. The tube was left to stand for 2 min, to initiate the separation of aqueous and organic phases. The tube was then briefly centrifuged (3,000g for 5 s) to complete the separation of the phases and compact the precipitates at the interphase. A wide bore pipette was used to transfer the upper aqueous phase to a clean 1.5 mL centrifuge tube, taking care not to transfer any of the interphase. A 600 µL aliquot (equal volume) of isopropanol was added to the supernatant. The two solutions were very gently inverted twice and the tube was incubated on ice for 10 to 15 min. The DNA precipitated from solution to form a cotton-wool like mass. DNA along with 300 µL of the solution was transferred using a wide bore pipette, to a 1.5 mL centrifuge tube containing 600 µL 80% (v/v) ethanol. The 1.5 mL centrifuge tube was then gently mixed by hand using a rocking motion (5 s). The DNA and a small amount of solution

were transferred to a clean 1.5 mL centrifuge tube containing 600 μL of 80% (v/v) ethanol. The process was repeated three to four times. The entire mixture was centrifuged for 5 s at 3,000 g. The ethanol was decanted to waste and the DNA pellet was left to air dry for 60 min. The DNA was resuspended in 20 to 50 μL sterile MilliQ water, and stored at 4°C.

A 2 μL aliquot of each sample of extracted DNA was combined with 1 μL of 10X loading buffer (25% [w/v] Ficoll 70 [Pharmarcia], 0.16% [w/v] bromophenol blue [Serva] and 0.16% [w/v] xylene cyanole FF [Sigma]) and 7 mL MilliQ water. The mixture was loaded onto a 0.8% (w/v) agarose LE (Roche)/1X Tris acetate EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.0]) gel. A 1 Kb plus DNA Ladder (Roche) and a High Mass Ladder (Gibco BRL) were included in the electrophoretic gel as size and quantity standards respectively. The 1 Kb plus Ladder was prepared by adding 4 μL 1Kb plus Ladder stock (1 μg μL^{-1}), 10 μL 10X loading buffer and 86 μL TE buffer (10 mM Tris [pH 8.0] and 1 mM EDTA [pH 8.0]). The High Mass Ladder was prepared by adding 2 μL of High Mass Ladder stock (130 ng μL^{-1}), 1 μL 10X loading buffer and 7 μL TE buffer. Samples were electrophoresed at 100 V in 1X TAE buffer for 1 h. Following electrophoresis the DNA samples were stained by placing the gel in an ethidium bromide solution (10 μg mL^{-1}) for 10 min and destained in MilliQ water for 2 min. The DNA samples were visualised by ethidium bromide fluorescence on a UV transilluminator (wavelength 302 nm, UVP Incorporated) and a digital photograph of the illuminated gel taken using a video camera (COHU) and ImagePC software (Scion).

4.2.1.2 Restriction endonuclease digestion of genomic DNA

The AFLP procedure used in this study was based on the method developed by Vos et al. (1995). Genomic DNA was digested in a reaction mixture containing 1X reaction buffer (50 mM potassium acetate [Sigma], 10 mM magnesium acetate [Sigma] and 10 mM Tris-HCl [pH 7.5]), 10U *Eco* RI restriction enzyme (Roche), 10U *Hpa* II restriction enzyme (Roche), 50 to 75 ng genomic DNA, and MilliQ water in a total volume of 25 μL. The reaction was incubated at 37°C for 2 to 3 h. The completeness of the genomic DNA digestion was determined by gel electrophoresis. A 10 μL aliquot of each genomic DNA digestion combined with 1 μL of 10X loading buffer (Section 4.2.1.1), was electrophoresed on a 0.8% (w/v) agarose/1X TAE buffer gel to ensure

complete digestion. Typically, a smear of low molecular weight DNA, between 100 bp and 1.5 Kb was observed. If digestion was not complete then a smear of higher molecular weight DNA was visible on the gel. In this case the remaining digest mixture was incubated at 37°C for a further 1 h. After the samples were determined to be completely digested by gel electrophoresis (Section 4.2.1.1), the tubes were incubated at 70°C for 15 min to irreversibly denature the restriction endonucleases. The restriction digestion reactions were either used immediately or stored at -20°C.

4.2.1.3 Preparation and ligation of oligonucleotide adapters

The double stranded oligonucleotide adapters were prepared by annealing two single-stranded oligonucleotides. The *Eco* RI and *Hpa* II adapters were prepared separately. The *Eco* RI reaction mixture contained 45% (v/v) TE buffer (pH 7.5), 5 pmol *Eco* RI Adapter I (prepared by Gibco BRL) and 5 pmol *Eco* RI Adapter II (prepared by Gibco BRL) in a total volume of 100 μL. The *Hpa* II reaction mixture contained 45% (v/v) TE buffer (pH 7.5), 50 pmol *Hpa* II Adapter I (prepared by Sigma Genosys, Australia) and 50 pmol *Hpa* II Adapter II (prepared by Sigma Genosys, Australia) in a total volume of 100 μL. The linker reaction mixtures were heated to 94°C for 4 min, and allowed to cool slowly on the bench. The oligonucleotide adapters were stored at -20°C. Sequences of the oligonucleotide adapters are provided in Appendix C.

Adapters were ligated to the digested genomic DNA fragments in a reaction mixture containing 1X T4 ligation buffer (Gibco BRL), 2.5 pmol *Eco* RI adapter, 25 pmol *Hpa* II adapter, 0.5U T4 DNA ligase (Gibco BRL) and 5 μL of *Eco* RI/*Hpa* II digested genomic DNA (Section 4.2.1.2), in a total volume of 10 μL. The tubes were mixed and then briefly centrifuged. Ligation reactions were incubated at 4°C overnight. The ligation reactions were either used immediately or stored at -80°C.

4.2.1.4 Pre-selective PCR amplifications of ligation PCR products

All pre-selective amplifications were performed using oligonucleotide primers that had a single base overhang, an adenosine on the *Eco* RI primer and a guanine on the *Hpa* II primer. Sequences of the pre-selective amplification PCR primers are provided in Appendix C. Each PCR reaction consisted of 1X reaction buffer (Qiagen), 250 μM of

each deoxy-nucleosidetriphosphate (dNTPs, Roche), 1X Q solution (Qiagen), 10 pmol *Eco* RI+A pre-selective primer (prepared by Sigma Genosys, Australia), 10 pmol *Hpa* II+G pre-selective primer (prepared by Sigma Genosys, Australia), 1U Taq DNA polymerase (Roche) and 1 μL ligated DNA, in a total volume of 20 μL. Thermocycling was performed in a PCR Sprint (Hybaid) using 20 cycles at 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. Ramping speed was limited to 1°C s^{-1}. At the completion of thermocycling the reactions were held at 4°C.

4.2.1.5 Selective amplification of pre-selective amplification PCR products

Selective amplifications were performed using the *Eco* RI selective primer with three selective bases and the *Hpa* II selective primer with four selective bases. Selective amplification reactions consisted of 1X reaction buffer, 250 μM dNTPs, 50 mM $MgCl_2$ (Qiagen), 10 pmol *Eco* RI+ANN selective primer (prepared by Sigma Genosys, Australia), 10 pmol *Hpa* II+TCAA selective primer (prepared by Sigma Genosys, Australia), 1U Taq DNA polymerase and 1 μL of pre-selective amplification product in a total volume of 20 μL. Sequences of the selective amplification PCR primer sequences are provided in Appendix C. Thermocycling was carried out in a DNA Engine, Peltier Thermal Cycler-2000. The PCR program consisted of an initial hold at 94°C for 2 min; 6 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s; 6 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 60 s; 24 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s and a final extension of 72°C for 5 min. The ramping speed for the program was limited to 1°C s^{-1}. At the completion of thermocycling the reactions were held at 4°C. The products were either used immediately or stored at -20°C.

4.2.1.6 Preparation of denaturing polyacrylamide gels

AFLP gels were poured, run and stained using Andrew Griffiths' method (A. Griffiths, personal communication, 2000). Griffiths' method is adapted from the AFLP method of Gibco BRL (c1988). Gels were silver stained after the protocol published by Promega (c1998).

Prior to pouring the polyacrylamide gel, the two glass plates were scrubbed with detergent, rinsed thoroughly and dried. Approximately 2 mL of 80% (v/v) ethanol was

wiped over the surface of the plate, which was then dried. This process was repeated so that each plate was cleaned twice with detergent and twice with ethanol. The cleaned face of the shorter plate was wiped with 2 mL Bind-Silane solution (95% [v/v] ethanol, 0.5% [v/v] glacial acetic acid and 0.05% [v/v] Bind-Silane [Pharmacia]). The plate was allowed to dry for 3 min, then washed and dried three times with 3 mL 95% (v/v) ethanol. The cleaned face of the longer plate was wiped with 2 mL of Rain-X (Blue Coral-Slick 50, USA), and then cleaned with 3 mL 95% (v/v) ethanol.

The plates were assembled using 0.4 mm spacers (Gibco BRL) to form a glass sandwich. It was important at this stage to ensure the faces of the long and short plates did not touch, as the resulting interaction would cause the gel to adhere to both plates. The bottom of the sandwich was sealed with electrical tape (3M) to prevent leakage of the non-polymerised gel solution. The glass sandwich was then placed in a S2 casting boot (Gibco BRL) to hold the plates securely.

A 5% denaturing polyacrylamide gel was prepared by firstly filtering 70 mL of the gel solution (8 M urea, 10% [v/v] Long Ranger gel solution [FMC BioProducts], 1X TBE [89 mM Tris, 89 mM orthoboric acid and 2 mM EDTA]) through two Whatman No 1 filters. Immediately prior to pouring the gel, 350 μL of 10% (v/v) ammonium persulphate (Sigma) and 35 μL of NNN'N'-tetramethylethylenediamine (TEMED, [BDH]) were added to the filtrate. The solution was mixed and the acrylamide solution dispensed into the glass sandwich using a syringe. Care was taken to ensure no bubbles formed in the gel. Pair of shark tooth combs (5.7 mm point to point spacing, Gibco BRL) were inserted, in reverse orientation, to form the space required for the wells. The combs were clamped in place with large bulldog clips. The top of the gel was sealed with cling film to prevent dehydration, laid at an angle of 5° and left 1 to 2 h to ensure complete polymerisation.

Once polymerised, the gel was set up for electrophoresis. The cling film, bulldog clips, casting boot, plastic tape and combs were removed from the gel sandwich. The sandwich was loaded into the S2 electrophoresis apparatus (Gibco BRL) and the upper and lower tanks filled with 1X TBE buffer. Excess urea and poylacrylamide fragments were flushed from the wells. The gel was pre-run at 45 W for 30 min.

4.2.1.7 Preparation of selective amplification PCR products for polyacrylamide electrophoresis

A 5.5 μL aliquot of selective amplification PCR product was mixed with 0.5 μL of formamide loading dye (98% [v/v] formamide [BDH], 10 mM EDTA, 0.05% [w/v] bromophenol blue and 0.05% [w/v] xylene cyanol). A 100 bp DNA Ladder was prepared by combining 99 μL formamide loading dye and 1 μL 100 bp DNA Ladder (Gibco BRL, 1μg μL^{-1}). Both the PCR product and the DNA Ladder were irreversibly denatured at 95°C for 4 min, before being rapidly cooled to 4°C. The ladder was stored at -20°C.

4.2.1.8 Electrophoresis and silver staining of denaturing polyacrylamide gels

After pre-running, the combs were inserted between the plates so the teeth rested on the gel surface to form wells, which were then flushed. A 6 μL aliquot of each denatured selective amplification PCR product was loaded. Replicate samples were run in adjacent lanes to allow easy analysis. A 6 μL aliquot of the 100 bp DNA Ladder was loaded before and after each primer combination. AFLP gels were run at 40 W until the xylene cyanol dye front had just run off the bottom of the gel, usually 4 h.

At the completion of the electrophoresis, the gel sandwich was removed from the apparatus, the spacers and combs removed, and the plates separated. The gel adhered to the short plate and the gel and the plate were transferred to a large tray containing 4 L of 10% (v/v) acetic acid, with the gel side uppermost. The tray was then gently agitated on a mechanical shaker for 1.25 h to remove urea from the gel. All remaining steps were also performed on a mechanical shaker. Next the gel was washed three times in 2 L of MilliQ water for 3 min. The gel was then soaked in 3 L of stain solution (6 mM silver nitrate [BDH] and 0.15% [v/v] formaldehyde [Sigma]), for 30 min. The gel was placed in 2 L of pre-chilled Milli-Q water for 5 s, then the gel was immediately transferred to 2 L of pre-chilled developing solution (4L of 280 mM anhydrous sodium carbonate [BDH] in MilliQ water, 0.16% [v/v] formaldehyde, and 50 μM sodium thiosulphate [BDH]). As soon as DNA bands became visible (approximately 5 min), the gel was transferred to a fresh 2L of developing solution. The gel was then developed until the bands reached the desired intensity. The developing reaction was stopped and the gel

fixed by the addition of 2L of chilled acetic acid. After 5 min the gel was transferred to chilled MilliQ water for 5 min, to remove excess acetic acid from the gel.

4.2.1.9 Excision and PCR amplification of polymorphic bands from polyacrylamide gels

The gel was transferred to a fluorescent light box to aid in the visualisation of DNA bands. Only bands of interest that were in both replicates of a plant selection were excised from the gel, using a sterile scalpel blade. The cut gel fragment was transferred to a 1.5 mL centrifuge tube containing 50 μL MilliQ water. Gel cuts were stored at 4°C for 12 h to allow diffusion of the DNA from the polyacrylamide gel. Periods longer than 24 h caused inhibitors (e.g. formamide) to diffuse from the fragment and prevent successful re-amplification.

Pre-selective amplification primers were used for the re-amplification of DNA from the polyacrylamide gel cuts. Each PCR reaction consisted of 1X reaction buffer, 250 μM dNTPs, 1X Q solution, 10 pmol of freshly diluted *Eco* RI+A pre-selective primer, 10 pmol freshly diluted *Hpa* II+G pre-selective primer, 1U Taq DNA polymerase and 1 μL AFLP band eluate, in a total volume of 20 μL. The thermocycling program consisted of an initial hold at 94°C for 2 min, 35 cycles at 94°C for 30 s, 50°C for 60 s and 72°C for 60 s, and a final extension of 72°C for 5 min. Ramping speed was limited to 1°C s^{-1}. After completion of the thermocycling, the reactions were held at 4°C.

For the purposes of DNA sequencing, it was necessary to identify the homogenous re-amplification PCR products that were composed of *Eco* RI-*Hpa* II based fragments. Re-amplification products were subjected to three PCR reactions: one containing both the *Hpa* II+G and *Eco* RI+A pre-selective amplification primers, one containing the *Hpa* II+G pre-selective amplification primer only and one containing the *Eco* RI+A pre-selective amplification primer only (as per Section 4.2.1.5).

A 5 μL aliquot of each re-amplified PCR product was combined with 1 μL of 10X loading buffer in a total volume of 10 μL. The mixture was loaded onto a 1.5% (w/v) agarose/1X TAE buffer gel. The gel contained 1 μg mL^{-1} ethidium bromide. A 10 μL aliquot of 1 Kb plus DNA Ladder (Section 4.2.1.1) and Low Mass Ladder were

included in the agarose gel as a size and mass standard respectively. The Low Mass Ladder was prepared by mixing 2 μL of Ladder (117.5 ng μL^{-1} [Roche]), 1 μL 10X loading buffer and 7 μL TE buffer. Samples were electrophoresed at 100 V in 1 X TAE buffer for 1 h.

4.2.1.10 Preparation, DNA sequencing and sequence analysis of PCR amplified polymorphic bands

Prior to sequencing the *Eco* RI-*Hpa* II re-amplification products, the PCR products were treated with shrimp alkaline phosphatase (SAP [USB]) and exonuclease I (EXO I [USB]) to remove the remaining dNTPs and single-stranded pre-selective amplification primers respectively. Each cleanup reaction consisted of the *Eco* RI-*Hpa* II re-amplification PCR product, 2U SAP and 10U EXO I. The reactions were incubated at 37°C for 30 min, then 80°C for 15 min. The latter incubation irreversibly denatured the enzymes. The reactions were used immediately or stored at –20°C until further use.

The *Eco* RI-*Hpa* II re-amplification PCR products were prepared for sequencing. Each sequencing reaction consisted of 1X sequencing buffer (400 mM Tris-Cl [pH 9.0] and 10 mM $MgCl_2$), 4 μL Big Dye Terminator Ready Reaction Mix Version 3 (Applied BioSystems), 3.2 pmol *Eco* RI-A or *Hpa* II-G pre-selective amplification primer and *Eco* RI-*Hpa* II re-amplification PCR product in a total volume of 20 μL. The amount of DNA to use in the reaction mixture was estimated as the size of the product divided by 20 (e.g. 300 bp/20 = 15 ng) (Applied BioSystems). The concentration of the PCR product was estimated from the Low Mass Ladder from the agarose gel. Sequencing reactions were carried out in a thermal cycler as follows: 28 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min; hold at 4°C. Ramping speed was set at 1°C s^{-1}.

Sequencing products were purified by ethanol precipitation. An 80 μL aliquot of 75% (v/v) isopropanol was added to the sequencing reaction. The sequencing product was incubated at room temperature for 20 min and centrifuged for 20 min at 35,000g. The supernatant was removed carefully, so as not to disturb the invisible pellet. A further 700 μL of 75% (v/v) isopropanol was added, and the pellet washed with gentle inversion. The mixture was centrifuged for 5 min at 35,000g and the supernatant decanted off. The pellet was air-dried overnight.

The Massey University DNA Analysis Service (MUSeq) carried out sequence determination using an ABI Prism 377-64 DNA Sequencer. For each re-amplification PCR product, DNA sequence information was obtained from digital electropherograms in both directions (i.e. 5' *Hpa* II-*Eco* RI 3' and 5' *Eco* RI-*Hpa* II 3'). A consensus sequence for band B was obtained from 5' *Hpa* II-*Eco* RI 3' and the reverse complement of 5' *Eco* RI-*Hpa* II 3', using ABI Prism MT Navigator PPC Software (Applied BioSystems). The sequences were screened against the EMBL/GENBANK databases using the BLAST program (website://www.ncbi.nlm.nih.gov/BLAST/).

4.2.1.11 PCR DNA methylation polymorphic marker primer design

PCR primers were designed from the consensus sequence of band B re-amplification PCR product using *OLIGO* 4.03 primer analysis software (National BioSciences). Primers were designed for both the 5' *Eco* RI and 5' *Hpa* II ends of the fragment and were 19 and 20 nucleotides in length respectively (Appendix C). Primers were designed, as close to the ends of the sequence as possible to ensure any polymorphism present would be included in the intervening region. Rules for good primer design were followed (Sambrook and Russell, 2001).

PCR amplification was performed using the Expand Long Template PCR system (Roche) on each DNA sample. The first master mix contained 1 mM of each dNTP, 800 nM of each primer, 100 to 500 ng template DNA (Section 4.2.1.1), in a total reaction volume of 25 µL. The second master mix contained 2X Expand Long Template PCR System Buffer 3 and 2.6U Expand Taq polymerase in a reaction volume of 25 µL. The second master mix was added to the first master mix. Thermocycling was carried out in a PCR Sprint Thermocycler. The PCR program consisted of an initial hold at 94°C for 2 min, 10 cycles of 93°C for 30 s, 55°C for 30 s and 68°C for 20 s, 24 cycles of 93°C for 30s, 55°C for 30 s and 68°C for 55 s, and a final extension of 68°C for 5 min. Ramping speed was limited to 1°C s^{-1}. At completion of thermocycling the reactions were held at 4°C.

A 5 µL aliquot of each re-amplified PCR product was combined with 1 µL of 10X loading buffer in a total volume of 10 µL. The mixture was loaded onto a 1.5% (w/v) agarose/1X TAE buffer gel. The gel contained 1 µg mL^{-1} of ethidium bromide. A

10 μL aliquot of 1 Kb plus DNA Ladder (Section 4.2.1.1) and Low Mass Ladder (Section 4.2.1.9) were included in the electrophoretic gel as a size and mass standards respectively. Samples were electrophoresed at 100 V in 1X TAE buffer. Gels were visualised by ethidium bromide fluorescence as detailed in Section 4.2.1.1.

4.2.2 cDNA AFLP

4.2.2.1 RNA isolation and quantification

Zantedeschia shoots were harvested from stored Best Gold, control and Dutch Florex Gold tubers. Two replicates were completed for Best Gold and Florex Gold control tubers and three replicates were completed for Dutch Florex Gold tubers. Each replicate was derived from several buds from the same tuber. Tubers had been harvested at the same time, and were held in identical storage conditions.

At least 0.5 g of shoot tissue was placed into liquid nitrogen and ground to a fine powder using a mortar and pestle. A 5 mL aliquot of Tri Reagent® (ProGENZ Ltd, New Zealand) was added to an Oakridge tube. The frozen ground tissue (0.5 g) was added to the Tri Reagent and mixed by inversion. The sample was centrifuged at 12,000g at 4°C for 5 min to remove insoluble material from the homogenate. The clear supernatant was transferred to a fresh Oakridge tube. The homogenate was incubated at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. A 1 mL aliquot of chloroform was added, and the tube shaken vigorously. The mixture was incubated for 10 min at room temperature and then centrifuged at 12,000g at 4°C for 15 min.

The upper aqueous phase, containing the RNA, was transferred to a fresh Oakridge tube and 1.25 mL isopropanol and 1.25 mL of a 0.8 M sodium citrate/1.2 M NaCl solution was added, mixed well and incubated for 5 to 10 min at room temperature. The mixture was centrifuged at 12,000g at 4°C for 15 min to pellet the RNA. The supernatant was discarded, the RNA pellet was washed with 5 mL 75% (v/v) ethanol, and centrifuged at 12,000g at 4°C for 5 min. The ethanol wash was again discarded and the pellet air dried for 3 to 5 min. A 50 μL aliquot of RNase-free water was added to the pellet and vortexed. The pellet was left at room temperature for 1 h to dissolve. The RNA

solution was centrifuged at 12,000g at 4°C for 30 s to bring the content into the bottom of the tube. The RNA and any loose pellet were transferred to a RNase free tube. A further 50 μL of RNAse-free water was added to the initial tube if the pellet had not dissolved sufficiently. The initial tube was vortexed and centrifuged at 12,000g at 4°C for 30 s. The second eluate was combined with the first eluate.

A 2 uL aliquot of RNA diluted in 198 μL of MilliQ water was quantified by measuring the absorbance at 260 nm, against a water blank, using an Ultraspec 300 (Pharmacia Biotech) in a 200 μL quartz curvette. The light path was 10 mm. For RNA, an OD_{260} of 1 corresponds to approximately 40 μg mL^{-1} (Sambrook and Russell, 2001). The absorbance at 280 nm was also measured. An estimate of purity of the RNA was calculated from the ratio of OD_{260}/OD_{280}. Pure RNA has an OD_{260}/OD_{280} absorbance of 2.0 (Sambrook and Russell, 2001).

4.2.2.2 mRNA isolation and quantification

The mRNA was isolated from total RNA using the PolyATtract mRNA Isolation System IV (Promega) according to the manufacturers instructions. A aliquot of 0.1 to 1.0 mg of total RNA was brought to a final volume of 500 μL in sterile MilliQ water. The RNA was denaturated by incubating at 65°C for 10 min, then 3 μL of the biotinylated-oligo(dT) probe (5' B-TTTTTTT 3') and 13 μL of 20X SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) were added to the RNA. The RNA was gently mixed and incubated at room temperature for 10 min to anneal.

The streptavidin-paramagnetic particles were resuspended by gently flicking the tube. They were then captured to the side of the tube by placing in a magnetic stand. The supernatant was discarded and the particles were washed three times with 300 μL 0.5X SSC. Each time the streptavidin-paramagnetic particles were captured using the magnetic stand and the supernatant discarded. After the three washes, the streptavidin-paramagnetic particles were resuspended in 100 μL 0.5X SSC.

The entire contents of the annealing reaction were added to the pre-prepared streptavidin-paramagnetic particles, mixed and incubated at room temperature for 10 min. The particles were washed four times with 300 μL 0.1X SSC. The particles were resuspended each time by gentle flicking and captured using the magnetic stand. After

the final wash, as much of the aqueous phase as possible was removed without disturbing the streptavidin-paramagnetic particles. The mRNA was eluted by resuspending the streptavidin-paramagnetic particle pellet in 100 µL sterile MilliQ water and flicking gently. The streptavidin-paramagnetic particles were magnetically captured and the eluted mRNA aqueous phase transferred to a RNase-free tube. The elution step was repeated by resuspending the streptavidin-paramagnetic particle pellet in 150 µL of sterile MilliQ water. The capture step was repeated, and the second eluate pooled with the first eluate.

The concentration and purity of each 200 µL aliquot of mRNA was determined by spectrophotometry as described in Section 4.2.2.1, but this time there was no dilution. The quartz curvette was first washed for 2 min in 50 mM NaOH followed by a rinse with sterile MilliQ water. After determination of mRNA concentration the mRNA eluate was frozen at -20°C for 10 min. The mRNA was dried in a Speed-Vac (Savant Speed Vac) and stored at -20°C until further use.

4.2.2.3 cDNA synthesis and quantification

A total of 50 to 100 ng mRNA and 20 to 100 pmols of biotinylated oligo(dT) primer (Sigma Genosys, Australia) (sequence information is provided in Appendix C) was prepared in a total volume of 4.5 µL in a 200 µL thin walled PCR tube. The mRNA was denatured for 10 min at 65°C and snap cooled on ice. The following reagents were added in order: 1X Expand reverse transcriptase buffer (first strand) (Roche), 10 mM DTT (Roche), 1 mM of each dNTP, 20U of RNase Inhibitor (Roche) and 50U Expand Reverse Transcriptase (Roche) in a total volume of 20 µL. The reaction mixture was incubated for 45 to 60 min at 42°C. The mixture was put on ice and the components for the synthesis of the second-strand were added. A 40 µL aliquot of a second-strand synthesis buffer was added (80 mM Tris-HCl [pH 7.5], 240 mM KCl and 10 mM $MgCl_2$), 130 µg mL^{-1} bovine serum albumin (MB grade, New England Biolabs), 0.13U RNase H (Roche), MilliQ water and 25U *Escherichia coli* DNA Polymerase I (Roche), in a total volume of 100 µL. The reaction mixture was mixed well and centrifuged at 4,000g for 10 s. The reaction mixture was incubated at 12°C for 60 min, 22°C for 60 min and 65°C for 10 min in a PCR Express Thermocycler (Science & Technology [NZ] Ltd). T4 DNA polymerase (Roche) (4U) was added to the reaction. The reaction was

centrifuged at 4,000g for 10 s and incubated for 10 min at 37°C. The reaction was stopped by the addition of 10 μL of 0.2 M EDTA solution (pH 7.2), and 2 μL 10% (w/v) N-Lauroylsarcosine. The cDNA was precipitated with 10% (v/v) 3M NaOAc (pH 5.6), and 200% (v/v) ethanol and incubated at -20°C for 1 h. The cDNA was centrifuged at 14,000g for 15 min. The supernatant was discarded. The pellet was washed in 500 μL ice cold ethanol (70% [v/v]) and centrifuged. The supernatant was discarded and the pellet air dried. The cDNA was resuspended in 25 μL sterile MilliQ water.

The cDNA was quantified using PicoGreen® (Molecular Probes) fluorescence dye, against a dilution series of PicoGreen fluorescence of λ DNA (Roche). Fluorescence was measured on a Polarstar fluorometer (BMG Labtechnologies), running Fluostar Galaxy software version 4.11-0. The dilution series of λ DNA were prepared in TE buffer to give 1, 5, 20, 50, 75 and 100 ng mL^{-1}. PicoGreen was used at 0.5% (v/v) dilution. Pico Green was diluted in buffer. The cDNA was diluted 0.5% (v/v) in TE buffer and compared to the λ DNA standard curve.

4.2.2.4 Digestion of cDNA and preparation and ligation of a *Taq* adapter

The cDNA was digested in a reaction mixture containing 1X OPA+ Buffer (Pharmacia), 5U of *Taq* I (Roche) and 55 to 115 ng of double stranded cDNA, in a total volume of 40 μL. The digest was incubated at 65°C for 1 h. The double stranded *Taq* I oligonucleotide adapter was prepared by adding 1 nmol of both *Taq* I adapter 1 (prepared by Sigma Genosys, Australia) (Appendix C) and *Taq* I adapter 2 (prepared by Sigma Genosys, Australia) (Appendix C) in a total volume of 20 μL. The adapter mix was incubated at 37°C for 10 min, then room temperature for 15 min and finally placed on ice. The ligation mixture was prepared by combining 1U T4 DNA ligase, 50 pmol *Taq* I adapter reaction, 5 mM ATP (Roche) and 1X OPA+ buffer, in a total volume of 10 μL. The 10 μL ligation mixture was added to the 40 μL digestion mixture and the reaction was incubated at 15°C overnight.

4.2.2.5 Selection of biotinylated cDNA fragments and quantification

The cDNA fragments containing a biotinylated T primer (i.e. fragments containing a *Taq* I site and a poly A tail) were isolated from non-biotinylated fragments (i.e. *Taq* I-*Taq* I and 5'cDNA-*Taq* I fragments) using M-280 streptavidin beads (Dynal). The streptavidin magnetic beads were prepared by resuspending the beads by gently inverting the vial to obtain a homogeneous suspension. A 10 μL aliquot of streptavidin beads (100 μg) was transferred to a fresh tube and placed in the Magnetic Particle Concentrator (MPC) for 1 to 2 min. While the tube remained in the MPC, the storage buffer was removed. The tube was removed from the MPC and 50 μL of 1X Binding and Wash Buffer (5 mM Tris-HCl [pH 7.5], 0.5 mM EDTA and 1 M NaCl), was added to wash the streptavidin beads. The tube was returned to the MPC for 1 to 2 min and the buffer removed. The streptavidin beads were resuspended in 50 μL of 2X Binding and Wash Buffer, to a concentration of 2 μg μL^{-1}. An equal volume of the biotinylated cDNA (50 μL) was added to the prepared streptavidin beads, diluting the Binding and Wash Buffer to 1X, the optimal salt concentration for binding. Beads were at a concentration of 1 μg μL^{-1}. The tube was incubated at room temperature for 1 h with gentle rotation. The tubes were placed in the MPC for 1 to 2 min, and washed twice with 100 μL 1X binding and wash buffer. All the buffer was removed from the beads after the final wash. The beads were resuspended in 100 μL of $TE_{0.1}$ buffer (10 mM Tris, 0.1 mM EDTA [pH 8.0]). The beads were PCR viable and no fragment elution was necessary. The resuspension volume was adjusted for each sample to give a final cDNA concentration of 1 ng μL^{-1}.

The biotinylated fraction was quantified using PicoGreen fluorescence and the Polarstar fluorometer (Section 4.2.2.3). The λ DNA standards were 0.25, 1.25, 5, 12.5 and 25 ng mL^{-1}. The cDNA was diluted 3% (v/v) in TE buffer and compared to the λ DNA standard curve.

4.2.2.6 PCR amplification of biotinylated cDNA fragments

PCR amplifications of biotinylated cDNA fragments were performed using a *Taq* I primer (Sigma Genosys, Australia), with two selective nucleotides at the 3' end, and a T primer that contained two or three selective nucleotides at the 3' end (Sigma Genosys, Australia). Sequences of the selective amplification primers are provided in Appendix

C. Each PCR reaction consisted of 1X Amplitaq Gold buffer (Applied Biosystems), 1.5 mM $MgCl_2$ (Roche), 0.2 mM of each dATP, dCTP, dGTP and dTTP (Roche), 15 pmol of each primer, 1U Amplitaq Gold (Applied Biosystems) and 350 pg of biotinylated cDNA fragments still bound to the beads, in a total volume of 25 µL. Samples were overlaid with paraffin oil and amplified in 96-well plates on a Techne PHC-3 thermal cycler (John Morris Scientific Ltd). PCR amplifications were preceded by a 9 min incubation at 92°C. Amplifications were performed for 34 cycles with the following cycle profile: 94°C for 30 s, 63°C for 30 s and 72°C for 60 s. PCR amplifications were followed by a 5 min incubation at 72 °C. At the completion of thermocycling the reactions were held at 10°C.

4.2.2.7 Non-denaturing acrylamide gels

Non-denaturing acrylamide gels were prepared by cleaning both a long and short plate with acetone and then ethanol. One milli metre spacers were placed on the long plate and the short plate flipped on top, with cleaned side down. The Bio-Rad sandwich clamps were finger tightened to lock the plates together, ensuring the plates and spacers were flush with the bottom of the clamp. The gel cassette was clipped into the pouring apparatus.

The 6% non-denaturing acrylamide gel mix was prepared by combining 6 mL 40% (w/v) acrylamide/bis solution (BioRad), 1X TBE buffer and 300 µL of 10% (w/v) ammonium persulphate in a total volume of 40 mL. A 3 mL aliquot of mix was removed, as a fast setting plug, and 12 µL of TEMED was added to the 3 mL aliquot. The plug was poured into the gel cassette, and air bubbles removed with tapping. The plug took about 10 min to set. To the remaining 37 mL of acrylamide mix, 40 µL of TEMED was added. This was swirled to mix and poured between the two plates at a 45° angle, running the mixture down the back plate. A 20 well comb was inserted into the gel cassette. The gel took approximately 1 h to set.

After the gel had set, the comb was removed and the wells flushed out with a water filled syringe to remove any unset acrylamide/salts, which could have affected the running of the samples. The gel cassette was removed from the pouring apparatus. The water cooling system was turned on so that there was a gentle stream of water running

through the internal chamber of the running apparatus. The internal chamber was removed from the running apparatus and the gel cassette was clipped onto the front of the internal chamber. If only one gel was run, a blank cassette with a plastic plate was required on the other side of the apparatus to form the buffer tank. The internal chamber was then returned to the running apparatus. TBE buffer (1X) was added to the top chamber, and the bottom reservoir filled so that the level was above the bottom of the gel.

A 10 μL aliquot of the PCR amplified biotinylated cDNA and 2 μL of orange dye (0.6% [w/v] Orange G [BDH], 30% [v/v] glycerol) were loaded onto the gel. A 1 Kb Ladder was included in the polyacrylamide gel as a size standard. A negative control containing only the PCR amplified PCR components was routinely included in the gel. The gel was run at 350 V for 1.75 h. Once the gel had run the cassette was removed from the running apparatus and the sandwich clamps were removed from the glass plates. The short plate was removed from the gel and the gel on the long plate was stained with ethidium bromide (250 $\mu g\ mL^{-1}$). The gel was destained in water for 5 min. The gel was viewed on a transilluminator (Eagle Eye II, Stragene, USA) running Stratgene Eagle Sight software. The gel was photographed and the differentially expressed bands labelled.

4.2.2.8 Excision and PCR amplification of differentially expressed bands from acrylamide gels

The differentially expressed bands were cut out of the gel using a sterile scalpel blade. The excised bands were placed in a tube with 50 μL of 2X PCR buffer II (Applied BioSystems) and incubated at 94°C for 90 min to elute the DNA from the gel.

A 5 μL aliquot of eluted band was re-amplified in a total volume of 25 μL using the *Taq* I 0 primer and the T primer 1 (Appendix C), that contained no selective nucleotides at their 3' ends. Each PCR reaction consisted of 1X Amplitaq Gold buffer, 0.75 mM $MgCl_2$, 0.1 mM dNTPs, 10 pmol *Taq* I 0 primer, 10 pmol T primer I, and 1.25U Amplitaq Gold. PCR amplifications were preceded by a 2 min incubation at 92°C. Amplifications were performed for 35 cycles with the following cycle profile: 94°C for

30 s, 63°C for 60 s and 72°C for 60 s. PCR amplifications were followed by a 5 min incubation at 72°C. Reactions were held at 4°C.

4.2.2.9 Preparation, DNA sequencing and sequence analysis of PCR amplified differentially expressed bands

A subset of 25 bands were sequenced. Preparation of PCR amplified polymorphic bands for DNA sequence determination was performed as outlined in Section 4.2.1.10. DNA sequence information was obtained from digital electropherograms in the 5' *Taq* I – TT 3' direction. For selected bands, DNA sequence information was also obtained from digital electropherograms in the 5' TT – *Taq* 3' direction. Sequences were screened against the EMBL/GENBANK data bases using the BLAST program (website://www.ncbi.nlm.nih.gov/BLAST and www.ncbi.nlm.gov/dbEST).

4.3 Results

4.3.1 Genomic DNA AFLP

4.3.1.1 Nucleic acid extraction, polyacrylamide gels and excision of polymorphic bands

DNA was extracted from leaf samples of Best Gold tubers B and C and Florex Gold tubers: control, Dutch tubers E and F, few-eyed tubers A and B and bushy tubers B and F. Aliquots of each DNA extraction was electrophoresed on an 0.8% (w/v) agarose gel/1X TAE buffer and the results are shown in Figure 4.1. DNA is presented in Figure 4.1 as discrete high molecular weight bands greater than 10 Kb in size.

The *Hpa* II+TCAA selective primer and six *Eco* RI selective primers combinations were investigated. With each combination of primers, approximately 50 discrete bands were detected. The primer combinations were:

Hpa II+TCAA and *Eco* RI+ATA	Figure 4.2
Hpa II+TCAA and *Eco* RI+AGG	Figure 4.3
Hpa II+TCAA and *Eco* RI+AGA	Figure 4.4
Hpa II+TCAA and *Eco* RI+ACG	Figure 4.5
Hpa II+TCAA and *Eco* RI+ACC	Figure 4.6
Hpa II+TCAA and *Eco* RI+AAG	Figure 4.7.

From the six *Eco* RI primer combinations with *Hpa* II+TCAA, three bands corresponding to putative polymorphic regions were excised. One band (A) was excised from the gel of the selective primer combination *Hpa* II+TCAA and *Eco* RI+AGA (Figure 4.4). Band A appeared absent in control Florex Gold and Best Gold. Two bands (B and C) were excised from the gel of the primer combination *Hpa* II+TCAA and *Eco* RI+ACC (Figure 4.6). These bands appeared present only in control Florex Gold.

The selective primer *Hpa* II+TCAA in combination with *Eco* RI+ATA, *Eco* RI+ACG and *Eco* RI+AAG showed polymorphic differences between Best Gold and Florex Gold (Figures 4.2, 4.5 and 4.7 respectively).

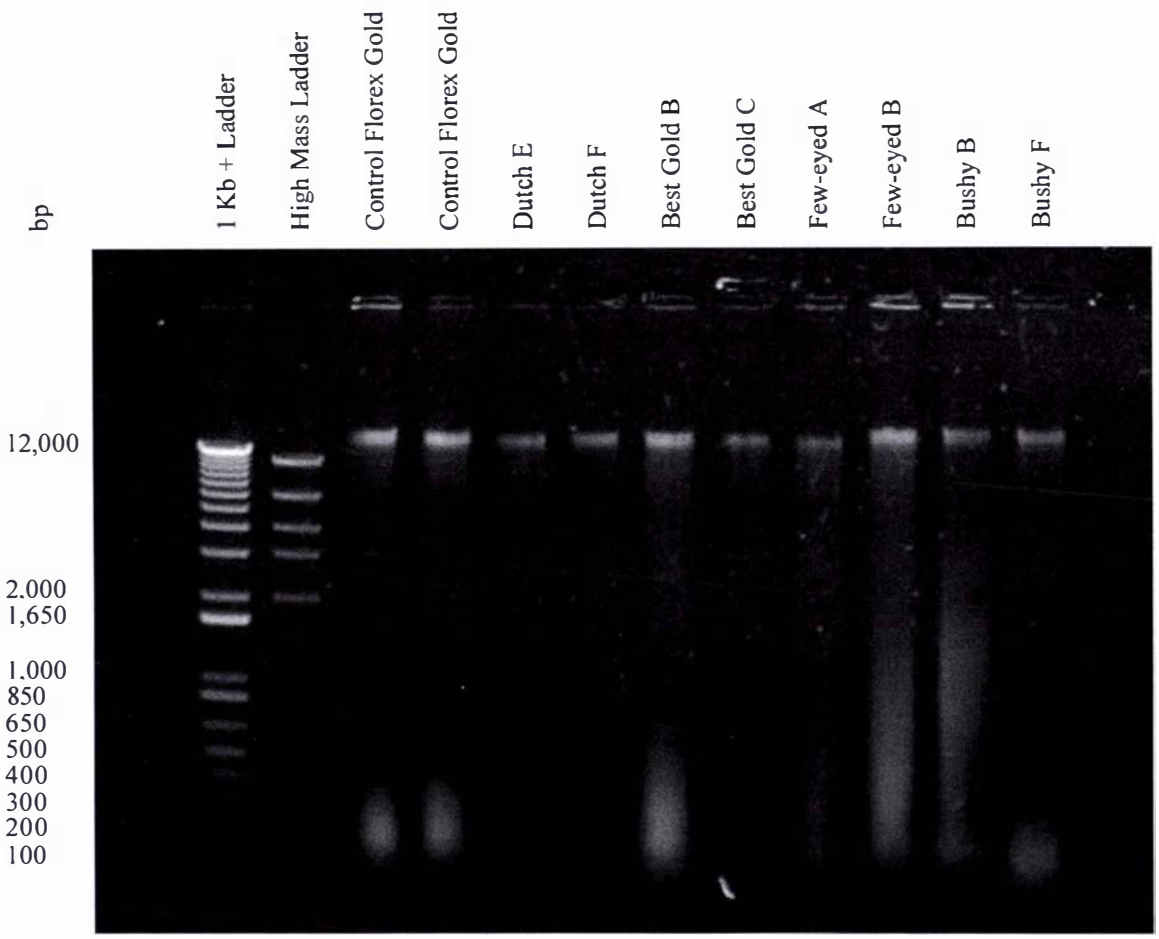


Figure 4.1: Agarose gel of DNA. A High Mass Ladder and a 1 Kb plus DNA Ladder were included in the agarose gel. DNA was visible as a high molecular weight band in each of the samples. RNA was visible as a low molecular weight smear. DNA samples were extracted from leaves of *Zantedeschia* plants in their second growing season. DNA was extracted from control Florex Gold, Dutch E and F, Best Gold B and C, few-eyed A and B and bushy B and F.

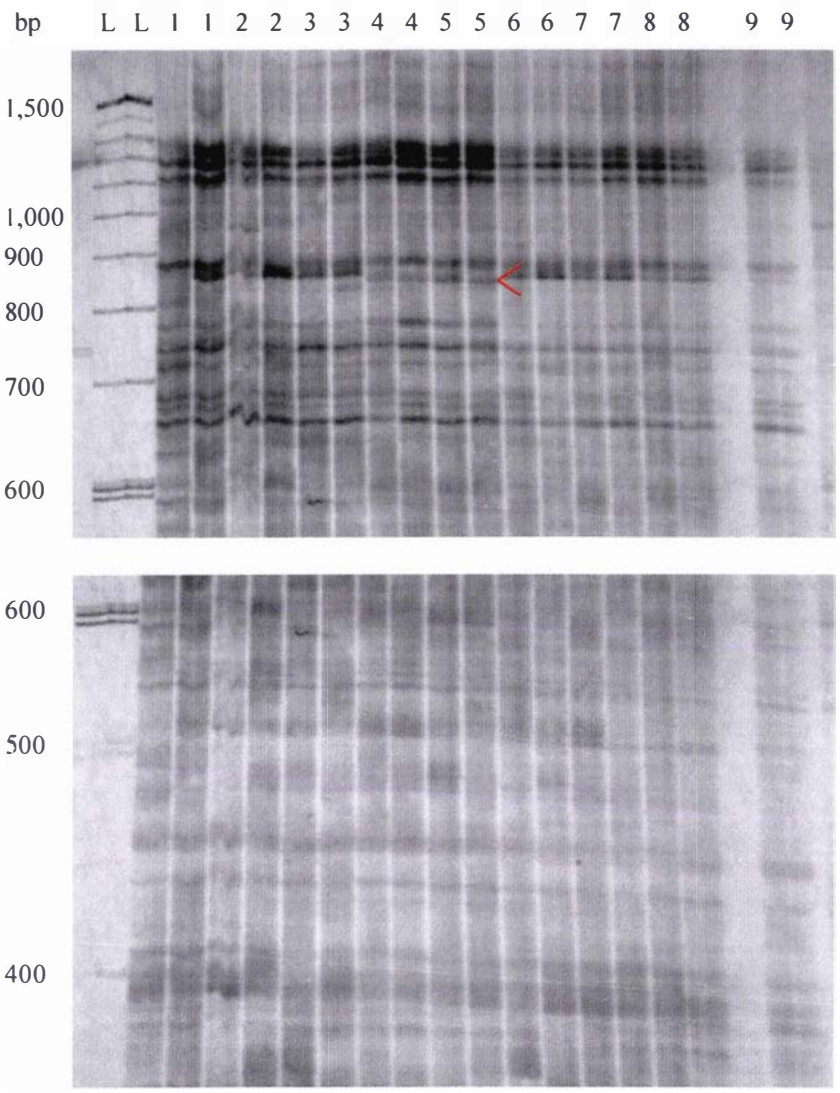


Figure 4.2: Polyacrylamide gel of selective amplification PCR products of the selective primers *Eco* RI+ATA and *Hpa* II+TCAA. Samples were control Florex Gold (1), Dutch E (2), Dutch F (3), Best Gold B (4), Best Gold C (5), few-eyed A (6), few-eyed B (7), bushy B (8) and bushy F (9). A 100bp DNA Ladder was included in the polyacrylamide gel (L). Polymorphic band in Best Gold is labelled (<).

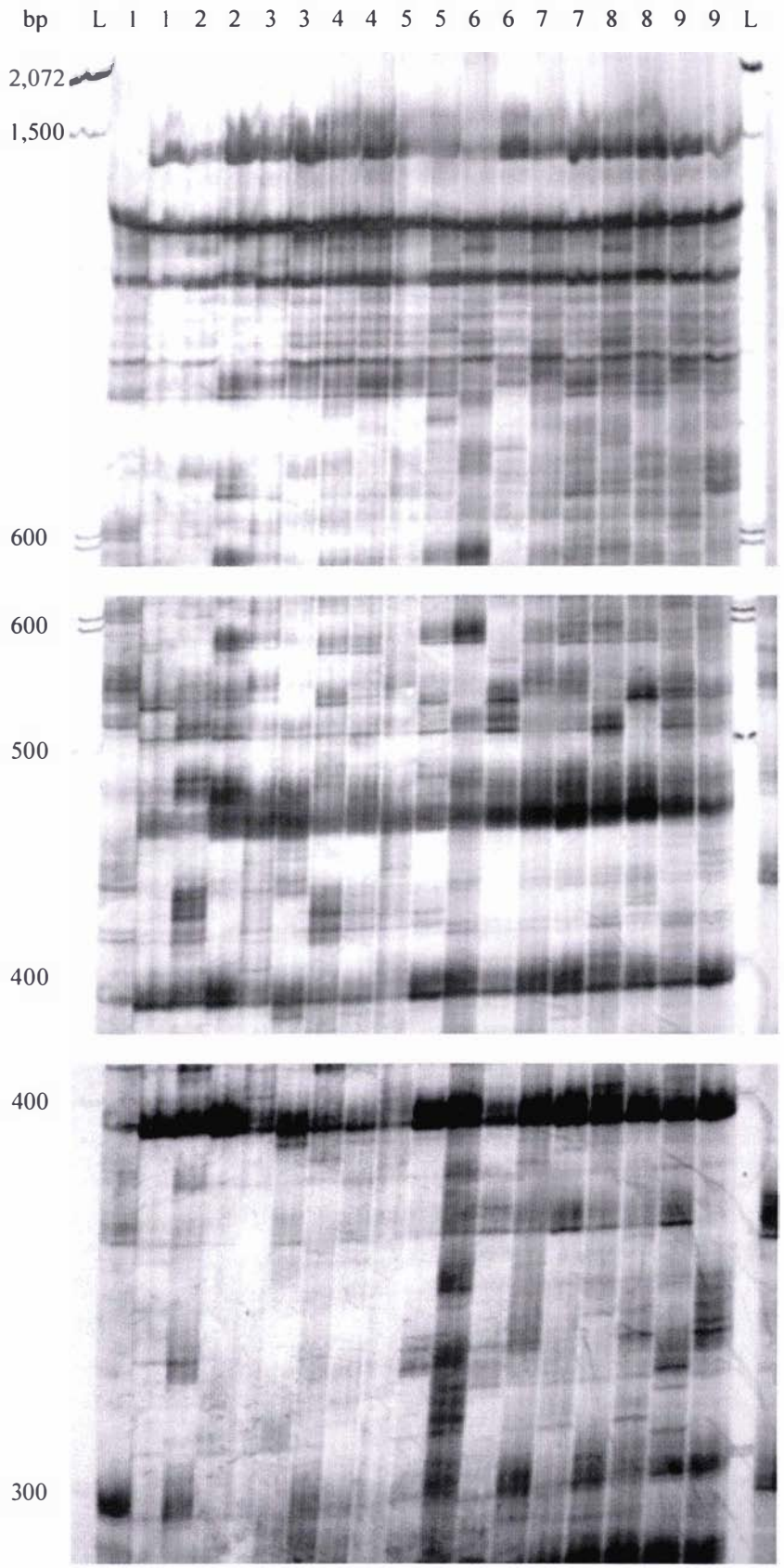


Figure 4.3: Polyacrylamide gel of selective amplification PCR products of the selective primers *Eco* RI+AGG and *Hpa* II+TCAA. Samples were control Florex Gold (1), Dutch E (2), Dutch F (3), Best Gold B (4), Best Gold C (5), few-eyed A (6), few-eyed B (7), bushy B (8) and bushy F (9). A 100bp DNA Ladder was included in the polyacrylamide gel (L).

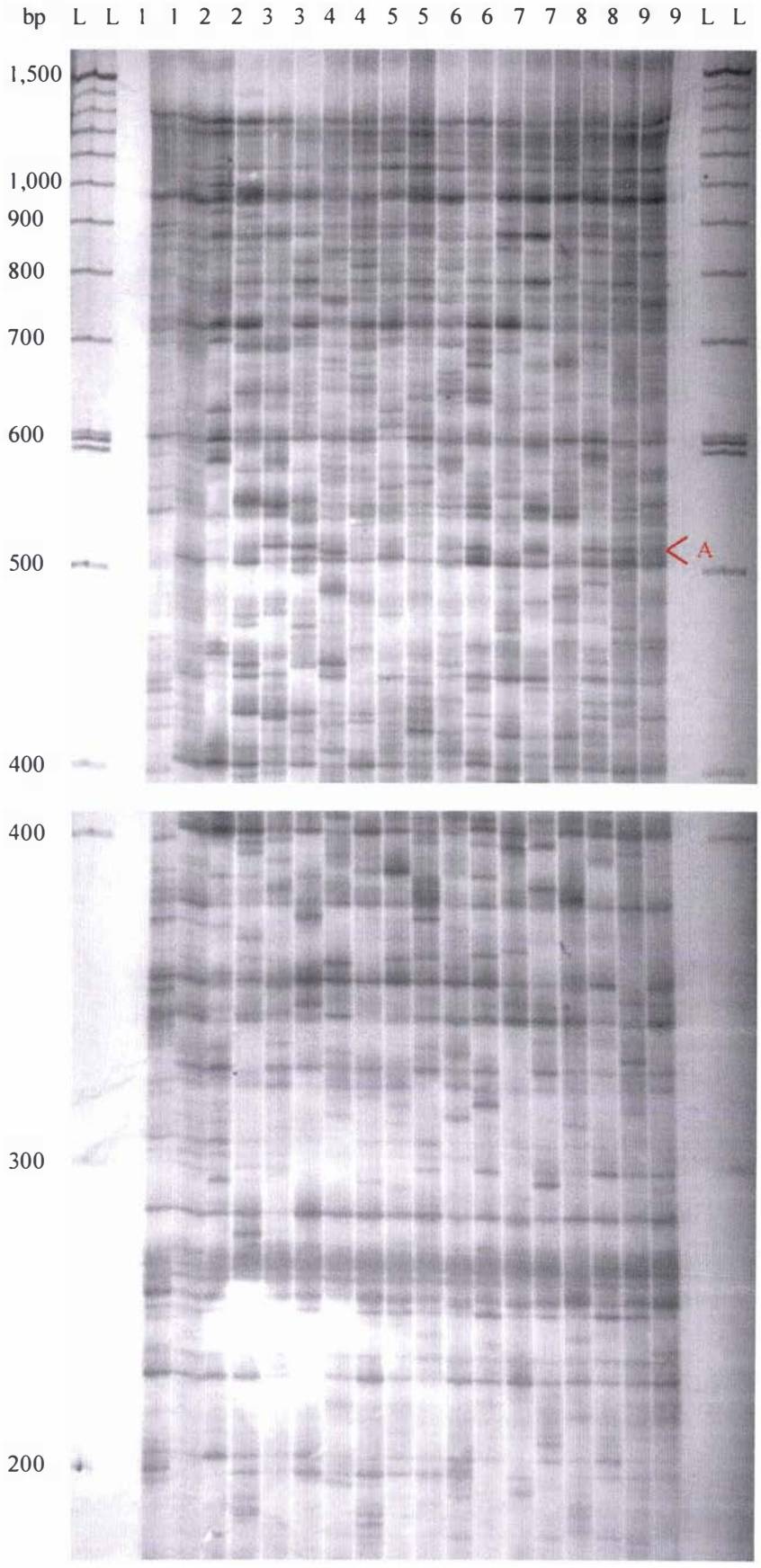


Figure 4.4: Polyacrylamide gel of selective amplification PCR products of the selective primers *Eco* RI+AGA and *Hpa* II+TCAA. Samples were control Florex Gold (1), Dutch E (2), Dutch F (3), Best Gold B (4), Best Gold C (5), few-eyed A (6), few-eyed B (7), bushy B (8) and bushy F (9). A 100bp DNA Ladder was included in the polyacrylamide gel (L). The polymorphic band present in Florex Gold, excluding control Florex Gold was excised and is labelled (<A).

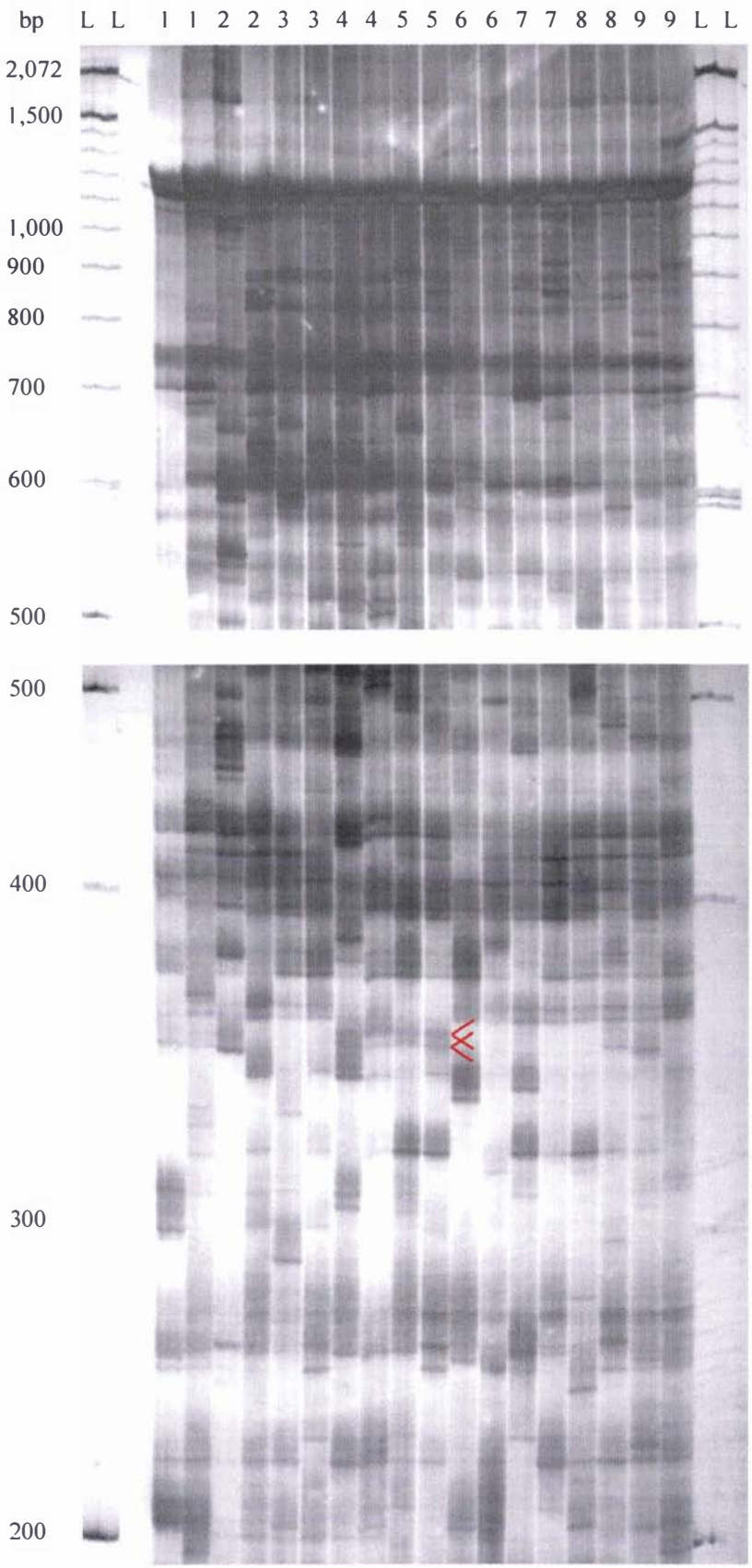


Figure 4.5: Polyacrylamide gel of selective amplification PCR products of the selective primers *Eco* RI+ACG and *Hpa* II+TCAA. Samples were control Florex Gold (1), Dutch E (2), Dutch F (3), Best Gold B (4), Best Gold C (5), few-eyed A (6), few-eyed B (7), bushy B (8) and bushy F (9). A 100bp DNA Ladder was included in the polyacrylamide gel (L). Polymorphic bands in Best Gold are labelled (<).

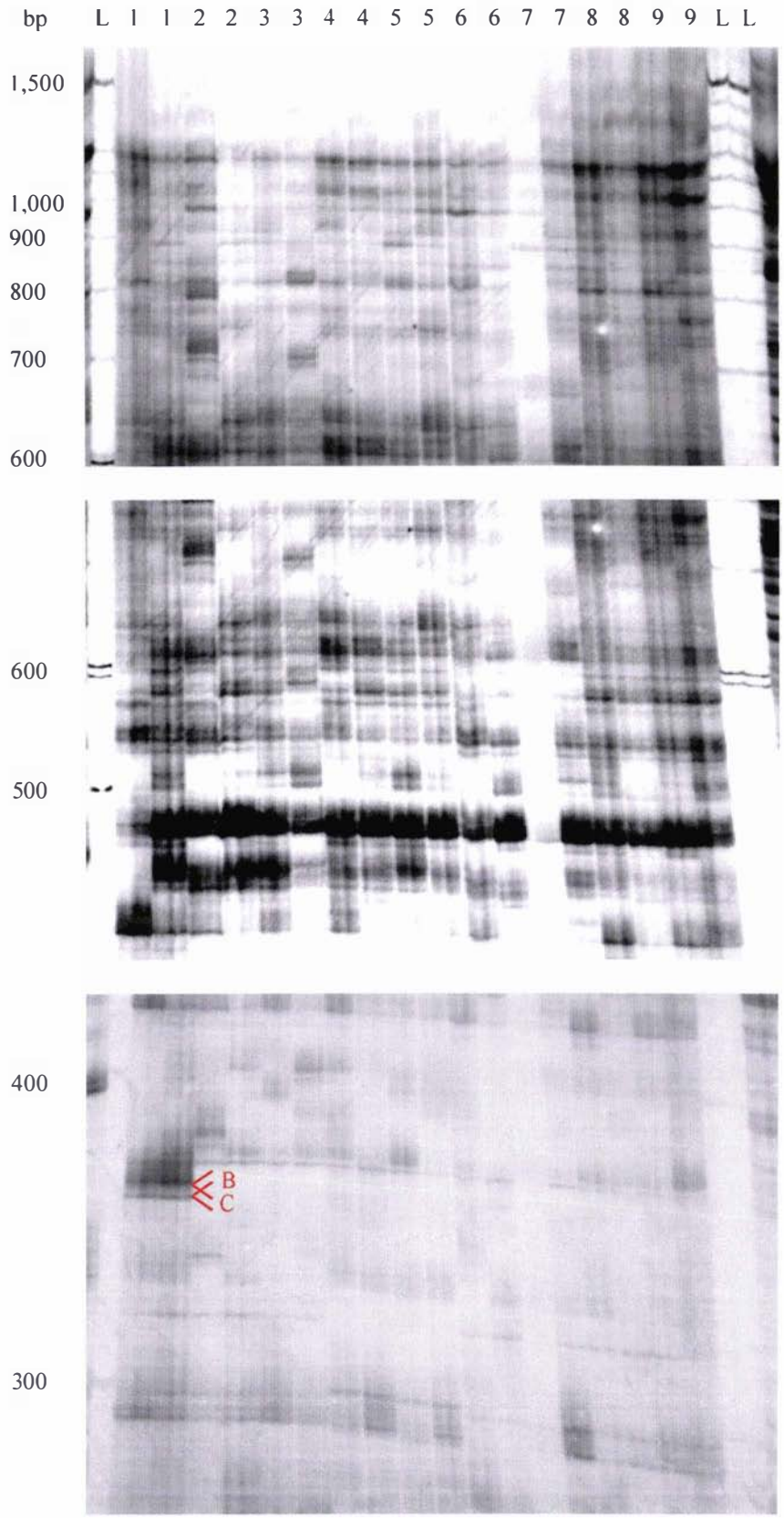


Figure 4.6: Polyacrylamide gel of selective amplification PCR products of the selective primers *Eco* RI+ACC and *Hpa* II+TCAA. Samples were control Florex Gold (1), Dutch E (2), Dutch F (3), Best Gold B (4), Best Gold C (5), few-eyed A (6), few-eyed B (7), bushy B (8) and bushy F (9). A 100bp DNA Ladder was included in the polyacrylamide gel (L). Polymorphic bands in control Florex Gold were excised and are labelled (<B and <C).

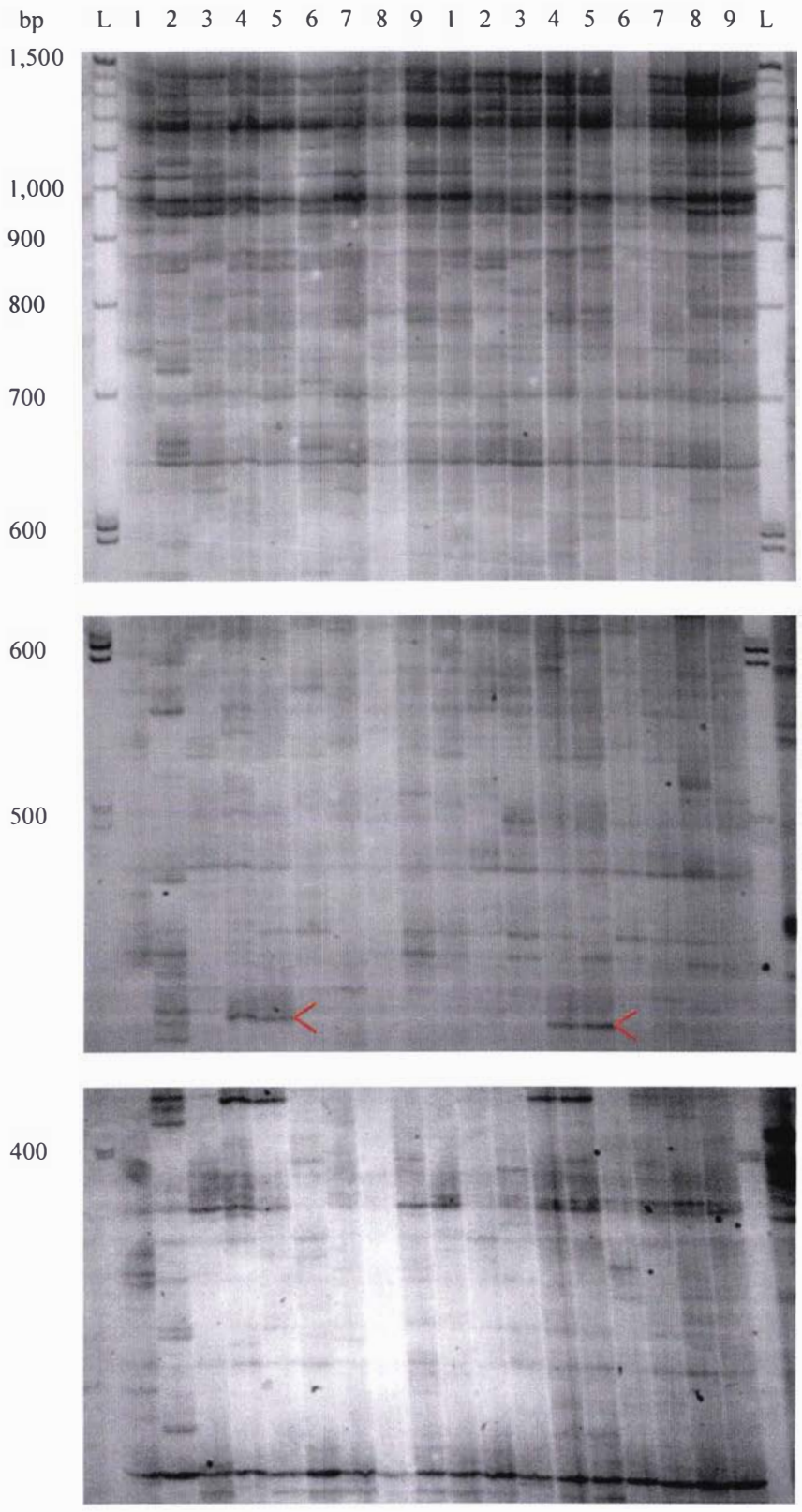


Figure 4.7: Polyacrylamide gel of selective amplification PCR products of the selective primers *Eco* RI+AAG and *Hpa* II+TCAA. Samples were control Florex Gold (1), Dutch E (2), Dutch F (3), Best Gold B (4), Best Gold C (5), few-eyed A (6), few-eyed B (7), bushy B (8) and bushy F (9). A 100bp DNA Ladder was included in the polyacrylamide gel (L). Polymorphic band present in Best Gold is labelled (<).

4.3.1.2 Re-amplification, DNA sequencing and sequence analysis of PCR amplified polymorphic bands

Only *Eco* RI/*Hpa* II products could be sequenced using the selective primers, so it was necessary to identify these products before sequencing was attempted. *Hpa* II/*Hpa* II and *Eco* RI/*Eco* RI based fragments could not be sequenced as it is not possible to sequence the two strands separately, and this would have resulted in a mixed sequencing signal. No PCR re-amplification of the PCR reactions containing only *Eco* RI or *Hpa* II selective primers was observed (Figure 4.8). PCR amplification was observed for the PCR reactions containing both the *Eco* RI and *Hpa* II selective primers.

The PCR re-amplification products of Bands A, B and C were sequenced. DNA sequence data is available in Appendix D. The DNA sequences of PCR amplified polymorphic bands represent genes of unknown function according to a BLAST alignment search of sequence similarity to genes in the NCBI database (Table 4.1).

4.3.1.3 DNA methylation polymorphic marker results

PCR primers were designed from the consensus sequence of the re-amplification product B. PCR amplification of each DNA sample, with the designed PCR primers, revealed that the DNA methylation polymorphic region B was present in all DNA samples investigated and of the same bp size (Figure 4.9).

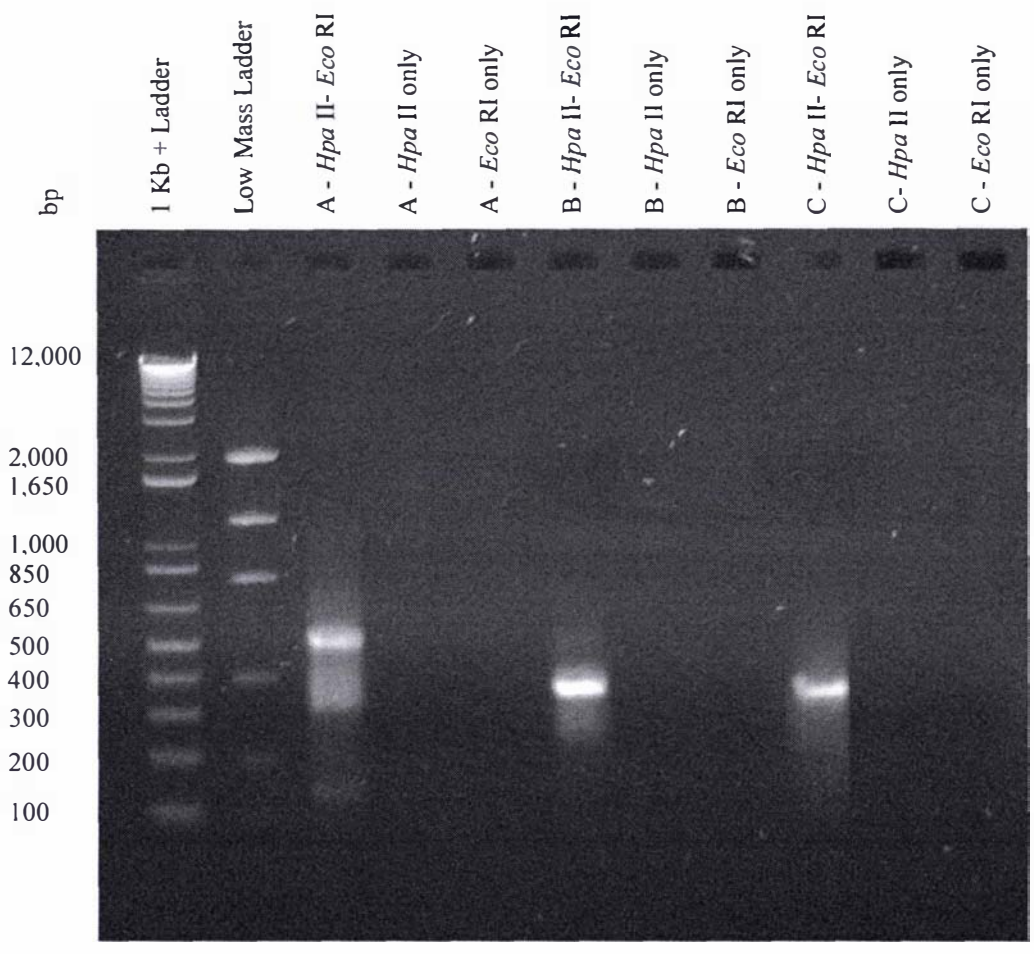


Figure 4.8: Agarose gel of re-amplification PCR products of both the *Hpa* II-*Eco* RI selective primers, only the *Hpa* II selective primer and only the *Eco* RI selective primer. Product A was a polymorphic band excised from the primer combination *Hpa* II+TCAA-*Eco* RI+AGA. Products B and C were polymorphic bands excised from the primer combination *Hpa* II+TCAA-*Eco* RI+ACC. A 1 Kb plus DNA Ladder and Low Mass Ladder were included in the agarose gel as size standards.

Table 4.1: Sequence similarity of genomic DNA AFLP fragments to sequences on the NCBI BLAST server.

Isolated fragment	Fragment derived from	Estimated bp length	Sequence similarity	Blast score
A	*Eco* RI+AGA and *Hpa* II+TCAA	510	No significant similarities found	
B	*Eco* RI+ACC and *Hpa* II+TCAA	350	No significant similarities found	
C	*Eco* RI+ACC and *Hpa* II+TCAA	350	No significant similarities found	

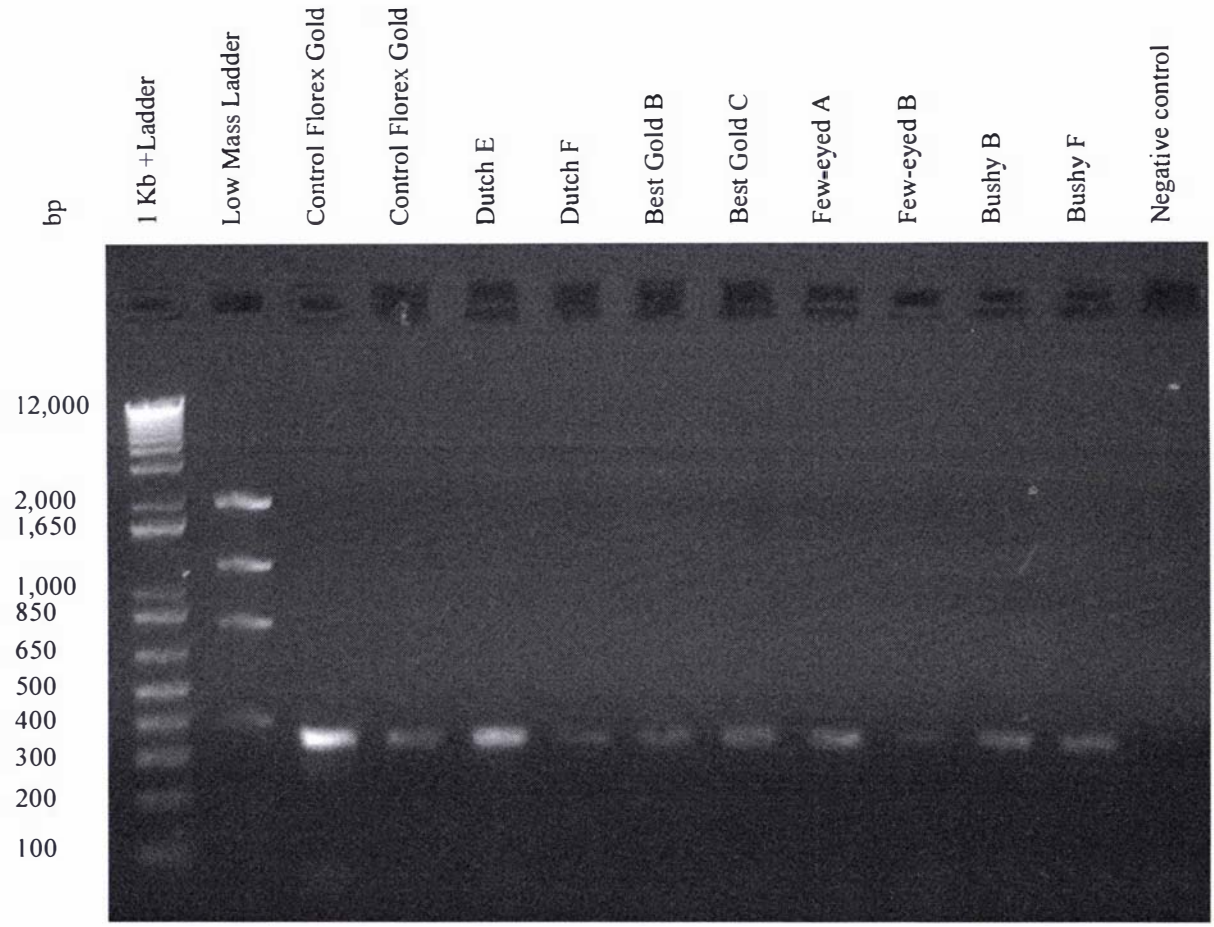


Figure 4.9: Agarose gel of PCR products of designed molecular marker primers. A 1 Kb plus DNA Ladder and Low Mass Ladder was included in the agarose gel. PCR was performed on DNA from control Florex Gold, Dutch E and F, Best Gold B and C, few-eyed A and B and bushy B and F. A negative control containing only the PCR components was included in the gel.

4.3.2 cDNA AFLP

4.3.2.1 cDNA AFLP gels of selective amplification PCR products

Twenty primer combinations were analysed in sprouting tuber bud tissue of two replicates of Best Gold and control Florex Gold and in three replicates of Dutch Florex Gold. Over 80 of the 500 bands detected differential expression between bushy and non-bushy selections. Some of the primer combinations investigated were subsets of other primer combinations. For example, the primer combination *Taq* I+CGA and TT+CAT displayed a subset of the bands seen for the primer combination *Taq* I+CG and TT+CA. Differential expression and differences in banding patterns were also observed between Best Gold and Florex Gold.

Two selective nucleotides on both the 3' ends of the *Taq* I and TT selective primers were investigated. The primer combinations were:

Taq I+AG and TT+CA	Figure 4.10
Taq I+AG and TT+AG	Figure 4.11
Taq I+CG and TT+AG	Figure 4.12
Taq I+CG and TT+CA	Figure 4.13.

Three selective nucleotides on both the 3' ends of *Taq* I and TT were also investigated. The primer combinations were:

Taq I+TAA and TT+CAT	Figure 4.14
Taq I+CGA and TT+CAT	Figure 4.15
Taq I+CGT and TT+CAT	Figure 4.16
Taq I+AGG and TT+AGC	Figure 4.17.

In general, three selective nucleotides produced smaller cDNA fragments, 50 to 150 bp. Most cDNA fragments from two selective nucleotides PCR were greater than 75 bp. A combination of two selective nucleotides on the 3' ends of the selective primer *Taq* I and three selective nucleotides on the 3' ends of the selective primer TT were examined. The primer combinations were:

Taq I+CG and TT+AGC	Figure 4.18
Taq I+AG and TT+AGC	Figure 4.19
Taq I+CG and TT+ACC	Figure 4.20

Taq I+CG and TT+CAT	Figure 4.21
Taq I+AG and TT+AAG	Figure 4.22
Taq I+AG and TT+AGA	Figure 4.23
Taq I+AG and TT+GGA	Figure 4.24
Taq I+AG and TT+CCT	Figure 4.25
Taq I+AG and TT+CAC	Figure 4.26
Taq I+AG and TT+CGT	Figure 4.27
Taq I+AG and TT+GCC	Figure 4.28
Taq I+AG and TT+GGT	Figure 4.29.

Several bands showing differential expression between bushy and non-bushy Florex Gold were excised, re-amplified and sequenced. To determine if the band isolated and re-amplified was the same as the one visualised in the original polyacrylamide gel, re-amplified bands were run on an agarose gel with a DNA size standard to check the product was of the expected size. Sequence data was also investigated to determine that the expected terminal sequences were found corresponding to the two or three selective base extensions used in the PCR. The length, pattern of expression and sequence similarity to known DNA sequences, of the differentially expressed bands, is shown in Table 4.2. Band I appeared slightly down-regulated in bushy plants and showed sequence similarity to *Chloranthus spicatus* 18S rRNA (CLURRE04), *Calycanthus occidentalis* 18S rRNA (CLYRRE04) and *Lillium superbum* 18S rRNA (AF206952). Band O was down-regulated in bushy plants and showed sequence similarity to a rice (*Oryza sativa*) 60S ribosomal protein mRNA (AF093630).

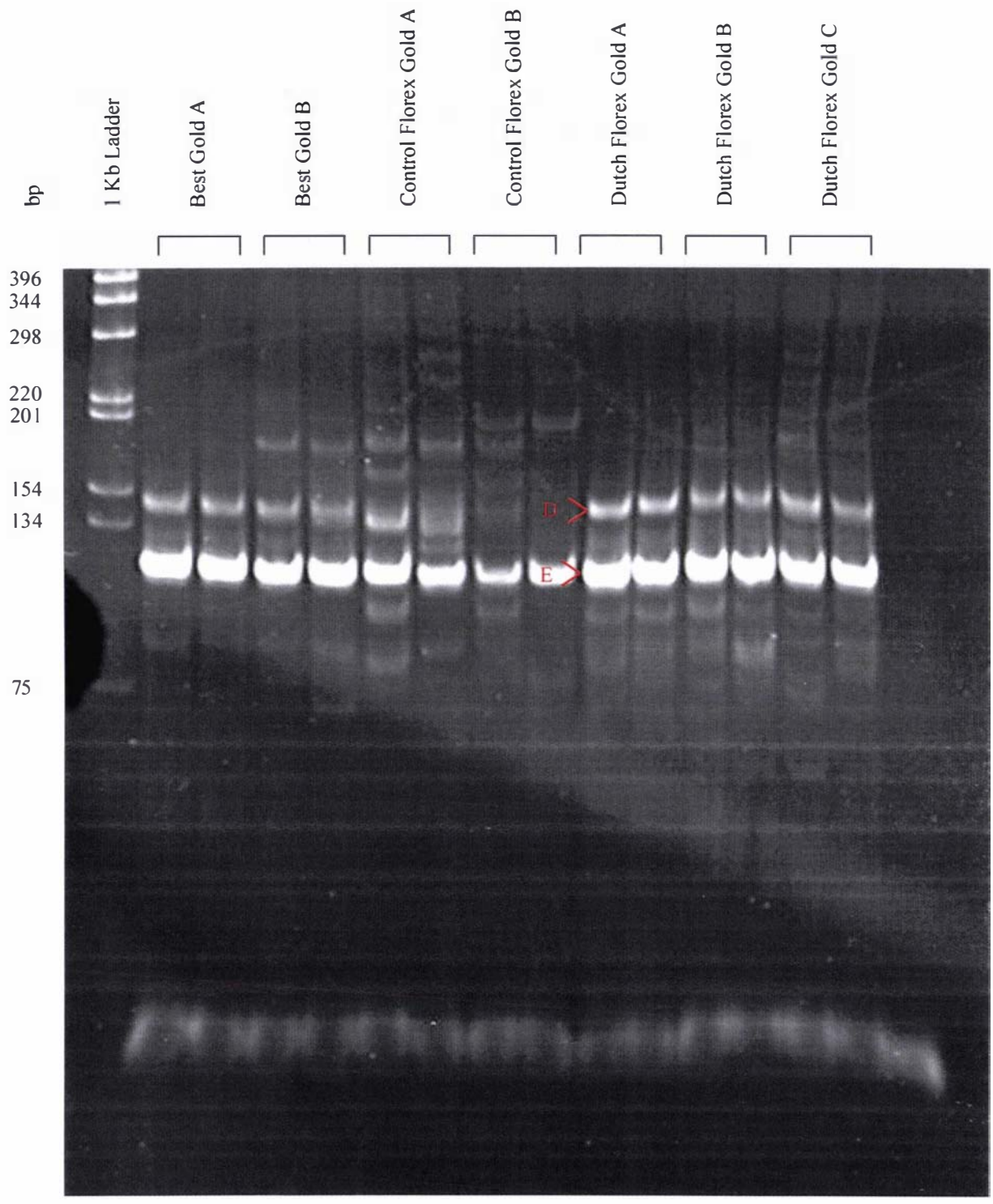


Figure 4.10: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+CA. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

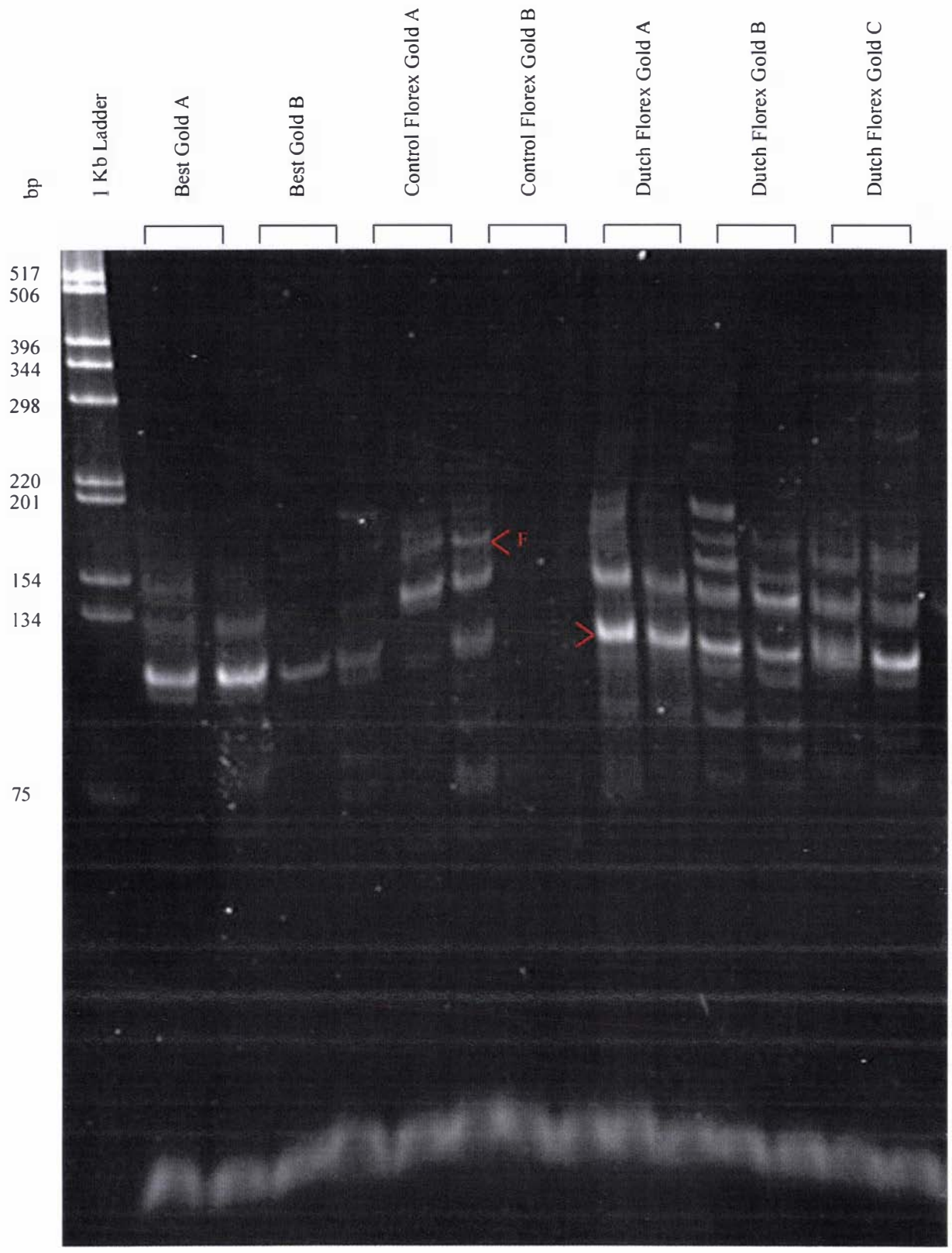


Figure 4.11: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+AG. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

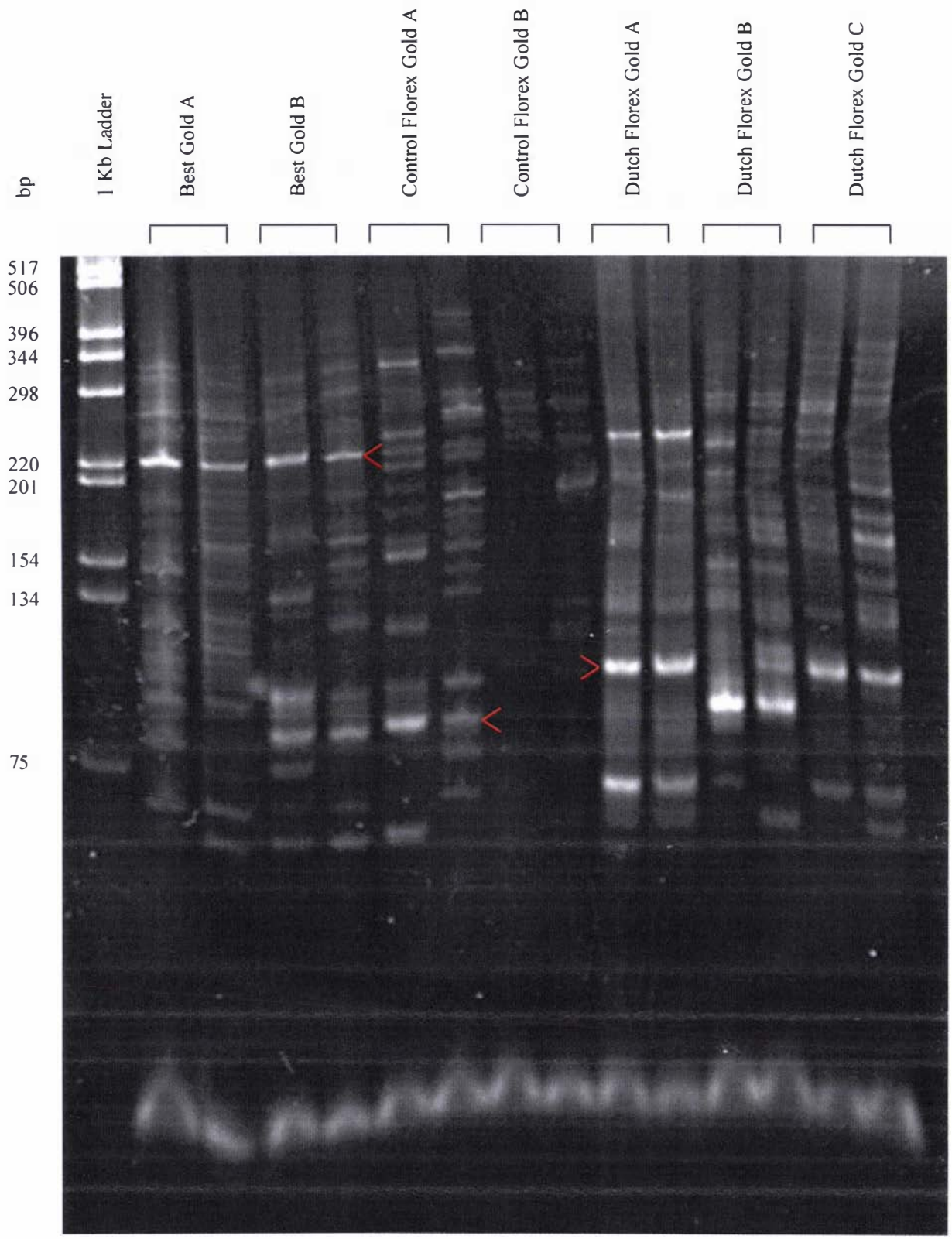


Figure 4.12: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+CG and TT+AG. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

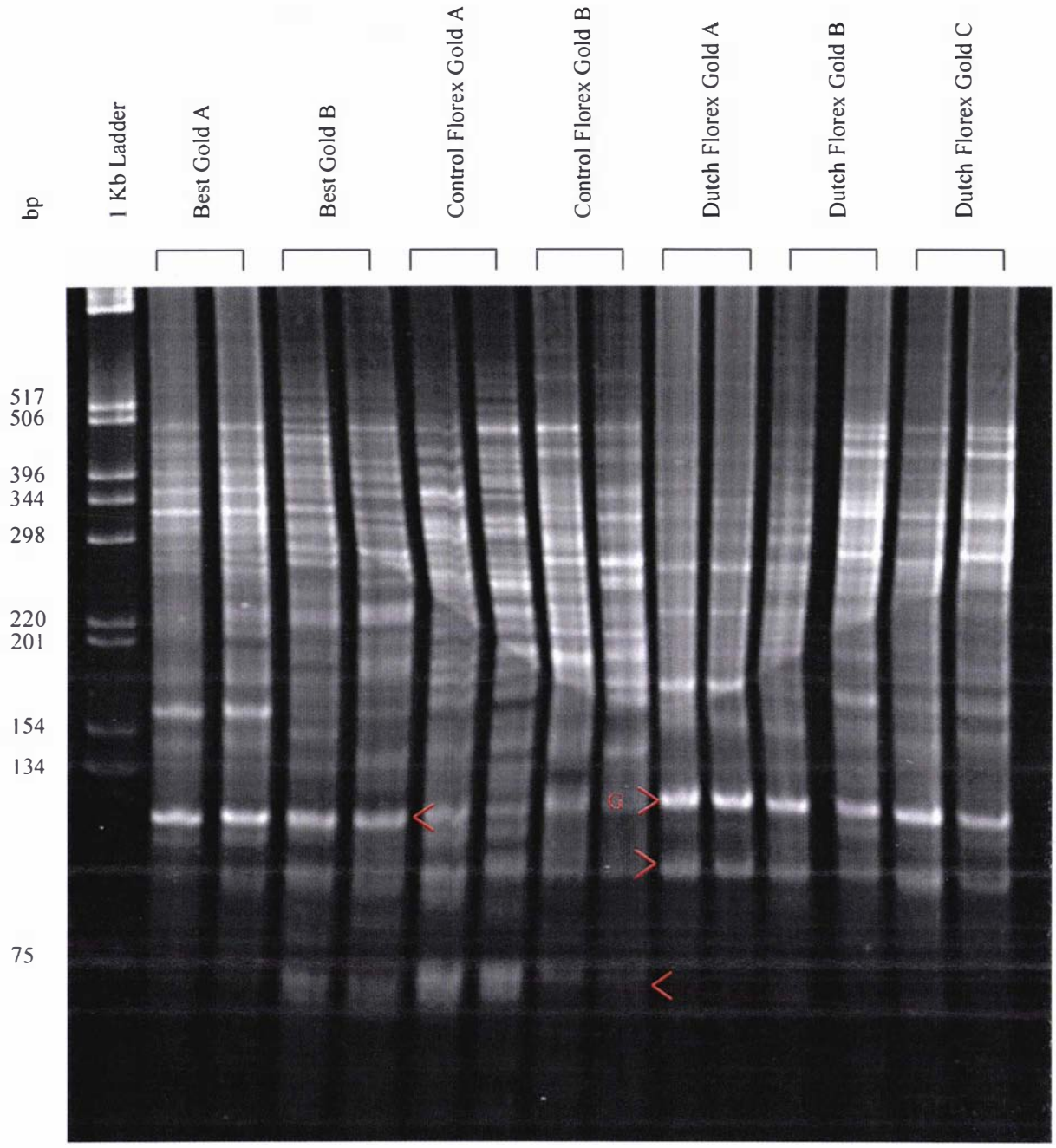


Figure 4.13: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+CG and TT+CA. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

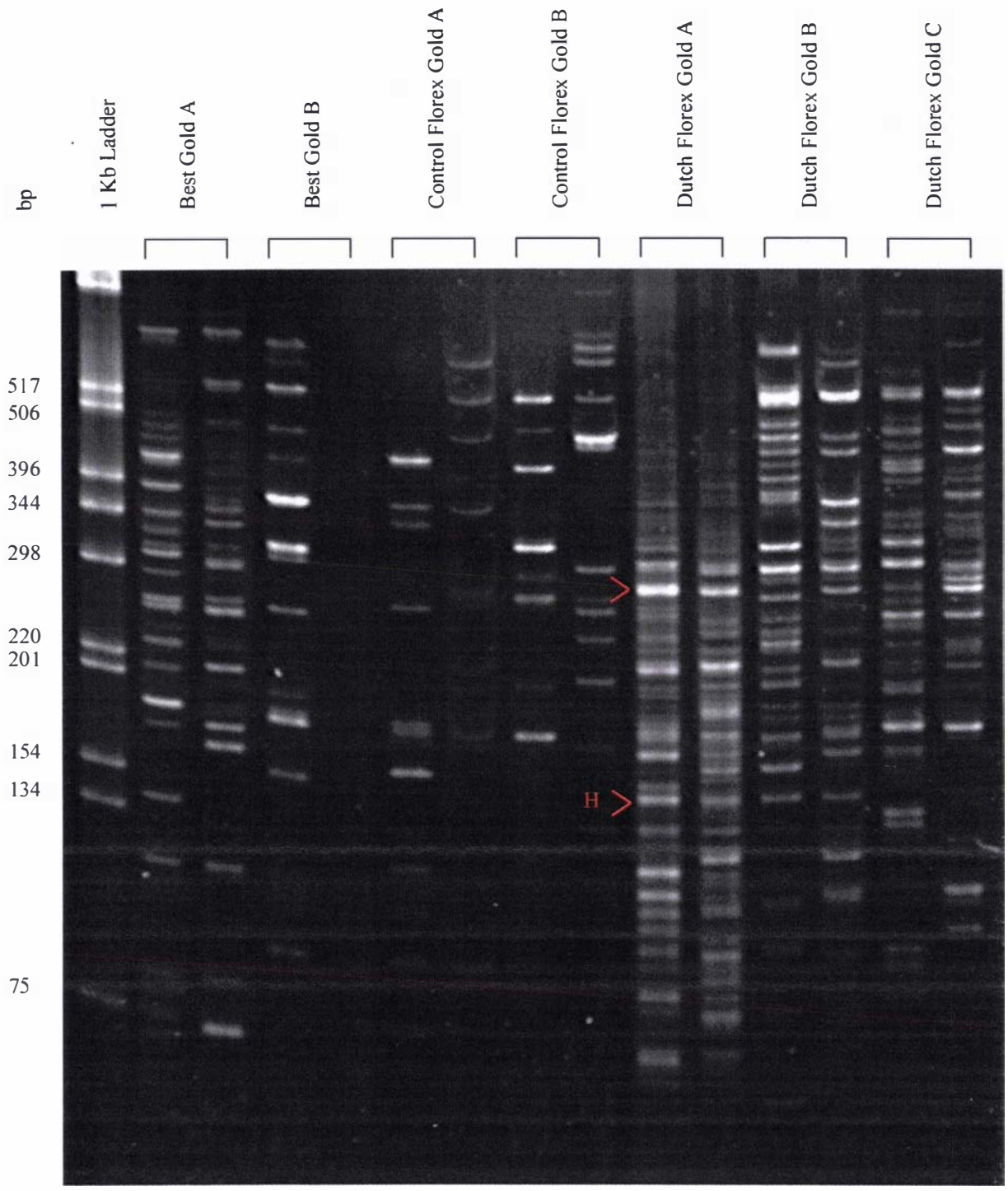


Figure 4.14: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+TAA and TT+CAT. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

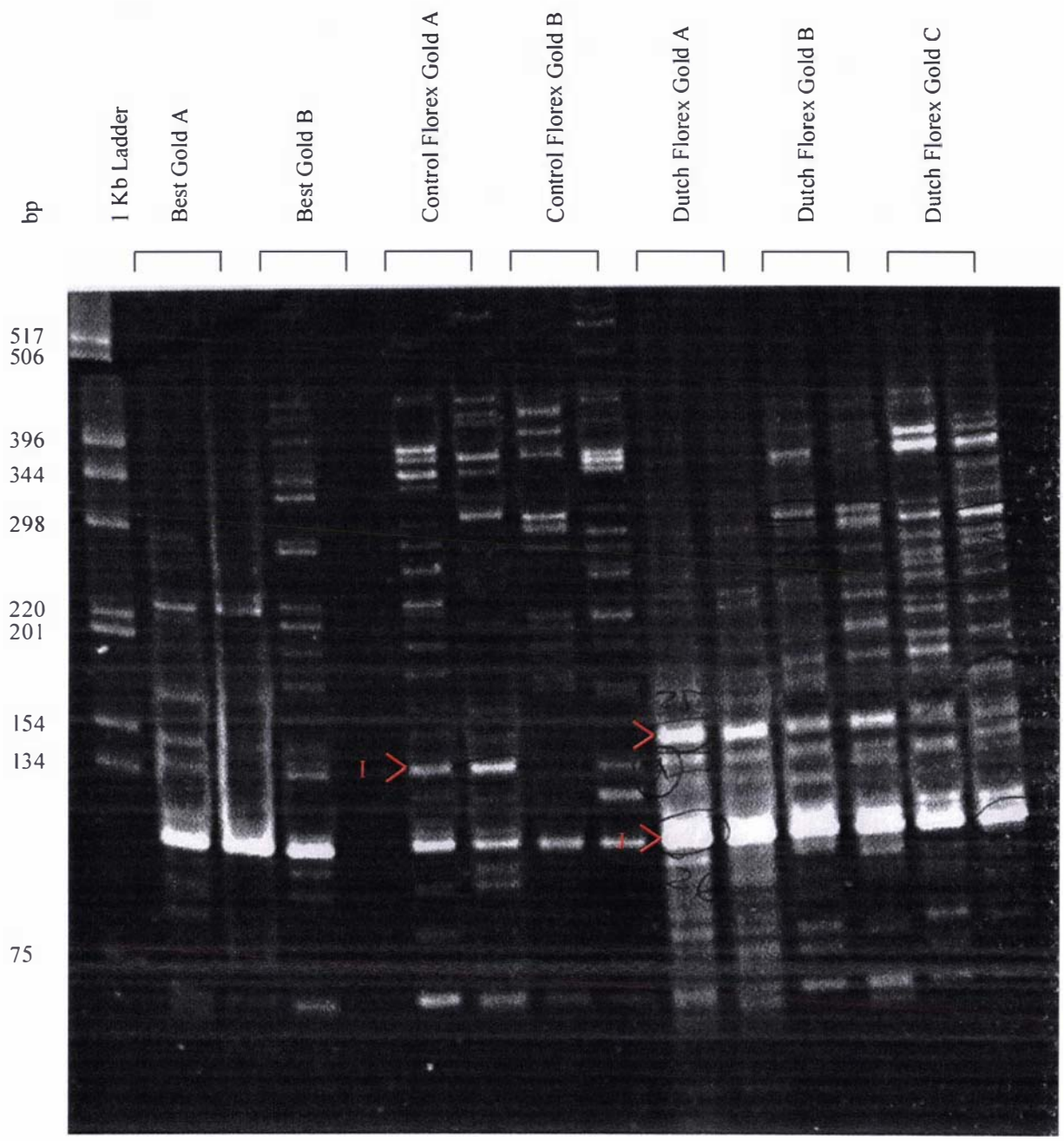


Figure 4.15: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+CGA and TT+CAT. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

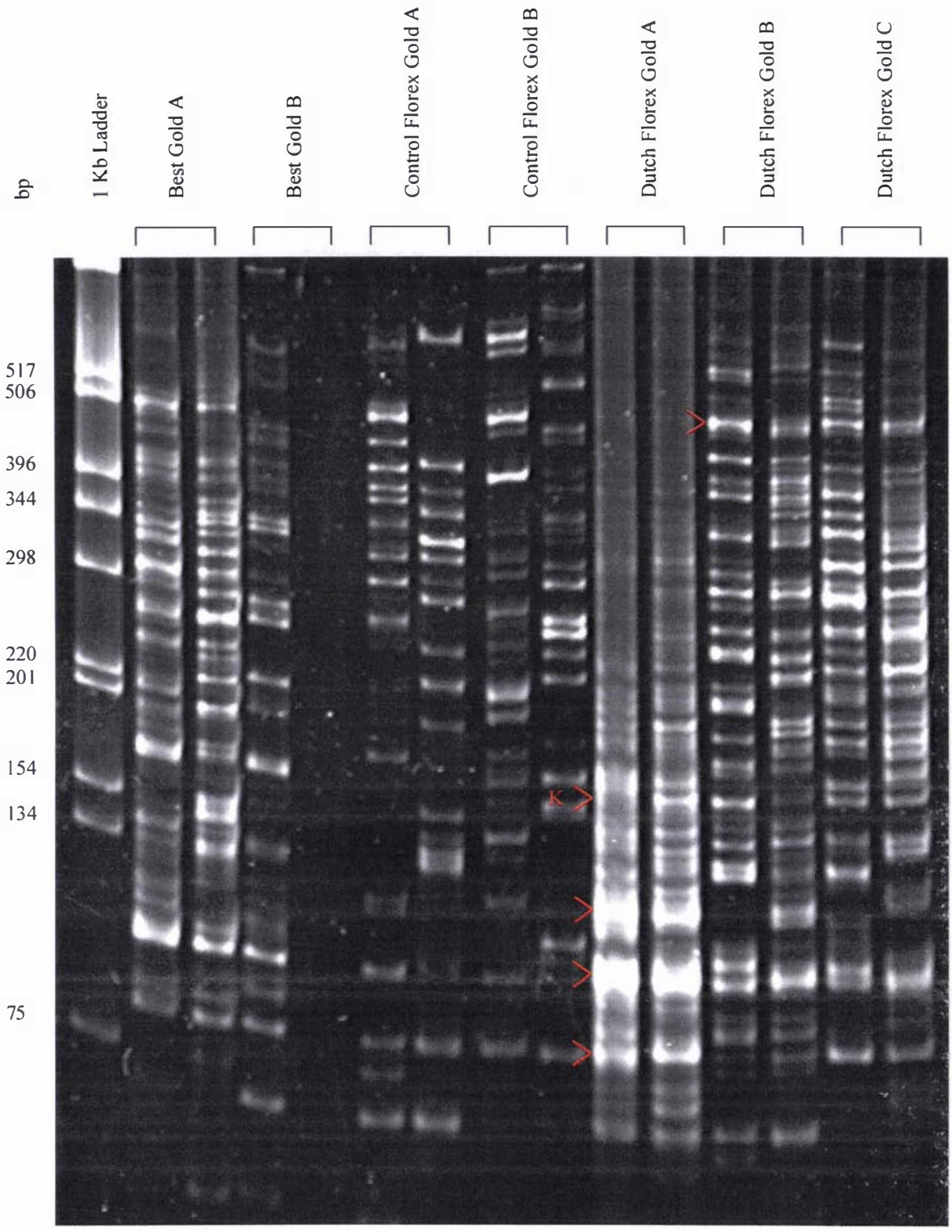


Figure 4.16: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+CGT and TT+CAT. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

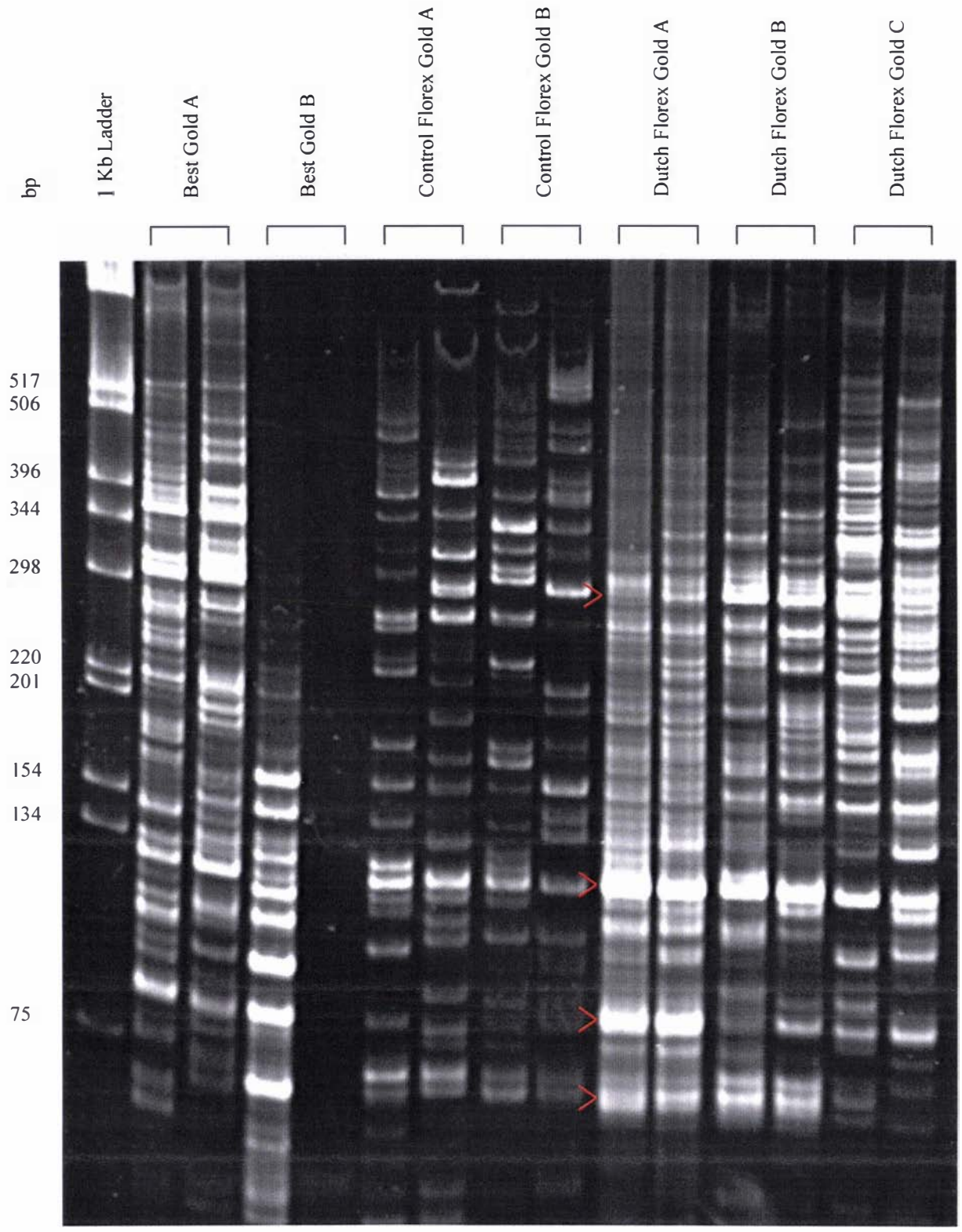


Figure 4.17: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AGG and TT+AGC. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on two replicates of sprouting Best Gold, control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

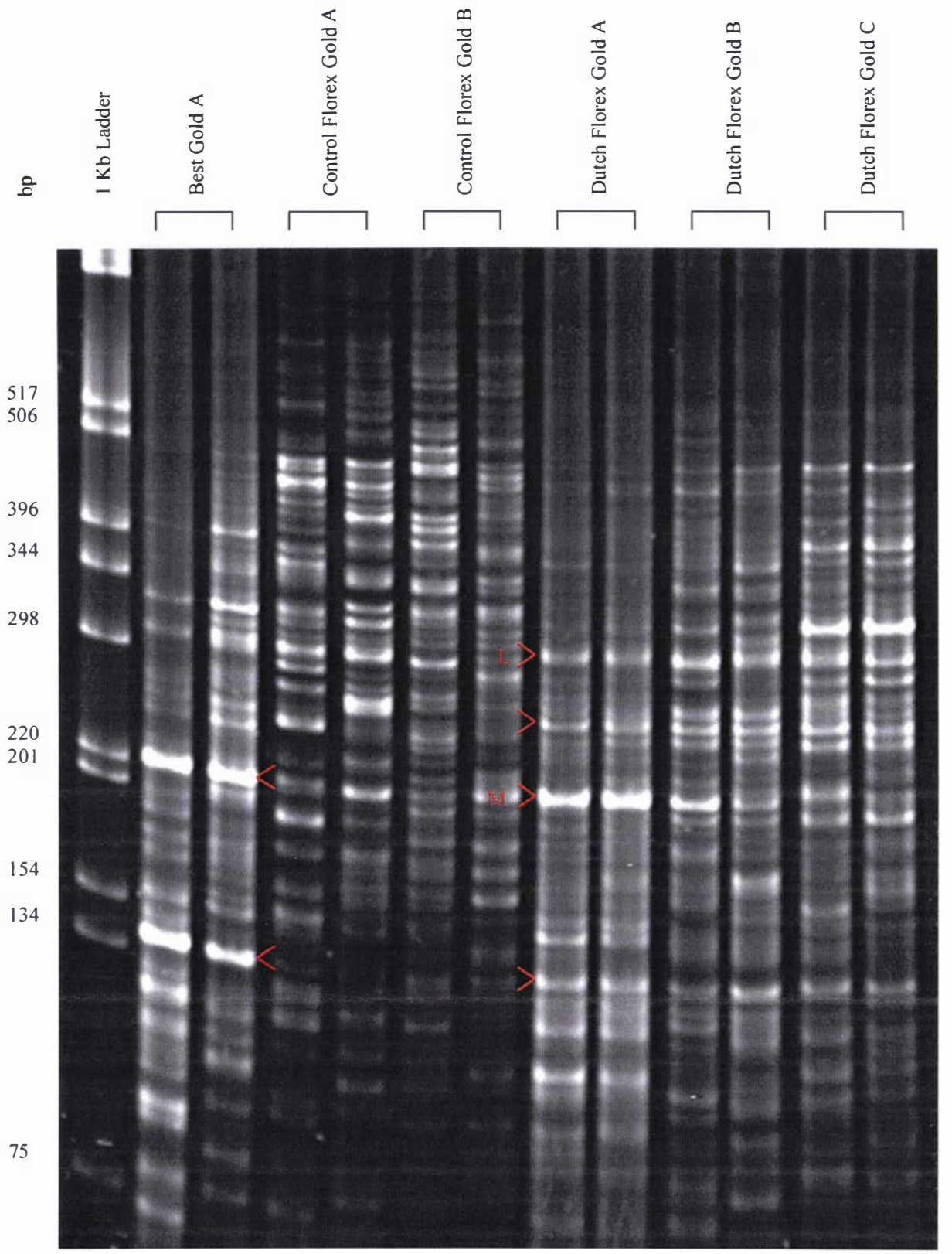


Figure 4.18: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+CG and TT+AGC. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

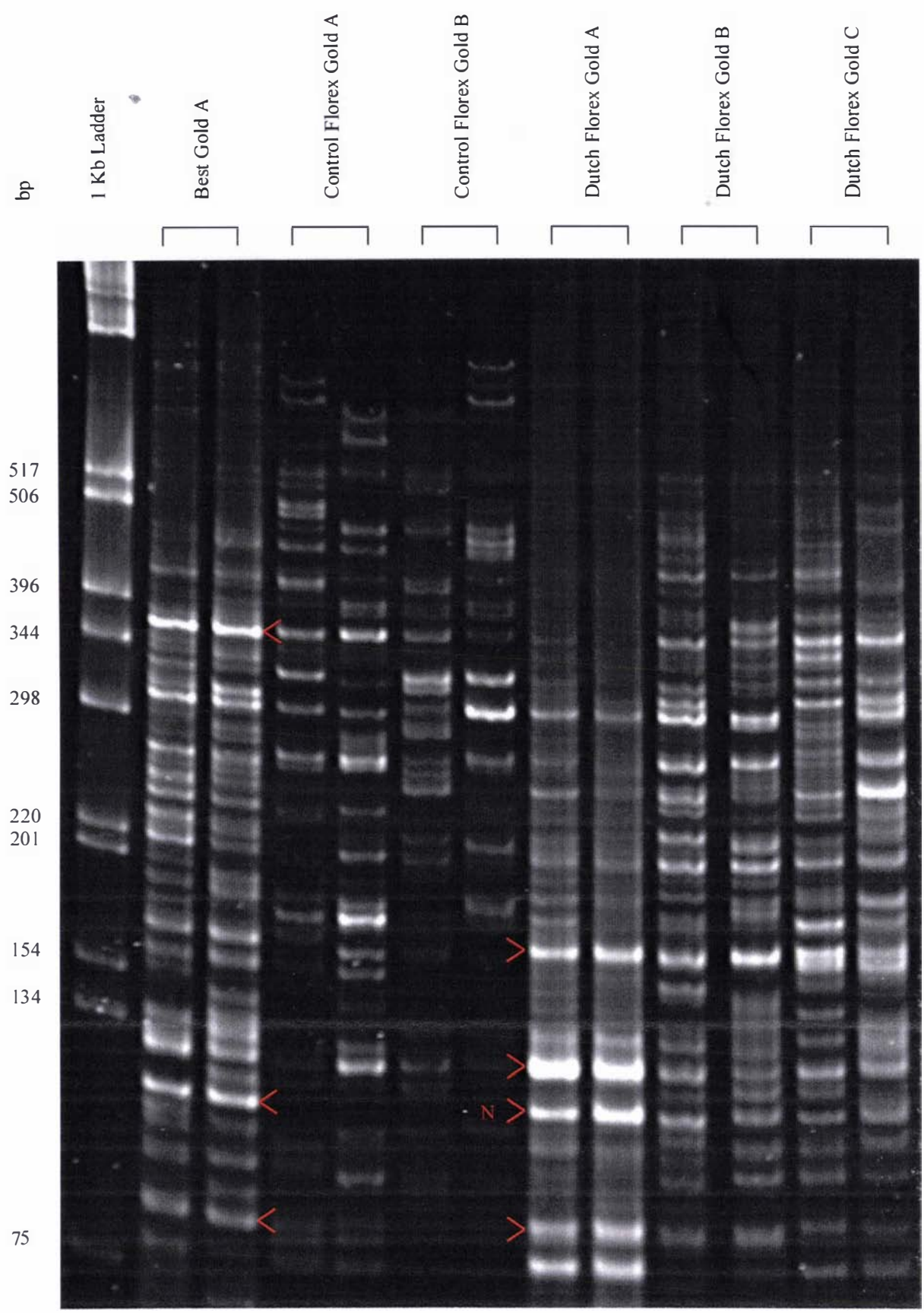


Figure 4.19: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+AGC. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

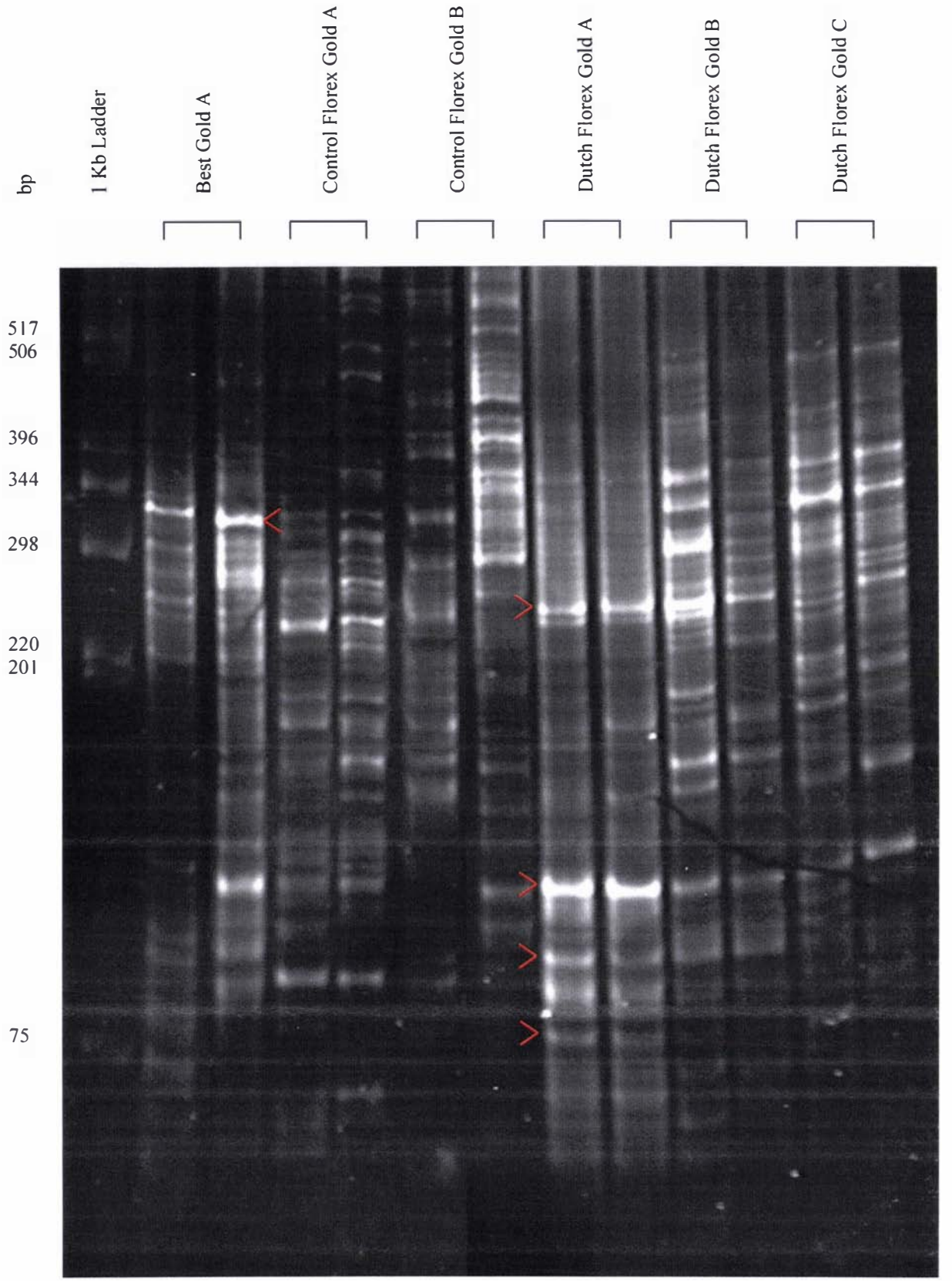


Figure 4.20: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+CG and TT+ACC. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

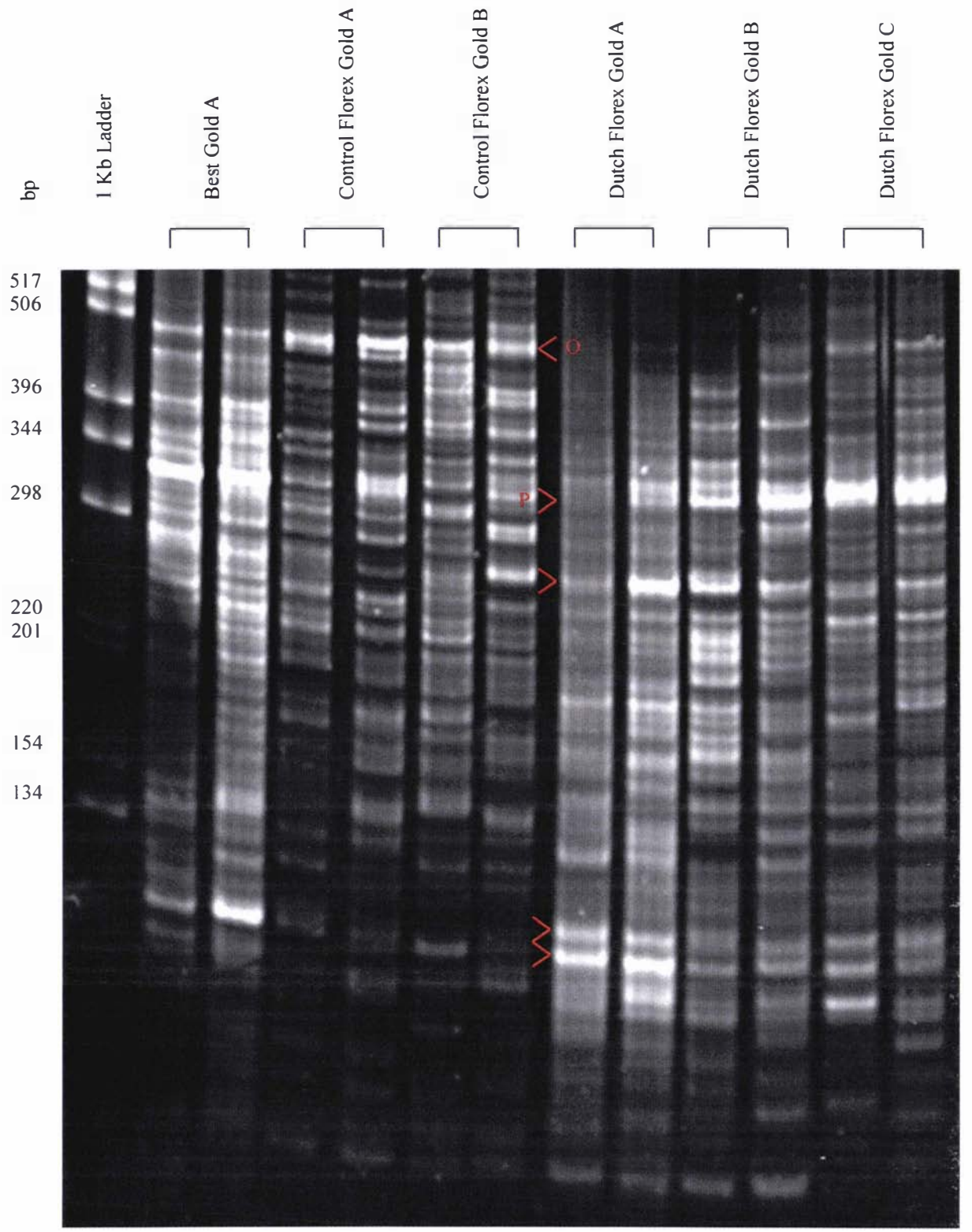


Figure 4.21: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+CG and TT+CAT. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

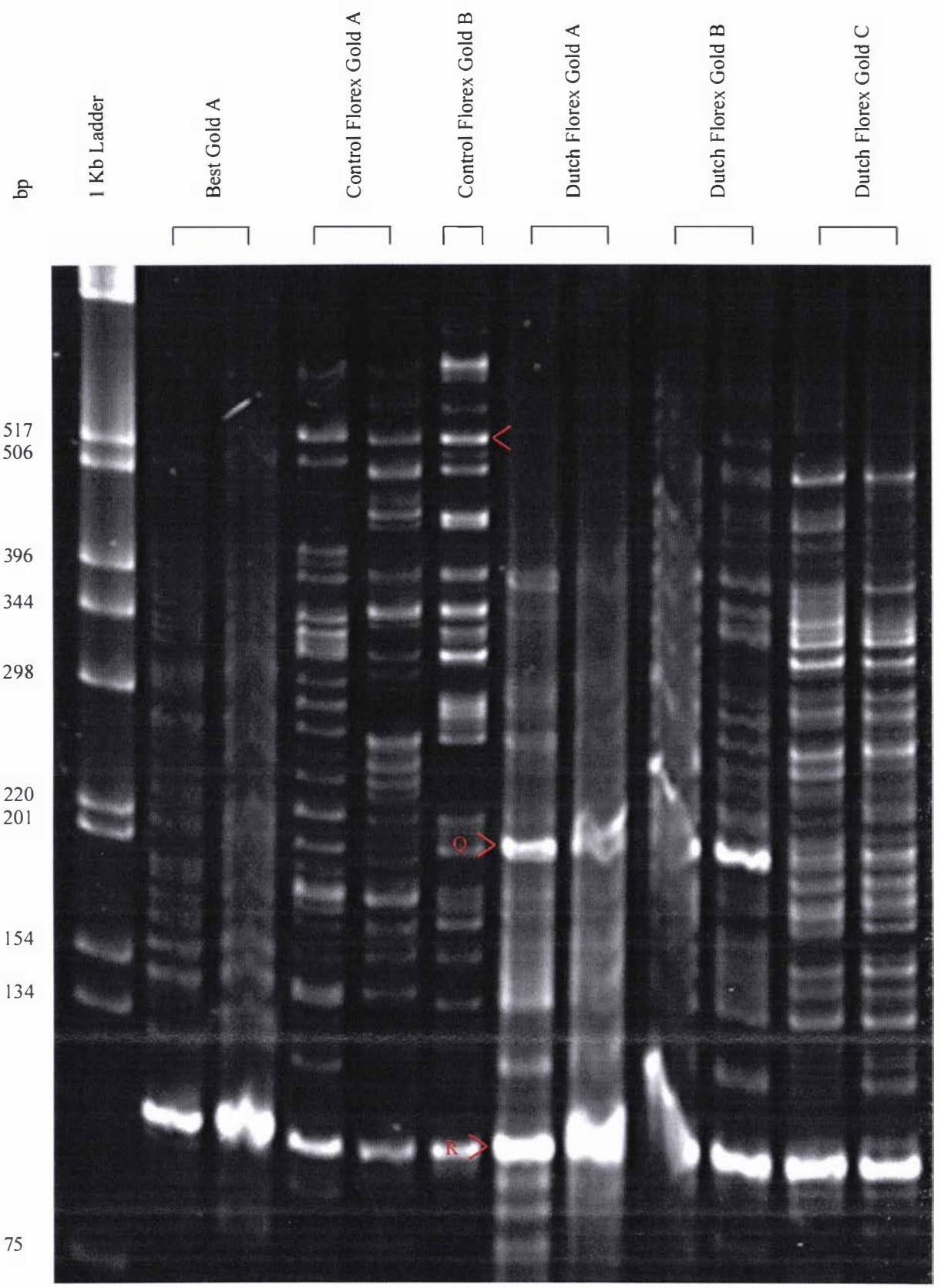


Figure 4.22: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+AAG. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

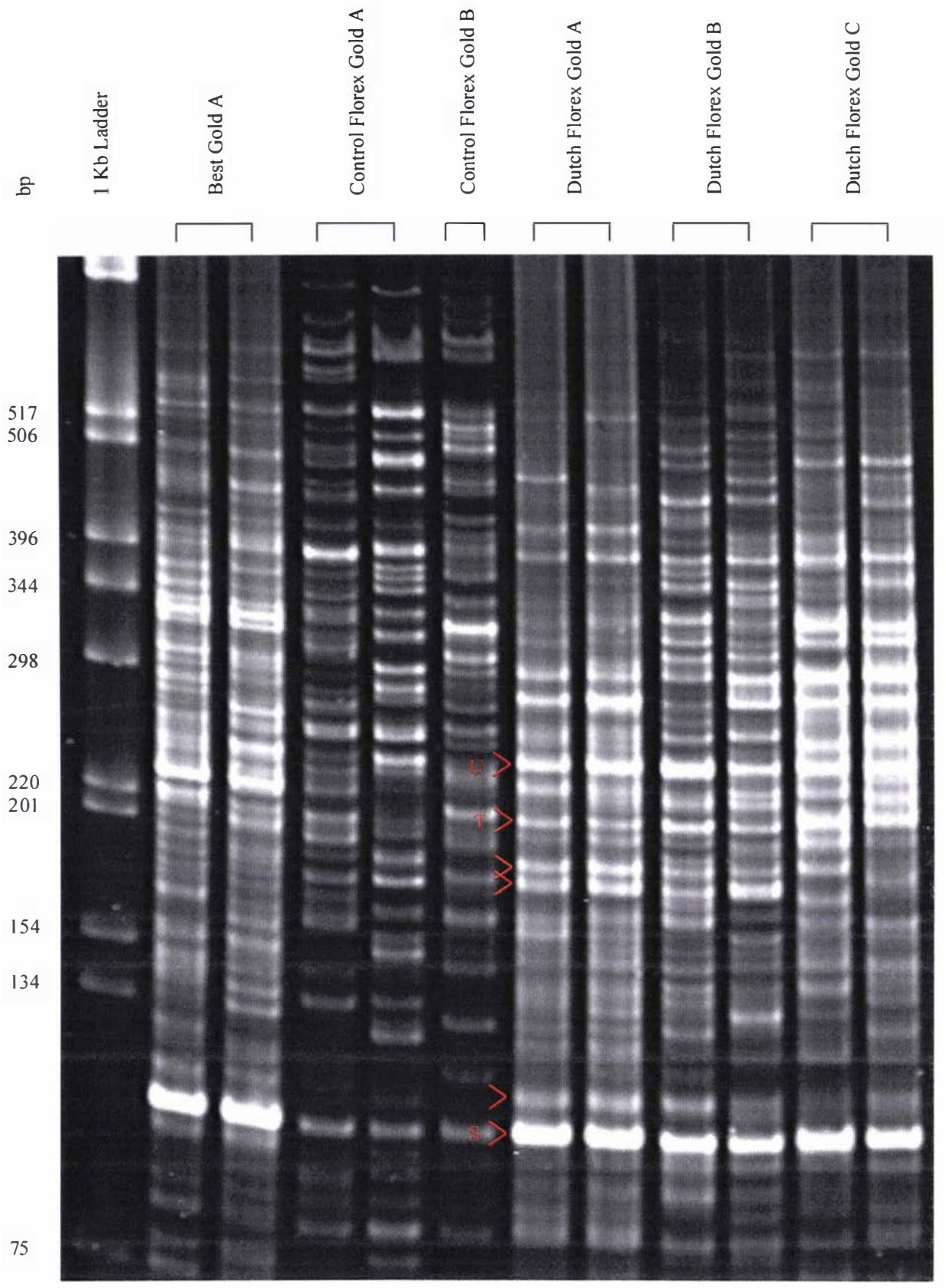


Figure 4.23: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+AGA. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

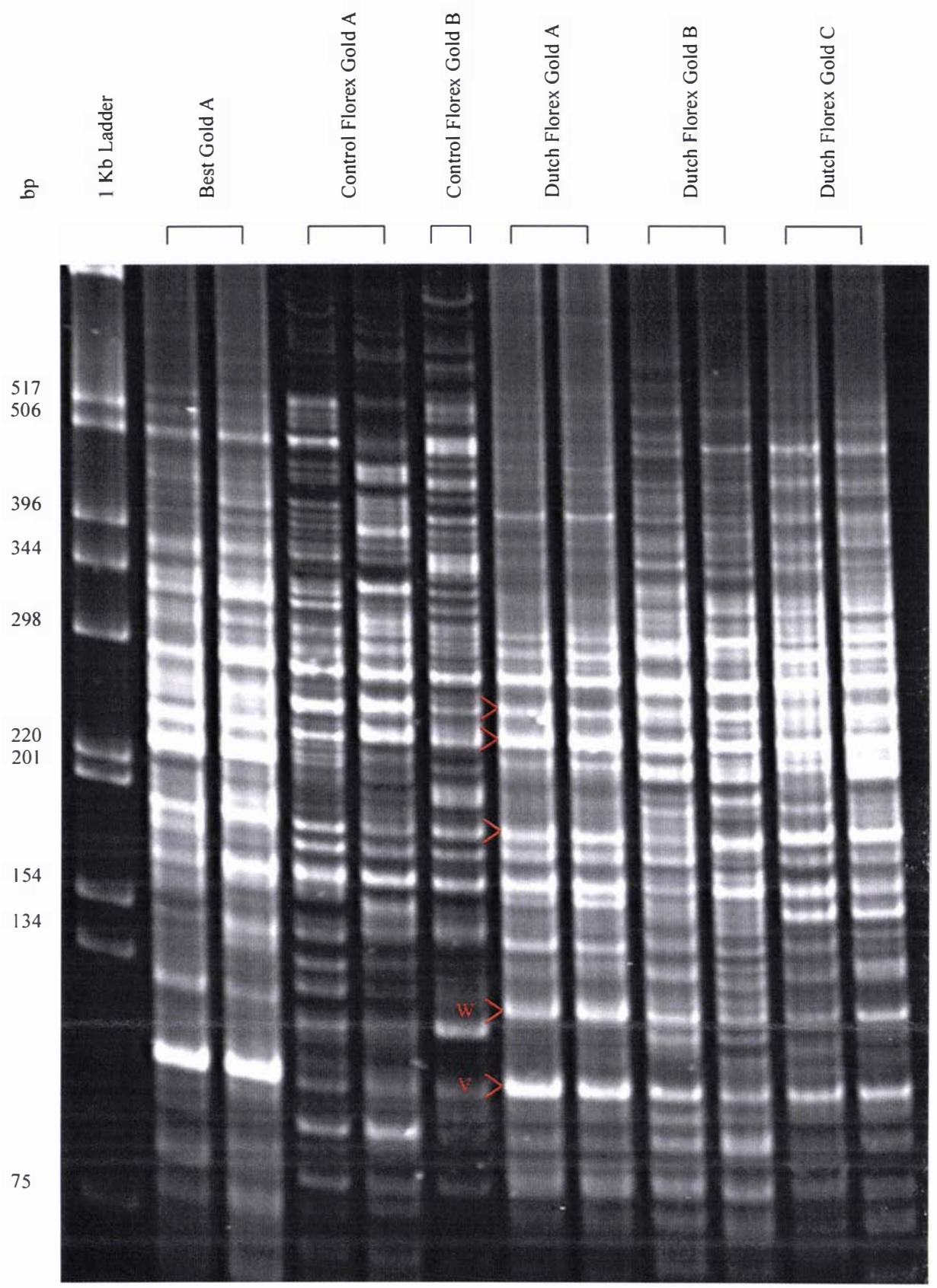


Figure 4.24: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+GGA. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

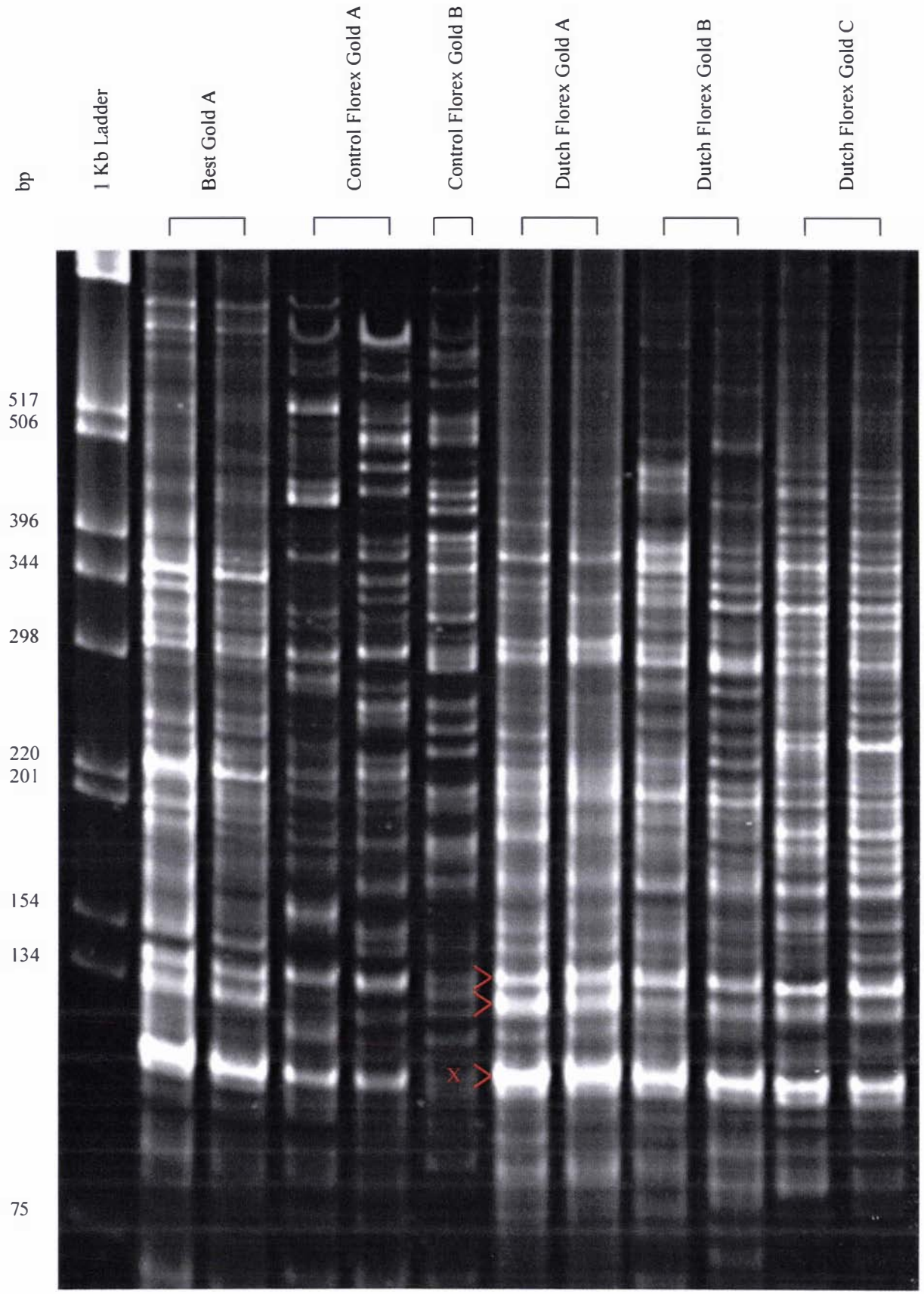


Figure 4.25: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+CCT. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold, two replicates of control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

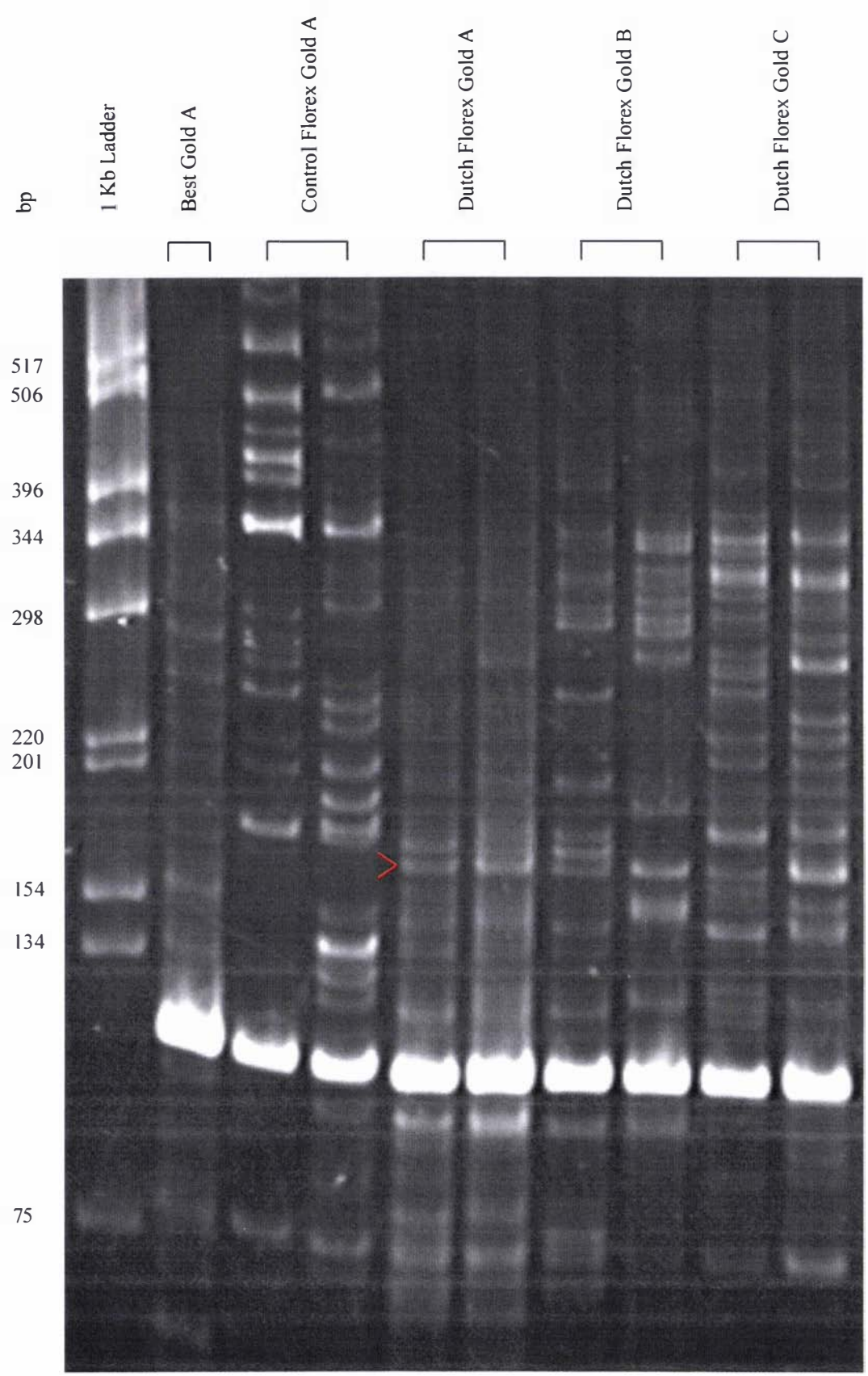


Figure 4.26: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+CAC. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold and control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (>) and bands excised.

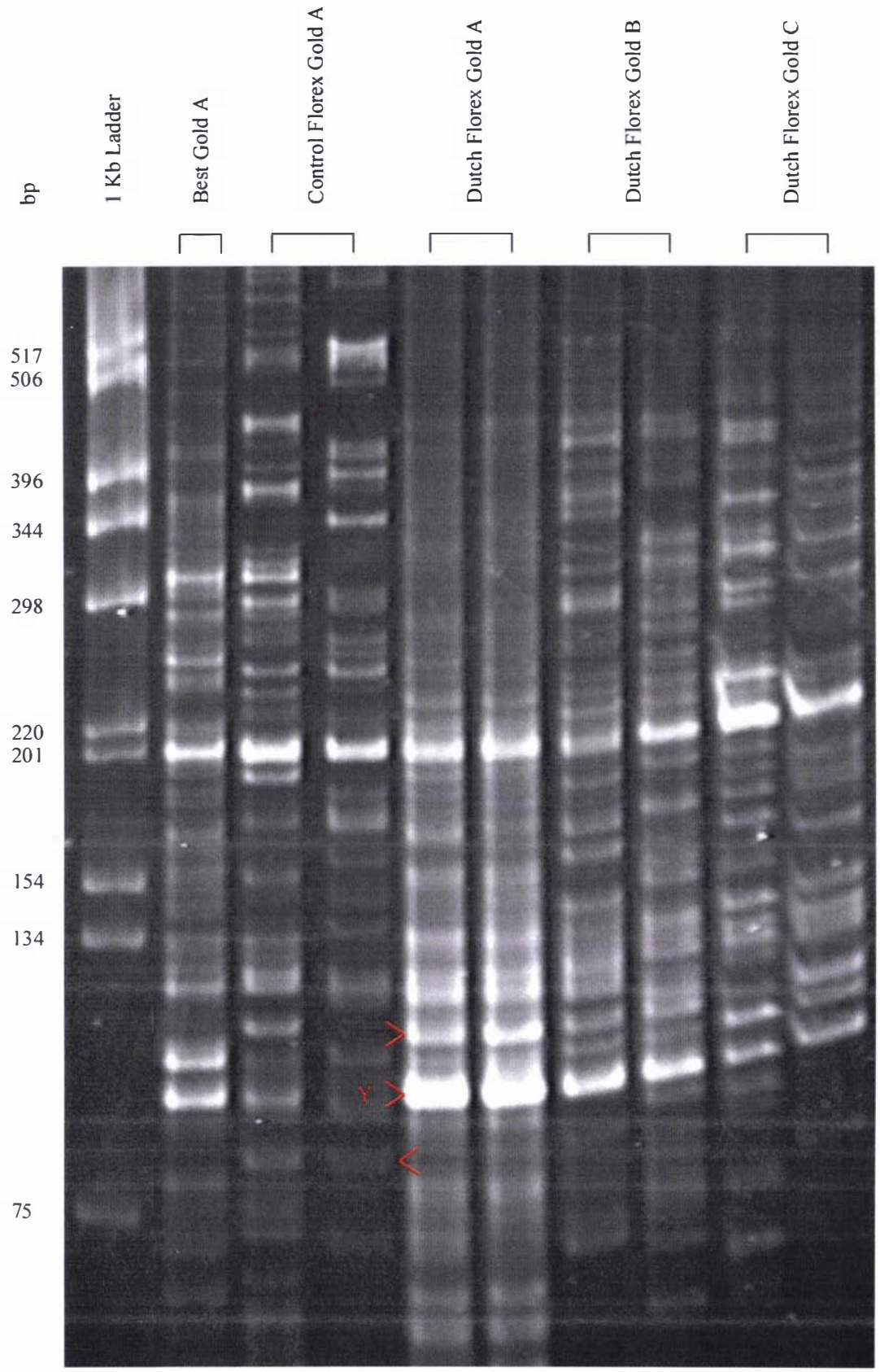


Figure 4.27: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+CGT. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold and control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

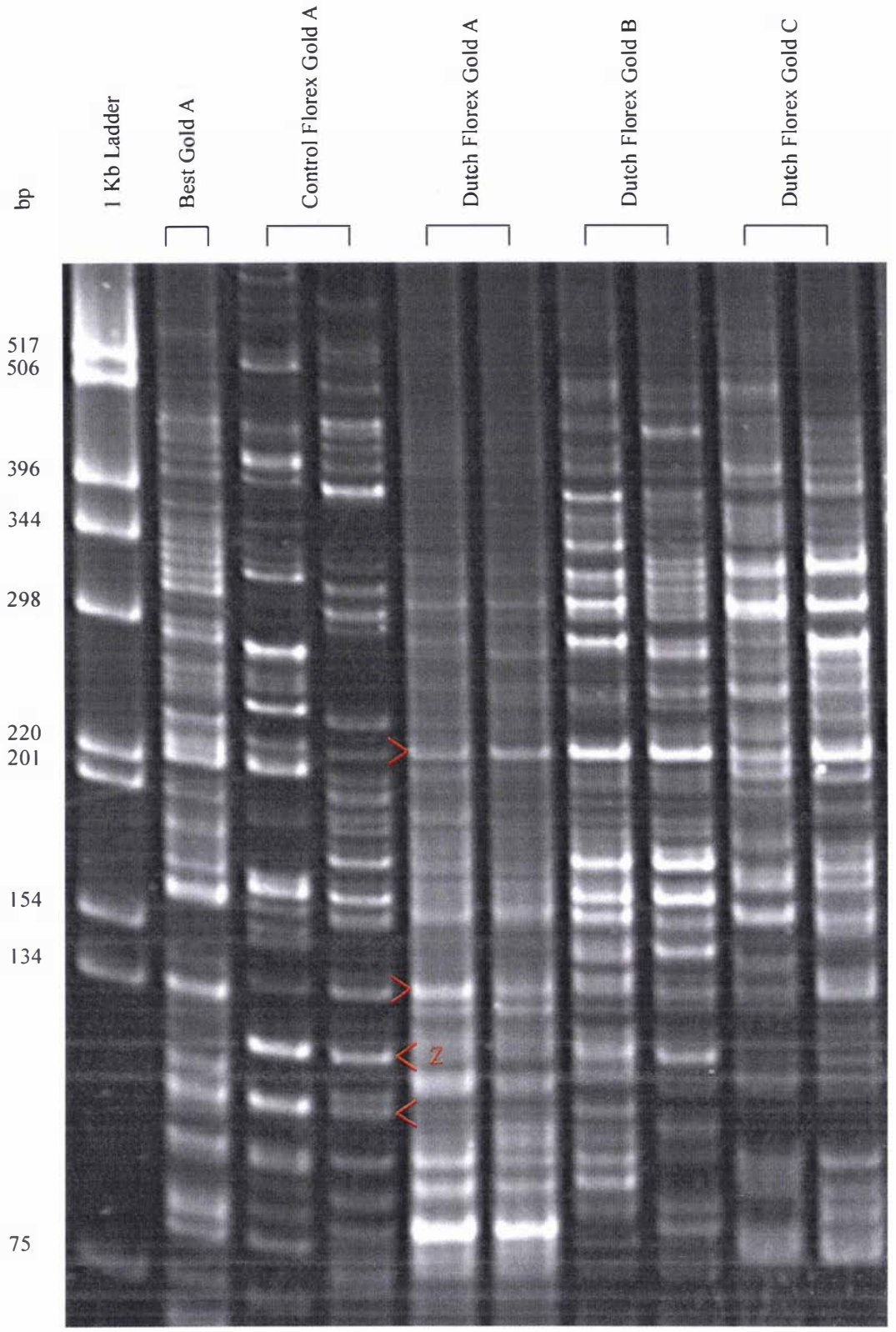


Figure 4.28: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+GCC. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold and control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

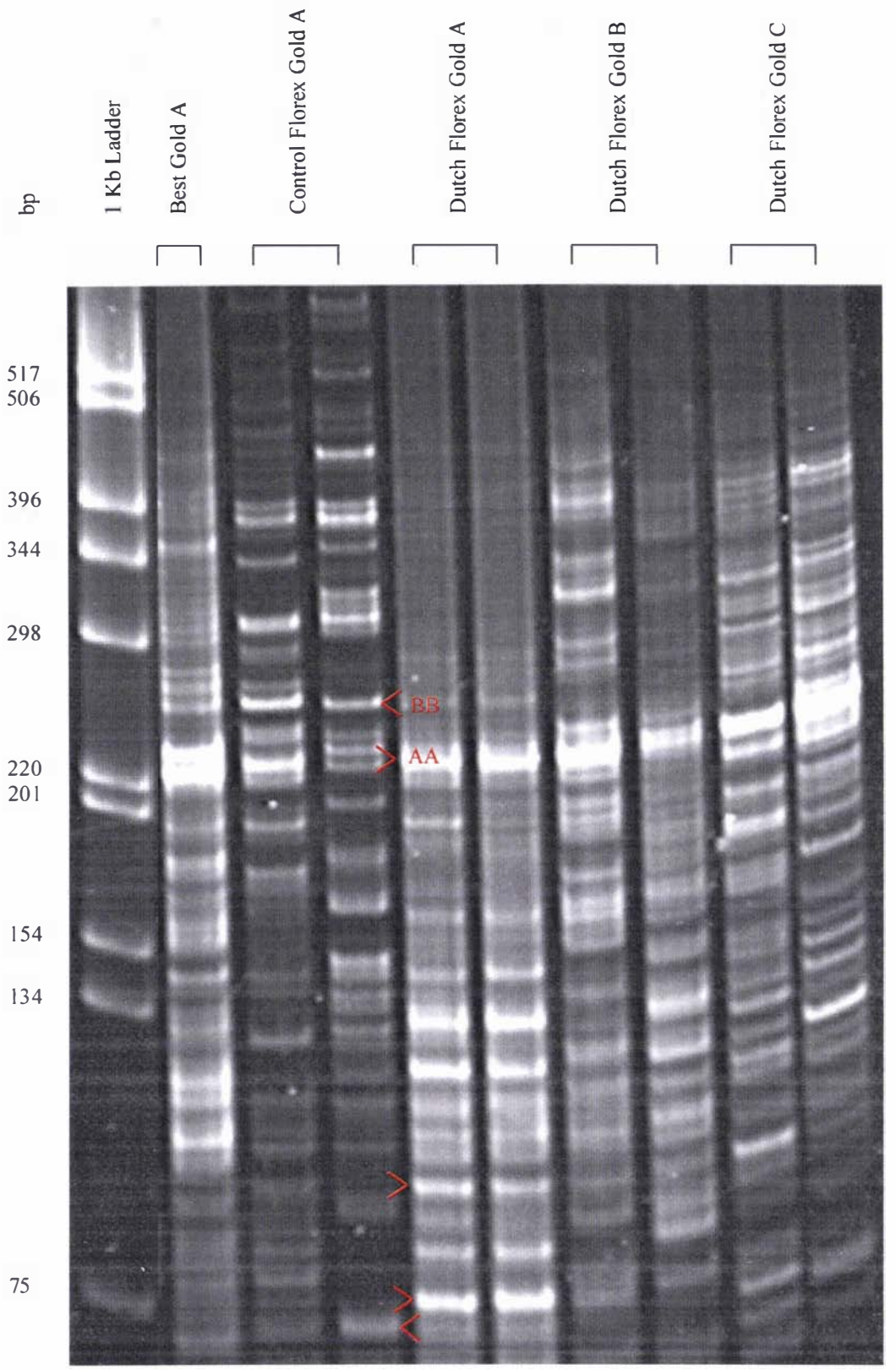


Figure 4.29: Polyacrylamide gel of PCR amplification of biotinylated cDNA fragments of the selective primers *Taq* I+AG and TT+GGT. A 1 Kb DNA Ladder was included in the polyacrylamide gel. PCR amplifications were performed on one replicate of sprouting Best Gold and control Florex Gold and three replicates of Dutch Florex Gold tuber shoots. Differentially expressed cDNA fragments are labelled (> or <) and bands excised.

Table 4.2: Sequence similarity of cDNA AFLP fragments to sequences on the NCBI BLAST server. Nucl = nucleotide BLAST search. EST = EST BLAST search.

Isolated fragment	Fragment derived from	Estimated bp length	Change in bushy	Sequence similarity	Blast score
D	*Taq* I+AG and TT+AC	154	Up	No significant similarities found	
E	*Taq* I+AG and TT+AC	110	Up	No significant similarities found	
F	*Taq* I+AG and TT+AG	154	Down	No significant similarities found	
G	*Taq* I+TCG and TT+CA	110	Up	No significant similarities found	
H	*Taq* I+TAA and TT+CAT	154	Up	No significant similarities found	
I	*Taq* I+CGA and TT+CAT	154	Down	(Nucl) *Chloranthus spicatus* 18S ribosomal RNA (CLURRE04) (Nucl) *Calycanthus occidentalis* 18S ribosomal RNA (CLYRRE04) (Nucl) *Lillium superbum* 18S ribosomal RNA (AF206952) (EST) Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues *Solanum* (BQ504859) (EST) *Capsicum annuum* cDNA (BM066181) (EST) Tomato flower, anthesis *Lycopersicon esculentum* cDNA (BI934049) (EST) Tomato flower, buds 0 to 3 mm *Lycopersicon esculentum* cDNA (BI923630)	4e-07 4e-07 4e-07 4e-07 4e-07 4e-07 4e-07
J	*Taq* I+CGA and TT+CAT	125	Up	No significant similarities found	

Isolated fragment	Fragment derived from	Estimated bp length	Change in bushy	Sequence similarity	Blast score
K	*Taq* I+CGT and TT+CAT	160	Up	No significant similarities found	
L	*Taq* I+CG and TT+AGC	300	Up	No significant similarities found	
M	*Taq* I+CG and TT+AGC	200	Up	No significant similarities found	
N	*Taq* I+AG and TT+AGC	110	Up	No significant similarities found	
O	*Taq* I+CG and TT+CAT	500	Down	(Nucl) *Oryza sativa* 60S ribosomal protein L21 (RPL21) mRNA (AF093630)	2e-04
				(EST) Peach developing fruit mesocarp *Prunus persica* (BU040083)	1e-11
				(EST) Populus petioles cDNA library *Populus tremula* (BU888779)	2e-06
				(EST) Populus bark cDNA library (BU816240)	2e-06
				(EST) Populus flower cDNA library (BI120120)	2e-06
				(EST) Sorghum halepense rhizome cDNA library (BQ656086)	9e-06
				(EST) Barley Leaf Library *Hordeum vulgare* cDNA clone (BE421378)	9e-06
				(EST) *Sorghum halepense* cDNA (AI724667)	9e-06
P	*Taq* I+CG and TT+CAT	320	Up	No significant similarities found	
Q	*Taq* I+AG and TT+AAG	200	Up	No significant similarities found	
R	*Taq* I+AG and TT+AAG	110	Up	No significant similarities found	
S	*Taq* I+AG and TT+AGA	110	Up	No significant similarities found	
T	*Taq* I+AG and TT+AGA	200	Up	No significant similarities found	

Isolated fragment	Fragment derived from	Estimated bp length	Change in bushy	Sequence similarity	Blast score
U	*Taq* I+AG and TT+AGA	220	Up	No significant similarities found	
V	*Taq* I+AG and TT+CCT	110	Up	No significant similarities found	
W	*Taq* I+AG and TT+CCT	130	Up	No significant similarities found	
X	*Taq* I+AG and TT+GGA	110	Up	No significant similarities found	
Y	*Taq* I+AG and TT+CGT	110	Up	No significant similarities found	
Z	*Taq* I+AG and TT+GCC	125	Down	No significant similarities found	
AA	*Taq* I+AG and TT+GGT	200	Up	No significant similarities found	
BB	*Taq* I+AG and TT+GGT	250	Down	No significant similarities found	

4.4 Discussion

4.4.1 Differences in DNA methylation patterns between bushy and non-bushy Florex Gold as determined by genomic DNA AFLP

The use of methylation-sensitive restriction enzymes during AFLP permits a wide sampling of the genome, whilst targeting the detection of polymorphisms specifically resulting from changes in methylation status (Matthes et al., 2001). Methylation-sensitive AFLP allowed the detection of putative DNA methylation polymorphic regions between bushy and non-bushy Florex Gold plants and between Florex Gold and Best Gold plants. Furthermore, any bands of interest could be excised from the gel, sequenced and characterised (Matthes et al., 2001). Three bands corresponding to putative DNA methylation polymorphic regions between bushy and non-bushy plants were excised, re-amplified and sequenced. Band A appeared absent in control Florex Gold and Best Gold. Bands B and C appeared present in only control Florex Gold. The variation in DNA methylation in Band B and C occurred between control Florex Gold, which had been micropropagated once, 12 years ago and both bushy and non-bushy Florex Gold, which had been successively micropropagated from T1 or T2 tubers. The DNA methylation polymorphism in Band A was absent in both control Florex Gold and Best Gold. Control Florex Gold has been micropropagated once and Best Gold has not been previously micropropagated. This may suggest that the DNA methylation polymorphisms A, B and C, were associated with the tissue culture process.

DNA was sequenced without cloning in this research. Other researchers using genomic DNA AFLP and cDNA AFLP fingerprinting techniques use cloning methods (Bachem et al., 1996; Bachem et al., 2001; Xiong et al., 1999; Suarez et al., 2000; Matthes et al., 2001). In New Zealand permission to clone PCR products requires approval from the Environmental Risk Management Authority (ERMA). A PCR-based method (Section 4.2.1.10) was used to obtain sequence information and therefore cloning was not necessary. The sequences represent DNA of unknown function according to a BLAST alignment search of similarity to DNA in public databases (Table 4.1). The differences in methylation profile may be in DNA with as yet unknown function. As sequence information is growing rapidly, information may become available in the future. Conversely, the differences in methylation profile may be in highly repetitive non-coding regions of the DNA (Matthes et al., 2001).

The role of DNA methylation in the regulation of gene expression has been extensively investigated in mantled oil palm derived from somatic embryogenesis (Jaligot et al., 2000; Rival et al., 2000; Matthes et al., 2001). Micropropagated mantled oil palm is a syndrome comparable to the bushy syndrome in *Zantedeschia*. Matthes et al. (2001) found that no polymorphism was consistently different between normal and abnormal clones in the oil palm samples tested by AFLP with cytosine methylation sensitive enzymes. Matthes et al. (2001) suggested that, whilst this method was an effective way of detecting variation in tissue culture-derived plants, different approaches are required to identify the causal basis of the mantled fruit abnormality.

Previous analyses of normal and abnormal oil palm clones using biochemical and cytogenetic markers did not reveal a simple basis to the production of abnormal clones (Rival et al., 1998a; Rival et al., 1998b; Jaligot et al., 2000). Using the RAPD's DNA profiling technique, Rival et al. (1998a) could not detect polymorphisms between normal and abnormal palms, although a total of 8,900 markers were scored. However, levels of 5mdC, measured by HPLC, have shown that global methylation in leaf DNA of abnormal regenerants was significantly lower than in their normal counterparts (Jaligot et al., 2000). This effect was variable in intensity from one genotype to another. The variant regenerants within each group of clonal progeny studied, were able to be distinguished from the normal ones. The methylation rate data, however, did not always allow discrimination between normal regenerants of one clonal group and abnormal regenerants of another. This inherent genotype-dependent variability associated with global DNA methylation rates made the demonstration of a direct cause and effect relationship between DNA methylation and the mantle flowering abnormality difficult. Measurement of adult palm leaf global DNA methylation rates also did not provide a predictive individual test for the early detection of the mantled abnormality (Jaligot et al., 2000).

When investigating differences in DNA methylation patterns, DNA samples are generally digested with isoschizomers (Xiong et al., 1999; Matthes et al., 2001; Peraza-Echeverria et al., 2001). Isoschizomers are restriction enzymes that have the same recognition sequence but may not share the same cut site due to cytosine methylation. For example, the isoschizomers *Hpa* II and *Msp* I, both cut the sequence C/CGG but *Hpa* II does not cleave ^{m}CCGG and C^{m}CGG (Xiong et al., 1999; Matthes et al., 2001; Peraza-Echeverria et al., 2001). The comparison of the different fingerprints obtained

with methylation sensitive isoschizomers, provide proof of methylation differences (Donini et al., 1997). *Hpa* II and *Eco* RI were chosen for this research as Matthes et al. (2001) and Xiong et al. (1999) found that a higher frequency of polymorphic fragments was obtained using the template generated with *Hpa* II/*Eco* RI in oil palm and rice respectively. A second isoschizomer, for example *Msp* I, was not chosen in this research, as preliminary investigations were concerned with whether differences in methylation patterns between bushy and non-bushy plants could explain the bushy syndrome. After there was evidence that differences in methylation patterns were present between bushy and non-bushy Florex Gold, PCR primers were designed from one DNA methylation polymorphic region.

PCR amplification using PCR primers designed from the consensus sequence of band B, revealed that the DNA methylation polymorphic band B was the same base pair size and present in all DNA samples investigated (Figure 4.9). Therefore, it can be inferred that no changes in the DNA sequence of band B had occurred. *Hpa* II is unable to cleave DNA at the relevant restriction site if one or both cytosines are fully methylated (Xiong et al., 1999). The absence of band B in bushy tubers B and F, Dutch tubers E and F, as well as few-eyed tubers A and B and Best Gold tubers B and C, suggests that the DNA methylation polymorphism band B was hypermethylated. There is evidence that cytosine methylation inhibits gene expression (Kaeppler and Phillips, 1993a). Therefore the gene(s) encoded by the DNA in band B, if present in coding regions of the DNA, may have decreased transcription in both bushy and Dutch bushy tubers, as well as few-eyed and Best Gold tubers.

4.4.2 Differences in gene expression patterns between bushy and non-bushy Florex Gold as determined by cDNA AFLP

Using the cDNA-AFLP technique, twenty primer combinations were investigated. More than 500 bands were detected and over 80 of bands were differentially amplified between bushy and non-bushy Florex Gold. A subset of 25 were re-amplified and sequenced. Sequence alignment was compared on the nucleotide and EST databases of NCBI and revealed mostly genes of unknown biological function (Table 4.2). An arbitrary E-value cut off of $5e^{-4}$ was used. This was based on the E-value cut off used by Ditt et al. (2001) during cDNA AFLP investigations into plant gene expression in response to *A. tumefaciens* infection. Band I showed sequence similarity to 18S rRNA

in *Chloranthus spicatus* (CLURRE04), *Calycanthus occidentalis* (CLYRRE04) and *Lillium superbum* (AF206952). Band I appeared down regulated in bushy Florex Gold. Band O showed sequence similarity to the 60S ribosomal protein in *Oryza sativa* (AF093630). Band O appeared down regulated in bushy Florex Gold.

McClelland et al. (1995) hypothesised that changes in gene expression due to the 'treatment' of a cell will usually affect tens or hundreds of genes. Therefore, the bushy phenotype is most likely the result of changes in gene expression, including changes in DNA methylation, in tens or hundreds of genes. These genes may include rRNA genes. The sequences encoding rRNA in eukaryotes are highly conserved (Eckenrode et al., 1985; Harding, 1991b). There are, however, reports of changes in gene expression of rRNA genes in plants such as micro-propagated potato (Harding, 1994), flax genotrophs (Schneeberger and Cullis, 1991; Cullis et al., 1999) and 'mantled' oil palm (Matthes et al., 2001). Harding (1991a) reported a change in the rRNA gene locus of potato (*Solanum tuberosum* L.) plants recovered from cultures maintained on mannitol-supplemented medium. Potatoes cultured on mannitol-supplemented medium, which is commonly used to prolong the transfer period of cultures in slow growing conditions, have stunted stems, shortened internodes, and vitrified leaves with a reduced surface area, which was attributed to hypermethylation (Harding, 1994). Several flax varieties have been shown to undergo environmentally induced heritable changes resulting in stable lines termed genotrophs. Small genotrophs had reduced plant weight, plant height, DNA content, copy number of rDNA genes, and variation in the 5S rRNA gene (Schneeberger and Cullis, 1991). Some genotrophs also have changes in the number of genes coding for the large rRNAs (Cullis et al., 1999). Matthes et al. (2001) reported a DNA methylation polymorphism with high sequence identity to the 25S ribosomal gene in 'mantled' oil palm. *A. thaliana* mutants with reduced rRNA methylation have also been reported. DNA methylation mutants of *A. thaliana*, *ddm1-1/ddm1-1* and *ddm1-2/ddm1-2*, contain severely hypomethylated 18S, 5.8S and 25S ribosomal genes (Vongs et al., 1993). Therefore, there is evidence that down regulation of rRNA genes (60S and 18S) may be two of those genes that play a role in the expression of the bushy phenotype.

A major advantage of the cDNA AFLP technique over the genomic DNA AFLP technique, is the determination of differentially expressed DNA in coding regions, as the cDNA is synthesised from RNA. Differences in methylation profile are often in

highly repetitive non-coding regions of the DNA (Matthes et al., 2001). In this study, genomic DNA AFLP combined with a high-resolution quantitative separation technique, usually required that an AFLP band was unique to a bushy or non-bushy parent. In cDNA AFLP, band intensity is a direct function of template concentration (Kuhn, 2001), and permits limited conclusions to be drawn on the quantitative relationships of individual bands (Bachem et al., 1996; Kuhn, 2001).

The cDNA AFLP technique has also proved to be a highly effective tool for displaying genes that are differentially expressed during the life cycle of an organism (Bachem et al., 1996; Suarez et al., 2000). Levels of specific gene expression vary at different stages of plant growth and development and in different tissues (Brown, 1989). In this research, sprouting tubers of the same age, and held in the same storage conditions were used. The cDNA was also quantified at two steps in the cDNA AFLP procedure: the double stranded cDNA fraction prior to digestion of cDNA and ligation of *Taq* I adapter, and the biotinylated cDNA fraction prior to PCR amplification. Any differences in the initial concentrations of the cDNA products are preserved as a ratio of intensities in the final fingerprint (McClelland et al., 1995). McClelland et al. (1995) reported that the ratio of starting materials between samples is maintained even when the number of cycles is sufficient to allow the PCR reaction to saturate. This is because the number of doublings needed to reach saturation are almost completely controlled by the invariant products that make up the majority of the fingerprint (McClelland et al., 1995).

Further advantages of the cDNA AFLP and genomic DNA AFLP techniques, is that they can be performed in the absence of DNA sequence data, in contrast to microarray analysis (Ditt et al., 2001). The cDNA-AFLP analysis also required a relatively low amount of starting material and small amounts of RNA (2 μg) when compared with the amounts needed for cDNA microarrays (50 to 200 μg) (Kuhn, 2001; Ditt et al., 2001). The use of a pre-amplification step after the production of the primary template during cDNA AFLP, allows, for practical purposes, virtually unlimited amounts of secondary template to be generated (Bachem et al., 1996). The pre-amplification step was not included in the present study as it was a preliminary look at differences in gene expression and only a small amount or primary template was needed.

The cDNA AFLP technique has long primers so more stringent annealing temperatures can be used. This increases the quality of individual amplification products in comparison to other RNA fingerprinting techniques (Bachem et al., 1996; Ditt et al., 2001). In cDNA AFLP primarily perfect matches between primer and substrate are tolerated in the amplification reaction. Rare instances of mismatch have been observed in the cases where transcript levels are extremely high (Bachem et al., 1996). In other RNA fingerprinting techniques, abundant cDNAs with poor matches to primers used, may outperform rarer species with perfect matches, during the course of PCR amplification (Bachem et al., 1996).

A significant disadvantage of the cDNA-AFLP method is the requirement for appropriate restriction sites on the cDNA molecules (Kuhn, 2001). In this preliminary investigation *Taq* I was used. The target sequence of *Taq* I is 5'-T/CGA-3', and the expected frequency of this sequence is on the order of 1/256, therefore most cDNA species should be digested into fragments (Habu et al., 1997; Xiong et al., 1999). To visualise every cDNA present in a plant cell with high probability, cDNA AFLP experiments using different enzymes would be necessary (Kuhn, 2001).

Replicates of PCR amplification during this research were not always reproducible, as cDNA replicates did not consistently produce the same banding pattern as seen in the polyacrylamide gels (Figures 4.10 to 4.29). The PCR protocol was optimised for *Zantedeschia*, but perhaps needs to be optimised further. Numerous steps were taken to optimise the PCR, including investigations of different cycle number, annealing temperature, two or three selective nucleotide primer combinations and cDNA template concentration. Differences in banding patterns between bushy and non-bushy Florex Gold were only investigated further if the band of interest was present in all replicates of a phenotype and differentially expressed in the other phenotype. The results of the two, or three in the case of Dutch bushy Florex Gold, independent cDNA samples were highly reproducible, as indicated by the reproducible banding patterns of analysis of two (or three) sets of cDNA samples per selection. Differences in banding patterns were also reproducible as determined when a subset of cDNA was investigated. For example banding patterns seen in the polyacrylamide gel of the selective primers *Taq* I+CGA and TT+CAT (Figure 4.15) were present in the polyacrylamide gel of the selective primers *Taq* I+CG and TT+CA (Figure 4.13). Kuhn (2001) found that cDNA-AFLP banding patterns are highly reproducible and almost free from false positives.

Some sequences from the re-amplified differentially expressed bands were very short, with a base pair size of 50 to 75 bp, but ranged up to 500 bp. This is similar to the range of sizes found by Gellatly et al. (2001), who found cDNA fragments that ranged in size from 100 to 400 bp. The shorter sequences proved hard to "call" on the ABI Prism 377-64 DNA Sequencer and some manual editing was required. Sequence data, however, contained the appropriate terminal sequences corresponding to the two or three selective base extensions used in the PCR. It was also difficult to make a consensus sequence from sequences obtained using both *Taq* I and TT primers, as the 3' overlap from each sequence direction was small. Short sequence data also proved difficult to characterize by nucleotide database searches. Sequence data was also compared to EST libraries on the public databases. ESTs are generated by sequencing random cDNA clones from libraries obtained from different tissues at various stages of an organism's development (Suarez et al., 2000). There were limited sequences (42) of *Zantedeschia* on the NCBI public database in December 2002, for direct comparison of the cDNA sequences obtained. *Zantedeschia* is a member of the Araceae family and there were a relatively small number of sequences (672) of members in the Araceae family in December 2002.

4.4.3 Future research

The cDNA AFLP technique was an effective way of detecting differential gene expression between bushy and non-bushy plants. Bushiness, however, may be a complex mechanism involving many different genes. Therefore, it would be beneficial to investigate more differentially expressed genes using cDNA AFLP, to gain a detailed understanding of the mechanisms underlying the bushy syndrome. Future research will also be needed to validate the differential expression patterns using sequence-specific primers and quantified cDNA in real time PCR analysis. Future research will also need to consider the effects of post-transcriptional events. All differential display techniques provide information about steady state mRNA concentrations and cannot detect post-transcriptional events, known to play a role in plant gene regulation, such as changes in mRNA stability, translation efficiency or protein (de)phosphorylation (Kuhn, 2001).

Chapter Five: Conclusions

In this study, a multiple-faceted approach, utilising environmental, physiological and molecular techniques, was taken to investigate the variables associated with bushiness in *Zantedeschia* Florex Gold. The criteria established for a plant to be considered bushy included the plant having a squat tuber with multiple nodes and many eyes, multiple primary shoots (≥5) that had reduced stem length and no flowers or flowers with reduced weight and stem length (<30 cm). Bushiness is a potentially serious problem in the commercial *Zantedeschia* industry, as previously high quality cultivars, including Florex Gold, were periodically displaying bushy symptoms that resulted in reduced financial return. If tubers of Florex Gold continue to produce bushy plants with multiple shoots, there will be a significant impact on New Zealand tuber exports.

Predisposing factors to the bushy syndrome were determined, although no one factor emerged as the sole cause of the syndrome. Predisposing factors to bushiness included the *Z. elliottiana* parentage, the Florex Gold genotype, the selection of tubers for initiation into tissue culture with multiple eyes, or tubers themselves recently from tissue culture. A variable temperature growing environment (screen house), a shortened storage time (11 weeks) and plant location accentuated individual bushy characteristics (including reduced flower weight, stem and spathe length) in those cultivars prone to the bushy syndrome.

Anecdotal suggestions favoured cytokinins as the cause of the bushy syndrome. However, the use of MS media supplemented with the standard cytokinin concentration (4.4 μM 6-BAP) during commercial micropropagation did not predispose plants to bushiness. The use of elevated cytokinin during micropropagation did not produce plants exhibiting the bushy syndrome. However, high levels of TDZ, but not 6-BAP, were shown to affect flower spathe length and primary shoot number. However, Florex Gold, when compared to a range of non-bushy *Zantedeschia* cultivars, displayed increased sensitivity to 6-BAP during micropropagation as assessed by a root length bioassay.

No role for endogenous cytokinin concentration or profile in the expression of the bushy syndrome was established. There were no measurable differences in the total cytokinin

levels between bushy and control Florex Gold tuber samples, whether dormant or sprouting. Therefore, the sum of biologically active cytokinins was not directly responsible for the expression of the bushy syndrome. An eight-fold higher concentration of the storage *O*-glucoside cytokinins was found in bushy compared to the control Florex Gold sprouting T1 tubers. However, the higher concentration of *O*-glucosides was detected in the New Zealand bushy tubers, but not in the Dutch Florex Gold tubers. Therefore, the high levels of *O*-glucosides do not seem to account for the expression of the bushy syndrome.

There were differences in DNA methylation patterns and differential gene expression levels between the recently micropropagated bushy Florex Gold and the control Florex Gold selection that had been micropropagated once, 12 years previously. This may indicate that somaclonal variation is an underlying cause of the bushy syndrome. Three putative DNA methylation polymorphic regions were detected between bushy and control Florex Gold. Sequence data of the DNA methylation polymorphic regions were not similar to any gene(s) currently listed on the NCBI database. As sequence information is rapidly growing, information may become available in the future. Conversely, the differences in methylation profile may be in highly repetitive non-coding regions of the DNA. Differential gene expression levels, in coding regions of the DNA, found 60S and 18S rRNA down-regulated in bushy Florex Gold compared with the control Florex Gold. The underlying cause of the bushy syndrome is most likely the result of changes in gene expression including changes in DNA methylation in tens or hundreds of genes including the 60S and 18S rRNA genes.

A preliminary investigation of differential gene expression utilised cDNA AFLP. The cDNA AFLP technique was an effective way of detecting differential gene expression between bushy and non-bushy plants. Bushiness, however, may be a complex mechanism involving many different genes. The extent to which changes in gene expression, including changes in DNA methylation, are associated with bushiness will only be revealed by investigation of more primer combinations and further genomic sequencing of other differentially expressed genes. Limited tuber material was available to investigate differential gene expression, as this was an additional piece of research completed at the end of the project. It would be beneficial to extend this investigation to a range of bushy and non-bushy selections and to compare bushy and non-bushy selections immediately after micropropagation. A further comparison of

bushy and non-bushy sprouting T1 tubers just prior to their second growing season, when the full range of bushy symptoms is first seen, would be appropriate.

This research has validated many of the current micropropagation protocols already used by Multiflora Laboratories Limited in *Zantedeschia* Florex Gold production. These protocols include tissue culturing Florex Gold on MS media containing low levels of 6-BAP, which would reduce any possible carry-over effect of cytokinins on bushy characteristics. This research also verified the anecdotal evidence that Florex Gold was more sensitive to the cytokinins used during tissue culture.

Obviously, the *Zantedeschia* tubers chosen for micropropagation need to come from plants displaying those characteristics considered desirable by commercial flower producers, including long flower stem and spathe length and desirable flower colour and shape. However, considering the bushy syndrome may be a response to a complex set of factors, avoiding any of the predisposing factors determined in the research is advisable. Consequently, it is recommended that *Zantedeschia* Florex Gold tuber material chosen for micropropagation is a deep, apically dominant tuber, with few eyes and nodes. Mature tubers should be chosen for micropropagation, especially not themselves recently from tissue culture. Only MS media containing low levels of the cytokinin, 6-BAP, should be used for micropropagation and TDZ should be avoided. Growing conditions need to be optimised for each step in the *Zantedeschia* life cycle, and it appears that growers need to choose to either optimise tuber weight or flower stem, spathe length and flower weight. The practise of exporting tubers overseas to be grown in the natural rest period (forcing) of the tuber needs to be reviewed especially with regard to those cultivars predisposed to the bushy syndrome.

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APPENDIX A

Composition of potting mix

50/50 Bark/Pumice

	gm per m^3	per 200L
Osmocote (8 to 9 month) (16-3.5-10+1.2mg)	4500	900
Dolomite	5000	1000
Superphosphate	1000	200
Calcium ammonium nitrate	200	40
Fritted trace elements	200	40
(Boron 0.875%, Copper 1.7%, Iron 3.35%, Manganese 1.7%, Molybdenum 0.023%, Zinc 0.6%)		
Iron (coated iron 5 month)	300	60
Potassium sulphate	500	100
Terrozole	100	20

General characteristics of tubers of different size grades (Welsh, 1994).

Tuber Size Grade (minimum diameter, cm)	General Comments	Approximate flower stem length (cm)	Approximate weight after curing (g)
5+	Generally, the top tuber grade offered for sale. Only a small percentage of tubers would reach this size in two seasons from seed or tissue culture.	60-100	50-90
4-5	Most cultivars will flower without gibberellin treatment at this size. It is often the minimum size for export tubers.	50-60	25-50
3-4	Gibberellin must be used for a high percentage of these tubers to flower.	40-50	15-25
2-3	If treated with gibberellin, posy grade flowers can be expected with a 4+ cm diameter tuber at the end of the season.	20-40	5-15
1-2	Usually seen as a tuber to be gown on, although a mini-posy flower may result following gibberellin treatment.	10-20	2.5-5

APPENDIX B

Standard Curve for RIA

ZR/iPA solutions:

Stock = 1x 10^{-3} M

Solution A 10 µL stock + 990 µL 50% methanol

Solution B 30 µL solution A + 2970 µL 50% methanol

Solution C 200 µL solution B + 1800 µL 50% methanol

Standard Curve

Each tube is repeated in triplicate = total of 51 or 60 tubes

Tube #	Solution	Amount (µL)	50% methanol (µL)	pmole
1	C	0	350	0.0
2	C	0	350	0.0
3	C	5	345	0.05
4	C	10	340	0.1
5	C	20	330	0.2
6	C	40	310	0.4
7	C	60	290	0.6
8	C	80	270	0.8
9	C	100	250	1.0
10	B	20	330	2.0
11	B	30	320	3.0
12	B	40	310	4.0
13	B	50	300	5.0
14	B	100	250	10.0
15	B	250	100	25.0
16	B	350	0	35.0
17	A	10	340	100.0
[18	A	35	315	350.0]
[19	A	70	280	700.0]
[20	A	100	250	1000.0]

The cross-reactivities of two monoclonal antibodies raised against ZR with selected cytokinins (Lewis, 1994).

	% Cross reactivity	
Cytokinin	Clone 16	Clone 12
ZR	100	2.4
Z	34	1.7
DZ	14	-
DZR	30	-
Z9G	39	-
DZ9G	8	-
Z7G	0	-
ZOG	0	-
ZROG	0	-
DZOG	0	-
DZROG	0	-
iPA	0.9	100
iP	0	93
iP9G	-	131

- = not tested

APPENDIX C

Oligonucleotides used in genomic DNA AFLP

Oligonucleotide group Oligonucleotide name	Oligonucleotide sequence (bold type denotes selective sequence)
Adapters	
Eco RI Adapter I	5' CTCGTAGACTGCGTACC 3'
Eco RI Adapter II	5' AATTGGTACGCAGTCTAC 3'
Hpa II Adapter I	5' GATCATGAGTCCTGCT 3'
Hpa II Adapter II	5' CGAGCAGGACTCATGA 3'
Pre-selective amplification	
Eco RI Primer	
Eco RI+A	5' GACTGCGTACCAATTC**A** 3'
Hpa II Primer	
Hpa II+G	5' ATCATGAGTCCTGCTCG**G** 3'
Selective amplification	
Eco RI Primers	
Eco RI+AAG	5' GACTGCGTACCAATTC**AAG** 3'
Eco RI+ACC	5' GACTGCGTACCAATTC**ACC** 3'
Eco RI+ACG	5' GACTGCGTACCAATTC**ACG** 3'
Eco RI+AGA	5' GACTGCGTACCAATTC**AGA** 3'
Eco RI+AGG	5' GACTGCGTACCAATTC**AGG** 3'
Eco RI+ATA	5' GACTGCGTACCAATTC**ATA** 3'
Hpa II Primer	
Hpa II+TCAA	5' CATGAGTCCTGCTCGG**TCAA** 3'
Polymorphic marker primers for band B	
Eco RI primer	5' GTACCAATTCACCGCTCAC 3'
Hpa II primer	5' CTGCTCGGTCAACTTGCTCC 3'

Oligonucleotides used in cDNA AFLP

Oligonucleotide group Oligonucleotide name	Oligonucleotide sequence (bold type denotes selective sequence)
Biotinylated-oligo(dT) primer	5' GCTGCAGACTTGTAGTTTTTTTTTTTTTVV 3'
Adapters	
Taq I Adapter 1	5' CCGGATGAGTCCTGAC 3'
Taq I Adapter 2	5' CGGTCAGGACTCATCCGG 3'
Selective amplification	
Taq I Primers	
Taq I+AG	5' CCGGATGAGTCCTGACCGA**AG** 3'
Taq I+CG	5' CCGGATGAGTCCTGACCGA**CG** 3'
TT Primers	
TT+AG	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**AG** 3'
TT+CA	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**CA** 3'
TT+AAG	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**AAG** 3'
TT+ACC	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**ACC** 3'
TT+AGA	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**AGA** 3'
TT+AGC	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**AGC** 3'
TT+CAC	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**CAC** 3'
TT+CAT	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**CAT** 3'
TT+CCT	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**CCT** 3'
TT+CGT	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**CGT** 3'
TT+GCC	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**GCC** 3'
TT+GGA	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**GGA** 3'
TT+GGT	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT**GGT** 3'
Re-amplification	
Taq I 0 Primer	5' CCGGATGAGTCCTGACCGA 3'
TT Primer 1	5' GCTGCAGACTTGTAGTTTTTTTTTTTTT 3'

APPENDIX D

Genomic DNA AFLP sequences of excised bands

A 5' *Eco* RI -*Hpa* II 3'

```
  1 AAGNANNGGC TTNCCTNNNC CTGAATCTCA NNAACCCGGA GNACGANATC ATGGCGANAT
 61 CNGAAGGGNA NNNGCCTTNT CGATTGATNC CTNCGANCGG GACTNNNNTA ANATGCTTNT
121 GCATNANGCT ATANCNGTGT TGATTCCTGT NCNNTAGCAC ATGNANCNGA TATCAANCTG
181 GAAATNAAGG NNCTGTTGCC ATAAGTGCAT ANTNTGTCGA ACAAATANNG ATTTGTCGNG
241 GTNGNAATNA GNTNNNANNC CTTTCTANCG ATGTAGATGA NCTACATGNG AGGGNCAATN
301 GTGTTNTAAT TTGTGNCCCA CCCTNACCGT TGTCCNTTTT GTTCNNTNGA CNNCCNTGTN
361 TTTTTTTCCA GNGGNNTAGG GGAGTTNANA AAAGGTNACN CGTNANCCCC TCTTTTNTTT
421 CTTGNTATAC NTNAGNTANA AAGCNCCCTT CGGGATNATG CAGGNGATNA AGGGTNGATT
481 NNTGNGTTTA NCCTNGCCNG ACTCNGAT
```

A 5' *Hpa* II -*Eco* RI 3'

```
  1 ANGCNCTGCT ATCCNCCNCA NCTCNNCGGC GTNTTATTGA TNCAANTGAC ATNAATTCAN
 61 GCATTNNTNT GNNNCTCTCT TCGTGNCTNG GTACTTNTCN ACCCCNCAGC ANCCTGAGAN
121 ANANCACGAT GGAGTACACA ATNTNTGCAA NTGGTNGTAN GGNGGGGTNG AANAATTNAA
181 CTCATTNTTN CCTAGCCCCT NANTTNCTTN NNACCNCTGN NATNAATCGC NTTNNGTNTC
241 TTNNCNAAAA GNCAGTATTN ACTGTTTTGT NAANCCCNAG NGNAATCTAA NCTCCTNCTT
301 TCTTNATAAT TTTCTGANGA AGGANNGCTT CACTGGGTGT TCAGAAAATT ANNTAGAATG
361 TNCTCTCTCC TNCCAAAAAA ATGATTGGCG ATTNTTGNCA ACNCCCCCNG ATGANGCCTT
421 NNTTCTNTTG GCNAAAATCG TAGACATGAT NGTGAAACNT GCNNTTCGGG GNGCTCTACA
481 ANTNTGAANC CNNTNCC
```

B 5' *Eco* RI -*Hpa* II 3'

```
  1 AAAGAAGGAT TCCATCCTTG TGCTATCACT TCGTTTCTTT AAGATGCTAG GAGCGAACCC
 61 ATTCATAGAT CTTCGTTTTC TACTTGCTTT TGAGTTTCCC CAGTTCGGTG GAGATCTTTA
121 CCTAATTCAC TACTCTACGT AGATTGGCTT CCGACAGCTC CNAAGAGATC CCGCCTTGCT
181 TGCCTGTGAT ACTAACCATA GGGGCCGAGC CCAGCCTCAT GTGCCGATGT AGTTTTCGAC
241 TTTCTCGAAT TGCCCTCAGA TGCCCATAGT TAATTTAGTT AGGTGAAAAA ACTGGAGCAA
301 GTTGACCGAG CAGGACTCAT GAA
```

B 5' *Hpa* II -*Eco* RI 3'

```
  1 AATNAACNTA NTGTCTNTNT CACTATGGGC ATCTGAGGGC AATTCGAAGC AAAGTCGAAA
 61 ACTACATCGG CACATGTAGG CTGGGCTCGG CCCCTATGGT TAGTATCACA GGCAAGCAAG
121 GCGGGATCTT TCGGAGCTGT CGGAAGCCAA TCTAGTAGAG TAGTGAATTA GGTAAAGATC
181 TCCACCGAAC TGGGGAAACT CAAAAGCAAG TAGAAAAGAA GATCTATGAA TGGGTTCGCT
241 CCTAGCATCT TAAAGAAACG AAGTGATAGC ACAATGATCT ACTTACTTAC TAAGATTAGT
301 GATTAAGTGA GCGGTGAATT GGTACNCAN
```

B Consensus sequence

```
  1 AGTCCTGCTC GGTCAACTTG CTCCAGTTTT TTCACCTAAC TAAATTAACT ATGGGCATCT
 61 GAGGGCAATT CGAGAAAGTC GAAAACTACA TCGGCACATG AGGCTGGGCT CGGCCCCTAT
121 GGTTAGTATC ACAGGCAAGC AAGGCGGGAT CTTTcGGAGC TGTCGGAAGC CAATCTAGTA
181 GAGTAGTGAA TTAGGTAAAG ATCTCCACCG AACTGGGGAA ACTCAAAAGC AAGTAGAAAA
241 GAAGATCTAT GAATGGGTTC GCTCCTAGCA TCTTAAAGAA ACGAAGTGAT AGCACAATGA
301 TCTACTTACT TACTAAGATT AGTGATTAAG TGAGCGGTGA ATTGGTAC
```

C 5' *Eco* RI -*Hpa* II 3'

```
  1 AATCAAGGGN TTCCTTACCN NGNGGTGCTA NCNCTTCNTT CCTTNNGAGA TGNNANGATN
 61 NTNCNCNTNN CATAGATCNN TTGTGTTTCT ANGTNNCTTT GNTNTCNTCC CCNNNTTNGG
121 TGGTAGTATG CGTGTTACCT AATCTCNCTA CCTCTACGTA TATTGGNTCT CNGACAGCCC
181 CAANGAATCC CGCCCCGCTT GCCTGTNATN NTAACCATAN GGGCCGAGNC CTNCCTNATG
241 TGCCGATGTN GTCTTCAANT TTCNCGAATT GCCCTCAGAT GCCCATAGTT NATTTAATNA
301 GGTGAAAANN NTGGAGCACG TTGACCGAGG ACTNATAA
```

C 5' *Hpa* II -*Eco* RI 3'

```
  1 AAANNANGGA TATCTCTTAN NCTGNNCATN ATNATGGCNC TTCGCANAAA ANTNAAAAAC
 61 TNACATCGGC ACATGAGGCT GGGCTCGGCC CCTATGGTTA GAATCACAGG CNANCTAGGN
121 GGGATCTTTC GGAGCTGTCT GGAAGCCAAT CTACGAGAGT AGTGAATTAG GTAAAGATCT
181 CCACCGANNT GGGGAAACTC AANAGCNNNT NGAAAATAAG ATCTATGAAT GGGTTCGCTC
241 CTATCATCTT AAAGAAACGA AGTGATAGCA CTCATGATCT ACATTACTTA CTACTATTAT
301 TGCATATTGG TGGAGCGGTG NATTGGTACN CAGNTCA
```

cDNA AFLP sequences of selected excised bands

D 5' *Taq* I - TT 3'

```
  1 AGGCTNTCTC TCTNCAATAA GAGAGCCCCA CATCTTATCT CTATGAGTGA AAAAAAAA
```

E 5' *Taq* I - TT 3'

```
  1 GGGGCTNTCT NTCTCCAATG AGAGAGCCCC ACATCTTTAT CCTTATGAGT GAAAAAAAAA
 61 AAAA
```

F 5' *Taq* I - TT 3'

```
  1 GTTCCCGCGN CNGACTANTA TGTCCTTCGC CTCCATATCN TGGANTCNCN TTTGTATTCT
 61 TGGGCTTTGG CTAAAAAAAA AAAAA
```

G 5' *Taq* I - TT 3'

```
  1 GTATCTCTTT CATNGGAGAG CCCCACATCT TATCTNCTGA GTGAAAAAAA AAAAAA
```

H 5' *Taq* I - TT 3'

```
  1 CTTTCNCNAG GCNGNNGCNN GNGGNTTTCN NCCCCNTTTA GTGTNCNTTC CNGNGNGAAG
 61 GTTNGGGTNG CTGTGNATNA TGAAAAAAAA AA
```

I 5' *Taq* I - TT 3'

```
  1 CTTNNCCCGA TGCCGACCNG GGATCGGCGG NNTGTGTGCT TTTAGGACTC CGCCGGCACT
 61 CTTATGAAAA AAAAAAAAAA
```

J 5' *Taq* I - TT 3'

```
  1 CCCANTNAGA GAGCCCCACA TCTTATCNTA TGAAAAAAAA AAAA
```

J 5' TT - *Taq* I 3'

```
  1 TNCNCTATAN TGCAAAAGAT AGAACACACG GATGCCAACA TCCTCGTCGG TCAGGACTCA
 61 TCCGG
```

K 5' *Taq* I - TT 3'

```
  1 CNCCANGGGT GCGTGGGNGA ANTNTGANCC CNCGNNTTGC TCTCTNCAGN GGGATCGCNA
 61 GCTGGCNTNC TTCGGGTAAG NATTTTGNAT GAAAAAAAAA A
```

L 5' *Taq* I - TT 3'

```
  1 CCNCCTGCTG TTCGCNCTGN ACACNCTNNG GGNTGTGCGG GGGGGGTTAA CGGNNACCCG
 61 TCTGCGNNCG CCTNTGNTTG TGTNGCAGNN NNCTAGTAAG NNAATAAAGN TGCCATGGNN
121 NNTACTCTGC TCNTNATNNG GGANCTTGAG GATGGGGGTG NCCCANGTTA NNTGNGNNTN
181 GTGGNNATAA ATGGTGACGC ANGGTTATGC CTGCATCACT TTGAGAAGCT AAAAAAAAAA
241 AAA
```

M 5' *Taq* I - TT 3'

```
  1 CAGNGGTTNC CGTCATTTNN NTTGCCNNNA CCTCGGTAGG AGATGAACAG NNGGGNAGCC
 61 ATANGAGACA CCCNNCNNTA GNAAAACCTT GGGAGCCATC TCGTCCCCTA CAGACCAAAA
121 CCTGCTAGAA GGNCGGCTGC TAAAAAAAAA AAAA
```

N 5' *Taq* I - TT 3'

```
  1 GGGGCTGTCG TCTTTCAATG NNGAGAGCCC CGACATCTTT ATCGTNATGA GCTAAAAAAA
 61 AAAAAA
```

O 5' *Taq* I - TT 3'

```
  1 CAGGGAATCT GNNGGTCGTG TTGAANCCCC GATGCATGCC TTCCNTGNGC ANCTGACGAG
 61 TTCCGCCACA GGGAAGATNG NGAATGAATG AGTCTGAACC NNGNCGNNNA GGCNCGTGTG
121 ATGANNANNC TTATCNCCAA GACGTGTCAG NNGGAGGAGT CCCAAGAGCN GGGATTCNTG
181 GTATTNAGGC ACCACTCCNT GCACACTGTC ACGCCCATTC CTTATGATGT CCAGGAATGA
241 TNTCAAAGGA NGTNACTNNA TTGCTACCCT ATCCAANGTC TTACNGNGTG NTGTGGTCTN
301 GTNGACACCT TTNGGGTAAN GNTGAACTTG NAGTGCTNNN CGNCTGNCTT CTNTTCGATC
361 GTTGGANNNN NCTNATTCTT GANCTTTNGA CCAACTTGGN CTACATATGG NCTTTNCTTG
421 GTCGANTNGA TGAAAAAAAA AAAAA
```

O 5' TT - *Taq* I 3'

```
  1 GGNTCATGCT TTGNCCAAAG GCTCNAGAAT AAGGCTTAAC CAACNTATCA AATAAAAGAC
 61 AGTNANACAG ACACTTAAAG TACNTCCTTA ACCAAAANGT GNCACNCNAG AACACAAAAC
121 NCNNTAAGAT GNTGGATAGG GTAGCAATTT AGTAACCTCC TTTGANATCA TTAACGACAT
181 NNTAAGGAAT GGGCNTGACA NTCTCNANTG TGGTGCCTTC NACCATNAAC CCTGGCTTGG
241 GACCCTCTGG NTGCCCCTTG GGGCTAANGA TCTCACCNCG TGCCTTCNGC CTCACGCTTC
301 AACTCATCNT TCTTTATCTT CCTCTGNCGG AACTCCTCAA TGCANCTGGA AGGCAANANN
361 TGCTCAANAC NAACGTGGAT TCTCTTCCCA NTAATCCTGT TCCCNACCTG CTTGTTGACG
421 TCGGTCAGGA CTCATCCGG
```

P 5' *Taq* I - TT 3'

```
  1 CCCAAGGGTT CCACCGTCCT ACTACTCCTG CTCCTGCNGG ATGGAAAGGG ACNGATGTGG
 61 GATGTCCAAC TGATCNGAGN AGAGCTCCNT NNTNGAANAA CCANCTGCTN TCAATNATGN
121 GGCTTNNGNC CCGNNAGACA NTNGNGNCTC NATTAATNAN GNGTNGTTNC ACGAGTACTN
181 CNCNCCTTGT NNNNAGCGGC NGCTATCCCC CCAANTAATN GTNNNCTGNC CNCTNCTNTC
241 CTTNTNATCG AAGNGCTGNT TTNGATGAAA AAAAAAA
```

P 5' TT - *Taq* I 3'

```
  1 ACCGACNGNG GCACGCATAC ATTTATTTNT TGGGCTTATA GCCCTTGCTT ACAAGCACGT
 61 ACGGTACTAC ATCAAGTCAA GCACTTATTT ATTTATATGC CAATCTTACC NTGCGCGCAC
121 GTAAGCCTCA TATATGAGCA GCTGGTTGCT CTACATAGTC TAGCGTACTC TCCNATCAGN
181 AGGAGGNCAN CCNCCTTCCC NNCNNCCCTG CTGGAGGAGC AGGANTAGGA GGAGGACNAT
241 CNTGGNCCTG GCNGGTTNNT NAATATACTC ATCCGG
```

Q 5' *Taq* I - TT 3'

```
  1 ATTTTATTGC TGTTNNTGTC TCTTCCCGTT ACATCGCGCT AGCTAATCTA TATATCTACG
 61 CGTACTGTCC GGATCTTGTG AATTGTGTGA ACTTCTCATT TTGAATAAAG TGTAATATTT
121 GCTTAAAAAA AAAAAAA
```

R 5' *Taq* I - TT 3'

```
  1 AAGAGAGCCC CACATCTTAT CTTAAAAAAA AAAAAA
```

S 5' *Taq* I - TT 3'

```
  1 AAGAGGAGCC CCACATCTTA TCTAAAAAAA AAAAAA
```

S 5' TT - *Taq* I 3'

```
  1 AATGNTTATA TNTTAANAAN NATAGNAACA CACGTATGTC TCTAACANTC CCTCTTCGGT
 61 CAGGACTCAT CCGG
```

T 5' *Taq* I - TT 3'

```
  1 AAGGGNTTNC ATTGCGGGTT TGATNCCCTG NCNGGAAAGG CTTGATATCA TCTNAACGAN
 61 ATATCAACCN GCGNCCCCTN GTCCANGATC GTGTGGTGCA GNCATNACNA NTGAGACATN
121 GTCTCGNCTC GTGTAAACAC TCGTAANTNG TAAGTATGTT NGNCTTTCTC ATCTAAAAAA
181 AAAAA
```

U 5' *Taq* I - TT 3'

```
  1 TCCCAGGNGC ANAGNGATCA TATGTTCCCC AANACATCTC CACTGCAATG GTGTTATCCG
 61 TACCTTATGT GCGTCGCTAA TAATGGGAGG TATGGTCTAT GAGTCCACTC TCTTGTTATG
121 TGGACGTATG GAGTCGATAC GTNTGGGGCT CTGGCTCCAT CNTATTTGTC TAAAAAAAAA
181 A
```

V 5' *Taq* I - TT 3'

```
  1 TCTCCAATNA GAGGAGCCCC ATCTTNTTCT CCAAAAAAAA AAA
```

V 5' TT - *Taq* I 3'

```
  1 CNCTCCCAAA AGGATAGAAC ACACGNATGT CCCACCCTCC CTCCTTCGGT CAGGACTCAT
 61 CCGG
```

W 5' *Taq* I - TT 3'

```
  1 CTTTCCCCTN TANGATGTGT GATATCTGGG TCCTCCCCTA CCTCGTGCNG TGNATCTTGT
 61 GCGCTCTCCC TCCAAAAAAA AA
```

W 5' TT - *Taq* I 3'

```
  1 ACCNANACNA ATGNCAANTT GAAACNACAT AAGCATGTCT TNGTCCTNAG NCAGTCCTTC
 61 GGTCAGGACT CATCCGG
```

X 5' *Taq* I - TT 3'

```
  1 CANTNGGGNN ANTNAGNCCC CCCCGAGCTA ATNCGTTTTA NTTCNTTTCC TGGGAGGAAA
 61 AAAAAAAA
```

X 5' TT - *Taq* I 3'

```
  1 AGNAATACNA TAGANACACG TTTTATGCCN ANANTCCTTC GGTCAGGACT CATCCGG
```

Y 5' *Taq* I - TT 3'

```
  1 TCNCATGGGA GAGCCCCTTA TCTTCTCACG AAAAAAAAAA AAA
```

Y 5' TT - *Taq* I 3'

```
  1 TGCAAAAAAA TAGAAACACT ACGNATGCCC AAACCAATCC CTCTTCGGTC AGGACTCATC
 61 CGG
```

Z 5' *Taq* I - TT 3'

```
  1 NGAATTGCCN CTGTATCATA AATCCCAAGT TTCTTTTGGA GTTACTGGCA AAAAAAAAAA
 61 AA
```

Z 5' TT - *Taq* I 3'

```
  1 NTCNCCAATA TTATGATACA GCTTCATTTC AGCNAATCAT ACAAGAATAT TCCTGCCTTC
 61 GGTCAGGACT CATCCGG
```

AA 5' *Taq* I - TT 3'

```
  1 TTCCCCNNGT CGGCTTTGGT GTTCCTGGTG TTCCTCCCCC CAGTGNTACC GANGTGTGGC
 61 ATTNANNTCA TGTGGGAACC AGGNCGNCTN CATCCTCCAA TCGTAGAATN AAAGATGCGT
121 CTGGTNNTGT CGTGAGTAGT ATTCTGNTCT CTTGTAGTAT AGGGCTGAGC NGTCCNTTAC
181 TTGCTCGGGN NTNNCCTATT TACCAAAAAA AA
```

BB 5' *Taq* I - TT 3'

```
  1 GAGGNCGGNT NNCTTGGCAT GGNCCCTCCG NGNNCTACAC TGCGAGTTGT GAGNATNCAT
 61 TCANGGAGTA TCAGACAGAN GCACTCCTGC GTCAGTCCTN NNNGGGTCAN AAANCTTNCA
121 TTGCGGCNNC NTTTCTTCCN GAACNNCNGC TNCGTNNAAT TCGGNGGNNG TANTTCTCNG
181 GTTGAGNTGG AANATCANNA TTTATCACCA AAAAAAA
```

BB 5' TT - *Taq* I 3'

```
  1 CCNNNGGAGN ACTTCCTCCA CCGAANTNNA ANAANTGNAC GTTTTNGANC AAANGGNGTT
 61 GCCANTCAAC TTTGNANNCC CCCNGCAGGG ATANAATGAA NCNTTTTTTN NNTNCGGAAC
121 NTCNANCANG ACNNGATTNN AGANGCGCCA CCCGANGNNN CCCTANCTNC NCTAANGGNT
181 TGATGGGTTT CTATTGNNTT CNCCTTCGGT CAGGACTCAT CCGG
```