Screening of plant suspension cultures for antimicrobial activities and characterization of antimicrobial proteins from *Arabidopsis thaliana*

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Summary

The continuously increase in resistance of human pathogenic microorganisms to the known antibiotics leads to the necessity for searching new sources for production of new active antimicrobial compounds from different natural sources especially plants, since many plants have been found to be able to produce antimicrobial compounds as a defense phenomenon against invading microorganisms. The aim of this work is to screen cultures for production of antimicrobial activity against representative of human pathogenic microorganisms and selection the most active cell culture producing antimicrobial protein(s) which are active against these pathogenic microorganisms and also isolation ,purification of the active protein(s) and cloning of its/their genes.

Ten different plant suspension cultures have been screened in presence of nine elicitors for their antimicrobial activity against five selected human pathogenic microorganisms, and it has been found that the heterotrophic cultures are more active against the tester isolates than the autotrophic ones. The intracellular fraction of the mixotrophic *Arabidopsis thaliana* culture elicited with salicylic acid showed the highest antimicrobial activity against the tester isolates.

The presence of proteinous antimicrobial activity has been elucidated by testing the activity of ammonium sulphate precipitate against *Candida maltosa.* High speed centrifugation technique has been used for partial purification of the active protein. The proteinous nature of the isolated compound has been confirmed by using bioautography technique and its molecular weight could be estimated to be around 26KDa. The active protein has been purified using gel filtration, and using mass spectrometry technique, for microsequencing of the active protein, it has been found that the function of the protein is unknown and we have termed it as AtPDP1 according to *Arabidopsis thaliana* Plat-Domain Protein1, since it contains a plant stress domain termed PLAT domain. It has been found that a second protein from the same plant with high homology level to AtPDP1 with the same domain, we termed it as AtPDP2. Genes for AtPDP1 and AtPDP2 have been cloned in *E. coli* using PGEM-T easy vector. The expression of both genes have been tested using Digital Northern program, and it has been observed that both genes are induced by different pathogens, chemicals known to induce defense in plant cells and also different hormones. We tried to clone the gene for AtPDP1 in PBI121

binary vector under the control of an elicitor inducible promoter of a proteinase inhibitor gene, to test its function in plant by overexpression, but we did not succeeded.

Also the work aims to cloning the different known thaumatin genes from *Arabidopsis thaliana* for future work which represented by testing their expression under different stimuli, since most thaumatins have antimicrobial activity and some of them are active against *Candida* spp..Thirteen genes of known thaumatins from *Arabidopsis thaliana* have been cloned in PGEM-Teasy vector in DH5α *E. coli* cells. The expression of the thirteen genes has been done using Digital Northern program and it has been found that different genes show different expressions under different stimuli and the expression of At1g75800 gene was the maximum under all stimuli. The minimum expression of genes was for At1g75050. The rest of thaumatin genes showed moderate expressions under different stimuli.

Zusammenfassung

Die zunehmende Resistenz humanpathogener Mikroorganismen gegen bekannte Antibiotika bedingt die Notwendigkeit, nach neuen Quellen für die Produktion antimikrobieller Stoffe zu suchen. Als eine solche Quelle gelten besonders Pflanzen, da viele antimikrobielle Stoffe bei der Abwehr gegen invasierende Mikroorganismen bilden. Das Ziel der vorliegenden Arbeit besteht in der Charakterisierung von pflanzlichen Zellkulturen im Hinblick auf ihre Fähigkeit, anitimkrobielle Aktivität gegen humanpathogene Mikroorganismen zu entwickeln. Dabei sollen aktive Proteine aufgereinigt und die kodierenden Gene isoliert werden.

Dazu wurden zehn verschiede pflanzliche Suspensionskulturen in Anwesenheit von neun Elicitoren auf ihre antimikrobielle Aktivität gegen fünf humanpathogene Mikroorganismen getestet. Dabei erwiesen sich die heterotrophen Kulturen im Vergleich zu den autotrophen als aktiver. Die höchste antimikrobielle Aktivität wurde bei der intrazellulären Fraktion der mixotrophen Kultur von *Arabidopsis thaliana* nach Elicitierung mit Salicylsäure nachgewiesen. Da in einem Präzipitat mit Ammoniumsulfat Aktivität gegen *Candida maltosa* nachgewiesen wurde, konnte angenommen werden, dass es sich bei der aktiven Komponente um ein Protein handelt. Durch Hochgeschwindigkeitszentrifugation wurde eine partielle Aufreinigung dieser aktiven Komponente erreicht. Die proteinoide Natur wurde durch Bioautographie bestätigt und das Molekulargewicht auf ca. 26kDa geschätzt. Mittels Gelfiltration und Massenspektrometrie wurde das Protein aufgereinigt. Die Mikrosequenzierung ergab ein Protein mit bisher unbekannter Funktion, das eine pflanzliche Stressdomäne (PLAT) enthält. Das Protein wurde daraufhin als AtPDP1 (Arabidopsis thaliana Plat-Domain Protein 1) bezeichnet. Das Gen und ein zweites mit hochgradiger Homologie (AtPDP2) wurden in *E. coli* kloniert. Der Digital Northern zeigt an, das beide Gene durch verschiedene Pathogene induziert werden, sowie von Chemikalien, die pflanzliche Abwehr hervorrufen und weiterhin von Phytohormonen. Der Versuch, AtPDP1

unter die Kontrolle eines Promors einer Proteinase zu stellen, der Induzierbarkeit durch Elicitoren vermittelt, blieb erfolglos.

Weiterhin wurden 13 Thaumatingene aus *Arabidopsis thaliana* in *E. coli* kloniert, da ihre antimikrobielle Aktivität bekannt ist, und ihre Expression durch verschiedene Stimuli induziert wird. Von diesen Genen zeigt der Digital Northern bei allen Stimuli eine maximale Expression für At1g75800, während At1g75050 minimal induziert ist. Diese Gene stehen für zukünftige Studien zur Verfügung.

1.Introduction

1.1 Overview of production of antimicrobial compounds by plants

The continuous use of antibiotics has resulted in multiresistant microbial strains allover the world and as expected, hospitals have become breeding grounds for human associated microorganisms (Mainous and Pomeroy, 2001). Among fungi associated with human diseases, the *Candida* spp. were, for many years, the most frequently isolated from clinical specimens (Ainsworth, 1986). Also the emerging of AIDS disease increased the risk of infections in the immunocompromised patients, caused by pathogenic yeast (known or emerging) which cause cutaneous or systemic infections (Anaissie and Bodey, 1989; Cimolai *et al*., 1987). Another serious risk is caused by increasing development of fungal resistance to currently available antimycotic agents (Masiá Canuta and Gutiérrez Rodero, 2002). This phenomenon, firstly described in bacteria, is becoming a looming threat also for yeast, occasionally developing resistance also towards azoles and polyenes, which represent the two major classes of known antimycotic drugs (Sanglard and Odds, 2002; Nguyen *et al*., 1996; Pfaller *et al*., 2001; Rex *et al*., 2000; Powderly *et al*., 1988). Also for bacterial strains, they have genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents (Cohen, 1992). The knowledge acquired in the past two decades and the discovering of new groups of antimicrobial proteins make natural antibiotics the basic element of bacterial and fungal infections (De Lucca, 2000; Hancock, 2000; Welling *et al*., 2000; Selitrennikoff, 2001). Among eukaryotes producing antimicrobial compounds, plants synthesize a vast array of secondary metabolites as defense mechanisms for protecting themselves against pathogen infections [bacteria, fungi and viruses (Osbourn, 1996; Mendoza *et al*., 1997; Skadhange *et al*., 1997; Tereschuk *et al*.,1997; Bois *et al*., 1999; Hou and Forman, 2000; Rauha *et al*., 2000)]. The use of plant extracts and phytochemicals can be of great

significance in therapeutic treatments. In the last years, a number of studies have been conducted in different countries to prove such efficiency (Almagboul *et al*., 1985,1988; Artizzu *et al*., 1995; Ikram and Inamul, 1984; Izzo *et al*., 1995; Kubo *et al*., 1993; Shapoval *et al*., 1994). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in secondary metabolism of the plant. Hence, more studies pertaining to use of plants as therapeutic agents should be emphasized, especially those related to control of antibiotic resistant microbes.

1.2 Overview of production of new pharmaceutical compounds by plant tissue cultures

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides (Balandrin and Klocke, 1988). The search for new plant-derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Phillipson, 1990). In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Roa and Ravishankar, 2002). Cell suspension culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted.

Plant tissue cultures were first established in 1939-1940. However, it was only in 1956 that the first patent for the production of metabolites by mass cell cultures was filed by the American pharmaceutical company, Pfizer Inc. (Pétiard and Bariaud-Fontanel, 1987). The potential of plant cell cultures to produce useful compounds, especially for drug development, was perceived in the late 1960s. Thus Kaul and Staba (1967) and Heble et al. (1968) isolated visnagin and diosgenin respectively from cell cultures

in larger quantities than from the whole plant. However, a large number of cultures failed to synthesize products characteristic of the parent plant. For instance, morphinan, tropane and quinoline alkaloids are synthesized only at extremely low levels in cell cultures (Berlin, 1986). That is why, after a surge of interest, the trend of research declined.

In 1976, at an international congress held in Munich, Zenk and his co-workers demonstrated the outstanding metabolic capacities of plant cells and highlighted the spontaneous variability of plant cell biosynthetic capacity, which could explain the contradictory results obtained earlier. This natural variability is exploited to identify high-yielding cultures for use on an industrial scale (Tabata et al., 1976; Zenk et al., 1977; Zieg et al., 1983; Yamada, 1984; Benjamin et al., 1986). Since the late 1970s, research and development in this area has seen a high increase in the number of patent applications field, especially by the scientific and corporate filed, and by the scientific and corporate sectors in the Federal Republic of Germany and Japan. In 1983, for the first time, a dye, shikonin, with anti-inflammatory and anti-bacterial properties, was produced by plant cell cultures on an industrial scale by Mitsui Petrochemical Industries Ltd (Fujita et al., 1982). However, although this was thought to be a major breakthrough, shikonin production is still the only plant product to be produced on a commercial scale by cell cultures.

Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the last few years. New physiologically active compounds of medicinal interest have been found by bioassay. It has been demonstrated that the biosynthetic activity of cultured cells can be enhanced by regulating environmental factors, as well as by artificial selection or the induction of variant clones. Some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture system not only by inducing specific organized cultures, but also by undifferentiated cell cultures. The possible use of plant cell cultures for the specific biotransformation of natural compounds

has been demonstrated (Cheethama, 1995; Scragg, 1997; Krings and Berger, 1998; Ravishankar and Ramachandra Rao, 2000). Due to these advances, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations.

The major advantage of cell culture system over the conventional cultivation of whole plants are:

(1) Useful compounds can produced under controlled conditions independent of climatic changes or soil conditions.

(2) Cultured cells would be free of microbes and insects.

(3) The cells of any plant, tropical or alpine, could easily be multiplied to yield their specific metabolites.

(4) Automated control of cell growth and rational regulation of metabolite processes would reduce of labor costs and improve productivity.

(5) Organic substances are extractable from callus cultures.

1.3 Overview of production of antimicrobial peptides by different organisms

1.3.1 Antimicrobial peptides and their mode of action

1.3.1.1 Antimicrobial peptides

Members of the major groups of antimicrobial peptides have been classified mainly on the basis of their biochemical (net charge) and/or structural features (linear/circular/amino acid composition), looking for common patterns that might help to distinguish them (Tossi and Sandri, 2002; Zasloff, 2002). The resulting most important groups are the following:

From eukaryotes

***Cationic peptides*:** This is the largest group and the first to be reported, being widely distributed in animals and plants. So far, more than a thousand of such peptides have been characterized and over 50% of them have been isolated from insects (Bulet et al., 1999; Andreu and Rivas,

1998). On the basis of their structural features, cationic peptides can be divided into the following:

1- ***Cecropins*:** This is a family of 3-4 kDa linear amphpatic peptides described in haemolymph of insects in the early 1980s (Hultmark et al., 1980; Andreu and Rivas, 1998; Boman 1998; Zheng and Zheng, 2002). These molecules are devoid of cysteine residues and contain two distinctive helical segments: a strongly basic N-terminal domain and a long hydrophobic C-terminal helix, linked by a short hinge. Shortly there after, other linear amphipatic peptides such as the magainins isolated from Xenopus skin, were isolated from vertebrate sand included in the same group (Zasloff, 1987; Bechinger et al. 1993; Simmaco et al. 1998). These were the first molecules used to evaluate their biochemical applications (Hancock, 2000).

2- ***Defensins*:** This is a highly complex group of 4 kDa open-ended cysteine-rich peptides arranged with different structural motifs. They have been mostly isolated from mollusk, acari, arachnids, insects, mammals and plants. Defensins are arranged in families, based on their structural differences. Invertebrates (Hubert et al 1996; Andreu and Rivas, 1998; Dimarcq et al 1998; Bulet et al 1999; Mitta et al 1999; Silva et al. 2000; Nakajima et al. 2001) and plant (Broekaert et al. 1997; Garcia-Olmedo et al 1998; Segura et al. 1998; Liu et al. 2000). Defensins are characterized by three and four disulfide bridges, respectively. They show a common structure comprising and α-helix linked to a ß-sheet by two disulfide bridges, distinctive structure known as CSab motif. In mammals, α- and ß-defensins are characterized by an antiparallel ß-sheet structure, stabilized by three disulfide bridges (Zasloff, 2002). Some of them naturally exist as cyclic molecules such as the theta-defensins (Tang et al. 1999; Lehrer and Ganz, 2002). It has been difficult to determine whether all molecules are homologous or have

independently evolved similar features, but evidences are in favor of a distant relationship. The best evidence of this relationship is structural, particularly from their overall three-dimensional structure and from the spacing of helf-cystine residues involved in intra-chain disulfide bonds.

3- ***Thionins*:** These are antimicrobial, and generally basic, plant peptides with a molecular weight of 5 Da, which contain 6 or 8 conserved cysteine residues. Their *in vitro* toxicity against plant pathothogenic bacteria and fungi indicates a role in the resistance of plants (Bohlmann, 1999). Ligatoxin B, a new basic thionin containing 46 amino acid residues has been recently isolated from the mistletoe *Phoradendron liga* (Li et al., 2002). Similarities observed by structural comparison of the helix-turn-helix (HTH) motifs of the thionins and HTH DNA-binding proteins, lead the authers to propose that thionins meight represent a new group of DNA-binding proteins.

4- ***Amino acid-enriched class:*** This is a distinctive class of antibacterial and antifungal cationic peptides, enriched in specific amino acids, with distinctive features depending on organism from which they are isolated. Those proline-glycine-rich are mostly from insects and active against Gram-negative bacteria (Bulet et al 1999; Otros, 2000); while cystein-rich peptides, not related to defensins, represent the most diverse family among arthropods (Dimarcq et al 1998). On the other hand those enriched in histidine are particularly basic, mostly from mammals (Pollock et al 1984). Among them, histatin recovered from saliva from humans and primates and primarily directed against fungal pathogens, outstands for its distinctive mechanism of action which does not involve channel formation in the fungal cytoplasmic membrane but rather translocates efficiently into the cell and targets the mitochondrion (Tsai and Bobek, 1998). Those enriched in histidine and glycine are quiet large, also affecting fungal pathogens and a distinctive feature

is that their residues are arranged in approximately regular but different structural repeats (Tossi and Sandri, 2002). Finally, only two peptides enriched in tryptophan residues have been described both drived from porcine cathelicidin precursors (Schibli et al. 2002). The outstanding feature through, is broad spectrum of activity including hundreds of Gram-positive and negative clinical isolates in addition of fungi and even the enveloped HIV virus (Gennano and Zanetti, 2000).

5- ***Histone derived compounds*:** This is a family of cationic helical peptides corresponding to cleaved forms of histones originally isolated from toad-(buforin) (Park et al 1996) and fish epithelia (Parasin) (Park,1998). These molecules are structurally similar to cecropins and quite active against bacteria and fungi. In the case of buforin II, at least, it was demonstrated that this molecule penetrates bacterial membranes and bind to nucleic acids thus interfering with cell metabolism and leading to rapid cell death (Park et al. 1998). AMPs are important factors in fish innate immunity (Iwanaga et al. 1994; Lemaitre et al. 1996; Zhou et al. 2002) and new contributions tend to demonstrate it. Recently, an active peptide was identified both in coho salmon mucus and blood, which display full indentity with the N-terminus of trout H1 histone (Patrzykat et al 2001). This is an indication that histone proteins may be a relatively ubiquitous component of host defenses (Hirsh, 1958). This assumption has been strengthened in recent years by the isolation of histone-like proteins in the cytoplasm of murine macrophages (Hiemstra et al 1993) and the characterization of histone H2B fragments in human wound fluids (Frohm et al. 1996)

6- ***Beta-hairpin:*** This class of cationic peptides includes a wide range of 2 to 8 kDa compounds containing beta-hairpin cross-linked by disulphide bridge(s). The smallest members of this class with one disulfide bridge, is represented by thanatin and brevinin. Those containing two disulfide bridges are represented by androctonin

(Mandard et al. 1999) tachyplesin and protegrin I (Mandard et al. 2002). Members of this latter group are 2-kDa hairpin-structured peptides, isolated from both invertebrates and vertebrates and show preferential antibacterial and antifungal activities (Dimarcq et al. 1998)

1.3.1.2 *Mechanism of action of cationic peptides*

In spite of the fact that the mechanism of action is not satisfactory established for all cationic peptides, the structural model established by Shai-Matzusaki-Huang (Matzuski,1999) provides a reasonable explanation for most antimicrobial activities of these compounds (Zasloff, 2002). The model proposes that these linear amphipatic-helical peptides interact with bacterial membranes and increase their permeability, either by the effect of their positive charges with anionic lipids of the target membrane or by membrane destabilization through lipid displacements due to drastic changes in the net charge of the composed system. A similar mechanism has been proposed for the cysteine-rich peptides such as defensins, which are suggested to form ion-permeable channels in the lipid bilayer. In contrast, some peptides penetrate into cells to exert their action over target molecules (Kragol et al. 2001). Several additional hypotheses have been proposed to explain the mechanisms by which peptides kill target; such hypotheses include induction of hydrolases which degrade the cell wall, disturbance of membrane functions and damage to crucial intracellular targets after internalization of the peptide (Zasloff, 2002).

Anionic peptides: This is a smaller noval group of molecules displaying antimicrobial activity which up to now, have been mostly isolated from mammals.

1- ***Neuropeptide derived molecules:*** This is the first class of anionic compounds recently found in infectious exudates of cattle and humans. They mostly include peptides derived from processing of

neuropeptide precursors such as pro-enkephalin-A, to yield active peptide B and enkelytin; some of them are phosphorylated (Salzet and Tasiemsky, 2001). These peptides are mainly active against Gram-positive bacteria at micromolar concentrations, like cationic peptides, and similar products have been reported in some invertebrate species (Salzet, 2001).

2- ***Aspartic-acid-rich molecules:*** Peptides of this class have isolated and characterized primarily from cattle pulmonary surfactants (Brogden et al. 1996; Bals, 2000; Fales et al 2002). They have a structure similar to the charge-neutralizing pro-peptides of group I serine proteases and have been proposed to regulate the activity of pulmonary enzyme systems in these animals. Recently, a novel anionic 47-amino acid peptide, named dermicidin, has been identified in human sweat, in response to a variety of pathogenic Gram-positive bacteria and ascribed to this class of molecules (Schittek et al 2001).

3- ***Aromatic dipeptides:*** The aromatic dipeptides comprise low molecular might antibacterial compounds primarily isolated from dipteran larvae. There are only two well characterized members: the N-alanyl-5-glutathionyl-3,4-dihyroxy-phenylalanine (573 Da), identified in flesh fly sarcophagi peregrine (Leem, 1999; Akiyama et al. 2000), and the *P*-hydroxycinnamaldehyde, isolated from the saw fly *Acantholyda parki* (Leem et al. 1999). The mode of action of these molecules is, at present, unknown.

4- ***Oxygen-binding proteins:*** Peptides derived from oxygen-binding proteins, or hemocyanin derivatives (Destoumieux-Garzon et al. 2001; Muñoz et al 2002; Muñoz et al. 2003), are the first representatives of the group of peptides derived from oxygen-binding proteins recently isolated from the hemolymph of arthropods and annelids species. Another molecule, detected in tick hemolmph, is a cleaved form of vertebrate hemoglobin, processed by the parasite after blood meal ingestion (Fogaca et al. 1999).

These proteins have been reported as bactericidal compounds and meight be considered as a reservoir of defense molecules to be used as integrative weapons to fight pathogens (Vizioli and Salzet, 2002). Bactericidal activity of anionic peptides, oxygen-binding protein derivatives and aromatic dipeptides are not as potent as cationic peptides, and their physiological relevance remains to be established in order to define their importance as components of innate response (Decker et al. 2001). These molecules, whose mode of action could differ from that of cationic peptides and other antibiotics, could complement the activity of other comounds and constitutive a useful base to develop noval synthetic derivatives.

From prokaryotes

The antimicrobial peptides produced by bacteria have been grouped into different classes based upon the producer organisms, molecular size, chemical structure and mode of action, which resulted in different names for putative compounds which turned out to be identical: (thiolbiotics, lantibiotic microcin, colicin, bacteriocin, to name a few) (Kolter and Moreno, 1992). The most relevant active-membrane peptides among them are produced by Gram-positive bacteria and classified taxonomically as bacteriocins (Oscáriz and Pisabarro, 2001). Some of them have been the center of attention because of their application as food preservatives (Schillinger et al. 1996). Bacteriocins, cationic, neutral and anionic in chemical nature, are all in the range of 1.9 (Actagardine) and 5.8 (Lactococcin B) kDa in molecular mass (Jack et al. 1995), cationic, neutral and anionic in chemical nature (Oscáriz and Pisabarro, 2001). The most thoroughly studied bacteriocins are those produced by lactic-acid bacteria, of which sakacins seem to be most unique (Jack et al. 1995; Simon et al. 2002), and the lantibiotics, which contain modified amino acid residues (Oscáriz and Pisabarro, 2001). Another representative, pediocins, are usually co-transcribed

with a gene encoding a cognate-immunity protein (Fimland et al. 2002). The 44-amino acid pediocin produced by *Pediococcus acidilactici* strains is encoded in an 8.9 kb plasmid.

1.3.2. Antimicrobial proteins and peptides from plants

Activation of signal transduction network after pathogen recognition results in reprogramming of cellular metabolism, involving large changes in gene activity. Plants contain many defense related proteins. In addition to resistance R genes and genes encoding signal transduction proteins, they possess downstream defense genes, such as pathogenesis-related proteins (PRs), enzymes of oxidative stress protection, tissue repair, lignification and others. It should be stressed that many of these genes are involved in secondary metabolism, such as shikimate and phenylpropanoids pathway (Somssich and Hahlbrock, 1998). Induction of defense gene transcripts is observed sometimes in the attached cells, but mostly in surrounding plant tissues (Scheel,1998). Accumulation of pathogenesis-related proteins represents the major quantitative change in protein composition that occurs in noninoculated plant parts that, upon challenge, exhibit acquired resistance (Van Loon,1997). Seventeen pathogenesis-related protein families from different plant species have been characterized and classified according to sequence similarities (Fritig et al.,1998), although additional pathogen-induced proteins with potential antipathogenic action keep being described (Table 1.1). Within one family several members may share similar biological activities but differ substantially in other properties such as substrate specificity, physicochemical properties or subcellular localization. The inducible pathogenesis-related proteins are mostly acidic proteins that are secreted into the intracellular space (Van Loon,1997). In addition, basic pathogen-related protein occur at relatively low levels in the vacuole.

Most pathogen-related proteins have a damaging action on the structure of the parasite: PR1 and PR5 interact with the plasma membrane, whereas ß -1,3 glucanases (PR2) and chitinases (PR3, PR4,

PR8 and PR11) attack ß -1,3 glucans and chitin, which are components of the cell walls in most higher fungi. PR5 proteins are thought to create transmembrane pores, and therefore been named permatins.
Chitinases can also display lysosyme activity and hydrolyze bacterial peptidoglycan. Microbial proteinases involved in pathogenesis are completely inhibited by a tobacco proteinase inhibitor. Plants also synthesize inhibitors of fungal polygalacturonases considered as pathogenicity factors. The PR10 family has sequence similarity to ribonucleases and is the only family consisting of cytoplasmic proteins.

In addition to pathogenesis related proteins, small peptides with antimicrobial activity, such as thionins, defensins and lipid transfer proteins also accumulate in infected plants and probably components of the induced defense system (Bergey et al., 1996; Broekaert et al., 1997; Fritig et al., 1998). Eight families of antimicrobial peptides, ranging in size from 2 to 9kD have been identified in plants. These are thionins, defensins, so-called lipid transfer proteins, hevein- and Knottin-like peptides, MBP1, IbAMP and the recently reported snakins (Francisco Garcia-Olmedo et al., 1998).

1.4. Induction of antimicrobial compounds by plant cells

1.4.1 Inducible defense

Activation of inducible defenses are triggered by specific recognition of pathogen invasion by plants (Fig 1.1). Perception in host specific resistance involves receptors with high degree of specificity for pathogen strains, which are encoded by constitutively expressed defense resistance (*R*) genes, located either on the plasma membrane or in the cytosol (Edreva,1991; Martin, 1999; McDowell and Dangl, 2000). Large repertoires of distantly related individual *R* genes with diverse recognitional specificities are found within a single plant species (Ellis et al., 2000). Individual R genes have narrow recognition capabilities and they trigger resistance when the invading pathogen expresses a corresponding (Avr) gene. Avr genes from different pathogen classes are

Table(1.1): Antimicrobial pathogenesis-related proteins in plants.

PR protein family	Activity	Pathogen target
PR1	?	Membrane
PR-2	1,3-β-glucanase	Cell wall glucan
PR-3	Endochitinase	Cell wall chitin
PR-4	Endochitinase	Cell wall chitin
PR-5	?	Membrane
PR-6	Proteinase inhibitor	Proteinase
PR-7	Proteinase	?
PR-8	Endochitinase	Cell wall chitin
PR-9	Peroxidase	*
PR-10	RNAase	?
PR-11	Endochitinase	Cell wall chitin
Unclassified	α-Amylase	Cell wall α-glucan
	Polygalacturonase inhibitor protein (PGIP)	Polygalacturonase
PR-12	Defensin	Membrane
PR-13	Thionin	Membrane
PR-14	Lipid-transfer protein	Membrane
PR-15	Oxalate oxidase	?
PR-16	Oxalate oxidase-like	?
PR-17	Unknown protein	?

* Peroxidase has indirect antimicrobial activity by catalyzing oxidative crosslinking of proteins and phenolics in the plant cell wall, and thus protects the host from degradation by pathogen's hydrolytic enzymes.

structurally very diverse and have different primary functions in the biology of these organisms.

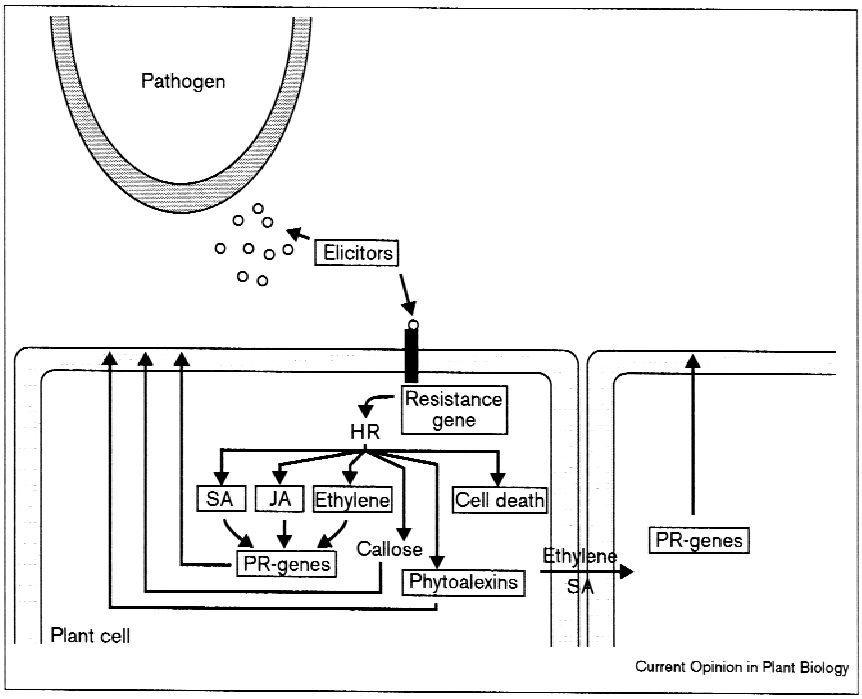


Fig (1.1): Schematic representation of defense responses induced in plant-pathogen interaction

Specific recognition of the aggressor by the plant requires the presence of matching Avr and R genes in the two species and is thought to be mediated by ligand receptor binding (Glazebrook, 1999). Over 20 R genes with recognition-specificity for defined Avr genes have been isolated from seven plant species, including both monocots and dicots (Milligan et al., 1998; Rossi et al., 1998; Martin, 1999). These genes are effective against bacterial, viral and fungal pathogens and against both nematodes and aphides species (Martin, 1999). Plant R genes encode proteins that both determine recognition of specific Avr proteins and initiate signal transduction pathway leading to complex defense responses (Zhou et al., 1998; del Pozo and Estelle, 1999; Martin, 1999). In addition to gene for gene recognition mediated by R and Avr genes, non-host resistance is achieved through the recognition of specific pathogen or plant cell wall derived signal molecules, termed exogenous or endogenous elicitors, respectively. These elicitors are often low-molecular weight compounds that are either synthesized as such or are liberated from polymeric

precursors during infection (Somssich and Hahlbrodk, 1998). The chemical structure of different elicitors is of great variety, such as glycoproteins, peptides and oligosaccharides. Some proteinaceous elicitors are directly produced by bacterial or fungal pathogens, whereas biologically active oligosaccharides are released from pathogen and plant cell walls by hydrolases secreted by the two organisms. Complex and largely unresolved perception systems exist for elicitors on the plant cell surface that activate multiple intracellular defense signaling pathways. In general the multicomponent response of plants to pathogens in host and nonhost resistance appears to be activated by ligand/receptor interactions, in which Avr gene and pathogen or plant surface-derived elicitors serve as ligands for plasma membrane located or cytosolic receptors.

1.4.2 Elicitor treatment

Microbial infections of intact plants often elicit the synthesis of specific secondary metabolites. The best understood systems are those of fungal pathogens in which case the regulatory molecules have been identified as glucan polymers, glycoproteins and low molecular weight organic acids (DiCosmo and Tallevi, 1985). The possible correlations between stress and secondary metabolism in cultured cells has been demonstrated (DiCosmo and Towers, 1984). Biosynthesis of flavonoids is induced by light via phytochrome or/and UV-photoreceptors and by infection with phytopathogenic organisms or compounds which induce the synthesis of antimicrobial compounds in plants. The addition of an extract of *Verticillium*, a parasitic fungus of plants, has induced the synthesis of gossypol by cell suspensions of *Gossypium arboreum* (Pétiard and Bariaud-Fontanel, 1987).

Examples also of microbial elicitor induction include psoralen production in parsley (Tietjen , 1983), Diosgenin production in the Mexican yam (Rokem , 1984). Tyler, (1989) described that a cell line of *Papaver somniferm* synthesizes and accumulates sanguinarine when exposed to homogenate of the fungus *Botrytis.*

Effect of elicitors on secondary metabolism have been investigated at the enzymatic levels to determine their mode of action. Eilert and Eolters (1989) added autoclaved culture homogenate of yeast, *Rhodotorula rubra* into the suspension culture of *Ruta graveolens* and found that S-adenosyl-L-methionine:anthranilic acid N-methyltransferase was elicited. Also a yeast polysaccharide preparation induced L-tyrosine decarboxylase in suspension cultures of *Thalictrum rugosum* and *Eschscholtzia californica*, the enzyme was induced after 5 hours after addition of the elicitor at a concentration of 30 to 40 ug/g-cell fresh weight (Marques and Brodelius, 1992).

Mazukami et al. (1992), reported a transient increase in rosmarinic acid content in cultured cells of *L. erythrorhizon* after addition of yeast extract to the suspension cultures and the maximum was reached in 24 hr. Also they found that when plant cells were treated with yeast extract on the 6^{th} day of cultivation, the level of rosmarinic acid increased 2.5 times and the activity of phenylalanine ammonia-lyase in cells rapidly increased before synthesis of rosmarinic acid.

Conrath, 1989, found that chitosan elicits a rapid deposition of the 1,3-ß-glucan, callose in suspension cultured cells of parsley, *Petroselinum crispum*.

Developments in phytochemical elicitation have shown that simple inorganic and organic molecules can induce product accumulation. Sodium orthovanadate (Hattori and Ohta, 1985) and vanadyl sulphate induced the accumulation of isoflavone glucosides in *Vigna angularis* cultures and indole alkaloid accumulation in *Catharanthus roseus* cultures, respectively. Other substances found to stimulate alkaloid accumulation in *C. roseus* include sodium chloride, potassium chloride and sorbitol (Smith et al, 1987a) as well as abscisic acid (Smith et al, 1987b). Processes such as these, employing simple and cheap elicitors have much promise in industrial scale plant cell cultures.

Davis et al. (1992), recognized that addition of oxalate to medium of *Gossypium hirsutum* suspension culture could reduce the amount of

Verticillium dahliae elicitor to be employed to stimulate metabolite synthesis. Addition of a fungal elicitor often inhibits the growth of plant cells but a combination of the elicitor and oxalate did not reduce the cell mass of the plant, therefore secondary metabolite synthesis was increased up to ten fold.

Dunlop and Curtis. (1991) reported that a combination of phosphate limitation and fungal elicitation synergistically increased production of secondary metabolites. They found that either phosphate limitation or elicitation with a mycelial extract of the fungus, *Rhizocotonia solani* alone results in increased production of the sesquiterpene solavetivone by *Agrobacterium rhizogenes* – transformed hairy root cultures of *Hyoscyamus muticus* . However, when phosphate limitation is coupled with fungal elicitation, the productivity increase is considerably greater than that obtained with either method alone.

Although the mechanism by which many elicitors increase the productivity of secondary plant metabolites has not been elucidated, their stimulating activity is quite significant if an appropriate elicitor is chosen to stimulate synthesis of a particular product. However, the use of microbial elicitors may not be economical since an elicitor-producing microorganism should be cultivated in a fermentor separately from cultivation of plant cells using another fermentor. The fermentation cost for an elicitor-producing microorganism is not always inexpensive. In this sense, a simple and cheap compound should be employed as an elicitor.

1.*4.3 Signal transduction*

Receptor-mediated recognition at the site of infection initiates cellular and systemic signaling processes that activate multi-component defense responses at local and systemic levels, resulting in rapid establishment of local resistance and delayed development of systemic acquired resistance (Scheel, 1988). The earliest reaction of plant cells include changes in plasma membrane permeability, (Fig 1.2), leading to

calcium and proton influx and potassium and chloride efflux (McDowell and Dangl, 2000).

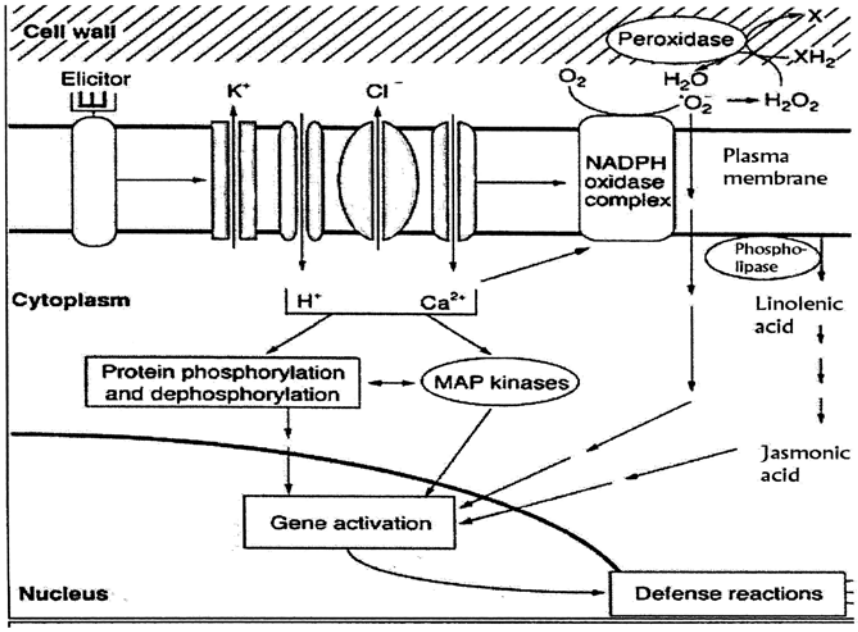


Fig (1.2):Major components of signal-transduction chain from elicitor perception to gene activation in plant cell.

Ion fluxes subsequently induce extracellular production of reactive oxygen intermediates, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl free radical ($OH^\bullet$), catalyzed by a plasma membrane-located NADPH oxidase and/or apoplastic-localized peroxidases (Somssich and Hahlbrock, 1998). The initial transient reactions are, at least in part, prerequisites for further signal transduction events resulting in a complex, highly integrated signaling network that triggers the overall defense response. The role of calcium is shown in experiments with calcium channel inhibitors, which, preventing increases of cytosolic calcium concentrations, delay the development of hypersensitive response. Heterortimeric GTP-binding proteins and protein phosophrylation/dephosophrylation are probably involved in transferring signals from the receptor (Legendre et al, 1992). The changes in ion fluxes trigger localized production of reactive oxygen intermediates and nitric oxide, which act as second messengers for hypersensitive response induction and defense gene expression (Piffanelli et al., 1999). Synergistic interaction between reactive oxygen intermediates, nitric oxide and

salicylic acid have been postulated (McDowell and Dangl, 2000). Other components of signal network are specifically induced phospholipases which act on lipid-bound unsaturated fatty acids within the membrane, resulting in the release of linolenic acid which serves as a substrate for the production of jasmonate, methyl jasmonate and related molecules via a series of enzymatic steps. Oxidative burst is a central component of machinery defense of plants (Lamb and Dixon, 1997; Alvarez et al., 1998). The brust of H_2O_2 production at the plant cell surface drives rapid peroxidase-mediated oxidative cross-linking of structural proteins in the cell wall, thereby reinforcing this physical barrier against pathogen ingress (Schell,1998). Additionally, low doses of reactive oxygen metabolites act as signals for the induction of detoxification mechanisms involving superoxide dismutases and glutathione-S-transferase, and activation of other defense reactions in neighboring cells.

Most of the inducible, defense-related genes are regulated by signal pathways involving one or more of the three regulators jasmonate, ethylene and salicylic acid (Delaney et al., 1994; Sticher et al., 1997; Van Loon, 1997; Reymond and Farmer, 1998; Knoester et al., 1998; Ananieva and Ananiev, 1999). The exact role of ethylene as a defense regulator is not clear, but it has been shown that this hormone preferentially induces basic pathogenesis-related proteins. Jasmonate is essential for the defense of tomato against tobacco hornworm larvae and for defense of *Arabidopsis* against fungal pathogens as *Phytum mastophorum* and the fly *Bradysia* (Reymond and Farmer, 1998). Jasmonate and ethylene co-operate to regulate the expression of many genes and at least some jasmonate- inducible genes are not inducible in plants unable to produce or sense ethylene (Reymond and Farmer; 1998). During systemic acquired response, salicylic acid level rise throughout the plant. Defense genes such as pathogenesis-related genes are expressed, and plant becomes more resistant to pathogen attack. Plants that cannot accumulate salicylic acid due to the presence of transgene that encodes salisylic acid- degrading enzyme develop hypersensitive response after

challenge to avirulent pathogens, but do not exhibit systemic expression of defense genes and do not develop resistance to subsequent pathogen attack (Glazebrook; 1999). Arabidopsis mutants, compromised in their ability to respond to jasmonate or to produce salicylic acid, have been used to demonstrate that the two pathways are utilized differentially against contrasting modes of attack (McDowell and Dangl, 2000). The ethylene-jasmonate dependent pathway is activated by pathogen that kill plant cells to obtain nutrients. In contrast salicylic acid-dependent response is triggered by pathogen that obtain nutrients from living plant tissue. It has been also suggested that ethylene-jasmonate and salicylic acid pathways are mutually inhibitory. Such cross-talk probably implies a capacity for a selective defense against specific types of parasites.

1.5 Thaumatin like proteins

1.5.1 Thaumatin-like proteins from plants

Seventeen PR protein groups have been isolated and characterized from various plants till now (Van Loon and Van Strien, 1999), and most of them are induced by phytopathogens or abiotic stresses and have antifungal activity in *in vitro* assays (Vigers et al., 1991). Among them, the PR5 proteins which are known as thaumatin–like proteins (TLPs) because their sequences are similar to thaumatin, a sweet-tasting protein isolated from the tropical plant *Thaumatococcus daniellii* (Cornelissen et al., 1986). They are divided into 3 subgroups: acidic (PR-S), basic (Osmotin) and neutral proteins (Osmotin-Like-Protein, OLP). PR-S is acidic extracellular form of thaumatin isolated from tobacco plants infected with tobacco mosaic virus, and it shows antifungal activity against some plant pathogenic fungi like *Cercospora beticola* (Vigers et al., 1992). Osmotin protein was first isolated from salt-adapted tobacco cell cultures (Singh et al., 1985 and 1989). It also shows antifungal activity against several phytopathogenic fungi in *in vitro* assays (Woloshuk et al., 1991). For example zeamatin, an osmotin isolated from *Zea mays*, shows antifungal activity against *Candida albicans*, *Neurospora crassa* and *Trichoderma reesei*, and causes leakage of cytoplasmic material from the fungi and

cause haphal rupture. It appears that high concentrations of TLPs can actively lyse fungal membrane, but at low concentrations they may cause membrane permeability changes that cause leakage of cell constituents (Roberts and Selitrennikoff, 1990). Osmotin overexpression in transgenic plants increases the plant resistance to several pathogens (Liu et al., 1994). OLP, the neutral isoform, has been identified in several plants like tobacco, potato and *Arabidopsis* (King et al., 1988, Melchers et al.,1993, Zhu et al.,1993, Hu and Reddy, 1997, Chen et al., 1996, Capelli et al., 1997). They respond to a variety of abiotic stimuli and to pathogenic fungi (Zhu et al., 1993). *Arabidopsis ATosm3* and tomato *Tomf21*, showed tissue specific expression (Chen et al., 1996 and Capelli et al., 1997).

Thaumatin like proteins (TLPs) have been purified from several plant sources especially from leaves of infected tobacco, tomato and soybean, grains of barley, maize and wheat, fruits of Cherries and *Diospyros texana* and floral buds of *Brassica napus*.

TLPs are generally highly soluble proteins that accumulate to high levels (1 to 12%) in selective tissues or subcellular compartments under appropriate conditions or are secreted into the medium or extracellular space. They remain soluble even under acidic conditions and are quite resistant to proteolysis. The molecular weights of mature TLPs fall into two size ranges. One group of proteins has a size range of 22 to 26 kDa (201 to 299 amino acids), while the members of the other group have sizes around 16 kDa (148 to 151 amino acids). The second group of TLPs has an internal deletion of 58 amino acids.

TLPs have pI values ranging from very acidic to very basic (range 3.4 to 12). Thus there are the acidic, neutral and basic TLPs. Generally, the extracellular TLPs tend to be acidic, while the vacuolar (or other vesicular) TLPs tend to be basic, but it is not clear whether there is any biological significance to this observation.

TLPs are generally resistant to proteases and pH- or heat- induced denaturation, and this may due to the presence of 16 cysteines, which

have been shown to be involved in formation of 8 disulfide bonds in thaumatin and zeamatin (Roberts and Selitrennikoff, 1990).

The three-dimensional structure of thaumatin I has been determined using X-ray crystallography (De Vos et al., 1985 and Ogata et al., 1992), and it has been found that 2 building motifs are utilized in thaumatin structure: a folded ß-sheet (which forms an identifiable domain I) and the ß-ribbons and small loops stabilized by disulfide bonds which form two separate domains II and III. The positions of the 8 disulfide bonds in thaumatin have been assigned. It has been proposed that domains II and III may be important for binding to the sweetness receptors on membranes of taste buds. Disruption of the disulfide bonds results in loss of the unique tertiary structure of the thaumatin molecule and loss of sweetness (Van der Wel and Loeve, 1972). The locations of the cysteines are highly conserved between thaumatin and the members of the class of larger TLPs. Interestingly; the low-molecular-weight TLPs are missing most of domain and several half cysteines which stabilize this domain. The crystal structure of zeamatin has also been determined (Batalia et al., 1996). Zeamatin, whose tertiary structure closely resembles that of thaumatin, has a ß-sandwich with 11 ß-strands from which an arm-like structure extends to form a large cleft lined by several acidic residues. This arm has a short α-helix and three disulfide bonds. The amino acid sequences constituting the acidic cleft are highly basic residues.

The crystal structure of zeamatin indicates that this protein associates to form a homodiner, but does not suggest the formation of a cyclic aggregate that is characteristic of channel forming proteins. It has been suggested that (Batalia et al., 1996) electrostatic interactions between zeamatin and some membrane channel proteins are responsible for destabilization of pressure gradients that maintain the hyphal tip in the extended state.

1.5.2 Thaumatins from Arabidopsis

It has been observed that *Arabidopsis* plants infected with tobacco mosaic virus (TMV), *Pseudomonas syringae* pv. Tomato (pst) DC3000 or treated with 2,6 dichloroisomicotinic acid (INA) or salicylic acid (SA) accumulate several PR proteins resulting in induction of systemic acquired resistance (Uknes et al., 1992 and Ward et al., 1991). Among these proteins PR5 mRNA and encoded extracellular protein (acidic PR5) are induced when *Arabidopsis* plants are challenged by pathogens or systemic acquired resistance-inducing compounds (Uknes et al., 1992). Also 2 cDNA clones for *Arabidopsis* thaumatin like proteins (ATLP-1 and ATLP-2) were isolated and sequenced (Hu and Reddy, 1995). The nucleotide sequence of ATLP-1 cDNA shows limited similarity with those of other thaumatin- like proteins from plants including *Arabidopsis.* The predicted amino acid sequence of ATLP-1 shows 52% identity to the above mentioned thaumatin-like protein PR5 from *Arabidopsis* and it shows significant homology (about 40-47% amino acid identity) to maize thaumatin-like antifungal protein and osmotin (King et al., 1988, Singh et al., 1989, Huynh et al., 1992). As mentioned above the known thaumatin-like protein from *Arabidopsis* is an acidic extracellular protein. However, ATLP-1 has been found (Hu and Reddy, 1995) to be basic protein with predicted pI value of 9.6. In this respect it is more similar to osmotins which are basic intracellular thaumatin-like proteins. The second cDNA, ATLP-2, was found to be identical to ATLP-1 except that it lacks 109 nucleotides in 3′ untranslated region in front of the poly (A) tail (Hu and Reddy, 1995). Hence, it is likely that ATLP-1 and ATLP-2 are derived from the same gene by differential processing of transcript but it has been reported that it is not known if the expression of ATLP-1 is induced by infection with pathogen or SAR-inducing compounds. It is also not known if the ATLP-1 gene product has antifungal activity or not. In addition, a receptor protein kinase (PR5K) with amino acid-terminal domain closely related to PR5 family of antifungal proteins has also been isolated from

Arabidopsis (Wang et al., 1996). The PR5K gene codes for predicted 665-amino acid polypeptide that comprises an extracellular domain related to the PR5 proteins, a central transmembranespanning domain, and an intracellular protein-serine/threonine kinase. The extracellular domain of PR5K (PR5-like receptor kinase) is most highly related to acidic PR5 proteins that accumulate extracellularly in plants challenged with pathogenic microorganisms. The kinase domain of PR5K is related to a family of protein-serine/threonine kinases that are involved in expression of self-incompatibility and disease resistance. It has been observed that PR5K transcripts accumulate at low levels in all tissues examined, although particularly high levels are present in roots and inflorescence stems, and also treatments that induce authentic PR5 proteins had no effect on the level of PR5K transcript, suggesting that the receptor forms part of pre-existing surveillance system. From the structural similarity between extracellular domain of PR5K and the antimicrobial PR5 proteins it is suggested that a possible interaction with common related microbial targets. Recently, a cDNA for *Arabidopsis* thaumatuin-like proteins 3 (ATLP-3) with antifungal activity has been isolated (Hu and Reddy, 1997), and it has been observed that it shares significant sequence similarity with acidic PR5 proteins from the same plant and other plants. It has been found that its expression is induced by SA and different pathogens. Lack of C-terminal extension and the presence of a signal sequence at the amino–terminus in ATLP-3 suggests that it can be secreted to the extracellular space. ATLP-3 shows strong antifungal activity against several fungal strains like *Fusarium oxysporium*, *Alternaria solani*, *Trichoderma reesei* and *Candida albicans.* It appears to inhibit the growth by inhibiting the germination of fungal spores. Boiling of ATLP-3 for 5 min results in complete loss of its antifungal activity. ATLP-3 cross react with antibodies for other thaumatin-like antifungal proteins like anti-osmotin and anti-zeamatin antibodies.

Recent studies on ATLP-1 (Hu and Reddy, 1997) indicate that it can be induced with SA and different pathogens and it also shows strong cross

reactivity with both anti-osmotin and anti-zeamatin antibodies, but it shows little or no effect on fungal growth.
Results from different published reports suggest that there are at least 4 (possibly more) related thaumatin-like proteins in Arabidopsis (Hu and Reddy, 1997).

1.6 Objective of this thesis

The objectives of this thesis are:

1- Screening different plant cell cultures for production of antimicrobial protein(s) in presence of different common elicitors. This point includes testing some heterotrophic and autotrophic plant cell cultures with and without addition of nine different elicitors for testing the presence and induction of antimicrobial proteins. Five human pathogenic isolates will be used for testing the presence of antimicrobial proteins produced by the different plant cell cultures.

2- Select the high producing cell culture and purification of the antimicrobial protein(s). In this point the most active plant cell culture elicited with different elicitors will be selected for further experimentation and testing the presence of antimicrobial proteins and not low molecular weight compounds will be done. In this point also the active antimicrobial protein(s) will be purified and the pure protein will be identified using microsequencing technique.

3- Cloning of the gene(s) for the active protein(s) for further biotechnological applications. In this point, after identification of the active protein(s), the genes coding for the active protein(s) will be cloned for further applications like testing the expression under

different stimuli and overexpression of the genes in plants or plant cell cultures for maximization of the protein productivity.

4- Cloning of thaumatin genes from *Arabidopsis thaliana*. Thaumatins are antimicrobial proteins most of them are active against fungi and some of them are active against the used tester isolate in this work which is *Candida* spp., so the cloning aims to testing the expression of different thaumatin genes under different stimuli for maximization of their production by *Arabidopsis*.

2.Results

2.3 Screening the antimicrobial activity of plant cell Cultures.

Ten different plant cell cultures were tested for their antimicrobial activity against five different human pathogenic isolates. Most of the used plant cell cultures are known to produce antimicrobial pathogenesis related proteins or peptides as a defense mechanism against plant pathogenic microorganisms. Three groups of plant cell cultures were used in the study, the first group includes a collection of plant cell cultures used in Prof Zenk lab which include (BY2, Ps, *Lycopersicon esculentum* [*heterotrophic*], Nico, and *Arabidopsis* cultures). The second group includes model cultures used in Prof Müller lab, which include (AST, ECO, and *Gypsophila arabia* cultures), and the third group includes model plant cell cultures used in Prof Roitsch lab, which include (*Chenopodium rubrum* and *Lycopersicon esculentum [autotrophic]* cultures). In the current study we have tested the difference in antimicrobial activity between the heterotrophic and autotrophic cultures against the five tested human pathogenic isolates. All plant cell cultures have been tested in absence (control) or in presence of nine different elicitors. Four types of elicitors were selected for testing their elicitation for all different plant cell cultures. The first type represents different fungal components and includes chitosan (fungal cell wall preparation), Alt elicitor (fungal spore suspension of boiled spores of *Alternaria brassiciola*) and fol-elicitor (mycelial suspension of *Fusarium oxysporium lycopersici*). The second type represents different plant components which includes MeJ (a signal molecule), PGA (plant cell wall preparation), SA (signal molecule) and ASA (analogue for SA). The third type represents different bacterial components which includes PRM1 (boiled bacterial suspension of avirulent strain of *Pseudomonas syringae*) and DC3000 (boiled bacterial suspension of virulent strain of *Pseudomonas syringae*). All screening experiments were done using five human pathogenic isolates which

include, *Candida maltosa* (a kerationlytic fungus), *Staphylococcus aurus* and *Bacillus subtilis* (Gram positive isolates) and *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative isolates).

Protein content was measured in all different elicited plant cell culture fractions as an indicator for induction of antimicrobial proteins and not low molecular weight ones.

Results of antimicrobial activity of aqueous extracts of ten plant cell cultures are presented in Table (2.1). Intracellular fraction of heterotrophic *Nicotinana tabacum* (BY2) cells treated with different elicitors was more active than extracellular and cell wall bound fractions for the same plant treated with the same elicitors. Different elicitor treatments induce different antimicrobial activity for the three aqueous plant cell culture fractions. Intracellular fraction treated with chitosan and Fol showed the highest antimicrobial activity against all tester isolates. Protein content of intracellular fraction was higher (moderate) than those for extracellular and cell wall bound fractions (low).

For heterotrophic *Petroselium crispum* (Ps) cell culture, all plant fractions could not inhibit all tester isolates and for example, the extracellular fraction treated with different elicitors has no activity at all on *B. subtilis* and *C. maltose* and the same for the intracellular fraction against *S. aureus*. Also for the cell wall bound fraction since no activity could be observed against *C. maltose, E. coli* and *B. subtilis.*

For the two autotrophic plant cell cultures used in the study; fractions of *Chenopodium rubrum* could not show high inhibitory activity against the tester isolates and the protein content for the intracellular fraction was higher than those for extracellular and cell wall bound fractions. The same was for the second autotrophic *Lycopersicon esculentum* culture, since the three fractions showed low activity against all tester isolates and slight changes in activity and protein content due to the treatment with elicitors observed in the three plant aqueous fraction extracts.

Table (2.1): Summary of screening of 10 different plant species against 5 human pathogenic isolates in presence of 9 different elicitors.

Plant sp. and Family	Elicitor	Extracellular fraction						Intracellular fraction						Cell wall bound fraction					
		Pathogen					Protein content	Pathogen					Protein content	Pathogen					Protein content
		C.m	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*	
Nicotiana tobacum (BY2) Solanaceae	Control	I,-	-,I	-,I	-,II	-,III	L,L	II,I	I,I	I,I	I,I	II,I	M,M	-,-	I,-	II,II	-,-	-,-	L,L
	Chitosan	II,II	II,II	III,I	II,I	II,I	L,L	IIII,III	I,I	II,II	IIII,IIII	I,II	M,M	III,III	I,II	II,-	-,-	I,I	L,L
	Mej	I,I	II,I	II,II	I,II	III,II	L,L	II,II	IIII,II	II,II	II,III	-,II	M,M	-,-	I,I	II,II	-,-	-,-	L,L
	PGA	I,I	III,I	II,I	I,II	-,I	L,L	IIII,III	I,II	III,III	-,III	-,III	H,M	II,III	I,-	II,-	-,-	-,-	L,L
	DC3000	I,I	II,II	II,II	II,II	II,II	L,L	II,II	III,II	II,III	-,I	-,II	H,M	-,III	I,I	-,-	II,III	-,I	L,L
	PRMI	-,-	I,I	II,I	-,II	I,I	L,L	IIII,III	I,I	II,II	III,I	II,II	H,M	-,-	I,I	-,II	-,-	-,I	L,L
	SA	I,I	I,II	II,I	I,I	-,-	L,L	-,II	IIII,II	II,II	-,II	I,II	M,M	-,-	I,I	II,II	-,-	-,II	L,L
	Alt	I,I	I,-	I,II	-,II	I,II	L,L	II,III	II,II	IIII,II	-,II	II,II	M,M	-,-	II,I	II,I	-,-	-,-	L,L
	Fol	II,I	III,I	III,III	-,III	III,III	L,L	II,II	IIII,III	IIII,II	II,II	IIII,II	M,M	II,II	III,II	II,I	III,-	III,I	L,L
	ASA	I,I	III,I	I,I	-,I	-,I	L,L	II,II	I,II	IIII,III	III,III	II,I	H,M	-,-	I,II	II,II	II,-	-,-	L,L
Petroselium crispum (Ps) Apiaceae	Control	-,-	III,-	II,-	-,-	I,I	L,L	-,-	-,-	II,II	-,-	II,II	M,L	-,-	II,I	-,-	-,-	-,-	L,L
	Chitosan	-,-	-,I	I,II	-,-	-,IIII	L,L	-,-	III,III	II,III	-,-	III,II	VH,M	-,-	I,II	-,-	-,-	II,I	L,L
	Mej	-,-	I,I	-,I	-,-	I,I	L,L	II,II	IIII,II	II,II	-,-	-,-	H,M	-,-	I,I	-,-	-,-	II,I	L,L
	PGA	-,-	-,-	-,II	-,-	-,-	L,L	-,-	II,II	II,II	-,-	III,II	M,L	-,-	I,I	-,-	-,-	II,II	L,L
	DC3000	-,-	II,-	II,I	-,-	I,I	L,L	-,-	III,II	-,-	-,-	-,-	H,M	-,-	II,II	-,-	-,-	I,I	L,L
	PRMI	-,-	II,-	II,I	-,-	-,I	L,L	-,-	-,II	-,-	-,-	II,II	H,L	-,-	II,I	-,-	-,-	I,I	L,L
	SA	-,-	I,II	I,III	-,-	-,-	L,L	II,II	-,-	II,II	-,-	II,II	M,L	-,-	II,I	-,-	-,-	I,I	L,L
	Alt	-,-	IIII,-	II,-	-,-	I,I	L,L	-,-	III,II	-,II	-,-	III,II	M,M	-,-	I,I	-,-	-,-	-,II	L,L
	Fol	-,-	III,-	III,IIII	-,-	III,III	L,L	-,-	II,II	II,II	-,-	II,II	M,L	-,-	I,II	-,-	-,-	I,I	L,L
	ASA	-,-	II,-	-,II	-,-	II,III	L,L	-,-	II,II	-,II	-,-	III,II	H,L	-,-	I,II	-,-	-,-	I,II	L,L

Plant sp. and Family	Elicitor	Extracellular fraction						Intracellular fraction							Cell wall bound fraction						
		Pathogen					Protein content	Pathogen					Protein content		Pathogen					Protein content	
		C.m	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*			*C.m*	*S.a*	*E.c*	*B.s*	*P.a*		
Chenopodium rubrum (Autotrophic culture) Chenopodiacea	Control	-,-	-,-	-,-	I,I	-,-	L,L	-,-	I,I	I,I	I,I	-,-	VH,VH		-,-	-,-	-,-	I,I	-,-	L,VH	
	Chitosan	-,-	-,-	I,I	I,I	I,I	L,M	-,-	-,-	-,-	-,-	-,-	H,VH		-,-	-,-	-,-	-,-	I,-	L,VH	
	Mej	-,-	-,-	-,-	I,I	-,-	L,L	-,-	I,I	I,I	I,I	-,-	M,VH		-,-	-,-	I,I	I,I	I,I	L,VH	
	PGA	-,-	-,-	-,-	-,-	I,I	L,M	I,I	I,I	I,-	I,I	-,-	H,VH		-,-	I,I	-,-	-,-	I,-	L,VH	
	DC3000	-,-	-,-	-,-	-,-	I,I	L,L	-,-	I,I	I,I	I,I	-,-	H,VH		-,-	-,-	I,-	-,-	-,-	L,VH	
	PRM1	-,-	I,I	I,I	I,I	-,-	L,L	-,II	I,I	-,-	II,I	-,II	H,VH		-,-	-,-	I,-	-,-	-,-	L,VH	
	SA	-,-	I,I	I,I	II,I	-,-	L,L	I,I	I,I	-,-	-,-	-,-	VH,VH		-,-	-,-	-,-	-,-	-,-	L,VH	
	Alt	I,I	-,-	I,I	I,I	-,II	L,M	I,III	I,II	I,III	I,I	-,-	M,VH		-,-	-,-	-,-	-,-	-,-	L,VH	
	Fol	-,-	I,I	I,I	-,-	-,-	L,M	-,-	I,II	-,-	I,I	-,-	H,VH		-,-	-,-	-,-	-,-	-,-	L,VH	
	ASA	-,-	-,-	I,I	-,-	-,-	L,L	-,-	I,II	-,-	I,I	-,-	VH,VH		-,-	II,I	-,-	II,I	-,-	L,VH	
Lycopersicon esculentum (Autotrophic culture) Solanaceae	Control	-,-	-,-	-,II	I,I	-,-	L,M	-,-	-,-	I,I	-,-	-,-	M,VH		-,-	-,II	-,-	-,-	I,I	L,H	
	Chitosan	-,-	II,I	-,-	I,III	-,-	L,M	-,-	-,-	-,-	-,-	-,-	VH,VH		-,-	I,III	I,I	-,-	I,I	L,VH	
	Mej	-,II	-,-	-,-	I,I	-,-	L,M	-,-	-,-	I,-	-,-	-,-	M,VH		-,-	I,I	I,I	-,-	I,I	L,VH	
	PGA	-,-	I,I	-,-	I,I	-,II	L,H	-,-	-,II	I,I	-,-	-,-	H,VH		-,-	I,III	I,III	-,-	I,I	L,VH	
	DC3000	-,-	-,-	I,I	I,I	-,-	L,H	-,-	-,II	I,I	-,-	-,-	H,VH		-,II	I,III	I,III	I,I	I,I	L,M	
	PRM1	-,-	-,II	-,-	I,-	-,II	L,L	-,-	-,II	I,I	-,-	-,-	H,VH		-,-	II,I	I,I	I,I	I,I	L,VH	
	SA	-,-	II,II	II,II	II,I	-,-	M,M	II,-	-,-	I,I	-,-	-,-	M,VH		-,-	I,I	I,I	I,I	I,I	L,M	
	Alt	-,-	II,II	I,I	I,-	-,-	L,L	-,-	-,-	I,I	-,-	-,-	VH,VH		-,-	-,-	I,I	I,I	-,-	L,VH	
	Fol	-,-	-,-	II,I	III,I	-,-	L,M	I,I	-,-	I,I	-,-	-,-	M,VH		-,II	I,I	I,I	I,III	I,I	L,H	
	ASA	-,-	I,II	I,II	I,II	-,-	L,M	-,-	-,-	I,I	-,-	-,-	H,VH		-,-	I,I	I,I	I,I	I,I	L,H	

Plant sp. and Family	Elicitor	Extracellular fraction						Intracellular fraction						Cell wall bound fraction					
		Pathogen					Protein content	Pathogen					Protein content	Pathogen					Protein content
		C.m	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*	
Nicotiana pulmbaginifolia Solanaceae	Control	-,II	-,-	I,-	-,-	I,I	L,L	-,I	-,-	-,-	III,II	-,II	L,L	-,I	-,I	-,I	-,I	I,I	L,L
	Chitosan	I,II	-,-	II,-	-,II	-,II	L,L	I,II	II,II	I,I	II,II	-,II	L,L	-,III	III,II	-,II	-,I	II,II	L,L
	Mej	I,III	-,-	-,-	-,-	-,-	L,L	-,I	-,I	-,I	-,II	-,I	L,L	-,II	II,I	II,-	I,I	II,I	L,L
	PGA	III,II	-,-	-,-	-,-	-,II	L,L	I,I	II,I	-,I	-,I	-,I	L,L	-,II	II,I	-,I	II,I	-,II	L,L
	DC3000	II,II	-,-	-,-	II,II	II,II	L,L	-,I	II,-	-,II	II,II	-,I	L,L	II,I	I,I	-,I	-,I	-,II	L,L
	PRM1	-,III	-,-	I,I	-,I	I,II	-,L	III,I	-,III	III,II	-,II	I,I	L,L	-,I	I,I	I,I	-,I	III,I	L,L
	SA	-,II	-,-	II,-	-,II	II,I	-,L	-,I	II,I	III,II	II,II	-,I	L,L	II,I	-,-	-,-	I,I	-,I	L,L
	Alt	II,-	-,-	II,I	-,-	-,-	L,L	-,I	-,II	-,I	-,I	-,II	L,L	-,I	-,I	II,I	II,I	II,I	L,L
	Fol	I,II	-,I	I,I	-,I	I,II	L,L	III,I	II,I	I,I	III,II	-,I	L,L	-,I	II,I	II,I	-,I	II,II	L,L
	ASA	-,III	-,-	I,-	-,III	I,II	L,L	-,I	-,I	-,II	II,II	-,I	L,L	-,III	-,I	-,I	-,II	-,I	L,L
Lycopersicon esculentum (Heterotrophic culture) Solanaceae	Control	-,I	I,-	-,I	-,I	I,I	M,M	-,-	I,I	I,I	-,I	I,-	V/L/H	-,II	-,I	-,II	-,I	-,I	L,L
	Chitosan	-,II	III,-	I,I	-,I	I,I	L,L	-,I	I,I	I,I	-,II	I,I	M,H	-,I	I,II	I,I	-,I	I,II	L,L
	Mej	-,II	I,-	I,I	II,I	-,I	M,M	-,I	I,-	I,I	-,-	I,-	V/L/VH	-,I	I,II	I,I	-,I	-,I	L,L
	PGA	-,II	I,-	-,I	-,I	III,I	L,L	-,I	I,II	I,I	-,II	I,I	V/L/VH	-,-	I,-	I,I	-,I	I,-	L,L
	DC3000	-,II	III,I	-,I	-,I	I,I	M,M	-,I	I,I	II,-	-,I	I,-	V/L/VH	-,II	I,II	II,II	-,I	I,I	L,L
	PRM1	-,II	I,-	I,I	-,I	I,I	M,M	-,I	I,-	II,I	-,I	I,-	V/L/VH	-,I	I,II	I,I	-,I	-,I	L,L
	SA	-,I	I,-	-,I	-,I	I,I	L,M	-,I	I,I	II,I	-,I	I,I	V/L/VH	-,I	I,II	I,I	-,I	-,I	L,L
	Alt	-,I	I,-	II,I	-,I	I,I	L,M	-,I	-,I	II,II	-,II	I,I	V/L/VH	-,II	III,I	I,II	-,I	I,I	L,L
	Fol	-,I	I,-	III,-	-,II	-,II	L,L	-,I	I,II	I,II	-,I	I,I	V/L/VH	-,II	-,II	-,I	-,I	-,II	L,L
	ASA	-,II	II,-	II,I	-,I	II,I	M,L	-,I	I,I	I,I	-,I	I,I	V/L/VH	-,III	-,I	I,I	-,I	-,II	L,L

Plant sp. and Family	Elicitor	Extracellular fraction						Intracellular fraction						Cell wall bound fraction						
		Pathogen					Protein content	Pathogen					Protein content	Pathogen					Protein content
		C.m	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*	
Eschscholtzia californica AST Papaveraceae	Control	III,II	III,II	II,II	II,III	-,II	L,L	-,-	-,-	-,-	-,II	III,II	L,L	-,-	I,II	II,I	-,I	II,III	L,L
	Chitosan	III,III	I,II	-,II	II,II	-,II	L,L	I,-	-,I	III,III	III,I	-,I	L,L	-,-	II,II	III,III	III,I	II,II	L,L
	Mej	II,II	I,II	-,I	-,I	-,I	L,L	-,-	-,II	I,-	I,I	-,III	L,L	I,-	II,II	III,II	I,II	II,II	M,L
	PGA	I,III	-,II	-,II	I,II	II,II	L,L	-,-	II,-	-,II	I,I	-,I	L,L	I,-	-,I	-,I	II,I	II,II	L,L
	DC3000	III,III	II,II	-,I	-,II	-,I	L,L	-,-	-,-	I,II	II,III	-,II	L,L	-,-	-,III	I,II	II,II	-,II	L,L
	PRM1	-,III	-,II	-,I	I,I	-,II	L,L	-,-	-,II	-,II	III,III	II,II	L,L	I,-	I,II	III,II	I,II	II,II	L,L
	SA	-,III	-,II	I,II	-,III	-,II	L,L	-,-	-,II	II,II	-,III	I,III	L,L	I,-	-,II	I,III	I,II	II,III	L,L
	Alt	III,III	I,II	II,I	II,II	-,II	L,L	-,-	-,II	I,II	-,II	-,II	L,L	-,-	I,I	I,II	-,I	-,II	L,L
	Fol	III,III	-,I	-,II	-,II	-,II	L,L	II,-	II,II	II,-	III,III	II,II	L,L	II,-	I,II	II,II	I,II	I,II	L,L
	ASA	III,III	-,II	-,I	II,II	-,II	L,L	II,-	III,II	II,-	III,III	-,II	L,L	I,-	I,II	I,II	I,III	II,III	L,L
Eschscholtzia californica * ECO Papaveraceae	Control	-	II	-	II	II	L	-	I	-	I	I	-	I	I	II	I	I	L
	Chitosan	II	II	II	II	II	-	-	-	I	I	I	-	II	II	-	II	I	-
	Mej	II	II	I	III	-	-	-	I	I	-	-	L	II	II	-	II	II	-
	PGA	-	II	-	II	II	L	-	-	III	II	-	L	I	I	II	I	II	-
	DC3000	-	III	II	II	-	-	-	-	II	I	II	L	I	II	II	II	II	L
	PRM1	I	II	-	I	-	L	-	-	II	I	I	L	I	I	-	I	II	-
	SA	III	-	-	III	III	L	-	-	II	-	I	L	I	II	II	II	II	-
	Alt	I	-	-	-	-	-	-	-	-	-	II	-	II	I	II	I	I	-
	Fol	II	III	-	-	-	-	-	-	II	II	I	L	I	I	-	I	I	-
	ASA	II	III	III	II	III	L	-	II	I	I	I	L	I	I	II	I	I	-

Plant sp. and Family	Elicitor	Extracellular fraction						Intracellular fraction						Cell wall bound fraction					
		Pathogen					Protein content	Pathogen					Protein content	Pathogen					Protein content
		C.m	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*		*C.m*	*S.a*	*E.c*	*B.s*	*P.a*	
Gypsophila arabia (Heterotrophic) Caryophyllaceae	Control	I,I	I,I	I,I	I,I	I,I	VH,VH	II,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,II	II,I	I,III	I,I	M,VH
	Chitosan	I,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,II	I,I	I,I	VH,VH	I,I	I,I	II,I	I,I	I,I	H,VH
	Mej	I,I	I,II	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	H,VH
	PGA	I,I	I,I	I,I	I,I	I,I	VH,M	I,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	VH,VH
	DC3000	-,I	I,I	I,I	I,I	I,I	H,VH	II,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	H,VH
	PRM1	-,I	I,I	I,I	I,I	I,I	H,VH	I,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	VH,VH
	SA	I,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	M,VH
	Alt	-,I	I,I	I,I	I,I	I,I	VH,VH	II,I	I,I	-,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	H,VH
	Fol	-,I	I,I	I,I	I,I	I,I	H,VH	I,I	I,II	I,I	I,I	I,I	VH,VH	III,I	I,I	I,I	I,I	I,I	VH,VH
	ASA	I,I	-,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	VH,VH	I,I	I,I	I,I	I,I	I,I	M,VH
Arabidopsis thaliana (Mexotrophic) Brassicaceae	Control	-,-	-,-	II,II	-,-	II,II	L,L	-,I	II,III	II,II	III,III	I,II	H,M	-,-	I,II	II,I	I,III	II,I	L,L
	Chitosan	I,II	II,II	I,III	II,II	II,I	L,L	III,III	I,III	I,III	II,III	II,I	H,M	II,III	III,III	I,II	II,IIII	II,III	M,L
	Mej	-,-	IIII,I	-,I	III,II	II,II	L,L	III,III	III,III	III,III	I,II	I,III	H,H	I,II	III,II	II,II	I,III	II,III	M,L
	PGA	-,-	-,-	-,-	I,III	-,-	L,L	I,II	III,III	I,II	II,II	II,III	H,M	II,III	III,II	II,III	-,II	II,II	L,L
	DC3000	-,-	II,II	-,I	I,III	II,III	L,L	III,IIII	II,III	III,III	III,II	IIII,III	H,H	II,III	-,III	-,II	I,III	III,III	L,L
	PRM1	-,-	II,I	I,I	III,II	I,II	-,L	II,IIII	IIII,III	III,III	IIII,II	III,III	H,M	IIII,II	III,II	-,II	I,II	II,II	L,L
	SA	-,-	II,I	III,II	II,II	I,II	-,L	IIII,III	IIII,III	II,III	IIII,II	II,III	H,M	IIII,II	-,-	-,I	-,-	-,III	L,L
	Alt	-,-	IIII,II	II,I	II,I	II,II	L,L	I,II	II,III	I,II	II,II	I,III	M,M	-,I	-,I	-,I	II,-	II,-	L,L
	Fol	-,-	I,II	-,I	-,II	I,II	L,L	II,II	II,III	III,III	III,III	III,IIII	H,M	III,I	-,II	II,II	-,III	II,III	M,L
	ASA	-,-	-,I	-,II	II,II	-,II	L,L	III,III	II,II	II,III	III,II	II,III	H,M	II,II	-,II	II,I	II,III	II,II	M,L

Abbreviations:

(-): Negative, (*I*): Diameter of inhibition zone from **0.6** to **0.8** cm, (*II*): Diameter of inhibition zone greater than **0.8** or equal to **1** cm, (*III*): Diameter of inhibition zone greater than **1** or equal to **1.2** cm, (*IIII*): Diameter of inhibition zone greater than **1.2** cm.
(**L**): Soluble proteins greater than **0** or equal to **0.2** ug/ul, (**M**): Soluble proteins greater than **0.2** or equal to **0.4** ug/ul, (**H**): Soluble proteins greater than **0.4** or equal to **0.6** ug/ul, (**VH**): Soluble proteins greater than **0.6**ug/ul.
C.m: *Candida maltosa*, ***S.a***: *Staphylococcus aureus*, ***E.c***: *Escherichia coli*, ***B.s***: *Bacillus subtilis*, ***P.a***: *Pseudomonas aerryginosa*
* Screening done for one time.
** (-,-) Values for two separated screening experiments.

Intracellular fraction of the heterotrophic *Nicotiana pulmbaginfolia* culture treated with different elicitors showed different antimicrobial activities against tester isolates and fraction treated with *Pseudomonas syringae* (PRM1) elicitor showed the highest antimicrobial activity against all tester isolates and the antimicrobial activity varied considerably in this fraction after treatment with different elicitors, and the same was for both extracellular and cell wall bound fractions, but the protein contents were low for the three plant fractions.

All fractions for the heterotrophic *Lycopersicon esculentum* cultures showed low response in presence of different elicitors and the protein content was higher for the intracellular fraction comparable to the extracellular and cell wall bound fractions.

Extracellular fraction of heterotrophic *Eschscholtiza californica* (AST) culture treated with different elicitors showed high antifungal activity against *C. maltosa* comparable to the intracellular and cell wall bound fractions, but intracellular fraction treated with all elicitors showed high activity against B. *subtilis* and also the cell wall bound fraction showed high antimicrobial activity against *E. coli.*

Eschscholtiza californica (ECO) heterotrophic culture showed different antimicrobial activity against all tester isolates comparable to *Eschscholtiza californica* (AST) culture and the extracellulr fraction treated with ASA showed antimicrobial activity against all tester isolates but the intracellular fraction did not show any antifungal activity in presence of different elicitors. Control for cell wall bound fraction for the same plant and fraction treated with SA, Alt and ASA showed low to moderate activity against all tester isolates.

The control of the three fractions of the heterotrophic *Gypsophila arabia* and all fractions treated with different elicitors showed nearly the same low to moderate activity against all tester isolates and also the protein content for all fractions was moderate to very high.

The intracellular fraction of the mexotrophic *Arabidopsis thaliana* culture treated with different elicitors was the most active fraction against all

tester isolates comparable to all tested fractions of all plant cell cultures used in the study and fraction elicited with the elicitors DC3000, PRM1, SA, ASA, Mej, Fol and chitosan (Fig 2.1,Fig 2.2,Fig 2.3 and Fig 2.4) was highly active against all tester isolates and also the protein content was varying from high to moderate in presence of all elicitors.

This fraction is followed by the cell wall bound fraction and ASA showed the highest antimicrobial activity against all tester isolates. Also for extracelllular fraction, the fraction elicited with chitosan showed high antimicrobial activity against all tester isolates.

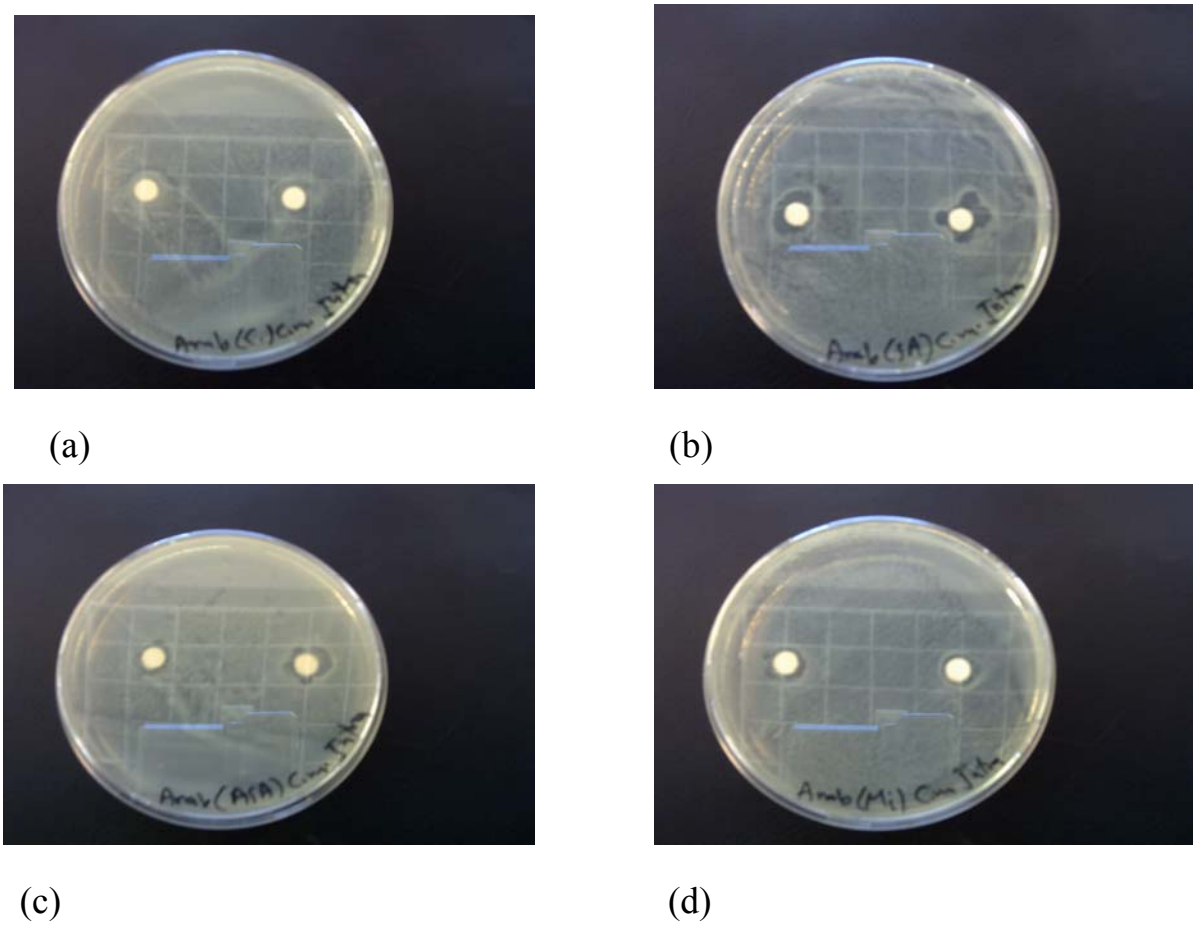
For reference standards, results in [Table 2.2] show that chloromephenicol inhibits both *Saphylococcus aureus* and *Escherichia coli* at high concentrations (50ug/ml) but has no effect on both *Bacillus subtilis* and *Pseudomonas aerryginosa*. In case of Nystatin, increased concentrations of the antibiotic lead to increasing of its inhibition of *candida maltosa* growth.

Table (2.2): Antimicrobial reference standards

Microorganism	**Concentration of antibiotic (ug/ml) [a] / Inhibition zone (cm)**					
	Control [b]	**5**	**10**	**25**	**50**	**100**
C. maltosa	-	0.9	1.1	1.5	1.6	2.5
S. aureus	0.7	0.7	0.7	0.8	1.0	1.4
E. coli	-	-	-	-	1.2	1.3
B. subtilis	-	-	-	-	-	-
P. aerryginosa	-	-	-	-	-	-

[a] : Nystatin and Chloromephenicol antibiotics were used as antifungal and antibacterial standards respectively.

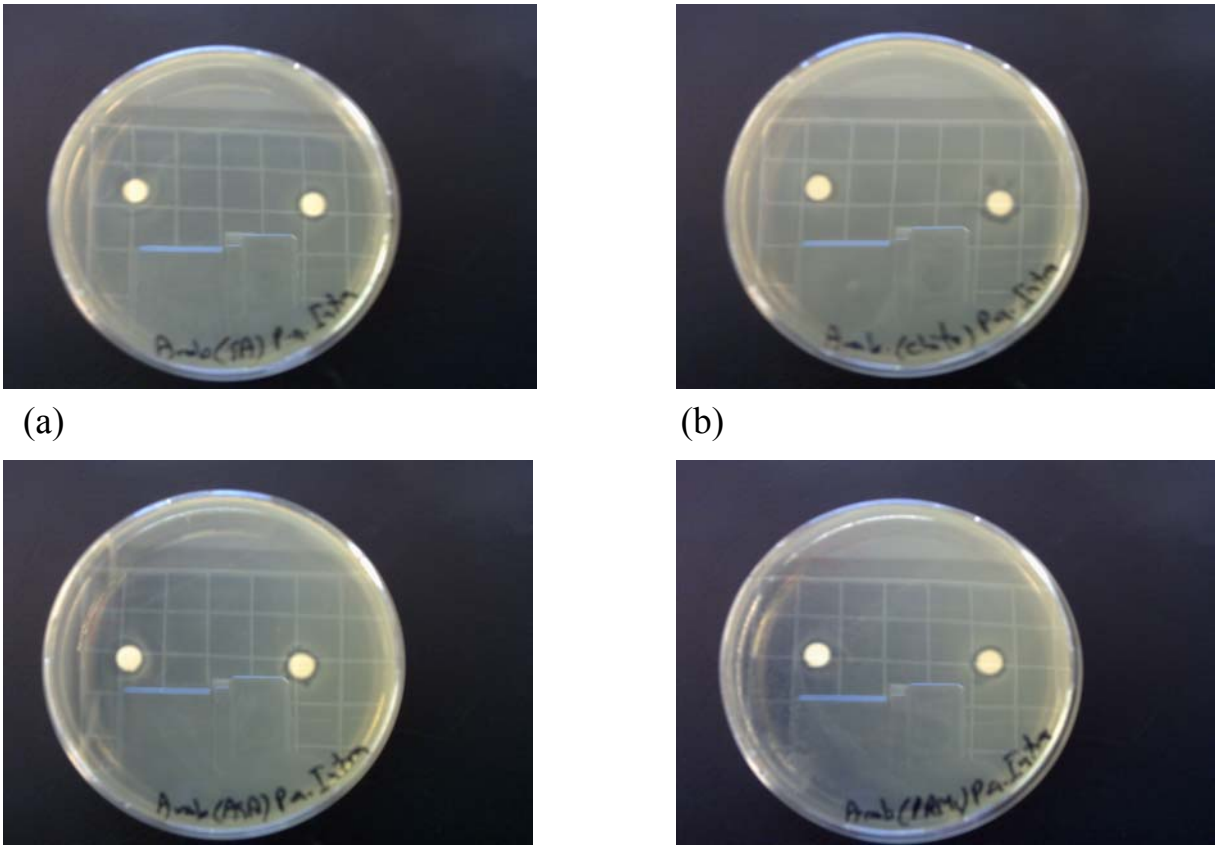
[b]: Antimicrobial activity of solvents used in dissolving the standard antibiotics (DMSO for Nystatin and MeOH for Chloromephenicol).



(a) (b)

(c) (d)

Fig (2.1):Antimicrobial activity of intracellular fraction of *Arabidopsis thaliana* elicited with different elicitors against ***C. maltosa***:
(a) Control (no treatment)
(b)SA
(c)ASA
(d)Mej

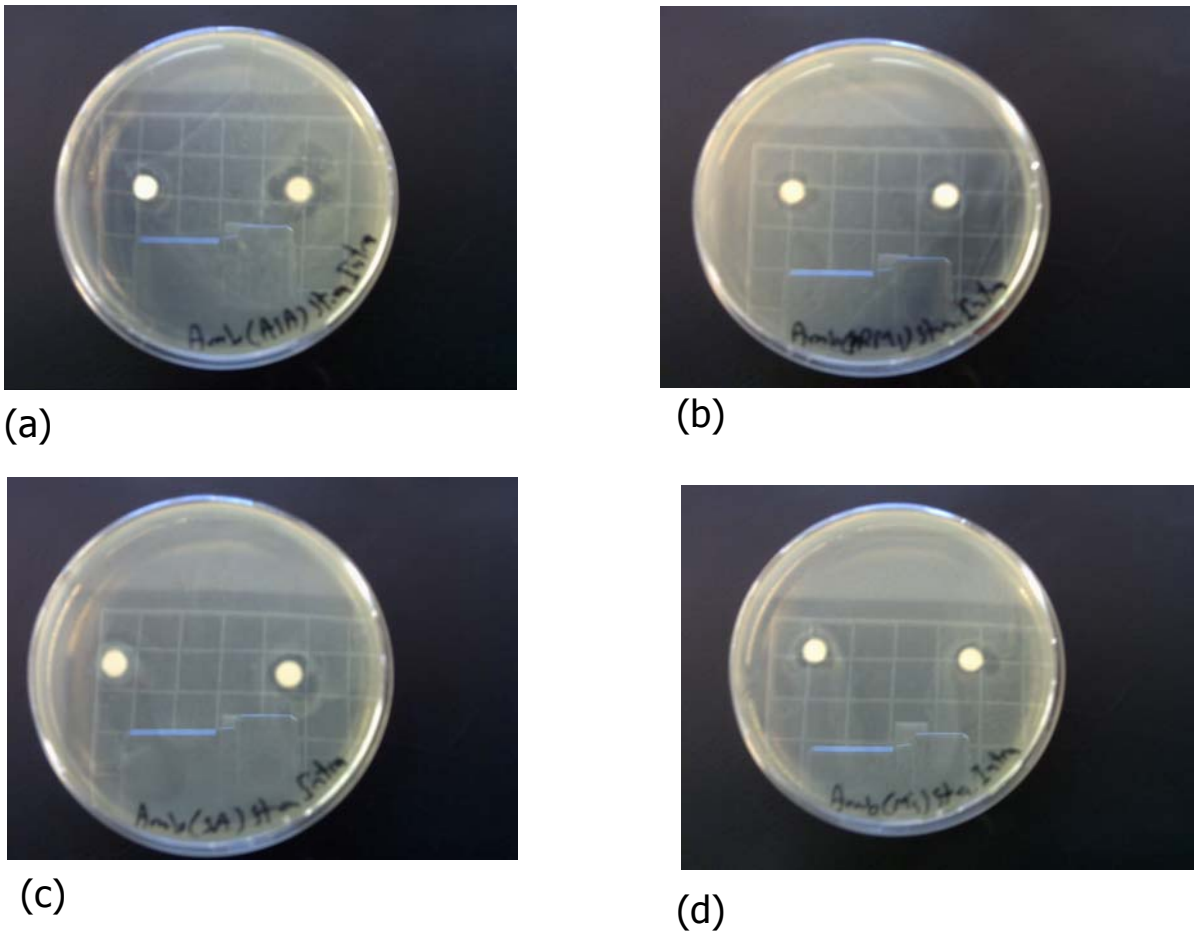


(a) (b)

(c) (d)

Fig(2.2):Antimicrobial activity of intracellular fraction of *Arabidopsis thaliana* elicited with different elicitors against ***P. aerryginosa***:

(a) SA
(b) Chitosan
(c)ASA
(d) PRM1



(a)

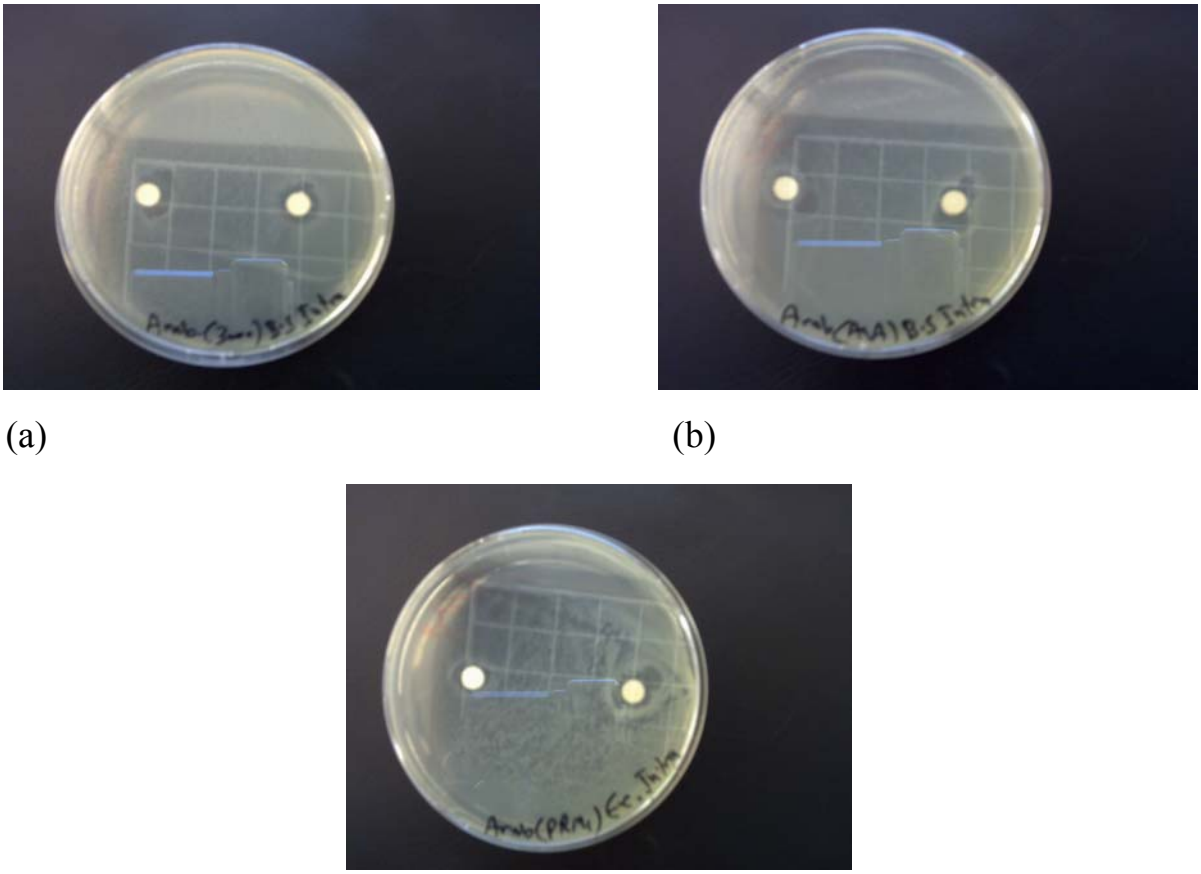
(b)

(c)

(d)

Fig (2.3): Antimicrobial activity of intracellular fraction of *Arabidopsis thaliana* elicited with different elicitors against ***S. aureus***:

(a) ASA
(b) PRM1
(c) SA
(d) Mej



(a) (b)

(c)

Fig (2.4):Antimicrobial activity of intracellular fraction of *Arabidopsis thaliana* elicited with different elicitors against ***other strains***:
(a) *B. subtilis*, DC3000
(b) *B. subtilis*, ASA
(c) *E.coli*, PRM1

2.4 Purification of antimicrobial activity from *Arabidopsis thaliana*

2.4.1 Ammonium sulphate precipitation

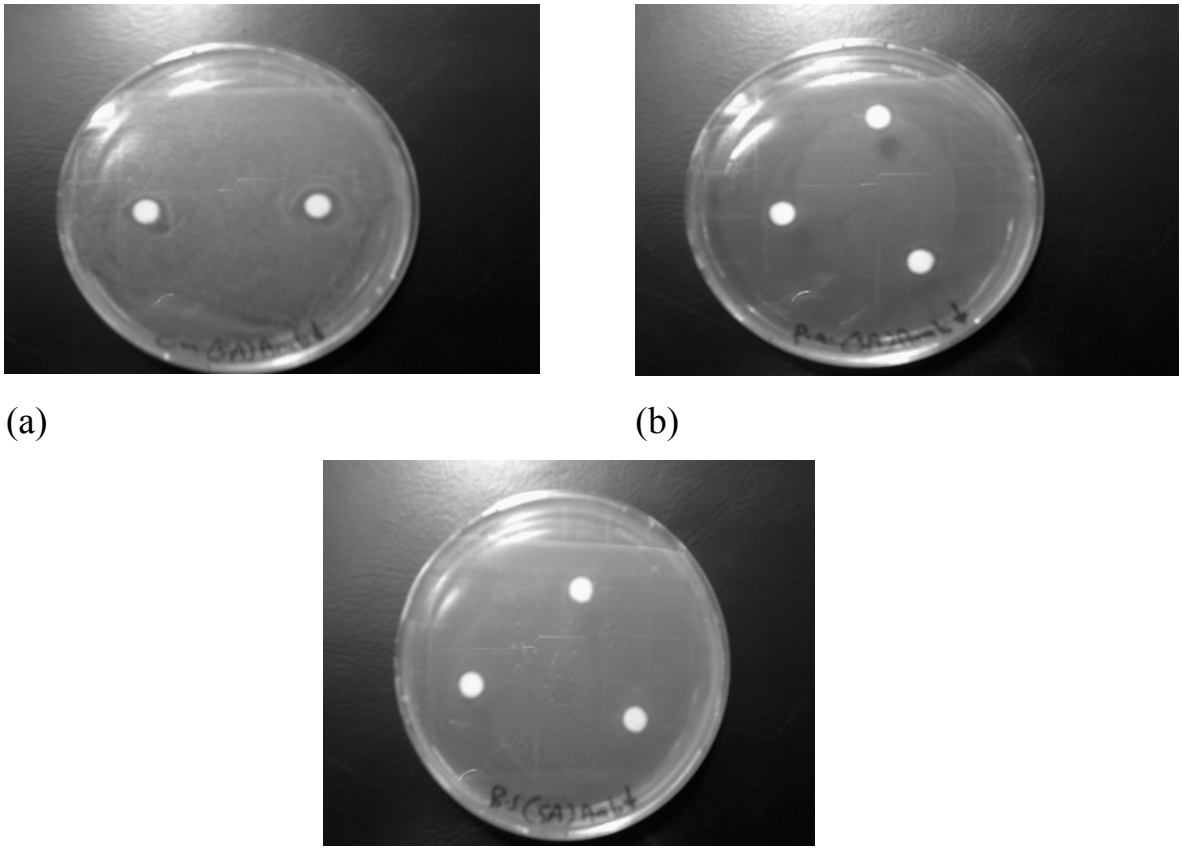
The intracellular fraction of *Arabidopsis thaliana* elicited with SA was selected for testing the presence of antimicrobial protein(s) responsible for its high antimicrobial activity against all used tester isolates and it was selected rather other elicited fractions because the high intensity of the inhibition zone of this fraction against all tester isolates. Definite volumes (5ml) of intracellular *Arabidopsis thaliana* fraction (control and SA elicited fraction) were precipitated with ammonium sulphate (80% saturation) and precipitate was dissolved in 1ml of Na-phosphate buffer after dialysis. Table (2.3) shows the antimicrobial activity of the precipitate for both the control (intracellular fraction without elicitation) and the SA elicited fraction, and from this experiment it could be noticed the presence of proteinous compound(s) responsible for the

Table (2.3):Antimicrobial activity [Inhibition zone (cm)] of ammonium sulfate precipitate of intracellular fraction of *Arabidopsis thaliana*.

Isolate	Elicitor*	
	Control	SA
C. maltosa	0.70	1.15
P. aerryginosa	0.83	0.86
E. coli	-	0.73
S. aureus	0.75	0.75
B. subtilis	0.93	0.80

***Experiment repeated twice with the same results**

antimicrobial activity. Table(2.3) also indicates that SA-elicited fraction is more active against tester isolates than the control fraction and that the antifungal activity [against *C. maltosa* (Fig 2.5a)] is the highest value of activity followed by the activity against *P. aerryginosa* (Fig 2.5b), *B. subtilis* (Fig 2.5c) and *S. aureus*. Also this fraction shows activity against



(a) (b)

(c)

Fig (2.5):Antimicrobial activity of ammonium sulphate precipitate of intracellular fraction elicited with SA of *Arabidopsis thaliana* against tester isolates**.**
(a): *C.maltosa.*
(b): *P. aerryginosa*
(c): *B. subtilis*

E. coli comparable to the control (negative). This experiment confirmed the presence of antimicrobial protein(s) in the intracellular fraction which is/are responsible for the antimicrobial activity and also indicate the activation of induction of this activity with SA elicitor.

2.4.2 High speed centrifugation

During the preparation of intracellular fraction of *Arabidopsis thaliana* it could be observed that the antimicrobial activity can be precipitated at high speed centrifugation (14000rpm) for 20min, so this phenomenon was used for isolation of the antimicrobial activity from the elicited intracellular fraction of *Arabidopsis thaliana,* since ammonium sulphate precipitation has the disadvantage of denaturation of part of the precipitated proteins. For the high speed precipitation of the antimicrobial activity, 60ml of SA-elicited fraction were exposed to centrifugation at 14000rpm for 20min and pellet was dissolved in 3ml of 8M urea and both the supernatant and urea solution were used for testing the antimicrobial activity against *C. maltosa*, since it was the most sensitive organism, among the used isolates, against the antimicrobial protein(s). Also to compare this method with the precipitation with ammonium sulphate, the same volume of the fraction (60ml) were precipitated with ammonium sulphate (80% saturation) and after dialysis the precipitate was collected and dissolved in the same volume (3ml) of 8M urea solution. Table (2.4) shows the antimicrobial activity of active proteins isolated by high speed centrifugation and ammonium sulphate precipitation against *C. maltosa* as a tester isolate, and from this table it could be observed that high speed centrifugation is more effective for isolation of the active protein than the ammonium sulphate precipitation since the inhibition zone diameter was higher [2cm for SA treated fraction and 1.35cm for control (Fig 2.6c)] comparable to ammonium sulphate precipitation [1.2cm for SA treated fraction and 1.1cm for control (Fig 2.6b)]. These

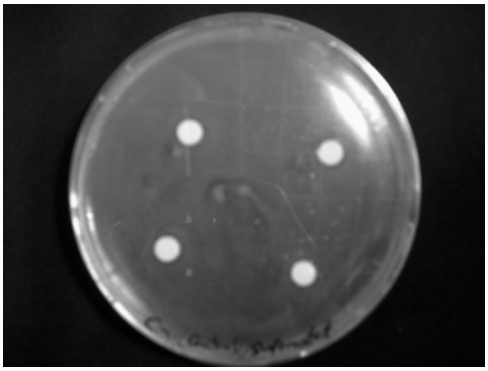
Table (2.4): Antimicrobial activity [Inhibition zone (cm)] of both high speed centrifugation precipitate and ammonium sulphate precipitate dissolved in 8M urea.

Fraction	Elicitor *	
	Control	SA
Supernatent	0.7	0.7
High S. Precipitate dissolved in 8M Urea	1.35	2.0
Ammonium S. Precipitate dissolved in 8M Urea	1.1	1.2

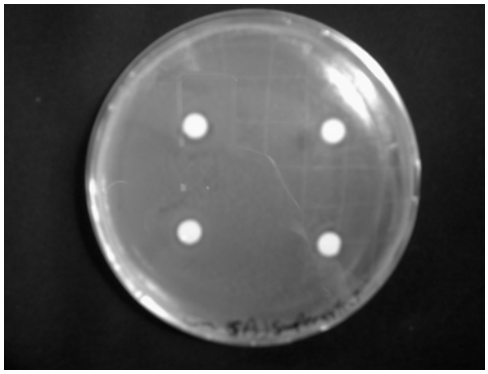
*** Experiment repeated twice with the same results**

results indicate the denaturation of part of the activity due to the use of ammonium sulphate precipitation and support the use of high speed centrifugation instead of salt precipitation. Slight activity of the supernatant after ammonium sulphate precipitation, (Fig 2.6a), of active protein was observed for both control and SA treated fraction which indicates the precipitation of not all of the activity by ammonium sulphate precipitation. Fig (2.7) shows that both high speed centrifugation (for control and SA-elicited fraction) and ammonium sulphate precipitate the same proteins from intracellular fraction of *Arabidopsis thaliana* as indicated from the similarity of protein bands on 15% SDS polyacrylamide gel, but by comparing the intensity of different protein bands for high antimicrobial activity after high speed centrifugation with the lower (moderate) after the ammonium sulphate precipitation it could be observed that one band (about 30kDa) is more strong, on gel, in case of high speed centrifugation comparable to ammonium sulphate. This band may be responsible for the antimicrobial activity of the intracellular *Arabidopsis* fraction. High speed centrifugation was selected for all further isolation

(a): ***Candida maltosa*** + Supernatant after ammonium sulphate precipitation

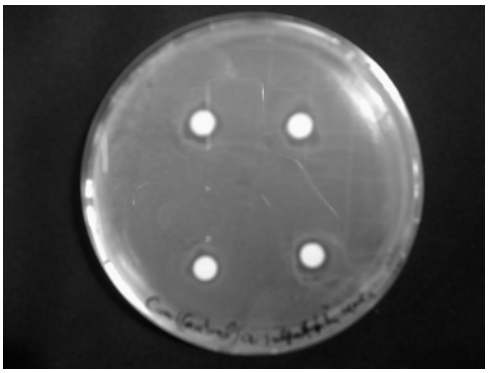


Control

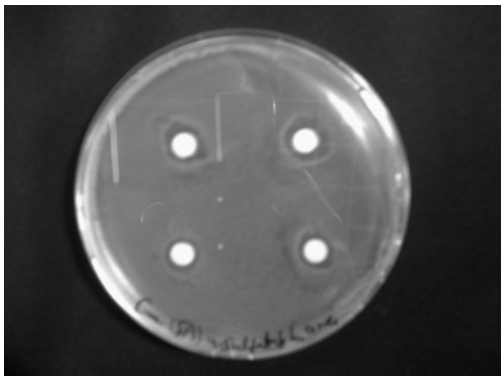


SA

(b): ***Candida maltosa*** + Precipitate of ammonium sulphate dissolved in 8M Urea

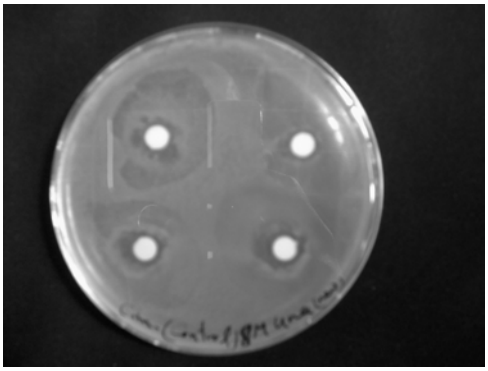


Control

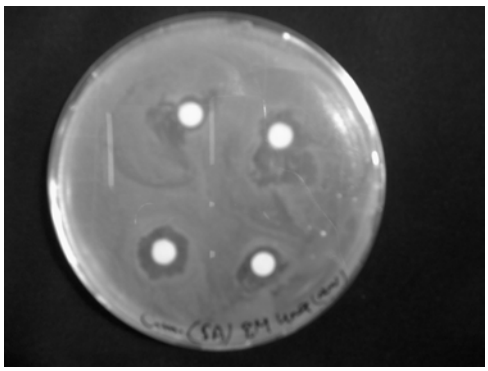


SA

(c): ***Candida maltosa*** + precipitate after high speed centrifugation dissolved in 8M Urea



Control



SA

Fig(2.6): Antimicrobial activity of both ammonium sulphate precipitate and high speed precipitation dissolved in 8M urea.

and purification experiments for the active protein(s) instead of ammonium sulphate precipitation.

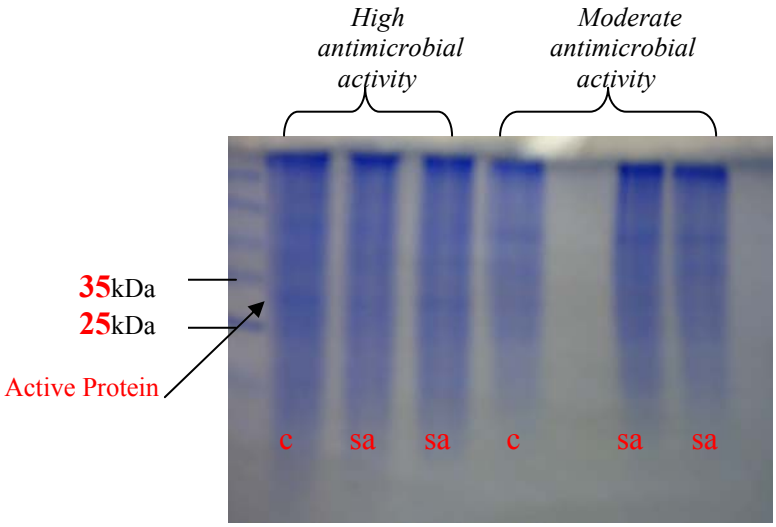
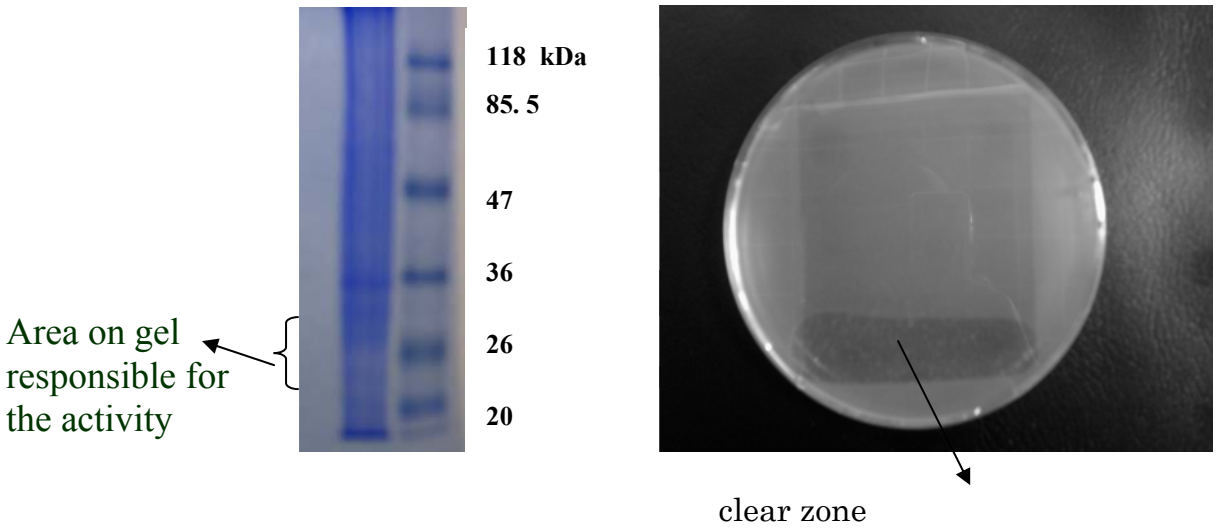


Fig (2.7): Comparison for protein content for both high speed centrifugation (High antimicrobial activity) and ammonium sulphate precipitate (Moderate antimicrobial activity) for control (c) and SA (sa) samples.

2.4.3 Bioautography

This experiment was done to confirm the presence of antimicrobial protein(s) in the crude extract of SA-elicited *Arabidopsis* intracellular fraction and also to calculate the molecular weight of the protein of interest to select a suitable purification method. Fig (2.8) shows that a clear zone of antimicrobial activity against *C. maltosa* could be observed after covering a gel (12.5%) with low strength LB medium (20%) seeded with cells of the tester fungus and incubation for overnight. By comparing the protein bands on a lane of gel running simultaneously with the tested gel in the petridish, it could be observed that the area on the gel responsible for the activity is between 26 and 36kDa.



Fig(2.8): Bioautography of *Arabidopsis thaliana* intracellular proteins

2.4.4 Gel filtration

Sephacryl S-200 gel filtration column was selected for the purification of the active antimicrobial protein. Fig (2.9a) shows the calibration of the used column with 4 standard proteins; ribonuclease A (13.7kDa), chymotrypsinogen (25kDa), ovalbumin (43kDa) and Bovine serum albumin in presence also of Dextran 2000 (2000kDa) and the elution volume (V_o) was calculated and then 5 beaks (corresponding to the 5 used standards) were selected using ÄKTA*prime* protein purification system. 4ml of plant sample dissolved in 8M urea were injected to the top of the same column connected to ÄKTA*prime* and 1ml fractions were collected. The antimicrobial activity
of all fractions were tested using the most sensitive isolate, *C. maltosa*, and two active fractions eluted in the area of 25kDa (chymotrypsinogen) were observed and their purity were confirmed by SDS-Polyacralymide gel stained with silver staining (Fig 2.9b).

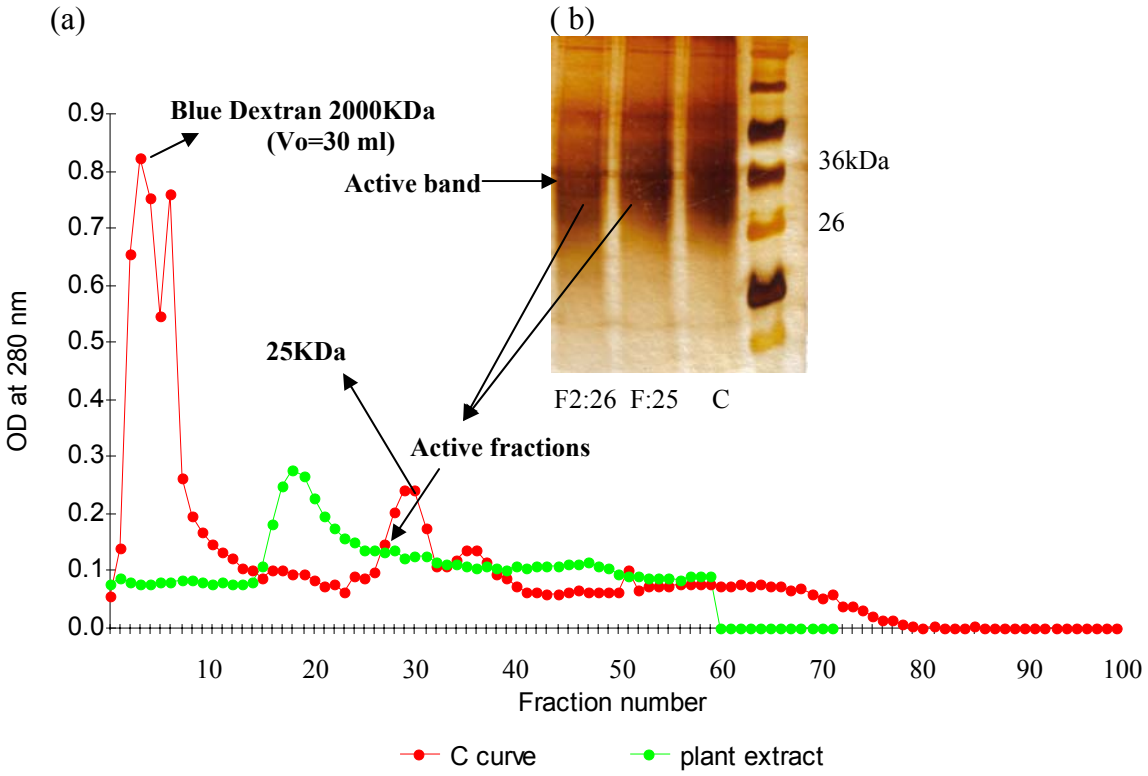


Fig (2.9): Gel filtration (Sephacryl S-200) for standard and plant intracellular proteins

2.2.5 Mass spectrometry and identification of the active protein

The protein band was excised from silver-stained gel and subjected (by Dr. Joerg Reinders) to trypsin digestion and the obtained peptides mixture was subjected to nano-LC- MS/MS analysis using Dionex Ultimate 3000 nano-LC-System directly coupled with ThermoElectron LCQDecaXP ion trap mass spectrometer. Fig (2.10) shows the mass spectrum for 12-long peptide of the digested protein with sequence (IYDKDGDYIGIK). By searching this results against the NCBInr database using the Mascot algorithm (Version 2.1; MatrixScience, London, GB), an unknown protein with accession number NP_195683 (GI:15236014) could be found and this protein has a calculated molecular weight of 20.005 kDa and characterized by the presence of plant specific domain which is termed PLAT- Plant Stress domain. This protein represent an endomembrane system with unknown function,

but it is considered as a lipid-associated family protein and it has the following sequence. The protein will referred to as AtPDP1,accrording to (Arabidopsis thaliana- Plat Domain Protein 1).

1 **marrdvllpfllllatvsavafaeddpdcvytfylrtgsiwkagtdsiisa riydkdgdyigiknlqawaglmgpdynyfergnldifsgrapclpspicalnl tsdgsgdhhgwyvnyveittagvhaqcstqdfeieqwlatdtspyeltavrnn cpvklrdsvsrvgseirkklswvv** 181

2.2.6 Antimicrobial activity of AtPDP1

Purified protein was tested for its antimicrobial activity against the five tester isolates used in the study and results in Table (2.5) show that the AtPDP1 is active against all tester isolates especially *C. maltosa* [Fig (2.11)] followed by the Gram negative bacteria *E. coli* and *P. aerryginosa* and lower activity against the Gram positive bacteria *S. aureus* and *B. subtilis*.

2.4.6 Homology with other known proteins and sequence analysis

To compare the sequence of AtPDP1 from intracellular fraction of *A. thaliana* elicited with SA with the other known proteins from other organisms, Blast-Aligorithms (Altschul et al.,1997) were used. Table (2.6) conclude the homology of AtPDP1 with known proteins from other organisms. The highest homology (100% identity, 100% similarity) was to the dehydration stress induced protein from *Arabidopsis thaliana* (GI_21593926) followed by the unknown protein (80% identity, 91% similarity) from *Arabidopsis thaliana* (GI_27754389), which will referred to as AtPDP2, and Lipoxygenase,LH2 (70% identity, 82% similarity) from *Medicago truncatula* (GI_92898164). Lower homology level was observed for other proteins. Fig (2.12) shows an alignment [done by Clustal X program (Thompson et al.,1997) of the isolated unknown protein from

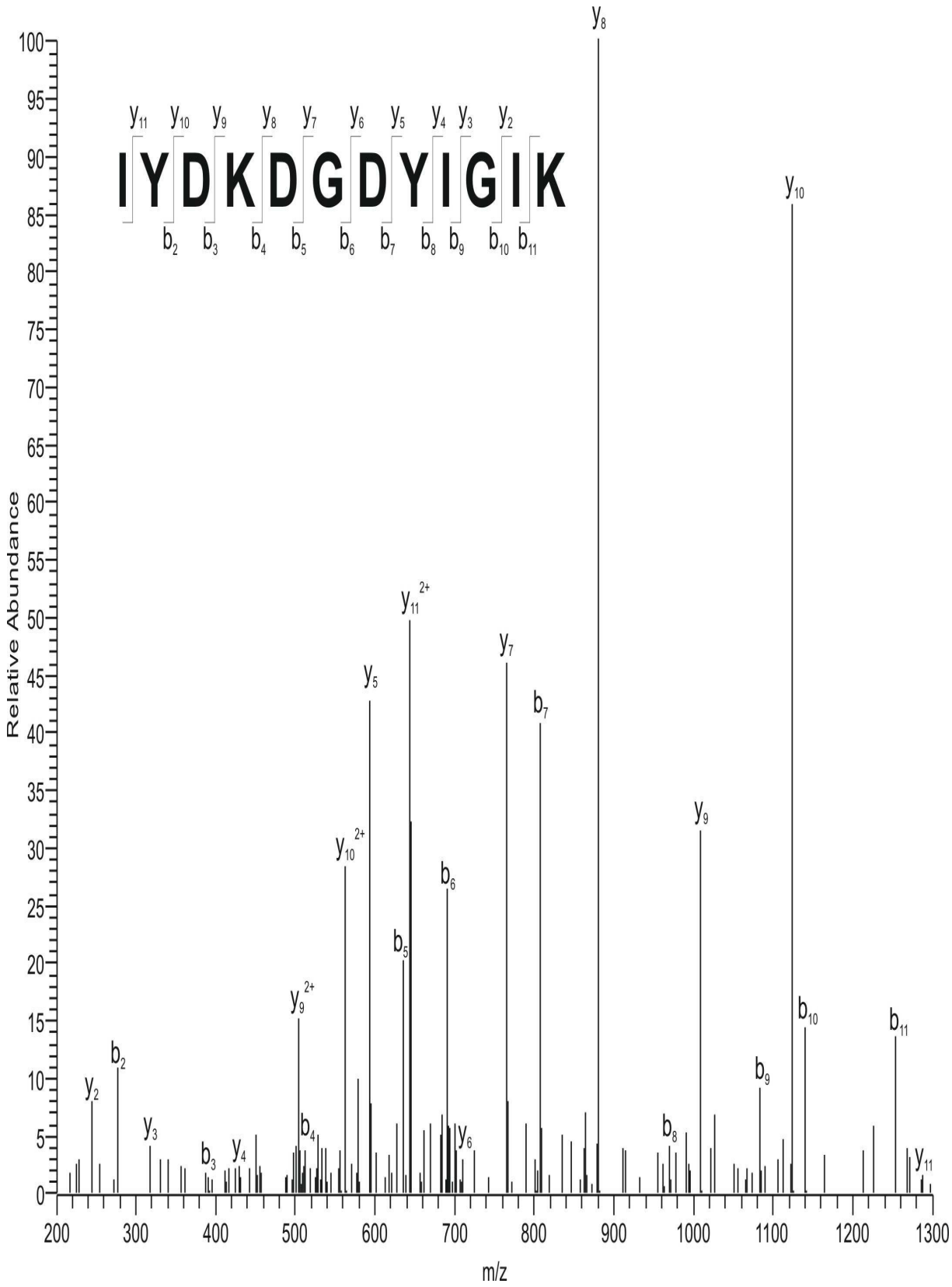


Fig (2.10): Mass spectrum for a peptide with 12 amino acids from the trypsin digested active protein from *Arabidopsis thaliana.*

Table (2.5): Antimicrobial activity [Inhibition zone(cm)] of purified AtPDP1

Isolate	AtPDP1*
C. maltosa	1.2
P. aerryginosa	1.0
E. coli	1.1
S. aureus	0.9
B. subtilis	0.9

***Experiment repeated twice with the same results**

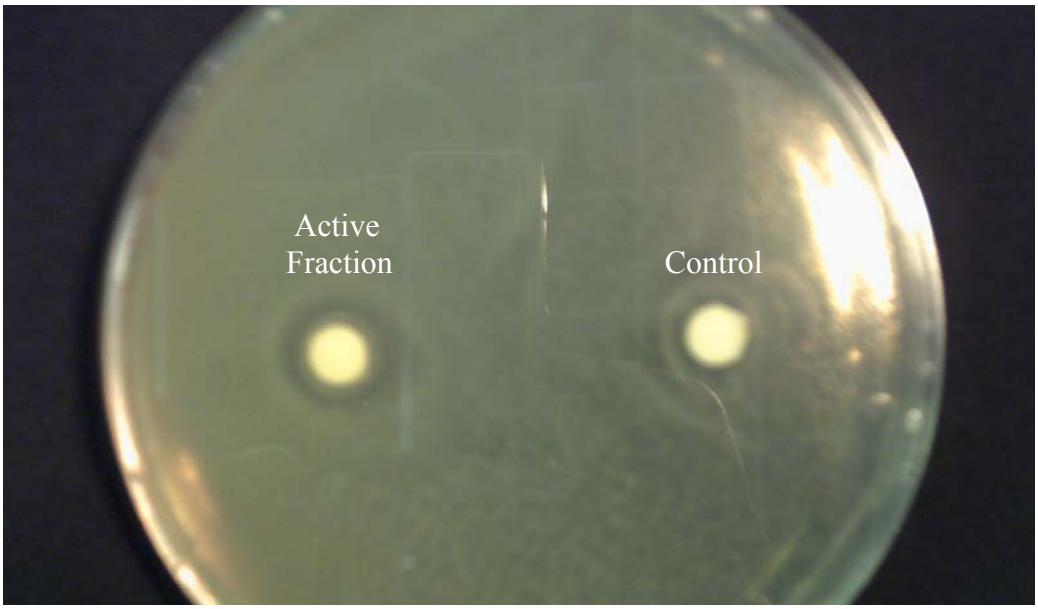


Fig (2.11): Antimicrobial activity of purified AtPDP1 against, *C. maltosa*, comparable to control (Buffer).

alignment [done by Clustal X program (Thompson et al.,1997) of the isolated AtPDP1 with other known proteins, from other plants, with PLAT-plant-stress domain and from the alignment it could be observed that the PLAT domain is the most homologous domain among all proteins. Fig (2.13) shows a cladiogram representing the relationship of sequences of proteins used in the alignment. From Fig (2.12)and Fig (13.2) it could be observed that sequence of AtPDP1 is more related to the dehydration stress induced protein from *Arabidopsis* followed by Lipoxygenase, LH2 from *Medicago truncatula*. It also has been observed that AtPDP1 has calculated molecular weight of 20.005 kDa which is different from the observed molecular weight on the gel (about 30kDa). Using PROSITE program (Hofmann et al., 1999) it could be found the presence of a glycosylation sit (NLTS) which may be responsible for the post-translation modifications of AtPDP1 that lead to change in its final molecular weight.

Table(2.6): Homology of isolated unknown protein with other proteins.

Protein	Organism	GI	Homology	
			Identity %	***Similarity %***
- Unknown protein of lipid associated family proteins (AtPDP1)	*Arabidopsis thaliana*	15236014	100	100
- Putative protein	*Arabidopsis thaliana*	7270957	100	100
- Putative protein	*Arabidopsis thaliana*	3080442	100	100
- AT4g39730/ T19P19_120	*Arabidopsis thaliana*	20147335	100	100
- AT4g39730/ T19P19_120	*Arabidopsis thaliana*	19310489	100	100
-Dehydration stress-induced protein	*Arabidopsis thaliana*	21593926	100	100
- Unknown protein (AtPDP2)	*Arabidopsis thaliana*	18399899	80	91
- Lipoxygenas, LH2	*Medicago truncatula*	92898164	70	82

-Elicitor - inducible protein EIG-J7	*Capsicum annuum*	40287496	67	79
- Wound /Stress protein	*Lycopersicon esculentum*	51457948	66	77
- TMV-induced protein	*Capsicum annuum*	7542598	48	69
-Elicitor - inducible protein	*Nicotiana tabacum*	10241929	55	68
- TMV-induced protein 1-2	*Capsicum annuum*	28628595	53	71
- LOXHD1 protein	*Homo sapiens*	28838367	27	47
-Polycystin 1-like 2	*Mus musculus*	31541842	28	40
- Polycystic kidney disease 1-like 3		87280850	28	39
- Polycystin		68431741	27	41
- 5-lipoxygenase	*Bos taurus*	13235074	30	41
- 11 R-lipoxygenase	*Gersemia fruticosa*	68519190	24	40
- Chain B, insights from X-Ray crystal lipoxygenase calcium activation via a C2-like domain and a structural basis of product chirality		90109546	29	42
- Arachidonate 15-lipoxygenase	*Strongylocentrotus Purpuratus*	72134572	27	40
-Embryo-specific protein 3 (ATS3)	*Arabidopsis thaliana*	7546697	29	42

```
                                                          ____PLAT domain_______
GI_15236014      ----MARRDVLLPFLLLLATVS--AVAFAED----DPDCVYTFYLRTGSIWKAGTDSIIS 50
GI_21593926      ----MARRDVLLPFLLLLATVS--AVAFAED----DPDCVYTFYLRTGSIWKAGTDSIIS 50
GI_92898164      ----MANPTTLFALFFLLSFCF--AGTVTSD------DCVYTVYVRTGSIIKAGTDSIMT 48
GI_40287496      MGVA-QVNHIWFYLMIILFSIS--ISSIAAS----EDDCVYTAYIRTGAIIKAGTDSNIS 53
GI_51457948      MGVAAQVNQMWFNLMIVLFFVS--ISSISA------EDCVYTAYIRTGSIIKAGTDSNIS 52
GI_7542598       -----MSNQFWFRLTIILISIS--VSSISGS----ELNCVYSVYVQTGQFFGAGTDSKIT 49
GI_51535090      ---MAPKIPIFFFLLALAAGVQGETGGVGGGGGNVEYNCVYTVFVRTGSAWKGGTDSTIG 57
GI_37533440      ----MNKLSLALASYLILFLAA--AATAATT------RCTFEIVVKTDGRRNAGTDARVS 48
GI_47419897      ----MALKAVLISPFFFLFLAG-IITLPAASKQLGEGECLYTIYVQTGDIADAATDAVVS 55
                           :    .                      * :   ::*.    ..**: :
                 _____________________PLAT domain_____________________________
GI_15236014      ARIYDKDGDYIGIKNLQAWAGLMGPDYNYFERGNLDIFSGRAPCLPSPICALNLTSDGSG 110
GI_21593926      ARIYDKDGDYIGIKNLQAWAGLMGPDYNYFERGNLDIFSGRAPCLPSPICALNLTSDGSG 110
GI_92898164      LTLYNADGYGILIRDLEAWGGLMGSGYNYFERGNLDIFSGRGPCLDGPVCNMNLTSDGSG 108
GI_40287496      LTLYDADGYGLRIKNIEAWGGLMGPGYNYFERGNLDIFSGRGPCLAGPICKMNLTSDGTG 113
GI_51457948      LTLYDANGYGLRIKNIEAWGGLMGPGYNYFERGNLDIFSGKGPCVNGPICKMNLTSDGTG 112
GI_7542598       LSLYDADGYGLRIKSLEAWGGLMGSGYDYFEFGKLDLFTGRGPCLNGPVCKMNLTSDGTG 109
GI_51535090      VEFAGADGRGVRIADLERWGGLMGAGHDYYERGNLDVFSGRGPCLPAAPCWMNLTSDGAG 117
GI_37533440      LQVRAARGPTLTVANLESWG-QMAAGHDYFEKGNLDRFRGAGDCMPSEPCNMVLTSDGSG 107
GI_47419897      LVISDHPG---------------AGGYDYFERGNLDFFSGQASCLSAPPCWMLLAHDNTG 100
                   .    *  : :  :       :. .::*:* *:** * * . *: .  * : *: *.:*
                 ____________PLAT domain_________
GI_15236014      DHHGWYVNYVEITTAG-VHAQCSTQDFEIEQWLATDTSPYELTAVRNNCP---------- 159
GI_21593926      DHHGWYVNYVEITTAG-VHAQCSTQDFEIEQWLATDTSPYELTAVRNNCP---------- 159
GI_92898164      SHHGWYCNYVEVTTTG-AHIPCAQQQFEVEQWLATDTSPYELSAIRNNCQYNNLGQAHHK 167
GI_40287496      PHHGWYCNYVEVTVTG-AHKQCIQQLFNVEQWLGTDVSPYKLTAIRNNCN---------- 162
GI_51457948      PHHGWYCNYVEVTVTG-AKKQCNQQLFTVNQWLGTDVSPYKLTAIRNNCKN--------- 162
GI_7542598       QHAGWYCNYVEVTSTG-EHKRCNQQLFTVEKWLGAGEFPDGLTAIKNNCG---------- 158
GI_51535090      AHHGWYCNYVEVTATG-PHRGCAQRRFDVEQWLATDASPYRLTAVRDQCR---------- 166
GI_37533440      NKPGWYVSYVMVTQLGQGRLPSMTHRWAVDQWLAIDEAPHMLTAERRGCG---------- 157
GI_47419897      NKPGWFVDHVLVTMAPLANAPVGQHLFRVNQWLARDESPNQLATTRNDCDNDS------N 154
                  : **: .:* :*     .     : : :::**. .  *  *:: :  *

GI_15236014      VKLRDSVSR-VGSEIRKKLSWVV------- 181
GI_21593926      VKLRDSVSR-VGSEIRKKLSWVV------- 181
GI_92898164      LKTVDAVSSESGSDFSILASTVHV------ 191
GI_40287496      KPKSSEEQPIYDSESYTTVDVI-------- 184
GI_51457948      KYESGELKPLYDSESFSIVDVI-------- 184
GI_7542598       -RKPNEQLSTYDPQSYHVVDVL-------- 179
GI_51535090      ----GHAAA--------------------- 171
GI_37533440      ---IGAAAP--------------------- 163
GI_47419897      IRMSSALQAAMGARNAGVSRRMGGSEPKPM 184
                     .               :
```

Fig (2.12): Alignment of isolated AtPDP1 (GI_15236014) with other known proteins containing PLAT-plant stress domain: GI_21593926: Dehydration stress-induced protein [*Arabidopsis thaliana*], GI_92898164: Lipoxygenase,LH2 [*Medicago truncatula*], GI_40287496: Elicitor-inducible protein EIG-J7 [*Capsicm annuum*], GI_51457948: Wound/Stress protein [*Lycopersicon esculentum*], GI_7542598: TMV-Induced protein I [*Capsicm annuum*], GI_51535090: Putative dehydration stress-induced protein [*Oryza sativa* (japonica cultivar-group], GI_37533440: Putative elicitor inducible protein [*Oryza sativa* (japonica cultivar-group)], GI_47419897: tuber-specific elicitor- inducible-protein [*Zantedeschia* hybrid cultivar]

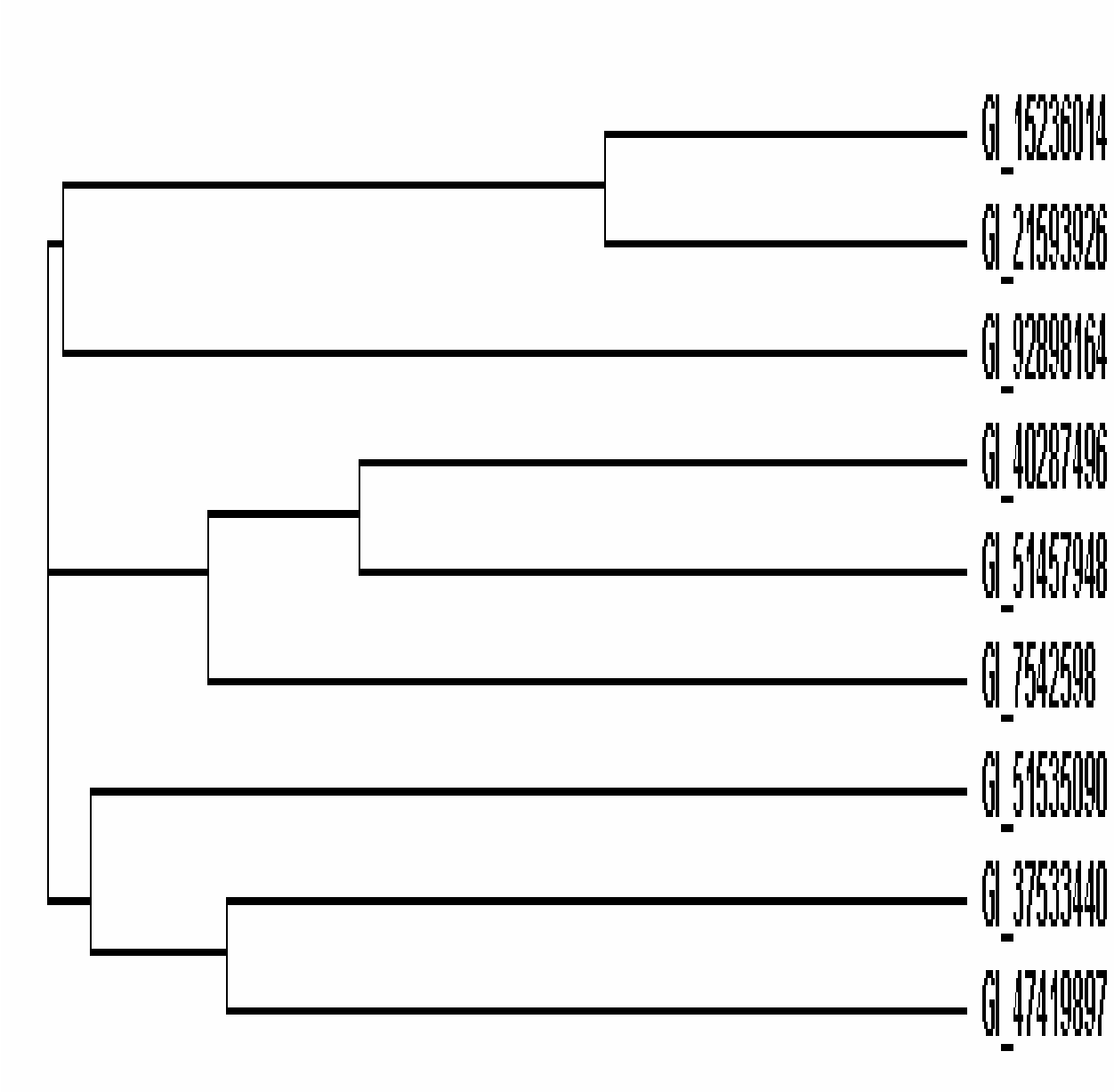


Fig (2.13): Cladiogram showing the relationship of sequences used in the alignment:
GI_15236014: AtPDP1.
GI_21593926: Dehydration stress-induced protein [*Arabidopsis thaliana*]
GI_92898164: Lipoxygenase,LH2 [*Medicago truncatula*]
GI_40287496: Elicitor-inducible protein EIG-J7 [*Capsicm annuum*]
GI_51457948: Wound/Stress protein [*Lycopersicon esculentum*]
GI_7542598: TMV-Induced protein I [*Capsicm annuum*]
GI_51535090: Putative dehydration stress-induced protein [*Oryza sativa* (japonica cultivar- group)]
GI_37533440: Putative elicitor inducible protein [*Oryza sativa* (japonica cultivar-group)]
GI_47419897: tuber-specific elicitor- inducible- protein [*Zantedeschia* hybrid cultivar]

2.5 Cloning of genes for AtPDP1 and AtPDP2.

cDNAs of AtPDP1 and AtPDP2 with PLAT-plant-stress domain were synthesized from RNA isolated from plant materials treated with SA using reverse transcriptase. PCRs were carried out at 57°C and 59°C annealing temperatures for AtPDP1 gene (At4g39730) and AtPDP2 gene (At2g22170) respectively. Fig (2.14a and 2.14b) show the (546 bp and 552 bp) PCR products for both genes respectively. PCR products were excised from gels and after cleaning, they were ligated in PGEM-T Easy vector (Appendix 6.3) and used for transformation of DH5α strain of *E. coli*. Blasts for both genes are shown in Appendix (6.4)

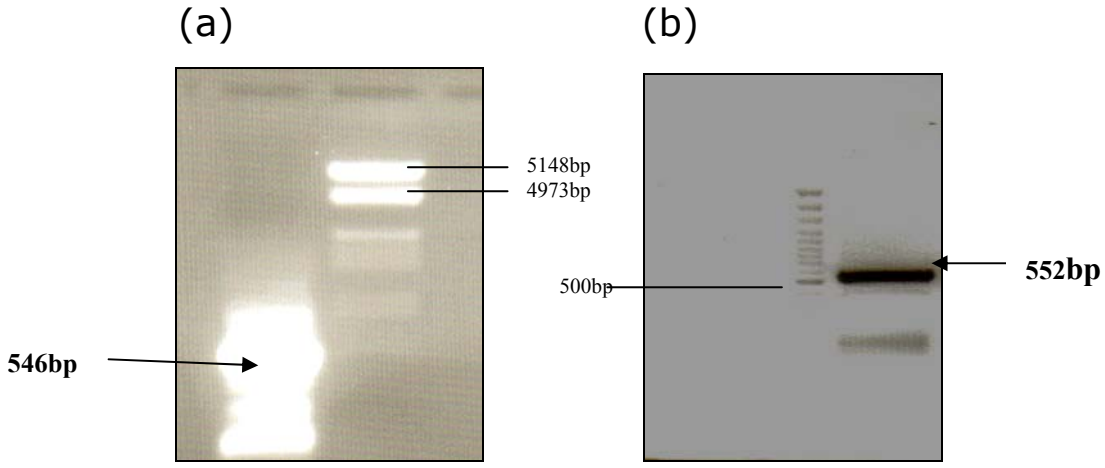


Fig (2.14): RT-PCR for both unknown protein genes:
(a): At4g39730 (for the isolated AtPDP1)
(b): At2g22170 (for AtPDP2)

2.6 Expression of AtPDP1 and AtPDP2 under different stimuli (Digital Northern).

The expression of the gene for AtPDP1 (At4g39730) and also for AtPDP2 (At2g22170) with the same PLAT domain was tested using Digital Northern (WWW.genevestigator.ethz.ch). Table (2.7) shows that At4g39730 is constitutively expressed by plant in shoot system more than roots and the reverse for At2g22170. Both genes are induced by different pathogens, chemicals known to induce defense in plant cells and different hormones. The maximum induction for At4g39730 was by pathogens followed by the effect of SAR (Systemic Acquired

Resistance), ABA and *P. syringae* (avr). For At2g22170 the maximum induction was by mycotoxins followed by Zeatin, GA3 and ABA.

Table(2.7): Expression of the unknown proteins under different stimuli (Digital Northern).

Experiment	Signal	
	At4g39730 (for the isolated AtPDP1)	At2g22170 (for AtPDP2)
Shoot apex	7019	3159
Roots	4346	7900
Chitin	10922.84	16355.4
Mej	9336.314	13506.13
DC3000	9845.152	263.4071
Psyr-avr	12755.76	1031.456
SA	11562.82	17973
Alt	8023.2	5204.1
Pathogen	22570.5	3336.5
Mycotoxin	13053	18433
SAR	14641.14	3120.424
Auxin (IAA)	11455.99	13497.2
ABA	12456.01	16896.91
GA3	11642	16993.18
Zeatin	11717.49	17062.93

2.5 Cloning of wound-inducible promoter

Genomic DNA from popular plant (*Populus trichocarpa* hybride) was used for isolation of proteinase-inhibitor-like gene win 3.12 (GenBank accession Nr L11233) in presence of pair of primers (Appendix 6.2) with 2 engineered [*Hind*III (forward primer) and *Xba*I (reverse primer)] restriction sites. PCR was performed at 55C annealing temperature for 40 cycles. After running 1% agarose gel, a 846-bp product (Fig 2.15) was excised and purified. PCR product then was ligated in PGEM-T easy vector

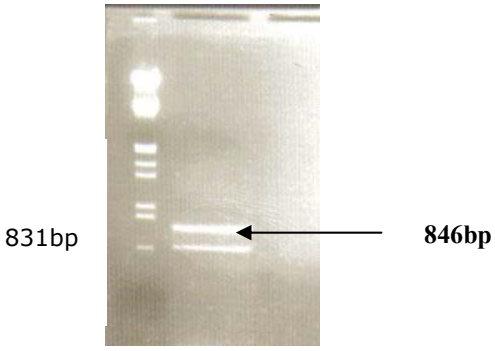


Fig (2.15): PCR for proteinase-inhibitor-like gene

and used for transformation of DH5α strain of *E. coli.* After plasmid mini-preparation, plasmids were digested with *Hind*III and *Xba*I restriction enzymes. Two positive colonies for the same gene were sequenced and new sequences were compared with the already known entries Blast-Algorthms of NCBI (http://www.ncb/.nih.gov/BLAST) and 2 different results were obtained for the 2 sequenced gene plasmids (Appendix 6.5.1 & 6.5.2) but both were identical for *Populus trichocarpa* wound responsive gwin3 gene and not to win 3.12 gene. Effort was done to test the expression of the cloned genes (the 2 different sequenced clones) in PBI121 plasmid after removal of 35S promoter from the binary vector, but unfortunately we could not obtain transformants (colonies) of *E. coli* with the new engineered PBI121 plasmid

2.6 Cloning of thaumatin genes from *Arabidopsis thaliana*

The objective for cloning of the thaumatin genes is for additional work which include testing the expression of different genes under different stimuli since thaumatin genes have defense role, in plants, against pathogens and also some of them are active against *Candida* spp.

Genomic DNA was isolated from *Arabidopsis thaliana* materials and was used as template for cloning of 13 genes of thaumatin. Pair of primers for every thaumatin gene was designed (Appendix 6.2) for cloning coding region of genomic DNA greater than 500 bp [(Table 2.8) ,(Appendix 6.6 & 6.7)]

Table(2.8): Selected coding regions of genomic DNA and cDNA for thaumatin genes

Gene Name	Coding region (genomic DNA)				cDNA
1-At1g200030	coding_region	426-1123	AGI-TIGR	2004-09-08	1-697=697
	coding_region	1392-1593	AGI-TIGR	2004-09-08	
2-At1g75800	coding_region	108-162	AGI-TIGR	2004-09-08	54-756=702
	coding_region	244-946	AGI-TIGR	2004-09-08	
	coding_region	1432-1666	AGI-TIGR	2004-09-08	
3-At1g75050	coding_region	29-77	AGI-TIGR	2004-09-08	48-772=724
	coding_region	251-975	AGI-TIGR	2004-09-08	
4-At1g75030	coding_region	28-76	AGI-TIGR	2004-09-08	48-739=691
	coding_region	174-865	AGI-TIGR	2004-09-08	
5-At5g02140	coding_region	1-55	AGI-TIGR	2004-09-08	56-727= 67
	coding_region	150-822	AGI-TIGR	2004-09-08	
	coding_region	898-1054	AGI-TIGR	2004-09-08	
6-At5g24620	coding_region	151-908	AGI-TIGR	2004-09-08	1-757=757
	coding_region	1018-1522	AGI-TIGR	2004-09-08	

Gene Name	Coding region (genomic DNA)				cDNA
7-At4g38660	coding_region	118-193	AGI-TIGR	2004-09-08	194-863=669
	coding_region	320-989	AGI-TIGR	2004-09-08	
	coding_region	1363-1654	AGI-TIGR	2004-09-08	
8-At4g24180	coding_region	1-70	AGI-TIGR	2004-09-08	71-768=697
	coding_region	169-866	AGI-TIGR	2004-09-08	
9-At1g19320	coding_region	47-101	AGI-TIGR	2004-09-08	54-742=688
	coding_region	210-898	AGI-TIGR	2004-09-08	
10-At4g36010	coding_region	166-929	AGI-TIGR	2004-09-08	1-763=763
	coding_region	1529-1670	AGI-TIGR	2004-09-08	
11-At2g17860	coding_region	1-762	AGI-TIGR	2004-09-08	1-762=761
12-At4g38670	coding_region	126-180	AGI-TIGR	2004-09-08	54-753=699
	coding_region	278-977	AGI-TIGR	2004-09-08	
	coding_region	1407-1497	AGI-TIGR	2004-09-08	
13-At1g70250	coding_region	11-299	AGI-TIGR	2004-09-08	421-1185=764
	coding_region	384-455	AGI-TIGR	2004-09-08	
	coding_region	819-881	AGI-TIGR	2004-09-08	
	coding_region	968-1732	AGI-TIGR	2004-09-08	
	coding_region	1818-1850	AGI-TIGR	2004-09-08	
	coding_region	1947-3124	AGI-TIGR	2004-09-08	

PCRs for all 13 thaumatin genes were done in 30ul volume each at different annealing temperatures (45.1, 47.7, 51.4, 55.3 and 59.7 C). After running gel, the optimal annealing temperature for every gene was selected and another PCR was carried out in 50ul volume the following expected sizes of PCR products were observed on gels:

At1g20030 :322bp, At1g75800:425bp, At1g75050:507bp,

At1g75030: 448bp, At5g02140:470bp, At5g24620:504bp, At4g38660: 468bp, At4g24180: 503bp, At1g19320:568bp, At4g36010: 430bp, At2g17860:403bp, At4g38670:522bp, At1g70250:451bp.

PCR product for every gene was excised from gel and after cleaning they were ligated in PGEM-Teasy vector and used for transformation of DH5α strain of *E. coli*. After plasmid mini preparation for every gene, plasmids were digested with the suitable restriction enzymes for selecting the positive colonies as following:

At1g20030:PstI&NcoI, At1g75800:ApaI&SacI, At1g75050:ApaI&SacI, At1g75030:PstI&NcoI, At5g02140:PstI&NcoI, At5g24620:PstI&NcoI, At4g38660:EcoRI, At4g24180:PstI&NcoI, At1g19320:PstI&NcoI, At4g36010:PstI&NcoI, At2g17860:PstI&NcoI, At4g38670:EcoRI and At1g70250:EcoRI.

One positive colony for every gene was sequenced and new sequences were compared with the already known entries Blast-Algorthms of NCBI (http://www.ncb/.nih.gov/BLAST) and different Blast results for the 13 thaumatin genes are represented in Appendix (6.8.1- 6.8.13).

2.7. Expression of different thaumatin genes under different stimuli (Digital Northern).

The expression of thirteen thaumatin genes under different stimuli (Digital Northern) (WWW.genevestigator.ethz.ch), is shown in (Table 2.9). Expression of At1g75800 gene was the maximum under all stimuli, than other genes, followed by At4g36010 and At4g38660. The minimum expression of genes was for At1g75050, At4g24180 and At2g17860. The rest of genes showed moderate expression under different stimuli.

Table (2.9): Expression of different thaumatin genes under different stimuli (Digital Northern):

Stimuli/Signal						
Gene	**SA**	**Mej**	**DC3000**	**H_2O (Seed)**	**ABA**	**GA_3**
At1g20030	181	226	1244.5	4.8	6.8	22.1
At1g75800	1919	2072	4246	10151	10327	8192
At1g75050	8	13.5	43	42.6	58	53
At1g75030	216	261	220	19.6	69.8	80.3
At5g02140	122	136.5	77.2	175	169	38
At5g24620	626	1907	1633	1432	1169	282
At4g38660	1062	1149	566	1775	1443	790
At4g24180	37	37	15.5	23	25	7
At1g19320	66	152.5	170.7	80.7	156	196
At4g36010	238	524.5	7589	2059	2908	3096
At2g17860	90	31	63	27	96	98.7
At4g38670	329	393	320	659	787	665
At1g70250	249.5	200	142.1	33	63	37

3. Discussion

3.1 Production of antimicrobial compounds by plant cell cultures

The antimicrobial activity of plant extracts against bacteria and fungi have been reported by other workers (Heisey and Gorham,1992; Caceres et al.,1991). In this study aqueous extracts of ten different plant cell cultures showed different antimicrobial spectra against five human pathogenic microorganisms, namely *Candida maltosa, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aerryginosa*. Most plant fractions observed to exhibit different spectra against the tester isolates in presence of different elicitors. Elicitors may be responsible for induction of antimicrobial compounds, as a natural defense, in plant cell cultures. Velazhahan et al. (2000) reported the induction of elicitor-inducible antifungal chitinase in rice suspension cultured cells when treated with a fungal elicitor isolated from *Rhizoctonia solani*. Also Thorsten et al., (1997) reported the phytoalexins synthesis and accumulation in elicitor stimulated parsley cell cultures. In general, accumulation of different antimicrobial PR proteins in infected plants are thought to be component of induced defense system in these plants (Broekaert et al., 1997; Stintzi et al., 1993; VanLoon, 1997), and use of different chemicals for induction of defense in plants have been reported by many workers (Gullino et al., 2000; Kessmann et al.,1994; Uknes et al., 1996). In the present study the heterotrophic cultures were more active against tester isolates than the autotrophic ones. The intracellular fraction of the mexotrophic *Arabidopsis thaliana* elicited with different elicitors was the most active fraction against all tester isolates especially those elicited with bacterial elicitors for *Pseudomonas syringae* strains DC3000 and PRM1 and also those treated with SA and ASA. The antifungal activity of all these fractions was higher than the effect of 10ug/ml of Nystatin solution which have been used as antifungal positive control. The antimicrobial activity of this fraction is followed by that of the intracellular fraction of the

heterotrophic *Nicotiana tobacum* (BY2), since not all elicitors induce the same antifungal activity but the most active fraction was that elicited with chitosan, PGA and PRM1 also, the activity was higher than that of 10ug/ml of Nystatin, and the same was observed for the extracellular fraction of the heterotrophic culture *Eschscholiza californica* (AST) elicited with chitosan, Alt and ASA. All the remaining plant cell culture fractions showed lower activity against the tested fungus. The susceptibility of this yeast to different plant extracts has been documented in the literature (Alonso-paz el al.,1995; Lemos et al., 1992; Martinez et al.,1994; Nascimento et al., 1990; Saxena et al., 1994).

For bacterial strains, also the intracellular fraction of the mexotrophic *Arabidopsis thaliana* elicited with different elicitors was the most active one followed by the cell wall bound fraction of the same plant and the intracellular fraction of *Nicotiana tobacum* (BY2). Using chloromephenicol as a positive antibacterial control, this antibiotic showed increased activity only against *Staphylococcus aureus* at different concentrations, but it inhibited *Escherichia coli* cells only at high concentrations and did not show any antibacterial activity against both *Pseudomonas aeruginosa* and *Bacillus subtilis* even at very high concentration (100ug/ml). So the antibacterial activity of som elicited fractions like *Arabidopsis thaliana* intracellular fraction elicited with Mej, DC3000, PRM1, ASA or SA was higher , against all tester bacterial strains than chloromephenicol. The antimicrobial properties of plants have been investigated by a number of researchers world wide, especially in Latin America. In Argentina, 122 known plant species were tested for therapeutic treatments (Anesini and Perez, 1993). It was documented that among the compounds extracted from these plants, twelve inhibited the growth of *Staphylococcus aureus* and ten inhibited *Escherichia coli*. A more detailed study on antimicrobial compounds was done evaluating extracts from 120 plant species from 28 different families (Santos Filho et al., 1990). It was documented that 81 extracts obtained from 58 plants were active against *S. aureus* and five extracts from four other plants inhibited the growth of *P. aeruginosa*.

Another study (Lemos et al., 1992) detected the antibacterial and antifungal (*C. albicans*) activities of essential oils obtained from *Croton triangularis* leaves. The antimicrobial activity from *Mikania triangularis* was tested against five genera of bacteria and three genera of yeast, and results showed that it had activity against *S. aureus, E.coli, P. aeruginosa* and *B. cereus* (Cruz et al.1996). Since few studies considering the production of antimicrobial proteins by plant cell cultures, the protein content for different elicited plant cell cultures, in the present study, was measured as an indicator for induction of antimicrobial protins or peptides. Among the screened plant cell cultures, the intracellular fraction of the mixotrophic *Arabidopsis thaliana* culture elicited with different elicitors showed the highest antimicrobial activity coincided with high protein content which may be responsible for the high antimicrobial activity of the fraction. Fraction elicited with SA have been selected for testing the presence of antimicrobial protein(s) and further work.

3.2. Isolation and characterization of an antimicrobial protein (AtPDP1)

Intracellular fraction of *Arabidopsis thaliana* elicited with different elicitors was the most active fraction comparable to other plants fractions elicited with the same elicitors, and the fraction elicited with SA showed the strongest and more intense inhibition zones against most of tester isolates especially *Candida maltosa*. So this fraction was selected for further experimentation for testing the presence of antimicrobial proteins responsible for the inhibition zones. Ammonium sulphate precipitation of proteins in this fraction indicated the presence of antimicrobial proteins responsible for the antimicrobial activity since the precipitated proteins were active against *C. maltosa*. The isolation of antimicrobial proteins or peptides from plant materials have been reported by many workers (Verburg and Khai Huynh, 1991; Khai Huynh et al., 1996; Yasnetskaya et al., 2003; Last and Llewellyn., 1997; Khai Huynh et al., 1992; Feng et al.,

2003; Chen et al., 2002; Lee et al., 1999; Tailor et al., 1997; Diz et al., 2003; Segura et al., 1998).

High speed centrifugation was selected instead of ammonium sulphate precipitation since it was more efficient for isolation of the antimicrobial activity. By testing the identity of protein profiles for both methods they were identical but the intensity of protein bands were more strong for high speed centrifugation isolation method than those for the ammonium sulphate precipitation. This indicated the insolubility of the active protein in the usual buffers, since it may be bound to other cellular components like different cellular membranes. Wang et al., (1996) have isolated an *Arabidopsis thaliana* gene that codes for a receptor related to antifungal pathogenesis related (PR) proteins. The PR5K gene codes for a predicted 665-amino acid polypeptide that comprises an extracellular domain related to PR5 proteins, a central transmembrane-spanning domain and an intracellular protein-serine/theronine kinase. The structural similarity between the extracellular domain of PR5k and the antimicrobial PR5-proteins suggested a possible interaction with common or related microbial targets.

Using bioautography method, the proteinsous nature of the antimicrobial compound was confirmed and its molecular weight could be estimated to be between 26 and 36 kDa. This was confirmed also by gel filtration since after calibration of the used column with 5 different standards, it could be isolated the active protein as a single band of 30 kDa molecular weight. Protein band was subjected to Mass Spectrometry after digestion with trypsin into small peptides. Spectrum for a peptide with 12-amino acids was compared with protein database [NCBInr database using the Mascot algorithm (Version 2.1; MatrixScience, London, GB)], and from the search it could be found that the active protein is similar to an unknown protein, (shows 100% identity to dehydration-stress indused protein from *Arabidopsis thaliana*), with a plant-stress induced PLAT (Polysystin- Lypoxygenase- Alpha toxin- Triacyal glycerol lipase domain). So the isolated protein will be referred to as Arabidopsis

thaliana Plat-Domain-Protein 1 (AtPDP1). The role of some pathogenesis-related (PR) proteins as stress proteins with functions that exceed their involvement in plant-pathogen interaction have been described by other workers. It has been reported that thaumatin-like PR5 family is expressed not only in response to pathogens, but also during osmotic stress. Thus, osmotin (tobacco PR-5c) and its homology (NP24 PR5a) in tomato were independently identified as being induced by salt stress (Singh et al., 1987) and infection (Stintzi et al., 1991). Also lipid transfer proteins can be induced by infection, have antifungal activity, and are expressed during drought stress (Kader, 1997) so, AtPDP1 (with 100% identity to dehydration-stress-induced protein from the same plant) may be similar to thaumatins and lipid transfer proteins from other plants, since it has antimicrobial activity.

By testing the antimicrobial activity of the AtPDP1 against the five tester isolates, it was active against all of them with different magnitude, since the highest activity was against *C. maltosa* followed by the Gram negative (*E. coli* and *P. aerryginosa*) and the least activity against Gram positive ones (*S. aureus* and *B. subtilis*).

Using Blast-Aligorithms program (Altschul et al., 1997) the isolated AtPDP1 showed different homology levels to other proteins from other organisms as shown in Table (6.2), the highest homology level (100% identity, 100% similarity) was to dehydration- stress- induced protein from *Arabidopsis thaliana* followed by an unknown protein (80% identity, 91% similarity) from the same plant [will be referred to as AtPDP2] and Lipoxygenase, LH2 (70% identity, 82% similarity) from *Medicago truncatula*. Lower homology level to other proteins from other organisms was found; elicitor inducible protein EIG-J7 (67% identity, 79% similarity) from *Capsicum annuum*, wound/stress protein (66% identity, 77% similarity) from *Lycopersicon esculentum*, TMV- induced protein (48% identity, 69% similarity) from *Capsicum annuum*, Elicitor inducible protein (55% identity, 68% similarity) from *Nicotaina tabacum* and TMV-induced protein 1-2 (53% identity, 71% similarity) from *Capsicum annuum*. All of

these proteins have the same PLAT- plant stress domain (PLAT/LH2 domain) and using Clustal X program (Thompson et al., 1997) it could be observed from the alignment that this PLAT-domain is the conserved region in all of these proteins. In general PLAT/LH2 domain consists of eight stranded beta-barrel and its proposed function is to mediate interaction with lipids or membrane bound proteins and it termed PLAT/ LH2 after (Polycystin-1, Lipoxygenase, Alpha toxin and Triacylglycerol lipase)/ (Lipoxygenase homology 2). The three dimensional structure of the PLAT domain of human pancreatic lipase (Van Tilbeurgh et al, 1993), rabbit-15 Lipoxygenase (Gillmor et al., 1997) and alpha toxin from *Clostridium perfringes* (Naylor et al., 1998) is characterized. Leyen et al. (1998) demonstrated a new structural fraction of 15-Lipoxygenase with PLAT domain since they found that 15-Lox integrates into membrane of various organelles and permeabilizes them by forming pores of 7-17 nm which are sufficient to release proteins with relative molecular masses as large as 400 kDa. This activity of 15-Lox is reminiscent of the ability of several bacterial toxins to form pores in membranes of eukaryotes. They also proposed that 15-Lox initiates organelle degradation by pore formation in all cellular organelle membranes, allowing their subsequent degradation. The same also was observed (May et al.,2000) for lipid body lipoxygenase (LBLOX) from cucumber seedlings, since they reported that a few isoforms of LOX bind or integrate into membranes of various organelles and in such case the function of LOX may be directed towards modification of the respective membrane. In case of LBLOX, both phospholipid monolayer and the bulk of triacylglycerols of lipid body structures were modified in the fat degrading cotyledons of cucumber, and they also found that LBLOX contains a ß-barrel-forming N-terminal domain (PLAT domain) which represents a membrane binding domain that is functionally comparable with the C2 domain of mammalian phospholipases. Within this domain a unique arrangement of glutamyl residues which could be used for co-ordination of Ca^{2+}, and this indicates a relationship between the LBLOX β-barrel and the members of Ca^{2+}

dependent membrane-binding domains. For Alpha toxin, the protein composed of 2 domain; the N-terminal domain contains the phospholipase C active site and the C-terminal domain which is putative phospholipid recognition domain (the PLAT domain) which shows structural homology with phospholipids-binding C2-like domain from a renge of eukaryotic proteins. Jepson and Titball, (2000) found that the ability to bind membrane phospholipids in Ca^{2+} dependent manner and toxicity is conferred by this PLAT domain (C-terminal domain) which also contributes to the sphingomyelinase activity. It has been reported (Lichtenheld et al., 1988; Liu et al., 1995) that a protein with 70 kDa found in secretory granules of cytotoxic T cells called perforin binds phosphorylcholine in Ca^{2+} dependent manner and inserts into cellular membranes to form pores that result in lysis of target cells. This protein has C2 domain similar to PLAT domain, which may be responsible for activity.

In plants, the PLAT domain may interact directly with membrane in Ca^{2+} dependent manner or the PLAT domain may be involved in protein-protein and/or protein-lipid interaction (Bateman and Sanford., 1998)

An ethylene-induced cDNA encodes a lipase that contains the PLAT domain was expressed at the onset of senescence in *Dianthus caryophyllus* (Hong et al.,2000), since there is evidence that the hydrolytic release of free linoleic and linolenic acids from membrane phospholipids results in enhanced lipoxygenase activity and an accumulation of peroxidized lipids in bilayers, leading to further destabilization of membrane structure that terminates by loss of cell function.

Shin et al., (2004) reported that overexpression of CaTin1 [*Capsicum annuum* tobacco mosaic virus (TMV)-induced clone1] (contains the PLAT domain) in tobacco plants in sense- or antisense orientation showed interesting characteristics such as the accelerated growth and the enhanced resistance to biotic (virus infection) as well as abiotic (drought and salt treatments) stresses and they suggested that this tolerance may caused by the elevated level of ethylene and H2O2 in transgenic plants,

but they did not refer to testing the antimicrobial action for the protein product of the gene. Thus the function of the isolated AtPDP1, isolated in the current work, may be the defense against some pathogens, tolerance against abiotic stress like osmotic stress and drought and it may be also responsible for protein-protein and/or protein lipid interaction in the plant.

The suggested action of the isolated AtPDP1 on tester isolates is by binding of the PLAT domain to the membrane of target cell and forming pores in these membrane which leads to leakage of the cellular contents and subsequent cell death. Using ProtParam program (www.expasy.org) it has been found that the protein is acidic (*p*I=4.97) but contains both acidic residues [(Asp+Glu)=22] and basic residues [(Arg+Lys)=16]. The basic residues are able to bind to the negatively charged phospholipids head groups of target cell leading to neutralization of net charge of membrane that leads to membrane fusion and aggregation. Chapman and Davis (1998) reported that the positively charged residues of synaptotagmin (a Ca^{2+} binding synaptic vesicle protein essential for rapid release of neurotransmitters) may mediate interaction with nearby anionic lipid head groups. In addition an NMR study of C2A of the compound demonstrated that Arg-233 and Lys-236 in loop 3, along with Arg-199 in loop 2, made contacts with a recombinant fragment of Syntaxin 1A (an essential component of the putative membrane fusion machinery) corresponding to residues 1-177.

Also the residues (Asp and Glu) are able to bind to negatively charged phospholipids and neutralize their charge but in presence of Ca^{2+} since Ca^{2+} bind first to the phospholipids and then to the negatively charged residues in the PLAT domain. In case of perforin, it is able to bind to membranes of target cell in a Ca^{2+} -dependent manner via its C2 –domain Once bound, perforin inserts into membranes where it polymerizes to form large pores (Chapman and Davis, 1998).

Using PROSITE program (Hofmann et al., 1999) it has been observed that the calculated molecular weight for the isolated AtPDP1 is about 20.005 kDa which is different from that observed during purification

steps of the protein (about 30 KDa). This difference may be due to the presence of glycosylation sites (NLTS) in the protein which may be responsible for post-translation modifications of the protein or this difference may be due to binding of protein to different cellular membranes in the plant cell.

3.3. Cloning of genes for AtPDP1 and AtPDP2 and their expression pattern under different stimuli (Digital Northern)

Gene for the active isolated AtPDP1 (At4g39730) and also for AtPDP2 (At2g22170) were isolated using RT-PCR using RNA samples isolated from *Arabidopsis* materials treated with SA. PCR products were 546 and 552bp respectively. PCR products were cloned in DH5α *E. coli* cells using PGEM-T easy vector and sequenced.

Further molecular biology work for cloning the two genes in *Agrobacterium tumefaciens* using PBI121 plasmid for overexpression of the two genes in *Arabidopsis thaliana* cell cultures under the control of both constitutive (*Ca*MV 35S) or inducible (*win 3.12T*) promoter was tried but we did not succeeded to get transformants of *E. coli* cells with the new PBI121 plasmid. Work should be continued on this point to maximize the productivity of plant cells for the active protein, and the overexpression also will aid to understanding the function of the considered protein(s) in the plant.

The expression of the gene for the isolated AtPDP1 (At4g39730) and also the expression for a second gene, for AtPDP2, (At2g22170) was tested using Digital Northern (www.genevestigator.ethz.ch). From results it could be noticed that both genes are constitutively expressed in *Arabidopsis* tissues, but the first gene (for AtPDP1) was found to be expressed mainly in plant shoot system more than roots and the reverse was observed in case of the second gene since it was expressed mainly in roots. Shin et al (2004) reported that CaTin1 (*Capsicum annuum* tobacco mosaic virus (TMV)-induced clone 1), with the same PLAT domain, is expressed only in roots and not in other parts of the plant. Both genes

were induced by different pathogens which reflect their role in defense against different pathogens like different other PR (Pathogenesis-related) proteins.
Also both genes were found to be induced by different chemicals and hormones which are known to induce defense and induction of different PR proteins in plants. PR proteins have been described to be induced by numerous conditions and chemical compounds, including senescence, callus culture, UV, light, wounding, plasmolysis, polyacrylic acid, Auxin, cytokinin, heavy metal salts, mannitol, amino acids, thiamine, arachidonic acid, ozone, hydrogen peroxide, etc (Van Loon, 1983; Kessmann et al., 1994; Ryals et al., 1996).
For At4g 39730 gene, the maximum induction was by pathogens followed by the effect of SAR (Systemic Acquired Resistance), ABA and *P. syringae* (avr). For At2g22170 gene, the maximum induction was by mycotoxins followed by zeatin, GA3 and ABA. High expression of both genes under different selected stimuli support their suggested roles in defense of *Arabidopsis* against different pathogens, and for At4g 39730 (gene for AtPDP1) the results of digital northern agree to high extent with results of screening of the intracellular fraction elicited with different elicitors, which support the antimicrobial activity of the isolated protein.

3.4. Cloning and expression of thaumatin genes from *Arabidopsis thaliana*

Most of thaumatins play role in defense of Arabidopsis against invading microorganisms and also some of them have antimicrobial activity against *Candida* spp, so they are further candidates for antimicrobial genes. Thirteen thaumatin genes were isolated using PCR using genomic DNA, as template, isolated from *A. thaliana* materials. PCR products were ligated in PGEM-Teasy vector and cloned in DH5α *E. coli* cells. Additional future work will be carried out in the lab which includes

testing the expression of different cloned thaumatin genes under different stimuli.

The expression of the thirteen cloned thaumatin genes was tested using Digital Northern (www.genevestigator.ethz.ch), and from the results (Table 9.2) it could be noticed that different thaumatin genes show different expressions under different stimuli. The maximum expression was for At1g75800 gene in presence of SA, Mej, DC3000, imbibition of water (seeds), ABA and GA_3, followed by At4g36010 and At4g 38660. Hu and Reddy, 1997 reported that a thaumatin like protein from Arabidopsis (ATLP-3) is induced by pathogen infection and by compounds that are known to elicit systemic aquired resistance like DC3000 and SA. The minimum expression of genes was for At1g75050, At4g24180 and At2g17860. The rest of thaumatin genes showed moderate expression under different stimuli. The variation in the expression of genes under different stimuli represents different roles of these genes against invading microorganisms and suggests that At1g75800, At4g36010 and At4g38660 play an essential role for defense of Arabidopsis against pathogens. The objective of this study is to select the most active genes against pathogenic microorganisms especially fungi since many authors have reported that most members of thaumatins have antifungal activity against broad spectrum of fungal pathogens (Huynh et al., 1992; Vigers et al., 1991, 1992; Woloshuk et al., 1991).

4. Materials and Methods

4.1 Materials

4.1.1 Suspension cell cultures

For antimicrobial screening, all cultures were elicited after 2 or 10 days incubation period (for heterotrophic and autotrophic cultures respectively) and harvested after another 2 days incubation period.

Arabidopsis thaliana

The mixotrophic suspension culture of *Arabidopsis thaliana* was used for different biochemical experiments in the study. The cell line was maintained by transferring 10ml of suspension culture to 50ml of B5 medium at interval of one week. The cell cultures were maintained by shaking at 26^{o}C in light. For antimicrobial screening, the subculture was done by transferring 10ml of one week old cultures to 50ml of the fresh B5 medium.

Chenopodium rubrum

The autotrophic suspension culture of *Chenopodium rubrum* was used for different biochemical experiments in the study. The cell line was maintained by transferring 12 ml of suspension culture to 16 ml of MS medium at intervals of 2 weeks. The cultures were maintained by shaking at 26^{o}C in light. For antimicrobial screening, the subculture was done by transferring 12 ml of 2 week old cultures to 16 ml of fresh MS medium .

Eschscholzia californica (AST)

The heterotrophic suspension culture of *Eschscholzia californica* (AST) was used for different biochemical experiments in the study. The cell line was kind gift from Prof Mueller (Pharmaceutical Biology,university

of wuerzburg). The cell line was maintained by transferring 20 ml of suspension cultures to 50 ml of LS medium at the interval of one week. The cell cultures were maintained by shaking at 26^{o}C in light. For the antimicrobial screening, the subculture was done by transferring of 20 ml of one week old cultures to 50 ml of fresh LS medium.

Eschscholzia californica (ECO)

The heterotrophic suspension culture of *Eschscholzia californica* (ECO) was used for different biochemical experiments in the study. The cell culture was kind gift from Prof Mueller (Pharmaceutical Biology, university of wuerzburg), and cell line was obtained by incubation of pant calli in 40 ml of LS medium for 2 weeks with continues shaking in light, then cell were transferred to 50 ml of LS medium and subcultured every one week by transferring of 10 ml of suspension culture to 50 ml of fresh LS medium. For antimicrobial screening the subculture was done by transferring of 10 ml of one week old culture to 50 ml of LS medium.

Gypsophila arabia

The heterotrophic suspension culture of *Gypsophila arabia* was used for different biochemical experiments in the study. The cell culture was kind gift from Prof Mueller (Pharmaceutical Biology, university of wuerzburg), and cell line was obtained by incubation of plant calli in 40 ml of LS medium for 2 weeks with continues shaking in light, then cells were transferred to 50 ml of LS medium and subcultured every one week by transferring of 10 ml of suspension culture to 50 ml of fresh LS medium. For antimicrobial screening, the subculture was done by transferring of 10 ml of one week old culture to 50 ml of LS medium.

Lycopersicon esculentum

The autotrophic suspension culture of *Lycopersicon esculentum* was used for different biochemical experiments in this study. The cell line was maintained by transferring 15 ml of suspension culture to 15 ml of MST medium at intervals of 2 weeks. The cell cultures were maintained by shaking at 26^{o}C in light. For antimicrobial screening the subculture was done by transferring 15 ml of 2 weeks old cultures to 15 ml of fresh MST medium.

Lycopersicon esculentum

The heterotrophic suspension culture of *Lycopersicon esculentum* was used for different biochemical experiments in the study. The cell line was maintained by transferring 10 ml of suspension cultures to 50 ml of C3 medium at the intervals of one week. The cell cultures were maintained by shaking at 26^{o}C in light. For the antimicrobial screening, the subculture was done by transferring of 10 ml of one week old culture to 50 ml of fresh C3 medium.

Nicotiana plumbaginifolia

The heterotrophic suspension culture of *Nicotiana plumbaginifolia* was used for different biochemical experiments in the study. The cell culture was kind gift from Prof Mueller (Pharmaceutical Biology, university of wuerzburg). The cell line was maintained by transferring 10 ml of suspension cultures to 50 ml of LS medium at intervals of one week. The cell cultures were maintained by shaking at 26^{o}C in light. For the antimicrobial screening, the subculture was done by transferring of 10ml of one week old cultures to 50ml of the fresh LS medium.

Nicotiana tobacum

The heterotrophic suspension culture of *Nicotiana tobacum* was used for different biochemical experiments in the study. The cell line was maintained by transferring 5 ml of suspension cultures to 50 ml of BY2 medium at intervals of one week. The cell cultures were maintained by shaking at 26^{o}C in light. For the antimicrobial screening, the subculture was done by transferring of 5ml of one week old cultures to 50ml of the fresh BY2 medium.

Petroselium crispum

The heterotrophic suspension culture of *Petroselium crispum* was used for different biochemical experiments in the study. The cell line was maintained by transferring 10 ml of suspension cultures to 50 ml of B5 medium at intervals of one week. The cell cultures were maintained by shaking at 26^{o}C in light. For the antimicrobial screening, the subculture was done by transferring of 10ml of one week old cultures to 50ml of the fresh B5 medium.

4.1.2 Tester isolates

Candida maltosa

The isolate *Candida maltosa* (SBUG 700) was used as a fungal tester isolate in the screening experiments. The culture was maintained on LB agar medium (PH7) at 30^{o}C incubation temperature, and subcultured every one month and stored at 4^{o}C. For antimicrobial screening experiments, *C. maltosa* was subcultured overnight in LB medium, then

diluted fungal cell suspension of OD_{600} =0.2 was used for inoculating the screening plates.

Staphylococcus aureus

The isolate *Staphylococcus aureus* (ATCC 6538) was used as a gram positive bacterial tester isolate in the screening experiments. The culture was maintained on LB agar medium (PH7) at 37°C incubation temperature, and subcultured every two weeks and stored at 4°C. For antimicrobial screening experiments, *S. aurus* was subcultured overnight in LB medium, then diluted bacterial cell suspension of OD_{600} =0.2 was used for inoculating the screening plates.

Escherichia coli

The isolate *Escherichia coli* (ATCC 11229) was used as a gram negative bacterial tester isolate in the screening experiments. The culture was maintained on LB agar medium (PH7) at 37°C incubation temperature, and subcultured every two weeks and stored at 4°C. For antimicrobial screening experiments, *E. coli* was subcultured overnight in LB medium, then diluted bacterial cell suspension of OD_{600} =0.2 was used for inoculating the screening plates.

Bacillus subtilis

The isolate *Bacillus subtilis* (ATCC 6051) was used as a gram positive bacterial tester isolate in the screening experiments. The culture was maintained on LB agar medium (PH7) at 37°C incubation temperature, and subcultured every two weeks and stored at 4°C. For antimicrobial screening experiments, *B. subtilis* was subcultured overnight

in LB medium, then diluted bacterial cell suspension of OD_{600} =0.2 was used for inoculating the screening plates.

Pseudomonas aeruginosa

The isolate *Pseudomonas aeruginosa* (ATCC 22853) was used as a gram negative bacterial tester isolate in the screening experiments. The culture was maintained on LB agar medium (PH7) at 37°C incubation temperature, and subcultured every two weeks and stored at 4°C. For antimicrobial screening experiments, *P. aeruginosa* was subcultured overnight in LB medium, then diluted bacterial cell suspension of OD_{600} =0.2 was used for inoculating the screening plates.

4.1.3 Medium

BY2 Medium (for one liter)	
MS-Medium	4.3g
MES buffer	2g
Inosit	100mg
Sucrose	30g
KH_2PO_4	200mg
Thiamin	1ml
1mg/ml 2,4 D in ethylalcohol	200ul

PH was adjusted to 5.8 with conc. KOH

C3 Medium (for one liter)	
MSIT-Medium	4.41g
KH_2PO_4	200mg
1mg/ml 2,4 D in ethylalcohol	100ul
0.40mg/ml Kinetin in dH_2O	50ul
Sucrose	30g

PH was adjusted to 6 with 2% KOH

MST Medium (for one liter)	
Murashige and Skoog Medium (with Vitamins)	4.41g
0.2mg/ml 2,4 D in ethylalcohol	100ul

PH was adjusted to 5.7 with 1N NaOH

LS Medium (for one liter)	
Linsmaier, Bednar& Skoog Medium	4.60g
Sucrose	30g

PH was adjusted to 6 with conc. KOH

MS Medium (for one liter)	
MS-Medium	4.30g

PH was adjusted to 5.7-5.8 with <1N NaOH

B5 Medium (for one liter)	
Gambory's B5 Basal Medium with animal organics	3.20g
1mg/ml 2,4 D in ethyl alcohol	1ml
Sucrose	20g

PH was adjusted to 5.5 with <1N NaOH

LB Medium*	
Bacto- Tryptone	1%
Yeast- Extract	0.5%
NaCl	1%

PH was adjusted to 7.0 with<1N NaOH

*1.5% agar added for solid medium.

*100ug/ml Ampecillin for plating.

4.1.4 Buffers

Autotrophic cultures

Potassium carbonate buffer (For 1 liter)	
K_2CO_3	55.3g
$KHCO_3$	160.2g

Mini Preps

Resuspension buffer	
Glucose	50mM
Tris-HCL pH 8	25mM
EDTA	10mM

Lysis buffer *	
NaOH	0.2N
SDS	1%

* Freshly prepared

Neutralization buffer (For 100ml)	
5M KAc	60ml
Glacial AcOH	11.5ml
Water	28.5ml

Agarose gel electrophoresis

5X TBE-buffer	
Tris HCl pH 8.0	890mM
H_3BO_4	890mM
EDTA	20mM

10X DNA loading buffer	
Glycerin	50%
EDTA	100mM
Bromophenol blue	0.25%
Xylen Cyanol	0.15%

RNA Isolation

RNA-denaturing solution	For 50ml
Guanidium – thiocyanat 4M	23.66ml
(Tri)-Na-citrate 25mM	0.37ml
Sarcosyl (N-Lauryl Sarcosine) (0.5%)	0.25ml
Add 7ul of β-ME freshly before use/ml	

Na-Acetate solution	
Na-Acetate PH= 4	2M

CIA	
Chloroform/Isoamylalcohol	24:1

5X RNA gel running buffer	
MOPS	41.86g/L
NaAc	6.8g/L
NaOH	7 pellets/L
EDTA	10ml/L
DEPC	1ml/L

RNA Loading dye	
5X RNA-loading buffer	76ul
Formaldehyde	126ul
Formamide	378ul
10X DNA-loading dye	76ul
Ethidium bromide (5mg/ml)	5ul

Formaldehyde gel	
Agarose	1.2g
Water (heat to dissolve agarose)	62.2ml
5X RNA running buffer	20ml
37% Formaldehyde	17.8ml

DNA Isolation

Extraction buffer	
Tris-HCL pH 8	100mM
EDTA	50mM
Nacl	500mM
SDS	1.5%
β-ME freshly before use	1%

SDS-PAGE

Separating gel		
	12%	15%
Acrylamide/Bis (30:0.8)	2.5ml	3ml
1.5M Tris pH 8.8/0.4% SDS	1.5ml	1.5ml
Water	2ml	1.5ml
10% APS	60ul	60ul
TEMED	10ul	10ul

Stacking gel	
Acrylamide/Bis (30:0.8)	400ul
1M Tris pH 6.8/0.4% SDS	750ul
Water	1.85ml
10% APS	25ul
TEMED	7.5ul

Running buffer	
Tris	25mM
Glycin	192mM
SDS	0.1%

10X SDS loading dye	
Tris-HCl pH 6.8	160mM
Glycerin	28%
SDS	3%
DTT	10mM

Gel Staining with Commassie brilliant blue

Commassie brilliant blue	
Commassie blue R 250	0.25%
AcOH	10%
MeOH	50%

Destaining solution(100ml)	
AcOH	10ml
MeOH	45ml
Water	45ml

Bradford-Protein estimation

Bradford Reagent (6X)	
Serva blue G	95mg
85% H_3PO_4	100ml

4.2 Methods

4.2.1 Antimicrobial Measurements

4.2.1.1 Treatment with various elicitors:

Control treatment

For control treatments of different plant cell cultures, no addition of any materials or solvents to the control flasks was done in all experiments.

Chitosan treatment

0.5% (w/v) chitosan preparation was made by dissolving in 1M acetic acid and dialyzed for 18 hours. A final concentration of 0.05% was used for experiments.

Methyl jasmonate

500mM stock solution was prepared by mixing methyl jasmonate in ethanol. 1ul/ml of culture medium was the final concentration used in different experiments.

Polygaracturonic acid (PGA)

1% (w/v) polygalacturonic acid preparation was made by dissolving in ddH2O. A final concentration of 0.05% was used for experiments.

Pseudomonas syringae (DC3000)

Pseudomonas syringae elicitor was prepared by boiling a suspension of the virulent strain of *Pseudomonas syringae* (DC3000) of OD600=0.2 for 10 min at 100^{o}C. The boiled suspension was stored at -20C. The elicitor used in concentration of 2ul/ml of culture medium.

Pseudomonas syringae (APRM1)

The same as (DC3000) elicitor but the averulant isolate of *Pseudomonas syringae* (APRM1) was used instead of *P. syringae* (DC3000). The elicitor used in concentration of 2ul/ml of culture medium.

Salicylic acid

500mM stock solution was prepared by dissolving salicylic acid in ddH2O. 1ul/ml of culture medium was the final concentration used in different experiments.

Alternaria brassiciola

Alternaria brassiciola elicitor was prepared by boiling a suspension of *A. brassiciola* conidia with spore concentration of 5X10^{5} (spores/ml) for 10min at 100^{o}C. The boiled spore suspension was stored at -20C. The elicitor used in a concentration of 1ul/ml of culture medium.

E-Fol (Fusarium oxysporium lycopersici)

E-Fol elicitor was prepared by suspending mycelial homogenate of the fungus *Fusarium oxysporium lycopersici* in ddH2O with concentration 1:1000 (w/v). 1ul/ml of culture medium was the used concentration for different experiments.

Acetylsalicylic acid (ASA)

50mM stock solution was prepared by dissolving Acetylsalicylic acid in ddH2O. 1ul/ml of culture medium was the final concentration used in different experiments.

4.2.1.2 Preparation of different fractions for screening

Different plant cell cultures were elicited using different elicitors and cells were collected, after 2 days (for heterotrophic) or 10 days (for

autotrophic) incubation period, by centrifugation and the supernatant was used as the extracellular fraction for testing the antimicrobial activity and protein content. Cells were immediately frozen in liquid nitrogen. 5gm amounts of cells were ground under liquid nitrogen and powder was suspended in 10ml of extraction buffer (10mM Na2HPO4, 15mM NaH2PO4 PH=7) and stirred overnight at 4^{o}C. After centrifugation the supernatant was used as the intracellular fraction for testing the antimicrobial activity and the protein content. Pellets were resuspended in 10ml of another extraction buffer (10mM Na2HPO4, 15mM NaH2PO4, 1M Nacl, 15mM EDTA PH=7) and stirred overnight at 4^{o}C. After centrifugation the pellets were discarded and the supernatant was dialyzed overnight, using dialysis tube of cut off=14 KDa, against 5 liter dialysis buffer (10mM Na2HPO4, 15mM NaH2PO4 PH=7) with 3 times change of buffer during the period of dialysis. The dialyzed fraction was used as the cell wall bound fraction for testing the antimicrobial activity and the protein content.

4.2.1.3 Antimicrobial activity assay

This assay was done according to a method modified from the that described by Vivanco *et al* (1999) as follows: The fungal and bacterial isolates were grown overnight at 30^{o}C (for the fungus) or at 37^{o}C (for the bacterial strains), with shaking, in LB broth medium. All the cultures were diluted to an optical density reading OD_{600}=0.2 and spread on 100mm-plates containing solid LB-agar medium. Sterile Whattman filter paper discs with a diameter of 0.5cm were saturated with different plant extracts and lied on the top of the inoculated plates. The plates were incubated for 24houres at 30^{o}C for the fungal culture and 37^{o}C for the bacterial ones, and inhibition was defined as bacteria- or fungus- free zone surrounding the filter discs after inoculation. Two replicates per treatment were included, and each experiment was done twice. [one time in case of *Eschscholtzia californica* (ECO)].

Two synthetic antibiotics [Nystatin (antifungal) and Chloromephenicol (antibacterial)] were tested dissolved in DMSO and MeOH respectively, at different concentrations, as references to compare their antimicrobial activities with those of plant cell culture fractions.

4.2.2 Molecular Biological Methods

4.2.2.1 DNA-Agarose gel electrophoresis

DNA was electrophorsed in 0.8% to 1.5% agarose gel made in TBE buffer with 3.5ug of ethidium bromide in 100ml total volume. The DNA sample was mixed with appropriate amount of loading buffer and subjected to electrophoresis performed in 1X TBE buffer at a voltage of 5 to 10 V/cm. As a DNA molecular weight marker either λDNA restricted with *Hind*III and *Eco*RI or PUC 19 restricted with HinfI was used.

4.2.2.2 Competent *E. coli* cells

500ul of overnight grown preculture of *E. coli* strain in LB (5ml) were used to inoculate 50ml LB medium. The cells were incubated until the absorbance at OD_{600} reached between 0.5-0.6 units. Cells were harvested at 2500rpm for 10min at 4C and then the pellets were resuspended in 15ml of cold $CaCl_2$ (100mM) and were incubated on ice for 40min. The cells were then harvested at 2500rpm for 5min at 4^{o}C. The pellets were resuspended in 5ml of $CaCl_2$ (100mM) and incubated on ice for 1-3 hours. 1ml of ice cold glycerol (87%) was added to cells and after mixing 200ul of cells were added to ice-cold tube and tubes were snap frozen in liquid N_2. The competent cells are stored at -80 ^{o}C.

4.2.2.3 Extraction of RNA

Total RNA isolation was done according to the following method: For RNA isolation care was taken that all buffers, glassware, pipette tips and

eppendorf tubes were free of any RNAse contamination. The plant cells were harvested by centrifugation and snap frozen in liquid nitrogen. The cells were ground in presence of liquid nitrogen. Total RNA was extracted from the ground material in a precooled eppendorf tube (2 replicates are prepared for each sample). To a 100mg of tissue powder, 500ul RNA denaturing solution, 50ul sodium acetate solution and 500ul acid phenol was added. The eppendorf tubes were immediately vortexed until the cells were completely thawed. Cells were incubated at RT for next 10min. The upper phase was taken out carefully in a new eppendorf tube following centrifugation at 13,000 rpm for 10min. The upper phase is once again extracted with 500ul of CIA and centrifugation at 10,000 rpm for 5min. To the upper phase, 500ul of isopropanol was added and incubated overnight for RNA precipitation in -20^{o}C. RNA was precipitated after centrifugation at 13,000 rpm for 30min at 4^{o}C. The supernatant was discarded and pellet was washed with 500ul of 85% ethanol. The pellets were dried at 37 ^{o}C for 10min. The RNA pellets were resuspended in DEPC treated water by shaking at 66 ^{o}C for 10min. The RNA were pooled from the replicate samples and the concentration was determined by measuring the absorbance in DNA/RNA calculator from Pharmacia (1 OD = 40ug ml^{-1} of RNA).

4.2.2.4 Extraction of genomic DNA

100 mg of homogenized plant material were mixed with 1ml of extraction buffer with vortex till all the material was suspended in the solution. The suspension then is incubated at 65 ^{o}C for 10min, then 2ul of KAc (5M) is added to the mixture. Then tubes on ice for 10-60min. After centrifugation at 14000rpm for 10min at 4 ^{o}C the liquid phase in every tube is mixed with phenol, Chloroform and Isoamylalchol (400ul:400ul:7ul per sample) with mixing followed by centrifugation for 5min at room temperature. Upper phase is mixed with 500ul isopropanol and incubated at -20 ^{o}C for overnight. DNA is precipitated by centrifugation for 10min at

14000rpm at 4 °C. Pellets are washed with 70% ethanol and dried at room temperature. DNA is then suspended in 50ul Tris buffer containing RNase (100:1 v/v) and incubated at 37 °C for 1 hour to obtain RNA-free DNA.

4.2.2.5 Polymerase chain reaction

Polymerase chain reaction was carried out in a volume of 50ul. 5ul of complementary DNA (cDNA) was used as template for each PCR reaction along with, 10uM of primer solutions (5ul of forward and reverse primers), 1ul of 10mM dNTPs, 1ul (1U) of Taq polymerase and 5ul of 10x PCR buffer. The volume was completed to 50ul by addition of 28ul of ddH2O. The PCR reaction was carried out with a 3min of denaturation at 94°C for one cycle, followed by 40 cycles of 30sec of denaturation at 94°C, 30sec of annealing at 57°C or 59°C (see the results section) and extension of 1min at 72°C. A final 10min extension cycle was carried at 72°C prior to halting the reaction at 4°C. All the reaction was loaded on agarose gel for further work.

PCR for proteinase inhibitor-like gene (win 3.12) was carried out using 5ul of genomic DNA (250ng) from popular plant as template along with, 10uM of primer solutions (6ul of forward and reverse primers), 1.7ul of 10mM dNTPs, 2ul (2U) of Taq polymerase, 5ul of 10x PCR buffer and 5ul of 5mM Mgcl. The volume was completed to 50ul by addition of 19.3ul of ddH2O.genomic. The same program mentioned above was used but the used annealing temperature was 55 °C.

PCRs for thaumatin genes were carried out using genomic DNA (250ng) from *Arabidopsis thaliana* materials using the same reaction mixture as for win 3.12 gene. The same program mentioned above but with different annealing temperatures for every gene, ranging from 45 to 60 °C for every gene was used, then another PCR was carried out for every thaumatin gene at the optimal annealing temperature.

4.2.2.6 Reverse Transcription-PCR

Reverse transcription was carried out using a kit for first strand cDNA synthesis from Fermentas (RevertAid™) on total RNA. 2ug RNA was used for reverse transcription. To the RNA, 2ul of antisense primer (1:10 diluted) was added and volume was adjusted up to 12ul with ddH2O. The mixture was incubated at 70 ^{o}C for 5min and immediately transferred on ice. To this, 4ul of 5x 1st strand buffer, 2ul of 10mM dNTPs and 1ul of Ribonuclease inhibitor was added and incubated after mixing for 5min at 37^{o}C. The reverse transcription reaction was then carried out at 42^{o}C for 60min after adding 1ul of reverse transcriptase (200U/ul). Then the reaction was incubated at 70 ^{o}C for 10min then immediately on ice to stop the reaction. cDNA formed was used for regular PCR using 5ul of the product.

4.2.2.7 Elution and purification of DNA fragments

The DNA band from 1% agarose gel was cut out under UV light and DNA was eluted and purified using a NucleoSpin Extraction Kit (Clontech). 500ul of NT buffer was added to the cut piece and agarose was melted at 65^{o}C for 10min. Solution was added to purification column and column was centrifuged at 11000g for 1min, and the eluted solution was discarded. 600ul of NT3 buffer was added to the column to wash the membrane followed by centrifugation of the column at 11000g for 1min. The membrane was dried by centrifugation of empty column for 2min at 11000g. For elution of the purified DNA from the column, 20-25ul of NE buffer was added to the membrane and after incubation for 1min at room temperature, the column was centrifuged for 1min at 11000g, the eluted buffer contains the purified DNA. 5ul of purified DNA was loaded on agarose gel for estimating DNA amount.

4.2.2.8 Ligation

Ligation was carried out in pGEM-T Easy Vector using pGEM-T Easy Vector kit from Promega. Ligation mixture consisted of 1ul (50ng) of vector DNA, 1ul of purified DNA, 5ul of 2x ligation buffer, 1ul of T4 ligase and 2ul of water, in total volume of 10ul. Ligation reaction was run at 21^{o}C for 2-3houres or overnight at 14^{o}C.

4.2.2.9 Transformation of *E. coli* cells

The competent cells were thawed on ice, one vial (200 ul) for each transformation was taken. 4ul of ligation mixture were added and cells incubated on ice for 15-20min. The cells were given heat shock at 24^{o}C for 90min then were chilled on ice for another 90min. 800ul of LB medium was added to cells and incubated them at 37^{o}C for 1hour. After centrifugation for 3min at 12000rpm, 870ul of cell suspension were plated on LB medium with specific selection marker and incubated at 37^{o}C for overnight.

4.2.2.10 Mini Prep and restriction analysis

The bacteria were grown in 2-3ml of LB medium with antibiotic (Amp., 100ug/ml) overnight at 37^{o}C. *E. coli* cells were harvested by centrifugation at 12000*g* for 3min. The residual medium was removed and cells were resuspended in 100ul of ice-cold resuspension buffer by vortexing. 200ul of lysis buffer was added on the resuspended cells and incubated on ice for 3min. 150ul of neutralizing buffer was added and again incubated on ice for 3min. Following this the cells were centrifuged at 12000*g* for 5min at 4^{o}C. The supernatant was transferred into a new tube and an equal volume of Phenol: Chloroform was added followed by vortexing. After centrifugation at 12000g for 8min at 4^{o}C, the supernatant was transferred into a fresh tube and double-stranded DNA was

precipitated with 2 volumes (800ul) of ice cold ethanol (100%). Tubes were kept at -20°C for 10-15min, then centrifugation at 12000g for 10min at 4°C. Supernatant was discarded and pellets were washed with 70% ethanol (500ul) and after vortex and centrifugation, ethanol was removed and pellets were dried at 37°C and resuspended in 25ul sterile water. For restriction analysis, 4ul of miniprep DNA was restricted in an end volume of 18ul using 2ul of appropriate buffer, 1ul RNase and 1ul of enzyme (10U). The reaction was incubated at 37°C for 3houres or overnight. The restriction digest was then analyzed by agarose gel electrophoresis.

4.2.2.11 Sequencing

Sequence information was obtained by Sanger-Coulson method using the Thermo sequence fluorescent primer cycle sequencing kit with 7-deaza-dGTP (Amersam Pharmacia Biotech, Freiburg, Germany). Software for the sequencing was Base Imagir 4.0 (L1-COR, MWG-Biotech GmbH, Ebersberg, Germany). To compare the new sequences with already known entries BLAST-Algorithms (Altschul et al. 1997) of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) were used.

4.2.3 Biochemical Methods

4.2.3.1 SDS PAGE (Polyacrylamide gel electrophoresis)

12% or 15% SDS- Polyacrylamide gel were prepared according to Laemmli (1970). SDS loading dye with 1/10 volume of protein sample was boiled at 95°C for 5min and all the sample (50ul) was loaded on gel. Gels were run in 1X running buffer at 20mM for 15min, then at 35mM until the blue band is just leaving the gel. As protein standard, the 14.4KDa protein molecular weight marker (Fermentas) was used.

4.2.3.2 Commassie brilliant blue staining

Gels were immersed in Commassie brilliant blue staining solution for overnight with continues shaking. Gels were destained by using destaing solution with continues changes of the solution till the appearance of protein bands with clear background of the gel.

4.2.3.3 Silver staining

Silver staining of gels was done according to the following meathod: Gels were fixed for 1 hour in 300ml of a fixative solution composed of 50% methanol, 12% glacial acetic acid and 0.5ml of 37% formaldehyde. Gels were pretreated with 300ml of 0.2g/l $Na_2S_2O_3.{}_5H_2O$ after washing with 50% ethyl alcohol 3 times for 20min each. Then gels were washed with water for 20 seconds for 3 times before impregnation in 300ml mixture of 2g/l $AgNO_3$ and 0.75ml/l of 37% acetic acid for another 20min. Gels were developed for (2-10) min in 300ml of a developer solution composed of 60g/l $NaCO_3$, 0.5ml/l, 37% acetic acid and 4mg/l $Na_2S_2O_3.{}_5H_2O$ after brief washing (for 20sec) in water for 2 times. The developer is removed and gels were washed twice with water for 2min each. The staining was stopped by immersing the gels in 300ml of a mixture of 50% methanol and 12% acetic acid. Gels were washed for 20min in 50% methanol then with 30% methanol solution for 30min and finally with 4% glycerin solution for 30min.

4.2.3.4 Bioautography

Bioautograghy method was performed according to the method of Vigers *et al* (1991) as follows: SDS-PAGE was performed following the method of Laemmli (1970) using 12% polacrylamide gel (100X140X0.75mm) with 5% stacking gel. Samples were boiled for 5min

in sample buffer (15% (w/v) sucrose, 2.5 (w/v) SDS, 125mM Tris, PH=6.8). Electrophoresis was done for 90min at room temperature at 35mA. The resulting gel was then incubated, with shaking, in 250ml of 1% (v/v) Triton X-100 in water for 20min at room temperature. After brief washing in water, gel was incubated in 100ml of 20% LB broth medium and gently shaken for 30min at room temperature. Finally, gel was placed in 100mm-diameter Petri dish and 20ml of warm agar (1.5% (w/v) agar, 20% LB medium and 4-6X10^5 *Candida maltosa* cells per milliliter of final concentration) was poured over the gel and allowed to solidify. After overnight incubation at 30^oC, the position of antimicrobial (antifungal) activity on the gel was detected as a clear band of growth inhibition against a background of fungal growth. For calculating the molecular weight value for the compound (protein) responsible for the antimicrobial activity, prestained MW marker was run in one lane of the gel, and in a second lane, 50ul of crude plant extract was also run simultaneously. After electrophoresis, these 2 lanes were cut and stained with Coomassie Brilliant Blue R-250 (overnight). By comparing, after destaining, the stained part of the gel with the gel in Petri dish, the band from the plant extract responsible for the activity can be detected, and also in presence of MW marker, the molecular weight of the active protein can be determined.

4.2.3.5 Purification of antimicrobial activity

Ammonium sulphate precipitation

5ml of intracellular fraction of Arabidopsis were treated with 4 gm of ammonium sulphate (80% saturation). Salt was added gradually with continuous stirring at 4^oC. The precipitate was collected at 14000rpm after keeping at 4^oC for overnight and it was resuspended in 1ml of Na-phosphate buffer (10mM Na_2HPO_4, 15mM NaH_2PO_4 PH=7) containing 2mM PMSF and 1mM Benzamidine hydrochloride as proteinase inhibitors and

dialyzed extensively against the same Na-phosphate buffer. The dialyzed suspension was centrifuged at 14000rpm for 20min and the pellets ware dissolved in 1m of 8M urea solution. Supernatant and urea solutions ware used for testing the antimicrobial activity of the intracellular proteins from mixotrophic Arabidopsis culture against the tester isolates.

High speed centrifugation

30 gm of Arabidopsis materials were extracted with Na-phosphate buffer (10mM Na_2HPO_4, 15mM NaH_2PO_4 PH=7) containing 2mM PMSF and 1mM Benzamidine hydrochloride for overnight at 4^oC with shaking. After low speed centrifugation, the supernatant was exposed to high speed centrifugation (14000rpm) for 20min and pellet was dissolved in 3ml of 8M urea. Both the supernatant and urea-extract were used for testing the presence of antimicrobial activity.

Gel filtration

Calibration of Sephacryl S-100 HR column

HiPrep Sephacryl S-100 HR column (Amersham Bioscience) was calibrated using molecular weight gel filtration calibration kit (Pharmacia) composed of four pure standard proteins; ribonuclease A (13.7KDa), Chymotrypsinogen (25KDa), Ovalbumin (43KDa) and Bovine serum albumin (67KDa) and the kit contains also Blue Dextran 2000 (2000KDa) marker. 1mg of each standard protein and 2mg of blue Dextran 2000 was dissolved separately in 1ml of 4M urea solution, and 200ul of each standard were mixed together and injected to the top of the Sephacryl S-100 column (connected to ÄKTA*prime* protein purification system) after washing the column with the running buffer (10mM Na_2HPO_4, 15mM NaH_2PO_4, 0.15M NaCl and 4M Urea PH=7). 1ml fractions were collected at a flow rate of 0.5 ml/min and V_o (the volume of mobile phase before

elution of blue Dextran 2000 standard) was calculated. 100 fractions were collected and the protein content of each fraction was measured using UV spectrophotometer and V_e (elution volume) was calculated for the five different standard markers. The calibration of the column was used for calculation of molecular weight of the protein of interest in the crude plant extract.

Purification of active protein from Arabidopsis

50 gm of Arabidopsis materials were extracted with Na-phosphate buffer (10mM Na_2HPO_4, 15mM NaH_2PO_4 PH=7) containing 2mM PMSF and 1mM Benzamidine hydrochloride for overnight at 4^oC with shaking. After low speed centrifugation, the supernatant was exposed to high speed centrifugation (14000rpm) for 20min and after discarding the supernatant, the pellet was dissolved in 5ml 8M urea and after another centrifugation at 14000 rpm for 30 min , the supernatant (4ml) was injected to the top of HiPrep Sephacryl S-100 HR column using 10ml-volume injection loop connected to ÄKTA*prime* protein purification system. The column was washed with the running buffer (10mM Na_2HPO_4, 15mM NaH_2PO_4, 0.15M NaCl and 4M Urea PH=7) before starting purification. After loading the protein sample, the instrument was run according to the following program:

Manual run → Press OK → Set method base → select (ml) →

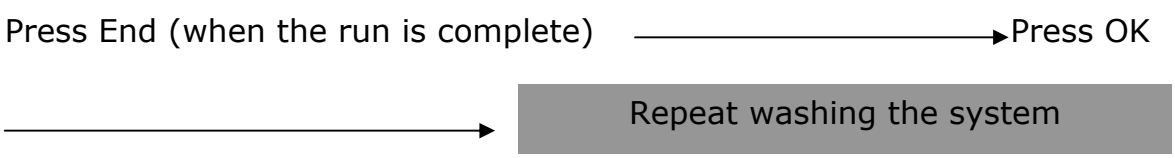
Set Concentration %B → Set Gradient →

Set flow rate → 0.5 (ml/min) → Set Fraction Base → select (ml) →

Set Fraction Size → select (1ml) → Set Pressure Limit → (0.15MPa) →

Set Buffer Valve Pos → Pos 1 → Set Inject Valve Pos → (Load) →

Inject your sample using syringe → (Inject) → Press OK to Start run →



1 ml fractions were collected at room temperature and protein concentration of every fraction was tested using UV spectrophotometer. The antimicrobial activity was tested for different fractions. The purity of different active fractions was tested using 15% SDS-PAGE and gels were stained with silver staining.

Mass spectrometry

The gel bands were excised, washed and subjected to in-gel-digestion with trypsin according to the method of Sickmann et al., 2000. Resulting peptides were eluted by incubation with 20 µL 5% formic acid for 15 min. The obtained peptide mixture was used for nano-LC-MS/MS analysis using a Dionex Ultimate 3000 nano-LC-system (Dionex GmbH, Idstein, Germany) directly coupled to a ThermoElectron LCQ DecaXP ion trap mass spectrometer (ThermoElectron, Dreieich, Germany) according to the method of Wilm *et al*., 1996).Tandem mass spectra were searched against the NCBInr database using the Mascot algorithm (Version 2.1; Matrixscience, London, GB). Significant protein identification with at least two different, significantly scored peptides were verified manually. 75 µm I.D., 15 cm length C18-column (LCPackings, Amsterdam, NL) with preconcentration (300 µm I.D., 1mm length, C18 Pepmap-column, LCPackings, Amsterdam, NL).

4.2.3.6 Bradford-Protein estimation

Protein content was estimated according to Bradford (1976), 100ul of sample was mixed with 900ul of Bradford reagent and was incubated at RT for 10 minutes. OD was measured at 578nm and standard was made with 0-10ug of BSA (Bovine albumin serum

4.3 Instruments

Protein purification system:	Äkta*p*rime, Amersham Pharmacia Biotech
Gel documentation system:	LTF M/WL 312nm, camera: CCD XC-ST50 Mitsubishi video copy processor P67E
SDS-Minigel-Apparatus:	Biometra
PCR-machine:	Hybaid: PCR*Sprint* and PCR*Express*
pH-Meter:	WTW pH525 with electrode Ingold 40557
Spectrophotometer:	Kontron Uvikon 860 with Plotter 800 Pharmacia Gene Quant II
Centrifuge:	Eppendorf 5415-C and 5417R, Heraeus Megafuge 1.0, Sorvall centrifuge RC-2B SS34, GSA and HB4

4.4 Chemicals and Enzymes

AppliChem, Darmstadt:	Dithiothreitol (DTT)
Biomers, Ulm:	Oligonucleotides
BioRad, Richmond:	Ammoniun persulphate, TEMED
Biozym, Hameln:	Agarose
Gibco BRL:	Ribonuclease Inhibitor
Fermentas, Germany:	Protein molecular weight marker
New England Biolabs:	Restrictionenzymes
Promega, Madison:	Reverse Transcriptase MMLV H(-)
Roth, Karlsruhe:	EDTA, Glycerin, Glycin, Chloroform p.a., Ethanol p.a., Phenol, RNAse, SDS, Tris
Serva, Heidelberg:	Acrylamid, Bisacrylamid
Sigma, Deisenhofen:	Ammonium-Persulfat, Ampicillin, DEPC, Formamid, HEPES, MOPS
USB, Cleveland	Ethidiumbromide

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6.1 Abbreviations

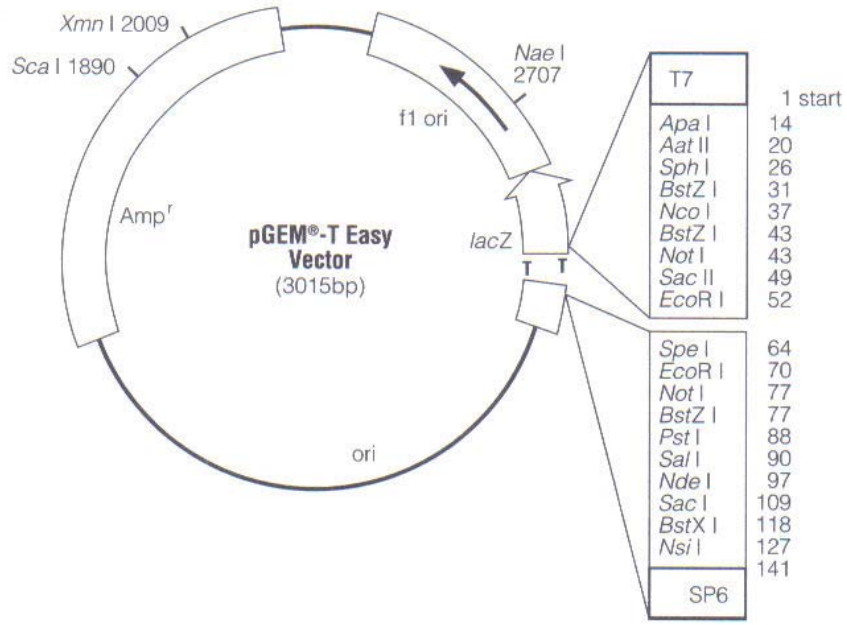
%	Percent
A	Adinine
ATP	Adenosine triphosphate
AtPDP	*Arabidopsis thaliana* Plate Domain Protein
APS	Ammonium per sulphate
bP	base pair
Bis	Bisacylamide
BSA	bovine serum albumin
C	Cytosine
Ca MV 35S	Cauliflower mosaic virus 35s promoter
cDNA	complementary DNA
cm	centimeter
ddH2O	double distilled water
DEPC	Diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleosid-5'-triphosphate
DTT	1,4- dithiothreitol
EDTA	ethylenediamine tetraacetic acid
G	Guanine
Ug	microgram
gm	gram
h	hour
kDa	kilo dalton
ul	microliter
LB	Lauria-Bertani
M	molar
mA	milli Ampere
ß-ME	bete mercaptoethanol
min	minute
mL	milliliter

mM	millimolar
MOPS	4-morpholinic-propansulfonic acid
mRNA	messenger RNA
ng	nanogram
O.D.	optical density
PAGE	polyacrylamide-gel electrophoresis
PCR	polymerase chain reaction
pH	potential of Hydrogen, pH scale is logarithmic
rpm	revolution per minute
RNA	ribonucleic acid
RAase	ribonuclease
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
T	Thymine
Taq	*Thermus aquaticus*
TEMED	N,N,N,N,-tetramethylaminomethane
T_m	annealing temperature
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
UV	ultraviolet
V	volt
V_e	elution volume
v/v	volume/volume
w/v	weight/volume
win	proteinase inhibitor-like gene

6.2 Oligo Nucleotide Primers

Name	Nucleotide Sequence (5' to 3')	T_m
At4G39730(5')	GATTGGATCCATGGCTCGTCGCGATGTTCT	**72**
At4G39730(3')	GATGAGCTCTTAAACGACCCAAGAAAGC	**62**
At2g22170(5')	ATAGGATCCATGATGCCTCGCCGTGACGTC	**72**
At2g22170(3')	TCAGAGCTCTCACACGATCCAAGAGAGAGT	**64**
Win(5')	ACTGCAGAAGCTTCCAACATCAATGAT	**61**
Win(3')	CGGGATCCTCTAGAATTTGTTGAATATGAG	**61**
At1g20030(5')	CTATCTAACGCCGGAGTTTCT	**50**
At1g20030(3')	ATTCTGACCGGAGCCGCCTTGA	**64**
At1g75800(5')	TTTTATCAAACGCCGGAGTTCCA	**59**
At1g75800(3')	TTGTTTACCTCTGCCGTCTAAAC	**52**
At1g75050(5')	TTGCTGTTTCCGCCGCCACCGT	**69**
At1g75050(3')	TCAGAATTAAACGCCGCACAAG	**57**
At1g75030(5')	CTCTCTCCGGCAACAGCATCAC	**58**
At1g75030(3')	TTCCGGCGAACTAAACGCCGCGC	**71**
At5g02140(5')	GGAGCTCAGCTTATAATAGTGAA	**47**
At5g02140(3')	ACAACCAACCACTTTCCCATCTC	**56**
At5g24620(5')	CCACTACCCACCACAGGGTTCA	**58**
At5g24620(3')	AAGAGTATAAAGACGGTCGGCATG	**56**
At4g38660(5')	CGAATGCTGGCTCACCTACT	**52**
At4g38660(3')	CAACTAGAAGGTGTAGCATA	**39**
At4g24180(5')	CAACTCCGGAAGCGGCGATATA	**60**
At4g24180(3')	ATCGATGGTTTACACTCGGTG	**52**
At1g19320(5')	CGGTACAACCTTCACCTTAACA	**51**
At1g19320(3')	TCTTCGAGTAATTCGTGGGCGG	**60**
At4g36010(5')	GGAACCTCTCCTTTACCTACAA	**49**
At4g36010(3')	GCTTTTGCAAGCTACTCCTTCA	**53**
At2g17860(5')	CACCGCTCCATTACCCACCACT	**60**
At2g17860(3')	TTCCAGCCGCTTCAACCGTTAC	**60**
At4g38670(5')	GCTCTCTGGTGCTAACTCA	**45**
At4g38670(3')	GAAGAGCGAGTAAACCGAAG	**49**
At1g70250(5')	CTGCAACCTACGGCTATAGGA	**52**
At1g70250(3')	AAGCTCTGGCAACTGGTATC	**49**

6.3 Vectors (PGEM –T Easy Vector)



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	**1**
multiple cloning region	**10-128**
SP6 RNA polymerase promoter (-17 to +3)	**139-158**
SP6 RNA polymerase transcription initiation site	**141**
pUC/M13 Reverse Sequencing Primer binding site	**176-197**
***lacZ* start codon**	**180**
***lac* operator**	**200-216**
β-lactamase coding region	**1337-2197**
phage fl region	**2380-2835**
***lac* operon sequences**	**2836-2996, 166-395**
pUC/M13 Forward Sequencing Primer binding site	**2949-2972**
T7 RNA polymerase promoter (-17 to +3)	**2999-3**

Source: http://WWW.promega.com

6.4 Blasts for AtPDP1 and AtPDP2

6.4.1 AT4g39730.1: Lipid-associated family protein, contains PLAT/LH2 domain from[Arabidopsis thaliana](AtPDP1)

```
Identities: 547/548 (99%)

Query: 68  cttaaacgacccaagaaagcttttcccgaatctcagacccgacccgactaacactatccc 127
           ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 598 cttaaacgacccaagaaagcttttttccgaatctcagacccgacccgactaacactatccc 539

Query: 128 taagcttgaccggacaattgttccgtacggcggtgagctcataaggagaagtatcagtag 187
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 538 taagcttgaccggacaattgttccgtacggcggtgagctcataaggagaagtatcagtag 479

Query: 188 cgagccattgctcaatctcaaaatcctgcgtcgagcactgtgcgtgaacaccagccgtcg 247
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 478 cgagccattgctcaatctcaaaatcctgcgtcgagcactgtgcgtgaacaccagccgtcg 419

Query: 248 tgatctcaacgtaattaacgtaccaaccatggtgatcgccggagccatcggaggttaggt 307
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 418 tgatctcaacgtaattaacgtaccaaccatggtgatcgccggagccatcggaggttaggt 359

Query: 308 ttaaggcacagatcggactaggtaaacacggtgctcttccactgaaaatgtcgagattac 367
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 358 ttaaggcacagatcggactaggtaaacacggtgctcttccactgaaaatgtcgagattac 299

Query: 368 ccctctcgaagtaattgtaatcaggtcccattaatccagcccaagcttgaaggtttttga 427
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 298 ccctctcgaagtaattgtaatcaggtcccattaatccagcccaagcttgaaggtttttga 239

Query: 428 ttccgatgtagtcaccgtccttatcgtagattcttgcgctgatgatcgaatcggttccgg 487
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 238 ttccgatgtagtcaccgtccttatcgtagattcttgcgctgatgatcgaatcggttccgg 179

Query: 488 ctttccagatcgatccggttctgaggtagaatgtgtatacacagtctggatcatcttcgg 547
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 178 ctttccagatcgatccggttctgaggtagaatgtgtatacacagtctggatcatcttcgg 119

Query: 548 cgaaagctacggcggagacggtggcgagaaggaggaggaaagggaggagaacatcgcgac 607
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 118 cgaaagctacggcggagacggtggcgagaaggaggaggaaagggaggagaacatcgcgac 59

Query: 608 gagccatt 615
           ||||||||
Sbjct: 58  gagccatt 51
```

6.4.2 AT2g22170.1: Lipid-associated family protein, contains PLAT/LH2 domain from[Arabidopsis thaliana](AtPDP2)

Identities: 554/555 (99%)

```
Query: 66  atgatgcctcgccgtgacgtcctcttcctctcactcctcctcgtcatcgccaccgtctcc 125
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 70  atgatgcctcgccgtgacgtcctcttcctctcactcctcctcgtcatcgccaccgtctcc 129

Query: 126 gctgtcgcattagccgacgatgaagcagattgtgtctacacattcttccttagaaccgga 185
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 130 gctgtcgcattagccgacgatgaagcagattgtgtctacacattcttccttagaaccgga 189

Query: 186 tcaaccttcaaagccggtacagattcgatcataagcgctagagtctacgacaaatacgga 245
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 190 tcaaccttcaaagccggtacagattcgatcataagcgctagagtctacgacaaatacgga 249

Query: 246 gactacatcgggatccgaaacctagaagcatggggtggtttaatgggaccaggttacaac 305
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 250 gactacatcgggatccgaaacctagaagcatggggtggtttaatgggaccaggttacaac 309

Query: 306 tactacgagaggggtaatctcgacattttcagtgggaaagcaccatgtcttccgagccca 365
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 310 tactacgagaggggtaatctcgacattttcagtgggaaagcaccatgtcttccgagccca 369

Query: 366 gtctgttcattaaatctgacctctgacggctccggtgaccaccacggttggtatgttaac 425
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 370 gtctgttcattaaatctgacctctgacggctccggtgaccaccacggttggtatgttaac 429

Query: 426 tacgtggaagtcacgacagccggagttcatgcgaagtgctcgtaccaaagctttgatgtt 485
           |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 430 tacgtggaagtcacgacagccggagttcatgcaaagtgctcgtaccaaagctttgatgtt 489

Query: 486 gagcaatggcttgcgtccgatacatcgccgtatgagctcagtgctgttcggaataactgt 545
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 490 gagcaatggcttgcgtccgatacatcgccgtatgagctcagtgctgttcggaataactgt 549

Query: 546 ccagtttcgcttagggaaagtgtcggtcgggtcgggtctgagatccggaagactctctct 605
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 550 ccagtttcgcttagggaaagtgtcggtcgggtcgggtctgagatccggaagactctctct 609

Query: 606 tggatcgtgtgagag 620
           |||||||||||||||
Sbjct: 610 tggatcgtgtgagag 624
```

6.5 Blasts for Win promoter region

6.5.1.A gi20946|emb|X15516.1|PTGWIN3 *Populus trichocarpa* wound responsive gwin3 gene

Identities: 718/735 (97%)

```
Query 73    ATTTGTTGAATATGAGTATTAGTTATCTCAATATTGTGTCTTGTTGATCTTGTGTTGAAG  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 732   ATTTGTTGAATATGAGTATTAGTTATCTCAATATTGTGTCTTGTTGATCTTGTGTTGAAA  673

Query 133   CAACGCATGGAGAGCGTATATTTATATGGAGCAAGCATTTGGATTATTTGGAATACAAAT  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 672   CAACGCATGGAGAGCGTATATTTATATGGAGCAAGCATTTGGATTATTTGGAATACAAAT  613

Query 193   TGGAGTGTTTTACTCGTGCTGATTTCCGACAAAACTAGAAATTATAGTCCTAGGGCGACG  252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  612   TGGAGTGTTTTACTCGTGCTGATTTCCGACAAAACTAGAAATTATAGTCCTAGGGCGACG  553

Query  253   AGTTGTCATATAGGTTTTGAGCTCTTGCCGCTTCCACCACTTGCTTACTTGATTTATCCC  312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  552   AGTTGTCATATAGGTTTTGAGCTCTTGCCGCTTCCACCACTTGCTTACTTGATTTATCCC  493

Query  313   ACAAAAGTGTTTTAATTACTCGTGCTTAAAATTGATCTGATCAATGAAATTGAAATTTTA  372
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  492   ACAAAAGTGTTTTAATTACTCGTGCTTAAAATTGATCTGATCAATGAAATTAAAATTTTA  433

Query  373   AATATCTTAAATTTAAATAAATAAACTATACTTTTTTGAAACGACTATCATTTTATTCAT  432
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  432   AATATCTTAAATTTAAATAAATAAACTATACTTTTTTGAAACGACTATCATTTTATTCAT  373

Query  433   TTATCGTGTAATGATTGGTGAACTTGATAACTCAAAAATAAACATCATGTATAAAATTGA  492
            |||||||||||||||||||||||||| |||||||||||||||| ||||||||||||||||
Sbjct  372   TTATCGTGTAATGATTGGTGAACTTGACAACTCAAAAATAAACACCATGTATAAAATTGA  313

Query  493   ACAAAACCCACTCAAATAGTCTACTTTATAAATAAGTTCAATTAAAACTACTAATTACAC  552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  312   ACAAAACCCACTCAAATAGTCTACTTTATAAATAAGTTCAATTAAAACTACTAATTACAC  253

Query  553   CTTCAAATCAAATTTAAGCTGTCAAGCTCATAATCAGTCATTTAAAATGAGATTACACGT  612
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  252   CTTCAAATCAAATTTAAGCTGTCAAGCTCATAATCAGTCATTTAAAATGAGATTACACGT  193

Query  613   TCAAGTATTCTAAAAACGTTTTGGTCCATTTATTTGCTCTGGACACTTCGGGAGACCTGA  672
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  192   TCAAGTATTCTAAAAACGTTTTGGTCCATTTATTTGCTCTGGACACTTCGGGAGACCTGA  133

Query  673   TTAATCAATAATATAGGTTAGTTTGCTTCAATAACTGGTTTTTCTTAGTTTTAATTCTAG  732
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  132   TTAATCAATAATATAGGTTAGTTTGCTTCAATAACTGGTTTTTCTTAGTTTTAATTCTAA  73

Query  733   AGTTTTGTTTTTGCACCGAAACCTATTCTAATCAAATTGTGATTATTAAGTGATAATCAT  792
            || |||||||||||||| |||| ||||| | | |||||  || ||||| || |||||||||
Sbjct  72    AG-TTTGTTTTTGCACAGAAA-CTATTTTGACCAAATCATGGTTATTGAGAGATAATCAT  15

Query  793   TGATGTTGGAAGCTT  807
            | |||||||||||||
Sbjct  14    T-ATGTTGGAAGCTT  1
```

6.5.1.B gi169460|gb|L11233.1|POPPROINH *Populus* x *generosa* isolate H11-11 serine proteiase inhibitor (win 3.12) gene

Identities: 718/735 (98%)

```
Query  73  ATTTGTTGAATATGAGTATTAGTTATCTCAATATTGTGTCTTGTTGATCTTGTGTTGAAG 132
           |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  823 ATTTGTTGAATATGAGTATTAGTTATCTCAATATTGTATCTTGTTGATCTTGTGTTGAAA 764

Query  133 CAACGCATGGAGAGCGTATATTTATATGGAGCAAGCATTTGGATTATTTGGAATACAAAT 192
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  763 CAACGCATGGAGAGCGTATATTTATATGGAGCAAGCATTTGGATTATTTGGAATACAAAT 704

Query  193 TGGAGTGTTTTACTCGTGCTGATTTCCGACAAAACTAGAAATTATAGTCCTAGGGCGACG 252
           |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  703 TGGAGTGTTTTACTCGTGCAGATTTCCGACAAAACTAGAAATTATAGTCCTAGGGCGACG 644

Query  253 AGTTGTCATATAGGTTTTGAGCTCTTGCCGCTTCCACCACTTGCTTACTTGATTTATCCC 312
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  643 AGTTGTCATATAGGTTTTGAGCTCTTGCCGCTTCCACCACTTGCTTACTTGATTTATCCC 584

Sbjct  313 ACAAAAGTGTTTTAATTACTCGTGCTTAAAATTGATCTGATCAATGAAATTGAAATTTTA 372
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Query  583 ACAAAACTGTTTTAATTACGCGTGCTTAAAATTGATCTGATCAATGAAATTAAAATTTTA 524

Sbjct  373 AATATCTTAAATTTAAATAAATAAACTATACTTTTTTGAAACGACTATCATTTTATTCAT 432
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Query 523  AATATCTTAAATTTAAATAAATAAACTATACTTTTTTGAAACGACTATCATTTTATTCAT 464

Sbjct 433  TTATCGTGTAATGATTGGTGAACTTGATAACTCAAAAATAAACATCATGTATAAAATTGA 492
           ||||||||||||||||||||||||||| |||||||||||||| |||||||||||||||
Query 463  TTATCGTGTAATGATTGGTGAACTTGACAACTCAAAAATAAACACCATGTATAAAATTGA 404

Sbjct 493  ACAAAACCCACTCAAATAGTCTACTTTATAAATAAGTTCAATTAAAACTACTAATTACAC 552
           |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Query 403  ACAAAACCCACTCAAATAGTCTACTTTATAAATAACTTCAATTAAAACTACTAATTACAC 344

Sbjct 553  CTTCAAATCAAATTTAAGCTGTCAAGCTCATAATCAGTCATTT  595
           |||||||||||||||||||||||||||||||| |||||||||
Query 343  CTTCAAATCAAATTTAAGCTGTCAAGCTCATAACCAGTCATTT  301
```

6.5.2.A gi20946|emb|X15516.1|PTGWIN3 *Populus trichocarpa* wound responsive gwin3 gene

Identities: 470/526 (89%)

```
Query 168 GTTATTGAAGCAAACCAACCTAGATTAGTGATTAATCAAGCCTCTCAAAGTGTCTAGTGC 227
          ||||||||||||||| |||||| |||| |||||||||||| ||| | ||||||| || ||
Sbjct 98  GTTATTGAAGCAAACTAACCTATATTATTGATTAATCAGGTCTCCCGAAGTGTCCAGAGC 157

Query 228 AAAGAAA-GGACCAAAACttttttttAGAATACTTGGACATGTAATCTCATTTTAAATGAC 286
          ||| ||| |||||||||| ||||| |||||||||| || ||||||||||||||||||||
Sbjct 158 AAATAAATGGACCAAAACGTTTTTAGAATACTTGAACGTGTAATCTCATTTTAAATGAC 216

Query 287 TGCTT-TGGGCTTGACAACTTGAATTTGATTTCGAAGATTTAATTAGTAGTTTTAATTGG 345
          || || || |||||||| ||| |||||||||| |||| | ||||||||||||||||||
Sbjct 217 TGATTATGAGCTTGACAGCTTAAATTTGATTT-GAAGGTGTAATTAGTAGTTTTAATTGA 275

Query 346 AGTTATTAATAGAGTAGACTATTTGTGTGCGTTTTGTTCAATTTTATACATGGTGTTTAT 405
          | ||||| ||| |||||||||||||| ||| ||||||||||||||||||||||||||||||
Sbjct 276 ACTTATTTATAAAGTAGACTATTTGAGTGGGTTTTGTTCAATTTTATACATGGTGTTTAT 335

Query 406 TTTCAAGTTGTCAAGTTCACCAATTATTACACGATAAATGAATACAATGATAGTCGTTTC 465
          |||  |||||||||||||||||| |||||||||||||||||| ||||||||||||||
Sbjct 336 TTTTGAGTTGTCAAGTTCACCAATCATTACACGATAAATGAATAAAATGATAGTCGTTTC 395

Query 466 AAAAAAGTATAGTTTATTTATTTAAATTTAAGATATTTAAAATTTTAAGTTCATTGATCA 525
          |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct 396 AAAAAAGTATAGTTTATTTATTTAAATTTAAGATATTTAAAATTTTAATTTCATTGATCA 455

Query 526 GATCAATTTTAAGCACGAG---TAAAACACTTTTGTGGGATAAATCAAGTAGGCAAGAG 581
          ||||||||||||||||||   |||||||||||||||||||||||||||| |||||| |
Sbjct 456 GATCAATTTTAAGCACGAGTAATTAAAACACTTTTGTGGGATAAATCAAGTAAGCAAGTG 515

Query 582 ---------GCAAGAGCTCAAAACCTATACGACAACACGTCGCCACAGGGCTATAATTTC 632
                  |||||||||||||||||||| |||||| |||||||  ||| ||||||||||
Sbjct 516 GTGGAAGCGGCAAGAGCTCAAAACCTATATGACAACTCGTCGCCCTAGGACTATAATTTC 575

Query 633 TAGTTTTGTCGGAAATCAGCACGAGTAAAACACTACAATTTATATT  678
          |||||||||||||||||||||||||||||||| |||||| ||||
Sbjct 576 TAGTTTTGTCGGAAATCAGCACGAGTAAAACACTCCAATTTGTATT  621
```

6.5.2.B gi169460|gb|L11233.1|POPPROINH *Populus* x *generosa* isolate H11-11 serine proteiase inhibitor (win 3.12) gene

Identities: 357/397 (89%)

```
Query 295  GCTTGACAACTTGAATTTGATTTCGAAGATTTAATTAGTAGTTTTAATTGGAGTTATTAA   354
           |||||||| ||| ||||||||| |||| | |||||||||||||||||| ||||||| |
Sbjct 317  GCTTGACAGCTTAAATTTGATTT-GAAGGTGTAATTAGTAGTTTTAATTGAAGTTATTTA   375

Query 355 TAGAGTAGACTATTTGTGTGCGTTTTGTTCAATTTTATACATGGTGTTTATTTTCAAGTT 414
           || |||||||||||| ||| ||||||||||||||||||||||||||||||||  ||||
Sbjct 376 TAAAGTAGACTATTTGAGTGGGTTTTGTTCAATTTTATACATGGTGTTTATTTTTGAGTT 435

Query 415 GTCAAGTTCACCAATTATTACACGATAAATGAATACAATGATAGTCGTTTCAAAAAAGTA 474
           ||||||||||||||| |||||||||||||||||| |||||||||||||||||||||||||
Sbjct 436 GTCAAGTTCACCAATCATTACACGATAAATGAATAAAATGATAGTCGTTTCAAAAAAGTA 495

Query 475 TAGTTTATTTATTTAAATTTAAGATATTTAAAATTTTAAGTTCATTGATCAGATCAATTT 534
           |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct 496 TAGTTTATTTATTTAAATTTAAGATATTTAAAATTTTAATTTCATTGATCAGATCAATTT 555

Query 535 TAAGCACG----AGTAAAACACTTTTGTGGGATAAATCAAGTAGGCAAGAG---------  581
           ||||||||    | ||||||| |||||||||||||||||||||| ||||| |
Sbjct 556 TAAGCACGCGTAATTAAAACAGTTTTGTGGGATAAATCAAGTAAGCAAGTGGTGGAAGCG  615

Query 582 GCAAGAGCTCAAAACCTATACGACAACACGTCGCCACAGGGCTATAATTTCTAGTTTTGT 641
           |||||||||||||||||| |||||| ||||||| |||| ||||||||||||||||||
Sbjct 616 GCAAGAGCTCAAAACCTATATGACAACTCGTCGCCCTAGGACTATAATTTCTAGTTTTGT 675

Query 642 CGGAAATCAGCACGAGTAAAACACTACAATTTATATT  678
           |||||||| |||||||||||||||| |||||| ||||
Sbjct 676 CGGAAATCTGCACGAGTAAAACACTCCAATTTGTATT  712
```

6.6 Genomic sequences for cloned thaumatin genes

(Red-shaded sequences used for design of forward primers and the green-shaded ones for the reverse primers)

>At1g20030

1 TTGAGATTTTTTCTTGAAGCCGACCAAAAAAAGGAGAAATCTAGCAGGCT
51 AATGTTTATTCTCTCTTTTCTTTTTGTCAGAGGCTCATTTGTCAGTAACC
101 ATTTGATCAACGGCTAAATCCTATATCCACTAAAATTCAACCACGTCATC
151 AGACATACCAATATACAGCAACACTTGATTGTAGCTAATAAAAAGATCGT
201 CAACAGAATTAACTCTTTAAATCACTTCTCTGTTATACCTCTCTGTTTCG
251 CTCCCTGAAGAAGAAGAAACAGAGCGTTCAGATTTCTCTTCAACATAATC
301 ATGGCATTGACCTTCTTCTTCTTGTTATCGAATTTGTTCTTCTCCGGTAA
351 GAATCTGCCTGATCTCACATATTTAAAGACTCTAATTATTTTTAAATTTC
401 TTCATTTTTTTCAATCTTAGGAGTTATGTCAAGGAGCTTCACATTTTCAA
451 ACAAATGCGATTACACAGTGTGGCCGGGAATTCTATCTAACGCCGGAGTT
501 TCTCCATTACCAACCACCGGCTTCGTCCTCCTGAAAGGCGAGACGCGTAC
551 AATCAACGCACCGTCATCATGGGGGGGTCGTTTCTGGGGAAGAACACTCT
601 GCTCAACCGATTCCGATGGAAAATTCTCGTGTGCCACCGGAGATTGCGGC
651 TCCGGAAAAATCGAATGCTCCGGCGCTGGAGCTGCTCCTCCTGCGACTTT
701 AGCTGAATTCACTCTCGATGGCTCCGGAGGACTCGATTTCTACGATGTCA
751 GTCTCGTCGACGGCTACAATGTCCAGATGCTGGTGGTTCCTCAAGGCGGC
801 TCCGGTCAGAATTGCAGCAGCACCGGCTGCGTCGTCGATTTAAACGGATC
851TTGCCCGTCGGAGCTAAGGGTGAATAGCGTCGACGGGAAGGAGGCGGTG
901CGATGGCGTGTAAAAGTGCGTGCGAGGCGTTTCGGCAGCCGGAGTATTGC
951 TGCAGCGGCGCGTTTGGATCGCCTGACACGTGTAAGCCGTCTACGTACTC
1001GCGGATCTTCAAGAGCGCGTGTCCACGCGCCTATAGCTACGCGTATGAG
1051 ATAAGTCTAGTACATTTACGTGCGCGAAATCTCCCAACTACGTCATCACT
1101 TTCTGTCCTTCTCCCAACACCAGGTAAAATTCCTTTAATACCCTTCTTCT
1151 ATTTACTTTCACCCATTTCGTTAAAAGGTGATTAGGAAATATTTACTCTT
1201 TCATAAGTAGGATTTTTTTGCTTGTTGTATAATCGAAGTTACTAGATACT
1251 CAATTCCATTGATAATCATTAATTTTGCTATACAAGTTTTGTTTTTCTTT
1301CAAGAGTTTTATGATAGACATTGTTTGTCAGGCTCCTATAAAAGAGAGAC
1351 ACGTGGGAACTAACTTCATTCACTTATTCCTTTATTTGCAGCCTAAAATC
1401 AGCTGAGGAACACAGCACAGAGACAATGACGACGACAAGTCCAAGTTCG
1451 GCAGTACGACGTCGTCGCAGATGGTCTACGAAGGTGCTCTGGACGAAAGC
1501 AGTGGTTCACCATCTACGTATCACGGCGTATCACGAGCAATCACAGTCGT
1551 TCTTCTCTCGCTTGCTTTTTGTCGTATGTGGTGGTTCTTCTGACCAGAGA
1601 TTATTTGTCTTAGTGTCTGCCACGTATGCGCAGAAACAAGAACGTATGGG
1651 CCTTTAGAAGGCCTTTTCTCACTATAGTAAAAATATTTCTAAAGTTTTTC
1701 CACATAATGTCAACTTTTTACTCCACATGACCAAACGTTGAATGATTAAA
1751 CCATTCTTAAGGAAAATCAAGAAAGTAGGCTATATTAAAGCATTAATATT
1801 GGTAGTATTTTAGATTAGTCTTAAGTCTAAACATAGTTTTTAGGAATAAT
1851 TTTATAATTAATTTTAAAAATTTTGATGTTAAAAAACTAAAATTTTGGCA
1901 ATATACAATGGTGGTTTTTA

>At1g75800 (S.for cDNA only, s. for DNA is not avilible)

1 CGCTCTCAGAATCCTCTTTTAAGTCTCTGTTTCATAAATAAAACTAATAA
51 AGAAGAAGAAGAAAACAGAGCAATTAAAGTCCTTAAATCTTCTTCTTCTC

101 CAATTCAATGGCTCTTCCGTTGCCATTGATCTTCCTTATCTTCTCTCATT
151 TGTTCGTTTCTGGAGTTAGATCAACGAGCTTCATTATGGTGAACAAATGC
201 GAATACACAGTCTGGCCTGGACTTTTATCAAACGCCGGAGTTCCACCACT
251 TCCGACGACCGGATTCGTTCTCCAAAAAGGCGAAGAACGAACAATCAGCG
301 CTCCAACTTCATGGGGAGGAAGATTCTGGGGGAGAACTCAATGTTCCACC
351 GACACCGACGGAAAATTCACTTGTCTCACCGGAGATTGCGGATCTGGTAC
401 CCTCGAATGCTCCGGATCCGGAGCAACACCACCAGCAACACTAGCGGAAT
451 TCACACTAGACGGATCTAACGGACTCGATTTCTACGACGTTAGTCTCGTC
501 GACGGTTACAACGTCCCGATGCTAGTGGCTCCACAAGGAGGCTCGGGTTT
551 AAACTGTAGCAGCACCGGATGCGTTGTAGATCTGAACGGTTCGTGTCCGT
601 CGGAGCTTAAAGTGACGAGTTTAGACGGCAGAGGTAAACAATCCATGGGA
651 TGTAAAAGCGGTTGTGAAGCTTTTCGTACGCCGGAGTATTGTTGCAGCGG
701 CGCCCACGGTACACCTGACACGTGTAAACCGTCGTCGTACTCGTTGATGT
751 TTAAAACTGCGTGTCCACGTGCTTACAGCTACGCTTACGATGATCAGAGT
801 AGTACCTTCACATGTGCTGAATCTCCTAATTACGTTATCACGTTTTGCCC
851 TACTCCTAACACCAGTCAAAAATCATCTCAAGATCAGAGCCCAGATCCCA
901 AACCGACGACACCAACCGGGACGTCGTCGACAACTCCTGCCGGAGATAGT
951 AGTACGACGTGGTCACCGGTAGATACATCAATGATATACGAAGGAGCTTT
1001 GGATCAAAACAAAGGATCACCGTCCACGTGTCATCTTTCGTTATGTGGAA
1051 TCACAGTCACACTTGCGCTGGCCTTTTGTCGGATGTGGCGGCTCTTTTGA
1101 CACCTAACTTTGTCTCCCGCGTAAAACACTGAAGAATGTGGGCCCTCACT
1151 GACGAAAGCCTTATCTCGTTCCGGCCCATGTGAACCGGTTATTCCCGAAC
1201 GACGTCGGATTGGGTCAGAGTTGAGAAAAAAAGTTAACAACACCCTACAG
1251 TTACGAAAAT ATCCTTCATT CATGTTCATG GGAGAATTCT TAATTTGGGT
1301 GAGGAACACG TGGGCAAGAA AGTGGGCTGC TGGTGGAGTT CATAAAAGT
1351 GGGAATGTTT AATTGTAAGG TCAGAATATT TATTTGATTT ATAATGTAAC
1401 TAGGATTAGA AGAATATTAT GTACACTATA TAAAGCAGTA ATCTATTTAG
1451 TTTAAAGCTA TATATTGGAA ACTATTGGTG GGAATATTTC ATTTAATTGT
1501 TATCTGC

>At1g75050
1 CACACACACA AAACTCGTTA AAACAAAGAT GGCGAATTAC TCAAGTATTC
51 ACATTCTCTT CTTGGTCTTC ATCACAAGTA AAGAGCAATC ACACTCTGTT
101 TCTGTTTCTG TTTCTCCTCT GTTTTATCTG TTTCTAAATG AGAATAATTT
151 CGATACTATT TCTTAAAACT ATATTTTTTT ATGTTCATTC GTCTTCAAAT
201 CACAACATAA TTCTCCAAAA CATATCGTGT TTTTTCTTCG GATTTAACAG
251 TTTTTATTGT TGTTGTTTGT TTTGAAATTG CAGGCGGCATTGCTGTTTCC
301 GCCGCCACCG TCTTCACTTT ACAGAACAGT TGTCCTTACA CCGTTTGGCC
351 TGGAATTCTC TCCGGCAACG ACAACACCCT CGGCGACGGC GGATTTCCCT
401 TAACACCAGG CGCTTCCGTA CAGCTCACGG CTCCTGCAGG ATGGTCAGGA
451 CGGTTCTGGG CTCGTACCGG CTGCAACTTC GACGCCTCCG GCCACGGTAA
501 CTGCGGCACC GGAGACTGCG GCGGTGTTCT TAAATGTAAC GGCGGCGGAG
551 TTCCACCAGT CACTCTGGCT GAATTCACAC TTGTAGGCGA CGGCGGCAAG
601 GATTTCTACG ACGTGAGTCT CGTCGACGGT TACAATGTCG AGATGGGGAT
651 TAAACCTCAA GGAGGTTCCG GTGATTGCCA TTACGCCGGC TGCGTCGCCG
701 ACGTCAACGC GGTTTGCCCT AACGAGCTTC GGCTCATGGA TCCACATACA
751 GGAATCATCG CGGCGTGTAA AAGCGCTTGTGCGGCGTTTAATTCTGAGGA
801 GTTCTGTTGT ACCGGTGCTC ACGCGACGCC GCAAACTTGT TCTCCGACGC
851 ATTACTCGGC GATGTTTAAA AGCGCTTGCC CTGGTGCTTA CAGTTACGCC

901 TACGACGACG CCACGAGCAC CTTCACTTGT ACCGGCTCTA ACTACTTGAT
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6.7 cDNA sequence alignments for thaumatin genes

(Red-shaded sequences used for design of forward primers and the green-shaded ones for the reverse primers)

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At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       ATGACAACGGCGATGATGATATTCGCGGTTTTGGTTACAGTGGTGGAGGTGGAAGCACAG 60


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       ACTGAGTGCGTGAGCAAGATAGTTCCCTGCTTCAGGTTCTTGAACACGACAACAAAGCCG 120


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       TCGACGGATTGTTGCAACTCGATCAAGGAAGCGATGGAGAAAGATTTTAGCTGTCTCTGC 180


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------ATTTTCAACTCAAATAGCTTCAC 23
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       ACCATCTACAACACTCCTGGTTTACTCGCTCAGTTCAACATCACTACAGATCAAGCTCTC 240


At1g200030      ------------------------------------------------------------
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At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       AGTTTTACTAATCTCTCTCATCTTCTTCCCAACATCAAAGAAGACCTTTACTTCTGGTTA 83
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GGTCTCAACCTTCGATGTGGAGTCAACACTGATCTATCCGCTTGTTCCGGTTCTGGTGCT 300


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ----------------------------ATGGCGAATTACTCAAGTATTCACATTCTCTTC 33
At1g75030       ----------------------------ATGGCGAAAATCTCCAGTATTCACATTCTCTTC 33
At5g02140       ----------------------------ATGACGAAATCTGCAACTCTATTCCTTCTCTTT 33
At5g24620       ----------------------------ATGGGAAGATCACCAATGGTTCCTATGGCTGTT 33
At4g38660       GAATAGACCAAGAACACTGAGTACGACAAAGAACCATGATGAATCTTTCTTCTCGTCGCG 143
At4g24180       ------------------------------ATGATCATAACAGTCCTTCATTCTCAT---G 28
At1g19320       ---------------------------------ATGGCGA--TTTTCTCCACATCACA 23
At4g36010       ----------------------------ATGGGTCAATCTCAAGTTTCTCTCTTTCTTCTT 33
At2g17860       ----------------------------ATGGATTTGGCTAAAGTCTCTTGCTTTCTCTTA 33
At4g38670       ------------------------------------------------------------
At1g70250       CCACCACCACCACCTGATCTATTTCCTCCACCTTCAGCTCAAATGCTCCCTCCACCTCCA 360


At1g200030      ------------------------------------------------------------
At1g75800       ------ATGGCTCTTCCGTTGCCATTGATCTTCCTTATCTTCTCTCATTTGTTCGTTTCT 54
At1g75050       TTGGTCTTCATCACAATTTTTATTGTTGTTGTTTGTTTTGAAATTGCAGGCGGCATTGCT 93
At1g75030       TTCGTCTTCATCACAA--------------------------------GTGGCATTGCT 60
At5g02140       CT---CACTATCTCAG-------------------------------------CCTTCACT 54
At5g24620       CTTGTTTATGTTTCTCTAC---------------------------------TGTTCTCT 60
At4g38660       CCTCTTCCACTCTCACATTGAG----------CTTCACTGTTCTCCTTTTAGCTTCCACA 193
At4g24180       TCTCATTCTATTTCAT----------------CATCCTTAGCTTCCTCTT--CTTCCATG 70
At1g19320       TCTTCTC----TTCAT----------------CTCCTTCATCATAGCCACATGTACAATT 63
At4g36010       CTAATTCTTGTTTT-------------------------------------CATCTAT 54
At2g17860       GCGATTATGTTTTT-------------------------------------CATCAAT 54
At4g38670       --ATGATGAATCGGATCCTCTTGTTTATGATCCTGGCTTTTGTCTCATCTCTTTCAGAA- 57
At1g70250       GCTTCGTCACCTGCACCTCCATCGCCACCTTCTTCATCCCGACCACGTCCATTGCCACGT 420


At1g200030      ------ATGTCAAGGAGCTTCACATTTTCAAACAAATGCGATTACACAGTGTGGCCGGGA 54
At1g75800       GGAGTTAGATCAACGAGCTTCATTATGGTGAACAAATGCGAATACACAGTCTGGCCTGGA 114
At1g75050       GTTTCCGCCGCCACCGTCTTCACTTTACAGAACAGTTGTCCTTACACCGTTTGGCCTGGA 153
At1g75030       GATTCCGCC---ACCGTATTCACTTTACAAAACAGTTGTGCGTACACCGTTTGGCCGGGA 117
At5g02140       GATGGAGCT-----CAGCTT-ATAATAGTGAATAACTGCCAGGAGAGCATTTGGCCGGGA 108
At5g24620       GTTTCTTATTCCTCCACGTTCGTCATCACAAATAACTGTCCCTTTACTATATGGCCTGGA 120
At4g38660       GGTTCGTATGGCTCAACATTCACATTCGCTAATCGGTGCGGCTACACCGTATGGCCTGGA 253
At4g24180       GAAGTG-ATGGTGCTACAATAACCATCGTAAATCGATGCAGCTTCACCGTGTGGCCGGGA 129
At1g19320       TCTGTCTCCGGTACAACCTTCACCTTAACAAACCATTGCGGCTCCACAATCTGGCCTGGA 123
At4g36010       GGAGTTTCCTCCACCACTTTCACCATCGTTAACCAGTGTAGCTACACCGTCTGGCCTGGA 114
At2g17860       GGTGGTTCCTCCACCACGTTCACCATCGTTAACCAGTGTAACTACACCGTCTGGCCGGGA 114
At4g38670       --GTTGA-GCCAATTTCGTTCAAGATCACGAATCGTTGCCGGAACACGATATGGCCCGGT 114
At1g70250       CCATCAATGTCAAGGAGCTTTACGATAGAAAACAAATGCCAATACACCATATGGCC---- 476
                             *    *   **    **       *   * *****


At1g200030      ATTCTATCTAACGCCGGAGTTTCTCCATTACCAACCACCGGCTTCGTCCTCCTGAAAGGC 114
At1g75800       CTTTTATCAAACGCCGGAGTTCCACCACTTCCGACGACCGGATTCGTTCTCCAAAAAGGC 174
At1g75050       ATTCTCTCCGGCAACGAC---AACACCCTCGGCGACGGCGGATTTCCCTTAACACCAGGC 210
At1g75030       ACTCTCTCCGGCAACAGC---ATCACCCTCGGCGACGGCGGATTTCCCTTAACTCCAGGC 174
At5g02140       ATTCTCGGCGGCGGAGGCCAAATCACACCAAGAAACGGCGGATTCCACATGGGAAGCGGC 168
At5g24620       ACTCTTGCAGGCTCTGGCACCCAGCCACTACCCACCACAGGGTTCAGGCTTGATGTGGGA 180
At4g38660       ATCCTATCGAATGCTGGCTCACCTACTCTCTCCACCACCGGATTCGAGCTCCCTAAAGGC 313
At4g24180       ATTTTGTCCAACTCCGGAAGCGGCGATATAGGCACCACCGGCTTCGAGCTCGTTCCCGGT 189
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At1g19320       ATCCTCACCGCAAACGGCGCAC---AACTCGGCGACGGCGGATTTGCCTTAGCTTCAGGA 180
At4g36010       CTTCTCTCCGGCGCCGGAACCTCTCCTTTACCTACAACCGGATTCTCCTTAAACCCTACC 174
At2g17860       CTTCTCTCCGGCGCCGGCACCGCTCCATTACCCACCACTGGTTTCTCCCTCAACTCCTTT 174
At4g38670       TTGCTCTCTGGTGCTAACTCAGCGCCGCTCCCCACAACTGGCTTTCGTCTCTCTAGAGGA 174
At1g70250       -TGCAACCTACGGCTATAGGAG-ATCACTAGAAACAACCGGTTTTGTTCTTGAGAAAGGA 534
                                                    ** **     *

At1g200030      GAGACGCGTACAATCAACGCACCGTCATCATGGGGGGGTCGTTTCTGGGGAAGAACACTC 174
At1g75800       GAAGAACGAACAATCAGCGCTCCAACTTCATGGGGAGGAAGATTCTGGGGGAGAACTCAA 234
At1g75050       GCTTCCGTACAGCTCACGGCTCCTGCAGGATGGTCAGGACGGTTCTGGGCTCGTACCGGC 270
At1g75030       GCTTCCGTGCAGCTCACGGCTCCTACCGGTTGGTCAGGCCGGTTCTGGGCTCGTACCGGC 234
At5g02140       GAAGAAACCATCATAGACGTACCGGATAAATGGTCCGGTCGAATCTGGGGTAGACAAGGC 228
At5g24620       CAGTCTGTTAGAATTCCCACGGCTCTAGGCTGGTCAGGTCGTATTTGGGCTAGGACTGGC 240
At4g38660       ACCTCTCGCTCTCTCCAAGCTCCCACCGGTTGGTCCGGTCGATTCTGGGCTCGTACCGGT 373
At4g24180       GGTTCACGTTCGTTCCAAGCTCCAGCTAGCTGGTCAGGTCGGTTTTGGGCACGAACCGGT 249
At1g19320       TCTTCCGTAACCTTCACCGTTTCTCCAGGTTGGTCCGGTCGATTTTGGGCTCGAACGTAT 240
At4g36010       GAGACACGAGTTATCCCGATCCCCGCAGCTTGGTCCGGTCGTATTTGGGGTCGTACTCTC 234
At2g17860       GAGTCACGACTCATCTCCATACCCGCCGCTTGGTCCGGCCGCATATGGGGACGCACGCTC 234
At4g38670       AAATCTAAAACCGTCGCAATACCAGAGTCGTGGTCCGGTCGTCTCTGGGCACGAACCTTG 234
At1g70250       GAGACGCGTACCATCAAAGCGCCGTCATCATGGATAGGTCGTTTCTGGGGTAGGACACTC 594
                         *       *       ***   **  *  * ****   *

At1g200030      TGCTCAACCGAT---TCCGATGGAAAATTCTCGTGTGCCACCGGAGATTGCGGCTCCGGA 231
At1g75800       TGTTCCACCGAC---ACCGACGGAAAATTCACTTGTCTCACCGGAGATTGCGGATCTGGT 291
At1g75050       TGCAACTTCGAC---GCCTCCGGCCACGGTAACTGCGGCACCGGAGACTGCGGCGGTG-- 325
At1g75030       TGCAACTTTGAC---GCCTCCGGTCACGGTACCTGCGTCACCGGAGACTGCGGCGGCG-- 289
At5g02140       TGCACCTTTAAC---CAAAACGGTAAAGGCTCATGCCAAACCGGAGACTGCAACGACGGC 285
At5g24620       TGCAACTTTGAT---GCTAACGGCGCGGGAAAATGTATGACTGGAGACTGCGGCGGAA-- 295
At4g38660       TGCAAGTTCGAC---AGTTCTGGCTCCGGTACATGCAAAACCGGTGACTGCGGCTCCAAC 430
At4g24180       TGCAACTTCAACTCAGACACAGGACAAGGCACCTGCCTAACCGGGGACTGCGGCTCTAAC 309
At1g19320       TGTAACTTCGAC---GCCTCAGGCTCAGGTAAATGCGGTACCGGGGACTGCGGCAGCAAG 297
At4g36010       TGCACACAAGACGCAACCACCGGAAGATTCACTTGCATCACCGGGGACTGTGGTTCTTCC 294
At2g17860       TGCAACCAAAACGAAATCACCGGAATATTCACTTGCGTCACCGGCGACTGCGGCTCATCC 294
At4g38670       TGTTCACAAGATCGGAGTTCCGGTTCTTTCGTCTGCTTAACCGGAGATTGCGGCTCGGGA 294
At1g70250       TGCTC-CACCAACTCAACAGGGGGTTTCTCATGTGCC--ACGGGAGACTGCACCTCCGGA 651
                **        *          **          **    ** ** ** **

At1g200030      AAAATCGAATGCTCCGGCGCTGGAGCTGCTCCTCCTGCGACTTTAGCTGAATTCACTCTC 291
At1g75800       ACCCTCGAATGCTCCGGATCCGGAGCAACACCACCAGCAACACTAGCGGAATTCACACTA 351
At1g75050       -TTCTTAAATGTAACGG---CGGCGGAGTTCCACCAGTCACTCTGGCTGAATTCACACTT 381
At1g75030       -TTTTAAAATGTACCGG---CGGCGGAGTTCCTCCAGCGACTCTAGCGGAATTCACCGTG 345
At5g02140       TCTATCAACTGCCAAGGAACCGGCGGTGTACCACCAGCAACCGTAGTAGAAATGACATTA 345
At5g24620       -AGCTGGAGTGTGCTGGTAATGGCGCTGCTCCGCCGACATCTCTTTTCGAGATCACACTC 354
At4g38660       TCTGTCGAATGCGCCGGGCTTGGTGCCGCACCGCCTGTAACTCTCGCGGAGTTCACTCTA 490
At4g24180       CAGGTCGAATGTAACGGTGCAGGAGCAAAACCGCCCGCTACACTCGCCGAATTCACAATC 369
At1g19320       T---TAAAATGCGCCGGCGCAGGAGGAGCTCCACCAGCCACACTCGCCGAATTCACAATC 354
At4g36010       ACCGTAGAATGCTCCGGATCCGGTGCTGCACCTCCGGCTACACTCGCTGAATTCACCTTA 354
At2g17860       CAAATCGAATGCTCTGGCGCAGGAGCAATCCCTCCAGCTACACTAGCCGAATTCACACTC 354
At4g38670       AAAGTCGAATGTTCAGGCTCCGGAGCTAAACCTCCAGCAACTCTCGCCGAGTTTACTCTC 354
At1g70250       AAAATCAAGTGCCTCGGTATCC---CCATAGATCCAACAACAGTGGTCGAGTTTAACCTC 708
                    *  * **    **                **     *  *    **  * *   *

At1g200030      GATGGCTCCGGAGGAC---------------TCGATTTCTACGATGTCAGTCTCGTCGAC 336
At1g75800       GACGGATCTAACGGAC---------------TCGATTTCTACGACGTTAGTCTCGTCGAC 396
At1g75050       G---TAGGCGACGGCGGCA------------AGGATTTCTACGACGTGAGTCTCGTCGAC 426
At1g75030       GGATCAAGCAATGCCGGCA------------TGGATTTCTACGACGTGAGCCTCGTCGAC 393
At5g02140       GGCTCATCTTCCTCTCCGC------------TTCATTTCTACGACGTAAGTCTCGTCGAC 393
At5g24620       GGTCACGGTTCTGATGACA------------AAGATTTCTATGATGTCAGTCTCGTTGAT 402
At4g38660       GGCACTGG--CGGCG----------------ATGATTTCTACGACGTTAGTCTCGTCGAC 532
At4g24180       GGGTCAGGTCCAGCAGACCCGGCCCGTAAACAAGACTTCTACGACGTAAGCCTCGTGGAC 429
At1g19320       GGCTCCTC--CGGAAAGAAAAACGC-CGTGCAAGATTTTTACGACGTGAGCCTCGTAGAT 411
At4g36010       AACGGAGCTAATGGTC---------------TCGATTTCTACGACGTCAGTCTCGTCGAC 399
At2g17860       AACGGCGCCGAAAATC---------------TAGATTTCTACGACGTTAGCCTCGTCGAT 399
At4g38670       AACGGCACAGGAGGAC---------------TAGATTTTTACGACGTTAGCCTCGTCGAC 399
At1g70250       ---GCTTCTTACGGTG---------------TCGACTATTATGTCGTCAACGTCTTCAAT 750
                                                 * *  ** *  ** *   ** *  *
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At1g200030      GGCTACAATGTCCAGATGCTGGTGGTTCCTCAAGGCGGCTCCG--------------GT 381
At1g75800       GGTTACAACGTCCCGATGCTAGTGGCTCCACAAGGAGGCTCGG--------------GT 441
At1g75050       GGTTACAATGTCGAGATGGGGATTAAACCTCAAGGAGGTTCC------------------ 468
At1g75030       GGTTACAATGTAAAGATGGGGATAAAACCACAGGGAGGATTC------------------ 435
At5g02140       GGTTTCAATCTTCCGGTGTCGATGAAACCAATCGGCGGTGGA------------------ 435
At5g24620       GGGTATAACCTTCCCATAGTTGCTCTCCCGACGGGTGGTGGAC----TGGTT-------- 450
At4g38660       GGTTACAACATTCCGATGATAGTTGAAGTCGCCGGTGGATCT-------GGG-------- 577
At4g24180       GGTTACAACGTTCCCATGTTGGTGGAAGCAAGTGGCGGGTCA-------GAA-------- 474
At1g19320       GGTTATAACGTTCAGATGAAAATCACACCGCAAGGTGGCTCA-------GGG-------- 456
At4g36010       GGTTACAACATCCCGATGACCATCGTCCCTCAGGGTGGAGGAGATGCCGGAGGCGTCGCC 459
At2g17860       GGTTACAACATCCCAATGACGATCGTTCCTTACGGCGGAGC---TATCGGAG------TC 450
At4g38670       GGTTACAATCTCCCGATGCTAATCTTGCCCAAGAAAATCGTCA--------------TT 444
At1g70250       GGTTACAACCTCCCTTTGCTTGTGACCCCCGAGAACA----------------------- 787
                ** *  **  *     *

At1g200030      CAGAATTGCAGCAGCACCGGCTGCGTCGTCGATTTAAACGGATCTTGCCCGTCGGAGCTA 441
At1g75800       TTAAACTGTAGCAGCACCGGATGCGTTGTAGATCTGAACGGTTCGTGTCCGTCGGAGCTT 501
At1g75050       GGTGATTGCCATTACGCCGGCTGCGTCGCCGACGTCAACGCGGTTTGCCCTAACGAGCTT 528
At1g75030       GGAAATTGCAAATACGCAGGCTGCGTCTCGGACATCAACGAGATTTGCCCTAGCGAGCTT 495
At5g02140       GTTGGATGTGGCGTTGCGGCTTGTGAAGTGAATTTGAACATTTGTTGTCC--ATCAGCTT 493
At5g24620       GGCGCTTGTAATGCTACAGGATGTGTTGCTGATATCAATATTAGCTGTCCAAAGGAGCTT 510
At4g38660       CA-GTGCGCTT--CTACCGGATGCACTACGGATCTTAACATTCAGTGCCCTGCTGAGCTG 634
At4g24180       GGTACTTGCTTAACCACGGGATGCGTAACGGATCTAAACCAAAAATGTCCAACGGAGCTC 534
At1g19320       GA---TTGTAAAACCGCGGGATGCGTTTCGGACGTGAACGCGATTTGTCCAAAAGAGCTA 513
At4g36010       GGAAACTGTACGACAACCGGCTGCGTTGCGGAGCTCAACGGTCCATGTCCTGCTCAGCTG 519
At2g17860       GGTAAGTGTAACGCAACGGGTTGCGCGGCGGATCTCAACGGCTTATGTCCGGAGCAGCTG 510
At4g38670       GGAGGATGCGGAGCCACCGGGTGTCTCGTGGACTTGAACGGCGCGTGTCCCAGAGACCTA 504
At1g70250       -AAAACTGCAGAAGCATTGAATGCGTTATTGACATGAACGAGACATGTCCCTCGGAGCTA 846
                       *          *  **        *  * **       ** **     * **

At1g200030      AGGGTGAATAGCGTCGACGGG---AAGGAGGCGGTGGCGATGGCGTGTAAAAGTGCGTGC 498
At1g75800       AAAGTGACGAGTTTAGACGGC---AGAGGTAAACAATCCATGGGATGTAAAAGCGCGTGT 558
At1g75050       CGGCTCATGGATCCACAT---------ACAGGAATCATCGCGGCGTGTAAAAGCGCTTGT 579
At1g75030       AGGATCATGGATCCGAAT---------TCGGGAAGTGTCGCGGCGTGTAAGAGCGCTTGC 546
At5g02140       TGGAAGTTAAG---AGAG---------ATGGGAAA-GTGGTTGGTTGTAAAAGTGCTTGC 540
At5g24620       CAAGTGATGGGAGAGGAAGAGGCTGAAAGAGGAGGGGTTGTTGCATGCAAGAGCGCTTGT 570
At4g38660       C--GTTTCGG------------------CGACGGA----GACGCTTGCAAAAGCGCGTGT 670
At4g24180       C--GGTTCGG------------------GTCCGGG----TCAGCATGCAAAAGCGCGTGT 570
At1g19320       CAAGTTACGGGC----------------CCATCGGGTGTTGCAGCGTGTAAGAGTGCTTGT 558
At4g36010       AAAGTGGCGA------CGACG------GGAGCTGAAGGAGTAGCTTGCAAAAGCGCGTGT 567
At2g17860       AAAGTAACGGTTGAAGCGGCT------GGAACGGGTGCTGTGGCGTGCAAGAGCGCTTGT 564
At4g38670       AAGCTGGTGACGCGTGGGAATGG------GAACGGT---GTTGCTTGCCGTAGCGCGTGC 555
At1g70250       ATGGTGAACAGTAGTGGCCTTGGA---TCGCACCATCCAATAGCCTGCATGACCACTTGT 903
                                                      *  **    *   * **

At1g200030      GAGGCGTTTCGGCAGCCGGAGTATTGCTGCAGCGGC------------GCGTTTGGATCG 546
At1g75800       GAAGCTTTTCGTACGCCGGAGTATTGTTGCAGCGGC------------GCCCACGGTACA 606
At1g75050       GCGGCGTTTAATTCTGAGGAGTTCTGTTGTACCGGT------------GCTCACGCGACG 627
At1g75030       GCGGCGTTTAGTTCGCCGGAATTTTGCTGTACTGGT------------GCTCACGCGACG 594
At5g02140       TTGGCGATGCAATCGGCTAAGTATTGTTGCACCGGT------------GAATATGCGAAT 588
At5g24620       GAGGCCTTTGGGTTGGACCAGTACTGTTGCAGCGGT------------CAGTTTGCTAAC 618
At4g38660       TTAGCGTTTCGGAGTCCTGAATACTGTTGCAGCGGC------------GCGTATGCTACA 718
At4g24180       GAGGCTTTTGGTAGTCCAGAATATTGTTGTAGTGGC------------GCGTACGCCTCA 618
At1g19320       GAGGCTTTTAACAAGCCGGAGTATTGTTGCACCGGC------------GCGTATAGTACA 606
At4g36010       GAAGCGTTTGGAACGCCGGAGTACTGCTGTAGCGGC------------GCGTTTGGAACG 615
At2g17860       GAGGCGTTTGGAACGCCGGAGTTTTGTTGTAACGGC------------GCGTTTGGAACG 612
At4g38670       GAGGCATTCGGAGACCCAAGGTATTGCTGCAGCGAT------------GCGTACGCCACG 603
At1g70250       CAGAGATACCAGTTGCCAGAGCTTTGCTGTATCGGACTCTCTTCTGGGGTGGTCGTACCA 963
                                        ** ** *  *

At1g200030      CCTGACACGTGTAAGCCGTCTACGTACTCGCGGATCTTCAAGAGCGCGTGTCCACGCGCC 606
At1g75800       CCTGACACGTGTAAACCGTCGTCGTACTCGTTGATGTTTAAAACTGCGTGTCCACGTGCT 666
At1g75050       CCGCAAACTTGTTCTCCGACGCATTACTCGGCGATGTTTAAAAGCGCTTGCCCTGGTGCT 687
At1g75030       CCGCAAACTTGTTCTCCGACGTATTACTCGTCCATGTTTAAGAACGCTTGTCCTAGCGCT 654
At5g02140       CCTCAGGCGTGTAAGCCGACTCTGTTCGCGAATCTGTTTAAAGCGATTTGTCCTAAAGCT 648
At5g24620       CCGACCACATGCCGACCGTCTTTATACTCTTCCATCTTCAAGAGAGCTTGTCCCAGAGCT 678
At4g38660       CCTTCTAGTTGTCGGCCTTCCGTGTATTCGGAGATGTTTAAAGCCGCGTGTCCTCGCTCC 778
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At4g24180       CCCACCGAGTGTAAACCATCGATGTACTCGGAGATATTCAAATCTGCGTGTCCAAGATCA 678
At1g19320       CCGGCGACTTGTCCGCCCACGAATTACTCGAAGATATTTAAACAAGCTTGTCCGAGTGCG 666
At4g36010       CCGGACACGTGTAAGCCAAGCGAGTACTCACAGTTTTTCAAAAACGCGTGCCCGAGAGCT 675
At2g17860       CCGGACACGTGTCAGCCGAGTGAGTACTCTGTTTTCTTTAAGAAAACTTGTCCGACGGCT 672
At4g38670       CCGGACACGTGTCAGCCTTCGGTTTACTCGCTCTTCTTTAAGCATGCGTGCCCACGAGCA 663
At1g70250       CCGGGGAATATGCAAGCGGACGATCTACTCGCGGACATTCAATAACGTGTGCCCAAGCGCT 1023
                **       **    *        *   *       ** **       ** **     *

At1g200030      TATAGCTACGCGTATGACGATAAGTCTAGTACATTTACGTGCGCGAAATCTCCCAACTAC 666
At1g75800       TACAGCTACGCTTACGATGATCAGAGTAGTACCTTCACATGTGCTGAATCTCCTAATTAC 726
At1g75050       TACAGTTACGCCTACGACGACGCCACGAGCACCTTCACTTGTACCGG---CTCTAACTAC 744
At1g75030       TATAGTTACGCTTACGACGACGCATCGAGCACTTTCACTTGTACCGG---ATCTAACTAC 711
At5g02140       TATAGTTATGCGTTTGATGATTCTAGTAGTTTGAATAAGTGTAGAGC---TTCTCGTTAT 705
At5g24620       TATAGCTATGCCTTCGATGATGGAACCAGTACTTTCACCTGCAAGGC---TTCTGAATAT 735
At4g38660       TATAGCTACGCTTACGACGATGCTACGAGCACTTTCACTTGCGCCGG---TGGAGATTAT 835
At4g24180       TATAGCTACGCGTTCGACGATGCTACAAGTACGTTCACATGCACTGA---TGCTGATTAC 735
At1g19320       TATAGCTATGCATACGATGATGCTTCCAGTACTTTTACTTGTACTAA---TGCTAATTAT 723
At4g36010       TATAGTTACGCTTACGACGACGGAACTAGTACCTTCACTTGTGGCGG---AGCTGATTAC 732
At2g17860       TATAGTTACGCTTATGACGACGGCACAAGCACTTTTACTTGCTCCGG---TGCTGATTAC 729
At4g38670       TACAGCTACGCCTATGACGATAAGACGAGCACTTACACTTGCGCCACCGGAGCCGATTAC 723
At1g70250       TATAGTTACGCCTACGACGTTGATAACAGCAGTTTCACTTGTCCAAACTTCTCCAACTTT 1083
                ** ** ** ** *  ** *        **       *  **                 *

At1g200030      GTCATCACTTTCTGTCCTTC-------TCCCAACACCAGCCTAAAATCAGCTGAGGAACA 719
At1g75800       GTTATCACGTTTTGCCCTAC-------TCCTAACACCAGTCAAAAATCATCTCAAGATCA 779
At1g75050       TTGATCTCTTTCTGCCCCA--------CCCGGA-ACTAA---------------------- 774
At1g75030       TTGATCACTTTCTGCCCTA--------CCCAGT-CTTAA---------------------- 741
At5g02140       GTTATCACTTTCTGTCCTC--------CAAAGTCATTGAATGAGAAACTGAGAAAATTTG 757
At5g24620       GCAATTATCTTCTGCCCTGG-------CAGGGTAAAAAGACCAGACAGTCTGAACTCGGA 788
At4g38660       ACGGTGACGTTTTGCCCTT----------CTTCCCCTAGTCAAAAATCGACTAGCTACTC 885
At4g24180       ACCATCACTTTTTGCCCTT----------CCTTGCCTAGGTAG----------------- 768
At1g19320       GAGATCAGTTTCTGTTCTT----------AG------------------------------ 744
At4g36010       GTCATCACTTTCTGTCCTT--------CTCCTAACCCAAGTGTGAAGAGTGCAACGAAGG 784
At2g17860       GTCATCACTTTCTGTCCTT--------CTCCT---TCAAG-GTAA--------------- 762
At4g38670       TTCATCATCTTCTGTCCGCC-------ACCGTAT-ACAAGCGAGAAGC--------TTTT 767
At1g70250       GTCATCACGTTTTGTCCGTCTAGCTCAACGGTCCCAGAGGCAGGAAACATCAATAGCTCA 1143
                    *    ** **  *

At1g200030      CAGCACAGAGACAATGACGACGACAA----------GTC-----CAAGTTCCG------- 757
At1g75800       GAGCCCAGATCCCAAACCGACGACACCAACCGGGACGTCGTCGACAACTCCTGCCGGAGA 839
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       AACTCAAAATAGAATCAAAATTTGGTCATTATACA-GTGGTTACCATCCTTTTAACTAGT 816
At5g24620       TCCTCCCAGCCCACCTCAGAATCAGTTAGGGCA-----ACCTATGGCTCCCCCAACTCAA 843
At4g38660       CCCTCCAGTGACAGACTCGTCGTCGACCTCCCAAG-GTTCCGATCCTGTGCCTGGTTCTG 944
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       GAGTCCAACCCGTAGCTGTTAGCTACTCTAAGGCA-TCTCCAAACGCGTCGCCAACTCTT 843
At2g17860       ------------------------------------------------------------
At4g38670       AGGATC---GAGGAAAGATGGTGCAACACTGCCACTAGTAAACAAGAGCATGATTCATTT 824
At1g70250       ACGGTCCCAGAGGCAGGAAACATCAAAACTGGAACAGAAGCAAAAGGAAATATTCCATTG 1203


At1g200030      --GCAGTACGACGTCGTCGCAG-----------ATGGTCTACGAAGGTGCTCTGGACGA 803
At1g75800       TAGTAGTACGACGTGGTCACCGGTAGATACATCAATGATATACGAAGGAGCTTTGGATCA 899
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       GTTCTAAAGACACAAGCTTTAGCTGGTGGGTCCAAAGACATAAAAGACGGTTTTAGTGCT 876
At5g24620       AATCAGTATGGTCAACCTATGGCTCCTGCACCTCAGTATCAGTACGGTCAACCTATGGCT 903
At4g38660       ATACTGGTTACGCTGGTCAGGGTCAACAGACTCCAGGTCAAGGTAATGTGTACGGGTCAC 1004
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       TCTGCCGTGTTTTTCGATTGGCGTTTTGGCTGTCGCGTCTTGGGTGATGCAACGCGTTTTG 903
At2g17860       ------------------------------------------------------------
At4g38670       -GCCGCATCCGCACAGCAGCTGA------------------------------------ 846
At1g70250       AGGTTAAAACTCATACTTGGAGTTTCATCAGTATTGGCTACAATGATCATTATTGTGATT 1263
```

```
At1g200030      AAGCAGTGGTTCACCATCTACGTATCACGGCGTATCACGAGCAATCACAGTCGTTCTTCT 863
At1g75800       AAACAAAGGATCACCGTCCACGTGTCATCTTTCGTTATGTGGAATCACAGTCACACTT-- 957
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       TACTCATGA---------------------------------------------------- 885
At5g24620       CCACCGACTCAAAATCAGTATGGTCAACCTCTGGCTCCTCCGACTCAAAATCAATACGAT 963
At4g38660       AGGGGACTGGGTCGGAGATGGGTACGGGTGAGACCATGTTACAAGACGGATCATGGATGG 1064
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       TGA---------------------------------------------------------- 906
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GTGGGAAAGGTTAGAGCAAATAATATGAGAAAGAGTGATTTGAATGAGAAGAACATGGAA 1323


At1g200030      CTCGCTTGCTTTTTGTCGTATGTGGTGGTTCTTCTGA----------------------- 900
At1g75800       -GCGCTGGCCTTTTGTCGGATGTGGCGGCTCTTTTGA----------------------- 993
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       CAACCTCTGGCTCCTCCGACTCAA--AATCAATATGGTCAACCTATGGCTCCACCAACTC 1021
At4g38660       CTGGTTTGGCCATGGGAGAGGCAAGCAGACCAGCCGGCGTTTCATTGACAGTGCTGCTTG 1124
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GCAGTGGTAATGTTGAAACGATTTAGTTATGTACAAGTCAAGAAGATGACAAAATCATTT 1383


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       CGCCCCCACCCGGACCAAACGAGGATTCAATGACTCCCCCACCTCAGAATCAGAATGGGG 1081
At4g38660       CTGCATTTACTTTTCCGTTTATTTTCTCGTAGCATCATCGTCTTTAGATTTGGTAGTCGT 1184
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GAGAATGTTCTTGGGAAAGGGGGATTTGGAACTGTCTATAAAGGGAAGTTACCTGATGGC 1443


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ATGGGCAGTTCATGCCTCCTCCCACTTTGGACCAAGATCCAAACGACCAGTATATGAATC 1141
At4g38660       TTTGTTAGCATTGCCTTTGTTTAGTTTTTGTTGATACTCGATGTTTATCGCTTAAGACTG 1244
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       AGCCGAGATGTTGCAGTGAAGATCTTGAAGGAGTCAAACGAGGATGGAGAAGACTTCATC 1503


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       CCCCAATTCAGGATCAGAGTCAAAGAGAGACTCAATCTTCATCCGATCTTCTTCGACCTT 1201
```

```
At4g38660       CGACAATTTGGGTTTTAGGCTTTTAGGGTTTTGCATTGTAGCTTTGTCGTGTACGCGCGT 1304
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       AATGAAATAGCTAGCATGAGTAGGACATCTCATGCTAATATCGTTTCTCTACTTGGATTC 1563

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ACCCTGTCTTGTTGCTTCTTGGCTTTAGTCTAACTGCTTTGAGGCAATCTGGCACGACAT 1261
At4g38660       GTGTGCTTGTATCATCTGTCATTGAAGATTGTTTATCATGTTCGAAATTGTGAGATATGC 1364
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       TGTTATGAAGGGAGGAAGAAAGCTATAATTTACGAGTTGATGCCGAATGGATCCCTCGAC 1623

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       GA---------------------------------------------------------- 1263
At4g38660       TTTGTGATTTAAAGAAAGATCTTTTGTGGCTTT---------------------------- 1397
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       AAGTTCATCTCCAAGAATATGTCGGCGAAGATGGAATGGAAAACGTTATACAACATTGCG 1683

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GTAGGTGTGTCTCATGGCCTAGAATACTTGCATAGCCATTGTGTATCAAGGATTGTACAT 1743

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       TTCGATATAAAGCCACAGAACATACTCATAGATGGAGACTTATGCCCTAAGATTTCGGAT 1803
```

```
At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       TTCGGTCTTGCTAAGCTTTGCAAAAATAATGAAAGCATCATTTCAATGCTACATGCAAGA 1863

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GGCACCATAGGGTACATTGCTCCAGAAGTGTTCTCCCAGAATTTTGGAGGAGTTTCTCAT 1923

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       AAGTCTGATGTGTATAGTTATGGAATGGTGGTTCTTGAGATGATTGGAGCAAGGAACATA 1983

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GGAAGAGCTCAAAATGCTGGATCCAGTAATACTTCAATGTACTTTCCAGATTGGATTTAT 2043

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
```

```
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       AAAGATCTTGAGAAAGGAGAAATCATGAGTTTTTTGGCAGATCAAATAACCGAAGAAGAA 2103

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GATGAGAAGATAGTAAAGAAAATGGTATTGGTGGGTCTGTGGTGTATTCAGACCAATCCA 2163

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       TATGATCGTCCACCAATGAGCAAAGTTGTTGAAATGTTAGAGGGAAGTCTAGAGGCTCTC 2223

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       CAGATTCCACCTAAGCCTCTTTTATGTTTACCCGCAATAACAGCTCCAATAACAGTTGAT 2283

At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
```

```
At1g70250       GAAGACATTCAAGAGACTTCAAGCTTCTTGAAACCAAGTCAAGATACTTCATATTATTCC 2343


At1g200030      ------------------------------------------------------------
At1g75800       ------------------------------------------------------------
At1g75050       ------------------------------------------------------------
At1g75030       ------------------------------------------------------------
At5g02140       ------------------------------------------------------------
At5g24620       ------------------------------------------------------------
At4g38660       ------------------------------------------------------------
At4g24180       ------------------------------------------------------------
At1g19320       ------------------------------------------------------------
At4g36010       ------------------------------------------------------------
At2g17860       ------------------------------------------------------------
At4g38670       ------------------------------------------------------------
At1g70250       GAACAAATAGTTCAAGATATTGTAGAAGAGAATCAAGATAGCTCAAGATCTTCCTAA 2400
```

6.8. Blasts for thaumatins

6.8.1 AT1G20030.1 Pathogenesis related thaumatin family protein, similar to receptor Serine/threonine kinase (PR5) [Arabidopsis thaliana]

```
Identities = 25/25 (100%)

Query: 61  attctgaccggagccgccttgagga 85
           |||||||||||||||||||||||||
Sbjct: 542 attctgaccggagccgccttgagga 518
```

6.8.2 AT1G75800 Arabidopsis thaliana unknown protein

```
Identities = 419/419 (100%)

Query  58  TTTTATCAAACGCCGGAGTTCCACCACTTCCGACGACCGGATTCGTTCTCCAAAAAGGCG 117
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 223  TTTTATCAAACGCCGGAGTTCCACCACTTCCGACGACCGGATTCGTTCTCCAAAAAGGCG 282

Query  118 AAGAACGAACAATCAGCGCTCCAACTTCATGGGGAGGAAGATTCTGGGGGAGAACTCAAT 177
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  283 AAGAACGAACAATCAGCGCTCCAACTTCATGGGGAGGAAGATTCTGGGGGAGAACTCAAT 342

Query  178  GTTCCACCGACACCGACGGAAAATTCACTTGTCTCACCGGAGATTGCGGATCTGGTACCC237
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  343  GTTCCACCGACACCGACGGAAAATTCACTTGTCTCACCGGAGATTGCGGATCTGGTACCC402

Query  238  TCGAATGCTCCGGATCCGGAGCAACACCACCAGCAACACTAGCGGAATTCACACTAGACG297
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   403 TCGAATGCTCCGGATCCGGAGCAACACCACCAGCAACACTAGCGGAATTCACACTAGACG462

Query 298   GATCTAACGGACTCGATTTCTACGACGTTAGTCTCGTCGACGGTTACAACGTCCCGATGC 357
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  463  GATCTAACGGACTCGATTTCTACGACGTTAGTCTCGTCGACGGTTACAACGTCCCGATGC 522

Query 358  TAGTGGCTCCACAAGGAGGCTCGGGTTTAAACTGTAGCAGCACCGGATGCGTTGTAGATC 417
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 523  TAGTGGCTCCACAAGGAGGCTCGGGTTTAAACTGTAGCAGCACCGGATGCGTTGTAGATC 582

Query 418  TGAACGGTTCGTGTCCGTCGGAGCTTAAAGTGACGAGTTTAGACGGCAGAGGTAAACAA 476
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 583  TGAACGGTTCGTGTCCGTCGGAGCTTAAAGTGACGAGTTTAGACGGCAGAGGTAAACAA 641
```

6.8.3 AT1G75050.1 thaumatin like protein, putative/ pathogenesis related protein, putative, similar to thaumatin like protein [Arabidopsis thaliana]

Identities = 403/407 (99%)

```
Query: 60  ttgctgtttccgccgccaccgtcttcactttacagaacagttgtccttacaccgtttggc 119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 117 ttgctgtttccgccgccaccgtcttcactttacagaacagttgtccttacaccgtttggc 176

Query: 120 ctggaattctctccggcaacgacaacaccctcggcgacggcggatttcctttaacaccag 179
           |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct: 177 ctggaattctctccggcaacgacaacaccctcggcgacggcggatttcccttaacaccag 236

Query: 180 gcgcttccgtacagctcacggctcctgcaggatggtctggacggttctgggctcgtaccg 239
           |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct: 237 gcgcttccgtacagctcacggctcctgcaggatggtcaggacggttctgggctcgtaccg 296

Query: 240 gctgcaacttcgacgcctccggccacggtaactgcggcaccggagactgcggcggtgttc 299
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 297 gctgcaacttcgacgcctccggccacggtaactgcggcaccggagactgcggcggtgttc 356

Query: 300 ttaaatgtaacggcggcggagttccaccagtcactctggctgaattcacacttgtaggcg 359
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 357 ttaaatgtaacggcggcggagttccaccagtcactctggctgaattcacacttgtaggcg 416

Query: 360 acggcggcagggatttctacgacgtgagtctcgtcgacggttacaatgtcgagatgggga 419
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 417 acggcggcaaggatttctacgacgtgagtctcgtcgacggttacaatgtcgagatgggga 476

Query: 420 ttaaaccccaaggaggttccggtgattgccattacgccggctgcgtc 466
           ||||||| |||||||||||||||||||||||||||||||||||||
Sbjct: 477 ttaaacctcaaggaggttccggtgattgccattacgccggctgcgtc 523
```

6.8.4 AT1G75030.1 Pathogenesis related thaumatin family protein, identical to thaumatin like protein [Arabidopsis thaliana]

Identities = 364/371 (98%)

```
Query: 59  ctctctccggcaacagcatcaccctcggcgacggcggatttcccttaactccaggcgctt 118
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 146 ctctctccggcaacagcatcaccctcggcgacggcggatttcccttaactccaggcgctt 205

Query: 119 ccgtgcagctcacggctcctaccggttggtcaggccggttctgggctcgtaccggctgca 178
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 206 ccgtgcagctcacggctcctaccggttggtcaggccggttctgggctcgtaccggctgca 265
```

```
Query: 179 actttgacgcctccggtcacggtacctgcgtcaccggagactgcggcggcgttttaaaat 238
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 266 actttgacgcctccggtcacggtacctgcgtcaccggagactgcggcggcgttttaaaat 325

Query: 239 gtaccggcggcggagttcctccagcgactctagcggaattcacccgtgggatcaagcaat 298
           |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct: 326 gtaccggcggcggagttcctccagcgactctagcggaattca-ccgtgggatcaagcaat 384

Query: 299 gccggcatggatttctacgacgtgagcctcgtcgacggttac-atgtaaagatggggat- 356
           |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct: 385 gccggcatggatttctacgacgtgagcctcgtcgacggttacaatgtaaagatggggata 444

Query: 357 aaaccacagggaggattcggaaattgcaaatacgcagcctgcgtctcgaacat-aacgaa 415
           |||||||||||||||||||||||||||||||||||||||| |||||||||| ||||  |
Sbjct: 445 aaaccacagggaggattcggaaattgcaaatacgcaggctgcgtctcggacatcaacg-a 503

Query: 416 gatttgcccta 426
           ||||||||||
Sbjct: 504 gatttgcccta 514
```

6.8.5 AT5G02140.1 thaumatin like protein, putative, similar to SP: P50699 thaumatin like protein precursor[Arabidopsis thaliana]

Identities = 474/474 (100%)

```
Query: 57  tggagctcagcttataatagtgaataactgccaggagagcatttggccgggaattctcgg 116
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 57  tggagctcagcttataatagtgaataactgccaggagagcatttggccgggaattctcgg 116

Query: 117 cggcggaggccaaatcacaccaagaaacggcggattccacatgggaagcggcgaagaaac 176
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 117 cggcggaggccaaatcacaccaagaaacggcggattccacatgggaagcggcgaagaaac 176

Query: 177 catcatagacgtaccggataaatggtccggtcgaatctggggtagacaaggctgcacctt 236
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 177 catcatagacgtaccggataaatggtccggtcgaatctggggtagacaaggctgcacctt 236

Query: 237 taaccaaaacggtaaaggctcatgccaaaccggagactgcaacgacggctctatcaactg 296
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 237 taaccaaaacggtaaaggctcatgccaaaccggagactgcaacgacggctctatcaactg 296

Query: 297 ccaaggaaccggcggtgtaccaccagcaaccgtagtagaaatgacattaggctcatcttc  356
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 297 ccaaggaaccggcggtgtaccaccagcaaccgtagtagaaatgacattaggctcatcttc 356

Query: 357 ctctccgcttcatttctacgacgtaagtctcgtcgacggtttcaatcttccggtgtcgat 416
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 357 ctctccgcttcatttctacgacgtaagtctcgtcgacggtttcaatcttccggtgtcgat 416
```

```
Query: 417 gaaaccaatcggcggtggagttggatgtggcgttgcggcttgtgaagtgaatttgaacat 476
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 417 gaaaccaatcggcggtggagttggatgtggcgttgcggcttgtgaagtgaatttgaacat 476

Query: 477 ttgttgtccatcagctttggaagttaagagagatgggaaagtggttggttgtaa 530
           ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 477 ttgttgtccatcagctttggaagttaagagagatgggaaagtggttggttgtaa 530
```

6.8.6 AT5G24620.1 thaumatin like protein, putative, similar to thaumatin like protein [Arabidopsis thaliana]

Identities = 502/505 (99%)

```
Query: 60  ccactacccaccacagggttcaggcttgatgtgggacagtctgttagaattcccacggct 119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 295 ccactacccaccacagggttcaggcttgatgtgggacagtctgttagaattcccacggct 354

Query: 120 ctaggctggtcaggtcgtatttgggctaggactggctgcaactttgatgctaacggcgcg 179
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 355 ctaggctggtcaggtcgtatttgggctaggactggctgcaactttgatgctaacggcgcg 414

Query: 180 ggaaagtgtatgactggagactgcggcggaaagctggagtgtgctggtaatggcgctgct 239
           ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 415 ggaaaatgtatgactggagactgcggcggaaagctggagtgtgctggtaatggcgctgct 474

Query: 240 ccgccgacatctcttttcgagatcacactcggtcacggttctgatgacaaagatttctat 299
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 475 ccgccgacatctcttttcgagatcacactcggtcacggttctgatgacaaagatttctat 534

Query: 300 gatgtcagtctcgttgatgggtataaccttcccatagttgctctcccgacgggtggtgga 359
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 535 gatgtcagtctcgttgatgggtataaccttcccatagttgctctcccgacgggtggtgga 594

Query: 360 ctggttggcgcttgtaatgctacaggatgtgttgctgatatcaatattagctgtccaaag 419
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 595 ctggttggcgcttgtaatgctacaggatgtgttgctgatatcaatattagctgtccaaag 654

Query: 420 gagcttcaagtgatgggagaggaagtggctgaaagaggaggggttgttgcatgcaagagc 479
           |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct: 655 gagcttcaagtgatgggagaggaagaggctgaaagaggaggggttgttgcatgcaagagc 714

Query: 480 gcttgtgaggcctttgggttggaccagtgctgttgcagcggtcagtttgctaacccgacc 539
           |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct: 715 gcttgtgaggcctttgggttggaccagtactgttgcagcggtcagtttgctaacccgacc 774

Query: 540 acatgccgaccgtctttatactctt 564
           |||||||||||||||||||||||||
Sbjct: 775 acatgccgaccgtctttatactctt 799
```

6.8.7 AT4G38660.2: Similar to pathogenesis-related thaumatin family protein [Arabidopsis thaliana]

Identities: 447/451 (99%)

```
Query: 58  tcgaatgctggctcacctactctctccaccaccggatttgagctccctaaaggcacctct 117
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 157 tcgaatgctggctcacctactctctccaccaccggatttgagctccctaaaggcacctct 216

Query: 118 cgctctctccaagctcccaccggttggtccggtcgattctgggctcgtaccggttgcaag 177
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 217 cgctctctccaagctcccaccggttggtccggtcgattctgggctcgtaccggttgcaag 276


Query: 178 ttcgacagttctggctccggtacatgcaaaaccggtgactgcggctccaacgctgtcgaa 237
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 277 ttcgacagttctggctccggtacatgcaaaaccggtgactgcggctccaacgctgtcgaa 336


Query: 238 tgcgccgggcttggtgccgcaccgcctgtaactctcgcggagttcactctaggcactggc 297
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 337 tgcgccgggcttggtgccgcaccgcctgtaactctcgcggagttcactctaggcactggc 396


Query: 298 ggcgatgatttctacgacgttagtctcgtcgacggttacaacattccgatgatagttgaa 357
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 397 ggcgatgatttctacgacgttagtctcgtcgacggttacaacattccgatgatagttgaa 456

Query: 358 gtcgccggtggatctgggcagtgcgcttctaccggatgcactacggatcttaacattcag 417
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 457 gtcgccggtggatctgggcagtgcgcttctaccggatgcactacggatcttaacattcag 516

Query: 418 tgccctgctgagctgcgtttcggcgac-gagacgcttgcaaaagcgcgttgtttagcgtt 476
           |||||||||||||||||||||||||| |||||||||||||||||| ||||||||||
Sbjct: 517 tgccctgctgagctgcgtttcggcgacggagacgcttgcaaaagcgcg-tgtttagcgtt 575

Query: 477  tc-gaatcctgaatactgttgcagcggcgcg 506
             || || ||||||||||||||||||||||||
Sbjct: 576  tcggagtcctgaatactgttgcagcggcgcg 606
```

6.8.8 AT4G24180.1 Similar to thaumatin, putative [Arabidopsis thaliana]

Identities = 445/469 (94%)

```
Query: 58  atcgatggtttacactcggtgggtgaggcgtacgcgccactacaacaatattctggacta 117
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 734 atcgatggtttacactcggtgggtgaggcgtacgcgccactacaacaatattctggacta 675
```

```
Query: 118 ccaaaagcctcacacgcgcttttgcatgctgacccggacccgaaccggagctccgttgga 177
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 674 ccaaaagcctcacacgcgcttttgcatgctgacccggacccgaaccggagctccgttgga 615

Query: 178 catttttggtttagatccgttacgcatcccgtggttaagcaagtaccttctgacccgcca 237
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 614 catttttggtttagatccgttacgcatcccgtggttaagcaagtaccttctgacccgcca 555


Query: 238 cttgcttccaccaacatgggaacgttgtaaccgtccacgaggcttacgtcgtagaagtct 297
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 554 cttgcttccaccaacatgggaacgttgtaaccgtccacgaggcttacgtcgtagaagtct 495


Query: 298 tgtttacgggccgggtctgctggacctgacccgattgtgaattcggcgagtgtagcgggc 357
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 494 tgtttacgggccgggtctgctggacctgacccgattgtgaattcggcgagtgtagcgggc 435

Query: 358 ggttttgctcctgcaccgtttacatttcgacctgg-tagagccgcagtccccggtttagg 416
           |||||||||||||||||  |||||  |||||||||  |||||||||||||||||  |||||
Sbjct: 434 ggttttgctcctgcaccg-ttaca-ttcgacctggttagagccgcagtccccgg-ttagg 378

Query: 417 caggtgcccttgtccctgtgtctgatttgaaagtttgcaccggtttctggccaaaaaccg 476
           ||||||  ||||||  ||||||||||  |||||  |||||  |  |  |||  |||  |||||||
Sbjct: 377 caggtg-ccttgt-cctgtgtctgagttgaa—gttgcaaccggttcgtgcccaaaaccg 322

Query: 477 cccttaccaactatccctgaagcttggaaccaacgtgaaccaccgggaa 525
           |||  ||||  |||  |||  |||||||||  |||||||||||||||||
Sbjct: 321 acctgaccagcta--gctggagcttggaacgaacgtgaaccaccgggaa 275
```

6.8.9 AT1G19320.1 Pathogenesis related thaumatin family protein, similar to SP: P28493 pathogenesis related protein 5 precursor (PR-5) from [Arabidopsis thaliana]

Identities = 566/569 (99%)

```
Query: 60  tcttcgagtaattcgtgggcggacaagtcgccggtgtactatacgcgccggtgcaacaat 119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 686 tcttcgagtaattcgtgggcggacaagtcgccggtgtactatacgcgccggtgcaacaat 627

Query: 120 actccggcttgttaaaagcctcacaagcactcttacacgctgcaacacccgatgggcccg 179
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 626 actccggcttgttaaaagcctcacaagcactcttacacgctgcaacacccgatgggcccg 567

Query: 180 taacttgtagctcttttggacaaatcgcgttcacgtccgaaacgcatcccgcggttttac 239
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 566 taacttgtagctcttttggacaaatcgcgttcacgtccgaaacgcatcccgcggttttac 507

Query: 240 aatcccctgagccaccttgcggtgtgattttcatctgaacgttataaccatctacgaggc 299
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 506 aatcccctgagccaccttgcggtgtgattttcatctgaacgttataaccatctacgaggc 447
```

```
Query: 300 tcacgtcgtaaaaatcttgcacggcgtttttctttccggaggagccgattgtgaattcgg 359
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 446 tcacgtcgtaaaaatcttgcacggcgtttttctttccggaggagccgattgtgaattcgg 387

Query: 360 cgagtgtggctggtggagctcctcctgcgccggcgcattttaacttgctgccgcagtccc 419
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 386 cgagtgtggctggtggagctcctcctgcgccggcgcattttaacttgctgccgcagtccc 327

Query: 420 cggtaccgcatttacctgagcctgaggcgtcgaagttacaatacgttcgagcccaaaatc 479
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 326 cggtaccgcatttacctgagcctgaggcgtcgaagttacaatacgttcgagcccaaaatc 267

Query: 480 gaccggaccaacctggagaaacggtgaaggttacggaagatcctgaagctaa-gcaaatc 538
           |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||| 
Sbjct: 266 gaccggaccaacctggagaaacggtgaaggttacggaagatcctgaagctaaggcaaatc 207

Query: 539 cgccgtcgccgagttgtgcgccgtttgc-gtgaggattccaggccagattgtggagccgc 597
           |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct: 206 cgccgtcgccgagttgtgcgccgtttgcggtgaggattccaggccagattgtggagccgc 147

Query: 598 aatggtttgtt-aggtgaaggttgtaccg 625
           |||||||||||| ||||||||||||||||
Sbjct: 146 aatggtttgttaaggtgaaggttgtaccg 118
```

6.8.10 AT4G36010.2 Similar to pathogenesis related thaumatin family protein [Arabidopsis thaliana]

Identities = 426/432 (98%)

```
Query: 61  ggaacctctcctttacctacaaccggattctccttaaaccctaccgagacacgagttatc 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 285 ggaacctctcctttacctacaaccggattctccttaaaccctaccgagacacgagttatc 344

Query: 121 ccgatccccgcagcttggtccggtcgtatttggggtcgtactctctgcacacaagacgca 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 345 ccgatccccgcagcttggtccggtcgtatttggggtcgtactctctgcacacaagacgca 404

Query: 181 accaccggaagattcacttgcatcaccggggactgtggttcttccaccgtagaatgctcc 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 405 accaccggaagattcacttgcatcaccggggactgtggttcttccaccgtagaatgctcc 464

Query: 241 ggatccggtgctgcacctccggctacactcgctgaattcaccttaaacggagctaatggt 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 465 ggatccggtgctgcacctccggctacactcgctgaattcaccttaaacggagctaatggt 524

Query: 301 ctcgatttctacgacgtcagtctcgtcgacggttacaacatcccgatgaccatcgtccct 360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 525 ctcgatttctacgacgtcagtctcgtcgacggttacaacatcccgatgaccatcgtccct 584

Query: 361 cagggtggaggagatgccggaggcgtcgccggaaactgtacgacaaccggctgcgttgcg 420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 585 cagggtggaggagatgccggaggcgtcgccggaaactgtacgacaaccggctgcgttgcg 644
```

```
Query: 421 gagctcaacggtccatgtcctgctcagctgaaagtggccacaac-ggagctgaaaggata 479
           |||||||||||||||||||||||||||||||||||| || || ||||||||| |  ||
Sbjct: 645 gagctcaacggtccatgtcctgctcagctgaaagtggcgacgacgggagctgaaggagta 704

Query: 480 gcttgcaaaagc 491
           ||||||||||||
Sbjct: 705 gcttgcaaaagc 716
```

6.8.11 AT2G17860.1 Pathogenesis related thaumatin family protein, similar to receptor Serine/threonine kinase (PR5) [Arabidopsis thaliana]

Identities = 230/231 (99%)

```
Query: 58  caccgctccattacccaccactggtttctccctcaactcctttgagtcacgactcatctc 117
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 132 caccgctccattacccaccactggtttctccctcaactcctttgagtcacgactcatctc 191

Query: 118 catacccgccgcttggtccggccgcatatggggacgcacgctctgcaaccaaaacgaagt 177
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct: 192 catacccgccgcttggtccggccgcatatggggacgcacgctctgcaaccaaaacgaaat 251

Query: 178 caccggaatattcacttgcgtcaccggcgactgcggctcatcccaaatcgaatgctctgg 237
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 252 caccggaatattcacttgcgtcaccggcgactgcggctcatcccaaatcgaatgctctgg 311

Query: 238 cgcaggagcaatccctccagctacactagccgaattcacactcaacggcgc 288
           ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 312 cgcaggagcaatccctccagctacactagccgaattcacactcaacggcgc 362
```

6.8.12 AT4G38670 Arabidopsis thaliana unknown protein

Identities = 525/525 (100%)

```
Query 59   GAAGAGCGAGTAAACCGAAGGCTGACACGTGTCCGGCGTGGCGTACGCATCGCTGCAGCA 118
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct764   GAAGAGCGAGTAAACCGAAGGCTGACACGTGTCCGGCGTGGCGTACGCATCGCTGCAGCA 705

Query 119  ATACCTTGGGTCTCCGAATGCCTCGCACGCGCTACGGCAAGCAACACCGTTCCCATTCCC 178
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 704  ATACCTTGGGTCTCCGAATGCCTCGCACGCGCTACGGCAAGCAACACCGTTCCCATTCCC 645

Query 179  ACGCGTCACCAGCTTTAGGTCTCTGGGACACGCGCCGTTCAAGTCCACGAGACACCCGGT 238
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SbjCt 644   ACGCGTCACCAGCTTTAGGTCTCTGGGACACGCGCCGTTCAAGTCCACGAGACACCCGGT 585

Query 239 GGCTCCGCATCCTCCAATGACGATTTTCTTGGGCAAGATTAGCATCGGGAGATTGTAACC 298
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 584 GGCTCCGCATCCTCCAATGACGATTTTCTTGGGCAAGATTAGCATCGGGAGATTGTAACC 525

Query 299  GTCGACGAGGCTAACGTCGTAAAAATCTAGTCCTCCTGTGCCGTTGAGAGTAAACTCGGC 358
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 524  GTCGACGAGGCTAACGTCGTAAAAATCTAGTCCTCCTGTGCCGTTGAGAGTAAACTCGGC 465
```

```
 Query 359  GAGAGTTGCTGGAGGTTTAGCTCCGGAGCCTGAACATTCGACTTTTCCCGAGCCGCAATC 418
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct 464  GAGAGTTGCTGGAGGTTTAGCTCCGGAGCCTGAACATTCGACTTTTCCCGAGCCGCAATC 405

 Query 419  TCCGGTTAAGCAGACGAAAGAACCGGAACTCCGATCTTGTGAACACAAGGTTCGTGCCCA 478
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct 404  TCCGGTTAAGCAGACGAAAGAACCGGAACTCCGATCTTGTGAACACAAGGTTCGTGCCCA 345

 Query 479  GAGACGACCGGACCACGACTCTGGTATTGCGACGGTTTTAGATTTTCCTCTAGAGAGACG 538
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 Sbjct 344  GAGACGACCGGACCACGACTCTGGTATTGCGACGGTTTTAGATTTTCCTCTAGAGAGACG 285

 Query  539  AAAGCCAGTTGTGGGGAGCGGCGCTGAGTTAGCACCAGAGAGCAA  58
              ||||||||||||||||||||||||||||||||||||||||||||
 Sbjct 284  AAAGCCAGTTGTGGGGAGCGGCGCTGAGTTAGCACCAGAGAGCAA 328
```

6.8.13 AT1G70250 Arabidopsis thaliana ATP binding/ protein kinase/ protein serine/threonine kinase/ protein-tyrosine kinase/ transmembrane receptor protein serine.

Identities = 452/452 (100%)

```
Query 59   CTGCAACCTACGGCTATAGGAGATCACTAGAAACAACCGGTTTTGTTCTTGAGAAAGGAG  118
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 486  CTGCAACCTACGGCTATAGGAGATCACTAGAAACAACCGGTTTTGTTCTTGAGAAAGGAG 545

Query 119  AGACGCGTACCATCAAAGCGCCGTCATCATGGATAGGTCGTTTCTGGGGTAGGACACTCT 178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 546  AGACGCGTACCATCAAAGCGCCGTCATCATGGATAGGTCGTTTCTGGGGTAGGACACTCT 605

Query 179  GCTCCACCAACTCAACAGGGGGTTTCTCATGTGCCACGGGAGACTGCACCTCCGGAAAAA 238
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 606  GCTCCACCAACTCAACAGGGGGTTTCTCATGTGCCACGGGAGACTGCACCTCCGGAAAAA 665

Query 239  TCAAGTGCCTCGGTATCCCCATAGATCCAACAACAGTGGTCGAGTTTAACCTCGCTTCT  298
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 666  TCAAGTGCCTCGGTATCCCCATAGATCCAACAACAGTGGTCGAGTTTAACCTCGCTTCT 725

Query 299  ACGGTGTCGACTATTATGTCGTCAACGTCTTCAATGGTTACAACCTCCCTTTGCTTGTGA 358
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 726  ACGGTGTCGACTATTATGTCGTCAACGTCTTCAATGGTTACAACCTCCCTTTGCTTGTGA 785

Query 359  CCCCCGAGAACAAAAACTGCAGAAGCATTGAATGCGTTATTGACATGAACGAGACATGTC 418
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 786  CCCCCGAGAACAAAAACTGCAGAAGCATTGAATGCGTTATTGACATGAACGAGACATGTC 845

Query 419  CCTCGGAGCTAATGGTGAACAGTAGTGGCCTTGGATCGCACCATCCAATAGCCTGCATGA 478
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 846  CCTCGGAGCTAATGGTGAACAGTAGTGGCCTTGGATCGCACCATCCAATAGCCTGCATGA 905
```

```
Query 479   CCACTTGTCAGAGATACCAGTTGCCAGAGCTT  510
            ||||||||||||||||||||||||||||||||
Sbjct 906   CCACTTGTCAGAGATACCAGTTGCCAGAGCTT  937
```

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Walid Wahid Ali

Curriculum Vitae

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Name: Walid Wahid Nazir Ali
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Marital status: Married

Education

2003-2007 Ph.D. student at University of Würzburg, Germany
Research project: Screening of plant suspension cultures for antimicrobial activities and characterization of antimicrobial proteins from *Arabidopsis thaliana.*
Supervisor: Prof. Dr. Thomas Roitsch

1994-2000 Master of Science
Botany Department, Faculty of Science, El-Minia, Egypt
Research project: Biodegradation of chicken feather by some keratinolytic fungi.
Supervisors: Prof. Dr. M.A. Elnaghy
Prof. Dr. E. M. Fadl-Allah
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1993-1994 Postgraduate studies for the partial fulfillment of the M.Sc. degree in the department of Botany, Faculty of Science, Minia University, El-Minia, Egypt.

1988-1992 Bachelor of Science
Department of Botany, Faculty of Science, Minia University, El-Minia, Egypt.

1985-1988 Mallawi Secondary School

Scholarship

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Language

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation in allen Teilen selbst angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ich habe die Dissertation weder in gleicher noch in ähnlicher Form in anderen Prüfungsverfahren vorgelegt.

Ich erkäre weiterhin, dass ich bislang noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, Mai 2007

.....