University of Alberta

Expression and purification of recombinant forms of two *Arabidopsis thaliana* PR-10 homologues (MLP423 and Bet v 1)

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

In

Plant Science

Department of Agricultural, Food and Nutritional Science

Fall 2013
Edmonton, Alberta

Abstract

Pathogenesis related 10 (PR-10) proteins have important roles in mediating plant abiotic and biotic stress tolerance. Most of the members of this family possess ribonuclease activity. The homologues of PR-10 (ABR17) protein, MLP423 and Bet v 1, may have similar biological and physiological functions. The deduced amino acid sequence of MLP423 and Bet v 1, when compared to ABR17, indicated several conserved amino acids and a conserved P-loop motif (GxGGxGxxK). In the present investigation, cDNAs encoding *Arabidopsis thaliana* MLP423 and Bet v 1 were isolated and cloned, recombinant proteins expressed in *E. coli*, and purified using Ni-NTA and size exclusion chromatography. Both in-solution and in-gel RNase activity assays were conducted to determine whether these recombinant proteins possessed RNase activity, which indicated that neither MLP423 nor Bet v 1 possessed RNase activity. Our results are discussed in the context of the possible functions of these PR-10 homologues in plants.

Acknowledgements

I take this opportunity to convey my deep sense of gratitude to my supervisor Prof. Nat Kav, Associate Dean (Academic) for his help, solicitude and for providing me the necessary inputs required for achieving a higher level of education and experience.

I also gratefully acknowledge Dr. Michael Deyholos and Dr. Stephen Strelkov for their valuable suggestions and critical evaluation of my research during my studies at the University of Alberta, Edmonton, Canada.

I wish to express my sincere heartfelt thanks to Dr. Muhammad Rahman, Dr. Shiv S Verma, Swati Megha, Mohammad Asif, and Atif Kamran for their extended help in research. I would also like to thank Jody Forslund, Dr. Urmila Basu, Bruce Alexander, Lynn Elmes, and Laura Smith for their help during my graduate study and research.

I would like to say thanks to the Islamia University of Bahawalpur and Pakistan Government (Higher Education Commission, Islamabad) for providing me an opportunity to continue my study here at the University of Alberta. I am also thankful to the Department of Agricultural, Food and Nutritional Science and Department of Biological Sciences, University of Alberta, for providing me required facilities during my studies.

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List of Abbreviations

ABA, abscisic acid

ABR17, abscisic acid responsive 17

A. thaliana, *Arabidopsis thaliana*

CaMV, cauliflower mosaic virus

CK, cytokinin

CSBPs, cytokinin specific binding proteins

DEPC, Diethylpyrocarbonate

dsRNAi, double-standed RNA interference

DREB, dehydration responsive element binding factor

EDTA, ethylene diaminetetraacetic acid

FAO, food and agricultural organization

FPLC, Fast protein liquid chromatography

GLP, germin-like proteins

IPTG, isopropyl-beta-D-thiogalactopyranoside

LB, Luria-Bertani

NAM, no apical meristem

F-Y, nuclear factor Y

Ni-NTA, nickel-nitrilotriacetic acid

NMR, nuclear magnetic resonance

ORF, open reading frame

PCR, polymerase chain reaction

PR, pathogenesis related

PVDF, polyvinylidene fluoride

qRT-PCR, quantitative real time polymerase chain reaction

RNase, ribonuclease

ROS, reactive oxygen species

RSOsPR-10, root specific *Oriza sativa* PR-10

RT-PCR, reverse transcriptase polymerase chain reaction

SDS, sodium dodecyl sulfate

TL, thaumatin-like

WT, wild-type

1.0 Introduction

Enhancing crop productivity to feed the ever-growing world population is a big challenge for the scientific community all over the world. In less than 50 years, the global population has doubled from 3 to over 6.9 billion (Bongaarts, 2009). Moreover, according to predictions by FAO (Food and Agricultural Organization), the world population will reach 9 billion by 2050, and to feed this population, food production must increase by 70 %.

Plants are continuously exposed to various environmental factors including biotic and abiotic stresses. Although there are different types of abiotic stresses, salinity and drought are of significant concern, because these stresses, collectively, may affect more than 10 % of the cultivated land resulting up to 50% to 70% yield losses (Boscaiu et al., 2012; Suzuki et al., 2005). Salinity, both in top and sub soil is affects crop yield (Grewal, 2010). It has been pointed out that salinity stress affected 800 million hectares of soil around the globe (FAO, 2008). Salinity adversely affects plant developmental processes from vegetative to reproductive stages, which reduces the quality and yield (Gorai et al., 2011).

Drought also can have devastating effects on plants, mostly resulting in a decline in crop yield. These crop yield losses subsequently lead to losses of communities. In 2010-2011, the driest period in the history of Texas, USA an estimated US7.5$ billion was lost as a result of crops being affected

(http://www.ate.tx.us). Some of the deleterious effects of drought on plants include a reduction in water status, leaf chlorophyll contents, transpiration and photosynthesis rate (Vaadia et al., 1961), and an accumulation of reactive oxygen species (ROS) which have damaging effect (Keunen et al., 2013). These physiological changes affect plant growth and development mainly due to altered biosynthesis of biomolecules like, chlorophyll, lipids, proteins and carotenoids, which lead to reduced cell division, cell expansion and ultimately reduction in overall crop yield (Fan et al., 1994; Jaleel et al., 2009; Zwiazek and Blake, 1990). Therefore, the above deleterious stresses have a negative correlation with increased global food demand. To feed the ever-growing population, there is a dire need to develop and improve the traits of agronomically important crops against environmental stresses.

Plants defend themselves in response to environmental stresses, through the induction of cascades of defense signalling molecules including calcium signalling molecules and proteins (Tuteja and Sopory, 2008). Pathogenesis related (PR) proteins are induced as a result of stresses (abiotic and biotic) and protect the plants from their deleterious effects (Briceno et al., 2012; Jwa et al., 2001; Lu et al., 2012; Van Loon et al., 1994).

PR proteins were first reported to be induced during pathogen invasion (Van Loon and Van Kammen, 1970). In 1983, it was observed that PR proteins were not only induced by pathogens but that they were also induced by abiotic stresses (Van Loon, 1983). PR proteins have been classified into 17 different

families (Liu et al., 2005; Van Loon and Van Kammen, 1970) on the basis of their biological function. Among PR proteins, the PR-10 protein family is a large group with more than 100 members (Liu and Ekramoddoullah, 2006). The first PR-10 protein was identified by (Somssich et al., 1988) from parsley(*Petroselinum crispum*). PR-10 proteins are present in almost all plant species including monocotyledonous and dicotyledonous plants (Liu and Ekramoddoullah, 2006). More than 100 members of this family have been identified and isolated from 70 different plant species (Matton and Brisson, 1989a; Warner et al., 1994; Wu et al., 2003).

A number of studies have revealed that PR-10 proteins increase in abundance following exposure to abiotic and/or biotic stresses and it is evident that PR-10 proteins might be involved in general defense mechanisms (Liu and Ekramoddoullah, 2006). For instance, proteomic analysis of pea plants under salinity stress showed an increased abundance of PR-10 protein (Kav et al., 2004). Similarly a rapid accumulation of RSOsPR-10 (root specific *Oryza sativa* PR-10) protein in the roots of rice (*Oryza sativa*) plants on exposure to drought, salinity and fungal infection was also reported (Hashimoto et al., 2004). In addition to drought and salinity PR-10 proteins are also induced by cold (Lee et al., 2012a; Zhang et al., 2010), toxic metals (Koistinen et al., 2002) and oxidative stress (Lee et al., 2012a). PR-10 proteins are also induced by fungal (Jwa et al., 2001) and bacterial pathogen invasion (Richard-Molard et al., 2004). PR-10 proteins are also known to be induced by the exogenous application of phytohormones such as cytokinins (Carpin et al., 1998), abscisic acid (Zhang et al., 2010), jasmonic acid

(Hashimoto et al., 2004). All these studies indicate that PR-10 proteins can be induced by a number of external stimuli including abiotic, biotic stress as well as the application of phytohormones.

Over expression of *PR-10* in plants has enhanced the plant adaptability against abiotic and biotic stresses. For instance, overexpression of *ABR17*(PR10) from pea (*Pisum sativum* L.),resulted an enhanced tolerance towards salinity stress cDNA in *Brassica napus*(Srivastava et al., 2004) and multiple abiotic stress tolerance in *Arabidopsis thaliana* (Srivastava et al. 2006b) Similarly, an over-expression of potato (*Solanum tubersum*) PR-10a protein in transgenic potato cell culture resulted to an increased salt and osmotic stress tolerance (El-Banna et al., 2010). Recently, PR-10a from potato was expressed in faba bean (*Vicia faba* L.) and a similar enhanced tolerance to drought and salt stresses in transgenic plants was observed (Hanafy et al., 2013). Furthermore, the members of the PR-10 protein family such as major latex protein (MLP) and birch allergen protein (Bet v1) were also identified from different plant species (Lytle et al., 2009). Like PR-10 proteins, Bet v 1 family is mainly acidic in nature with molecular weight around 17 kDa (Breiteneder et al., 1989; Ipsen and Lowenstein, 1983). It is therefore hypothesized from the above facts that MLP and Bet v 1 protein may have similar biological functions. However, the molecular function of these PR-10 proteins (MLP423 and Bet v 1) is not well understood, and several studies have reported that these proteins are responsible for protecting the plants against pathogen attack and abiotic stresses (Flores et al., 2002; Ruperti et al., 2002).

Based on the above information, we developed experiments to explore the role of MLP and Bet v 1 with respect to PR-10 protein. We isolated the cDNAs encoding MLP423 and Bet v 1 from *Arabidopsis thaliana* and expressed them in *Escherichia coli* (*E. coli*) to characterize their RNase activity.

2.0 Review of literature

2.1 Classification of PR proteins

PR proteins are categorized into 17 (PR-1 to PR-17) different families (Van Loon and Van Strien, 1999a). The PR-1 family is the most abundant and in infected tissue can be induced to 10,000-fold, comprising 1 to 2% of the total leaf protein (Alexander et al., 1993). PR1 proteins were accumulated in tobacco and rice on fungal (*Magnaporthe grisea*) invasion (Agrawal et al., 2001; Gordon-Weeks et al., 1997). PR-2 proteins are known as β-1,3-glucanases and are widely distributed in seed-plant species (Meins et al., 1992; Stone and Clarke, 1992). These proteins possess antimicrobial activity and are involved in diverse developmental and physiological processes (Fulcher et al., 1976; Hou et al., 2012; Seo et al., 2008). PR3 proteins were identified from pepper and citrus and exhibited chitinase-like activities (Young and Byung, 1996 (Porat et al., 2001). PR4 proteins were also reported to possess chitinase (Margis-Pinheiro et al., 1991) and RNase (Caporale et al., 2004) like properties. PR5 proteins were induced by biotic and abiotic stresses (El-kereamy et al., 2011) and were identified as thaumatin like protein (Hu and Reddy, 1997).PR6 proteins exhibited

chitinase and proteinase inhibitor like properties (Jwa et al., 2006), and are known to be involved in targeting nematodes and insect pests of plant (Van Loon et al., 2006). PR7 family members are endoproteinase-like proteins with antimicrobial activities (Van Loon and Van Strien, 1999b). Members of the PR8 family have been identified as chitinase type III like proteins (Jung et al., 1993; Park et al., 2004). PR9 protein identified from pearl millet (*Pennisetum glaucum* L.) exhibited peroxidase activity (Shivakumar et al., 2003). PR-10 family of PR proteins is widely studied and most of its members possess RNase activity (Lo et al., 1999; Shivakumar et al., 2000; Srivastava et al., 2006a; Zhou et al., 2002)and are induced by various abiotic stress conditions (Srivastava et al., 2006b; Swoboda et al., 1996; Van Loon et al., 2006). PR12 proteins identified from barley (Penninckx et al., 1996) and radish (Terras et al., 1995) are categorized as defensin-like proteins with antimicrobial activity (Rayapuram et al., 2008). Similarly, PR13 protein from *A. thaliana* also exhibited defensin-like properties with antimicrobial activity (Epple et al., 1995). PR15 and PR 16 have been classified as germins and germin-like proteins (GLPs) and PR16 and these proteins were isolated during the response to bacterial and viral infection (Park et al., 2004).In summary, PR proteins, of which PR-10 is a family, have been classified into the aforementioned 17 families based on sequence and functional similarities.

2.2 PR-10 proteins

PR-10 proteins were first identified in pea in response to fungal pathogen attack (Riggleman et al., 1985). Currently, the PR-10 protein family is comprised of more than 100 members which have been identified and isolated from 70 different plant species including monocotyledonous and dicotyledonous plants(Matton and Brisson, 1989a; Warner et al., 1994; Wu et al., 2003).

As indicated earlier, PR-10 proteins are induced by various abiotic and biotic stress conditions (Van Loon and Van Strien, 1999a) including, for example, drought (Hashimoto et al., 2004), salinity (Hashimoto et al., 2004; Kav et al., 2004), fungal pathogens (Jwa et al., 2001) and bacterial pathogens (Richard-Molard et al., 2004). A PR-10 protein from western white pine (*PmPR-10*) was significantly induced upon wounding, suggesting a defense- related function for this protein (Liu et al., 2003). A number of PR-10 proteins characterized till date have been reported to possess RNase activity(Lo et al., 1999; Shivakumar et al., 2000; Srivastava et al., 2006a; Zhou et al., 2002).For example, a PR-10 protein from *Crocus sativus* (*Cs*PR-10), which has RNase activity, showed inhibitory activity against different fungal pathogens (*Verticilliumdahilae, Penicillium sp.* and *Fusarium oxysporum*) (Gomez-Gomez et al., 2011). PR-10 proteins are not always induced by environmental stresses but some PR-10 members are also constitutively expressed in vegetative parts, flowers, pollen grains and fruits, which is an indication of their role in normal plant growth and development (Biesiadka et al., 2002; Breda et al., 1996; Breiteneder et al., 1989; Sikorski et al.,

1999). It is clear from the reports in the literature that PR-10 proteins may have complex and diverse functions during normal plant growth and development as well as during responses to stress.

Major latex proteins (MLPs), as the name suggests, are proteins present in natural rubber latex. These proteins were first identified in the latex of opium poppy (*Papaver* somniferum) by (Nessler et al., 1985). MLPs are found only in plant and members of this protein family have been identified from different plants such as *Arabidopsis*, cucumber, melon, peach and soybean. In Arabidopsis 24 members of MLPs have been identified (Osmark et al., 1998). The function of MLP Proteins is still unknown, though they have been associated with fruit and flower development as well as in plant defense responses. However, the MLP proteins from Bell Pepper are known to be induced by wounding (Pozueta-Romero et al., 1995). MLPs on the basis of modest sequence similarity have been characterized as members of the Bet v 1 protein superfamily (Osmark et al., 1998). Bet v 1, a major allergenic protein of birch pollen, was originally identified from birch pollen allergic patients by screening a pollen cDNA library with serum IgE (Breiteneder et al., 1989). On the basis of deduced amino acid sequences, Bet v 1 protein family members range from 154 to 160 residues with masses ranging from 16.6 to 17.5 kDa (Wen et al. 1997). The deduced amino acid sequence of a cDNA clone coding for Bet v l of white birch pollen showed 55% sequence identity with a pea disease resistance response gene, suggesting that Bet v l may be involved in pathogen resistance of pollen (Breiteneder et al., 1989). In addition, Bet v 1 family proteins on the basis of similar three-

dimensional (3D) structure are believed to be homologous to the PR-10-like protein family (Radauer et al., 2008). Further, when the 3D structure of birch Bet v 1 and yellow lupine PR-10 protein were compared, it was observed that amino acids E96, E148 and Y150 were close together and thus predicted as active sites for RNase catalytic activity (Wu et al., 2003). It has been further demonstrated that Bet v 1, MLPs and PR-10 subfamilies show low sequence similarities (25%), but they have similar Y-shaped hydrophobic cavity, which suggested that they all have some common role in plant defense (Osmark et al., 1998).

2.3 Structure of PR-10 proteins:

PR-10 proteins are small proteins with molecular weights ranging from 6-43 kDa (Liu and Ekramoddoullah, 2006; Van Loon and Van Strien, 1999a). Structures of a few PR-10 proteins were investigated using nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction (Markovic-Housley et al., 2003). PR-10 proteins consist of 3 α helices, one long C-terminal (α3) helix, two short N-terminal helices (α1 and α2) and a seven-stranded β-sheet (β1 to β7) (Gajhede et al., 1996). The orientation is in such a way that the single long C-terminal, i.e., α3 helix, is encircled by a seven-stranded β-sheet and two α helices (α1 and α2), which are present between β1 to β2. In this overall 3D structure, as a result of connection sequences between α-helix and β-strands, nine short loop structures and a hydrophobic cavity are formed. This hydrophobic cavity is presumed to have a crucial role in the biological activity of PR-10proteins

(Gajhede et al., 1996; Markovic-Housley et al., 2003). Moreover, the hydrophobic cavity is presumed to be involved in their ligand (e.g. cytokinins and flavonoids) binding activity and has a crucial role in the biological activity of PR-10 proteins (Fernandes et al., 2013; Fernandes et al., 2008; Hoffmann-Sommergruber et al., 1997; Zubini et al., 2009). Although the deduced amino acid sequence alignment from different species of PR-10 proteins varies considerably, the phosphate binding (P)-loop motif (GXGGXGXXK) is highly conserved (Gajhede et al., 1996; Wu et al., 2003). Besides this (P)-loop motif, some other amino acid residues including tyrosine (K53) histidine(H69), lysine (Y80) and glutamic acid (E148) are conserved, which we speculated to be involved in RNase activity (Lebel et al., 2010). Furthermore, site directed mutagenesis studies of the conserved amino acids residues showed alteration in RNase activity (Krishnaswamy et al., 2011; Zhou et al., 2002). The above facts suggest that these conserved amino acid residues may be essential for the biochemical activity.

2.4 Ligand binding properties of PR-10 protein

It has been demonstrated that the hydrophobic cavity present in PR-10 proteins has the affinity to bind with cytokinins (CKs), fatty acids and brassinosteroids (Fujimoto et al., 1998; Markovic-Housley et al., 2003). The cytokinin specific binding proteins (CSBPs) were classified as PR-10 proteins due to their predicted secondary-structure and to a lesser extent, their sequence

similarity (Fujimoto et al., 1998).It was found that PR-10 protein from white birch (Bet v 1) can bind various compounds (including fatty acids, flavonoids, and cytokinins) and NMR data indicate that the ligands are bound in the internal cavity. Crystallographic studies have also shown that Betv1 can bind two deoxycholate molecules in the cavity (Markovic-Housley et al., 2003). The crystallographic model of PR-10 protein from yellow lupine (*Lupinus luteus* L) PR-10 protein shown to have ability to bind different cytokinins (Fernandes (Fernandes et al., 2009; Fernandes et al., 2013) suggest that this protein, and perhaps other PR-10 proteins as well, can act as a reservoir of cytokinin molecules in the aqueous environment of a plant cell. These facts therefore here suggested that PR-10 protein have affinity to binds plant hormones, such as CK which during the stress conditions may help the plant to sustain in adverse environment.

2.5 Role of PR-10 in abiotic stress

The pathogenesis related proteins (PRs) are induced in tobacco and other plant species by both abiotic and abiotic stresses, such as necrotizing and non-necrotizing viruses, viroids, fungi, bacteria, specific physiological conditions and a variety of chemicals (Van Loon, 1983).A homologue of PR-10 protein, Bet v 1, was found to be accumulate in mid-winter and thereby conferring freeze tolerance in cortical parenchyma cells in mulberry trees(Ukaji et al., 2001). Moreover, the proteome analysis of pea (*Pisum sativum*) roots under saline conditions showed

increased in the abundance of PR-10 proteins (Kav et al., 2004). Also, the proteome analysis of rice roots under salt, drought and blast fungus infection showed increased in expression or accumulation of *RSOsPR-10* (Hashimoto et al., 2004) and suggesting a pivotal role of PR-10 proteins during stress.

Furthermore, the over-expression of PR-10 (ABR17) protein from pea (*P. sativum*), when constitutively expressed in *B. napus*, resulted in enhanced seed germination under salinity stress (Srivastava et al., 2006b). Moreover, the transcriptional profiling of cDNA encoding *ABR 17* under saline conditions revealed the abundance and up-regulation of several abiotic stress responsive genes (Krishnaswamy et al., 2008) and transcription factors in transgenic *Arabidopsis* plants, which suggests the impact of ABR17 in protecting the plants against abiotic stress (Krishnaswamy et al., 2011). Recently, the PR-10 protein from ginseng (PgPR-10) was shown to have ribonuclease activity (Lee et al., 2012b), which further supported the role of PR-10 protein against abiotic stress. In the same year (El-Banna et al., 2010)reported the role of PR-10 protein in enhancing osmotic tolerance of a transgenic cell culture of potato (*Solanum tuborosum*). The above scientific studies have shown an association between abiotic stress and increased abundance of PR-10 proteins.

2.6 Role of PR-10 proteins in biotic stress

As the name indicates, PR proteins are induced by pathogens. The first PR-10 protein was also identified in pea in response to fungal pathogen attack (Riggleman et al., 1985). Later, a number of PR-10 proteins were found to be induced by pathogen invasion in various plant species; including potato, (Matton and Brisson, 1989b), pea (Fristensky et al., 1988), soybean (Crowell et al., 1992), sorghum and rice (Lo et al., 1999; McGee et al., 2001). An elevation in the level of PR-10 proteins was observed in response to viruses (Puhringer et al., 2000) and bacteria (Park et al., 2004); PR-10 protein from western white pine has been found to be significantly induced upon wounding, and suggesting the role of PmPR-10 protein in the defense response (Ekramoddoullah et al., 1998).

The over-expression of the pea PR-10 gene (*DRR49*) in potato has conferred resistance against the early attack of dying disease (Chang et al., 1993). Similarly *A. thaliana* plants expressing the PR-10 cDNA from *Pinus monticola* (*PmPR10-1.13*) showed enhanced expression in response to wounding stress and the pathogen. The above studies have demonstrated the importance of PR-10 proteins in mediating plant responses to biotic stresses. However, their precise roles in these responses are still unclear.

2.7 PR-10 proteins and RNase activity

RNases are the proteins that hydrolyze ribonucleic acid and are usually present in the extracellular space and secretory pathways of plants (Deshpande and Shankar, 2002). PR-10 protein isolated from *Capsicum annuum,* showed RNase activity and was directly linked with its antiviral activity (Park et al., 2004). Recombinant peaPR-10.4 (ABR-17) protein purified from *E. coli* showed RNase activity (Srivastava et al., 2006a), and its expression in *Arabidopsis* showed tolerance against salinity (Srivastava et al., 2007). RNase activity of Bet v 1, a homolog of PR-10 in the birch pollen allergen has also been reported (Bufe et al., 1996; Swoboda et al., 1996).

Several researchers have investigated the mechanistic function of PR-10 proteins, and it has been reported that most of the PR-10 protein members protect the plants under different abiotic and biotic stresses through their RNase activity, which possibly relate to plant defense mechanism (Bufe et al., 1996; Liu and Ekramoddoullah, 2006; Park et al., 2004). Moreover, the RNase activity of PR-10 proteins may limit pathogen invasion at the site of pathogen attack through the direct degradation of pathogen RNA (Park et al., 2004). The SsPR-10 protein from yellow fruit nightshade (*Slanum surattense*) showed RNase activity and also inhibited the hyphal growth of *Pyricularia oryzae* (Liu et al., 2006). Recently, PR4 protein from *Capsicum Chinese* (*C. chinese*) has been demonstrated to possess RNase and DNase activity and its constitutive expression in *Nicotiana langsdorfii* conferred resistance against most of the tobamoviruses (Guevara-

Morato et al., 2010). The role of the RNase activity of PR-10 proteins in protection of plants during programmed cell death around infection sites and self-incompatibility during fertilization has also been suggested (Huang et al., 1994; Liu and Ekramoddoullah, 2006; Swoboda et al., 1996). These studies suggest that host cell damage as a result of fungus attack triggers the synthesis of PR-10 proteins and, thus, they play an important role in plant defense mechanisms. These observations suggest a functional link between the role in defensive mechanisms of PR proteins and their RNase activity.

2.8 Objectives

The aim of this research project was to determine whether two PR-10 protein homologs from *A. thaliana,* MLP423 and Bet v 1, were ribonucleases and the first three activities were directed towards achieving this goal. The final objective was to generate plant expression constructs in order to express pea ABR17 (a PR-10 protein) under the control of an inducible promoter. This objective was to lay the foundation for future studies, which would be directed at exploring the role of RNase activity of pea ABR17 in mediating the observed phenotypic effects including multiple stress tolerance in transgenic plants expressing pea *ABR17*.The objectives were to:

(i) Isolate cDNA encoding MLP423 and Bet v1.from *A. thaliana*.

(ii) Express and purify MLP423 and Bet v 1 in *E. coli*.

(iii) Determine the RNase activity of recombinant forms of MLP423 and Bet v 1.

(iv) Generate plant expression vectors to ultimately achieve the inducible expression of pea ABR17 and its site-directed mutants.

3.0 Materials and Methods

3.1 Phylogenic relationship and amino acid sequence alignment

To investigate the phylogenetic relationship between ABR17, MLP423 and Bet v 1 the amino sequences for these proteins were first aligned using the Constraint-based Multiple Alignment Tool (COBALT) from NCBI. Conserved sequence motifs were identified from the amino acid sequence alignments. The alignment information was subsequently used to construct a phylogenetic tree using ClustalW2 program (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/)

3.2 Isolation of cDNAs encoding MLP423 and Bet v 1

All plastic and glassware used for RNA extraction were treated with 0.1 % diethylpyrocarbonate (DEPC) solution (v/v) to eliminate possible RNase contamination. DEPC treated water was used for making all reagents and solutions used for RNA isolation. Total RNA was isolated from two-week old *A. thaliana* (ecotype Columbia) tissues using TRI-Reagent (Ambion, USA) as per the manufacturer's instructions. Briefly, TRI-reagent (1 ml) was dispensed into a pre-cooled 2 ml Eppendorf tube. Frozen plant leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. The ground leaf powder (100 mg) was transferred to the Eppendorf tube containing TRI-reagent, mixed by vortexing thoroughly and incubated at room temperature (RT; 21 ± 2ºC) for 3-4

minutes. Chloroform (200 µl) was added the tubes and the mixture inverted a few times and incubated at RT for an additional 10 minutes. After incubation, the preparation was centrifuged at 12,000 xg for 10 minutes at 4°C. A clear aqueous upper layer was observed which was transferred to a new tube and 0.7 volumes of isopropanol was added to the transferred aqueous phase, mixed thoroughly and incubated at RT for 10 minutes. The tubes were then centrifuged at 12,000 xg for 10 minutes at 4°C, and the supernatant was discarded. The RNA pellet was washed with 1 ml of 75% ethanol followed by centrifugation for 5 minutes at 12,000 xg. The ethanol was discarded and the RNA pellet was briefly air dried for 5-10 minutes at RT after which it was dissolved in nuclease free water. Isolated RNA was quantified using a Nanodrop 2000(Thermo Fisher Scientific, Wilmington, DE, USA), aliquoted in clean tubes and stored at -80 °C until further use.

3.3 cDNA synthesis and RT-PCR

cDNA was synthesized using 1µg of the isolated RNA, using the iScript cDNA synthesis kit (BioRad, Mississauga, ON, Canada) as per the manufacturer's instructions. The synthesized cDNA was then used as the template for PCR to amplify the cDNA encoding MLP423 and Bet v 1 using gene-specific primers (Table 1).

Table 1. List of primers for amplification of *Bet v 1* and *MLP423*

Primers	**Sequence (5'-3')**	**T_m (^oC)**	**Expected size (bp)**
Forward Bet v1	CGATAC***GGATCC***ATGAAAACATCTCAAGA ACAGCATGTA	64	480
Reverse Bet v1	GCTATG***CTTCAG***TTAAGTGAGCTCCATCAT CTTCTCCGA	65	
Forward MLP423	CGATAC***GGATCC***ATGGGGTTGAGTGGTGT TCTTCATGT	60	468
Reverse MLP423	GCTATG***CTTCAG***TTAGGCACTAGTTTGCTT AAGAAGAT	63	

PCR reaction mixtures (25 μL) consisted of 0.1 mM dNTP, 0.5 mM of each primer, 0.5 units of *Taq* polymerase and the standard *Taq* polymerase buffer (New England Biolabs, Toronto, Canada). PCR cycling conditions consisted of an initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 45 Seconds, annealing at 55°C for 45 Seconds and extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. Amplified PCR products were analyzed using an agarose gel (1.2 % w/v) and purified using a QIAquick gel extraction kit (Qiagen Inc., Ontario, Canada) according to the manufacturer's instructions.

3.4 Cloning of cDNAs into the protein expression vector

Plasmid pET22b+ (NovagenInc. Madison, USA) and the newly purified PCR products were restriction enzyme-digested with *Bam*HI and *Xho*I (New England Biolabs, Toronto, Canada) according to the protocol suggested by the supplier of these restriction enzymes. Briefly, the reaction mixtures were incubated at 37^{o}C for 1 hour. After digestion, the products were visualized on an agarose (1 %) gel and the products were gel-purified as described above for use in ligation reactions.

The gel purified DNA (for the amplification products obtained using primers for *MLP423* and *Bet v 1*) was ligated separately to linearized pET22b+ vector using T4 ligase ((Roche Diagnostics Ltd., Lewes, England)as per the supplier's instructions. A 1:3 (insert: vector) ratio (50 ng vector DNA and 150 ng insert DNA) was used in the ligation reaction, in a total volume of 21 µl. The mixture was gently centrifuged for 10 seconds to mix the components and incubated at room temperature for 1 hour.

An aliquot (5 µl) of ligation mix was used for bacterial transformation and the remainder was stored at -20 ºC for future use if required. DNA was transformed into *E. coli* strain *(DH5α)* using heat shock transformation. Briefly, competent DH5α cells were removed from -80 ºC (50 µl aliquots) and thawed on ice for 5 minutes. This was followed by addition of 2 µl of the ligation mixture into cells. Subsequently, the tubes were placed into a water bath at 42 ºC for 90

seconds following which, they were once again placed on ice for 5 minutes. Luria Bertani (LB) medium (1 ml without antibiotics) was added to the cell suspension and incubated in a shaker incubator set at (37 ºC and 250 rpm for 1 hour. After incubation the culture was centrifuged at 1500 xg for 1minuteand 900 µl of the supernatant was removed with a pipette without disturbing the pellet. The pellet was then re-suspended in the remaining medium, spread on LB plates containing ampicillin (100µg/ml) and incubated at 37 ºC overnight.

In order to select the successful transformants containing the recombinant plasmids, colony PCR was carried out using T7 universal promoter sequence primer as a forward primer and a reverse primer specific for the cDNA of interest. For setting up colony PCR large but fairly isolated colonies were selected by "stabbing" the pipette tip into the colony. The stabbed tip was touched to an already labelled plate (master plate) and then placed in a PCR tube containing PCR reaction mixture. The bacterial culture on the tip was used as a template for colony PCR, using *MLP423* and *Bet v 1* specific reverse primers. Master plate of the colonies used for the PCRwas incubated overnight at 37 °C overnight.

3.4.1 Plasmid extraction

Bacterial colonies which were positive for the presence of recombinant plasmids based on colony PCR results were selected and inoculated into LB medium (5 ml) containing ampicillin (100 µg/ml). Inoculated cultures were

incubated at 37 °C overnight in a shaker incubator set at 250 rpm. Bacterial cultures were harvested the following morning by centrifugation at RT for 30 minutes at 2000 xg. Bacterial pellets were re-suspended in 250 µl re-suspension solution (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and mixed thoroughly. An equal volume of the lysis buffer (200 mM NaOH, 1% SDS (w/v)) was added and mixed thoroughly by inverting the tubes (4-6 times) and allowing at least 2-3 minutes for cell lysis at RT. Following cell lysis, the neutralization buffer (3.0 M potassium acetate, pH 5.5) was added and the mixture incubated for 10 minutes at RT. The tubes were then centrifuged at 10,000 xg for 10 minutes at RT. The supernatant was applied to the QIAprep spin column by decanting followed by centrifugation at 10,000 x g for 1 minute and flow-through was discarded. The QIAprep spin column was washed by adding 0.75 ml Buffer PE, followed by centrifugation for 1 minute and flow-through was discarded. To remove residual wash buffer, the column was centrifuged for an additional 1 minute. The QIAprep column was then placed in a clean 1.5 ml microcentrifuge tube and 50 µl elution buffer (10 mM Tris-HCl, pH 8.5) was added to the center of QIAprep spin column to eluate the plasmid DNA. Let stand for 1 minute, and centrifuged 10,000 x g for 1 minute. The concentration of the isolated plasmid DNA was determined using a Nanodrop2000 (Thermo Fisher Scientific, Wilmington, DE, USA).

3.4.2 Determination of the sequence of inserts

Recombinant plasmids isolated from bacterial cells were confirmed to harbor the appropriate cDNA of interest using PCR analysis. The plasmid was prepared for sequencing reaction using BigDye termination kit (Life technologies, USA). The plasmid (400 ng) was used in this set of reaction, with forward primer (10µM) and BigDye master mix 2 µl (Life technologies, USA) and the final reaction volume was adjusted to 20 µl with nuclease free water. The reaction was set up in PCR tubes with 200mMTris buffer (pH 7.6) and 0.5 mM of forward primer. PCR thermo-cycling conditions consisted of an initial denaturation step at 96 ℃ for 30 seconds, and annealing at 50℃ for 15 seconds and 60℃ for 2 minutes following which, the reactions were held at 4℃. The reaction products were precipitated using $1/10^{th}$ volume of 1.5M NaOAc/250 mM EDTA and two volumes of ethanol (100 %). The precipitated reaction products were washed with 70 % ethanol, and air dried at RT. Sequencing reaction products were then submitted to the Molecular Biology Service Unit (MBSU) at the University of Alberta, further analysis and sequence information was obtained.

3.5 Expression of MLP423 and Bet V1 in *E. coli*

Plasmids containing the cDNA encoding *Bet v 1* and *MPL423* as confirmed by DNA sequence analysis were transformed into chemically competent *E. coli* Rosetta expression cells (Novagen, California, USA) by heat

shock transformation as described previously. These transformed *E. coli* Rosetta cells were selected on LB plates with appropriate antibiotics (ampicillin100µg/ml and chloramphenicol 34µg/ml). A single, isolated colony was inoculated into 50 ml 2 x YT medium (Messing J, 1983) as a starter culture with appropriate antibiotics and further used as inoculums for larger cultures (1.5 liter). The inoculated media was placed in the shaker incubator set at 250 rpm and 37°C. The optical density at 600 nm (OD_{600}) was checked at regular intervals and when the OD reached 0.4-0.5 protein expression was induced by adding a solution of isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Induced cultures were incubated overnight in the shaker incubator set at previously described conditions.

3.5.1 Preparation of periplasmic extracts

Bacterial cells were harvested by centrifugation at 8300 xg for 10 minutes at 4ºC. Pellets were re-suspended in a small volume (5 % v/v of the cell culture) of extraction buffer (Tris-HCl 200 mM, Sucrose 20 %, EDTA 1 mM, pH-7.0). The suspended cultures were mixed by inverting the tubes at regular intervals to facilitate the release of periplasmic material into the extraction buffer. The extract was then centrifuged at 10,000 xg for 30 minute at 4 ºC to sediment the impurities. The supernatant containing the periplasmic proteins was collected and dialyzed against a buffer containing Tris-HCl 50 mM, EDTA 0.5 mM, DTT 1

mM, and pH 8.0 at 4 ºC using a dialysis membrane with a cut off of 8000 Da (Spectrum Laboratories, Inc., Rancho Dominguez, CA)

3.5.2 Purification of recombinant proteins from periplasmic extracts

The recombinant proteins were purified by metal-chelation affinity chromatography using a Ni-NTA Agarose (5 ml) column (Qiagen Inc., Ontario, Canada). The column was equilibrated by washing with 5 bed volumes of equilibration buffer (Tris-HCl 50 mM, NaCl 100 mM, pH 7.0). The dialyzed periplasmic extracts were applied to the equilibrated column. Weakly interacting proteins were desorbed from the column by washing with 20 bed volumes of wash buffer I (Tris-HCl 50 mM, NaCl 100 mM, Imidazole 15 mM, pH 7.0) and the eluted material was collected in separate flask. The column was then washed with 10 bed volumes of wash buffer II (Tris-HCl 50 mM, NaCl 100 mM, Imidazole 30 mM, pH 7.0), followed by 5 bed volumes of wash buffer III (Tris-HCl 50 mM pH 7.0, NaCl 100 mM Imidazole 60 mM,).After the final wash, the proteins remaining adsorbed to the Ni-NTA matrix was first eluted with 5 bed volumes of elution buffer I(Tris-HCl 100 mM pH 7.0, NaCl 100 mM, Imidazole100 mM,) followed by elution with 5 bed volumes each of elution buffer II (Tris-HCl 50 mM, NaCl 100 mM, Imidazole 200 mM, pH 7.0), & elution buffer III (Tris-HCl 50 mM, NaCl 100 mM, Imidazole 300 mM, pH 7.0). The material eluted from the column during each stage was collected separately. To check the purity of the recombinant protein, the fractions were electrophoressed on 12 % SDS PAGE gel.

In order to further purify the recombinant MLP423 and Bet v1 proteins, size exclusion chromatography on an AKTA FPLC (Fast protein liquid chromatography) System (GE Healthcare Biosciences, Pittsburgh, USA) was performed using a Superdex 75 10/300GL column (GE Healthcare Biosciences, Pittsburgh, USA). The protein was diluted in phosphate buffer (100mM) pH-7.4; the column was subsequently also washed with the same buffer. The sample (500 µl) was loaded using the micro-injector. Fractions of 300 µl each were collected and relative purity was evaluated through electrophoresis on 12 % SDS PAGE gel. The desired fractions of proteins were quantified using the Bradford method of protein quantification, employing BSA as a standard as described by Marion M. Bradford (1976).

3.5.3 Western blot analysis

Western blotting was used to confirm the expression of recombinant proteins MLP423 and Bet v 1. The purified recombinant protein was loaded on a 12 % SDS-PAGE gel and electrophoresis was carried out at 150 Volts for 1 hour. The separated proteins was electro-blotted onto polyvinylidene fluoride(PVDF) membrane (Millipore Corp., Burlington, MA, USA) in 25 mM Tris pH 8.2, 192 mM glycine, and 20 %(v/v) methanol. The membrane was blocked for 2 hours in 5 % (w/v) non-fat dry milk (BioRad, Mississauga, ON, Canada) in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl containing 0.05 %(v/v) Tween-20. Immunodetection was performed using purified rabbit anti-His tag (1:500 dilution) as the primary

antibody and goat-anti-rabbit HRP-conjugated secondary antibody (ab6721, Abcam, San Francisco, CA, USA, 1:10000 dilutions). Antigen-antibody complexes were visualized with a peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA) and the images recorded using a Bio-RadGS-800-calibrateddensitometer (Bio-Rad, Hercules, CA, USA).

3.6 Characterization of the RNase activity of the purified recombinant proteins

3.6.1 In-solution RNase activity

Purified MLP423 and Bet v 1 proteins were used for the RNase activity assay as described by Srivastava et al (2006a) with some modifications. The assay was conducted in a 1.5 ml microcentrifuge tube. To set up the reaction, 3.25µg of total RNA was isolated from two week old *Arabidopsis* seedlings as described earlier (Srivastava et al., 2004) and used as a substrate. Controls included reactions in which only RNA was present and in which RNA and buffer were present. To determine the RNase activity of the purified proteins, tubes containing 6, 9, 12 or 15 µg of recombinant protein (MLP423 and Bet v 1) were also set up in duplicate. An additional negative control, containing 15 µg of boiled recombinant proteins was also included in these experiments. All the reaction mixtures were incubated at 37ºC for 3 hours. After the incubation, an equal volume of 1:1 phenol-chloroform was added and mixed thoroughly. The mixture was centrifuged at 10,000 xg at 4 ºC and the top aqueous layer was

analyzed on a 1.2% agarose gel to determine the extent of RNA degradation in these samples.

3.6.2 In-gel RNase activity

In-gel RNase activity assays were performed using the recombinant proteins (MLP423 and Bet v1), essentially as described earlier (Yen and Green, 1991) with minor modifications. To avoid RNase contamination, the apparatus was washed with 0.1 % DEPC treated water and autoclaved. Yeast tRNA (2.5mg/ml) was mixed with the reagents (40% Acrylamide, 1.5M Tris pH 8.8, 10% SDS, 10% APS, Temed) to generate a 15 % polyacrylamide gel. The protein was separated by electrophoresis at 150 Volts for 1 hour. After electrophoresis the gels were washed twice with 25 %isopropanol to remove SDS. To remove the isopropanol, the gel was washed thrice with 0.01 M Tris-HCl pH 7.4 for 10 minutes each at room temperature (RT). After washing, the gel was incubated in a buffer containing Tris-HCl 100 mM, pH 7.4 at 51ºC for 50 minutes. The gel was stained with 0.2 %(w/v) toluidine blue in Tris-HCl10 mM, pH 7.4for 5 minutes at RT and de-stained by incubating four times with Tris-HCl 10 mM, pH-7.4 for 5 minutes each after which the gels were rinsed and stored in 10 % glycerol in Tris-HCl 10 mM, pH 7.4. Gel images were documented using a Bio-RadGS-800-calibrated densitometer (Bio-Rad, Hercules, CA, USA).

4.0 Results

4.1 Phylogenetic relationship and amino acid sequence alignment

A phylogenetic tree was constructed based on the amino acid sequences of ABR17, MLP423 and Bet v1. The results obtained indicated that as these proteins belong to the same clade so they have close genetic relationship (Figure 1a), however, the deduced amino acid sequence alignment showed less sequence similarity (Figure 1b). Sequence alignment of these proteins showed conserved motifs (GXGGXXG), which was designated as P-loop motifs. Moreover, it showed some conserved amino acids such as aspartic acid (D-75), glycine (G-87-88), leucine (L-24), lysine (K-34), proline (P-33) and serine (S-84) (Figure 1b).

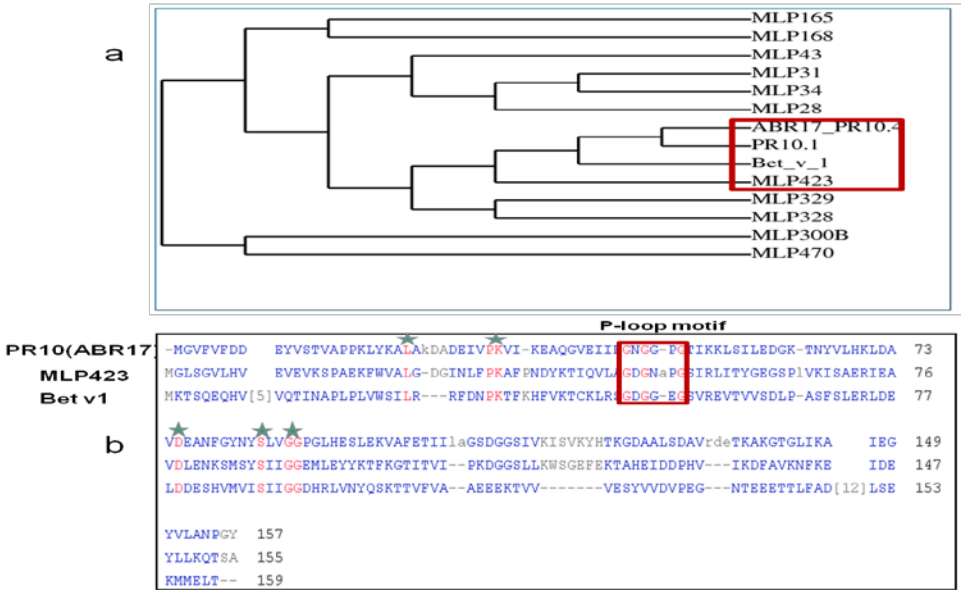

Figure 1. Phylogenetic relationship studies of ABR17 (a) with MLPs, and Bet v 1 from *A. thaliana* and (b) amino acid sequence alignment of ABR17 with MLP423 and Bet v 1

4.2 Synthesis of cDNA encoding Bet v 1 and MLP 423

As described in the materials and methods section, total RNA isolated from *A. thaliana* was used to synthesize cDNA encoding both the PR-10 protein homologues. The RNA used in these experiments was of sufficient quality as indicated by agarose gel electrophoresis (Figure 2a). Results of RT-PCR revealed the amplification of a 480 bp fragment, which corresponds to Bet v1 and 468 bp which corresponds to MLP423 (Figure 2b). Control PCR reactions where the RNA had not been reverse transcribed to DNA did not yield any amplification product indicating that the RNA used for RT-PCR was not contaminated with genomic DNA (Figure 2b). Our results thereby suggest that we were able to

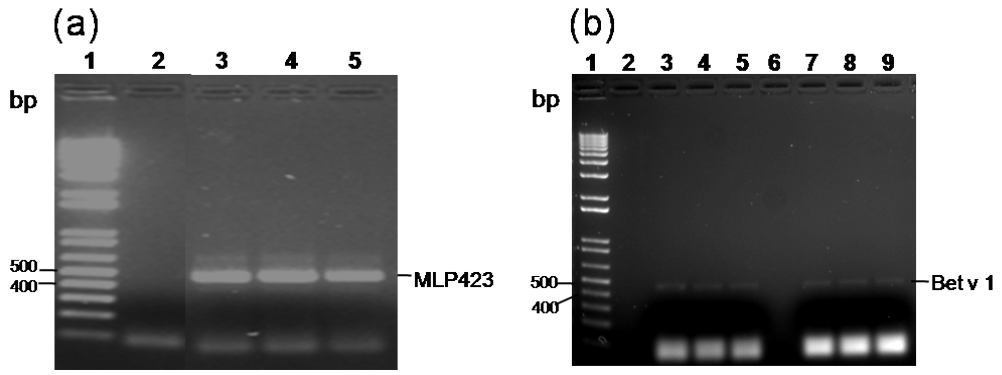

Figure 2. Amplification of cDNA encoding MLP423 and Bet v1. (a) Agarose gel showing the cDNA amplified with primers specific for MLP423.Lane 2 contains the no-RT control; lanes 3-5, amplification products of RT-PCR reactions using primers specific for MLP423. (b) Agarose gel showing the cDNA amplified with primers specific for Bet v1.Lanes 2 and 6 are empty lanes. Lanes 3-5 and 7-9 are amplification products of RT-PCR reactions using Bet v 1 specific primers (no RT control was not used)

amplify putative cDNA encoding MLP423 and Bet v1.

4.3 Cloning of MLP423 and Bet v 1cDNAinto protein expression vector and confirmation

cDNAs for *MLP423* and *Bet v 1*isolated as described in the previous section were cloned into the expression vector, pET22b+ and the positive clones were confirmed using colony PCR. PCR results indicated amplification of DNA fragments of the expected size; 468 bp for MLP 423 (Figure 3a) and 480 bp for Bet v 1 (Figure 3b). The position of the inserts relative to the promoter and terminator sequences in the plasmid are illustrated using the schematic diagrams above Figure 3a and 3b for *MLP423* and *Bet v 1*, respectively.

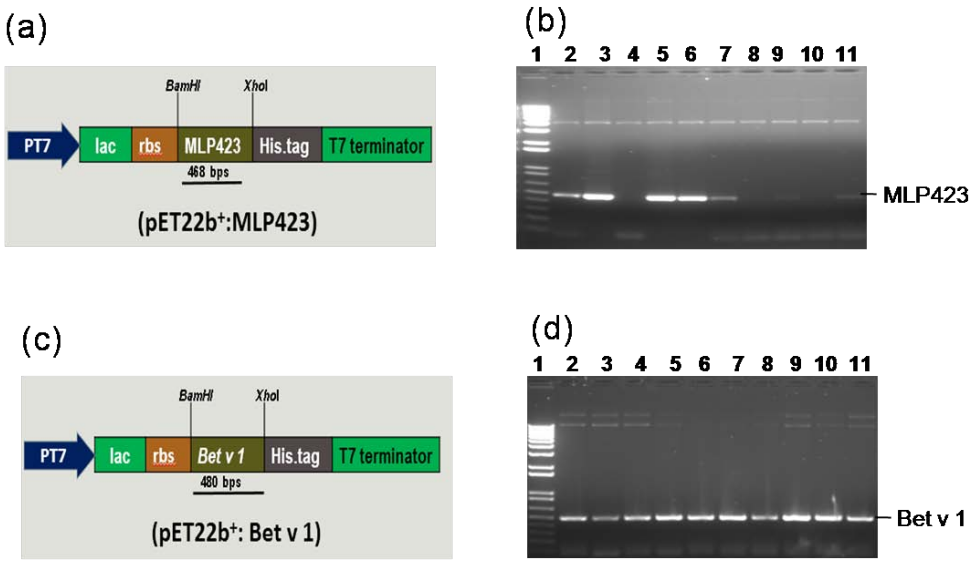

Figure 3. Confirmation of the presence of *MLP423* and *Bet v 1* cDNAs in the protein expression vectors. Putative *MLP423* (b) and *Bet v 1* (d) amplification products are observed in the various lanes containing amplification products from PCR reactions using individual colonies. The position of the putative *MLP423* and *Bet v 1* fragments in the expression vectors are shown in the schematic diagrams (a and c, respectively)

4.4 Sequence confirmation of constructs (MLP423 and Bet v 1)

Plasmid DNA isolated from three clones confirmed by colony PCR as containing the appropriate inserts (putatively encoding either MLP423 or Bet v1) was isolated and subjected to automated DNA sequence analysis using a Sanger DNA Sequencer model 3730 (Applied Biosystems, USA). Analysis of sequence data was carried out usingClustW2 (http://www.ebi.ac.uk/Tools/clustalw2/). Analysis of the sequence data revealed that the open reading frame (ORF) of *MLP423* and the *Bet v 1*, cloned in pET22b+ were in the correct reading frame.

4.5 Expression in and purification of recombinant forms of Bet v 1 and MLP423 from *E. coli.*

Recombinant plasmids containing sequence verified *MLP423* and *Bet v1* inserts were used for expression of the protein as described in the Materials and Methods. Periplasmic extracts were prepared as described earlier and used for isolation of the protein.

4.6 Western blot analysis

Western blots were used to confirm the expression of recombinant MLP423 and Bet v 1 proteins. Since the recombinant protein was fused with a poly histidine tag, anti-poly histidine antibody was used as a probe to hybridize with recombinant protein. After hybridization we observed the hybridization signals which corresponded to the protein expression of MLP423 (Figure 6a&b) and Bet

v 1(Figure 6c & d). This result here clearly indicated that the recombinant protein of MLP423 was successfully expressed in *E. coli*.

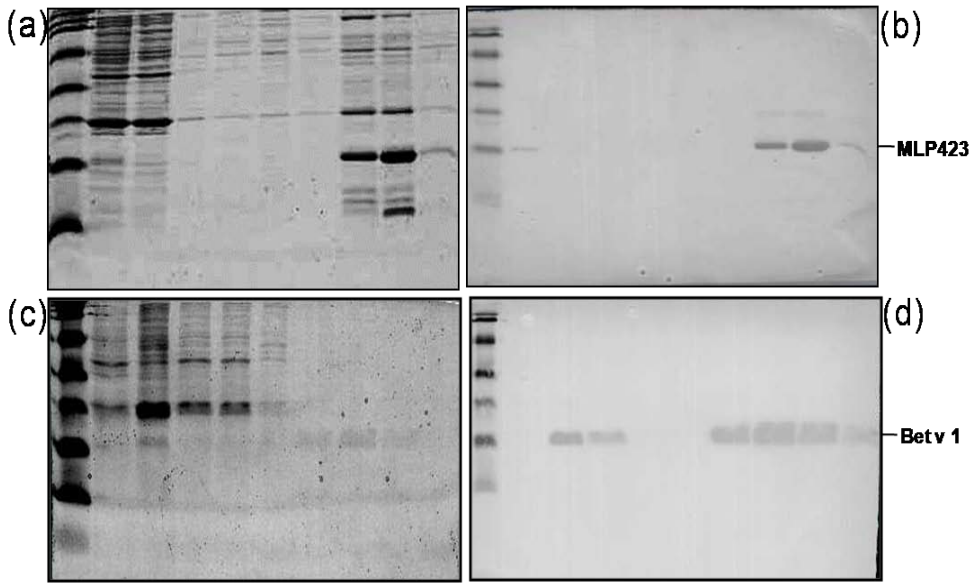

Figure 4. Western blot analysis of MLP423 and Bet v 1. The upper images show the western blot analysis of MLP423 which consists of an SDS-PAGE gel (a) and the corresponding immuno blot image (b). The lower figures show Western blot analysis of Bet v 1 which consists of an SDS-PAGE gel (c) and the corresponding immuno blot image (d). Anti-His antibody was used as a probe in the experiment.

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The first step used for the purification of the recombinant proteins was a metal-chelation affinity chromatography step with stepwise elution of the adsorbed proteins. This step did not result in purification of the recombinant protein to homogeneity as evidenced by the presence of multiple protein bands on gels after SDS-PAGE (Figure 5).

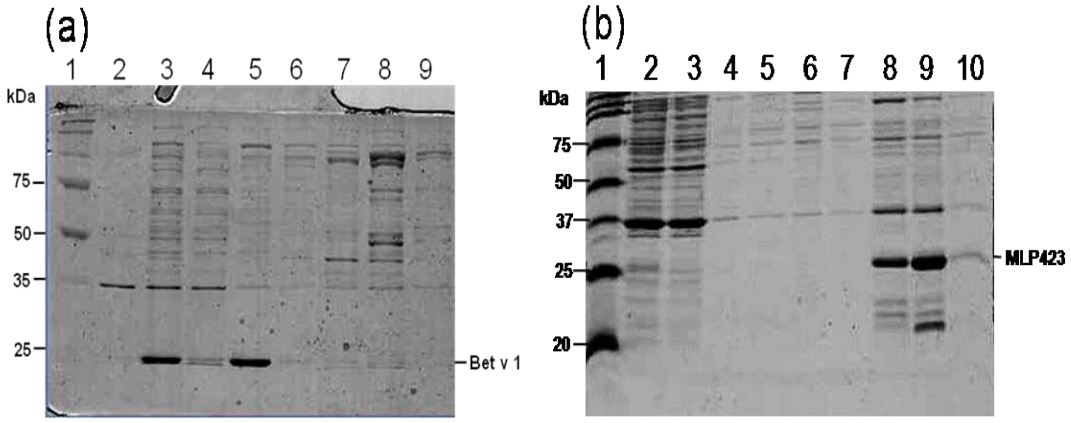

Figure 5. SDS-PAGE image showing purification of Bet v 1 and MLP423 using Ni-NTA chromatography. Lane 2 is the periplasmic extract and lane 3 is dialyzed periplasmic extract, lane 4 is flow through. Lanes 5-7 are different washes Lane 8-10 are eluted fractions with different imidazole concentrations.

Since many of these impurities differed in molecular weights when compared to MLP423 or Bet v1, we resorted to size exclusion chromatography on an FPLC system. Several fractions of Bet v1 eluting from the size exclusion column appeared to be homogeneous (Figure 6) and were used for further characterization of its RNase activity.

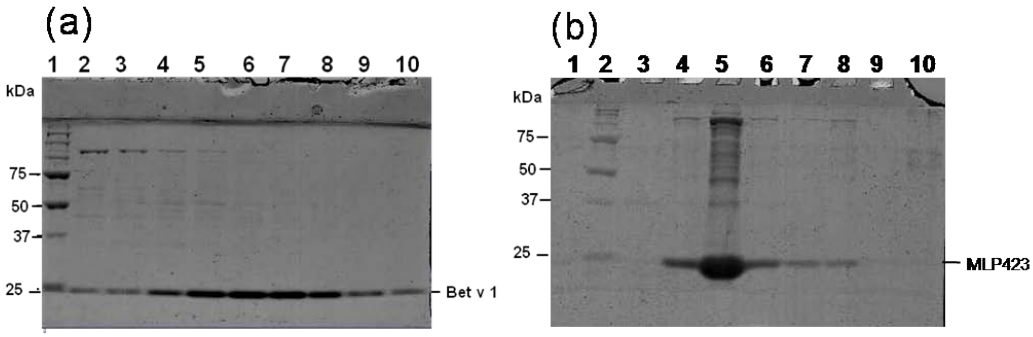

Figure 6. SDS-PAGE images showing different SEC, FPLC purified fractions or eluates of Bet v 1 and MLP423.Different fractions of Bet v 1(a) as eluted from the column. Fractions in lanes 1-4 contain some impurities, while fractions in lanes 5-10 show the pure protein of expressed Bet v 1.(b) Consists of different fractions MLP423 eluted from the FPLC column. Lanes 1 and 3 are empty lanes. Lanes 4-5 have some impurities, while lanes 6-10 are nearly pure eluates of MLP423.

4.7 In-solution RNA degradation assay of MLP423 and Bet v 1

The in-solution RNase activity was performed using the purified, recombinant proteins of MLP423 (Figure 7) and Bet v1 (Figure 8) as described in the Materials and Methods. Reaction products were analyzed using an agarose gel and the results indicated that MLP423 showed RNase activity (Figure 7) whereas; Bet v 1 did not show any RNase activity (Figure8).

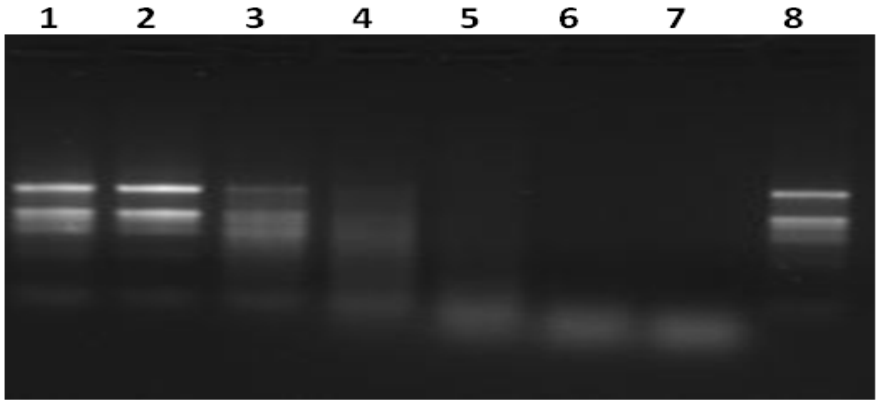

Figure 7. In solution RNase assay with a preparation of MLP423. RNA isolated from *A. thaliana* was incubated in the presence or absence of the MP423 protein preparation and the reaction products analyzed on an agarose gel. Lane 1 is total RNA from *Arabidopsis* incubated for the same time without any added protein preparation. Lane 2 is total RNA dissolved in the elution buffer used for preparing recombinant MLP423. Lanes 3-7 are the different concentrations (3-15 µg) of the MLP423 preparation and lane8contains the products from incubation of RNA with 15µg of boiled/denatured recombinant MLP423 preparation.

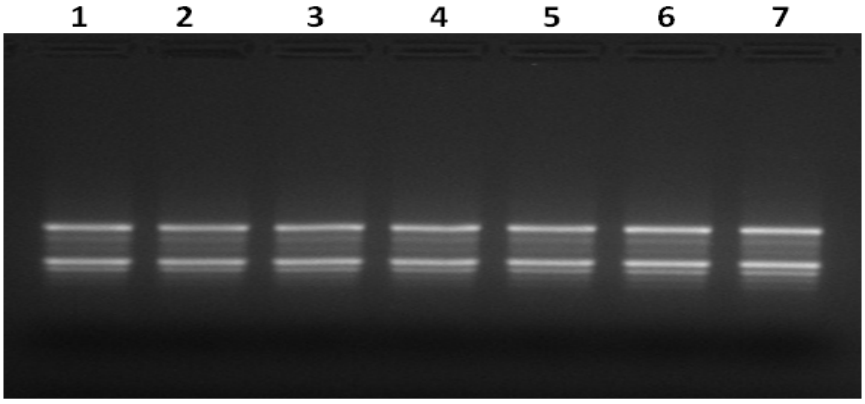

Figure 8. In solution RNase assay with a preparation of Bet v1. RNA isolated from *A. thaliana* was incubated in the presence or absence of the Bet v 1 protein preparation and the reaction products analyzed on an agarose gel. Lane 1 is total RNA from *Arabidopsis* incubated for the same time without any added protein preparation. Lane 2 is total RNA dissolved in the elution buffer used for preparing recombinant protein Betv1. Lanes 3-6 are different concentrations (3-12 µg) of Bet v 1 protein preparation and lane 7 contains the products from incubation of total RNA with 12 µg of boiled/denatured

4.8 In-gel RNA degradation assay of MLP423 and Bet v 1

In-gel RNA degradation assay was performed for MLP423 and Bet v 1.The results of in-gel RNA assay was analyzed on SDS-PAGE gels, in which the RNase activity was observed in total protein of *E. coli* indicating that the in-gel assay system was working, whereas the RNase activity was not exhibited by MLP423 and Betv1 (Fig. 9).

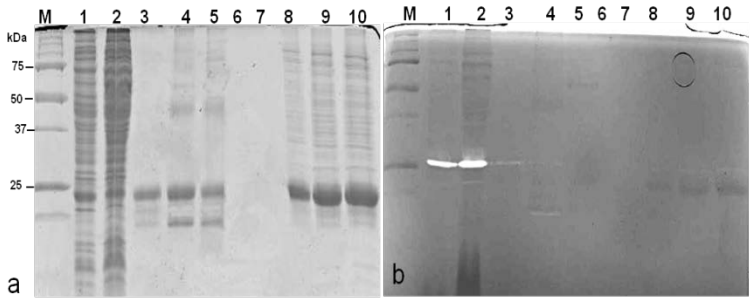

Figure 9. In-gel RNA degradation assay with recombinant MLP423 and Bet v 1. (a) The left panel of the image shows an SDS-PAG E gel and the (b) right panel shows the gel image after the RNA degradation assay; Lanes 1-2 contain total protein from *E. coli* where the RNA degradation was observed as a white band in the lane. Lanes 3-5 are recombinant protein (Betv1). Lane 6 is empty and lanes 7-9 are the recombinant protein MLP423.

5.0 Discussion

PR-10 proteins are present in almost every plant species. MLP423 and Bet v 1 are considered to be homologues of PR-10 proteins because of the significant similarities that these proteins exhibit at the amino acid sequence level. As discussed in the introductory chapters, these proteins may be important during normal growth and development of plants as well as in response to various stresses, both biotic and abiotic. Some PR-10 proteins are known to possess RNase activity including those extensively studied in our laboratory, ABR17 from pea (Krishnaswamy et al., 2008; Srivastava et al., 2006a). However, there is no information on the biological activities of MLPs or Bet v1s from *A. thaliana*. In order to determine whether these proteins possess RNase activity, we expressed one MLP (MLP423) and one Bet v1 (AT5G45870.1) in *E. coli*, purified the recombinant proteins and assayed their RNase activities.

The desired cDNAs were amplified and expressed in *E. coli* and purified using the chromatography method (Ni^{+}-NTA and size exclusion chromatography). In solution RNase activity assays using the recombinant MLP423 preparation showed RNase activity (Figure 7), whereas the Bet v1 preparation did not exhibit any RNase activity (Figure 8). In order to confirm that it was indeed the MLP423 protein in the preparation which exhibited the RNase activity, we performed an in-gel RNase activity assay (Yen and Green 1991).These assays have the ability to confirm that the observed RNase activity in-solution is indeed the result of the specific MLP423 band possessing this enzymatic activity. Where the protein

purity is in question, as it is perhaps in this case, it is imperative to obtain confirmation that the observed RNase activity is due to the activity of the protein in question, in this case, MLP423. In the case of Bet v 1, our in-gel assays confirmed the lack of RNase activity (Figure 9). On the contrary, the results from the in-gel assays were not in agreement with our in-solution assays and we could not attribute the observed RNase activity to the MLP423 band (Figure 9). In fact, it is clear from the results presented that another band which is present in this preparation as an impurity exhibits RNase activity (Figure 9) and this impurity must have been responsible for the observed activity of this preparation in-solution.

These preliminary findings need to be confirmed through additional experiments aimed at generating preparations of MLP423 and Bet v 1 which are near homogeneity. It is also possible that the observed lack of RNase activity of these two proteins is due to the fact that the recombinant forms of this protein did not fold into the correct three-dimensional structure. It is also conceivable that the RNase activity of these proteins depend on some form of post-translational modification (e.g. phosphorylation). In fact, it is known that the activities of some PR-10 proteins are indeed modulated by phosphorylation (Jain et al., 2011). If we were to assume that these proteins do not possess RNase activity, as indicated by our in-gel activity assays, despite the presence of the conserved P-loop motifs, the question must be asked as to why they do not possess RNase activity. It may be because they do not possess the conserved amino acids histidine (H)-69 and glutamic acid (E)-148, which were reported to have an important role for the

observed RNase activity of a number of PR-10 proteins (Wu et al. 2003, (Krishnaswamy et al., 2011). It is possible, therefore, that the absence of these two amino acids in MLP423 and Bet v1 is one of the reasons for the lack of RNase activity. An interesting future experiment might be to replace corresponding amino acids in Bet v1 and MLP423 with histidine and glutamic acid to see if these proteins can be converted to RNases. Another line of investigation in the future should be to determine, if indeed these proteins lack RNase activity, whether other members of the MLP and Bet v1 family possess RNase activity and if the native proteins isolated from plants exhibit such activity. Finally, if these proteins are demonstrated to lack RNase activity after a considerably more thorough analysis, experiments should be directed towards understanding their biological activities and role(s) *in planta*.

6.0 Appendix I

Generation of transgenic plants for inducible expression of pea ABR17 and its site-directed mutants

As described in the main body of this thesis, the PR-10 family of PR proteins is a large family with more than 100 members and its presence has been reported in different plant species. These proteins may be important for the protection of plants against from biotic and abiotic stresses (Liu and Ekramoddoullah, 2006; Van Loon and Van Strien, 1999a). Most of the members characterized, possess RNase activity (Bufe et al., 1996; Mauch and Staehelin, 1989).Over expression of PR-10 in plants has enhanced the plant performance especially against abiotic and biotic stresses. For example; the PR-10 protein such as, abscisic acid responsive 17 (ABR17/ PR10.4), a member of PR-10 protein family from pea (*Pisum sativum*), when over-expressed in Canola (Srivastava et al., 2004) and *Arabidopsis* (Srivastava et al., 2006b) showed tolerance against salinity stress. However, the molecular function of these PR-10 proteins are not well understood, and several studies have reported that these proteins are responsible for protecting the plants against pathogen attacks and abiotic stresses (Van Loon et al., 2006).

It has been reported that the pea PR-10 protein, ABR17, possesses RNase activity (Srivastava et al. 2006). Amino acid sequence alignment of different PR-10 proteins indicated the presence of several conserved amino acids (Liu and Ekramoddoullah, 2006). It has also been established through research from our group and that of others that, among the conserved amino acids, Histidine (H)

69and Glutamic acid (E) 48 are important for the observed RNase activity of ABR17 (Krishnaswamy et al. 2008). In fact the site-directed mutation of H69 to Leucine (L) and E48 to Alanine (A) alters the RNase activity of pea ABR17 with the H69L mutation lacking RNase activity and the E48A mutation leading to enhanced activity (Krishnaswamy et al. 2011).

It has also been demonstrated that the constitutive expression of pea ABR17 elevates the endogenous CK levels in this species and enhances germination and early seedling growth even under abiotic stress conditions (Srivastava et al.2006). It has also been reported that the constitutive expression of pea ABR17 confers multiple stress tolerance in *A. thaliana* (Srivastava et al.2006). However, it is not clear whether the observed RNase activity of pea ABR17 is important for both the elevation of endogenous CK levels, presumably as a result of tRNA degradation (Zubini et al., 2009), and the observed abiotic stress tolerance. In order to investigate the role of RNase activity of pea ABR17 in mediating the observed effects in *A. thaliana* (the increased endogenous CK and multiple stress tolerance), we wished to express, under the control of an inducible promoter, ABR17 and two of its site-directed mutants H69L and E48A in *A. thaliana* and characterize those transgenic plants. As a first step towards achieving this goal, we describe the isolation of an inducible promoter and the generation of several plasmid constructs. In addition, the transformation of plants with some of these constructs and the progress made are described in this appendix.

AI.1.1 Isolation of ethanol-inducible promoter (alc A promoter)

In order to develop a chemically inducible plant expression system, alcA promoter (an ethanol inducible promoter) was isolated from *Aspergillus nidulans* (fungal spores were kindly provided by Dr. Strelkov's lab) using standard molecular biological techniques. The primers used for various PCR amplification processes to generate the plasmid constructs are described in Table 2.

Table 2. List of primers used for amplification of alcA, ABR17, M1 and M2

Primers	**Sequence (5'-3')**	**Size of the amplicon (bp)**
Forward alc A (GUS) Reverse alc A (GUS)	ATAAGCTTTAAGTCCCTTCGTATTTCTCCG ATGGATCCTTTGAGGCGAGGTGATAGGAT	468
Forward alc A Reverse alc A	ATGGCGTAATTTAAGTCCCTTCGTATTTCTCCG CTTTCAAGGTATGGGTGTCTTTGTT	468
Forward M1, M2 and ABR17 (WT) Reverse M1, M2 and ABR17 (WT)	GTGGTCGCATATGGAAAATTTGTACTTTCAAGGTATGGGTGTCTTTGTTTTTGATGATGAATAC TATATAGCTCGAGTTAGTAACCAGGATTTGCCAAAACGTAACC	473

AI.1.2 Development of plasmid constructs

Following constructs were developed

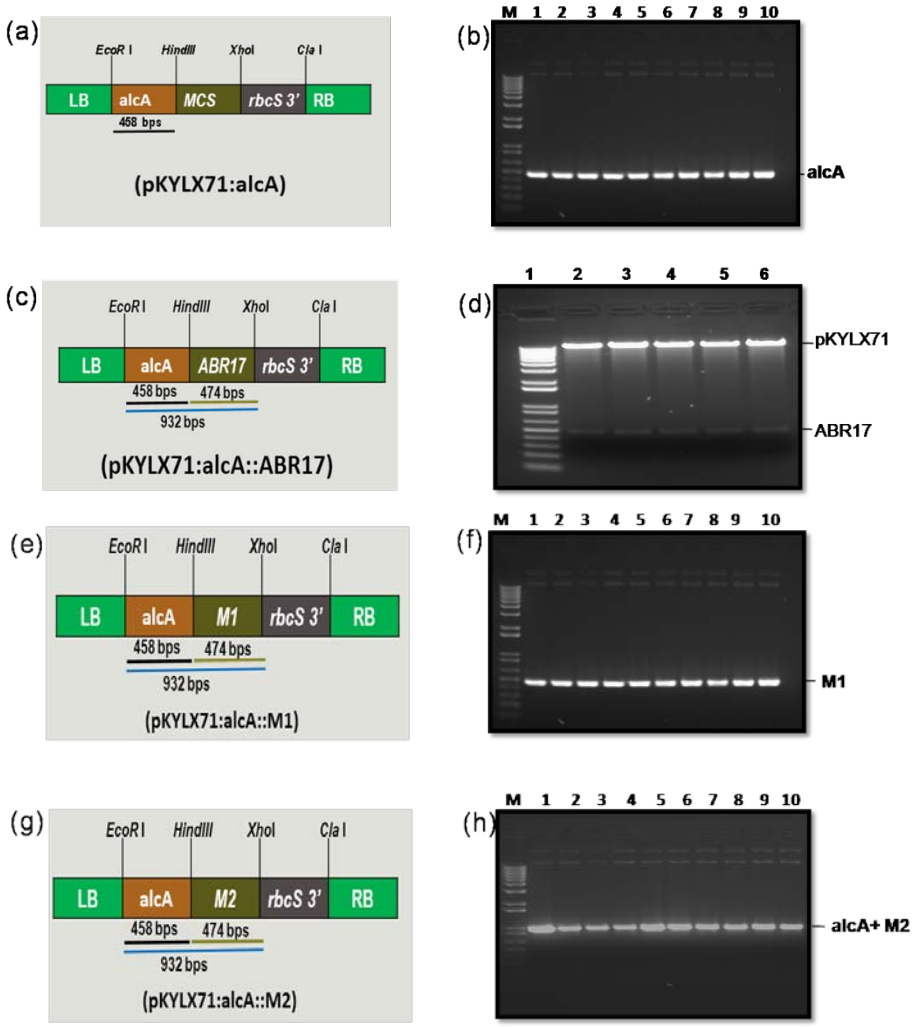

Figure.A. 10. Confirmation the presence of different inserts (alcA, ABR17, M1 and M2) into plant expression vector; pKYLX71. Left panels are the schematic diagrams (a, c, e and g) illustrating the positions of the putative fragments (alcA, ABR17, M1 and M2) respectively. While the agarose gel panels (b, f and h) on the right are confirmation of inserts through colony PCR using gene specific primers of alcA, M1 and M2 respectively. The agarose gel (d) shows the confirmation of ABR17 insert through restriction digestion.

AI.1.3 Plant transformation and confirmation of transgenic lines

The *Agrobacterium* strain harbouring the desired constructs was used for the plant transformation. The plant transformation protocol was used as described by (Clough and Bent, 1998) T_0 seeds were screened on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing kanamycin (50µg/ml). The putative transformants were screened using genomic PCR.

AI.2 Conclusion

We have isolated the alcohol-inducible promoter from *A. nidulans* to be potentially used for the inducible expression of pea ABR17 and its site-directed mutants. We have mobilized this promoter into the plant expression vector (pKYLX-71; An et al. 1985) to potentially express ABR17 and its mutants in an alcohol inducible manner. These constructs have been used to transform *A. thaliana*.

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