Deciphering Kunitz proteinase inhibitors in white clover (*Trifolium repens* L.): A transcriptional study

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Abstract

This thesis investigates the *Kunitz Proteinase Inhibitors (KPI)* gene family in white clover (*Trifolium repens* L.) as this family of inhibitors is one of the most abundant among the serine proteinase inhibitor families in legume species. In other studies, these proteins have mainly been shown to serve as storage proteins and to also act as potent defensive factors against insect herbivory. As they are involved in regulating proteolytic activity, the question arises as to how much they are also involved with regulating plant growth and development and how they respond to different stresses other than insect herbivory? Here, in this thesis effort has been under taken to answer these questions using the perennial legume white clover which is a major contributor to pasture productivity in New Zealand. However, as yet, very little is known about the occurrence of *Kunitz proteinase inhibitor (Tr-KPI)* genes or the functions of these genes in white clover. In this study, therefore, the spectrum of *Tr-KPI* genes is characterized, and the regulation of expression at the transcriptional level of different members of the gene family is examined.

To obtain *KPI* genes from white clover, degenerate primers were designed based on known legume *KPI* sequences. Four full length cDNA were obtained using degenerate and later gene-specific primers. Blast searching of the JCVI and NCBI database showed that they encode for proteins fall into the soybean trypsin inhibitor super-family (STI) and were named *Tr-KPI1*, *Tr-KPI2*, *Tr-KPI4* and *Tr-KPI5.* The expression in the transcript level of these four genes showed that *Tr-KPI1*, *Tr-KPI2, Tr-KPI5* are constitutively expressed in vegetative and reproductive parts whereas *Tr-KPI4* is more organ-specific such that it is expressed in the root and mature seed. A leaf and root developmental study showed that *Tr-KPI2* and *Tr-KPI5* are more developmentally regulated and transcript abundance during a germination time course study also suggests the involvement of *Tr-KPI1* and *Tr-KPI5* during seedling establishment.

To explore the function of these genes further, different forms of biotic and abiotic stress were applied to white clover. A mechanical wounding study revealed the possible involvement of *Tr-KPI1*, *Tr-KPI2* and *Tr-KPI5* in plant defense in both local and systemic tissues, and *Tr-KPI4* in the systemic tissue. A shoot herbivore (*Spodoptera litura*) and root herbivore (root knot nematode *Meloidogyne trifoliophila* and cyst nematode *Heterodera trifolii*) were also used to characterize the involvement of the *Tr-KPI* genes in plant defense response. Expression of the *Tr-KPI* genes against the generalist herbivore *S. litura* further supported the view that the *Tr-KPIs* in white clover are involved in plant defense responses where local (leaf), basipetal (root) and acropetal

(apical tissue) tissues were compared. The expression results suggest that *Tr-KPI1, Tr-KPI2* and *Tr-KPI5* are induced by herbivore attack and *Tr-KPI1* was found to be most involved (1600-fold at 24 h) followed by *Tr-KPI2* and *Tr-KPI5*. In the nematode experiment, inoculation by a cyst nematode was able to trigger the expression of *Tr-KPI1, Tr-KPI4* and *Tr-KPI*5 in the root tissue at day 4 and a systemic response of nematode feeding was also observed in the leaf tissue for these genes at day 8. Invasion by the root-knot nematode did not result in any significant up-regulation for *Tr-KPI* genes at day 4 and day 8. This finding suggests that *Tr-KPIs* might be involved in defense against cyst nematode invasion but not by root-knot nematodes. To further elucidate the involvement of *Tr-KPI* genes under cyst nematode attack, a resistant line 17R and a susceptible line 23S were used. In the resistant line 17R, all four *Tr-KPI* genes were significantly expressed by day 4 and day 8, and in the susceptible line 23S, high transcript abundance was observed only at day 4. Therefore, it can be proposed that *Tr-KPIs* in white clover are important in defense against white clover cyst nematode in combination with other defense genes.

For an abiotic stress study, water deficiency and limited phosphorus (Pi) treatments were employed to examine the expression of the *Tr-KPI* genes in white clover. For the water deficiency trials, two treatments were imposed: a pre-stressed (PS) treatment in which plants were subjected to a water deficit for 7 days, followed by watering for a further 7 days before the experimental water deficit was applied, or a non-pre stressed (NPS) treatment in which plants were subjected immediately to a water deficit. The level of *Tr-NCED1* (9-cis-epoxycarotenoid dioxygenase) expression, coding for an enzyme involved in ABA biosynthesis, was also investigated to prove that a water deficit is perceived by the plants. The *Tr-NCED1* level was found to be up-regulated in the NPS treatment when compared with the level observed in fully hydrated tissue. Under the NPS and PS treatments, the transcription level of *Tr-KPI1* and *Tr-KPI5* were induced significantly in the leaf tissue when compared with the control. Interestingly, the pre-stressed treatment triggered the expression of all three genes studied which were significantly higher compared to the expression level under the NPS treatment. To further characterize the role of *Tr-KPIs* under water stress, a drought tolerant ecotype Tienshan and drought susceptible cultivar, Kopu was used. A clear upregulation of *Tr-KPI1* in Tienshan and *Tr-KPI5* in Kopu was observed under the PS treatment when compared with the initial moisture content and NPS treatments indicating some selective pressure on the *Tr-KPIs* under water stress in susceptible and resistant plants.

In a macro-nutrient (Pi) limitation experiment, where the growing root is divided in different developmental regions comprising the elongation zone (EZ), the visible lateral root zone (VL)

and the mature zone, a higher level of *Tr-KPIs* expression was observed in the growing zone rather than mature root zone. Although expression of all four *Tr-KPIs* was up-regulated in the EZ region, only *Tr-KPI2* and *Tr-KPI4* showed an extended level of expression in the visible lateral root zone indicating a possible involvement in lateral root formation as Pi limitation does induce a higher number of lateral root primordia. In leaf tissue, the down regulation of *Tr-KPIs* was observed up to 12 h of the Pi starved treatment and the transcript level started to increase from 24 h onward indicating that *Tr-KPIs* are not early response genes in leaf tissue.

Finally, the *cis*-binding elements in the promoter regions of four *Tr-KPI* genes indicate that this gene family in white clover is controlled by different transcription factors. A number of growth and development-related transcription factor binding sites such as AREF, ASRC, LFY, MADS and biotic and abiotic stress responsive transcription factors binding sites such as EINL, MYBL, MYBS, MYCL, and WNAC have been identified in all the four promoter sequences, although differences in the pattern and frequency were observed across the four *Tr-KPI* genes were observed. This further highlights that this gene family is regulated by a complex network of hormonal and other stress induced cues.

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Abbreviations

°C	Degree Celsius
µg	Microgram
µL	Microlitre
µmol	Micromole
A_{260}	Absorbance at 260 λ nm
A_{280}	Absorbance at 280 λ nm
BLAST	Basic Logical Alignment Search Tool
bp	Base-pair
ca.	*circa* (approximately)
cDNA	DNA complementary to a RNA, synthesized from RNA by the reverse transcription *in vitro*
DEPC	Diethyl pyrocarbonate
DMF	*N,N*-dimethyl formamide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleotide 5'triphosphate
DTT	Dithiothreitol
E.coli	*Eschercia coli*
EDTA	Ethylenediaminettetra acetic acid
EST	Expressed sequence tag
g	Acceleration due to gravity (9.8m s^{-2})
g	Gram
h	hour
IPTG	Isopropyl-β-$_D$-thiogalactopyranoside ($C_9H_{18}O_5S$)
Kb	Kilo base-pairs
kDa	Kilo Daltons
L	Litre
LB	Lauria-Bertani (media or broth)
M	Molar (moles per litre)
mg	Milligram
Milli-Q water	Water purified by a Milli-Q ion exchange column
min	Minute
mL	Millilitre
mol	mole (amout of a substrate, Avogadro's number)
MPa	Mega Pascal
NBT	*p*-nitro blue tetrazolium chloride
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nmol	Nanomole
NPS	Non pre-stressed
ORF	Open reading frame

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline (50 mM sodium phosphate, pH 7.4 containing 250 mM NaCl)
PCR	Polymerase chain reaction
pH	-Log (H^+)
pmol	Picomole
PS	Pre-stressed
PVP-40	Polyvinyl pyrrolidone
qRT-PCR	Quantitative real-time PCR
RH	Relative Humidity
RNA	Ribunucleic acid
RNase	Ribonuclease
RO	reverse osmosis
RT-PCR	Reverse Transcriptase-polymerase chain reaction
sec	second
SEM	Standard error mean
T_m	Melting temperature at which DNA strands separate in preparation for annealing
Tris	Tris (hydroxymethyl) aminomethane
Tween-20	Polyoxyethylenesorbitan monolaurate
U	Unit (commercial enzymes are in U μL^{-1}, where unit is based on enzyme activity)
UTR	Untranslated region
UV	Ultra violet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X-Gal	5-Bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside