

CHARACTERIZATION OF GLUCAN HYDROLASE ACTIVITY IN PUTATIVE β -
GLUCAN-BINDING PROTEIN 4 IN *MEDICAGO TRUNCATULA*

by

DANA L. CHAFIN

(Under the Direction of Michael G. Hahn)

ABSTRACT

The hepta- β -glucoside elicitor, (HG), consisting of 1 \rightarrow 6, 1 \rightarrow 3- β - linkages is derived from glucans found in the cell wall of the pathogen, *Phytophthora sojae*. The HG is capable of eliciting defense responses in legumes leading to phytoalexin production. High affinity β -glucan-binding sites (GBPs) for the HG elicitor found in soybean have been shown to be plasma-membrane protein complexes. The soybean GBP also has glucanohydrolic properties toward β -1,3 glucans found in pathogen cell walls. This project had two principal goals. First, to clone full-length cDNA sequences corresponding to three EST contig sequences (*MtKV0*, *MtKV2*, and *MtKV3*) from *Medicago truncatula* that are highly similar to the soybean GBP and to study the expression patterns of these genes in *M. truncatula* tissues. The full-length cDNA sequence for *MtKV3* was determined. Gene expression studies in eight wild type tissues demonstrated that *MtKV0*, *MtKV2*, and *MtKV3* are most highly expressed in roots. The second goal was to identify and characterize single nucleotide polymorphisms (SNPs) leading to amino acids changes in *MtKV3* that might alter the glucanohydrolytic properties of this protein. The wild type and mutant genes were expressed in *Nicotiana benthamiana*. One mutant protein lost glucan hydrolytic activity suggesting amino acid variations in one of the putative conserved glucanase domains could be responsible for the loss of activity.

INDEX WORDS: Glucan-binding proteins, *Medicago truncatula*, 11427, 20054, 12897,
Elicitor-Hepta- β -glucoside, *Phytophthora sojae*, β -1,3 glucanase.

CHARACTERIZATION OF GLUCAN HYDROLASE ACTIVITY IN PUTATIVE β -
GLUCAN-BINDING PROTEIN 4 IN *MEDICAGO TRUNCATULA*

by

DANA L. CHAFIN

B.S., University of Georgia, 2003

A Thesis to the Graduate Faculty of The University of Georgia in Partial Fulfillment of
the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2008

© 2008

Dana L. Chafin

All Rights Reserved

CHARACTERIZATION OF GLUCAN HYDROLASE ACTIVITY IN PUTATIVE β -
GLUCAN-BINDING PROTEIN 4 IN *MEDICAGO TRUNCATULA*

by

DANA L. CHAFIN

Major Professor: Michael G. Hahn

Committee: Alan Darvill
R. Kelly Dawe

Electronic Version Approved

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2008

DEDICATION

I dedicate this thesis in memory of my Grandfather with his memorable words; *the limiting factor of discoveries in science is often the use of one's imagination.*

ACKNOWLEDGEMENTS

I would like to give special thanks to all the people who supported me during these past three years making this research project possible. Dr. Hahn, I would especially like to give gratitude for the opportunities, guidance, and funding over the past years. Having the chance to work at The University of California at Davis not only allowed me to gain experience and materials for this project, but I was able to meet Rebecca with whom I am still very close friends with to this day. I thank you for your patience and concerns for me during my HCV treatments. That was by far the longest and most grueling 48 weeks of my life. To my dear friend, Julie, who was the silver lining to my dark cloud during this time, we must always hope for the best. I give much thanks to Dr. Sivakumar Pattathil in the Hahn lab whose tremendous help and friendship made analyzing gene function in *Medicago truncatula* possible and fun. You truly are an amazing scientist. I would like to thank Dr. Yingzhen Kong and Dr. Gongke Zhou for their help in the lab; Ying I am so glad you introduced me to RACE technique, because I was hitting a brick wall with TAIL PCR. I really enjoyed all of our conversations. To my dear friends Heather, Rebecca, Michael, and Sarah, I will always have a warm place in my heart for you all. You helped me so much through graduate school whether over late night phone conversations, blowing off steam over dinners, or Michael, getting stuck in OBX after a nine hour drive. Sarah, I give special thanks for allowing me to stay with you during the summer of 2007 and for providing a home for Andromeda. Athens has not been the same since you left. I wish Brad and you all the best in life. I would like to thank my wonderful parents who are always so supportive and loving. I feel so blessed to have you both as parents. You two always give such level-headed advice. To my brother, Justin thanks for your support; I really enjoyed our phone

conversations within this past year. Last but not least in the family, I am so grateful for my twin sister, Laurel, who will always be my soul mate. Finally, Dr. Alan Darvill and Dr. Kelly Dawe I appreciate you both giving your time as committee members. I acknowledge there is a lot of unmentioned family and friends who have helped push me along the way, and I would like to say your support has not gone unnoticed.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
CHAPTER	
1 INTRODUCTION AND LITERATURE REVIEW.....	1
2 MATERIALS AND METHODS.....	6
3 RESULTS.....	17
4 DISCUSSION.....	34
REFERENCES.....	39

LIST OF TABLES

	Page
Table 1: Quantitative RT-PCR Primers.....	8
Table 2: Relative Root Expression of <i>MtKVO</i> , 2, and 3 in RT-PCR.....	22

LIST OF FIGURES

	Page
Figure 1: Heterologous Expression pCambia35SEGFPS2 Vector.....	13
Figure 2: ClustalX Protein Alignment of Full Length <i>MtKV3</i> cDNA.....	17
Figure 3: RT-PCR Amplification Curves.....	19
Figure 4: Linear Regression Line of Root cDNA Dilution Series.....	20
Figure 5: Quantitative RT-PCR Results of <i>MtKV0</i> , 2, and 3.....	20
Figure 6: <i>mtkv3_11427</i> mutant plant.....	23
Figure 7: <i>mtkv3_12897</i> mutant plant.....	23
Figure 8: <i>mtkv3_20054</i> mutant plant.....	23
Figure 9: Agarose Gel Analysis of <i>MtKV3</i> Clones.....	24
Figure 10: ClustalX Protein Alignment of Full Length <i>MtKV3</i>	26
Figure 11: Clustal X Protein Alignment of GH-81 Domains.....	27
Figure 12: C-75 Superdex HPLC Column Analysis.....	29
Figure 13: C-75 Superdex HPLC Column Analysis.....	30
Figure 14: MALDI TOF-MS.....	32

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Of the many possible pathogens including bacteria, viruses, fungi, and animals, only few species are effective in overcoming plant defenses. Plant pathways involving a successful defense system against pathogen invasion requires an ability for plant cells to recognize the potential pathogen as non-self (Hoch *et al.* 1991). In order for cellular communication to take place between host and pathogen, mutual identification of structures between both organisms must take place. Successful resistance from pathogen attack is a highly evolved system where recognition systems of both species takes place with a phenomenon involving a series of signal transduction events leading to transcription of genes whose products are vital for pathogen resistance (Boller 1995; Côté *et al.* 2000; Dixon *et al.* 1995). Once plant cellular reactions establish recognition of non-self, plant genes and their products of disease resistance are produced and released during the infection process.

Signaling events between the plant host and potential pathogens have been extensively studied in order to identify and characterize molecules involved in recognition between the two species. Biochemical investigations have proven to be invaluable in identifying elicitors capable of inducing defense signal transduction pathways in plants. Pathogenic elicitors interact with plant receptors with high specificity and can range structurally from carbohydrates to glycoproteins, lipids, and sterols (Boller *et al.* 1995; Ebel *et al.* 1998; Hahn *et al.* 1996). Elicitors were once referred to as signal molecules that induce the synthesis and accumulation of antimicrobial compounds, phytoalexins, in plant cells (Keen 1975). Today, however, elicitors

are commonly known as molecules stimulating any plant defense mechanism (Dixon *et al.* 1986; Hahlbrock *et al.* 1987).

Oligosaccharide elicitors found in fungal cell walls were among the first elicitors identified that could be shown to be responsible for inducing the synthesis and accumulation of phytoalexins in plants (Ayers *et al.* 1976; Darvill *et al.* 1984). Isolation and structural characterization of oligosaccharide elicitors provided important information about the crucial roles oligosaccharides play in eliciting biological signaling responses in plants (Ayers *et al.* 1976; Sharp *et al.* 1984). The hepta- β -glucoside (HG) elicitor, found in the cell wall of the oomycete, *Phytophthora sojae*, the agent of root and stem rot in soybean, is one of the smallest known elicitor-active oligosaccharides and consists of a 1,6- β -glucan backbone carrying 1,3- β -branches (Sharp *et al.* 1984). The HG elicitor has been shown to be the most active elicitor capable of promoting phytoalexin accumulation in soybean (Sharp *et al.* 1984). Based on the wide array of existing glucans, structural characterization of active glucan elicitors over twenty years ago established the minimum structural element required responsible for eliciting defense responses in legumes by oligoglucosides.

Plant receptors responsible for perceiving glucan elicitor signals during defense processes leading to phytoalexin production in legumes have been studied in detail. High affinity β -glucan-binding proteins (GBPs) found in legumes have been extensively studied as putative receptors in order to understand the signal pathways involved in plant-pathogen interactions. Studies have shown that most legumes possess binding sites for the hepta- β -glucoside elicitor (Cheong *et al.* 1991; Cosio *et al.* 1990; Côté *et al.* 2000). The defense mechanisms studied most frequently in legumes containing the high affinity binding sites for the β -glucan elicitor is phytoalexin production (Cheong *et al.* 1991; Cline *et al.* 1978; Cosio *et al.* 1996; Côté *et al.*

2000; Ebel *et al.* 1986). Binding studies in soybean and the model legume, *Medicago truncatula*, have shown β -glucan-binding proteins (GBPs) are present in both species and are capable of recognizing the fungal cell wall elicitor with high specificity and affinity (Cheong *et al.* 1991; Cosio *et al.* 1990; Côté *et al.* 2000). Other attempts to detect high affinity sites or biological activity of β -glucan fractions and their homologues in species outside the Fabaceae family have failed (Côté *et al.* 2000).

Three disparate EST (expressed sequence tags) sequences represented as *MtKVO*, *MtKV2*, and *MtKV3* had been identified by Francois Côté in the Hahn lab representing a multigene family encoding putative glucan-binding proteins in *M. truncatula* and were found to be highly similar to the GBP identified in soybean. Recent studies in soybean have also shown the putative GBP elicitor receptor has enzymatic activity, specifically the ability to hydrolyze β -1,3-glucans found in cell walls of pathogenic fungi (Fliegmann *et al.* 2005). The most abundant polysaccharides commonly found in fungal cell walls are polysaccharides predominately made of a large β -1,3 glucan backbone (Bartnicki-Garcia 1968). β -1,3 glucanases have been targets in understanding hydrolytic enzymes involved in fungal cell wall morphogenesis during growth and development and during fungal infection (Fisher *et al.* 2000; Mouyna *et al.* 2002). Identification of genes encoding fungal β -1,3 glucanases led to the classification of the soybean and the French bean GBP as members of Family GH-81 glycoside hydrolases (Henrisatt *et al.* 1996). Members of the GH-81 family share two conserved peptide domains which have been shown to be responsible for the hydrolysis of β -1,3 glucans (Baladrón *et al.* 2002; Mouyna *et al.* 2002). Mutant studies have shown the loss of β -1,3 glucanase activity in microbial hydrolases and in the soybean GBP occurring after amino acid variations within the two putative glucanase domains (Baladrón *et al.* 2002; Fliegmann *et al.* 2004; Henrissat 1991; Mouyna *et al.* 2002).

My initial goals in this study were to identify full length cDNA sequences of genes *MtKVO*, *MtKV2*, and *MtKV3* based on unpublished EST (expressed sequence tags) contig sequence analysis performed by Côté that represented a putative *M. truncatula* GBP gene family. Based on the cloning results of *MtKV3* full-length cDNA (unpublished data) using A17 wildtype leaf tissue, I showed in this study that *MtKV3* is currently represented as *MtGBP4* (DQ190943) in the Genbank database (Leclerq *et al.* 2007). Unpublished EST contig sequence results of *MtKV2* and *MtKV0* performed by Côté are now represented as *MtGBP1* and 3 (DQ190940 and DQ190942), respectively. I studied and characterized the expression patterns of *MtKV0*, *MtKV2*, and *MtKV3* by reverse transcriptase Real Time PCR in eight, different wild type- A17 *M. truncatula* tissues. In addition, I set out to characterize EMS (ethyl methylsulfonate) treated *M. truncatula* A17 TILLED (targeting induced local lesions in genomes) populations while working with collaborators at The University of California at Davis (UCD) in order to identify mutant lines containing mutations in *MtKV3*. I obtained three *mtkv3* seed lines from UCD and performed full-length cloning and sequencing of *MtKV3* in the mutant lines which led to the identification of single nucleotide polymorphisms (SNP) encoding amino acid substitutions within this gene. Based on *MtKV3* sequence homology with the soybean GBP elicitor receptor ortholog, my goal was then to determine whether wild type *MtKV3* contained glucan hydrolytic activity as described for the soybean GBP (Fliegmann *et al.* 2004). Furthermore, I wanted to characterize and study the variations of β -1,3 glucanase activity, if any, within *mtkv3* TILLED populations based on the identified SNP encoding amino acid substitutions.

Using a heterologous expression system in tobacco (*Nicotiana benthamiana*) leaves followed by analysis of hydrolyzed glucan oligosaccharides by HPLC and MALDI TOF-MS technique, I confirmed *MtKV3* β -1,3 glucan hydrolase activity. Two of the three *mtkv3* lines

had amino acid substitutions within the putative conserved glucan hydrolase domains. I showed loss of activity in one *mtkv3* line suggesting that the amino acid change within the putative glucosidase domain was responsible for the loss of hydrolytic activity.

CHAPTER TWO

MATERIALS AND METHODS

Plant material and RNA extractions

The initial lots of ecotype A17 seeds were bulked up by the following procedure: The A17 pods were broken, seeds were removed and acid scarified in concentrated H₂SO₄ for 7 min., and then rinsed 3 times with sterile dH₂O. Seeds were then surfaced-sterilized with ~50% (v/v) Clorox bleach+dH₂O for 7 min. and followed by thorough rinsing with sterile dH₂O. Sterile seeds were then placed on sterile H₂O-agar plates (1 g of agar per 100 mL of dH₂O), parafilm, and placed in a dark, cold room at 4 C for ~2 weeks. After 2 weeks, seedlings were planted in pots and placed in a growth chamber at 24-25 C with a 16 hr. day/8 hr. night photoperiod. Tissue collected included young leaves, old leaves, young petioles, old petioles, hydroponically grown leaves, apical buds, flowers, and roots and were immediately frozen in liquid nitrogen and stored at – 80 C. Young tissue was collected within two weeks after planting seedlings; old tissue was collected ~ 5-7 weeks after seedling planting. Seedlings were grown for root and hydroponic leaves in a caisson humidifying container with 9 L growth media after acid scarification. Roots and hydroponic leaves were collected after ~3 weeks. Tissue was immediately frozen in liquid nitrogen and stored at -80 C. RNA was extracted from tissue using Qiagen RNeasy Mini Kit (Cat. No. 74104).

Expression of *MtKV0, 2, and 3*: cDNA synthesis and qRT-PCR analysis

To confirm absence of RNA degradation prior to cDNA synthesis, the integrity and size distribution of the 28 S and 26 S rRNA was observed using 1 µg total RNA obtained from Qiagen RNeasy Mini Kit protocol and running RNA samples on a 1.2 % agarose ethidium

bromide gel. cDNA synthesis from each tissue was prepared by Polymerase Chain Reaction (PCR) amplification using 2 µg RNA with Qiagen Omniscript RT Kit (Cat. No. 205111); the amplification was 2 min. at 94 C followed by 29 cycles at 30 s at 94 C, 30 s ramping at 52-59 C, 1 min. at 72 C, and extension time was at 72 C for 6 min. Primers generated for *MtKV0*, *MtKV2*, and *MtKV3* were designed based on sequences obtained from Michael G. Hahn, -The Institute of Genomic Research (TIGR) (<http://www.medicago.toulouse.ifra.fr/Mt/EST/>), and/or from Genbank (<http://www.ncbi.nlm.nih.gov/>). SYBR-Green specific primer pairs (Table 1) for each *M. truncatula* gene was designed using Primer3 program software available online (<http://frodo.wi.mit.edu/>). To confirm the presence of cDNA templates, PCR was performed using a PTC-225 Peltier Thermal Cycler with gene specific primers cycling at 95 C for 3 min. followed by 35 cycles at 94 C for 20s, 55 C for 20 s, 72 C for 1 min. and 15 s, and extension took place at 72 C for 3 min. PCR products were run on 1.2 % agarose ethidium bromide gel to confirm presence of amplified products. After PCR confirmation of cDNA templates with gene specific primers, qRT Real Time PCR (quantitative reverse transcriptase) analysis took place using BioRad IQ 96-well PCR plates (Cat. # 223-9441) in a BioRad Icyler machine (Richmond, CA). PCR cocktail included a 20 µL total volume reaction mix using 5 µL cDNA, 2.5 µL of forward and reverse primers (each 2.5 µM working concentrations), and 10 µL BioRad 2X SYBR-Green IQ Supermix (Cat. # 170-8860). PCR cycle parameters were set to the following: Denaturing was set for 3 min. at 95 C followed by 40 cycles of 20 s at 95 C, 30s annealing at 56 C, and extension 72 C for 1 min. Melt curve analysis was performed at 95 C for 1 min. followed by 1 min. at 55 C, and photo capture with 80 cycles starting at 55 C for 10s and capturing every 0.5 degree centigrade change. Standardization of qRT PCR took place using a 14 degree annealing gradient starting at 52 C to find the optimal annealing temperature. After annealing

temperature optimization, cDNA samples were run in a 10X dilution series (1:10, 1:100, and 1:1000) with each dilution series run in triplicates to generate a standard curve to determine whether the amplification efficiencies (E) of primers were between 90-105 %. A linear regression line using serial dilutions series was plotted to determine whether sample data from serial dilutions had a coefficient of determination (R^2) value of ≥ 0.980 . Relative quantification of the three *M. truncatula* genes (*MtKV0*, *MtKV2*, and *MtKV3*) in the eight wild type tissue was determined using the *Mt* β -Actin reference gene and calibrated using root cDNA. Calculations of the relative fold expression in all samples were determined using the ΔC_T and $2^{-\Delta\Delta C_T}$ (Livak) Method.

Table 1: List of the *M. truncatula* GBPs primer sequences used in qRT PCR. +*M.t.* Actin- β were reference gene positive control primers, \pm *M. t.* Actin- β primers were gDNA contamination controls, and -*A.t.* (*Arabidopsis thaliana*) primers served as negative controls.

<u><i>Medicago truncatula</i> Gene</u>	<u>Accession #</u>	<u>cDNA primers</u>
<i>Mt</i> GBP1	DQ190940	KV2-F: 5'-AGCCTTGGCTTGATGGAAC KV2-R: 3'-CACCACCACCATCATTAGACC
<i>Mt</i> GBP3	DQ19042	KV0-F: 5'-ATGCTGATTCCGGTTTCG KV0-R: 3'-ACCCCATGCAGTGTCAATCT
<i>Mt</i> GBP4	DQ190943	KV3-F: 5'-ACCACCATCTCCAACAATGC KV3-R: 3'-AAGAAGTTTGTGGGGTCAGG
<i>Mt</i> Actin- β	AY372368	+Actin- β -F: 5'-CAAGTATTGTGGGCCGACCT +Actin- β -R: 3'-CTCTTTTGTATTGGGCTT \pm Actin- β -F: 5'-CAGATGCTGAGGATATTCAACCCC \pm Actin- β -R: 3'-CGACCACTTGCATAGAGGGAGAGG
<u><i>Arabidopsis thaliana</i> Gene</u>	<u>Accession #</u>	<u>cDNA primers</u>
- <i>At</i> Fut1	At1g19300	<i>At</i> Fut1-F: 5'-ATCTCAGCTCCGGTACGATG <i>At</i> Fut1-R: 3'-TGGGTGTTGGTGAAAGATCA

Cloning of full length *MtKV3* cDNA

Cloning of the 5' end of *MtKV3* was performed using TAKARA 5'-full RACE (rapidly amplifying cDNA ends) core set (Cat. # 6122) with A17 young leaf RNA. Phosphorylated primer 5'-/5Phos/CTAAGAATTGCATGG-3' was used in the initial PCR for cDNA synthesis. The four *MtKV3* primers used for the formation concatemers include S1-FF: 5'-CCATCTCCAACAATGCATCA-3', S2-2F: 5'-CAAAAATGGTGACCAACCTG-3', A1-3R: 5'-AACCGCAGATTGATGAAGG-3', and A2-1R: 5'-GAGGGGTGTGGATAGCAGAT-3'. After PCR, templates were run on a 1.2 % agarose ethidium-bromide gel. PCR products were recovered using Qiagen Qiaquick Gel Extraction Kit (Cat. # 28704) and sequenced using A2-1R: 5'-GAGGGGTGTGGATAGCAGAT-3' primer. To double check for PCR sequences errors, cloning of PCR 5' end products were ligated to Promega pGEM-T Easy Vector System using *E. coli* JM109 High Efficiency Competent Cells (Cat. 3 L2001), and transformants were plated on LB (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 10 g agar per 1 L of dH₂O) plates containing carbenicillin (100 µg mL⁻¹), IPTG (0.5 mM), and X-Gal (80 µg mL⁻¹) at 37 C overnight. Ligations were performed at 25 C for 2 hrs, 16 hrs at 16 C, and holding at 4 C. White colonies were selected and grown in 3 mL LB medium containing carbenicillin (100 µg mL⁻¹) and cultured at 250 rpm at 37 C overnight. Long-term cultures were prepared by pipetting 750 µL of culture into sterile microfuge tubes, mixing with 250 µL of 80 % (v/v) glycerol, and then placed in the -80°C freezer. Plasmids from 3 mL cultures were isolated using Qiagen QIAprep Spin Miniprep Kit (Cat. # 27106). After confirmation of positive inserts with single restriction digest using *Bst*ZI, plasmids were sequenced using universal T7 Promoter: 5'- TAATACGACTCAC TATAGGGC-3'.

TAKARA 3'-Full RACE Core set was used for cloning 3'-' *MtKV3* cDNA (A17 young leaf) using *MtKV3* adapter primer KV3_AD3': 5'-CTGATCTAGAGGTACCGGATCCG GCTTGGGCTTGGGATTCAGCTCTTA-3'. A Qiagen MinElute Reaction Cleanup Kit (Cat. # 28204) was used for recovery of 3' PCR products. Products were ligated into pUC118 DNA (TAKARA Bio Inc., Cat. # 3318). Prior to ligation, pUC118 plasmid DNA was *BamHI* digested, dephosphorylated using Promega alkaline phosphatase (Cat. # M182A), and recovered using Qiagen MinElute Reaction Cleanup Kit (Cat. # 28204). Ligation reaction solution was transformed using Promega *E. coli* JM109 Competent Cells (Cat. # 9052). White colonies were selected 18-24 hrs at 37 C on LB plates under ampicillin (50 µg mL⁻¹)/IPTG/X-Gal selection. Plasmids were collected and sequenced with universal M13 forward primer: 5'-GTAAAACGACGGCCAGT-3'. Deep freeze cultures were prepared using previous mentioned methods.

Cloning of *MtKV3* wild type and mutant A17 lines: *mtkv3_11427*, *mtkv3_20054*, and *mtkv3_12897*

Three mutant seed lines *mtkv3_11427*, *mtkv3_20054*, and *mtkv3_12897* and parental genomic DNA from each line were obtained from Doug Cook's laboratory at The University of California at Davis. Mutant lines were grown for tissue collection and RNA extractions; bulking of seed lots was done by previously mentioned methods. Cloning of the full length wild type *MtKV3* cDNA and the three *mtkv3* lines was performed using gene specific primers *MtKV3*-F: 5'-CACAAACAACCTCAGTTA-3' and *MtKV3*-R: 5'-CATAAGCAAACACAACA TAC-3'. High fidelity PCR was conducted using Invitrogen Platinum Pfx (Cat. # 11304-011) with denaturing for 2 minutes at 94 C followed by 35 cycles at 94 C for 20 s, annealing at 55 C for 30s, and extension at 68 C for 45 s followed by elongation for 10 minutes at 68 C. Insert DNA

from each line was ligated to pGEM-T Easy Vector Systems using previous methods for cloning of 5' and 3' *MtKV3* cDNA ends. Five milliliter cultures were grown using previous methods and 750 μ L from each culture line was used for preparing deep freeze stocks (previous methods). Plasmids containing cloned inserts were extracted from 3 mL cultures using a Qiagenprep Spin Miniprep Kit and single digests using *NotI* were performed for confirmation of inserts; plasmids were sequenced using gene specific primers spanning the entire gene using previous mentioned *MtKV3* forward and reverse primers and EBPKV35-F: 5'TGGTTAGGATTGCTTTGTTGC-3'.

Cloning of pCambia β *MtKV3* lines for heterologous expression

Agrobacterium tumefaciens strain GV3101PM90 containing vector pCambia35SLEGFPS2#4 (Figure 1) with a GFP tag located downstream from the cloning site was provided by Sivakumar Pattathil (Hahn laboratory) and used for heterologous expression of tobacco (*Nicotiana benthamiana*) leaves. pCambia35SLEGFPS2#4 plasmids were isolated and cut using restriction endonucleases *NcoI* and *Sall*. Double digestions took place at 37 C for 2 and .5 hours and run on a 1.2 % ethidium bromide gel to confirm the vector cleavage. Cut pCambia plasmids were dephosphorylated (previous methods) and ligated to *NcoI* and *Sall* digested pGEM-T Easy Vectors containing each clone of interest including *mtkv3_11427*, *mtkv3_20054*, *mtkv3_12897*, *MtKV3*, and pCambia35legfpNI (no insert) negative control plasmids. Transformation of the ligated clones was performed by heat shocking inserts into JM109 cells at 42 C for 50 s and plating the *MtKV3* culture lines and empty vector on kanamycin 50 μ g per mL⁻¹ LB plates overnight at 37 C. Single colonies from each line were grown at 37 C overnight in 3 mL LB medium containing kanamycin 50 μ g mL⁻¹. Plasmids were isolated and sequenced using *MtKV3* gene specific primers *MtKV3*-F: 5'-CACAAACAACCTCAGTTA-3', EBPKV35-F: 5'-TGGTTAGGATTGCTTTGTTGC, *MtKV3*-R: 5'-

CATAAGCAAACACAACA TAC-3', and green fluorescent protein gene specific primer EGFP-R: 5'-CGTTTACGTCGCCGT CCAGCTC-3'. Each of the pCambia35SMiKV3 plasmids and empty vector controls were transformed into *Agrobacterium tumefaciens* GV3101PM90 via electroporation. Electroporation was conducted using 3 μ L plasmid per 100 μ L GV3101. The mixtures were transferred to 3 pre-cooled 0.15 cm electroporation cuvettes and were kept on ice. Cuvettes and holder were wiped off and placed quickly in the BRL Electroporation System. The system was set at 250 μ F, 200 Ohms, and the time of shock was 3.8 s. Shocked cells were rapidly placed into 1 mL of liquid YEP (10 g yeast extract, 10 g peptone, and 5 g NaCl per liter of dH₂O, pH 7.0) medium in culture tubes and placed on a 29 C shaker at 250 rpm for 3 hrs. Afterwards, 100 μ L of the YEP-cell mixture was aliquoted onto YEP plates containing rifampicillin (50 μ g mL⁻¹), gentamycin (25 μ g mL⁻¹), and kanamycin (50 μ g mL⁻¹). Plates were placed in dark at 27 C for 3 days. Single colonies from each plate were inoculated into 5 mL of liquid YEP medium containing rifampicillin (50 μ g mL⁻¹), gentamycin (25 μ g mL⁻¹), and kanamycin (50 μ g mL⁻¹) and placed on a 29 C-250 rpm shaker for 2 days. Remaining cultures from each line (750 μ L) were pipetted into sterile microfuge tubes, mixed with 250 μ L of 80 % (v/v) glycerol, and placed in the -80 C.

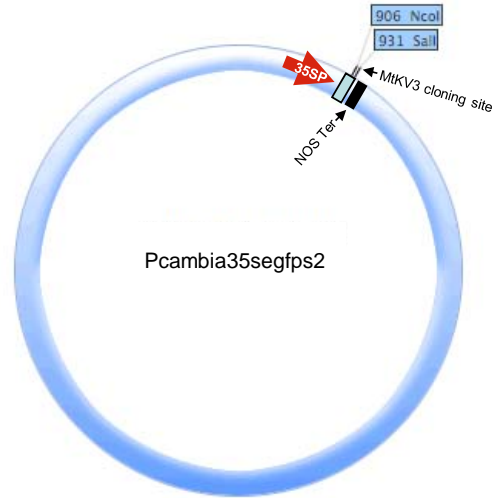


Figure 1: pCambia vector used for heterologous expression in *Nicotiana* leaves. The vector contains a 35S promoter (red arrow). *MtKV3* cloned lines were ligated between the *NcoI* and *SalI* sites denoted by black arrows. Four, full-length *MtKV3* clone lines were constructed consisting of wildtype *MtKV3* and mutant lines *mtkv3_11427*, *mtkv3_20054*, and *mtkv3_12897*.

Inoculation of pCambiaβeta*MtKV3* cultures for leaf infiltration

From the cultures grown from single colonies of *mtkv3_11427*, *mtkv3_20054*, *mtkv3_12897*, wild type *MtKV3*, and empty vector pCambia35legfpNI in YEP rifampicillin (50 $\mu\text{g mL}^{-1}$), gentamycin (25 $\mu\text{g mL}^{-1}$), and kanamycin (50 $\mu\text{g mL}^{-1}$) for 2 days at 27 C in dark, 2 mL of culture from each line was added to 20 mL LB medium containing antibiotics and cultured for 16 hrs under same conditions mentioned above. Twenty-two milliliter cultures were spun at 6000 g for 20 min. After centrifugations, pellets were resuspended with 15 mL of filter

sterilized infiltration media (2.033 g MgCl₂, 1.952 g MES, pH 5.7 dissolve in 1 L deionized H₂O) containing 20 μM final concentration of acetosyringone (29.4 g acetosyringone dissolved in 100 % ethanol, store at – 20 C). Cultures were then incubated on a shaker for 18-24 hrs at 27 C-250 rpm. After 18-24 hrs, aseptically grown cultures were transferred to sterile tubes and spun at 6000 rpm at 4 C for 20 min. using a Beckman J2-21 centrifuge. Pellets were resuspended with 15 mL infiltration media with 50 μL of 50 mM acetosyringone by gently inverting tubes, and the cultures were incubated for 21-24 hrs in dark 25C/250 rpm.

Transformation via leaf infiltration

After the 5th day, 3 mL cultures of each of the GV3101+*MtKV3* lines and empty vector control were placed in a sterile 15 mL Falcon Tube. Healthy 7 week old *Nicotiana* leaves were selected and each culture line was placed on a tobacco leaf using a 1 cc syringe with no needle. Without causing leaf injury and cross contaminating leaves, each culture line was infiltrated into the entire leaf. A volume of 2-4 mL from each cultured line was used for infiltration. Each leaf was properly labeled, and plants were placed in the growth chamber for three days. After 72 hrs, leaf tissue was immediately frozen in liquid nitrogen and stored at -80 C until needed.

Total protein isolation

Protein isolation was performed at sterile, 4 C conditions. Approximately 2 grams of frozen leaf tissue was ground in a cold mortar with 4 mL of cold Buffer (100 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 400 mM sucrose). After complete homogenization, inhibitors DTT (dithiothreitol) and PMSF (phenylmethanesulphonyl fluoride) were added to final concentrations of 1 mM and 0.1 mM, respectively. Small aliquots of homogenate were saved as total protein followed by differential centrifugation of the remaining homogenate samples. Soluble supernatant S₁ fractions were collected by 1000 g centrifugation of crude homogenate for 10 min.

at 4 C and aliquoted to 300 μ L samples. P₁ pellet (membrane bound) were resuspended in lysis buffer (150 mM NaCl₂ and 1% tritonX-100) followed by 3000 g centrifugation for 10 min. at 4 C to obtain soluble S₃ and pellet P₃ fractions. S₃ fractions were collected in the same manner as S₁ fractions and P₃ pellets were resuspended by the same methods for P₁ fractions. All soluble and pellet (S₁, P₁, S₃, and P₃) fractions were stored at -20 C.

***MtKV3* β -glucan hydrolase enzymatic studies**

Assays for 1,3- β -glucanase activity were conducted using Sigma laminaran (Cat. #. L9634) as substrate. Laminarin is a β -1,3 linked glucan with 2-3 % β -1,6 branched side chains. In a Falcon tube, 7.0 mg laminaran was incubated with 25 mg/mL NaCNBH₃ in 1 M NH₄OH at 25 C overnight to reduce all terminal sugars. Enzymatic assays were performed in sterile 1.5 mL eppendorf tubes using 25 mM MES/KOH buffer (pH 6.5), 335 μ g laminaran, and with S₁, P₁, S₃, and P₃ protein preps each at concentrations of 10 μ g, 50 μ g, and 100 μ g, respectively. Protein quantifications were performed using a 280 nm absorbance assay. Total reaction volume for each fraction was 500 μ L. After 24 hrs incubation at 37 C, protein samples were denatured in a 65 C water bath for 10 min., centrifuged for 5 min. at 8000 g, and 0.2 μ M sterile filterized before HPLC analysis.

HPLC and MALDI analysis

Analysis of the hydrolyzed laminarin substrate from the enzymatic activity of *MtKV3* using protein from the soluble and pellet (membrane bound) protein fractions used during the enzyme assays were collected using Dionex HPLC (high performance liquid chromatography) apparatus with a 75-Superdex column (75kDa). Substrate peaks were collected according to laminaran standards and eluted using 50 mM ammonium formate buffer (pH 5.0, formic acid) at a flow rate of 0.4 mL/min. Hydrolyzed laminarin substrate peaks were collected and lyophilized

overnight followed by two days of resuspending in 5 mL deionized H₂O and freeze drying overnight. Final polysaccharide products were resuspended in 5 µL of water to methanol in a ratio of 1:1. For hydrolyzed glucan analysis, samples were then prepared in a 1:1 ratio with Superdex DHB matrix (2,5- dihydroxybenzoic acid). Superdex matrix solution was prepared using a 9:1 (v/v) mixture of solution A (10 g/L DHB in 20 % {w/v}) to solution B (10 g/L 5-methoxysialic acid in 50 % methanol {w/v}). Hydrolyzed oligosaccharides were analyzed using MALDI Voyager-DE. MALDI mass spectrometry took place using the positive digitizing mode analyzing molecules within an atomic mass range (amu) of 500-3000 Da.

CHAPTER THREE

RESULTS

Full-length cDNA *MtKV3* sequence

The full length cDNA sequence of GBP *MtKV3* was obtained using RNA from young leaves of wild type *Medicago truncatula* A17 and RACE 5' and 3' techniques (see Methods). Figure 2 shows the full length cDNA *MtKV3* sequence that I obtained (DCKV3) compared to the partial *MtKV3* sequence data (CKV3) that had been obtained by Francois Côté in the Hahn lab (unpublished data). Comparisons between the two sequences reveal my *MtKV3* full length cDNA clone containing an additional 5 amino acids (MHHFT) at the 5' end of *MtKV3*. Figure 2 also shows significant differences between the two *MtKV3* sequences at amino acid positions 543-556.

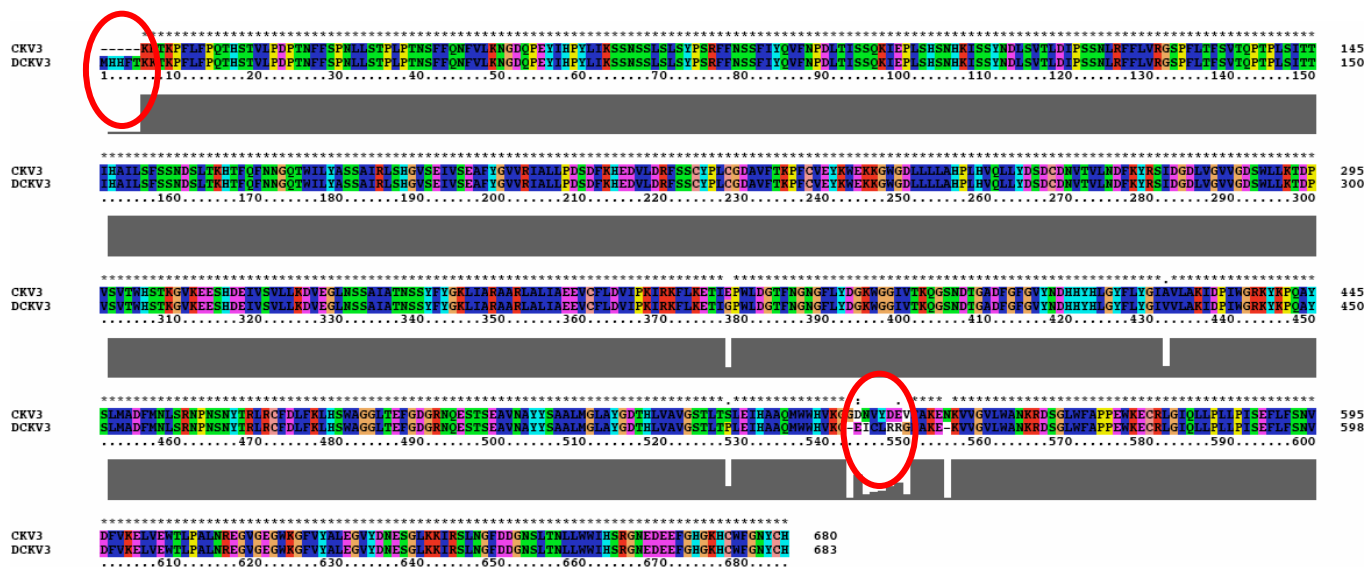


Figure 2: Amino acid alignments of *MtKV3* full length cDNA sequence results (DCKV3) compared to *MtKV3* sequence provided by Côté and Hahn (CKV3). Significant differences in alignments between the two sequences are denoted by red circles.

RT Real-Time PCR

Quantitative Real-Time PCR (qPCR) was performed to determine the relative expression levels of GBP genes *MtKV0*, *MtKV2*, and *MtKV3* in eight different tissues of wild type *M. truncatula*. The initial qPCR experiment was performed in a single 96 well plate to optimize and determine the fold difference of expression of *MtKV0*, *MtKV2*, and *MtKV3* compared to the relative expression of the reference gene *Mtβ-Actin* in wild type root cDNA.

Figure 3 shows the amplification curves of the dilution series of wild type *M. truncatula* root cDNA (2 μg total RNA) using gene specific primers (Table 1) for *Mtβ-Actin*, *MtKV0*, *MtKV2*, and *MtKV3*, respectively. The plots show an optimized qPCR assay with a 10X dilution series being evenly spaced and separated ~ 3.5 cycles apart as expected when an approximate doubling of product takes place during each cycle.

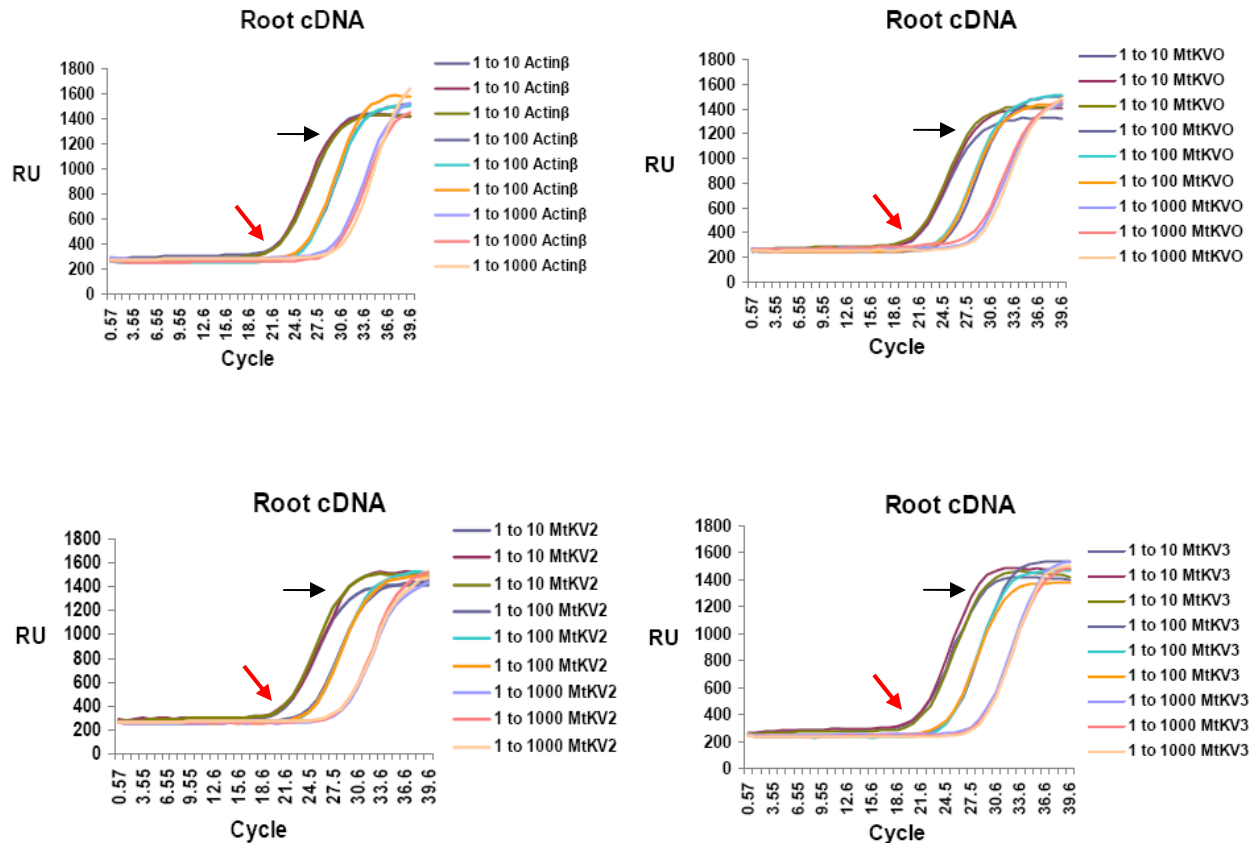


Figure 3: Amplification curves for the products obtained using the *Mtβ*-Actin, *MtKV0*, *MtKV2*, and *MtKV3* primers. For the qPCR, gene primers were used together with wild type root cDNA using a three point 10X dilution series run in triplicates. Plots show the fluorescence curves of the dilution series being separated ~3.5 cycles apart. The threshold cycle (C_T) where amplified products yield a detectable fluorescent signal above background is denoted by red arrows. The non-exponential phase (black arrows) indicates the point at which the PCR reagents are consumed and products are no longer being doubled in each cycle.

The R² value of the standard curve generated from the 10X serial dilutions (Figures 3) was 0.984 showing that the experimental data fit a linear regression line. Figure 4 shows a standard curve with the C_T (during exponential phase) plotted against the log of the starting quantity of cDNA (2 μg RNA) for the triplicate 10X dilution series using all four *M. truncatula* primer pairs.

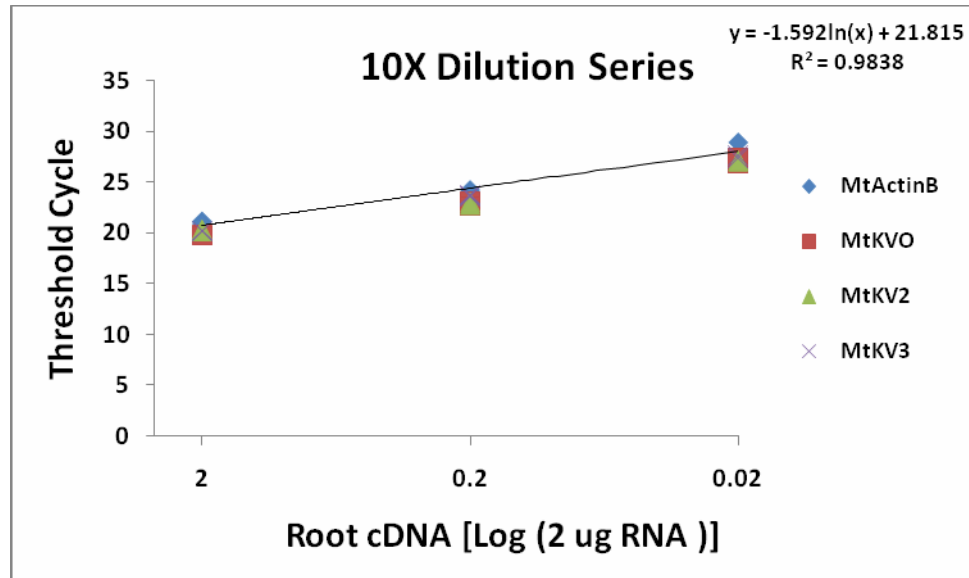


Figure 4: Linear regression line of triplicate runs of 10X serial dilutions using root cDNA (2 μ g RNA) with the four *M. truncatula* primer pair during qPCR. Amplification efficiencies ($E = 10^{-1/\text{slope}}$) of gene primers were ~90 % with the coefficient of determination (R^2) value of 0.982.

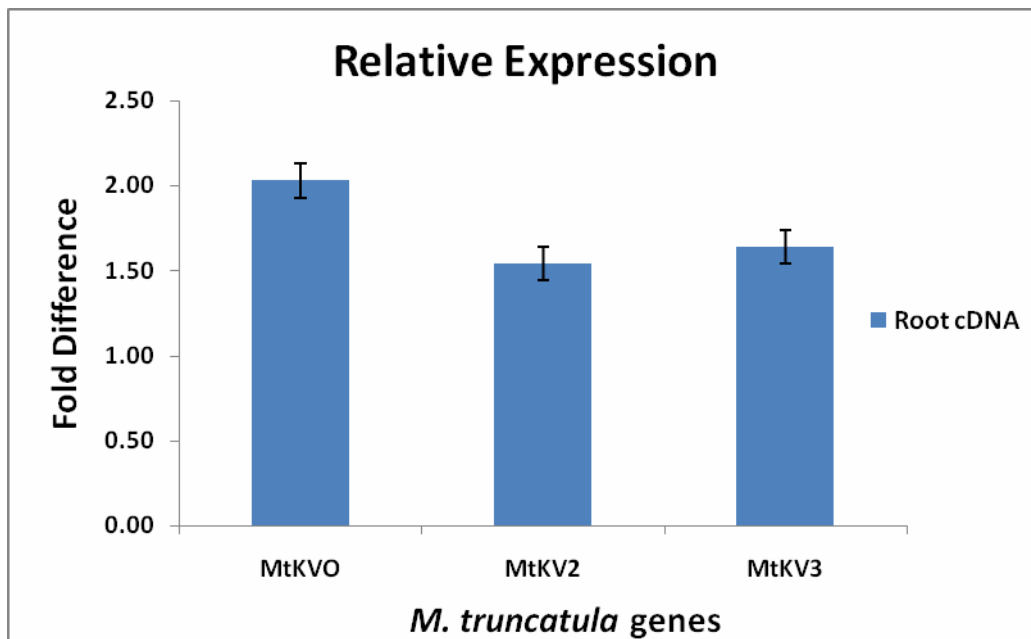


Figure 5: Difference in expression of each of the GBP *M. truncatula* genes compared to the expression of β -Actin in root cDNA. All three genes were more highly expressed in root than β -Actin. The expression of *MtKV0*, *MtKV2*, and *MtKV3* is 2.03, 1.54, and 1.64, fold higher respectively, in wildtype root cDNA than β -Actin expression.

The fold difference of root expression of *MtKV0*, *MtKV2*, and *MtKV3* was compared to the *Mtβ-Actin* (Figure 5). All three genes showed a slightly higher fold of expression in root compared to *Mtβ-Actin*. Figure 5 shows *MtKV0*, *MtKV2*, and *MtKV3* having a 2.03, 1.54, and 1.64, respectively fold higher expression in roots than *Mtβ-Actin*.

The relative expression of *MtKV0*, *MtKV2*, and *MtKV3* in other wild type cDNA samples using RNA prepared from young leaves, old leaves, young petiole, old petiole, hydroponically-grown leaves, apical buds, and flowers were determined in a subsequent qPCR assay based on the optimized qPCR protocol shown in Figures 4-5. The differences in fold expression levels of *MtKV0*, *MtKV2*, and *MtKV3* in all samples were compared to *MtKV0*, *MtKV2*, and *MtKV3* expression levels in root. The expression of the three *M. truncatula* genes in the seven samples were calibrated to root expression and normalized using the β -Actin reference gene (see Methods). The difference of expression of *MtKV0*, *MtKV2*, and *MtKV3* in all seven samples was considerably lower than the expression of each gene in root. *MtKV2* was poorly expressed in young and old leaf tissues showing a 344 and 169 fold lower level of expression, respectively compared to the *MtKV2* expression level in roots. *MtKV2* expression levels were significantly lower in all tissues except young petiole compared to the expression levels in root. Expression of all three *MtGBP* genes was much lower in young and old leaf samples compared to the expression levels observed in root. *MtKV0* showed poor expression levels in young petioles and apical buds. *MtKV3* levels of expression did not vary significantly among petioles, flowers, hydroponic leaves, and apical buds.

Table 2: Relative quantification of *MtKV0*, *MtKV2*, and *MtKV3* expression in seven wild type tissues relative to the expression of the three GBP genes in wild type root. All three genes showed a lower fold expression in all samples compared to root expression. *MtKV2* was poorly expressed in the seven tissues except young petioles while expression levels of *MtKV0* were the lowest in young petioles. *MtKV3* showed a more constitutive pattern of expression in tissues aside from young and old leaf.

	<i>MtKV0</i>	<i>MtKV2</i>	<i>MtKV3</i>
<u>Sample</u>	<u>Fold Difference Lower</u>	<u>Fold Difference Lower</u>	<u>Fold Difference Lower</u>
Young Leaf	16-17 ± 0.9	344-345 ± 0.1	9-10 ± 0.4
Old Leaf	19-20 ± 0.9	169-170 ± 0.7	17-18 ± 0.8
Young Petiole	51-52 ± 0.9	6-7 ± 0.1	4-5 ± 0.1
Old Petiole	1-2 ± 0.9	30-31 ± 0.1	1-2 ± 0.3
Hydroponic Leaf	8-9 ± 0.9	24-25 ± 0.6	3-4 ± 0.1
Apical Bud	20-21 ± 0.9	58-59 ± 0.2	6-7 ± 0.1
Flower	3-4 ± 0.9	76-77 ± 0.4	2-3 ± 0.1

Growing of *mtkv3* lines F₁ and F₂ generations

Three *MtKV3* mutant seed lines from UCD collaborators were obtained to grow mutant populations in order to clone full length *mtkv3* cDNAs (synthesized from RNA in young leaves) and identify the SNPs encoding amino acid variations in this gene. Successful F₁ and F₂ seed populations were collected from *mtkv3_11427* and *mtkv3_12897* mutant lines. Figure 6 and 7 show healthy *mtkv3_11427* and *mtkv3_12897* plants, respectively. Both plants showed no phenotypical variation from wild type except for the loss of prominent magenta markings commonly found in wild type A17 trifoliolate leaves. I was unable to successfully grow *mtkv3_20054* populations from the seed lot provided by UCD collaborators; no F₁ seed

population was collected from this line. Figure 8 shows *mtkv3_20054* 1-month-old seedling with a severe dwarf phenotype.



Figure 6: Healthy, 2 month-old *mtkv3_11427* plant.



Figure 7: Flowering *mtkv3_12897* plant.



Figure 8: The *mtkv3_20054* seedling has a severe dwarf phenotype.

Sequencing of full length *MtKV3*, *mtkv3_11427*, *mtkv3_20054*, and *mtkv3_12897* pCambia35SLEGFPS2 clones for heterologous expression

Cloning of the full length wild type and three *mtkv3* cDNA lines into pCambia35SLEGFPS2 vectors was performed for heterologous expression in *Nicotiana* cells for studying *MtKV3* β -1,3 glucan hydrolase activity in wild type and mutant lines. Full length cDNA cloning of wild type A17 *MtKV3*, *mtkv3_11427*, and *mtkv3_12897* was achieved (see Methods). Due to the unsuccessful collection of *mtkv3_20054* populations, I was unable to collect tissue for RNA isolation to perform full-length cDNA cloning in this line. Therefore, the full length cloning of *mtkv3_20054* was performed using parental gDNA provided by UCD collaborators. Isolated plasmids (Figure 9) from the pCambia35SLEGFPS2 vectors carrying the full-length *MtKV3* clones used for heterologous expression in *Nicotiana* leaf cells yielded ~2250 bp products.

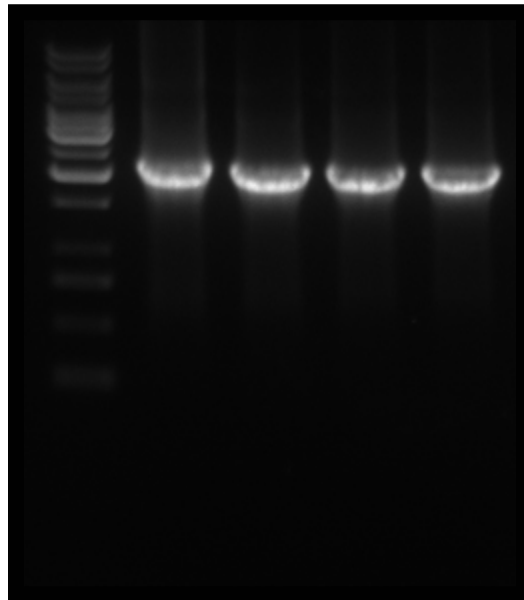


Figure 9: 1.2 % agarose gel of ~2250 bp full-length *MtKV3*A17, *mtkv3_11427*, *mtkv3_20054*, and *mtkv3_12897* clones, respectively. Plasmids were isolated from pCambia35SLEGFPS2 vectors used for heterologous expression of *MtKV3* in *Nicotiana* leaf cells for studying β -1,3 glucan hydrolase activity. Gel shows PCR of plasmids using gene specific forward primer at the 5' end of *MtKV3* and using reverse primer for the GFP gene located downstream from the cloning site in the pCambia vector (see Methods).

Full-length sequences of plasmids (Figure 9) containing the four *MtKV3* clones were aligned with *M. truncatula* GBP4 (Figure 10). Results in Figure 10 show the wild type *MtKV3* sequence missing 322 bp from the start at the 5' end yielding a loss of 104 amino acids. However, based on the similar banding patterns of the plasmid PCR products (Figure 9) of the full length *MtKV3* plasmid compared with the full length mutant *MtKV3* clones, suggests this region was likely present in the wild type clone and was a result of sequencing errors. No introns appear within *MtKV3* based on the similar sequence alignments of the full length cDNA *MtKV3* clones compared to the full length gDNA *mtkv3_20054*. Sequence results of interest were found in the *mtkv3_11427* and *mtkv3_12897* mutants (Figure 10). Nucleotide substitutions yielding amino acid variations were found (Figure 10-11) in *mtkv3_11427* at position 425 showing an extra aspartic acid (D) residue and *mtkv3_12897* showed a glutamic acid to alanine (E→A) substitution at amino acid position 503. These two amino acid changes lie within two regions (amino acid positions 424-430 and 503-510 in *MtKV3*) previously identified as being part of the glucan hydrolase domains in the French bean and soybean GBP and microbial GH-81 hydrolases (Baldrón *et al.* 2002; Fliegmann *et al.* 2004; Mouyna *et al.* 2002).

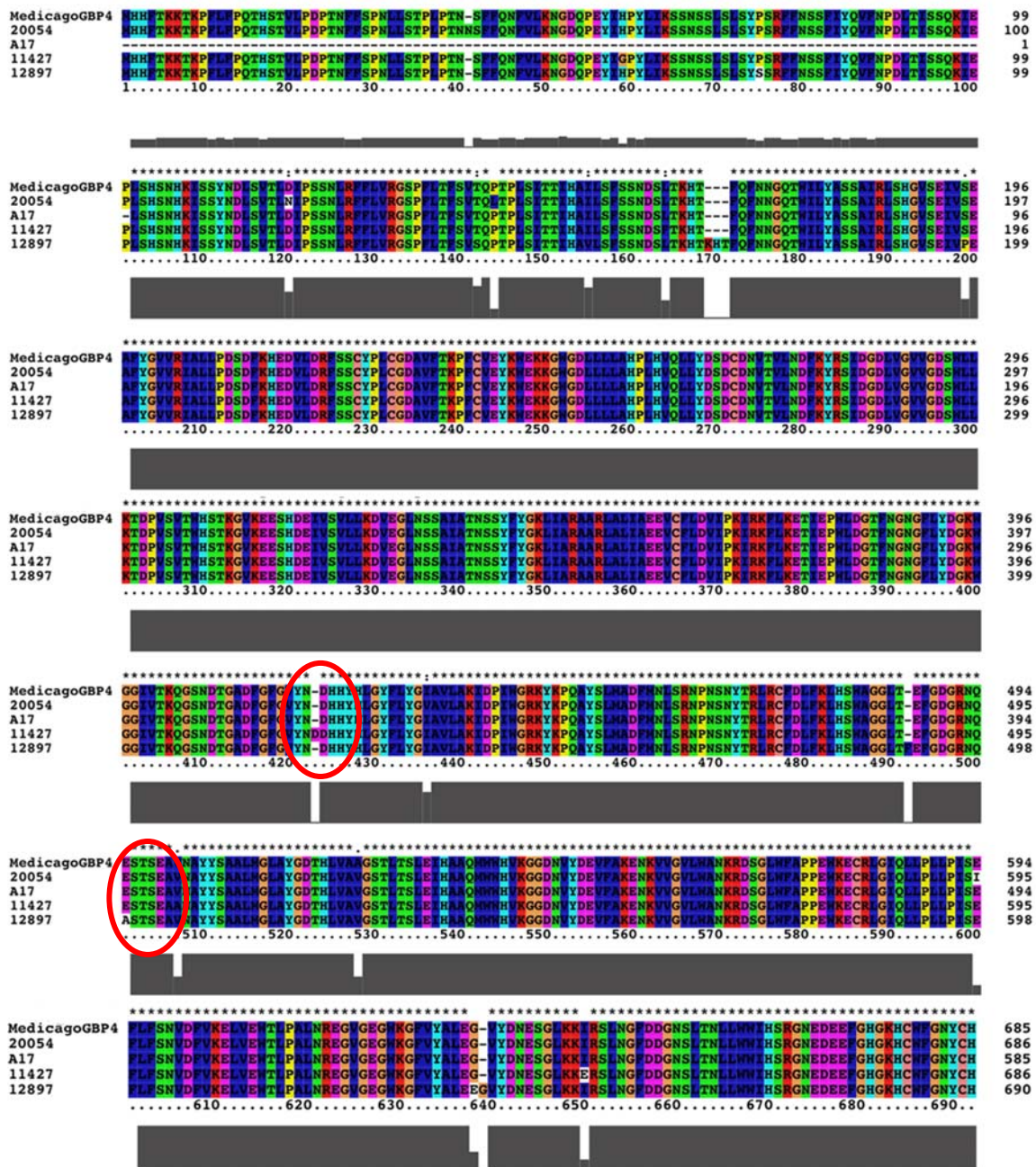


Figure 10: ClustalX protein alignment of sequences of pCambia35SLEGFPS vectors containing cloned inserts of *MtKV3* A17 and *mtkv3* mutant lines. Clones are aligned with the *M. truncatula* GBP4 sequence found in Genbank. Amino acid variations within the putative glucan hydrolase domains found in *mtkv3_11427* and *mtkv3_12897* mutants are denoted by red circles.



Figure 11: Protein alignments of the two putative-gluconase regions located in *MtKV3* lines and in soybean. *mtkv3_12897* has an E→A substitution at position 503 and *mtkv3_11427* shows an extra D insertion at amino acid position 425. Peptide regions YNDH and ESTSE represent putative gluconase domains also found the French bean GBP and in microbial β-1,3 hydrolases.

β-1,3 glucan hydrolase activity of *MtKV3* lines

Heterologous expression of the wild type *MtKV3* and three *MtKV3* mutants in *Nicotiana benthamiana* leaf cells was achieved (see Methods). Heterologous expression technique was used to determine whether the wild type *MtKV3* showed enzyme activity by hydrolyzing 1,3-β-glucans found in the laminarin substrate, and to determine the effects, if any, of the amino acid variations in *mtkv3* mutants on possible glucan hydrolase activity. To determine levels of enzyme activity in each *MtKV3* line, 10, 50, and 100 μg, respectively of protein from the soluble and pellet (membrane bound) fractions (see Methods) were used during incubations. All *MtKV3* lines using soluble fractions S₃ and pellet fractions P₁ and P₃ (see Methods) showed no hydrolysis of the laminarin substrate as evidenced by HPLC analysis of the products suggesting *MtKV3* was either not present or inactive in these protein preparations (data not shown). Glucanase activity was observed in *MtKV3*, *mtkv3_20054*, and *mtkv3_12897* lines (Figure 12) using 10 μg, 50 μg and 100 μg of total protein preparations and S₁ soluble fractions for the enzyme assays (data not shown for 50 μg and 100 μg assays using total and S₁ fractions). However, when 10 μg and 50

μg of total and S_1 protein fractions were used during incubations, I observed no hydrolase activity (Figure 13) for *mtkv3_11427*. HPLC results in Figure 12 show *MtKV3*, *mtkv3_20054*, and *mtkv3_12897* glucanase activity with a distinct size reduction in the laminarin substrate peak in the HPLC profiles using 10 μg of S_1 protein during incubations. Figure 13 shows no reduction of the laminarin substrate peak in the HPLC profile when 10 μg of *mtkv3_11427* S_1 protein was used during the enzyme incubation. Thus, Figure 13 results show no glucan hydrolase activity present in this mutant line. Empty vector and untransformed tobacco controls showed no size reduction of the substrate in any of the soluble or pellet fractions using 10, 50, or 100 μg protein concentrations during the incubation. Figure 13 shows an intact laminarin substrate peak collected \sim 20 minutes using 10 μg S_1 in untransformed tobacco and empty vector protein preps during incubations, respectively. HPLC analysis showed the laminarin substrate control standard (Figure 12) with no protein added during incubation also eluting from the column after 20 minutes.

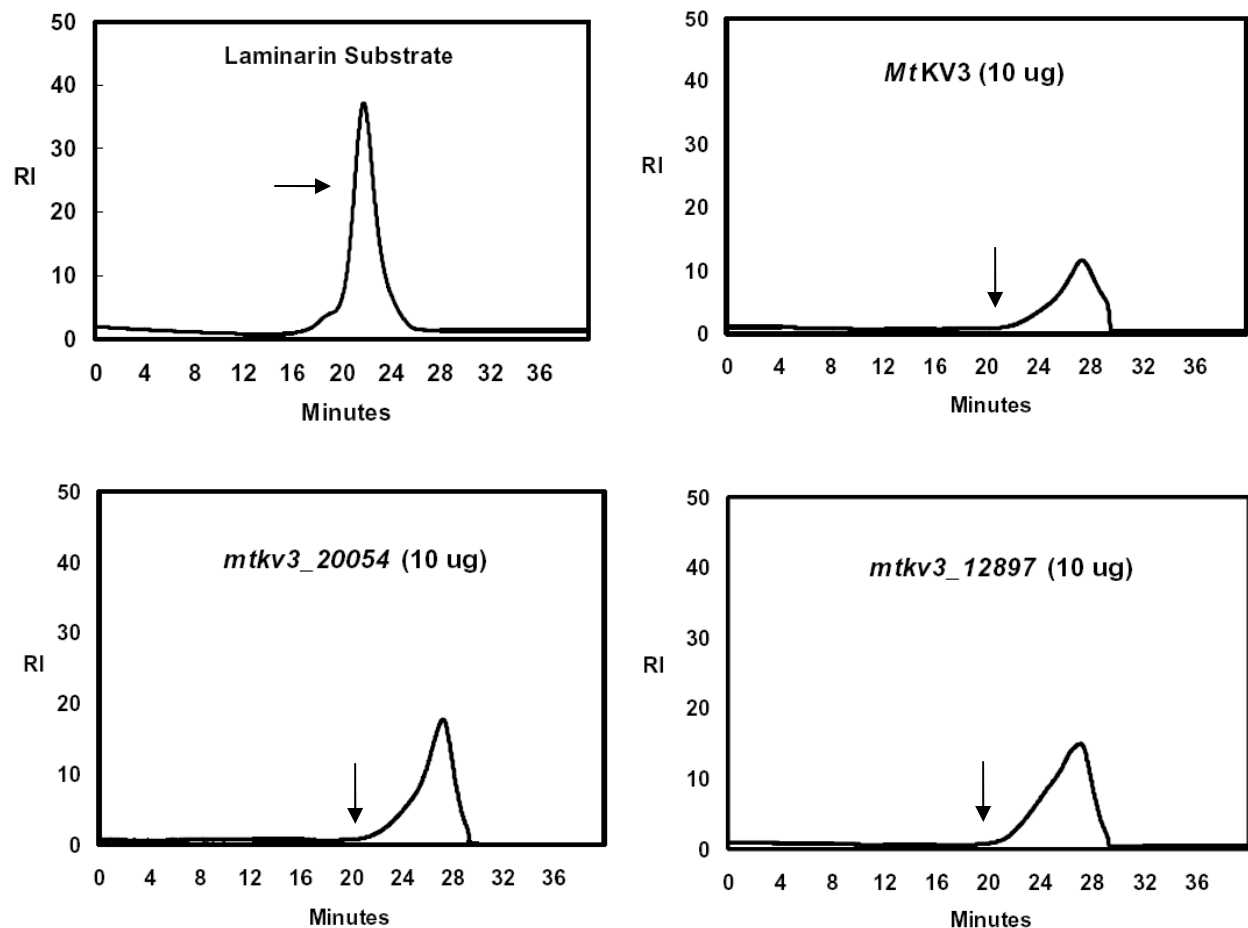


Figure 12: HPLC profiles showing elution of laminarin substrate and the hydrolyzed peaks in the *MtKV3* active lines. HPLC column analysis shows the β -1,3 glucan laminarin standard eluting from the column at ~ 18 minutes. Collection of laminarin took place ~ 18-28 minutes. *MtKV3*, *mtkv3_20054*, and *mtkv3_12897* profiles show glucanase activity using 10 μ g S₁ protein during incubations; the laminarin substrate peak is not present at 20 minutes and the hydrolyzed products were collected at ~ 23-28 minutes. Samples were applied to a C-75 Superdex HPLC column and eluted with 50 mM ammonium formate buffer over 40 minutes with a flow rate of 0.4 mL/min.

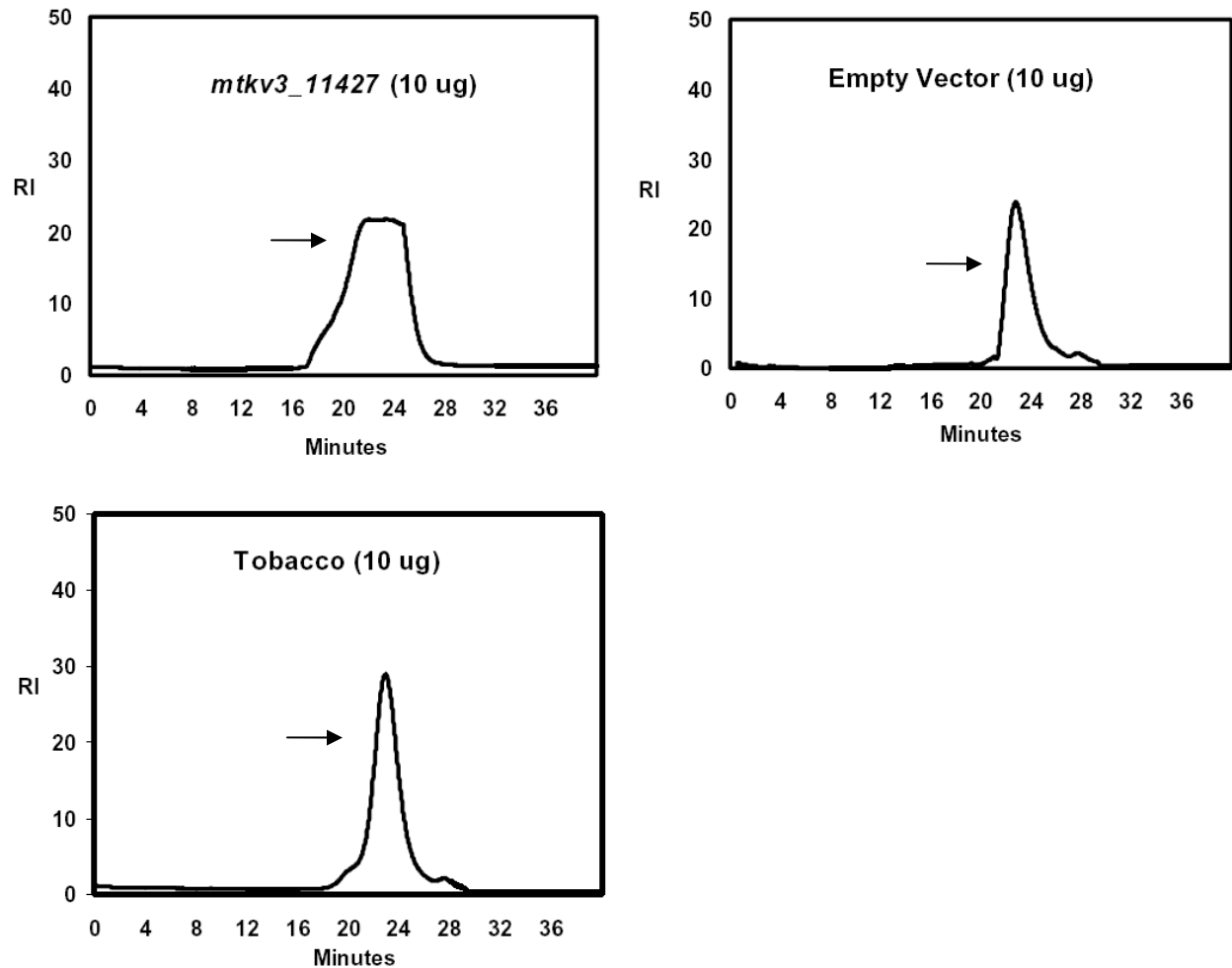


Figure 13: HPLC column analysis of the inactive *mtkv3_11427* mutant and negative controls. HPLC profiles show no glucan hydrolase activity in the *mtkv3_11427* mutant, empty vector negative control, and the untransformed tobacco control using 10 μ g of S_1 protein during incubations. All profiles show no size reduction of the laminarin substrate (black arrows) which elutes from the HPLC column at ~20 minutes. Substrate peaks were collected from the inactive lines ~18-28 minutes.

MALDI analysis of reduced oligosaccharides

MALDI (matrix-assisted laser desorption ionization) mass spectrometry was used to analyze the peak products collected during the HPLC column analysis (Figure 12-13). To characterize the glucans liberated by hydrolysis of the laminarin substrate by *MtKV3* glucanases, MALDI mass spec took place analyzing the molecular weights of products within an amu (atomic mass unit) range of 500-3000 Da which would yield hydrolyzed sugar products with a DP (degree of polymerization) of ~4-18. Figure 14 shows MALDI data of the glucans liberated

by the glucanase active wild type *MtKV3* and glucanase active *mtkv3_20054* and *mtkv3_12897* mutant lines. As would be expected if hydrolysis of laminarin β -1,3 glucans occurred, the MALDI data (Figure 14) shows oligosaccharides liberated from the wild type *MtKV3*, *mtkv3_20054*, and *mtkv3_12897* samples within the expected amu range. The spectrometry data in Figure 14 show all *MtKV3* active lines liberating sugar products within a range of DP 4-10. Thus, oligosaccharides within a range of \sim 850-1661 amu were observed in active *MtKV3* lines during MALDI analysis. No evidence of β -1,3-glucanase activity in the *mtgbp4_11427* mutant line, untransformed tobacco, or empty vector controls was observed during HPLC analysis (Figure 13). Therefore, I was not expecting to identify any oligosaccharides within a range of DP 4-18 in these samples. Figure 14 shows no liberated glucan products within a range of DP 4-18 in the laminarin standard, untransformed tobacco control, empty vector control, and the *mtkv3_11427* mutant line. Liberated glucan products from the *MtKV3* glucanase active lines were compared with oligosaccharide standards with molecular weights equivalent to glucans with DP 5-10 (Figure 14).

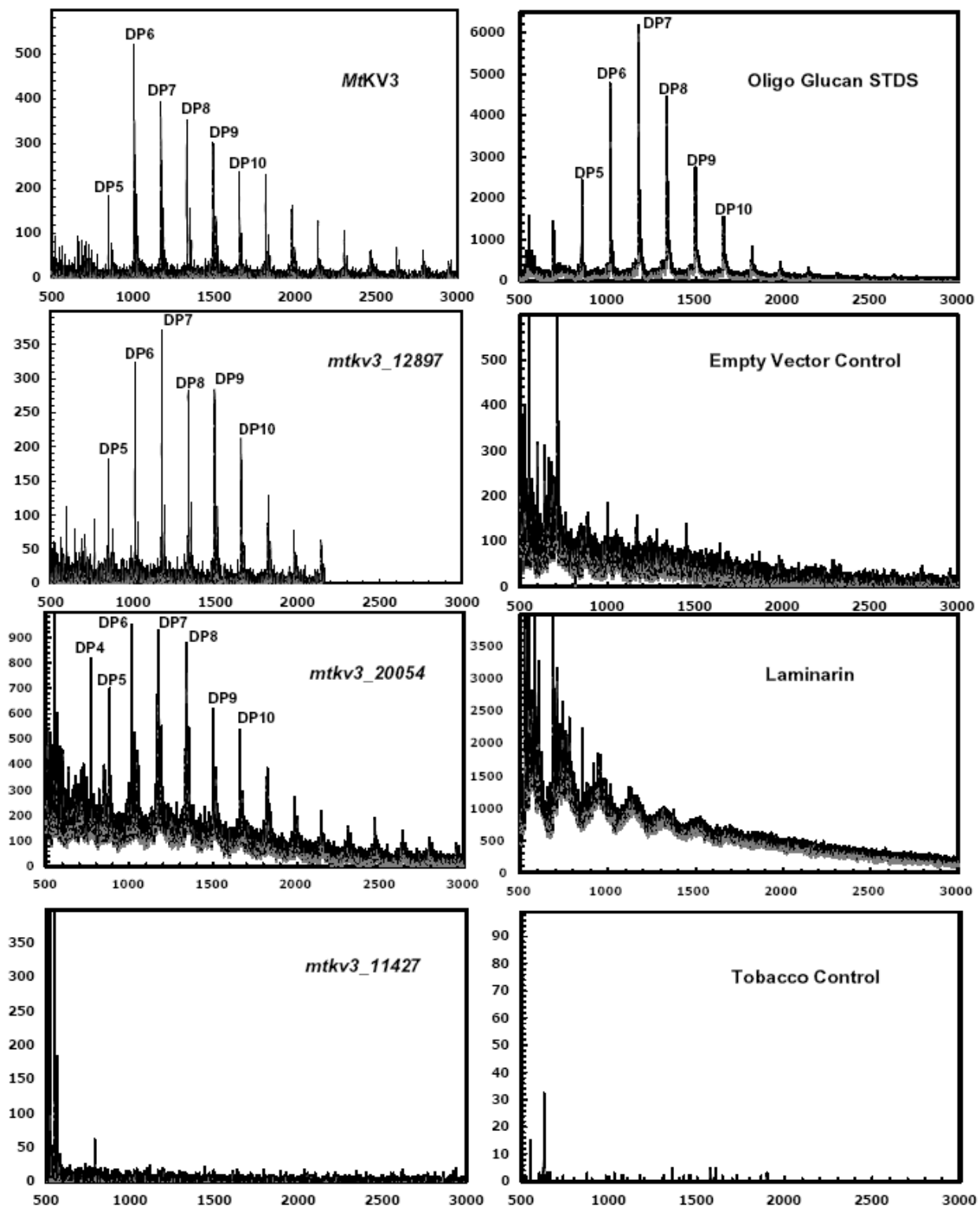


Figure 14: Degree of polymerization (DP) of glucan oligosaccharides recorded by MALDI TOF-MS from peaks collected during 75-Superdex HPLC analysis of glucan hydrolases. *MtKV3*, *mtkv3_20054*, and *mtkv3_12897* incubations contain molecules with amu values equivalent to oligoglucans of DP 5-10, consecutively (~850-1161 amu). In the oligo standards and *MtGBP* active lines, each oligosaccharide is separated by the equivalent of

one glucose monomer. Laminarin substrate, untransformed tobacco and empty vector controls, and *mtgpb4_11427* show no evidence of glucan hydrolase activity yielding 1→3-β-linked oligoglucans within the range of DP 4-10.

CHAPTER FOUR

DISCUSSION

One of the goals of this project was to determine the full-length cDNA sequences of three ESTs taken from TIGR database (<http://www.medicago.toulouse.inra.fr/Mt/EST/>) representing a putative β -glucan-binding protein family in *Medicago truncatula*. The ESTs under study represented three disparate sequences (KV0, KV2, and KV3) that had been previously identified by Francois Côté in the Hahn lab at The University of Georgia. While this study was underway, Leclerq and collaborators submitted full length cDNA sequences of four putative GBPs from *M. truncatula* to Genbank (Leclerq *et al.* 2007). Comparison of my full length *MtKV3* sequence (Figure 11) with the Leclerq submissions revealed that the sequence identified in my study corresponded to *MtGBP4*. Furthermore, *MtKV0* and *MtKV2* EST sequences were found to correspond to the *MtGBP3* and *MtGBP1*, respectively, reported by Leclerq *et al.* (2007). Thus, further efforts to clone additional *M. truncatula* GBPs were abandoned.

In parallel with the efforts to isolate the full length cDNA sequences of *MtKVO*, *MtKV2*, and *MtKV3* genes, a further aim of my studies was to examine the expression levels of each of the *MtGBPs* in different wild type *M. truncatula* tissues. Data from qPCR analysis showed that all three genes (*MtKV0*, *MtKV2* and *MtKV3*) have the highest expression in root tissue. *MtGBPs* were identified as genes encoding putative glucan-binding proteins for the HG elicitor found in fungal cell walls, so a high level of expression of these proteins in roots might be expected. The first line of defense in *M. truncatula* against fungal attack would need to be the recognition of the pathogen in roots where fungal infection frequently first takes place. Given

that the three *Mt*GBPs have similar expression patterns in roots (Figure 3-5) there is a good likelihood that *Mt*KV0, *Mt*KV2, and *Mt*KV3 may have redundant functions in root tissues.

In contrast to the similar levels of expression of the three *Mt*GBPs in root tissue, qRT PCR results revealed the differences in expression levels for the three *Mt*GBPs in the other seven tissues analyzed. None of the *Mt*GBPs were highly expressed in old and young leaves. *Mt*KV2 was poorly expressed in all above-ground tissues except young petioles, while *Mt*KV0 showed the lowest expression levels in young petioles (Table 2). *Mt*KV3 showed more similar levels of expression in flowers, apical buds, and petioles compared to *Mt*KV0 and *Mt*KV2. These varying patterns of *Mt*GBP expression in above-ground tissues suggest that there might be different functions for these three genes in these tissues above and beyond their possible role in plant defense.

One approach to gain information regarding gene function is to isolate mutants in the genes of interest. In June of 2005, I worked with collaborators in Doug Cook's laboratory at The University of California at Davis to screen *M. truncatula* EMS TILLING populations in an effort to identify GBP mutants in order to analyze the function of the glucan-binding proteins in *M. truncatula*. Due to time constraints on my stay in the Cook lab, Varma Penmetsa in the Cook lab continued the screening of the TILLING populations in parental *M. truncatula* lines and identified three lines carrying mutations in the *Mt*KV3 gene. Penmetsa's initial sequencing during screening had identified SNPs located at the following positions in each of the *Mt*KV3 mutant lines: *mtkv3_12897* mutant contained a P→S substitution at amino acid position 20, a D→N substitution occurred at amino acid position 65 in *mtkv3_20054*, and a L→F substitution at position 109 occurred in *mtkv3_11427*. In October of 2006, I returned to Davis, California to obtain sufficient seed of the three *mtkv3* lines (11427, 20054, and 12897) to continue studies of

these mutants at The University of Georgia. After cloning the full length cDNA sequence results from each *mtkv3* line, I was able to verify the partial sequencing efforts done by Penmetsa. I also identified an extra insertion in the *mtkv3_11427* line at amino acid position 425 and a SNP in the *mtkv3_12897* mutant located at amino acid position 503; both mutations were located within the two putative-conserved glucanase domains (Figure 10 and 11).

Two functions have been ascribed to the GBP isolated and characterized from soybean, one being a receptor for the HG elicitor (Cosio *et al.* 1991; Côté *et al.* 2000) and the other being an endoglucanase capable of releasing the HG elicitor from fungal cell walls (Fliegmann *et al.* 2005). Given the high degree of sequence similarity between *MtKV3* and the soybean gene, *MtKV3* could also have both of these functions. The mutations in *MtKV3* were located in two peptide domains reported as catalytic sites for members of the GH-81 family of glycosylhydrolases (Baladrón *et al.* 2002; Lammerts van Bueren *et al.* 2005; Mouya *et al.* 2002). Members of Family GH-81 show a catalytic activity of hydrolyzing β -1,3 glucans similar to polysaccharides found in fungal cell walls. Thus, I decided to test whether or not *MtKV3* has β -1,3 glucanase activity and determine if the mutations in its catalytic domains had any effect on this activity.

I chose to test the β -1,3 glucan hydrolase activity of the *MtKV3* by expressing the full-length wild type gene and the three *mtkv3* mutant genes in *Nicotiana benthamiana* leaves. The results of these heterologous expression studies demonstrated, for the first time, that *MtKV3* has β -1,3 glucan hydrolase activity. In addition, I was able to demonstrate that two of the three mutant proteins (*mtkv3_20054* and *mtkv3_12897*) were unaffected in their glucanase activities (Figure 13). That is these three proteins were capable of hydrolyzing the β -1,3 glucose backbone of the laminarin substrate and reducing its molecular size. All three proteins yielded glucose

oligomers within a range of DP 5-10 (Figure 15). However, the *mtkv3_11427* protein showed no glucan hydrolase activity towards the laminarin substrate even when protein amounts as high as 10 μ g and 50 μ g (Figure 14) were used in the enzyme assays. The results suggest that the additional D (aspartic acid) residue at amino acid position 425 interfered with the ability of the mutant protein to function as a glucanase. Asp 425 is located within the conserved peptide domain region proposed to be part of the β -1,3 glucanase active site found in Family GH-81 members. Mutations in this domain in Family GH-81 proteins from other organisms also affected glucanase activity. *Saccharomyces cerevisiae eng 1p* mutants showed phenotypes related to cell separation during mitosis establishing Eng 1 p as a β -1,3 glucanase involved in septum dissolution (Baladrón *et al.* 2002). In soybean a D substitution in this same peptide at the active site was shown responsible for loss enzyme activity (Lelcerq *et al.* 2007)

It was somewhat surprising that the *mtkv3_12897* mutant protein retained its glucanohydrolytic activity despite the E to A substitution at amino acid position 503 (Figure 12). This glutamic acid residue has been shown to be a putative nucleophile in the peptide active site located in the β -1,3 glucanase ENGL 1 of *Aspergillus fumigatus* (Mouyna *et al.* 2002). Moreover, substitutions of the two glutamic residues in the soybean GBP (located at positions at 503 and 507 in the orthologous *M. truncatula* sequence) resulted in the loss of β -1,3 glucanase activity in that protein (Fliegmann *et al.* 2005). However, *mtkv3_12897* had no amino acid substitution occurring at the glutamic acid residue located at the 507 position and this is possibly the reason for why this mutant retained hydrolase activity.

In conclusion, *MtKV3* does have glucan hydrolase activity towards β -1,3 glucans similar to the types of glucans found in the cell wall of fungal pathogens. Thus, *MtKV3* can legitimately be classified as belonging to Family GH-81 in the CaZY classification scheme (Henrissat *et al.*

1997). The *MtKV3* protein can therefore participate in at least one of the functions ascribed to the soybean GBP, namely the cleavage of fungal cell wall glucans thereby liberating elicitor-active oligoglucosides. However, additional enzymatic studies would be needed, for example, using cell walls from known pathogens of *M. truncatula* as substrates, to verify this role for *MtKV3* in the plant-pathogen interactions. In addition, further studies of the *MtKV3* and *mtkv3_11427* proteins are necessary to determine whether these proteins can also function as HG elicitor binding proteins during fungal infection. Such a dual role for GBPs has been hypothesized based on data obtained from studies on the soybean GBP (Fliegmann *et al.* 2005). Finally, it would be very interesting to analyze *mtkv3_11427* to see if the mutation in this protein has any effect of the response(s) of *M. truncatula* to infection by fungal pathogens.

REFERENCES

- Anderson AJ (1989) The biology of glycoproteins as elicitors. In *Plant-Microbe Interactions. Molecular and Genetic Perspectives*, ed. T Kosuge, E Nester, pp. 87-130. New York: McGraw Hill
- Ayers AR, Ebel J, Finelli F, Berger N, Albersheim P (1976) Host-Pathogen Interactions: IX. Quantitative Assays of Elicitor Activity and Characterization of the Elicitor Present in the Extracellular Medium of Cultures of *Phytophthora megasperma* var. *sojae*. *Plant Physiol* 57: 751-759
- Baladron V, Ufano S, Duenas E, Martin-Cuadrado AB, del Rey F, Vazquez de Aldana CR (2002) Eng1p, an endo-1,3-beta-glucanase localized at the daughter side of the septum, is involved in cell separation in *Saccharomyces cerevisiae*. *Eukaryot Cell* 1: 774-786
- Bhandal IS, Paxton JC (1991) Phytoalexin biosynthesis induced by the fungal glucan polytran L in soybean, pea, and sweet pepper tissues. *J Agric Food Chem* 39: 2156-2157
- Bell AA (1981) Biochemical mechanisms of disease resistance *Ann. Rev. Plant Physiol.* 32: 21-81
- Blondon F, Marie D, Brown S, Kondorosi A (1994) Genome size and base composition in *Medicago sativa* and *M. truncatula* species. *Genome* 37: 264-270
- Boller T (1995) Chemoperception of microbial signals in plant cells. *Annu Rev Plant Physiol Plant Mol Biol* 46: 189-214
- Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254
- Cheong JJ, Hahn MG (1991) A specific, high-affinity binding site for the hepta-beta-glucoside elicitor exists in soybean membranes. *Plant Cell* 3: 137-147
- Cheong JJ, Alba R, Cote F, Enkerli J, Hahn MG (1993) Solubilization of functional plasma membrane-localized hepta-beta-glucoside elicitor-binding proteins from soybean. *Plant Physiol* 103: 1173-1182

- Cheong JJ, Birberg W, Fugedi P, Pilotti A, Garegg PJ, Hong N, Ogawa T, Hahn MG (1991) Structure-activity relationships of oligo-beta-glucoside elicitors of phytoalexin accumulation in soybean. *Plant Cell* 3: 127-136
- Cline K, Wade W, Albersheim P (1978) Host-pathogen interactions. XV. Fungal glucans which elicit phytoalexin accumulation in soybean also elicit the accumulation of phytoalexins in other plants. *Plant Physiology* 62: 918-921
- Cosio EG, Ferger M, Miller CJ, Antelo L, Ebel J (1996) High-affinity binding of fungal β -glucan elicitors to cell membranes of species of the plant family Fabaceae. *Planta* 200: 92-99
- Côté F, Roberts KA, Hahn MG (2000) Identification of high-affinity binding sites for the hepta-beta-glucoside elicitor in membranes of the model legumes *Medicago truncatula* and *Lotus japonicus*. *Planta* 211: 596-605
- Darvill AG, Albersheim P (1984) Phytoalexins and their elicitors- a defense against microbial infection in plants. *Annu. Rev. Plant Physiol.* 35: 243-275
- Dixon RA, Lamb CJ (1990) Molecular communication in interactions between plants and microbial pathogens. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41: 339-367
- Dixon RA, Harrison MJ, and Paiva NL (1995) The isoflavonoid phytoalexin pathway: From enzymes to genes to transcription factors. *Plant Physiology* 93: 385-392
- Dixon RA (1986) The phytoalexin response: elicitation, signaling and control of host gene expression. *Biol. Rev.* 61: 239-291
- Ebel J, Mithöfer A (1998) Early events in the elicitation of plant defense. *Planta* 206: 335-348
- Fliegmann J, Mithofer A, Wanner G, Ebel J (2004) An ancient enzyme domain hidden in the putative beta-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. *J Biol Chem* 279: 1132-1140
- Fliegmann J, Montel E, Djulic A, Cottaz S, Driguez H, Ebel J (2005) Catalytic properties of the bifunctional soybean beta-glucan-binding protein, a member of family 81 glycoside hydrolases. *FEBS Letters* 579: 6647-6652
- Fontaine T, Hartland RP, Diaquin M, Simenel C, Latge JP (1997) Differential patterns of activity displayed by two exo-beta-1,3-glucanases associated with the *Aspergillus fumigatus* cell wall. *J Bacteriol* 179: 3154-3163
- Fontaine T, Hartland RP, Beauvais A, Diaquin M, Latge JP (1997) Purification and characterization of an endo-1,3-beta-glucanase from *Aspergillus fumigatus*. *Eur J Biochem* 243: 315-321

- Hahlbrock K SD (1987) Biochemical responses of plant to pathogens. In *Innovative Approaches to Plant Disease Control*, ed. I Chet, : pp. 229-254. New York: Wiley
- Hahn MG, Albersheim P (1978) Host-Pathogen Interactions: XIV. Isolation and Partial Characterization of an Elicitor from Yeast Extract. *Plant Physiol* 62: 107-111
- Hahn MG (1996) Microbial elicitors and their receptors in plants. *Annu Rev Phytopathol* 34: 387-412
- Hahn MG, Bonhoff A, Grisebach H (1985) Quantitative Localization of the Phytoalexin glyceollin I in Relation to Fungal Hyphae in Soybean Roots Infected with *Phytophthora megasperma* f. sp. *glycinea*. *Plant Physiol* 77: 591-601
- Hahn M, Darvill A, Albersheim P, Bergmann C, Cheong J-J, Koller A, Lò V-M (1992) Preparation and characterization of oligosaccharide elicitors of phytoalexin accumulation. In: Gurr S, McPherson M, Bowels DJ (eds). *Mol Plant Pathol (volume II): a practical approach*. Oxford University Press, Oxford UK, pp 103-147
- Ham K-S KS, Albersheim P, Darvill AG (1991) Host-pathogen interactions XXXIX. A soybean pathogenesis-related protein with β -1,3-glucanase activity releases phytoalexin elicitor-active heat-stable fragments from fungal walls. *Mol. Plant Microbe Interact.* 5: 545-552
- Hartland RP, Fontaine, JP, P Debeauvais, C Simenel, M Delepierre, and JP Latgé (1996) A secreted endo- β -glucanase from *Aspergillus fumigatus* that catalyzes the transfer of a segment of β -(1-3)-glucan to another β -(1,3) glucan molecule. *J Biol Chem* 271: 26843-26849
- Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* 280 (Pt 2): 309-316
- Henrissat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. *Curr Opin Struct Biol* 7: 637-644
- Hoch HC SR (1991) Signaling for infection structure for formation in fungi. In *The Fungal Spore and Disease Initiation in Plants and Animals*, ed. GT Cole, HC Hoch, pp. 25-42. New York: Plenum
- Jin W, Horner HT, Palmer RG, Shoemaker RC (1999) Analysis and mapping of gene families encoding beta-1,3-glucanases of soybean. *Genetics* 153: 445-452
- Keen NT (1975) Specific Elicitors of Plant Phytoalexin Production: Determinants of Race Specificity in Pathogens? *Science* 187: 74-75

- Kim Y-S RJ-H, Han S-J, Choi K-H, Nam K-B, Jang I-H, Lemaitre B, Brey PT, Lee W-J (2000) Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and β -1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. *J Biol Chem* 275: 32721-32727
- Kobayashi A TA, Kanzaki H, Kawazu K (1993) Elicitor-active oligosaccharides from algal laminaran stimulate the production of antifungal compounds in alfalfa. *Z Naturforsch* 48c: 575-579
- Lammerts van Bueren A, Morland C, Gilbert HJ, Boraston AB (2005) Family 6 carbohydrate binding modules recognize the non-reducing end of β -1,3-linked glucans by presenting a unique ligand binding surface. *J Biol Chem* 280: 530-537
- Leclercq J, Fliegmann J, Tellstrom V, Niebel A, Cullimore JV, Niehaus K, Kuster H, Ebel J, and A Mithofer (2008) Identification of a multigene family encoding putative beta-glucan-binding proteins in *Medicago truncatula*. *J Plant Physiol* 165: 766-776
- Ma C, Kanost MR (2000) A beta-1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade *J Biol Chem* 275: 7505-7514
- Mithofer A, Lottspeich F, Ebel J (1996) One-step purification of the beta-glucan elicitor-binding protein from soybean (*Glycine max* L.) roots and characterization of an anti-peptide antiserum. *FEBS Lett* 381: 203-207
- Mithofer A FJ, Neuhaus-Url G, Schwarz H, Ebel J (2000) The hepta-beta-glucoside elicitor-binding proteins from legumes represent a putative receptor family. *Biol Chem* 381: 705-713
- Mithofer A EJ, Arvind A, Bhagwat A, Boller T, Neuhaus-Url G (1999) Transgenic aequorin monitors cytosolic calcium transients in soybean cells challenged with β -glucan or chitin elicitors *Planta* 207: 566-574
- Montesano M BG, Palva ET (2003) Pathogen derived elicitors: searching for receptors in plants. *Mol Plant Pathol* 4: 73-79
- Mouyna I, Sarfati J, Recco P, Fontaine T, Henrissatz B, Latge JP (2002) Molecular characterization of a cell wall-associated beta(1-3) endoglucanase of *Aspergillus fumigatus*. *Med Mycol* 40: 455-464
- Ochiai M AM (2000) A Pattern-recognition Protein for -1,3-Glucan The Binding Domain and the cDNA Cloning of β -1,3-Glucan recognition protein from the silkworm *Bombyx mori* *J Biol Chem* 275: 4995-5002
- Ram SP HK, Romana LK, Shepard MG, and PA Sullivan (1988) The β -glucanases and β -glucosidase of *Candida albicans*. *Life Sci Adv* 7: 379-383

- Richter TE, Ronald PC (2000) The evolution of disease resistance genes. *Plant Mol Biol* 42: 195-204
- Sharp JK, Albersheim P, Ossowski P, Pilotti A, Garegg P, Lindberg B (1984) Comparison of the structures and elicitor activities of a synthetic and a mycelial-wall-derived hexa(beta-D-glucopyranosyl)-D-glucitol. *J Biol Chem* 259: 11341-11345
- Sharp JK, McNeil M, Albersheim P (1984) The primary structures of one elicitor-active and seven elicitor-inactive hexa (beta-D-glucopyranosyl)-D-glucitols isolated from the mycelial walls of *Phytophthora megasperma* f. sp. *glycinea*. *J Biol Chem* 259: 11321-11336
- Sharp JK, Valent B, Albersheim P (1984) Purification and partial characterization of a beta-glucan fragment that elicits phytoalexin accumulation in soybean. *J Biol Chem* 259: 11312-11320
- Schmidt WE, Ebel J (1987) Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions from soybean *Glycine max*. *Proc Natl Acad Sci U S A* 84: 4117-4121
- VandenBosch K CD, De Bruijn F, Huguet T (1996) Satellite meeting report. Emerging model legume systems: tools and recent advances. In: Stacey G, Mullin B, Gresshoff PM (eds) *Biology of plant-microbe interactions*. International Society for Molecular Plant-Microbe Interactions, St. Paul, Minn., pp 557-562