

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

A thesis presented in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Plant Molecular Biology

At Massey University, Palmerston North, New Zealand

Afsana Islam, 2013

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-KPI5* 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.

Acknowledgement

First of all, I would like to express my heartfelt respect to my supervisor Professor Michael T McManus for his continuous support, worthy guidance, valuable suggestions, incisive criticisms, encouragement and helpful comments during the research and writing period- I cannot thank you enough. I also thank my co-supervisor Paul Dijkwel for his encouragement, insightful comments, and helpful advice during the course of my study.

I am grateful for the support from Libby Burgess and her team from Plant and Food Research, Auckland during the herbivore experiment. I thank Chris Mercer, AgResearch Grasslands, Palmerston North, for helping me sorting out the nematode experiment. Tons of thanks go to Kim Richardson and his 'working bees' from AgResearch Grasslands, Palmerston North, for white clover transformation. Thanks to Steven Ray for his help in Plant Growth Unit, Massey University. Huge thank to Ann Turter, Cynthia Cresswell and James Connel who were always there to sort out any problems staying behind the scene.

Thank you my previous lab-mates: Aluh and Alvina for supporting me from the very beginning of my study. Thank you Susanna for your kindness and support and making my life easier in the lab. And Jay Jaya, thanks for your multitude help, I will always remember our 5.10 pm meeting. Thanks to you Srishti, will really miss the interesting topics about documentaries and movies. Thanks to the support team-Julia, Ameesha, Sashini, Uttara and Natalie for making the research easier. Thanks to my other lab-mates Mat, Diantha, Caleb, Rubina, Jibran, Faisal, Sam, and Jen.

I convey my special acknowledgement to Massey University for the Massey Doctoral Scholarship and Institute of Molecular BioSciences for funding the final year of my study and financial support for attending conferences.

Last but not the least; I thank my parents for always giving me mental support, my husband, brother and sister for inspiration and encouragement during the period of study.

Contents

	Page
Abstract	i
Acknowledgements	iv
Contents	v
List of Figures	x
List of Tables	xiii
Abbreviations	xiv
Chapter 1 Introduction	
1.1 Peptidase, protease, proteinase and proteinase inhibitors- A note on nomenclature	2
1.2 Proteinase Inhibitor (PI) -The natural antagonists of proteinases	2
1.3 The protein 'proteinase inhibitor' in plants	3
1.4 Plant serine proteinase inhibitors	4
1.4.1 Localization of plant serine proteinase inhibitors	5
1.4.2 Mechanism of inhibition	6
1.5 Plant Kunitz proteinase inhibitors - the diversified inhibitor family	7
1.6 Kunitz Proteinase Inhibitor (KPI) –Are they one unit many functions family?	9
1.6.1 Kunitz Proteinase inhibitors as storage proteins	9
1.6.2 Kunitz Proteinase inhibitors as regulators of proteinases during germination	10
1.6.3 Defense against insect herbivores as endogenous insecticides	11
1.6.4 Kunitz proteinase inhibitors during nodulation	12
1.6.5 Kunitz proteinase inhibitors in Programmed cell death (PCD)	13
1.6.6 Kunitz proteinase inhibitors in control of flowering	14

1.6.7	Kunitz proteinase inhibitors in plant defenses against abiotic stressors	15
1.7	Regulation of plant kunitz proteinase inhibitors	16
1.8	Concluding statement and research hypothesis	19
1.8.1	Why study Kunitz proteinase inhibitors in white clover	19
1.8.2	Hypothesis	20
1.9	Research objectives	20

Chapter 2 Methods

2.1	Plant Material	23
2.1.1	Establishment and maintenance of stock plants	23
2.1.2	Vegetative propagation of plant material for experimental use	23
2.1.3	Experimental plants and treatment	23
2.1.3.1	<i>Spodoptera litura</i> feeding experiment	24
2.1.3.2	Nematode feeding experiment	25
2.1.3.3	Water deficiency experiment	25
2.1.3.4	Phosphorus limiting experiment	26
2.2	Chemicals used	26
2.3	Molecular Biology Protocol	27
2.3.1	Nucleic acid isolation	27
2.3.1.1	Total RNA isolation	27
2.3.1.1.1	DNase treatment	28
2.3.1.2	Isolation of genomic DNA (gDNA) using the CTAB method	29
2.3.1.3	Quantification of nucleic acid	29
2.3.2	Synthesis of cDNA	30
2.3.3	Polymerase chain reaction (PCR)	31
2.3.3.1	Primer design	31
2.3.3.2	General PCR protocol for amplification of cDNA	31
2.3.3.3	Agarose gel electrophoresis	32
2.3.3.4	Quantitative RT-PCR (qRT-PCR)	33
2.3.4	Isolation of promoter region using genome walking technology	34

2.3.4.1	Digestion of genomic DNA	34
2.3.4.2	Purification of genomic DNA	34
2.3.4.3	Adaptor sequence, primers and ligation of genomic DNA to adaptor	34
2.3.4.4	PCR protocol to amplify upstream region of the selected genes by genome walking	35
2.3.5	DNA recovery	37
2.3.6	Plasmid cloning and transformation	38
2.3.6.1	Ligation of DNA into the pGEM® T Easy vector	38
2.3.6.2	Preparation of competent cells for plasmid transformation	39
2.3.6.3	Transformation of <i>E. coli</i> with pGEM®-T Easy vector	39
2.3.6.4	Isolation of plasmid DNA from <i>E. coli</i>	40
2.3.7	Automatic sequencing of DNA	41
2.3.7.1	Sequence analysis	42
2.4	Generation of Transgenic Plants	42
2.4.1	Developing RNAi Knockdown Lines in white clover	42
2.4.1.1	Growing <i>Agrobacterium</i> for transformation of RNAi constructs in white clover	43
2.4.1.2	Dissection and transformation of cotyledonary explants	43
2.5	Statistical analysis	44

Chapter 3 Results

3.1	Identification of <i>Kunitz proteinase inhibitor</i> genes in white clover	46
3.1.1	Approach I: Searching the AgResearch EST database	46
3.1.2	Approach II: Using different sets of degenerate primers	47
3.1.3	Identification of full length <i>Tr-KPI</i> genes	49
3.1.4	<i>Tr-KPI</i> genes share sequence similarity with <i>KPI</i> genes from other plant species	54
3.1.5	Structure of <i>Kunitz Proteinase Inhibitor</i> Gene Family in <i>Medicago truncatula</i>	58
3.2	Expression of <i>Tr-KPIs</i> in different tissues of white clover	62
3.2.1	Expression of <i>Tr-KPI</i> genes in leaves at different developmental stages	62

3.2.2	Expression of <i>Tr-KPI</i> genes in different regions of the root	64
3.2.3	Expression of <i>Tr-KPIs</i> during nodule development	64
3.2.4	Expression of <i>Tr-KPI</i> genes during germination	66
3.3	<i>Tr-KPI</i> genes and biotic stress	70
3.3.1	Response of <i>Tr-KPI</i> genes by mechanical wounding	70
3.3.1.1	Local response of <i>Tr-KPIs</i> in leaf tissue	70
3.3.1.2	Systemic response of <i>Tr-KPIs</i> in root tissue in response to wounding in the leaves	72
3.3.2	Changes in <i>Tr-KPI</i> gene expression in response to insect herbivory	76
3.3.2.1	Response of <i>Tr-KPIs</i> in local tissues by insect herbivory	78
3.3.2.2	Response of <i>Tr-KPIs</i> in systemic tissues by insect herbivory	78
3.3.3	Response of <i>Tr-KPI</i> genes in white clover to nematode feeding	83
3.3.3.1	Response of <i>Tr-KPI</i> genes in white clover to root-knot and cyst nematode feeding	83
3.3.3.2	Response of <i>Tr-KPI</i> genes in white clover resistant and susceptible lines infested with <i>Heterodera trifolii</i>	86
3.4	<i>Tr-KPI</i> Genes and Abiotic Stress	89
3.4.1	The influence of water deficit on <i>Tr-KPI</i> gene expression	89
3.4.1.1	Change in moisture content of the media	90
3.4.1.2	Expression of <i>Tr-NCED1</i> in the first fully expanded leaf of white clover in response to water deficit	92
3.4.1.3	Expression of <i>Tr-KPIs</i> in the first fully expanded leaf of white clover in response to water deficit	93
3.4.1.4	Expression of <i>Tr-KPIs</i> in roots of white clover in response to a water deficit	95
3.4.1.5	Expression of <i>Tr-KPIs</i> in the first fully expanded leaf of white clover ecotype Tienshan and cultivar Kopu in response to a water deficit	97
3.4.2	Changes in the expression of the <i>Tr-KPI</i> genes in response to change in phosphorus supply	100
3.5	Exploration of <i>Tr-KPI</i> Promoter Sequences	106
3.5.1	Cis – Binding elements in the Promoter Sequence	112
3.6	Knock-down of <i>Tr-KPI</i> genes expressing in white clover using RNAi	119
3.6.1	<i>Tr-KPI</i> genes expression in the RNAi lines	120

Chapter 4 Discussion

4.1	Occurrence of <i>KPIs</i> in White Clover	125
4.2	<i>Tr-KPI</i> genes differ in tissue specific expression and are developmentally regulated at the transcriptional level	128
4.3	Tr-KPI genes and biotic stress	131
4.4	Tr-KPI genes under abiotic stress	135
4.5	Cis-Regulatory elements of <i>Tr-KPIs</i>	141
4.6	<i>Tr-KPIs</i> RNAi lines	143
4.7	<i>Medicago</i> vs. white clover Kunitz proteinase inhibitors	143
4.8	Summary and Conclusion: The Bigger Picture	145
4.9	Future Directions	149
4.9.1	Growth and development analysis of <i>Tr-KPIs</i> RNAi knockdown lines	149
4.9.2	Analysis of <i>Tr-KPIs</i> inhibitory activity against different Proteinases	150
4.9.3	Identification of other <i>Tr-KPIs</i> members	151
4.9.4	Growth and development analysis of <i>Medicago</i> knockout lines	151
	References	152
	Appendices	165
	Appendix 1: Families of Proteinase inhibitors from angiosperm listed in the MEROPS database	166
	Appendix 2: Sequences from AgResearch white clover EST database gave hits against <i>M. truncatula</i> <i>KPI</i> gene	166
	Appendix 3: Sequence Alignment for designing four Different sets of Degenerate Primers	172
	Appendix 4: Sequence alignment for designing primers to obtain 3'UTR region	176
	Appendix 5: Sequence of Primers	177
	Appendix 6: Primers for genome walking to get the promoter region	179
	Appendix 7: Sequences of primers used for qRT-PCR	180
	Appendix 8: Hoagland's Medium (Gibeaut <i>et al.</i> , 1997)	181
	Appendix 9: Tissue culture media used for RNAi white clover lines	182

List of Figures	Page
1.1 Schematic representations of interactions between a proteinase and protein proteinase inhibitor	7
1.2 Points of convergence between environmental cues and the induction of plant Kunitz proteinase inhibitors	18
2.1 Schematic representation of <i>Tr-KPI</i> RNAi construct in pRNA69 vector	43
3.1 (A) Alignment of Tr-KPI partial amino acid sequences using ClustalW. (B) Identity of partial sequence of Tr-KPIs at the amino acid level	48
3.2 Alignment of identified full length <i>Tr-KPI</i> genes	51
3.3 (A) Alignment of Tr-KPIs deduced amino acid sequences. (B) Identity of Tr-KPI protein at the nucleotide and amino acid level. (C) Theoretical pI/Mw	52
3.4 Reactive site loop for Tr-KPI1 (A), Tr-KPI2 (2), Tr-KPI4 (C) and Tr-KPI5 (D)	53
3.5 Alignment of Tr-KPI deduced amino acid sequences with other Trypsin Inhibitors	56
3.6 Phylogenetic relationship with <i>M. truncatula</i> KPI and Tr-KPI proteins.	61
3.7 Expression of <i>Tr-KPI</i> genes in different plant parts of white clover	63
3.8 A. Leaf developmental stages along a single stolon of white clover. B. Expression of <i>Tr-KPIs</i> in different leaf developmental stages as indicated	65
3.9 A. Stages of root development in white clover used for expression studies. B. Expression of <i>Tr-KPIs</i> in the different root parts	66
3.10 Nodule development stages (A) and expression of <i>Tr-KPIs</i> during the stages of nodulation, as indicated (B)	67
3.11 A. Imbibed and germinated seeds of white clover. . B. Expression of <i>Tr-KPIs</i> in germinating seeds and seedlings of white clover at the post-imbibition time points indicated	69
3.12 Stolons of the white clover cultivar 'Huia' with the leaf and root tissues used to study wounding response of <i>Tr-KPI</i> genes, as indicated	71
3.13 Changes in <i>Tr-KPI</i> gene expression, as indicated, in response to mechanical wounding of the first fully expanded leaf (FFE)	73
3.14 Changes in <i>Tr-KPI</i> gene expression, as indicated, in roots subtending from the third node in response to mechanical wounding on the first fully expanded leaf (FFE)	74

3.15	Changes in <i>Tr-KPI</i> gene expression, as indicated, in roots subtending from the fourth node in response to mechanical wounding on the first fully expanded leaf (FFE)	75
3.16	A. Experimental setup for herbivory trial by <i>Spodoptera litura</i> . B. The first fully expanded leaf after feeding at 1 h, 6 h, 12 h and 24 h	77
3.17	Changes in <i>Tr-KPI</i> gene expression, as indicated, in response to insect herbivory of the first fully expanded leaf (FFE)	80
3.18	Changes in <i>Tr-KPI</i> gene expression, as indicated, in apical tissue in response to insect herbivory of the first fully expanded leaf (FFE)	81
3.19	Changes in <i>Tr-KPI</i> gene expression, as indicated, in roots in response to insect herbivory of the first fully expanded leaf (FFE)	82
3.20	Stolon of white clover cultivar Huia at day 4 after inoculation by cyst and root knot nematodes	84
3.21	Changes in <i>Tr-KPI</i> gene expression in response to nematode infestation, as indicated, in root tissue	85
3.22	Experimental setup for nematode inoculation of the resistant (17R) and susceptible line (23S)	87
3.23	Changes in <i>Tr-KPI</i> gene expression, as indicated, in root tissue in the resistant (17R) and susceptible (23S) lines in response to cyst nematode (<i>Heterodera trifolii</i>) infestation	88
3.24	Schematic depiction of the two different water deficit treatments imposed on white clover cultivar Huia grown in a vermiculite and perlite mixture (1:1)	90
3.25	Experimental set up of the water deficiency study and moisture content of the media	91
3.26	Relative expression of <i>Tr-NCED1</i> in the first fully expanded (FFE) leaves of NPS and PS treated plants at the moisture content of the media, as indicated	92
3.27	Expression of <i>Tr-KPIs</i> , as indicated, in the first fully expanded (FFE) leaves in response to NPS and PS treatments at the moisture content of the media, as indicated in cultivar Huia	94
3.28	Relative expression of <i>Tr-KPIs</i> , as indicated, in root tissue of NPS and PS treated plants at the moisture content of the media as indicated in cultivar Huia	96
3.29	Relative expression of <i>Tr-KPI</i> genes, as indicated, in the first fully	

	expanded (FFE) leaves of NPS and PS treated ecotype Tienshan at the moisture content of soil (SWC), as indicated	98
3.30	Relative expression of <i>Tr-KPIs</i> , as indicated, in the first fully expanded (FFE) leaves of NPS and PS treated cultivar Kopu at the moisture content of soil (SWC), as indicated	99
3.31	Experimental set up for phosphorus deficiency experiment	100
3.32	Relative expression of <i>Tr-KPIs</i> , as indicated, in the elongation zone (EZ) in control (P+) and Pi deficient (P-) treated plants using qRT-PCR	102
3.33	Relative expression of <i>Tr-KPIs</i> , as indicated, in the visible lateral root zone (VL) in control (P+) and Pi deficient (P-) treated plants using qRT-PCR	103
3.34	Relative expression of <i>Tr-KPIs</i> , as indicated, in the mature root zone (MR) in control (P+) and Pi deficient (P-) treated plants using qRT-PCR	104
3.35	Relative expression of <i>Tr-KPIs</i> , as indicated, in the first fully expanded leaf (FFE) in control (P+) and Pi deficient (P-) treated plants using qRT-PCR	105
3.36	Isolation of full length <i>Tr-KPI</i> genes, as indicated, spanning the promoter region, reading frame and part of the 3'UTR region using PCR	107
3.37	Full length <i>Tr-KPI1</i> gene, as indicated	108
3.38	Full length <i>Tr-KPI2</i> gene, as indicated	109
3.39	Full length <i>Tr-KPI4</i> gene, as indicated	110
3.40	Full length <i>Tr-KPI5</i> gene, as indicated	111
3.41	Putative transcription factor binding sites upstream of the ATG of the promoter region of <i>Tr-KPI1</i> , <i>Tr-KPI2</i> , <i>Tr-KPI4</i> and <i>Tr-KPI5</i> genes, as indicated, as identified by MatInspector	114
3.42	Transcript abundance of <i>Tr-KPI1</i> , <i>Tr-KPI2</i> , <i>Tr-KPI4</i> and <i>Tr-KPI5</i> in their respective RNAi lines, as indicated	121
3.43	Expression of the <i>Tr-KPI</i> genes in the leaf tissues of low category RNAi KD lines, as indicated	122
3.44	Expression of <i>Tr-KPI1</i> , <i>Tr-KPI2</i> and <i>Tr-KPI5</i> in the root tissues of <i>Tr-KPI4</i> RNAi lines, as indicated	123
4.1	<i>KPI</i> gene expression data (Affymetrix Medicago Gene Chip [®]) in <i>M. truncatula</i> (Jemalong A17)	144
4.2	Proposed tri-functional role of <i>Kunitz Proteinase Inhibitors</i> in white	

clover. Abiotic (black), biotic (blue) stress response and under normal growth and development (green) 148

List of Tables		Page
3.1	Predicted localization of Tr-KPI proteins from different plant species	57
3.2	Result of JCVI BLAST search using Tr-KPI1 aa sequences	59
3.3	Result of JCVI BLAST search using Tr-KPI2 aa sequences	59
3.4	Result of JCVI BLAST search using Tr-KPI4 aa sequences	60
3.5	Result of JCVI BLAST search using Tr-KPI5 aa sequences	60
3.6	Frequency of occurrences of Cis-binding elements in the promoter region of <i>Tr-KPI</i> genes, as indicated as predicted by MatInspector	118
4.1	Grouping of <i>Tr-KPIs</i> based on the expression in growth and development; biotic and abiotic stress response at the transcriptional level	147

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.	<i>circa</i> (approximately)
cDNA	DNA complementary to a RNA, synthesized from RNA by the reverse transcription <i>in vitro</i>
DEPC	Diethyl pyrocarbonate
DMF	<i>N,N</i> -dimethyl formamide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleotide 5'triphosphate
DTT	Dithiothreitol
<i>E.coli</i>	<i>Eschericia coli</i>
EDTA	Ethylenediaminettetra acetic acid
EST	Expressed sequence tag
<i>g</i>	Acceleration due to gravity (9.8m s ⁻²)
g	Gram
h	hour
IPTG	Isopropyl-β- <i>D</i> -thiogalactopyranoside (C ₉ H ₁₈ O ₅ S)
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	<i>p</i> -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	Ribonucleic 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