Analysis of Inducible Anti-Herbivore Defenses and Signals in *Populus*

by

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

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ABSTRACT

Plants are continuously subjected to biotic stresses such as herbivory and pathogens. Consequently they have evolved many defense mechanisms. Inducible defenses that are activated only after insect infestation are one type of plant adaptation to herbivory. Many plant species possess arrays of inducible defenses, including the accumulation of toxic phytochemicals and antinutritive proteins that function to deter herbivory. Inducible defenses are generally activated at the transcriptional level and they can occur at the whole plant level, which presumably protects the plant from future herbivory.

The genus *Populus*, which includes both aspens and poplars, is an important tree for forestry but often undergoes severe defoliation by herbivores. Outbreaks of forest tent caterpillar (*Malacosoma disstria*, FTC) and the subsequent massive defoliation of its natural host, trembling aspen (*Populus tremuloides* Michx.), are known to periodically occur in North America. Within aspen populations, however, individual clones show variation in susceptibility to FTC, and this suggests the importance of innate defenses of aspen. Although it has been known that aspen leaves contain phenolic phytochemicals as defensive compounds, the involvement of defensive proteins was not known when this work began. Therefore, one aim of this study was to investigate protein-based induced defenses in trembling aspen, using a molecular approach.

In order to initiate investigation of protein-based induced defenses in trembling aspen, genes for polyphenol oxidase (PPO) and trypsin inhibitor (TI), known defense-related genes in other plant species, were isolated and characterized. Both PPO and TI were transcriptionally activated in aspen foliage by FTC herbivory, artificial tissue damage, and methyl jasmonate, a signal molecule for inducible defenses. In time course analyses, it was demonstrated that PPO and TI mRNAs accumulated within several hours in both wounded leaves and unwounded leaves of the same plant. This was consistent with the wound response previously reported from other plant species including hybrid poplar (*Populus trichocarpa x P. deltoides*), and is indicative of the presence of signaling mechanisms for systemic induction of defense proteins in trembling aspen.

To further obtain insight into mechanisms for inducible defenses, signal molecules for induction of defenses were investigated using a model system, poplar suspension cultures, based on the observation that plant cell cultures often show rapid alkalinization of the medium in response to defense-related signal molecules. Using the alkalinization assay system, two different alkalinization factors were purified from poplar leaf extracts. First, three 5 kD peptides causing rapid alkalinization, the rapid alkalinization factors (RALFs), were isolated and further characterized at the molecular level. RALF appears to be a novel hormone-like peptide that was also recently characterized from tobacco. In contrast to other known alkalinization factors, RALF did not induce defenses such as the expression of phenylalanine ammonia lyase. Based on the expression profile of RALF genes, it was predicted that RALF may be involved in general cellular signaling such as growth and development rather than defense signaling.

A second alkalinization peptide causing slower alkalinization, slow alkalinization factor (SALF), was also isolated and partially sequenced by Edman degradation. Database searches of the obtained peptide sequence revealed that SALF seems to be derived from the N-terminus of a known protein, photosystem I centre protein subunit D. Although it is not yet clear whether the SALF peptide is a defense-related signal in poplar, it is hypothesized that this breakdown product of a known protein may act as a biologically active signal in plants.

Overall, this thesis presents: 1) the first demonstration of protein-based inducible defenses in trembling aspen at molecular level; 2) the discovery of novel peptide molecules with alkalinization activity in suspension cultures of poplar cells.

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ABA:	abscisic acid
AOC:	allene oxide cyclase
AOS:	allene oxide synthase
ATP:	adenosine 5'-triphosphate
bp:	base pairs
BLAST:	basic local alignment search tool
cDNA:	complementary DNA
CH_3CN:	acetonitrile
CHS:	chalcone synthase
DFR:	dihydroflavonol reductase
DNA:	deoxyribonucleoside triphosphate
ESI:	electrospray ionization
EST:	expressed sequence tag
EtBr:	ethidium bromide
FTC:	forest tent caterpillar
HCl:	hydrochloric acid
HPLC:	high performance liquid chromatography
JA:	jasmonic acid
KCl:	potassium chloride
KOH:	potassium hydroxide
MALDI-MS:	matrix assisted laser desorption ionization-mass spectrometry
MeJa:	methyl jasmonate
MeOH:	methanol
mRNA:	messenger RNA
MS medium:	Murashige and Shoog medium
LiCl:	lithium chloride
LOX:	lipoxygenase
NaCl:	sodium chloride
NCBI:	National Center for Biotechnology Information
NO:	nitric oxide
PAL:	phenylalanine ammonia lyase
PCR:	polymerase chain reaction
PI:	proteinase inhibitor
PPO:	polyphenol oxidase
Pmg:	*Phytophthora megasperma* elicitor
PS:	photosystem
RALF:	rapid alkalinization factor
RNase:	ribonuclease
rpm:	rotations per minute
SA:	salicylic acid
SALF:	slow alkalinization factor
SCX:	strong cation exchange
SDS:	sodium dodecyl sulphate
SR:	systemin receptor

SSC:	saline sodium citrate
TFA:	trifluoroacetic acid
TI:	trypsin inhibitor
UTR:	untranslated region
Vsp:	vegetative storage protein
WAX:	weak anion exchange

ACKNOWLEDGMENTS

I would like to extend my greatest appreciation to Dr. Peter Constabel for his guidance and funding to carry out this research work and his direction to hone my skills for good science.

I thank my graduate study committee members at University of Victoria for their roles: Dr. Bob Olafson for advising strategies to characterize peptides, Dr. Patrick von Aderkas for generously providing the HPLC system, the rotary evaporator system and space for inoculating cultures, and Dr. Francis Choy for general discussions. I also thank my former graduate study committee members at University of Alberta for their supervisory: Dr Susan Jensen for consulting procedures of peptide purification, Dr. Joceryn Ozga for discussing plant hormones, Dr. Allen Good and Dr. David Gifford for helping to practice scientific communication skills.

I also would like to show my appreciation to Gregory Pearce and Dr. Clarence Ryan, Washington State University, for discussing peptide purification and sharing their data prior to publication.

This work was carried out with the assistance from many people who kindly helped me. I appreciate Barry McCashin, University of Alberta, for HPLC maintenance, Dr. Randy Whittal, University of Alberta, for discussing mass spectrometry analyses, Dr. Mary Christopher, and Dr. Lynn Yip for technical assistance for molecular techniques, Brett Polis and Scott Schultz, University of Victoria, for HPLC assistance, Sean Brendall and Derek Smith for tutoring mass spectrometry analysis, and Darryl Hardie for discussing general peptide chromatography.

I appreciate the help of other graduate students in the lab, Joe Patton and Darren Peters for proofreading my writing, general discussion for conducting research and providing experimental materials, Jiehua Wang and Ian Major for maintenance of plant materials and sharing life of my graduate study. I also appreciate many people whom I could interact in Constabel lab and learnt many things: Mary and Charlotte for helping western blot, Trung, Sean, Jody, Jennifer, Naomi, and Cory for their delightfulness, Robin and Manoela for their remarkable enthusiasm for science.

Lastly, I would like to show my best appreciation to Daniel Bushey for his patience continuous encouragement, and love.

Chapter 1

Introduction

Induced biochemical defense is an important adaptive mechanism of plants to insect herbivory. In response to tissue damage caused by herbivory, plants develop resistance by producing anti-herbivore defensive molecules (Karban and Baldwin, 1997). These defensive molecules can be proteins or small organic compounds of plants, called phytochemicals. The induced defense occurs at a whole-plant level, which is called systemic induced defense. In systemic induced defense, local wounding by herbivory is recognized as a cue and the wound stimuli are signaled to the surrounding areas and further distal parts of the plant, where defense mechanisms are then also activated. Though systemic inducible defenses are observed in a broad range of plant species, signals that systemically convey wound stimuli have not been established in general. The peptide hormone, systemin, and the lipid-derived phytohormone, jasmonate, are proposed to be primary signals for systemic wound-signaling in tomato (*Lycopersicon esculentum*), in which induced defenses have been intensively investigated (Ryan and Moura, 2002). In other plant species, however, the nature of systemic signals is not understood.

Populus spp including aspen and poplar, are widely distributed in North America and are commonly consumed by herbivorous insects. The ecologically important species trembling aspen (*Populus tremuloides* Michx.) is a known host of forest tent caterpillar and regularly experiences severe defoliation. Extensive studies by Lindroth and coworkers have shown that trembling aspen possesses toxic phytochemicals that are constitutively present in foliage and accumulate more upon herbivory and wounding (Lindroth and Hwang, 1996). Wound accumulation of defense-related phytochemicals, condensed tannin, in trembling aspen but less in hybrid poplar makes trembling aspen a unique experimatl system to investigate phytochemicals as anti-herbivore defenses in *Populus* (Peters and Constabel, 2002). Lindroth's studies exclusively focused on phytochemical-based defenses and did not include protein-based induced defenses that are expected to be important parts of induced defenses in trembling aspen. In order to expand our knowledge in induced defenses in this tree species, this study was the first to characterize inducible protein-based defenses in trembling aspen. This study adds new

insights, inducible peortein-based defenses, into anti-herbivore defenses in trembling aspen.

In contrast to trembling aspen, hybrid poplar (*Populus trichocarpa x P. deltoides*) that is generated during the breeding program is important in tree plantations and is often used as an experimental model species for studying deciduous trees (Eckenwalder, 2001). Research on induced defenses in hybrid poplar was initiated in the 1980's by M. Gordon and coworkers using molecular approaches (Bradshaw et al., 1991). Their studies showed that several genes are systemically induced upon wounding, indicating the presence of systemic signals. Characterizing primary signals for wound-inducible defenses was a major interest in hybrid poplar at this point. Induction patterns of systemically induced defenses in hybrid poplar are stronger than trembling aspen (Christopher and Constabel, unpublished), and comparable to that in tomato in which several signals for induced defenses have been characterized. Therefore, it was hypothesized that hybrid poplar is likely to use similar molecules to transmit herbivore-wound signals. The second phase of this research program focused on surveying signals involved in wound signaling, specifically the isolation of peptides with signal-like functions from hybrid poplar.

In conclusion, this thesis deals with induced defenses in two different *Populus* species, molecular characterization of protein-based induced defenses in trembling aspen and potential signals for wound signaling in hybrid poplar.

1.1. Overview of Plant Induced Defenses against Herbivory

Plants, as stationary organisms, are often subject to herbivore damage, they consequently evolved defense mechanisms to resist herbivory (Gatehouse, 2002, Agrawal, 1998). Plant anti-herbivore defenses involve biochemical and physical traits, and their distributions depend on plant species and their tissue types (Hammerschmidt and Schultz, 1996). Anti-herbivore defenses may be always expressed (constitutive defense) or activated by insect herbivory (induced defense). Induced defenses that are rapidly activated after herbivory are dynamic responses involving changes in gene expression and metabolic processes which help to adapt to herbivory. Major types of inducible defense are accumulation of proteins with defensive functions and toxic

phytochemicals (Constabel, 1999). The two defense systems may differ in their speed of induction and specificities against different herbivores. Although enormous numbers of toxic phytochemicals are known to date, demonstration of phytochemical accumualtion as a result of herbivory is limited. On the other hand, induction of proteins with insecticidal activity is more characterized due to their standardized analytical methods and applicability for biotechnology. In addition to these two types biochemical defenses, it has recently been recognized that plants use indirect defenses at a third level by releasing volatile phytochemicals that attract insect predators and parasitoids (De Moraes, 1998). In the following section, proteins and phytochemicals with anti-herbivorous activities are reviewed with respect to their defensive roles against herbivory. Additionally, the kinetics of induced defense and signals involved in regulation of induced defenses are discussed later in this section. The plant-wide, or systemic, aspects of defense signaling will be discussed in section 1.2.

1.1.1. Proteins Demonstrating Insectisidal Activities or Relating to Defenses against Herbviory

Biochemical and molecular studies have identified components of protein-based inducible defenses from various plants. Several plant-derived proteins are found to demonstrate anti-herbivore activities (reviewed in Carlini and Grossi de Sá, 2002). Protease inhibitors (PIs) are among the most characterized group of proteins with anti-herbivore defensive functions (Ryan, 1990). PIs are considered to exhibit anti-herbivore functions by inhibiting proteolytic enzymes in the herbivore gut, causing oversecretion of digestive enzymes and subsequent anti-nutritive effects on the herbivores. Ingestion of high concentration of PIs lead to reduced growth rates in some insect species (Broadway et al., 1986). The study of PIs as a part of inducible defense was first carried out in tomato and it demonstrated that PIs accumulate in response to herbivory within hours after damage (Green and Ryan, 1972). In further molecular studies, genes encoding PIs were shown to be induced in response to wounding in many plant species (reviewed in Constabel, 1999; see Chapter 3 for further detail of PIs).

In addition to PIs, the involvement of oxidative enzymes in defense has been recognized primarily through the studies of Duffey and coworkers (Felton et al., 1989;

Duffey and Felton, 1991). Oxidative enzymes are proposed to demonstrate defensive roles by modifying or destroying nutrients through oxidative processes. Polyphenol oxidase (PPO) and peroxidase modify proteins through oxidation of phenolics, resulting in poor digestivity of proteins and thus reduced availability of essential amino acids for herbivores (see Chapter 2 for further details of PPO). Other oxidative enzymes include lipoxygenase, which modifies unsaturated fatty acids and generates free radicals (Duffey and Felton, 1991; Siedow, 1991). All these oxidative enzymes are known to be induced by herbivore damage (Thaler et al., 1996).

An additional group of proteins with anti-herbivore functions are the proteases. In corn callus cultures, a specific cysteine proteinase activity correlates with resistance to feeding by fall armyworm (*Spodoptera frugiperda*) (Jiang et al., 1995). In feeding tests, larvae fed with callus of the susceptible genotype that is transformed with a cysteine proteinase gene showed reduced growth rate, indicating the insecticidal activity of this protein (Pechan et al., 2000). Furthermore, it was shown that the cysteine protease is induced by larval infestation. Insect-feeding induction of another protease, leucine amino peptidase, was also observed in tomato (Pautot et al., 1993). Proteases may interact with feeding insects directly or could be involved in signaling pathways during induction of defenses.

Lectins, carbohydrate-binding proteins, are often found to have insecticidal activity (Chrispeels and Raikhel, 1991). The lectin of the snowdrop plant *Galanthus nivalis* inhibits growth of sucking insects, *Nilaparvata lugens* (Powell et al., 1993; 1998). Legume lectin found in *Griffonia simplicifonia* is also known to have a negative effect on the growth of cowpea weevil (Zhu et al., 1996). Carbohydrate-binding proteins presumably interfere with the digestive process by binding to the peritrophic membrane in the herbivore gut. Though a lectin was found to be induced by pathogenic fungi or wounding, induction by herbivory has not been reported (Cammue et al., 1990; Taipalensuu et al., 1997b).

Additionally, inhibitors of α-amylase are known to have insecticidal effects (Franoco et al., 2002). Some proteins with similarities to proteinase inhibitors or lectin were found to demonstrate α-amylase inhibitory activities and negative effects on insect growth (Grossi de Sá et al., 1997). Other types of proteins similar to pathogen related

proteins, maize thaumatin and sorghum thionin, are also found to have inhibitory functions of insect α-amylase (Richardson et al., 1987; Bloch et al., 1991).

In addition to proteins that are known to directly interact with insect herbivores performance, many other proteins are likely to be involved in inducible anti-herbivore defense mechanisms based solely on the induction of their genes upon wounding. In studies of inducible anti-herbivore defenses, many experiments conventionally employ mechanical wounding in order to simulate herbivore damages, which may also mimic pathogen invasion or abiotic stresses. Although plants can differentiate mechanical wounding and herbivory (Turling et al., 1990), many genes that are induced by mechanical wounding and likely involved in anti-herbivore defenses have been identified from microarray analyses (Reymond et al., 2000). Using differential display and large-scale microarray analyses, Baldwin's group identified sets of putative defense-related genes induced in tobacco foliage by *Manduca sexta* larvae feeding (Hermsmeier et al., 2001; Schittko et al., 2001). These studies have shown that there are likely many genes which are induced by herbivory.

Genes that are induced by wounding and thought to play roles during induced defenses include genes for the signaling pathway for induction of defenses (see 1.1.6), proteolytic enzymes (Chao et al., 2000), cell wall proteins (hydroxyproline-rich glycoproteins and glycine-rich proteins) (Wycoff et al., 1995; Merkouropoulos et al., 1999), and biosynthetic pathways of phytochemicals. Genes for phenylpropanoid pathway, phenylalanine ammonia lyase and chalcone synthase, are known to be induced by wounding (Fukasawa et al., 1996; Richard et al., 2000).

1.1.2. Phytochemicals with Defensive Functions against Herbivory

Phytochemicals, also called secondary metabolites, can play very important roles in plant defense. Plants produce a diverse assortment of small organic compounds, the majority of which do not appear to participate directly in normal growth and development (Croteau et al., 2000). Instead they have a variety of functions such as protection from stresses and attractant for pollination. Many phytochemicals are known to have defensive functions against herbivores and pathogenic microorganisms (Harborne, 1999;

2001). Low-molecular-mass antimicrobial compounds that accumulate in plant tissues upon pathogen infection are called phytoalexins. Similarly, several phytochemicals with anti-herbivore functions accumulate upon wounding and herbivory. As part of defense mechanisms, phytochemicals that could be present constitutively and inducibly function as anti-feedants, trapping herbivorous insects, toxic compounds, and physical barriers, and signals (Hammerschmidt and Schultz, 1996). Phytochemical categories that are known to have anti-herbivore defense functions include phenolic compounds, alkaloids, terpenoids, cyanogenic glycosides, and glucosinolates (Constabel, 1999; see below).

Phenolic compounds are a major group of phytochemical with stress-related functions and are ubiquitously found in plant kingdom. Phenolics are characterized as aromatic metabolites that posses one or more hydroxyl groups and are generally synthesized through the phenylpropanoid pathway where aromatic amino acids are enzymatically hydroxylated. Phenolics could be present in plant tissues as monomers, polymers, or conjugated form such as phenolic glucosides. Phenolics commonly accumulate in tissues after stresses such as UV, low temperature, and attacks from pathogens and herbivores (Dixon and Paiva, 1995). The increases in phenolic concentration are often mediated by up-regulation of the phenylpropanoid pathway. Phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) that are members of the phenylpropanoid pathway are known to be induced by wounding (Hahlbrock and Scheel, 1989). Some phenolic acid conjugates, such as chlorogenic acid themselves have moderate anti-feedant activity by making complexes with proteins (Beart et al., 1985), but their anti-herbivore activities are further enhanced by their oxidation (see Chapter 2). Phenolic polymers, tannin and lignin, are also inducible by wounding and participate in defense as feeding deterrents and in wound repair (Kahl, 1982).

Alkaloids are nitrogen-containing compounds and usually derived from amino acids. Their toxicity based on interfering with the nervous system suggests an important function for anti-herbivore defense (Hartmann, 1991). In *Nicotiana*, its was shown that an artificial induction of foliar nicotine concentration is correlated with increased protection of plants from herbivory by *Manduca sexta* (Baldwin, 1999). The increase in nicotine concentration was mediated by up-regulation of its biosynthesis in roots and its transport from roots to shoots. A gene for a key regulatory enzyme in nicotine

biosynthesis, putrescine *N*-methyltransferase is reported to be induced by herbivory (Winz and Baldwin, 2001).

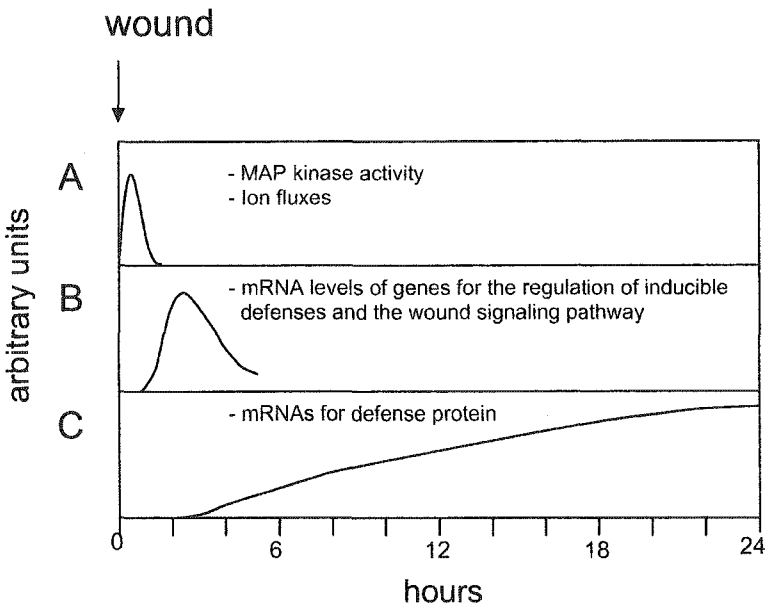
Terpenoids, which are composed of isoprene units, are known to have various physiological functions in plants. Defensive roles of terpenoids are well studied in conifers in the context of defense mechanisms against bark beetle (reviewed in Trapp and Croteau, 2001). Conifer oleoresin consisting a mixture of terpenoids accumulates upon wounding and plays a defensive role in wound sealing, extruding insects, and also direct toxic effects. Recent studies have proposed that herbivore-induced volatile terpenoids from tobacco act as specific signals to attract natural predators of the attacking herbivores, indicating that the importance of air-borne signals in indirect defenses (De Moraes et al., 1998). Furthermore, herbivore-induced terpenoids such as β-ocimene act as endogenous signals for induction of defense-related genes such as lipoxygenase (LOX) and phenylalanine ammonia lyase (LOX) (Arimura et al., 2000). Similarly, a diterpene was found to be a signal for wound-induction of defenses that was observed as the activation of mitogen activated protein (MAP) kinase and induction of defense-related genes (Seo et al., 2003). In grand fir, wounding induces the expression of terpene synthase genes and the accumulation of terpenoids (Steele et al., 1998).

Many phytochemicals involved in defense are constitutively presents in plants, while some of them are induced under stress condition such as wounding. The accumulation of phytochemicals is controlled by the precise regulation of their biosynthetic pathway. Accumulation of enzyme proteins involved in the biosynthesis of phytochemicals appears to be also regulated at the transcriptional level in the same manner as proteins with defensive functions described earlier (1.1.1). The next section discusses the kinetics of induced defenses by describing activation of the signaling pathways, components for induced defense, and the subsequent expression of defense-related genes.

1.1.3. Kinetics of Wound-Induced Responses

The expression level of genes involved in anti-herbivore defense is regulated in the context of complex defense mechanisms involving recognition of wound stimuli and signal transduction. The rapidity of changes in gene expression levels depends on their

Figure. 1.1. Time course representation of the induction of wound signaling components and defense proteins in wounded leaves in response to wounding. Wounding causes rapid cellular responses, including an increase in MAP kinase activity and ion fluxes such as calcium influx, within minutes (A). Accumulation of mRNA of genes for the wound signaling pathway is induced within 30 min (B). mRNAs of genes for defense-related proteins start accumulating approximately 2 h after wounding (C). Modified from Ryan (2000).


wound
A
- MAP kinase activity
- Ion fluxes
B
- mRNA levels of genes for the regulation of inducible defenses and the wound signaling pathway
C
- mRNAs for defense protein
arbitrary units
0
6
12
18
24
hours

functions in induced defense. In systematic studies in tomato, the induction of suites of genes involved in defense was surveyed by analyzing the timing of gene expression after wounding leaf tissues (reviewed in Ryan, 2000; Fig. 1.1). In the very early phase of wound responses, activities of enzymes involved in intracellular signaling increase and ion fluxes across the plasma membrane occur (Fig. 1.1A). The activation of mitogen-activated protein kinase (MAP kinase) and phospholipase D are first detectable within about 5 min after wounding (Stratmann and Ryan, 1997; Narváez-Vásquez et al., 1999; also see 1.1.5). This is followed by the transcriptional activation of genes involved in the wound signaling pathway (Fig. 1.1B). For example, the gene for the anti-herbivore peptide hormone, prosystemin (McGurl et al., 1992; see also 1.2.3) and a gene for calmodulin that binds calcium and mediates intracellular signaling are induced within 30 min of wounding (Bergey and Ryan; 1999). Other genes with regulatory functions, encoding for enzymes of the octadecanoid wound signaling pathway also start accumulating within 30 min after wounding (Fig. 1.1B; see 1.1.6). The genes of this pathway include lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) (Heitz et al., 1997; Laudert et al., 1996; Ziegler et al., 2000). The induction of genes involved in the signaling pathway occurs rapidly and reaches 2-3 hr after wounding. After the activation of the wound signaling pathway, genes encoding defensive proteins such as PIs and PPO become activated approximately 2 hr after wounding (Fig. 1.1C). The difference in timing for the induction of functionally different groups of proteins (i.e. signaling versus insecticidal) suggests that the wound signal transduction pathway is initially activated upon wounding, and consequently the expression of genes for defense proteins is induced. In such wound signaling, the flow of the signal transduction is likely controlled by regulatory or signal molecules. The known signal molecules involved in regulation of the herbivore defenses are reviewed in following section.

1.1.4. Overview of Signals Involved in Induced Anti-Herbivore Defenses

In induced defenses, the expression of defense-related genes is tightly regulated by wound signaling mechanisms. Upon tissue damage, wounding is recognized by surrounding tissues and the wound stimuli are signaled to intact tissues where genes for

defense proteins are induced. During the signaling process, signal molecules play key roles by conveying wound stimuli between and within cells. Plants produce a diverse array of signals that play regulatory roles at specific locations and times. Signal molecules have been identified in physiological experiments by monitoring their abilities to regulate the expression of defenses in plants. Most signal molecules were characterized in tomato by measuring the accumulation of PI as a maker of induced defenses.

A. Oligosaccharides

In the search for inducers of tomato PIs, factors that induce PIs in excised tomato plantlets were purified and identified from wounded leaf extracts. Oligo- and polygalacturonide fragments with degree of polymerization of between 2 to 20 were found to induce accumulation of PIs at a concentration of 2 mg/ml when they were applied to tomato plantlets through the cut stem (Bishop et al., 1981; 1984). The polygalacturonides are thought to be generated from the cell wall upon wounding and further fragmented into smaller oligogalacturonides by the polygalacturonase that is induced by wounding (Bergey et al., 1999). In addition to endogenous oligosaccharides, an oligosaccharide derived from fungal cell walls, chitosan, was also found to induce PIs in excised tomato leaves (Doares et al., 1995b). However, chitosan is expected to be primarily involved in defense against fungal pathogen rather than herbivores.

B. Systemin

Additionally, systemin, an 18 amino acid peptide, was isolated as an inducer of PIs from wounded leaves of tomato (Pearce et al., 1991; see also 1.2.2, 1.2.3). Related peptide molecules, systemin-like peptides, were also recently isolated from tobacco and induce PIs in tobacco plants (Pearce et al., 2001a; also see 1.2.4). Tomato systemin induces accumulation of PIs at extraordinary low concentration (10 fmol/plant; Pearce et al., 1991), suggesting a hormone-like function of this peptide in defense. In addition to PIs, tomato systemin induces at least 20 genes encoding other defense-related proteins including enzymes for the wound signaling pathway and major anti-herbivore proteins such as PPO (Bergey et al., 1996). Furthermore, transgenic tomato plants overexpressing

the gene for prosystemin show constitutive expression of PIs in the absence of wounding (McGurl et al., 1994). Additionally, transgenic plants expressing prosystemin in an antisense orientation inhibited the accumulation of PIs upon wounding, resulting in reduced resistance against *Manduca sexta* (McGurl et al., 1992; Orozco-Cardenas et al., 1993). This evidence indicates that systemin is a key signaling molecule in induced defense against herbivory in tomato, and it is also thought to act as a systemic signal (see 1.2.2).

C. A second messenger, hydrogen peroxide

In the study of systemin-regulated signaling, it was observed that systemin potentiates the oxidative burst that is normally associated with plant defenses against disease-causing microbes (Stennis et al., 1998). Hydrogen peroxide accumulates in response to both wounding and application of systemin (Orozco-Cardenas et al., 2001). The treatment of tissues with hydrogen peroxide also induces the expression of genes for defense proteins such as PIs. Genes for enzymes involved in wound signaling such as prosystemin and LOX, however, were not transcriptionally activated by hydrogen peroxide, implying that hydrogen peroxide acts as a second messenger in the later wound signaling events (i.e. downstream of the octadecanoid pathway, see 1.1.6).

D. Jasmonic acid and other lipid-derived compounds

The involvement of the lipid-derived compound, jasmonic acid (JA), as well as related compounds in inducible anti-herbivore defense has been studied in various plant species, mainly tomato and *Arabidopsis* (reviewed by Weber, 2002; Gatehouse, 2002). Though JA is known to have regulatory roles in a broad range of physiological processes including development (Creeman and Mullet, 1997), it is best characterized as a signal molecule in defense responses. The importance of JA as a signal molecule for induced defenses was demonstrated by several key studies. The treatment of tomato plants with methyl jasmonate, a volatile methyl ester of jasmonic acid, has been shown to induce the accumulation of PIs (Farmer and Ryan, 1990). A mutant of tomato that is deficient in JA biosynthesis was compromised in the wound-accumulation of PI and in resistance to *Manduca sexta* (Howe et al., 1996). Jasmonic acid content of leaves increases after

wounding, and mRNAs of defense related genes accumulate accordingly (Creelman et al., 1992). Genes encoding enzymes for the jasmonate biosynthetic pathway were found to be induced by wounding in several plant species, indicating the important role of *de novo* synthesis of jasmonates during induced defense (see 1.1.6). However, like the oligosaccharides and hydrogen peroxide, jasmonates are also known to induce defenses against pathogens.

Lipid-derived six-carbon (C_6) alcohols and aldehydes such as hexenol and hexenal also act as inducers of anti-herbivore defenses (Bate and Rothstein, 1998). *Arabidopsis* plants treated with C_6-volatile aldehydes and alcohols accumulate mRNAs of herbivore defense-related genes involved in phytochemical production: the phenylpropanoid-related genes including chalcone synthase and dihydroflavonol reductase. In contrast to jasmonates, the C_6-volatiles induce fewer defense-related genes and at a lower level. It may indicate that plants differentially utilize jasmonates and C_6-volatiles in response to different types and degrees of biotic stresses. Another recent study has also shown that hexenal activates the expression of pathogen-related genes (Almeras et al., 2003). It also should be mentioned that a C_6-alcohol is released from herbivore-damaged tobacco and cotton, and likely acts as a volatile signal for indirect defenses and attracts enemies of insect herbivores together with the terpenoids described above (De Moraes et al., 1998; see. 1.1.2).

E. Other phytohormones important for induced defense

While systemin and jasmonates are required to induce defenses, they may not be sufficient. Other phytohormones were also reported to be important in induced defenses. A tomato mutant with reduced abscisic acid (ABA) biosynthesis was found to be deficient in the wound response, and the response could be restored by exogenously applied ABA (Pena-Cortes et al., 1989; 1996). Although ABA is required for defense response, it is not a primary signal but appears to play an overall role in determining whether a plant can mount a defense response (Birkenmeier and Ryan, 1998). In another study, it was observed that wounding causes the accumulation of ethylene, and the inhibition of ethylene action impaired defense responses in tomato (O'Donnell et al., 1996). Furthermore, the treatment of suspension cells with systemin induces the

expression of a gene for ethylene biosynthesis and the accumulation of ethylene (Felix and Boller, 1995). Therefore, ethylene is likely a part of defense signaling as well as ABA. Recently, it was also shown that pretreatment of plants with brassinosteroid enhances disease resistance in tobacco and rice; however, it is not known whether brassinosteroids are involved in anti-herbivore defenses (Nakashita et al., 2003).

F. Signals that suppress induced anti-herbivore defenses

In addition to signals that positively regulate wound-induced defenses, some molecules that negatively regulate induced herbivore defenses have been reported. The negative effects of salicylic acid (SA) and nitric oxide (NO) on wound-induction of anti-herbivore defenses were shown (Doares et al., 1995a; Orozco-Cardenas and Ryan, 2002). SA and NO are known to act as positive regulators in disease resistance mechanisms (Shirasu et al., 1997; Delledonne et al., 1998). Thus, there may be regulatory mechanisms to coordinate induced defenses against herbivory and pathogens (Felton et al., 1999). The inhibitory activity of auxin on defense responses was also reported (Kernan and Thornburg, 1989).

Overall, it can be concluded that plants have diverse signal molecules to regulate their defense signaling pathways in response to wounding. Involvement of various of signal molecules imply the presence of integrated signaling events that are not only associated with herbivore defenses but also with a broad range of physiological processes such as development. It has been shown that signaling components of stress responses are also interacting with signaling components for development in a large-scale analysis of protein-protein interactions and gene expression in rice (Cooper et al., 2003).

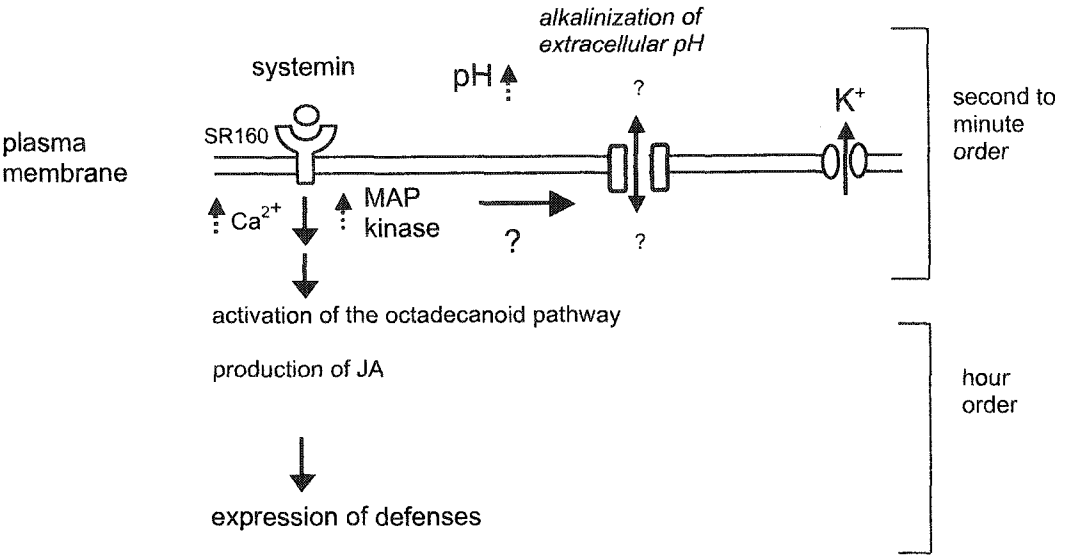
Upon tissue damage caused by herbivory or pathogen invasion, plants release signals to induce defense responses. However, specific downstream events of signal molecules are largely unknown. Of the many signal molecules described above, a peptide-based molecule, systemin has been extensively studied (Ryan, 2000). Systemin-regulated intracellular signaling has been partially characterized. In the next section, systemin-mediated intracellular signaling is reviewed.

1.1.5. Systemin and Its Cellular Signaling Events

The anti-herbivore defense hormone, systemin, is synthesized as a 200 amino-acid precursor protein, prosystemin, and is processed into a smaller 18 amino-acid active peptide (McGurl et al., 1992). As a signal for inducible defenses, systemin is recognized by target cells where it activates intracellular defense signaling events (Fig. 1.2). Recently, the systemin receptor (SR160) was isolated and characterized from the plasma membrane of cultured cells of tomato (Scheer and Ryan, 2002). SR160 is a 160 kD glycosylated receptor protein consisting of an extracellular leucine rich repeat domain, transmembrane domain, and intracellular serine/threonine protein kinase domain. Binding of systemin with SR160 correlates with the rapid activation of a phosholipase D and a 48 kD MAP kinase, which can be observed within 1-2 min after systemin treatment of cells (Narváez-Vásquez et al., 1999; Stratmann and Ryan, 1997; Scheer and Ryan, 1999). Simultaneously, ion fluxes across the plasma membrane were observed as early cellular responses to systemin: a transient pH increase (alkalinization) in the extracellular space, potassium efflux, and calcium influx (Felix and Boller, 1995; Moyen et al., 1998). The presence of an inhibitor, suramin that is known to prevent a peptide hormone from binding on its receptor, blocked systemin binding on its receptor, MAP kinase activation, and extracellular pH alkalinization, suggesting the tight links of systemin-receptor binding with the activation of MAP kinase and ion fluxes in early signaling events (Stratmann et al., 2000).

Recognition of systemin by its receptor triggers cellular signaling cascades and generates intracellular signal molecules called second messengers that further regulate downstream signaling events. By action of systemin-activated phospholipase A, cells accumulate lysophosphatidylcholine that is a known intracellular regulator of protein kinase and H^+-ATPse in other systems of intracellular signaling (Nárvaez-Vásquez et al., 1999; Munnik et al., 1998). Systemin also induces the accumulation of free fatty acid, mainly linoleic acid and linolenic acid in tomato leaf tissues. These fatty acids are likely used as precursors for lipid-derived signal molecules including jasmonates (Conconi et al., 1996; see 1.1.6). Hydrogen peroxide is also produced by as a result of systemin treatment, and may act as a second messenger (Orozco-Cardenas et al., 2001; see 1.1.4.C).

Figure.1.2. Systemin-regulated cellular signaling events. Upon the perception of systemin by the systemin receptor, SR160, within minutes cells activate early cellular events including an increase in MAP kinase activity, calcium influx, potassium efflux, and extracellular pH alkalinization. These early events further activate the wound signal pathway, including the octadecanoid pathway that produces JA, an inducer of genes for defensive proteins. Modified from Schaller (2000).

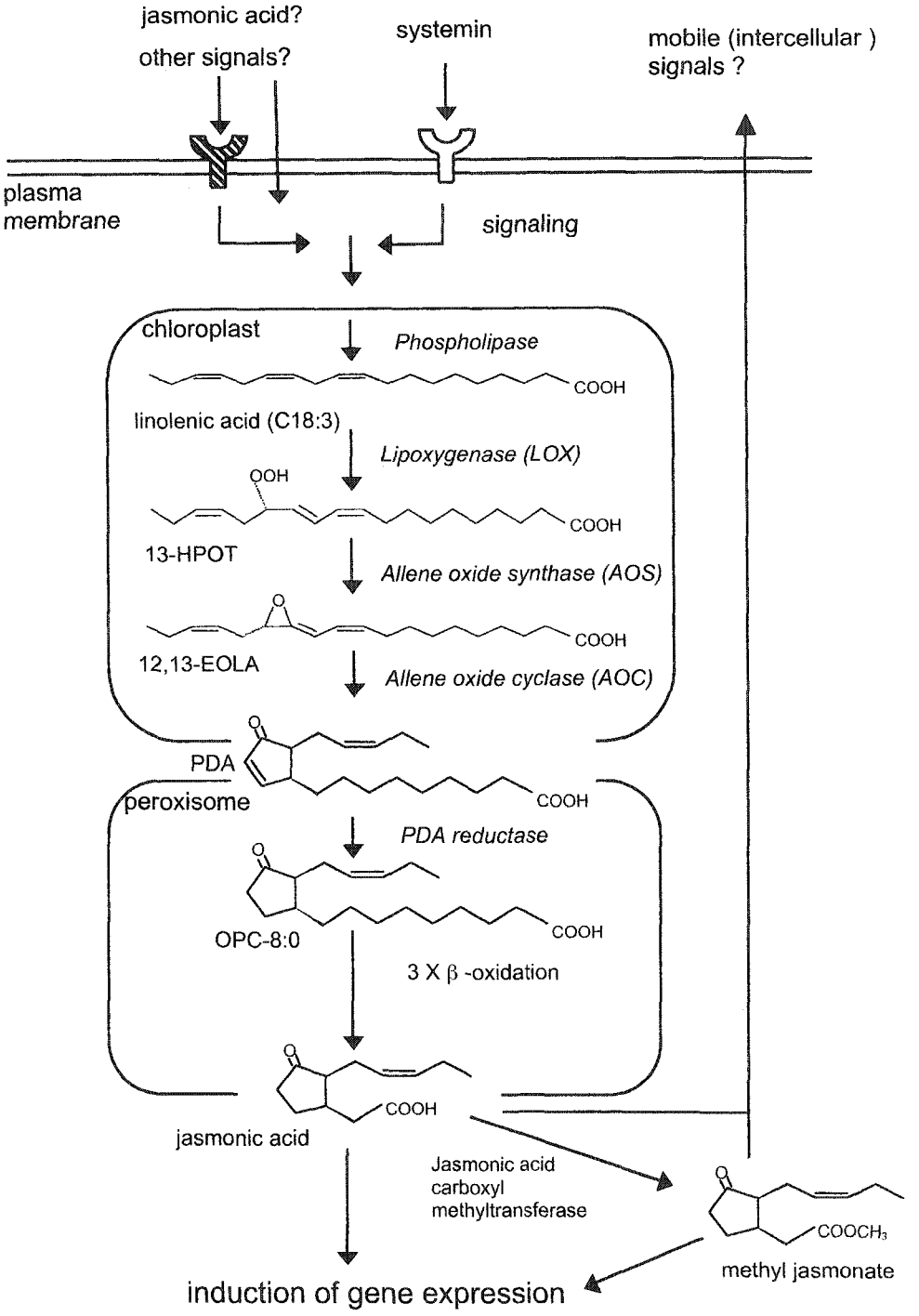

alkalinization of
extracellular pH
systemin
pH
?
K+
second to
minute
order
plasma
membrane
SR160
Ca2+
MAP
kinase
?
?
activation of the octadecanoid pathway
production of JA
hour
order
expression of defenses

Upon perception of systemin with its specific receptor, cells trigger early intracellular signaling cascades involving protein phosphorylation and ion fluxes (Felix and Boller, 1995). This further activates the downstream cellular signaling events, such as the production of jasmonates. In the next section, an overview of jasmonate biosynthetic pathway is described with regards to the intracellular signaling of induced defenses.

1.1.6. Jasmonates and the Octadecanoid Pathway

During signaling for induced defense, the production of jasmonic acid (JA) is a critical process. Upon wounding, JA accumulates in the tissues and acts as an inducer of defense-related genes such as PIs and PPO. Thus, the JA biosynthetic pathway is considered to be a major intracellular signaling pathway for induced defenses. JA and structurally related compounds, collectively called jasmonates, are known to be biosynthesized through the octadecanoid pathway, where linolenic acid is enzymatically converted to JA (Fig. 1.3; Vick and Zimmerman, 1984). Molecular studies of enzymes involved in JA synthesis have been carried out in the context of induced defenses using tomato and *Arabidopsis* (Weiler, 1997; Turner et al., 2002). Most members of genes for the enzymes in the pathway have been cloned and characterized. Genes encoding lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), and oxophytodienoic acid (OPDA) reductase (OPDA reductase) are all known to be transcriptionally activated by wounding or systemin and to function as members of wound-signaling components (Heitz et al., 1997; Sivasankar et al., 2000; Stenzel et al., 2003; Strassner et al., 2002). Recently, a gene for an enzyme that converts jasmonic acid to its volatile methyl ester, methyl jasmonate, was isolated and confirmed its importance in wound signaling (Seo et al., 2001). The absence of gene products of the members of the octadecanoid pathway causes a failure to exhibit defense responses. Antisense suppression of LOX reduced wound-induced accumulation of JA and the induction of defense-related genes in *Arabidopsis* (Bell et al., 1995). Furthermore, *Arabidopsis* plants with mutations in OPDA-reductase gene that cannot produce JA show impaired resistances against some herbivores (Stintzi et al., 2001). Wound-production of JA through the octadecanoid pathway appears necessary for induced anti-herbivore defense.

Figure. 1.3. The octadecanoid pathway in the context of wound signaling. Wound signals such as systemin activate phospholipase which releases linolenic acid from chloroplast membrane. Linolenic acid is enzymatically converted to 12-oxo-phytodienoic acid (PDA) through the action of lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) in chloroplast. PDA is reduced by PDA reductase to 3-oxo-2-(2'(Z)-pentenyl)-cyclo-pentane-1-octanoic acid (OPC8:0) that is subject to β-oxidation by yet unidentified mechanisms and converted to JA. JA can be converted to methyl jasmonate (MeJa) by jasmonic acid carboxyl methyltransferase. 13-HPOT, 13-hydroperoxylinolenic acid; 12, 13-EOLA, 12, 13-enoxylinolenic acid; OPC-8:0, 3-oxo-2-(2'(Z)-pentenyl)-cyclo-pentane-1-octanoic acid. Modified from Weiler (1997).


jasmonic acid?
other signals?
systemin
mobile (intercellular) signals ?
plasma membrane
signaling
chloroplast
Phospholipase
COOH
linolenic acid (C18:3)
Lipoxygenase (LOX)
OOH
COOH
13-HPOT
Allene oxide synthase (AOS)
O
COOH
12,13-EOLA
Allene oxide cyclase (AOC)
PDA
O
COOH
peroxisome
PDA reductase
O
COOH
OPC-8:0
3 X β -oxidation
O
COOH
jasmonic acid
Jasmonic acid carboxyl methyltransferase
O
COOCH3
methyl jasmonate
induction of gene expression

The cellular localization of the enzymes involved in the octadecanoid pathway is predicted from the deduced protein sequences of their cDNA sequences. It was revealed that JA biosynthesis likely takes place in several different cellular compartments, the chloroplast, cytosol, and peroxisome (Fig1. 3). In unwounded tissues, the activities of the enzymes are at a low level, and the precursors and the intermediates of JA are present in the tissues at low concentrations as well. It is thought that tissue damage causes these precursors and intermediates for JA to come into contact to the enzymes, which results in the production of JA within minutes (Weiler, 1997 and reference therein). This JA production is very rapid, but at a very low level and followed by a large JA burst approximately 60 min later (Dietmar et al., 1996; Dietmar and Weiler, 1998). The first locally-produced small amount of JA may move away from the wounded site and induce the expression of the genes for the octadecanoid pathway itself in the locally wounded areas and the further production of JA in surrounding tissues. For instance, a rapid minor JA peak occurs within minutes after wounding, and mRNA for allene oxide synthase (AOS) starts accumulating within 15-30 min. This is followed by an increase in AOS protein level and enzymatic activity at approximately 60 min after wounding. The second, larger, JA burst starts at approximately 60 min, which is the same timing as an increase in AOS activity (Dietmar et al., 1996). In this manner, wound stimuli are likely amplified through the octadecanoid pathway and generate signal molecules, jasmonates, which presumably enhances the induction of defenses. This hypothesis is supported by the observation that exogenously supplied MeJA itself induces sets of genes involved in the octadecanoid pathway in *Arabidopsis* (Sasaki et al., 2001, and references therein).

1.1.7. Post-Translational Regulation of JA Signaling

The effects of JA on the induction of defenses in a various plant species indicate the presence of conserved JA-regulated signal pathways in higher plants including *Arabidopsis*. Genetic analyses of *Arabidopsis* mutants in JA responses have been used to identify components of JA perception. Analyses of the COI-1 (coronatine insensitive) mutant that is insensitive to coronatine, a JA analog, and shows impaired defenses, revealed that F-box protein is important for JA-regulated signaling (Xie et al., 1998). With analogy to other organisms including yeast and human, it appears that the F-box

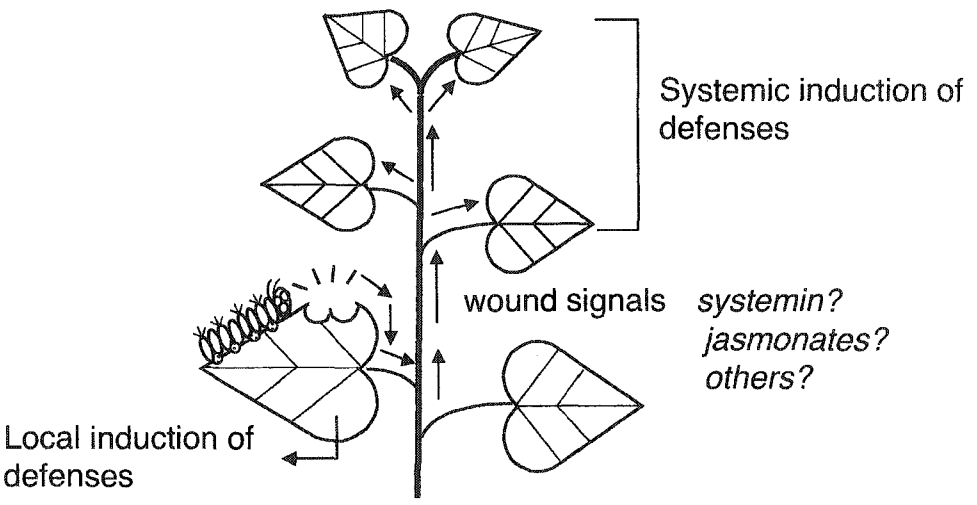
domain of the COI-1 protein functions protein-protein interactions and is involved in ubiquitin-ligase mediated proteolytic pathways (Xu et al., 2002). Further searches for proteins to interact with COI-1 protein using yeast two-hybrid system identified candidate target proteins, a repressor of transcription, histone deacetylase and a small subunit of Rubisco (Devoto et al., 2002). Given that acetylation and deacetylation of histone is an important mechanism in the regulation of gene transcription in eukaryotes (Lusser et al., 2001), perhaps JA regulates the expression of genes through histone deacetylase. Detailed mechanisms of ubiquitin-ligase mediated JA responses remain to be elucidated.

JA accumulates in wounded tissues by wounding, acts as a signal of induced defenses, and induces the expression of defense-related genes. In a similar manner to the peptide-based signal, systemin, JA activates the wound-signal pathway and induced defenses. However, the spatial distribution of these signals during induced defense is not known. It remains to be seen how and how far these signals can move from wounded sites to the surrounding area. Though the mobility of these signals (i.e. how far) is largely unknown, many studies have been shown that plants are capable of inducing defenses in distal areas from the wounded sites. This indicates that plants can generally transmit local wound stimuli to distal areas. The following sections review systemic-wound induction of defenses and discuss putative signals for this induction.

1.2. Systemic Induction of Anti-Herbivore Defenses and Signaling Mechanisms

As outlined in section 1.1, plants have evolved inducible defenses to deter future herbivore damage. In response to herbivory, plants synthesize a variety of defense-related proteins and phytochemicals within hours. Remarkably, many induced defenses were found to occur "systemically" that is, in the whole plant. Upon local tissue damage, many plants accumulate defense-related compounds in undamaged tissues of the plant as well as in the damaged tissue (Fig. 1.4). It is proposed that damaged tissues release wound signals that systemically move to intact tissues and activate defenses there. Systemic activation of defenses may be detected as the induction of gene expression, the accumulation of proteins, an increase in enzymatic activity, or the accumulation of

Figure. 1.4. Systemic induction of anti-herbivore defenses in plants. A damaged tissue induces the accumulation of defensive proteins. Wound signals that are released from the damaged tissues move to distal tissues through vascular system and induce defenses. Systemin and jasmonates are currently proposed to be systemic-wound signals in tomato.


Systemic induction of defenses
wound signals
systemin?
jasmonates?
others?
Local induction of defenses

phytochemicals. To date, the systemic induction of defense-related genes has been observed in a broad range of plant species. The distribution of systemic wound-induction of genes in plant kingdom, as well as aspects of systemic wound signals, is reviewed in the following section.

1.2.1. Occurrence of Systemically Induced Wound-Responses in the Plant Kingdom

Systemic induction of a defense-related protein was first demonstrated in tomato (Green and Ryan, 1972), when it was shown that PIs accumulated in both wounded and unwounded leaves in response to herbivory. PIs are the most characterized proteins among wound inducible proteins and used as marker proteins in many studies of wound responses. In analyses of gene expression, systemic induction of PIs were found in leaves of many plant species including tomato, potato, tobacco, soybean, alfalfa, poplar, and maize (Botella et al., 1996, Bradshaw et al., 1989; Cordero et al., 1994, McGurl et al., 1995).

In addition to PIs, other putative defense-related genes were also observed to be systemically induced upon wounding. Analyses of genes that are systemically induced by mechanical wounding have been used in order to identify genes possibly involved in systemic induced defense. Bergey et al. (1996) demonstrated that wounding tomato leaves causes systemic induction of over 20 genes including leucine aminopeptidase, threonine deaminase, and acyl CoA-binding protein. The biological roles of some wound-induced genes are as yet unclear; however, the occurrence of systemic wound induction is strong evidence for the communication system between wounded and unwounded tissues. In *Arabidopsis* leaves, genes that are systemically induced by wounding involve genes for the octadecanoid pathway such as allene oxide synthase (Laudert and Weiler, 1998), RNase (LeBrasseur et al., 2002), and arginine decarboxylase (Perez-Amador, 2002). In the related species *Brassica*, a gene for myrosinase-associated protein was induced systemically in response to wounding (Taipalensuu, et al., 1997a). In leaves of passion fruit (*Passiflora edulis*), wounding lower leaves systemically induces the accumulation of lipoxygenase protein and the increase in its enzymatic activity in unwounded upper leaves (Rangel et al., 2002). In chickpea (*Cicer arritinum*), injuring an internode of a seedling cause local and systemic induction of the accumulation of copper

amine oxidase protein and its enzymatic activity throughout the stem and leaf (Rea et al., 2002). Moreover, a systemic wound response was observed in a gymnosperm, white spruce (*Picea glauca*), where systemic induction of the defense-related chalcone synthase (CHS) gene by wounding was demonstrated (Richard et al., 2000).

The systemic induction of defenses in plants may be similar to immune mechanisms in animals. A plant that has activated defenses at a whole plant level would be competent to defend itself against herbivores and presumably prevent future herbivory. The occurrence of a systemic wound-response in the broad range of plant species suggests the presence of common systemic signaling mechanisms in plant kingdom. Identifying signals would be essential to fully understand the dynamics of inducible defenses at a whole plant level. Some signals previously mentioned in section 1.1.4. are also thought to act as systemic signals. Proposed signals for systemic induction of wound-responsive genes are discussed in the next section.

1.2.2. Signals for Systemic Induction of Defenses

There are several proposed signals for systemic induction of wound-responsive genes: electric pulse, hydraulic pressure that is physically generated by wound stimuli, systemin, and jasmonates (Wildon et al., 1992; Malone and Alarcon, 1995; Pearce et al., 1991; Li et al., 2002). Although there is a possibility that several signals may work as systemic signals at different times and tissues, the bulk and clarity of the experimental data available to date suggest that systemin and jasmonates are intercellular signals (Ryan and Moura, 2002).

A. Hypothesis that systemin is the systemic signal in tomato

Several key experiments have shown that systemin, a powerful inducer of PIs in an excised tomato leaf (see 1.1.4. *B*), may be a systemic signal for induced defense. The mobility of systemin in tomato plants was directly studied by applying radio-labeled systemin onto a wounded site of a tomato terminal leaflet (Nárvaez-Vásquez et al., 1995). Within 90 min after application, systemin moved into vascular systems of the main stem as observed by autoradiography. The intact radio-labeled systemin was recovered from

the phloem exudate of the leaf to which radio-labeled systemin had been applied. Systemin is thus capable of moving through the phloem.

In the other key experiment, transgenic tomato plants expressing the prosystemin gene in an antisense orientation showed impaired systemic responses to wounding (McGurl et al., 1992). Exogenously supplied systemin complements the systemic expression of wound responsive genes in the antisense transgenic plants. Furthermore, using grafted plants with a wild-type scion on top of a prosystemin-overexpressing rootstock, McGurl et al. (1994) demonstrated that unwounded leaves of wild scions constitutively express defense-related genes in the absence of wounding lower leaves. This indicates that systemin itself is a long-distance wound signal or that systemin induces the release of long-distance wound signals.

The expression pattern of the prosystemin gene also indicates the likelihood of systemin as a phloem-mobile signal. Using transgenic tomato plants harboring the prosystemin promoter fused with the β-glucuronidase reporter gene, Jacinto et al. (1997) demonstrated that the prosystemin is constitutively expressed in vascular bundles of petiole and stem tissues at a low level, and is strongly induced by wounding and MeJa treatment. The vascular localization of the prosystemin gene expression and its induction by wounding and MeJa would be consistent with its role in phloem-mediated systemic wound signaling.

B. Jasmonates as signals for systemic induction of wound-responsive genes

In grafting experiments, Li et al. (2002) investigated the essential roles of jasmonates in transmission and recognition of systemic wound signals using two mutant tomato plants: *jai-1* (deficient in JA response) and *spr-1* (deficient in JA biosynthesis). In several combinations of grafted plants with wild-type, *jai-1*, and *spr-1* as either scion or rootstocks, the induction of PI mRNA in scion leaves was monitored upon wounding of rootstock leaves. It was demonstrated that production of JA in rootstock is necessarily for induction of PI in unwounded upper leaves. Furthermore, it was shown that biosynthesis of JA in unwounded upper leaves is not necessary for the induction of PI.

In other studies of gene expression analyses, it was also found that the gene involved in the octadecanoid pathway, OPDA reductase (see 1.1.6.; Fig. 1.3), is inducible

in wounded leaves but not in upper unwounded leaves (Strassner et al., 2002). Furthermore, JA content dramatically increases in wounded leaves but not in upper unwounded leaves. These observations indicate that JA or other related lipid derived molecules are generated in local wounded tissues and possibly move to systemic unwounded leaves.

Since independent experimental systems demonstrated that both systemin and jasmonates are likely mobile signals, it is important to determine effects of JA and systemin in a single experimental system. In additional grafting experiments using systemin-overexpressed rootstock and *spr-1* scion, the relationship of systemin and jasmonates were investigated. The level of PI mRNA in *spr-2* scion leaves onto systemin-overexpressing rootstock is comparable to that in wild-type scion onto systemin-overexpressing rootstock, indicating that effects of translocated systemin could be depending on JA production in systemic leaves (Li et al, 2002). From these experimental results, currently it is hypothesized that both systemin and jasmonates move away from wounded sites upon wounding. They likely regulate wound signaling in a synergistic manner. It is reported that systemin and JA mutually stimulate their biosynthesis, which presumably amplifies wound signaling during induced defenses (Pearce et al., 2001a; Ryan, 2000 and reference therein).

In conclusion, two different molecules, jasmonic acid and systemin, likely both act as mobile signals for systemic induction of defenses in tomato. One may not be sufficient and both are probably mutually required. However, it also should be mentioned that *spr-1* mutant plants are not affected in the expression of a subset of rapidly induced signaling genes such as lipoxygenase and allene oxide synthase, indicating that the presence of systemin-and JA-independent pathway for wound signaling (Lee and Howe, 2003). It was reported that tobacco MAP kinase is systemically induced within 1 min after wounding (Seo et al., 1995). Considering the rate of phloem transport, which is estimated approximately 300 cm h^{-1} (Christy and Fisher, 1978), signals that do not require phloem transport (such as electrical signals) are likely involved in this very rapid wound signaling.

Jasmonates are known to induce herbivore defenses in many plant species, indicating the ubiquitousness of jasmonates and their regulatory mechanisms in many plants. On the other hand, to date systemin and its homologs have been found only in the Solanaceae family. The molecular characterization of tomato systemin was carried out, which subsequently provided a tool to reveal the presence of systemin homologs. The following sections describe characterization of systemin homologs found in the Solanaceae family (1.2.3).

1.2.3. Investigation of Systemin Homologs

Since various plant species show systemic wound responses (See 1.2.1), it could be hypothesized that many plant species may use systemin-like molecules for wound signaling. The search for systemin homologs was carried out using both molecular and biochemical approaches. Southern blot hybridization with the DNA probe for the tomato prosystemin gene could detect the presence of related DNA sequence in potato but not in tobacco, alfalfa, and *Arabidopsis* (McGurl et al., 1992). Consequently, cDNAs encoding systemin homologs were isolated by reverse-transcription polymerase chain reaction from potato, black nightshade, and bell pepper, all Solanaceous species (Constabel et al., 1998). They are all synthesized as ~ 200 amino-acid precursor proteins, and sequence conservation was found throughout the full-length protein. A unique feature of systemin peptide, a pair of -PP- at palindromic position, was conserved in all 18 amino-acid systemin homologs (Table 1.1). Moreover, cross-reactivity of systemin homologs was confirmed among tomato, potato, black night shade, and bell pepper, but not in tobacco (Constabel et al., 1998).

While the experiments in this thesis were in progress, a novel family of tobacco systemin-like peptides was isolated (Pearce. et al., 2001a). Based on the observation that the addition of tomato systemin induces alkalinization of the medium pH in cultured cells of tomato (see section 1.1.5; Fig. 1.2), tobacco leaf extracts were tested in alkalinization assays with tobacco cell culture and found to contain medium pH alkalinizing systemin-like peptides (Pearce et al., 2001a). Tobacco systemin-like peptides induce the accumulation of PI protein in tobacco plants and activate MAP kinase in tobacco suspension cells, as tomato systemin does in tomato plants and tomato suspension cells.

Table 1.1. Structural comparisons of systemin and systemin-like peptides found in the Solanaceae family

Peptide	a.a. sequence	Mass	modification by pentose	Mass without sugar	references
Tomato systemin	+AVQSKPPSKRDPPKMQTD-	2,010	ND		Pearce et al. 1991
Potato systemin-I	+AVHSTPPSKRDPPKMQTD-	1,992	ND		Constabel et al. 1998
Potato systemin-II	+AAHSTPPSKRDPPKMQTD-	1,964	ND		Constabel et al. 1998
Nightshade systemin	+AVRSTPPPKRDPPKMQTD-	2,021	ND		Constabel et al. 1998
Pepper systemin	+AVHSTPPSKRPPPKMQTD-	1,974	ND		Constabel et al. 1998
Tobacco sys-likeI	+RGANLPOOSOASSOOSKE-	3,060	9 units	1,869	Pearce et al. 2001a
Tobacco sys-likeII	+NRKPLSOOSOKPADGQRP-	2,784	6 units	1,992	Pearce et al. 2001a

a.a.: amino acid; O: hydroxyproline; ND: not determined

Tobacco systemin-like peptides also appear to be 18 amino-acid peptides, but show a divergent structure with post-translational modifications including hydroxylation of proline residues and glycosylation, not found in tomato systemin peptide (Table 1.1). Importantly, the sugar side chains of tobacco systemin-like peptides were determined to be required for biological activity. Furthermore, molecular studies revealed that tobacco systemin-like peptides were derived from a 165 amino-acid precursor protein that generates two related systemin-like peptide molecules. The tomato prosystemin and the precursor protein of the tobacco systemin-like peptides do not show similarity in their predicted protein sequences. In particular, tomato prosystemin does not have any signature to indicate the cellular localization of the mature systemin molecule. In contrast, the precursor protein for tobacco systemin-like peptides has a signal sequence to direct mature peptide molecules outside cells through the secretory pathway.

It is concluded that although there is not a high degree of sequence conservation between tomato systemin and tobacco systemin-like peptides, the link of peptide recognition by cells with cellular alkalinization responses and induction of defenses is conserved between tomato and tobacco. This suggests that alkalinization tests in suspension cell cultures may provide an alternative means to detect systemin activities in other plant species.

1.3. Extracellular Signals and Alkalinization-Associated Cellular Responses

Recognition of systemin by its receptor protein triggers rapid cellular responses including a pH increase in the extracellular space (Fig. 1.2). This was the basis of the isolation of tobacco systemin-like peptides (Pearce et al., 2001a). Alkalinization of extracellular space, which can be observed as an increase in the medium pH of suspension cell cultures, appears to be necessary for systemin-activated signaling of downstream events. By modulating the activity of the H^+-ATPase which regulates the plasma membrane potential, Schaller and Oecking (1999) showed that blocking apoplastic pH alkalinization suppressed the induction of anti-herbivore defense. Alkalinization of extracellular pH, however, is not only associated with systemin signaling but also known to be involved in other cellular responses (Felle, 2001). Other endogenous signal molecules, certain abiotic stimuli, and signal molecules derived from

microorganisms called elicitors are also capable of inducing pH alkalinization in the medium of plant suspension cultures (Pearce et al., 2001b; Felix et al., 2000; Yalamanchili and Stratmann, 2002; Nürnberger et al., 1994; Granado et al., 1995). Therefore, a rapid pH increase in extracellular space may be part of a common cellular process to sense the environment. Several mechanisms were proposed to be involved in extracellular alkalinization: the transient inhibition of the plasma membrane H^+-ATPase, proton influxes coupled with other ion channels, effluxes of anions such as bicarbonic acid, and activation of enzymes localized in the cell wall or plasma membrane (Mathieu et al., 1994; Otte et al., 2001; Felle, 2001 and reference therein). Specific mechanisms causing alkalinization in response to each environmental stimulus are as yet unknown; however, the rapid pH change seems to be associated with the further activation of the corresponding downstream signaling pathways including the induction of gene expression. Thus, measuring pH alkalinization has often been used as a device to detect changes in physiological status or the presence of extracellular signals. Several such characterized signals are reviewed below.

1.3.1. Defense-Related Signals

In addition to the anti-herbivore hormone, systemin, several other endogenous defense-related molecules are also known to induce pH alkalinization in the medium of plant suspension cultures. By analogy to systemin, oligogalacturonides that are also known to be inducer of PIs *in planta*, cause rapid pH alkalinization responses in tobacco suspension cultures (See 1.1.4. *A*; Mathieu et al., 1991). Cutin, an important component of cell walls and a polymer consisting of phenylpropanoid and esterified hydroxyfatty acids, can cause alkalinization in the medium of potato cell cultures (Schweizer et al., 1996). Alkalinization caused by cutin is followed by the production of ethylene and the induction of PAL and other defense related genes.

Plant cells are also known to show extracellular pH alkalinization responses upon recognition of signal molecules that are derived from potential pathogenic microorganisms (Boller, 1995). For instance, microorganism-derived glycoproteins, sterols, and lipo-chitooligosaccharides are known to induce pH alkalinization in culture medium. The most characterized elicitor is flagellin, a structurally conserved protein

found in the motile structure of many bacteria (Gomez-Gomez and Boller, 2002). The presence of the peptides from a highly conserved domain of flagellin induces pH alkalinization in suspension cell cultures of several plant species including tomato and *Arabidopsis* (Felix et al., 1999). Flagellin causes the alkalinization in a specific receptor-mediated manner (Meindl et al., 2000), which is followed by an oxidative burst and ethylene production that are indicative of defense responses (Felix and Boller, 1995). Similarly, pep13, a conserved peptide domain residing in transglutaminase from *Phytophthora* species, induces pH alkalinization in the medium of parsley suspension cultures and triggers defenses responses including the accumulation of phytochemicals (Nürnberger et al., 1994).

1.3.2. Other Alkalinization-Inducing Signals

Although many known alkalinization responses are related to defense signaling, signals causing alkalinization that are not related to defense signaling have also been described recently. During the purification procedure for tobacco systemin-like peptides, a novel peptide causing medium alkalinization, rapid alkalinization factor (RALF), was discovered (Pearce et al, 2001b; also see Chapter 4). The 5 kD peptide, RALF that is structurally unrelated to systemin, causes more rapid and intense alkalinization in tobacco culture medium than do the systemin-like peptide. Additionally, cells treated with RALF activate MAP kinase in a similar manner to cells treated with systemin-like peptides. Strikingly, RALF does not induce defenses, and instead shows inhibitory effects on the root growth of seedlings. In contrast to the diverse structure of systemin within the Solanaceae, RALF peptides found throughout the plant kingdom showed very conserved peptide sequences (Pearce et al., 2001b).

The analysis of a full length RALF cDNA indicates that the ~ 50 amino-acid RALF peptide is synthesized as a ~ 120 amino-acid precursor protein, with a signal peptide to sort mature peptide to secretory pathway. The mature RALF peptide contains two pairs of disulfide bridges that are shown to be structurally important for biological activity, as determined by the pH alkalinization test. Recently, a RALF-binding protein with a molecular weight of 120 kD, a putative RALF receptor protein, was identified from the plasma membrane of tomato cells (Ryan et al., 2002). RALF likely activates

receptor-mediated intracellular signaling events including pH alkalinization and activation of MAP kinases, which is independent from systemin-triggered signaling yet shows some resemblance to it (see Chapter 4 for further details on RALF).

1.4. Overview of Anti-Herbivore Defenses in *Populus*

The genus *Populus*, including poplars and aspens, is widespread in North America. In particular, trembling aspen (*Populus tremuloides*) is an early-successional species and one of the first woody plants to colonize after logging and fire, exhibiting its vital role in forest ecology (Dickmann, 2001). However, the fast growth of *Populus* appears to come at an expense, as a trade-off with other desirable traits such as resistance against stress. For example, some rapid-growth trees exhibit poor stress resilience and high susceptibility to pathogens and herbivores (Mattson et al., 2001 and reference therein). *Populus* is known to be susceptible to several leaf-feeding insects, shoot-feeding aphids, and wood-feeding borers, and insect-caused damage of *Populus* could affect the fitness of the entire forest. The means for protection of *Populus*, from insect herbivory have been proposed and evaluated including the use of insecticides, the introduction of known insect resistance genes that are derived from other organisms or plant species (McCown et al., 1991; Kang et al., 1991), and the use of insect predators or natural enemies of insects (Burkot and Benjamin, 1979; Mattson et al, 2001 and reference therein). For both practical and scientific benefits, it is important to understand innate defense mechanisms of *Populus*. Studies of an ecologically important *Populus* species, trembling aspen (*Populus tremuloides*), have shown the importance of phytochemical defenses against insect herbivory as described below (1.4.1).

In addition to the ecological importance of *Populus*, it has been used as a model organism for studying deciduous trees, due to its rapid growth and vegetative propagation that produces genetically identical clones. For experimental purposes and industrial application, a variety of poplar clones and hybrids have been developed (Eckenwalder, 2001). Some fast-growing hybrid poplars have been extensively used as experimental materials and for plantations. In the area of insect defense in hybrid poplar, several studies have been undertaken using *Populus trichocarpa x P. deltoides* hybrid. These

studies indicate the presence of significant, yet mostly unrevealed, protein-based defenses in hybrid poplar (1.4.2).

1.4.1. Studies of Anti-Herbivore Phytochemical Defenses in Trembling Aspen (*Populus tremuloides*)

Trembling aspen, which has a wide distribution in North America, is often subject to severe defoliation by a variety of insect herbivores such as the forest tent caterpillar (*Malacosoma disstria*), large aspen tortrix (*Choristoneura conflictana*) (Bechwith, 1968), and gypsy moth (*Lymantria dispar*). It has been observed that populations of forest tent caterpillars rose and fell in approximately 10-year cycles in Minnesota, Manitoba, Saskatchewan, and Alberta, and these outbreaks cause significant defoliation of aspen (Duncan and Hodson, 1958; Cerezke and Volney, 1995). However, aspen trees are not uniformly susceptible to insect herbivory, and the production of defense compounds is thought to be important in resistance against herbivores.

The interaction of herbivores and trembling aspen has been extensively studied in regards to phytochemical-based defenses by Lindroth's group (reviewed in Lindroth and Hwang, 1996). Foliar secondary metabolites in aspen that have anti-herbivore roles include the phenolic glycosides and condensed tannins. Phenolic glycosides, a suite of salicylate compounds, are distinguishing chemical feature of aspen and include four major compounds, salicin, salicortin, tremuloidin, and tremulacin (Lindroth et al, 1987a). These phenolic glycosides can be present in foliage up to 7 % dry weight depending on seasons (Lindroth et al., 1987b). In insect feeding experiments, it was shown that artificial diets containing phenolic glycosides have negative effects on larval performance of forest tent caterpillar and gypsy moth (Lindroth and Bloomer, 1991; Lindroth and Hemming, 1990). These phenolic glycosides are expected to be metabolized in herbivore guts and their enzymatically decomposed products analyzed in feeding tests also showed negative effects on larval growth (Clausen et al., 1989).

The condensed tannins, flavonoid polymers and thus products of phenylpropanoid metabolism, are also important for phytochemical defense in trembling aspen. The phenolic groups of the tannin bind to proteins and this is the basis of their anti-herbivore fundtions, which includes reducing digestivity of protein or interacting with proteins in

the herbivore digestive tracts (Barbehenn and Martin, 1994). Although condensed tannins have little impact against aspen-adapted herbivores (gypsy moths and forest tent caterpillar) as noted by Lindroth (Lindroth and Hwang, 1996; Hwang and Lindroth, 1997; Hemming and Lindroth, 1995), high concentrations of tannin limit herbivory by leaf beetles in the order of Coleoptera (Gruppe et al., 1999).

Dynamic changes in the content of phenolic glycosides and condensed tannin in aspen foliage were observed when aspen leaves were wounded. Aspen leaves accumulate salicortin and tremulacin upon mechanical leaf damage (Clausen et al., 1989). In a recent study, artificial defoliation induced an increase in the tannin concentration in aspen leaves (Osier and Lindroth, 2001). Regardless of the amount of experimental data on aspen phytochemical defense, molecular mechanisms that underlie induction of the phytochemical-based defenses are largely unknown. A recent molecular study conducted by Darren Peters in this laboratory showed that genes involved in the tannin biosynthetic pathway were induced by herbivory and mechanical wounding (Peters and Constabel, 2002). In addition to this thesis, to date this study is the sole example that shows the induction of aspen anti-herbivore defenses at the transcriptional level (Chapter 2 and Chapter 3). In contrast to annual species such as tomato and *Arabidopsis*, the molecular dynamics of aspen induced defenses are largely unknown. However, in a related species, poplar, molecular studies have been initiated and shown that poplar has an induced defense, similar to tomato. Molecular studies demonstrating induced defenses in poplar are described below.

1.4.2. Molecular Studies of Inducible Anti-Herbivore Defenses in Hybrid Poplar (*Populus trichocarpa x P. deltoides*)

Hybrid poplars (*Populus trichocarpa x P. deltoides*) that were produced during breeding program have been used as research experimental materials in the biology of poplar. Some hybrid poplar clones have also been used for poplar plantations due to their rapid growth and good form, as well as for phytoremediation due to their strong uptake of soil materials (Heilman and Stettler, 1985; Eckenwalder, 2001). Though hybrid poplars provide many potential applications, their commercial use is sometimes limited due to

due to their susceptibility to herbivory caused by poplar tent caterpillar (*Clostera inclusa*) and cottonwood borer (*Plectrodera scalator*) (Mattson et al., 2001).

The molecular biology of induced defenses in poplar was initiated by the identification of wound responsive genes by Bradshaw et al. (1991). Hybrid poplars (*Populus trichocarpa x deltoides* A. Henry '11-11') respond to mechanical wounding (simulating insect chewing) by accumulating specific mRNAs. A differential screening experiment identified a suite of *w*ound-*i*nducible (win) genes whose mRNAs accumulate systemically in response to mechanical wounding. Several *win* genes were further characterized as candidates of inducible defense genes. The *win*3 gene showed sequence similarity to known protease inhibitors that are a component of protein-based induced defenses in other plant species such as tomato (Bradshaw et al., 1989). The *win*4 gene showed similarity to a vegetative storage protein (VSP), whose mRNA also accumulated in response to wounding (Davis et al., 1993). However, the function of VSP and *win*4 during wound-induced response is not understood. The other *win* genes, *win*6 and *win*8 are similar in amino acid sequence to chitinase which catalyze the hydrolysis of chitin, a polymer of N-acetyl-D glucosamine found in fungal cell walls, and are thought to be important in defense against pathogens (Parsons et al., 1989; Davis et al., 1991a). Thus the discovery of wound-inducible genes from poplar identified several putative defense-related genes; however, specific roles of *win*4, *win*6, and *win*8 in anti-herbivore defenses have not been demonstrated.

Recently, additional work on inducible anti-herbivore defense was conducted in the Constabel laboratory by analyzing a known marker gene for induced defense, polyphenol oxidase (PPO) in hybrid poplar (*Populus trichocarpa x deltoides)* (Constabel et al., 2000). Upon mechanical wounding and forest tent caterpillar damage, leaf tissues of hybrid poplar saplings induce the accumulation of PPO mRNA and an increase in its enzymatic activity. MeJa-treatment induced poplar PPO gene expression as well. In a similar manner to tomato, hybrid poplar PPO can be systemically induced in distal unwounded leaves when their lower leaves were wounded. Systemic wound-induction of hybrid poplar PPO and *win* genes strongly suggests the presence of similar long-distance signaling mechanisms in tomato and poplar.

In the previous studies of the above-described *win* genes, the movement of wound signals was investigated in hybrid poplar saplings by Davis et al. (1991b). In the anatomy of poplar, every fifth leaf on a sapling is attached to a common vascular connection, and wounding a particular leaf causes the greatest accumulation of *win*3 mRNA in the wounded leaf and leaves that share a strong vascular connection with the wounded leaf. Moreover, blocking photoassimilate transport from a wounded leaf by shading abolished *win*3 gene expression in the remote leaves. Additionally, a recent study showed that jasmonic acid induced the translocation of carbon from source leaves to sink leaves, where biosynthesis of the defense-related compound, tannin, is induced (Arnold and Schultz, 2002). Those studies suggest the importance of phloem transport in the systemic induction of anti-herbivore defenses in poplar. The involvement of phloem transport in systemic wound signaling has been reported in the studies of mobility of systemin in tomato. However, the nature of systemic wound signal in poplar is unknown.

1.5. Objectives and Rationale of this Study

Although protein-based induced defenses have been extensively characterized in annual plant species, especially in tomato, some studies have clearly shown that trees also use similar induced defenses. Trees, as perennial species, are the largest and longest-lived organisms and perhaps have sophisticated protection mechanisms to defend themselves against biotic stresses. Understanding defense mechanisms in trees will provide new insights into plant defense. The genus *Populus* is a good model system for studying angisperm trees in both molecular biology and biochemistry, as it grows fast, is easily propagated, is amenable to tissue culturing, and is amenable to genetic transformation. Furthermore, whole genomic sequencing has been roughly completed in poplar and expressed sequence tags (EST) database from various mRNA pools are available for aspen (Wullscheger and Jansson, 2002; Sterky et al., 1998; Bhalerao et al., 2003). Therefore, *Populus* species was chosen as a plant material in this study.

When this research started, anti-herbivore defenses had been investigated only from the aspect of phytochemical defense in trembling aspen. It was therefore of interest to determine if, in addition, protein-based defenses were expressed in this native species, since several studies had previously shown that hybrid poplar demonstrates inducible

protein-based defenses including wound-induction of PIs and PPO. Additionally, it was also shown that hybrid poplar possesses systemically induced defenses activated upon tissue damage, implying the presence of systemic wound signaling and signals. For this study, I planned to conduct extended studies of protein-based induced defenses in *Populus*. The objectives of this study were: 1) to isolate and characterize a wound-inducible PPO gene (Chapter 2) and trypsin inhibitor gene (Chapter 3) in trembling aspen to demonstrate protein-based defenses in this important *Populus* species, and 2) to search for systemin-like peptide hormones from hybrid poplar, based on preliminary evidence that tomato and tobacco have similar peptides molecules. In order to survey systemin-like peptide hormones from hybrid poplar, a conventional assay (pH alkalinization test) that enabled me to indirectly detect the presence of systemin-like peptides was developed. Using the pH alkalinization test, I attempted to purify putative systemin-like peptides. Strikingly, the purification of alkalinizing factors resulted in the isolation of the two different alkalinizing peptides: RALF (Rapid Alkalinization Factor) (Chapter 4) and SALF (Slow Alkalinization Factor) (Chapter 5). RALF is apparently not a primary signal for induction of defense, but appears to be a novel peptide with hormone-like properties. SALF is likely derived from a photosystem I center complex subunit protein D (PSI-D). Considering the fact that only few biologically active small peptides with hormone-like properties have been isolated and characterized from plants to date (Ryan and Pearce, 2001), the isolation of RALF in this thesis is novel and also indicates the presence of more complex cellular mechanisms for recognition and transmission of extracellular signals than first expected. Ultimately, this thesis reports the characterization of inducible protein-based defenses and the discovery of peptides with alkalinization activities in the *Populus*.

Chapter 2

Isolation and Characterization of a Wound-Inducible Polyphenol Oxidase cDNA from Trembling Aspen

The contents of this chapter was published as primary data in the article:

Miyoshi Haruta, Jens A. Pedersen and C. Peter Constabel (2001) Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, potential substrates. Physiologia Plantarum 112: 552-558

2.1. Introduction

Polyphenol oxidase (PPO; EC 1.10.3.2) is a copper-containing enzyme that catalyzes the oxidation of *o*-diphenols to *o*-quinones using molecular oxygen (reviewed in Steffens et al., 1994). The quinoid products of PPO are highly reactive molecules that covalently modify cellular macromolecules, resulting in formation of melanin-like brown condensation polymers. PPO is found in a broad range of plant families (Mayer and Harel, 1979). However the physiological function of PPO still remains to be fully clarified. PPO has been proposed to participate in plant defenses against insects and pathogens (reviewed in Constabel et al., 1996 and references therein), possibly other cellular processes such as photosynthesis (Vaughn et al., 1988; Trebst and Depka, 1995), and flower coloration (Nakayama et al., 2000).

The hypothesis of PPO functioning in anti-herbivore defenses was tested in insect feeding experiments by Felton et al. (1989). Beet army worm larvae (*Spodoptera exigua*) fed with artificial diets containing casein, PPO, and its substrate, chlorogenic acid, showed reduced growth rate compared to those fed with diets without PPO. Allowing PPO and its substrate react prior to addition to the diet (casein) does not have significant impact on larval growth, indicating that the interaction of PPO-generated quinone with dietary protein is the basis of reduced growth rates of larvae. Therefore, PPO-caused modification of protein results in anti-nutritive effects on larval growth. In additional experiments, it was observed that cysteine, lysine, histidine, and methionine residues are preferentially alkylated by quinones, which prevent their assimilation (Felton et al., 1992a).

Furthermore, the inducibility of PPO enzymatic activity in potato leaves by insect damage supports the hypothesis of PPO functioning in defense against herbivores (Felton et al., 1992b). In a series of molecular studies, mechanical wounding mimicking insect feeding induces PPO mRNA within 24 hr in foliage of potato, tomato, and poplar (Thipyapong et al., 1995; Constabel et al., 1995; Constabel et al., 2000). In those experiments, the accumulation of PPO mRNA was also observed in upper, unwounded leaves of the plants whose lower leaves were wounded (systemic induction). PPO enzymatic activity increased accordingly. Those observations imply the importance of induced PPO activity in anti-herbivore defense in those plants.

Despite the high polyphenolic content in aspen foliage, PPO activity in trembling aspen has only been reported from a single study in the context of pathogen resistance (Takai and Hubbes, 1973). The possible involvement of PPO in phenolic glycoside toxicity (Chapter 1) and aspen defense against herbivory was therefore investigated in trembling aspen. Preliminary tests in this laboratory showed that trembling aspen grown in experimental growth chambers had significant PPO activity (Joe Patton and Peter Constabel, unpublished data). Therefore, using experimental plant materials available in the laboratory, aspen PPO as an anti-herbivore defense was studied in this chapter.

PPO cDNAs have been isolated from a number of plants including poplar (Constabel et al., 2000). The availability of the poplar PPO cDNA provided an opportunity to investigate PPO in trembling aspen at molecular level. Objectives of the studies described in this chapter were i) to isolate and characterize an aspen PPO cDNA, ii) to analyze PPO gene expression in response to herbivory and mechanical wounding, iii) to examine MeJa-inducibility of PPO gene expression in aspen. The work conducted here is the first step in examining the anti-herbivore role of PPO in trembling aspen.

2.2. Materials and Methods

Plant Materials and Treatments

Native trembling aspen (*Populus tremuloides* Michx.) were obtained locally and grown in a peat (Terra-light Redi-Earth, WR Grace, Ajax, Ontario, Canada). All plant material was maintained in the University of Alberta Biotron's growth chambers under 16 h of light (250 mmol m-2 s-1) at 18 C and 75 % relative humidity. Plants were watered daily with a solution containing 1 g L^{-1}20-20-20 fertilizer (Plant-Prod, Plant Products Co., Bramton, Ontario, Canada). Experiments were generally carried out with 10-12-week-old plants having 20-25 leaves, although for some experiments, larger plants were substituted. Leaves were numbered sequentially beginning with the youngest unfolded leaf, and leaves 9-15 were used for experiments.

Three types of induction treatments were performed. Wounding treatment was carried out by crushing leaf margins with a hemostat three times at 2-h intervals. Plants were exposed to MeJa by placing them in sealed glass boxes with 20 µl of a 10 % solution of MeJa in ethanol on a cotton wick. Tissues treated with forest tent caterpillar

(FTC) were provided by Joe Patton, University of Alberta. FTC treatment was performed by placing plants in 75-cm cages with 10 fourth and fifth instar larvae. Leaves were harvested 24 h after wounding and MeJa treatments and 36 h after FTC treatment, then frozen in liquid nitrogen and stored at – 80 °C until analyzed. For time course experiments, 6 leaves per plant were wounded, and both wounded and upper unwounded leaves were harvested at various times. All experiments were performed at least three times.

cDNA Library Screening, Sequencing, and Hybridization Analyses

A wounded leaf cDNA library was constructed from 5 μg poly (A)+ RNA, isolated from aspen leaves harvested 24 h after wounding, in Lambda ZAP II (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The library (5 x 10^5 plaques) was screened with a ^{32}P-labeled poplar PPO cDNA according to standard methods (Constabel et al., 2000; Sambrook et al., 1989). Positive plaques were purified, excised in vivo, and sequenced in both directions with the Thermosequenase dye terminator cycle sequencing system (Amersham, Baie d'Urfé, QC, Canada).

RNA was extracted from 500 mg crushed leaves using the extraction protocol of Chang et al. (1993), with the addition of one phenol and one phenol/chloroform extraction step following the LiCl precipitation. For northern analyses, 20 μg of total RNA was electrophoresed through 1.2 % agarose-formaldehyde gels, and blotted on Zeta-probe membranes (Bio-Rad). Hybridization was carried out with a full-length ^{32}P-labeled PPO cDNA at 65 °C using standard procedures (Sambrook et al., 1989). Hybridization signals were detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or recorded by autoradiography.

For Southern blots, 10 μg of genomic DNA isolated using the method of Doyle and Doyle (1990) was digested with 50 U of *Hin* dIII, *Xba* I, *Bam* HI, *Eco* RI, or *Eco* RV (Gibco/BRL, Rockville, MD) for 30 min at 37 °C. An additional 25 U of the restriction enzymes was added and digestion allowed to proceed for 24 h. Restriction fragments were desalted by ethanol precipitation, electrophoresed, and blotted on membrane. Hybridization was carried out as described above.

2.3. Results

cDNA Cloning and Sequence Analysis of Trembling Aspen PPO

Preliminary experiments of aspen PPO gene expression study showed that mechanical wounding induces PPO mRNA in aspen. Therefore, to maximize chances of obtaining PPO cDNAs, the cDNA library was generated from wounded leaf tissues where PPO mRNA should be abundant. Using poplar PPO cDNA as a probe, 138 clones showed positive hybridization signals from 5 X 10^5 screened (0.028 % of total clones). Three clones that had large insert sizes were purified and subjected to DNA sequence analysis. The largest clone was selected, and its entire nucleotide sequence determined on both strands. Database comparison confirmed its identity as trembling aspen PPO, and it was named PtPPO. The full-length cDNA was 1922bp in length and included an open reading frame of 563 amino acids with a predicted molecular mass of 64.5 kDa. The deduced amino acid sequence contained the sequence of a transit peptide for chloroplast thylakoid membrane at the N-terminus (Fig. 2.1). Removal of the predicted chloroplast transit peptide would give rise to a mature protein of 59 kDa. The deduced amino acid sequence shows significant identity with other plant PPOs, in particular those from the woody plants poplar (91.9 %; Fig. 2.2), apple (56.6 %), apricot (54.2 %), and grape (54.0 %). The copper-binding domains within the sequence are more conserved; for example, the CuA and CuB regions of PtPPO show 86.5 % and 76.3 % identity with those of apple, respectively.

Analysis of the PtPPO Gene Family

In order to determine whether or not PtPPO belongs to a multigene family, Southern blot analysis was carried out. As trembling aspen from natural populations (as used in this study) have high levels of heterozygosity, two aspen genotypes were tested; both gave the identical banding pattern (Fig. 2.3). Southern blots, washed at high stringency, showed two bands when DNA was digested with *Hin* dIII and *Xba* I and four bands after digestion with *Eco* RV. The PtPPO sequence contains a single internal *Eco* RV site but no *Hin* dIII or *Xba* I sites. This indicates that two copies of the PPO gene are present in the aspen genome. It is concluded that each band most likely represents a different PPO gene rather than different alleles at the same locus, as in wild aspen most

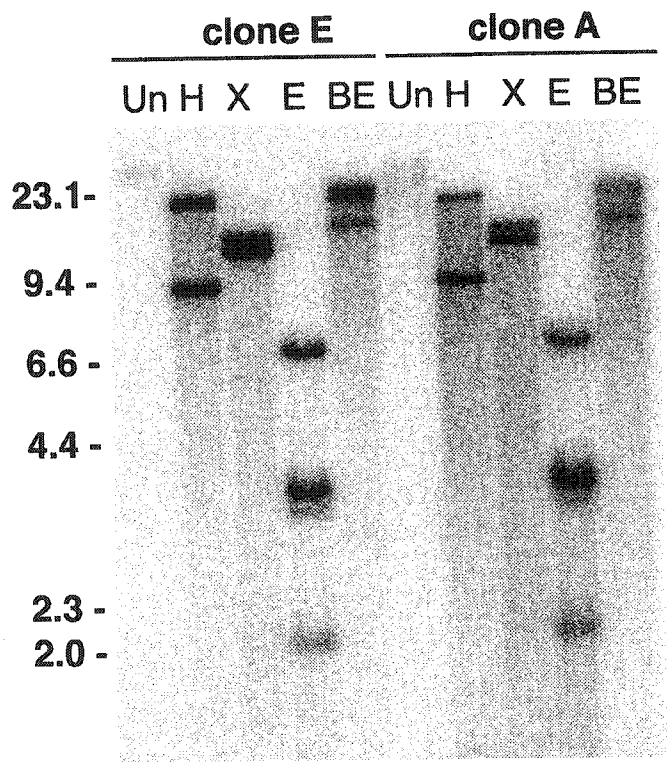
Figure 2.1. Nucleotide and predicted amino acid sequence of PtPPO. Double-underlined sequence corresponds to the predicted chloroplast transit peptide; single-underlined sequences are copper-binding domains. The PtPPO nucleotide sequence has been deposited in GenBank, accession no. AF 368291.

```
  1 CTGAACCACAGCCTCTGATTTCTCTCCTACTTTCAGCCTCGCCGTAATGTCCACCTTATC  60
  1                                                   M  S  T  L  S
    TTTCAGTCCTTTCTTTCCAAAACCACAGCAAGTTACCAAATCCAAAAGGCTGAATCACAC 120
  6 F  S  P  F  F  P  K  P  Q  Q  V  T  K  S  K  R  L  N  H  T
    ATACGTCCCTAGGGTTTCATGCAAAGCCACAGATGATACACAAAACCCTGCCACGAGAAG 180
 26 Y  V  P  R  V  S  C  K  A  T  D  D  T  Q  N  P  A  T  R  R
    AGACGTCCTCATTGGTCTAGGAGGCCTATATAGCGCTACCAATCTTGCTGACCGAACGGC 240
 46 D  V  L  I  G  L  G  G  L  Y  S  A  T  N  L  A  D  R  T  A
    CTATGCTAAGCCAATCACCTCTCCAGACTTGACCAATTGTAAGTTGGTGGACTTACCAAA 300
 66 Y  A  K  P  I  T  S  P  D  L  T  N  C  K  L  V  D  L  P  N
    CCCCGAAAATCCAACAGACTGCTGCTCTCCATTACCCAGAAAGATCATAGACTTCAGACC 360
 86 P  E  N  P  T  D  C  C  S  P  L  P  R  K  I  I  D  F  R  P
    CCCTTCTCCGTTCTCCCCGCTGCGCACTAGACGTGCGGCCCATTTAGTTGACGAAGACTA 420
106 P  S  P  F  S  P  L  R  T  R  R  A  A  H  L  V  D  E  D  Y
    CGTGGCCAAATATGCTGAAGCCATTTCCTTAATGAAAAGTCTCCCCGAAGATGATCCACG 480
126 V  A  K  Y  A  E  A  I  S  L  M  K  S  L  P  E  D  D  P  R
    TAACTTCTACCAACAAGCCAATGTCCATTGTGCCTATTGCGATGATGCCTACGAACAAGT 540
146 N  F  Y  Q  Q  A  N  V  H  C  A  Y  C  D  D  A  Y  E  Q  V
    GGGGTTTCCAAAATTAGAGCTTGATGTTCATTTCTGTTGGCTCTTCTTTCCTTGGCACAG 600
166 G  F  P  K  L  E  L  D  V  H  F  C  W  L  F  F  P  W  H  R
    ATACTATCTGTACTTCTATGAAAGAATCTTGGGCAAACTGATTAATGACCCCACTTTTGC 660
186 Y  Y  L  Y  F  Y  E  R  I  L  G  K  L  I  N  D  P  T  F  A
    TCTGCCTTTCTGGAACTGGGATTCCCCCAGTGGTATGCAAATGCCTTACATCTTTACCGA 720
206 L  P  F  W  N  W  D  S  P  S  G  M  Q  M  P  Y  I  F  T  D
    CCCTAAATCTCCACTCTATGACCAGTTCCGCGACCAGAATCACCAACCTCCTGTATTGCT 780
226 P  K  S  P  L  Y  D  Q  F  R  D  Q  N  H  Q  P  P  V  L  L
    AGATCTTGATTATGCAGCGGGAGACCCCAACCCCACAAACGCAAATCAGGTGTACTCTAG 840
246 D  L  D  Y  A  A  G  D  P  N  P  T  N  A  N  Q  V  Y  S  S
    TAATCGTAGAGTAATGTACAAGCAAATGGTGTCTGGTGCAGCAAAACCAACACTCTTTTT 900
266 N  R  R  V  M  Y  K  Q  M  V  S  G  A  A  K  P  T  L  F  F
    TGGAAAACCATACCGTGCTGGTGACGATGCTCGTCCTGGAGCTGGGACGATTGAGAGCAG 960
286 G  K  P  Y  R  A  G  D  D  A  R  P  G  A  G  T  I  E  S  S
    CCCTCACAATAATATTCACAGATGGACCGGTGATCCAACTCAAGAAAATACCGAAGACAT 1020
306 P  H  N  N  I  H  R  W  T  G  D  P  T  Q  E  N  T  E  D  M
    GGGCAATTTTTACTCAGCTGCGAGAGATCCAATATTTTTTTGCCATCACTCAAATGTAGA 1080
326 G  N  F  Y  S  A  A  R  D  P  I  F  F  C  H  H  S  N  V  D
    CCGAATGTGGACGATATGGAAGACTATACCTGGAGGACTTAGGAGGGATATCACTGATCC 1140
346 R  M  W  T  I  W  K  T  I  P  G  G  L  R  R  D  I  T  D  P
    TGATTGGCTTAATTCAGAGTTTCTTTTCTATAATGAGAATGCAGAGCTTGTTCGTTGTAA 1200
366 D  W  L  N  S  E  F  L  F  Y  N  E  N  A  E  L  V  R  C  K
    GGTTAGAGATTGTCTTGACAATAGAAGGCTAAGGTATACTTATCAAAATGTTGAAATTCC 1260
386 V  R  D  C  L  D  N  R  R  L  R  Y  T  Y  Q  N  V  E  I  P
    TTGGCTAAAATCAAAACCAATTCCAAGAAGGTTGGGAAAGAAAGCAGCTGAAACAAAAAC 1320
406 W  L  K  S  K  P  I  P  R  R  L  G  K  K  A  A  E  T  K  T
    TGCATTAACACCGATCACTGCATTCCCTTTAGTCTTAGACAAAACCATAGTTACTGTAGT 1380
426 A  L  T  P  I  T  A  F  P  L  V  L  D  K  T  I  V  T  V  V
    TTCAAGACCAAAGAAATCAAGAAGCAGGAAAGAGAAAGAAGAGGAAGATGAAGTTTTGGT 1440
446 S  R  P  K  K  S  R  S  R  K  E  K  E  E  E  D  E  V  L  V
    GATAGAAGGGATAGAATACGACAATGACAAATTCGTGAGGTTTGACGTGTTCATAAATGA 1500
466 I  E  G  I  E  Y  D  N  D  K  F  V  R  F  D  V  F  I  N  D
    TGACCTTGAGATACCTTCTAAACCAGAAAATACAGAGTTTGCTGGGAGCTTTGTTAATGT 1560
486 D  L  E  I  P  S  K  P  E  N  T  E  F  A  G  S  F  V  N  V
    TTCTCATAAGCGTGCGAAGAAGTCCAAGACAAGATTGATATTGGGGATTACAGAATTGTT 1620
506 S  H  K  R  A  K  K  S  K  T  R  L  I  L  G  I  T  E  L  L
    GGAAGACTTAGAAACTGATGGTGATGATAGCATTGTGGTGGCTTTGGTGCCCAGGTCTAA 1680
526 E  D  L  E  T  D  G  D  D  S  I  V  V  A  L  V  P  R  S  N
    TGGTGTTAGTGATCCTGTTGTCATCTCTGGCGTTAAGATTGAGTTTGTTAAAGACTGATT 1740
546 G  V  S  D  P  V  V  I  S  G  V  K  I  E  F  V  K  D  *
    ATTATCAAGGACATCTCGTTTTTTCTTATCCTTCAGGAGATCGATGTAGGTCTTTTAGTG 1800
    TTTGTTAAGATGTAGGTCTGCTGCTTAATATCATGCACGTGTAATTCAACTCTGTACAAT 1860
    CTACAATTAAAGTAATGGCAGCTGATTAATTTTTTTGATTGTTCAAAAAAAAAAAAAAAA 1920
```

Figure 2.2. Alignment of PtdPPO (poplar) and PtPPO (trembling aspen) peptide sequences. Identical amino acids are shown with asterisks and similar amino acid substitutions are shown with dots.

```
PtPPO   MSTLSFSPFFPKPQQVTKSKRLNHTYVPRVSCKATDDTQNPATRRDVLIGLGGLYSATNL 60
        **************.***.*****.****************.******************
PtdPPO  MSTLSFSPFFPKPQHVTKTKRLNHPYVPRVSCKATDDTQNPPTRRDVLIGLGGLYSATNL 60

PtPPO   ADRTAYAKPITSPDLTNCKLVDLPNPENPTDCCSPLPRKIIDFRPPSPFSPLRTRRAAHL 120
        *****.*****.****.*.**********..**.***.**********************
PtdPPO  ADRTAFAKPITPPDLTKCELVDLPNPENPSNCCTPLPKKIIDFRPPSPFSPLRTRRAAHL 120

PtPPO   VDEDYVAKYAEAISLMKSLPEDDPRNFYQQANVHCAYCDDAYEQVGFPKLELDVHFCWLF 180
        ********************.****************..***********.*** **.*
PtdPPO  VDEDYVAKYAEAISLMKSLPENDPRNFYQQANVHCAYCNGAYEQVGFPKLEIDVHSCWFF 180

PtPPO   FPWHRYYLYFYERILGKLINDPTFALPFWNWDSPSGMQMPYIFTDPKSPLYDQFRDQNHQ 240
        ************************************************************
PtdPPO  FPWHRYYLYFYERILGKLINDPTFALPFWNWDSPSGMQMPYIFTDPKSPLYDQFRDQNHQ 240

PtPPO   PPVLLDLDYAAGDPNPTNANQVYSSNRRVMYKQMVSGAAKPTLFFGKPYRAGDDARPGAG 300
        **.******.***********.****  ******************.****.*..****
PtdPPO  PPILLDLDYGAGDPNPTNANQLYSSNLTVMYKQMVSGAAKPTLFFGKSYRAGEDTSPGAG 300

PtPPO   TIESSPHNNIHRWTGDPTQENTEDMGNFYSAARDPIFFCHHSNVDRMWTIWKTIPGGLRR 360
        ***..****************.********************************** **
PtdPPO  TIETTPHNNIHRWTGDPTQENNEDMGNFYSAARDPIFFCHHSNVDRMWTIWKTIPGGTRR 360

PtPPO   DITDPDWLNSEFLFYNENAELVRCKVRDCLDNRRLRYTYQNVEIPWLKSKPIPRRLGKKA 420
        **.**********************.*****  *************.************
PtdPPO  DISDPDWLNSEFLFYNENAELVRCKVSDCLDNTGLRYTYQNVEIPWLESKPIPRRLGKKA 420

PtPPO   AETKTALTPITAFPLVLDKTIVTVVSRPKKSRSRKEKEEEDEVLVIEGIEYDNDKFVRFD 480
        ************************************************..***.**
PtdPPO  AETKTALTPITAFPLVLDKTIVTVVSRPKKSRSRKEKEEEDEVLVIEGIEYDKGKFVKFD 480

PtPPO   VFINDDLEIPSKPENTEFAGSFVNVSHKRAKKSKTRLILGITELLEDLETDGDDSIVVAL 540
        ******.*.******************.********************.**********
PtdPPO  VFINDDVEMPSKPENTEFAGSFVNVSHKHAKKSKTRLILGITELLEDLESDGDDSIVVAL 540

PtPPO   VPRSNGVSDPVVISGVKIEFVKD 563
        *****.****************.
PtdPPO  VPRSNSVSDPVVISGVKIEFVKE 563
```

Figure 2.3. Southern analysis of the PtPPO gene family in trembling aspen. Genomic DNA from two different genotypes was digested with restriction enzymes, electrophoresed and blotted according to standard protocols. Clones E and A represent different genotypes of aspen. Un, undigested DNA; H, *Hin* dIII; X, *Xba* I; E, *Eco* RV; BE, *Bam* HI and *Eco* RI.


clone E
clone A
Un H X E BE Un H X E BE
23.1-
9.4 -
6.6 -
4.4 -
2.3 -
2.0 -

alleles would be variable and produce restriction fragment polymorphisms.

Induction of PtPPO Expression by Wounding, Herbivory, and Methyl Jasmonate

Preliminary experiments indicated that undamaged leaves contain little detectable PPO mRNA when analyzed by northern blot, but that it accumulates following tissue damage. Therefore, different types of treatments to examine induction of PPO mRNA were tested. Feeding by FTC for 36 h resulted in a dramatic increase in PPO mRNA, as did mechanical wounding with a hemostat to simulate chewing insect herbivory (Fig. 2.4). In control leaves, no signal for PPO mRNA was detected. These experiments demonstrated the inducibility of PtPPO by both mechanical- and herbivore-wounding, a first indication of an anti-herbivore defensive role of PPO in trembling aspen. In additional experiments, MeJa, a known chemical inducer for wound-responsive genes in many plant species including poplar (Constabel et al., 2000), also induced PtPPO mRNA (Fig. 2.4).

The induction of PPO mRNA was characterized further. In order to study the kinetics of PtPPO mRNA induction, time course experiments were carried out. In preliminary experiments, accumulation of PPO mRNA was observed in unwounded (systemic) leaves in the wounded aspen sapling. Therefore, PPO gene expression was monitored in both wounded and unwounded leaves of the wounded plants. The accumulation of PtPPO mRNA in the wounded leaves was first detected 6 h after wounding and reached a maximum between 12 and 24 h (Fig. 2.5). In unwounded leaves, PtPPO was observed after 36 h.

2.4. Discussion

Cloning and Characterization of Trembling Aspen PPO cDNA

Resistance of trembling aspen to insect herbivory is known to involve multiple factors including physical defenses (i.e. the cuticle) and biochemical defenses (i.e. phenolic glycosides) (Lindroth and Hwang, 1996). In the study described in this chapter, protein-based induced defenses were investigated in trembling aspen using molecular techniques. A gene encoding polyphenol oxidase was first characterized as a marker

Figure 2.4. Induction of PtPPO mRNA by mechanical wounding, FTC treatment, and MeJa treatments. Plants were treated as described in Materials and methods, and leaves harvested 24 h (wounding and MeJa treatment) or 36 h (FTC herbivory) later for RNA extraction and northern analysis. Twenty micrograms of total RNA was separated on agarose gels, blotted, and hybridized with ^{32}P-labeled PtPPO cDNA. C, control; T, treated.

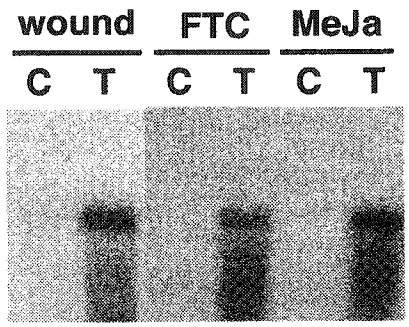
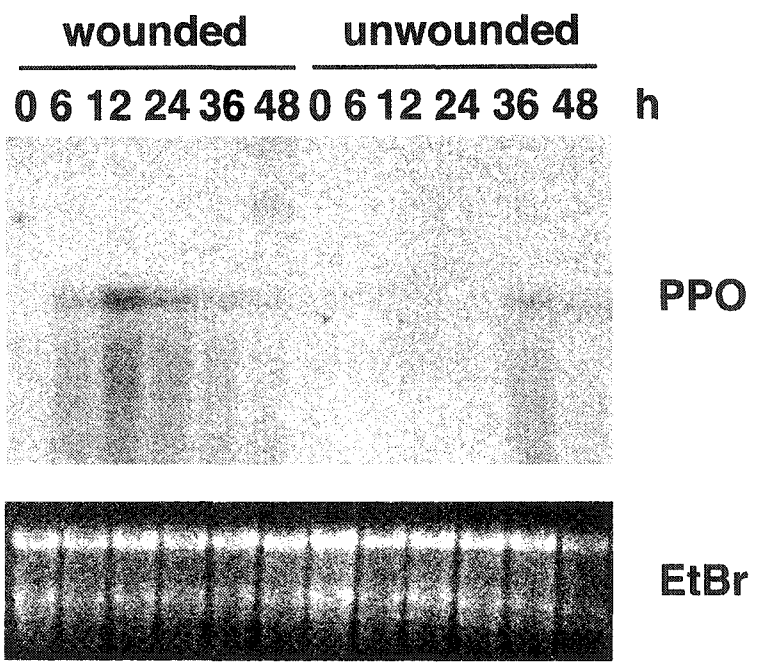

wound
FTC
MeJa
C T C T C T

Figure 2.5. Time course of wound-induced PtPPO gene expression. Leaves were wounded as described in Materials and Methods, harvested at the indicated times, and analyzed by northern blot. Unwounded leaves are from wounded plants. Upper panel, image of blot hybridized with ^{32}P-labeled PtPPO cDNA. Bottom panel, the ethidium bromide stained gel as a loading control.


wounded
unwounded
0 6 12 24 36 48 0 6 12 24 36 48 h
PPO
EtBr

gene for induced defenses in this species: a cDNA encoding PPO protein was cloned and characterized. Induction of PPO gene expression was examined following insect herbivory and mechanical wounding. In addition, the effect of a known inducer of herbivore defense genes, MeJa, on the expression of the PPO gene was analyzed.

Using a poplar PPO cDNA as a probe, a full length PtPPO gene was isolated from cDNA library constructed from wounded leaf tissues. Database analysis of PtPPO sequence confirmed that the cDNA encodes PPO. The highest sequence similarity to PtPPO was found from PPO cDNA sequences from woody plants, poplar and apple. The two copper-binding domains (CuA and CuB) showed higher sequence similarities, consistent with essential role of catalytic sites of this enzyme. The copper-binding domains of PtPPO include sets of histidine residues that are predicted to chelate two copper ions (Steffens et al., 1994).

The PtPPO-encoded protein includes a 5.5 kD transit peptide for the chloroplast thylakoid membrane at the N-terminus as predicted with the computational analysis of protein localization (http://psort.nibb.ac.jp). This prediction shows agreement with experimental observation; the plastid localization of PPO has been determined by immunocytochemical and biochemical studies in broad bean (Lax and Vaughn, 1991). The chloroplast localization of PPO supports the model of PPO reaction in defense as described (Duffey and Felton, 1991; Steffens et al., 1994). PPO located in chloroplasts of intact tissues is physically separated from its substrates, polyphenolic compounds located in vacuoles. Upon breakage of cells such as by chewing insects, PPO comes in to contact with its substrates released from vacuoles. Consequently, the PPO enzymatic reaction produces quinones which can crosslink cellular macromolecules including proteins, or form polyphenolic complexes.

Expression Analysis of Aspen PPO

In order to investigate the kinetics of PtPPO mRNA induction, time course experiments were carried out. The accumulation of PtPPO mRNA in the wounded leaves was first detected 6 h after wounding and reached a maximum between 12 and 24 h (Fig. 2.5). In contrast, unwounded (systemic) leaves, PtPPO induction was observed after 36 h. The delay in systemic PPO expression may be a result of the additional time required

for the wound signal to move to distal leaves. Induction of PPO in the systemic leaves was weaker than in the wounded leaves (Fig. 2.5) but was clearly demonstrated in several independent experiments. When wounded induction of PtPPO was monitored in leaves of differing ages, a strong dependence on leaf age was observed. Young leaves showed a stronger response to wounding than older leaves, with very old leaves not responding to wounding with PPO induction at all (data not shown). This pattern of expression is likely to be important in defense, as young leaves are more vulnerable to many herbivores than are older, tougher leaves (Coley, 1980). Induction of PPO activity in aspen leaves following wound treatment was also observed, with an average induction of approximately two-fold (data not shown). While relatively modest, this increase was consistently observed in several independent experiments, indicating that the observed induction of PPO mRNA does result in increased PPO protein accumulation as well. The PPO assays were complicated by significant inter-plant variability and a high level of background PPO activity from constitutive PPO expression. In some control leaves, PPO activity was as high as 800 μmol substrate oxidized min/mg soluble protein (using 3,4-dihydroxyphenylalanine as the substrate), which made it difficult to measure a strong increase in PPO activity above the basal level. PPO assays using methyl catechol gave the same results (data not shown). Therefore, monitoring PPO mRNA levels was considered to be a more sensitive indicator for PPO induction.

The inducible PPO gene expression observed here is consistent with a role for PPO in trembling aspen defense against herbivores, and parallels the pattern of expression for Kunitz trypsin inhibitors (Chapter 3) and dihydroflavonol reductase (Peters and Constabel, 2002). Like other plant species, trembling aspen appears to induce a number of defense genes in response to herbivore attack, which are likely regulated by a signaling system that includes jasmonates and octadecanoid signaling molecules (Weiler, 1997). Overall, the pattern of expression of PPO in trembling aspen is similar to that of hybrid poplar PPO (Constabel et al., 2000). Significant PPO expression may be common within the Salicaceae, as in a *Salix* species very high PPO levels were also measured although no wound induction was observed (Constabel and Ryan, 1998). Systemic induction of PPO mRNA was also previously reported in potato and tomato (Thipyapong et al., 1995; Constabel et al, 1996).

In conclusion, trembling aspen PPO cDNA was cloned and sequenced. Using northern analyses, it was demonstrated that PPO gene expression is induced systemically by wounding, by forest tent caterpillar feeding, and by MeJa. Together with previously reported studies by Lindroth's group (Lindroth and Hwang, 1996), anti-herbivore defense in trembling aspen therefore involves both protein- and phytochemical-based components. Such complexity resulting from an interaction of phytochemicals and plant proteins in a defense context is becoming increasingly apparent in many plant-herbivore interactions (Duffey and Stout, 1996). The presence of well-characterized phytochemicals and inducible protein-based defenses makes trembling aspen an interesting experimental system for studies of plant-herbivore interactions.

Chapter 3

Isolation and Characterization of Wound-Inducible Trypsin Inhibitor Genes from Trembling Aspen

The content of this chapter was published as part of the article:

Miyoshi Haruta, Ian T. Major, Mary E. Christopher, Joseph J. Patton and C. Peter Constabel (2001) A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. Plant Molecular Biology 46: 347-359

3.1. Introduction

Proteinase inhibitors (PIs) are a group of proteins that form complexes with proteases and inhibit their proteolytic activity. Several families of PIs are found in the plant kingdom and are classified based on the type of protease to which they are targeted (reviewed by Ryan, 1990); serine PIs, cysteine PIs, aspartic PIs, and metallo PIs. PIs are among the most studied anti-herbivore proteins against insects, and serine and cysteine PIs are more commonly found than aspartic and metallo PIs (reviewed by Constabel., 1999). PIs are often found in high concentration in plant storage organ such as seeds or tubers (Tovar et al., 1985; Mitsumori et al., 1994), possibly reflecting that they are protecting agents against invading organisms of storage tissues. Green and Ryan (1972) first demonstrated that PIs accumulate in tomato and potato leaves in response to herbivore attacks within hours of damage, indicating the dynamic nature of plant defense against insects.

The efficacy of PIs on insects has been shown in biochemical tests. Early studies of the effects of PIs on insect diet found that purified Bowman-Birk trypsin inhibitor at 5% of the diet inhibited growth of meal worm larvae (*Tribolium confusum*) (Birk, 1985). In other studies, trypsin inhibitor at 10% of the diet was toxic to larvae of *Callosobruchus maculatus* and *Manduca sexta* (Gatehouse et al., 1983; Shukle et al., 1983). In addition to those biochemical tests, more direct studies of transgenic experiments have demonstrated the effects of plant PIs in defense against herbivorous insects. Transgenic tobacco plants expressing a cowpea trypsin inhibitor gene and producing about 1% of the leaf protein as the inhibitor were more resistant to feeding larvae of *Heliothis virescens* than wild type plants (Hilder et al., 1987). Johnson et al. (1989) tested the effects of tobacco plants expressing potato trypsin inhibitor on larvae of tobacco hornworm, *Manduca sexta*. Larvae feeding the transgenic plants showed severe inhibition of growth compared to larvae feeding on control plants.

The involvement of PIs in defense against insects in *Populus* was first studied by Bradshaw et al. (1989; Chapter 1), who isolated a *win*3 (*w*ound *in*duced 3) cDNA encoding a protein similar to Kunitz type trypsin inhibitor. Transcripts of *win3* and *win3* family members were induced by mechanical wounding in poplar (Bradshaw et al., 1989; Hollick and Gordon, 1993). In other tree species, *Salix viminalis*, a gene encoding

trypsin inhibitor, *swin1.1* has been characterized and shown to be induced by wounding (Saarikoski et al., 1996). In the work described in this chapter, a gene for trypsin inhibitor (TI) from trembling aspen (*P. tremuloides*) was isolated and characterized. The purpose of this work was 1) to obtain a TI cDNA, a tool to investigate defense mechanisms using in trembling aspen, 2) to characterize trembling aspen induced defense at the molecular level using this probe. This study also extends previous work on poplar TI by demonstrating that aspen TI is induced by insect feeding and MeJa treatment. The pattern of expression of TI is consistent with a role as an inducible defense in trembling aspen.

3.2. Materials and Methods

Plant Material and Treatments

Trembling aspen were grown as described in Chapter 2, 2.2. Materials and Methods. Plant treatments by FTC, mechanical wounding, and MeJa were also conducted using the same methods described.

Screening, Cloning, and Sequencing of TI cDNA

A PCR fragment of aspen TI genomic DNA (PtTI1) was obtained from Joe Patton to use as a probe for cDNA library screening (Haruta et al., 2001a). The cDNA library constructed in the experiments in Chapter 2 was screened using standard methods (Sambrook et al., 1989). Purification of positive clones and DNA sequencing were carried out described in Chapter 2, Materials and Methods.

Hybridization Analyses

For Southern hybridization, previously Southern-blotted membrane prepared in Chapter 2 was hybridized with TI probes as described in Chapter 2, followed by washing at low stringency (1X SSC, 0.1 % SDS, 65 °C), and exposed to a phosphorimager screen. After developing image on the phosphorimager, the blot was also exposed to X-ray films. The same blot was rewashed at high stringency (0.1X SSC, 0.1 % SDS, 65 °C) and the image was again developed using the phosphorimager followed by X-ray film. For rehybridization of the membrane, the hybridized probe was stripped from the membrane

by washing in a solution containing 0.1 X SSC and 0.5% SDS at 95 °C for 20 min. Efficacy of stripping was confirmed by exposing membranes to phosphorsimager.

Northern blot analysis of TI genes was carried out using the protocol described in the Chapter 2.

3.3. Results

Isolation and Characterization of TI Genes in Trembling Aspen

Previous work in the Constabel laboratory provided two TI genes (PtTI1 and PtTI2) amplified from genomic DNA of trembling aspen using PCR with degenerate oligonucleotide primers. In order to obtain additional wound-inducible aspen TIs, the aspen cDNA library was screened in this study using PtTI1 as a probe. As a result of this screening, both weakly and strongly hybridizing plaques were identified. Three strongly hybridized plaques were identified by exposing hybridized membranes to X-ray films at room temperature for 18 hr. Weakly hybridized plaques (308 clones) were identified by exposing membranes to X-ray films at room temperature for 46 hr. Sequence analysis of inserts from both types of plaques indicated that the strongly hybridizing inserts contained sequences identical to PtTI2 that were previously cloned from PCR fragment of genomic DNA by Joe Patton. Weakly hybridizing plaques contained a distinct sequence differing from either PtTI1 or PtTI2. A full-length cDNA of this distinct type of TI was isolated, sequenced on both strands, and named PtTI3.

Conceptual translation and computer analysis of the sequence data predicted TI proteins with N-terminal signal sequences, which upon cleavage would give rise to mature polypeptides with molecular masses of 20.3, 20.0, and 18.8 kDa for PtTI1, PtTI2, and PtTI3, respectively. Sequence comparisons confirmed that PtTI1 and PtTI2 are very closely related, showing 91.7 % identity at the nucleotide level, and 83.1 % similarity at amino acid level (Fig. 3.1, Table 3.1). By contrast, PtTI3 appears quite distinct from both of these, with 52.2 % and 53.7 % amino acid similarity and 75.2 % and 75.8 % nucleotide identity with PtTI1 and PtTI2, respectively. In searches of similar sequence to PtTIs from GenBank databases, BLASTX analysis with the PtTI1 identified gwin3 and swin1.1 from hybrid poplar and willow, respectively (Altschul et al., 1997). Pairwise comparisons of the three new PtTI coding sequences with the two Salicaceae TIs

Figure 3.1. Multiple sequence alignments of three trembling aspen trypsin inhibitors with similar sequences from the Salicaceae. The three PtTI nucleotide sequences have been deposited in GenBank (accession nos. AF349441-AF349443). gwin3 (GenBank accession number P16335) and swin1.1 (AA68962) are from hybrid poplar (*P. trichocarpa x P. deltoides*) and *Salix viminalis*, respectively. The asterisks indicate regions of identity among all proteins.

```
PtTI1      1:MKITKFLGLSFLLFAFAAT-SFPEGVQAEDPEAVLDFYGDKVKAGAPYLIQDLTFIPHDN 59
PtTI2      1:MKITKFLGLSFLLFAFAAT-LFPEGVHAEDTAAVLDFYGREVQAGTPYLIQDLSYEP-GN 58
PtTI3      1:MKISNFLVLSFLLFAFTATSIFPRAVHA---AVVIDAFGDEVKAGDRYVIGAASNDFAIT 57
gwin3      1:MKITKFLGLSFLLFAFAAT-SFPDGVHAEDPAAVLDFYGREVQAGASYLI-D-----QED 53
swin1.1    1:MKITKFLALSFLLFAFTATS-FPHAVHAEDPAAVLDFYGNEVTAGASYFID-HEDSLAVS 58
             ***  ** ******** **  **  * *     * *  *  * **  * *

PtTI1     60:TTNYVVGATKSNDGVNRDVILSYGNEGLPVTFSPVTKSTDGVIREGSLITVSFDADTCKM 119
PtTI2     59:TSNFVVGAT-INPICNSDVVLSYENDGLPVTFSPVTESTDGVIREGTLITVSFDAATCKM 117
PtTI3     58:ATSPII----CN---S-DVVFSPMSDGLPVIFSKVVESNDSVINEDSYLNVDFDAPSCRM 109
gwin3     54:-F-RVVNAT-INPICNSDVILSTGIEGLPVTFSPVINSTDGVIREGTLITVSFDASTCGM 110
swin1.1   59:AATRII----CN---S-DVTLSPMSDKLPITFSPVVESTDSVIREGAYLNVNFNAILCRM 110
                     *     **  *     **  ** *  * * ** *     * * *  * *

PtTI1    120:AGVTPMWKIGFNSTGTGYIVTTGGVDQLNQFTITKYEKEGSFYQLSYCPNSDPFCECSCV 179
PtTI2    118:ADVTPMWKIGFNSTGTGYIVTTGGVDQLNQFMITKDKNESSFYQLSYCPKSDPFCECSCV 177
PtTI3    110:AGVSTMWKIELRLTARGFVVTTGGVAGLNRFTITKYEGGTNQYQLSYCPISEPICECSCV 169
gwin3    111:AGVTPMWKIGFNSTAKGYIVTTGGVDRLNLFKITKFESDSSFYQLSYCPNSEPFCECPCV 170
swin1.1  111:AGVTTMWKIELRATMRGFVVTTGGVDRLNLFKITKLEGDNSLYQLSYCPVSDPFCECSCV 170
             * *  ****    *  *  ******  ** * ***       ******* * * *** **

PtTI1    180:PVGANDDKYLAPKAADVVQVRFKPELNIYGDKMVSE                          215
PtTI2    178:PVGATNDKYLAPKAAVVVDVRFKPELNIYGDKMVSE                          213
PtTI3    170:PLGNVVNR-LAPST-VPFPVVFIPSDRASKIEYKMM                          203
gwin3    171:PVGANSDKYLAPNVSY-ADFRFKPDARIEST-----                          200
swin1.1  171:PVGNSCNR-LVPNARTPLLVVFEPDTDTAS------                          199
             * *      * *         * *
```

Table 3.1. Comparison of protein sequence identities (%) of three trembling aspen trypsin inhibitors with similar sequences from hybrid poplar (gwin3) and *Salix viminalis* (swin1.1).

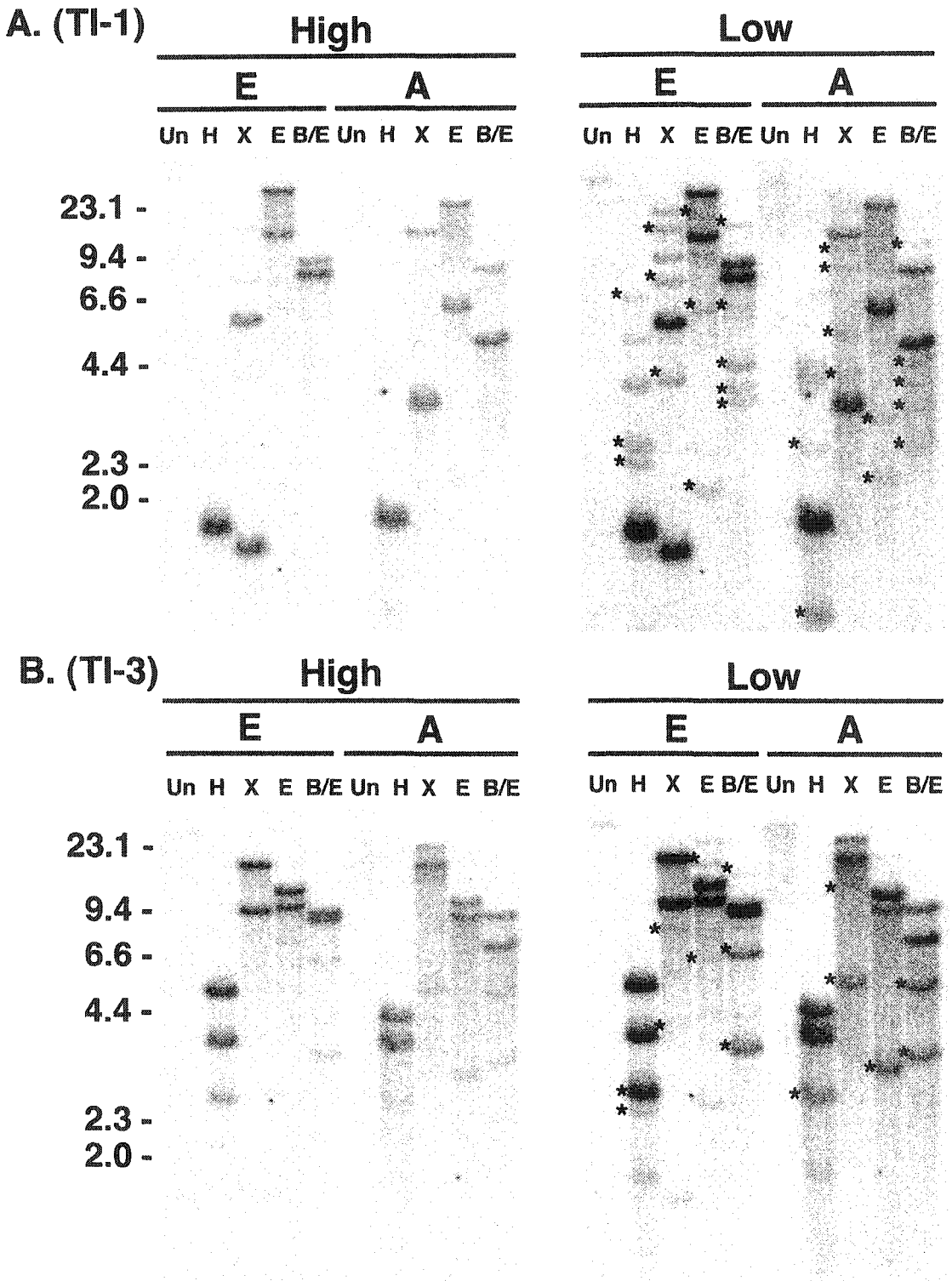
	PtTI1	PtTI2	PtTI3	gwin3	swin1.1
PtTI1	100	83.1	52.2	75.0	58.5
PtTI2		100	53.7	76.0	60.8
PtTI3			100	53.0	63.3
gwin3				100	65.3

indicated that PtTI1 and PtTIs are much more similar to gwin3 (75-76 % identity) than to PtTI3 (53% identity; Table 3.1). This suggests that PtTI1/2 and gwin3 could be orthologues and PtTI3 represents a divergent member (paralogue) with within this gene family. By contrast, swin1.1 is slightly more similar to PtTI3 than are PtTI1 and PtTI1 (Table 3.1). Among the next closest matches to the PtTIs in the databases are tobacco NF34 isolated during a screen for hypersensitivity-inducing proteins (Karrer et al., 1998), the nematode-induced LeMir gene from tomato (Brenner et al., 1998), and storage proteins from cocoa and sweet potato (Hattori et al., 1985; Spencer and Hodge, 1991). These all show identities of between 28% and 39% with the three PtTIs (not shown). However, only for swin1.1 and the sweet potato protein (sporamin) has trypsin inhibitor activity of the gene product been demonstrated (Saarikoski et al., 1996; Yeh et al., 1997).

Analysis of the TI Gene Family in Trembling Aspen

In order to estimate the size of the PtTI gene family in trembling aspen, Southern analyses of genomic DNA was carried out. Restricted DNA from two trembling aspen genotypes (genotypes 'A' and 'E') was used, and hybridized with PtTI1. This probe is 91.7 % identical to PtTI2 at the nucleotide level, and thus will hybridize to all PtTI2 genes as well. After high stringency washes, one or two bands were detected in both genotypes, depending on the restriction enzyme (Fig. 3.2). The pattern observed for each of the genotypes was distinct, although some bands were present in both. Since the plant material used is from wild, outbreeding individuals and highly heterozygous, these bands likely represent different alleles. The blot was then stripped and reprobed with PtTI3. With this probe, generally two bands were detected (Fig. 3.2B). The bands were clearly different from those observed with PtTI1 (compare to Fig. 3.2, panel A and B). As with PtTI1, the two genotype again gave distinct patterns, suggesting that distinct alleles are being detected. Under low stringency washing conditions, the same Southern blots revealed additional bands which are not recognized by either PtTI1 or PtTI3 probes under high stringent washing conditions (Fig. 3.2, right panels; see bands marked with an asterisk). Therefore, there appear to be additional TI-like genes in the aspen genome.

Figure 3.2. Southern analysis of PtTI gene family in trembling aspen. Genomic DNA from two different genotypes was digested with restriction enzymes, electrophoresed and blotted according to standard protocols. The same blots were probed with PtTI1 (top panels) and PtTI3 (bottom panels) and washed under high and low stringency conditions (left and right panels, respectively). Stars indicate those bands which did not hybridize strongly to either PtTI1 or PtTI3, and thus represent other TI-like sequences. E and A are different genotypes of aspen. Un, undigested DNA; H, *Hin* dIII; X, *Xba* I; E, *Eco* RV; B/E, *Bam* HI and *Eco* RI.


A. (TI-1)
High
Low
E
A
Un H X E B/E Un H X E B/E
23.1 -
9.4 -
6.6 -
4.4 -
2.3 -
2.0 -
B. (TI-3)
High
Low
E
A
Un H X E B/E Un H X E B/E
23.1 -
9.4 -
6.6 -
4.4 -
2.3 -
2.0 -

Herbivore-Induced Expression of TI Genes

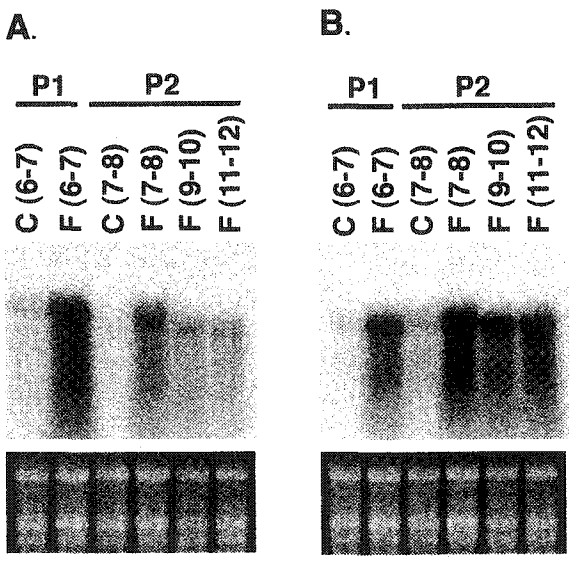
In order to determine if the PtTI genes act as inducible anti-herbivore defenses in trembling aspen, the effects of herbivory on PtTI gene expression were tested. Herbivore treated leaf tissues were obtained from Joe Patton in this laboratory. Two-month old aspen plants were subjected to herbivory by forest tent caterpillar (FTC) larvae for 36 hr. Since the FTC were allowed to move and feed on the entire plant, the amount and distribution of damage was difficult to control, and by their own preference the larvae fed mostly on leaves 6-12 (not shown). Therefore, only leaves with significant herbivore damage [leaves 6-7 for Plant 1(P1), leaves 7-12 for Plant 2 (P2)] were harvested for RNA analysis.

Analysis of the accumulation of TI mRNA by northern blot indicated that PtTI gene expression is strongly stimulated by FTC feeding. Replicate blots were hybridized with both PtTI1 and PtTI3 probes, and in both case, the patterns of expression was similar. Both probes clearly show that FTC damage results in an induction of both PtTI1/2 and PtTI3 mRNA in damaged leaves, with low transcript levels in control leaves (Fig. 3.3A and B). In general, PtTI mRNA accumulation was proportional to the amount of tissue removed by the herbivores (Fig. 3.3C). However, the relative signal profiles detected by the PtTI 1and PtTI3 probes were found to differ somewhat (compare panels A and B, Fig. 3.3). This suggests that although both genes are FTC damage-induced, there may be some differential expression of the two genes. While PtTI1 and PtTI3 cross-hybridize under the hybridization conditions used in this study (see Fig. 3.2), this was weak and unlikely to contribute significantly to the signals detected in Fig. 3.3. It was concluded that both PtTI1 and PtTI3 are herbivore-inducible, but that there are minor differences in expression levels between them. Additional blots probed with both types of probes showed similar profiles as well, suggesting that both types of transcripts accumulate in parallel (data not shown). Therefore, in subsequent RNA analyses, only PtTI1 was used as a probe.

Time Course Analysis of Wound-Induced TI Gene Expression

In order to determine kinetics of TI induction in aspen, time course analyses were carried out. To better control the timing and extent of damage, we simulated herbivory

Figure 3.3. Induction of PtTIs by herbivory in trembling aspen. Plants were subjected to herbivory by FTC for 36 h and analyzed by northern blot. Panel A was probed with PtTI1, panel B with PtTI3. Replica ethidium bromide-stained gels are shown as RNA loading controls.



C.

Plant	leaf number	damage (%)
P1	6-7	10-15%
P2	7-8	15-20%
P2	9-10	8-10%
P2	11-12	~ 5%

by the use of pliers. Previous work in poplar and aspen had indicated that the defense response is activated plant-wide, i.e., systemically (Parsons et al., 1989; Constabel et al., 2000; Chapter 2). Therefore, induction of PtTI was analyzed in both wounded and unwounded (systemic) leaves from wounded plants. Both wounded and unwounded leaves were harvested for northern analysis at time points of 0, 6, 12, 24, 36, and 48 hr. Fig. 3.4 shows that PtTI mRNA accumulated within 6 hr of damage in the wounded leaves, with a maximum at 24 hr. By contrast, in the unwounded leaves the response was visible only after 36 hr, and less intense than in the wounded leaves. Overall, this experiment demonstrated that TI expression is induced rapidly in response to wounding and the induction is systemic, consistent with an adaptive role in plant defense.

Induction of TI Gene Expression in Response to MeJa

Jasmonates are common inducers of defense responses in a variety of plants (Weiler, 1997). Previous experiments on wound-induced trembling aspen genes, PPO, suggested that methyl jasmonate (MeJa) is as an inducer of the defense response in trembling aspen (Chapter 2). Therefore, the effect of MeJa on TI expression was studied using aspen leaves from young (4-week old) and older (12-week old) plants. For comparison, a second set of trees was mechanically wounded. In both younger and older trees, MeJa induced TI gene expression, but the response was consistently stronger in the leaves from younger trees (Fig. 3.5). A similar age-dependent pattern was observed for wounded leaves, although overall response levels were stronger for wounded than MeJa-treated leaves. In conclusion, MeJa induction of aspen TI gene expression indicates that wound-activation of defenses in trembling aspen is regulated by a similar signaling pathway to tomato (see Discussion).

Wound-Induction of TI Gene Expression in Stem Tissues

In addition to leaf tissues, wound-induction of TI expression was analyzed in aspen stem tissues. Understanding expression of TI in stem tissues may provide insight into the transmission of wound signal to adjacent tissues. Stems from older and younger plants were wounded using a razor, and mRNA from both the bark and developing wood were analyzed individually by northern blot. In both tissues, TI mRNA was wound-induced

Figure 3.4. Time course of wound-induced TI gene expression in aspen leaves. Leaves were wounded as described in Materials and Methods (section 3.2), and harvested at the indicated times and analyzed by northern blot. Systemic leaves are unwounded leaves on wounded plants. The blot was probed with PtTI1. A portion of the ethidium bromide-stained gel is shown for a loading control.

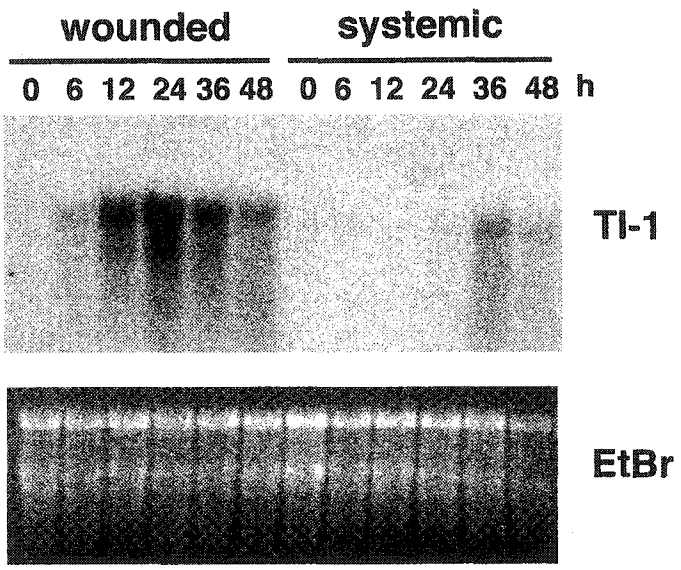
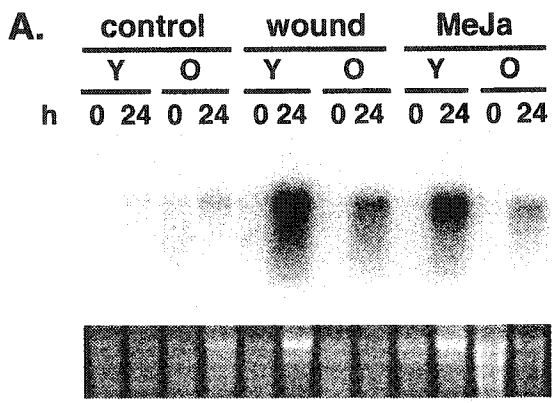
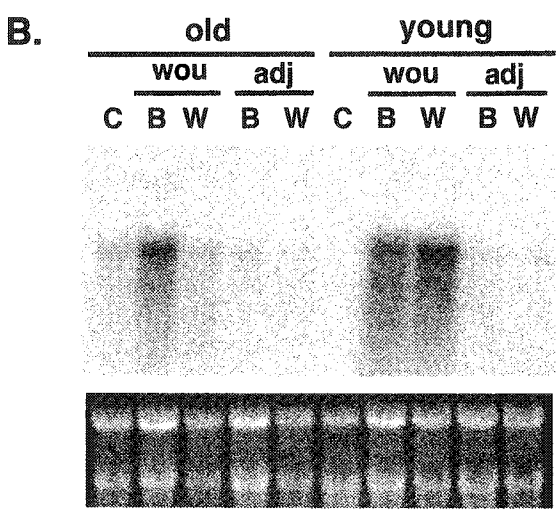

wounded
systemic
0 6 12 24 36 48 0 6 12 24 36 48 h
TI-1
EtBr

Figure 3.5. Northern analysis of TI1 gene expression by wounding and methyl jasmonate at different ages. (A) Induction of TI gene by wounding and methyl jasmonate in leaves at different ages of trembling aspen. Y, 4-week old; O, 12-week old. 0, before treatments; 24, 24 h after wounding or MeJa treatment. (B) Northern analysis of TI gene expression by wounding in aspen stem tissues. Y, 4-week old plants; O, 12-week old plants. Replica ethidium bromide-stained gels are shown as RNA loading controls. wou, wounded stem; adj, adjacent unwounded tissue on wounded stems; C, control (entire stem); B and W indicate bark and wood, respectively, from wounded stems.


A.
control
wound
MeJa
Y
O
Y
O
Y
O
h
0 24 0 24 0 24 0 24 0 24 0 24


B.
old
young
wou
adj
wou
adj
C B W B W C B W B W

and again in younger tissues the response was stronger (Fig. 3.5). Both bark and developing wood appeared to have wound-induced TI mRNA. However, the induction was restricted to the wounded area, as no TI mRNA induction could be detected in adjacent unwounded stem tissues (Fig. 3.5B). These results suggest that systemic wound signal is likely transmitted through vascular system in trembling aspen.

3.4. Discussion

Cloning and Characterization of Trembling Aspen TI cDNA

In order to characterize protein-based defenses in trembling aspen, TI genes were isolated and characterized in the study described in this Chapter. Using a PCR fragment of PtTI1 which was previously cloned in this laboratory, cDNA library screening was carried out. Subsequently, two TI sequences (PtTI2 and PtTI3), belonging to two classes, were isolated. PtTI1 and PtTI2 constitute one class and may be alleles at the same locus; the previously isolated hybrid poplar gwin3 genes are most similar to this group. PtTI3 defines a second, divergent class which has not been previously described. Southern analysis suggests that the PtTI gene family of trembling aspen is large; in addition to the genes characterized here, there appear to be additional TI-like genes, as indicated by weakly hybridizing bands on Southern blots (Fig. 3.2). Altogether it was concluded that there are at least three to four Kunitz type TIs in trembling aspen. In hybrid poplar, Hollick and Gordon (1993) identified five distinct Kunitz TI (gwin3-like) sequences in a genomic library, and there are at least three TI genes (swin1-like) in *Salix viminalis* (Saarikoski et al., 1996). This multiplicity of TI genes is consistent with a defensive function, since a single TI is effective against proteases found in some but not other herbivores (Ryan, 1990; Richardson, 1991). A battery of TIs with differing specificities for gut proteases could be important for defending against different herbivores with distinct digestive enzymes. Furthermore, several insect pests have been shown to contain a diversity of trypsin-like enzymes which may protect them against the effects of PIs. Jongsma et al. (1995) demonstrated that feeding *Spodoptera exigua* tobacco foliage over-expressing potato PI2 caused these herbivores to switch to PI2-resistant proteases, and thus resist the effects of PI2 (Jongma et al., 1995). A similar shift to PI-resistant protease was seen with several other lepidopteran pests including *Lymantria dispar* and

Helicoverpa zea when fed diets containing cabbage PI (Broadway, 1995). The presence of several related TI genes in trembling aspen may therefore be a counteradaptation to diversity of digestive enzymes in insects.

At the population level, additional TI diversity could reside in multiple TI alleles, as suggested by the restriction fragment polymorphisms detected on Southern blots (Fig. 3.2). Wild populations of trembling aspen have been shown to contain substantial genetic diversity (Yeh et al., 1995). TI genes appear to be particularly divergent, since Southern blots using the defense enzyme polyphenol oxidase as a probe detected no restriction fragment polymorphisms between the genotypes tested here (Chapter 2). Furthermore, PPOs from aspen and hybrid poplar are 92 % identical; the highest scores for the most similar TIs from these plants (PtTI2 and gwin3) is only 76% (Table 3.1). It thus appears that the TI genes are evolving more rapidly than other genes. Previous studies of hybrid poplar and *S. viminalis* had described a hypervariable region near the C-terminus of gwin3 and swin1 (Hollick and Gordon, 1993; Saarikoski et al., 1996). Given the adaptability of insects to their multiple digestive enzymes described above, rapid evolution of TI gene could be very adaptive. A similar situation has been described for disease resistance genes from crop plants, where resistance genes have been shown to evolve more rapidly than other region of the genome, facilitating rapid adaptation to new pathogen races (Richter and Roland, 2000).

Induction of TI Gene Expression in Trembling Aspen

As demonstrated for hybrid poplar and *S. viminalis*, the PtTI genes of aspen are systemically wound- and herbivore-induced. Northern blots with both PtTI1 and PtTI3 as probes indicated that both classes of TI are inducible, although there appear to be some differences in expression pattern. Such differences will have to be further defined using gene-specific probes, since the full length cDNA probes used here show some weak cross-hybridization (Fig. 3.2). Induction of Kunitz TI genes by wounding and herbivory has been previously documented in hybrid poplar and *S. viminalis*, as well as in sweet potato (Bradshow et al., 1989; Saarikoski et al., 1996; Yeh et al., 1997).

Similar to other inducible herbivore defense systems, PtTIs appears to be regulated via the octadecanoid pathway (Farmer and Ryan, 1992), as MeJa induced the TI genes in

aspen leaves (Fig. 3.5). Octadecanoid-mediated defense in other species, such as tomato, is complex, and at least 19 induced genes are up-regulated by this pathway (Bergey et al., 1996). Most likely, trembling aspen induced defense will be equally diverse, and in addition to TIs, other defense proteins may also be induced. Besides the already mentioned polyphenol oxidase, other up-regulated defense genes including PAL, CHS, and DFR, have been identified in trembling aspen (Peters and Constabel, 2002).

TIs as Inducible Defensive Proteins in Trembling Aspen

The significance of identifying the PtTI gene family in trembling aspen is in clearly establishing the presence of an inducible, protein-based defense system in this forest tree. Since toxic phytochemicals, such as phenolic glycosides and condensed tannin, are already well characterized in trembling aspen, this provides an opportunity for studying both inducible protein-based defenses and constitutive secondary metabolite-based defenses within the same experimental system. Potential trade-offs have been proposed to exist between constitutive and induced defense strategies (discussed in Agrawal et al., 1999), and this could be tested in trembling aspen. A high level of genotype-dependent variability in secondary metabolites has been observed in trembling aspen; for example, condensed tannin levels can vary by a factor 8-10 (Lindroth and Hwang, 1996; Constabel and Spence, unpublished data). It will be interesting to assay inducible protein defenses in these genotypes.

Alternatively, constitutive and induced defenses may not represent a trade-off, but rather perform complementary functions. This is consistent with Havill and Raffa (1990) who reported no negative correlation in constitutive and induced resistance in a group of diverse poplar hybrids. Multiple defense mechanisms should help to slow the evolution of resistance within herbivore populations, or may represent defense specialized against particular pests. Furthermore, a different defense strategy may be required in different plant organs or at different developmental stages. For example, young leaves are generally preferred by herbivores because of higher nutritive content and lower structural components, such as lignin (Coley, 1980). For trembling aspen, there is direct evidence that FTC are adapted to these leaves, since under natural conditions, FTC hatch out synchronously with spring leaf flush and feed on the very young leaves. If the hatch is

delayed and the larvae are forced to feed on older leaves, development is significantly slowed (Parry et al., 1998). Since the first leaves of the season contain lower levels of condensed tannin than later ones (Lindroth and Hwang, 1996; Constabel and Spence, unpublished), it is tempting to speculate that the young leaves rely on rapidly inducible protein-based defenses, rather than the more slowly accumulating secondary metabolites, for their defense. This idea is supported by the data shown in this study indicating that the youngest leaves show strongest wound induction of PtTIs. Further studies of PtTI expression in trembling aspen in relation to phytochemical defense in a variety of genotypes should lead to a clear picture of the interaction of constitutive and induced defenses of trembling aspen.

In summary, we have characterized a family of Kunitz TI genes from trembling aspen, a species for which PIs had not previously been described. The presence of a family of rapidly divergent TI genes in trembling aspen, their induction in response to herbivory, wounding, and MeJa suggest a defensive role of PtTI genes. Trembling aspen appears to have evolved multiple defense strategies which includes inducible defensive proteins as well as secondary metabolites. Future work will focus on insect bioassays of transgenic plants that overexpressing TI protein.

Chapter 4

Search for Extracellular pH Alkalinization Peptides from Poplar I. Rapid Alkalinization Factors in Poplar Cell Cultures

The chapter was published essentially in this form as the article cited below. Three Figures (4.1B, 4.2, and 4.4) that were not shown in the publication have been added in this version.

Miyoshi Haruta and C. Peter Constabel (2003) Rapid Alkalinization Factors (RALFs) in Poplar Cell Cultures: Peptide Isolation, cDNA cloning, and Differential Expression in Leaves and Methyl Jasmonate-Treated Cells. Plant Physiology 131: 814-823

4.1. Introduction

An essential feature of all plant cells is the electrochemical proton gradient across the plasma membrane, generated by the plasma membrane H^+-ATPase, which uses ATP to pump H^+outside the cell. This H^+gradient is important for many physiological processes including ion uptake, solute transport, and cell wall growth (Sanders and Bethke, 2000). Moreover, transient changes in extracellular or intracellular concentrations of H^+, and the accompanying plasma membrane depolarization or hyperpolarization, are implicated in the rapid responses of cells to environmental stimuli; for example, changes in turgor, gravity, and pathogen attack (Blumwald et al., 1998; Felix et al., 1999, 2000; Johannes et al., 2001). A common observation is the rapid alkalinization of the extracellular solution, which can be conveniently observed in suspension cell cultures; as a consequence, stress-induced culture medium alkalinization has become a useful tool for monitoring the rapid events that accompany stress signal transduction (Felix et al., 1993; Blumwald et al., 1998; Schaller and Oecking, 1999). Depending on the system, these rapid increases in medium pH could potentially be caused by several mechanisms, including activation of K^+/H^+antiporters, H^+/solute cotransporters, and other ion channels, as well as an inhibition of the plasma membrane H^+-ATPase (Mathieu et al., 1994).

The response of cells to plant pathogens and pathogen-derived signal molecules called elicitors has been extensively studied using changes in culture medium pH. For example, glycoproteins, peptides, sterols, lipo-chitooligosaccharides, and oligosaccharide elicitors can all induce alkalinization of culture media (Boller, 1995, and references therein). A well-characterized elicitor is bacterial flagellin, which induces medium alkalinization of tomato (*Lycopersicon peruvianum*) cell cultures within minutes, and active oxygen species several hours later (Felix et al., 1999). Active oxygen and the oxidative burst are known to be important components of plant defense against pathogens and are induced in many plant-pathogen interactions (Bolwell, 1999). In parsley (*Petroselinum crispum*) cells, a 13-amino acid peptide called pep-13, derived from a glycoprotein of *Phytophthora megasperma*, also induces rapid medium pH alkalinization. This is later followed by the induction of phenylalanine ammonia lyase (PAL) and the formation of defensive phytoalexins (Nürnberger et al., 1994), suggesting a link between

alkalinization and the defense response.

In addition to pathogen elicitors, plant-derived signals can cause culture medium alkalinization. Felix and Boller (1995) found that a tomato cell culture homogenate induced alkalinization in tomato cultures, as did the peptide wound hormone systemin. Alkalinization is associated with the specific binding of systemin to a receptor (Scheer and Ryan, 1999), and is followed by the induction of ethylene production and PAL activity (Felix and Boller, 1995). By using the fungal toxin fusicoccin to activate the H^+-ATPase and block extracellular alkalinization in tomato seedlings, Schaller and Oecking (1999) showed that the alkalinization is required for the induction of tomato defense genes. Therefore, the rapid pH changes observed in cell culture are essential components of signal transduction pathways. Furthermore, other plant peptides have been shown to trigger cell culture alkalinization: a recent report described the purification of a novel peptide from tobacco (*Nicotiana tabacum*) that causes rapid culture medium alkalinization (Pearce et al., 2001b). However, this peptide, named the rapid alkalinization factor (RALF), does not induce defense-signaling pathways, but inhibits root growth when present in the surrounding medium. Homologs to RALF-encoding genes are found in many plant expressed sequence tag (EST) databases. Although the functions of these peptides and genes is as yet unknown, their high degree of sequence conservation and wide representation in plant sequence databases suggests a fundamental function in plants (Pearce et al., 2001b).

Populus as a genus is very amenable to tissue culturing, and cell culture lines derived from hybrid poplar (*Populus trichocarpa x Populus deltoides*) have been maintained for many generations (de Sá et al., 1992). Due to its rapid growth, ease of vegetative propagation, and tractability to *Agrobacterium tumefaciens*-mediated genetic transformation, *Populus* has become a model organism for tree biotechnology, genomics, and proteomics (Sterky et al., 1998; Mijnsbrugge et al., 2000). These features also make poplar an economically important tree species, and poplar is grown in large-scale plantations for pulp production. Based on our interest in poplar and its responses to environmental stress, we began an investigation of pH alkalinization activities in hybrid poplar leaf extracts. In this study, we describe the isolation and characterization of several culture medium-alkalinizing peptides from poplar leaves, the cloning of two

corresponding cDNAs and analysis of homologous genes in the databases, and their expression in poplar saplings and cell culture. While the work was in progress, we learned that a peptide alkalinizing factor, RALF, had been identified and characterized in tobacco (Pearce et al., 2001b), and that our peptides were closely related to it. Thus, our work on RALF from poplar extends the characterization of these biologically active peptides to another plant family. It also provides additional data on the expression of the poplar RALF genes, which should help in elucidating in planta function of these novel peptides.

4.2. Materials and Methods

Plant Material

Poplar hybrid H11-11 (*Populus trichocarpa x Populus deltoides*) was propagated from green cuttings in peat (Terra-Lite Redi-Earth, WR Grace, Ajax, ON, Canada) in 15-cm-diameter pots as described (Constabel et al., 2000). Plants were maintained in environmental chambers under 16-h days at 18 C and 75 % relative humidity. Light intensity was 300 mEm^{-2} s^{-1} at pot height, composed of approximately 20 % incandescent (2,700-W) and 80 % cool-white (11,880-W) light. Plants were watered daily with solution containing 1g L^{-1} 20-20-20 Plant-Prod complete fertilizer (Plant Products, Brampton, ON).

Cell Cultures, Alkalinization Assays, and Elicitor Treatments

Hybrid poplar (H11-11) suspension cells were obtained from Dr. Carl Douglas (University of British Columbia) (de Sá et al., 1992) and maintained in Murashige Skoog medium (Sigma, St. Louis, MO) adjusted to pH 5.5-5.6 with KOH. For routine maintenance, 5 ml of a 1-week-old culture was transferred into 40 ml of medium in 200 ml flasks and maintained on an orbital shaker at 100 rpm in dark at room temperature. A 2-ml aliquot of cells was transferred into each well of 12-well tissue culture plates (Corning, Corning, NY) and allowed to equilibrate on an orbital shaker at 120 rpm for 50 min. Fraction (2 µl) were added to the cells and the change in pH of the medium was measured every 5 min for 35 min using an Accumet pH meter with an Accuphast pH electrode (Fisher Scientific, Nepean, ON, Canada).

Flg22 peptide and Pep-13 were kindly provided by Dr. Georg Felix, (Friedrich Miescher-Institut, Basel) and Dr. Thorsten Nürnberger (Institut für Pflanzenbiochemie, Halle, Germany), respectively. Partially acid hydrolyzed chitosan was obtained from Dr. Armand Seguin (Canadian Forest Service, Ste.-Foy, QC). Stock solutions for flg22, Pep-13, and chitosan were prepared at concentration of 1mM, 5mM, and 1mg mL^{-1}, respectively. Two microliters of elicitor stock solution was added into 2 mL of poplar cell for the pH alkalinization assay.

For northern analysis, 3-d-old cultures were treated with the elicitors for a 5-h period. Treatments consisted of final concentrations of 2.5 mg L^{-1} benzyl adenine, 2.5 mg L^{-1} naphthalene acetic acid, 50 μM MeJa (Bedoukian Research Danbury, CT), 9 % (w/v) Suc, 16.5 mg mL^{-1} NH_4NO_3, 0.5 mg mL^{-1} chitosan, 0.1 % (v/v) *Phytophthora megasperma* elicitor (Lisker and Kuc, 1977), or 5 x 10^{-5} N HCl. For MeJa time course experiments, eight flasks of 40 mL of culture were individually treated with 50 μM MeJa and cells were harvested at different time points. Treated cells were harvested by centrifugation at 1,600 g for 15 min, frozen in liquid nitrogen, and stored at – 80 °C until analyzed.

Peptide Isolation

Hybrid poplar leaf and petiole tissue (800g) was collected from 2-month-old poplar saplings. The tissues were homogenized in 200-g batches in a blender with 1 L of 1 % (v/v) trifluoroacetic acid (TFA), filtered through two layers of Miracloth (Calbiochem, La Jolla, CA), and the homogenate centrifuged at 10,000 g for 15 min. The supernatant was separated on C18 media (J.T. Baker, Phillipsburg, NJ) using open column reverse-phase chromatography (2.5 X 20 cm). The 60 % (v/v) methanol-eluting fraction showing rapid medium pH-alkalinizing activity was lyophilized, the active material was redissolved in 60 % (v/v) methanol/0.1 % (v/v) TFA, and separated on a Sephadex G-25 (Pharmacia, Uppsala) gel chromatography column (2.5 X 55.5 cm). A portion of the active fraction was lyophilized and further purified on a cation exchange Macro-spin tube (PolySulfoethyl A, The Nest Group, Southboro, MA). The 1 M KCl-eluting fraction was further separated using SCX HPLC (PolySulfoethyl A, 200 X 4.6 mm, The Nest Group) running a 350 to 650 mM KCl gradient in 25 % (v/v) acetonitrile/5mM potassium

phosphate buffer (pH3.0). Activity peaks 1, 2, and 3 were applied to a C18 HPLC column (Bondpack C18, 3.9 X 300 mm, Waters, Milford, MA) and separated with an acetonitrile gradient in 0.1 % (v/v) TFA. The purified peptides were analyzed by MALDI-MS at the University of Alberta's MS Facility. N-terminal sequencing was carried out using Edman chemistry at Washington State University (Pullman, WA).

Cloning and Sequence Analysis

All molecular cloning procedures were carried out following standard protocols (Sambrook et al., 1989). PCR primers for amplification of a RALF fragment for library screening were designed based on the sequence of an aspen EST in GenBank (accession A163551), and consisted of sense promer ATGGTGATGGGCTTGCCAT and antisense primer GCACCTTGTAATGCGACTGCA. The RALF cDNA fragment was amplified by PCR using a hybrid poplar leaf cDNA library as template (Constabel et al., 2000). The PCR fragment was cloned, sequenced, and used for library screening (approximately 5 X 10^5 plaques). The ten positive clones isolated from the secondary screening were excised into the pBluescript phagemid, and seven clones were sequenced using a fluorescently labeled dideoxyterminator sequencing kit (Thermosequenase, Amersham, Buckinghamshire, UK) on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA). Similarity searches (BLAST) were carried out at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment for RALF sequences from poplar and aspen was conducted using the Clustal program at http://Searchlauncher.bcm.tmc.edu/multi-align/multi-align.html, and pair-wise similarities calculated using Peptool and Genetool Software (BioTools, Inc., Edmonton, Canada).

RNA and DNA Hybridization Analysis

For Southern-blot analysis, genomic DNA was isolated from poplar leaves as described (Haruta et al., 2001). DNA (10 μg) was digested with HindIII, XbaI, and EcoRV (Life Technologies/Gibco-BRL, Gaitherburg, MD), electrophoresed through 0.8 % (w/v) agarose, and blotted onto Zeta Probe membranes (Bio-Rad, Hercules, CA) using standard protocols (Sambrook et al., 1989). The DNA was cross-linked to membranes

using a GC gene Linker UV chamber (Bio-Rad) before prehybridization for 2 h at 65 °C in 6 X SSC, 5 X Denhardt's solution, 0.5 % (w/v) SDS, and 100 μg mL^{-1} denatured salmon sperm DNA. DNA probes of PtdRALF1 and PtdRALF2 were prepared using the Rediprime II DNA labeling kit (Amersham Biosciences, Piscataway, NJ), and hybridization carried out for 18 h. The membranes were rinsed twice with 2 X SSC at room temperature, washed once with 1 X SSC/0.1 % (w/v) SDS at 65 °C for 30 min, and washed once with 1 X SSC/0.1 % (w/v) SDS at 65 °C for 10 min. Hybridizing bands were revealed by exposure to X-ray film or on a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA).

For northern analysis, total RNA was extracted from tissues or cell culture using the protocol described by Haruta et al. (2001a). Three-month-old poplar saplings were used as a source of different tissues for the gene expression study. Total RNA (20 μg per lane) was loaded onto 1.4 % (w/v) agarose-formaldehyde gels in MOPS buffer (pH 7.0) and transferred onto Zeta Probe membranes (Bio-Rad) using standard procedures (Sambrook et al., 1989). Hybridization and analysis was carried out as described above.

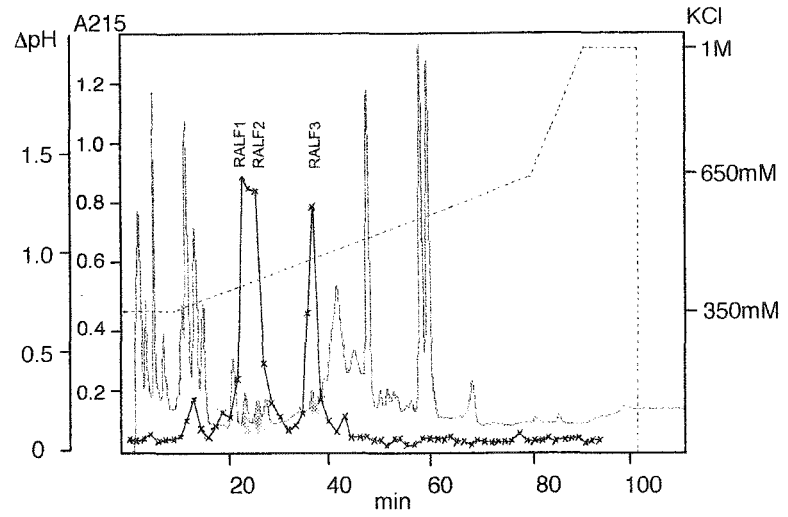
4.3. Results

Isolation and Characterization of RALF Peptides from Poplar Leaves

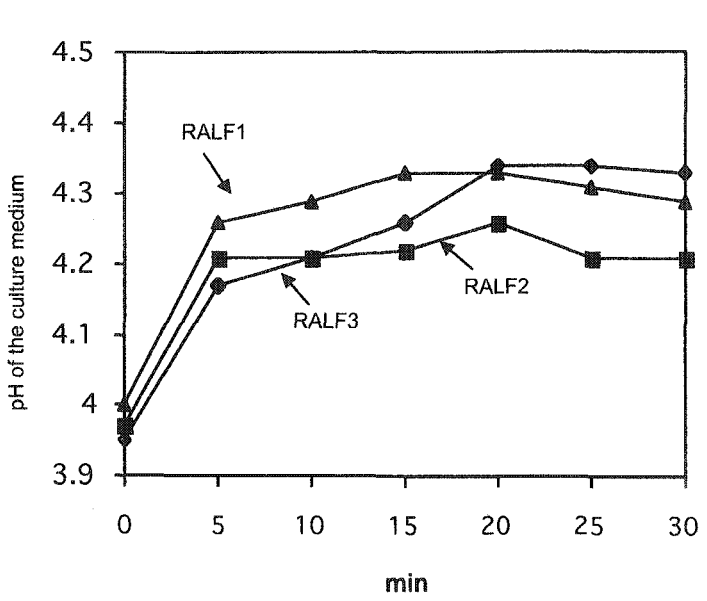
In order to investigate alkalinization factors in poplar, we obtained a poplar suspension cell culture (de Sá et al., 1992). Preliminary tests showed that the cells respond to the addition of fungal elicitors with culture medium alkalinization. The addition of crude poplar leaf extracts to the culture also induced a rapid alkalinization in the medium pH. Using C18 open column chromatography, we separated this alkalinization activity from leaf extracts into two active fractions, eluting with 60 % (v/v) and 20 % (v/v) methanol, respectively. The 60 % (v/v) MeOH fraction was further fractionated using Sephadex G-25 chromatography, strong cation exchange (SCX) HPLC, and C18 HPLC. The SCX HPLC elution profile showed that the presence of three activity peaks including a rapid alkalinization response (Fig. 4.1). These three activity peaks all showed identical alkalinization kinetics, suggesting they all contained similar alkalinization factors (data not shown). Incubation of active fractions with proteolytic enzyme proteinase K caused a loss of the alkalinizing activity of greater than

Figure 4.1. Strong cation exchange HPLC of RALFs (A) and pH alkalinization by three RALFs in poplar suspension cultures (B). A. Active compounds were separated using a 350 to 650 mM KCl gradient (dashed line). Medium pH alkalinization activity was assayed by adding 2 μL of each fraction to 2 ml of cells and measuring the pH every 5 min for 35 min. The maximum pH increase (ΔpH) was calculated and plotted with the elution profile monitored at 215 nm. HPLC peaks 1 through 3 (small solid peaks in the elution profile) that corresponded to the highest alkalinization activity were recovered and further purified by C18 HPLC. (B) Three activity peaks containing RALF1, RALF2, and RALF3 were analyzed by pH alkalinization assay.

A


ΔpH
A215
KCl
1M
650mM
350mM
RALF1
RALF2
RALF3
min

B


pH of the culture medium
RALF1
RALF2
RALF3
min

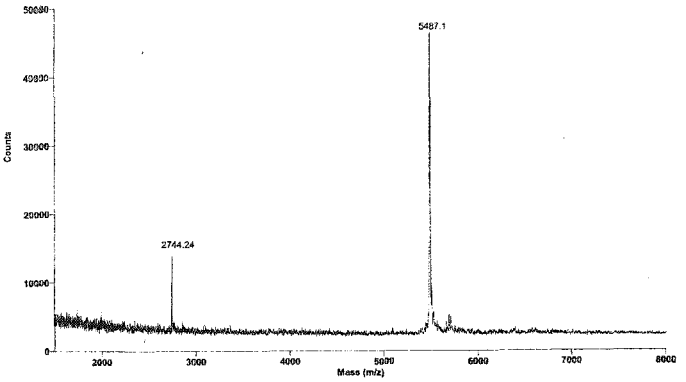
90 %, suggesting that the active compounds were peptides. The three SCX column peaks were each further purified by C18 HPLC, from which they all eluted with identical retention times (data not shown). Peak 1 yielded the most active compound, which we estimated at 200 pmol by comparison with chromatographic peaks of known peptides, and which corresponded to about 94.8 g of leaf tissues. The yield was later confirmed by the peaks obtained during the Edman sequencing.

The three active peaks were subjected to matrix-assisted laser desorption ionization (MALDI)-mass spectrometry (MS) analysis (Fig. 4.2). This analysis indicated that HPLC peaks 1 and 2 were pure compounds, with molecular masses of 5,488.7 and 5,402.7 mass units, respectively (Table 4.1). MS analysis of peak 3 revealed that it was a mixture of three different molecules of 5,514.4, 4979.8, and 4,966.0 mass units each. The compound in peak 1 was subjected to N-terminal Edman sequencing, which yielded ATTKYVSYGALQ(W)NXVPXSSXGASY(Y)N (X = unreadable residue) as a sequence. Querying GenBank databases with this amino acid sequence using BLAST revealed that many plant EST databases contained similar predicted peptides. The highest BLAST score was obtained with an EST of unknown function from hybrid aspen (*Populus tremula x P. tremuloides*; EST clone AI163551; Sterky et al., 1998). Furthermore, we learned that our peak 1 peptide was very similar (14 of 17 amino acids identical) to a peptide that had been purified and characterized from tobacco (RALF; Pearce et al., 2001b). Therefore, we named the peptide that we had identified poplar RALF1. We inferred that peaks 2 and 3 likely contain similar RALF peptides also, based on their similar masses, susceptibility to proteolytic inactivation, similar chromatographic behavior on reverse phase and cation exchange HPLC, and identical kinetics of alkalinization. We named these other peptides poplar RALF2, RALF3-1, RALF3-2, RALF3-3, respectively.

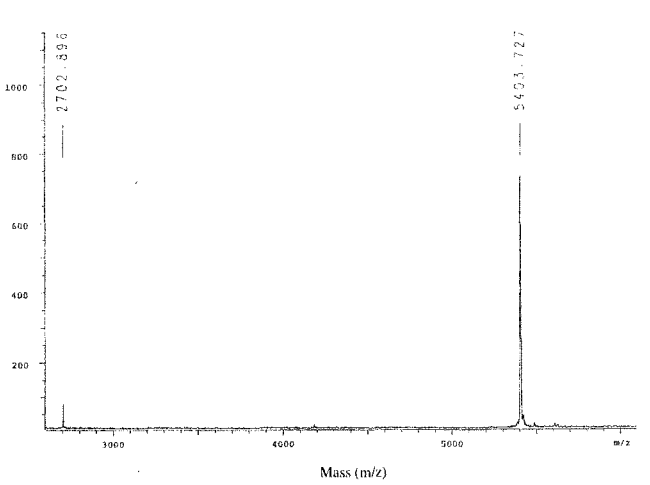
To gain more insight into the nature and function of these alkalinization factors, we compared the alkalinizing activity of poplar RALF1 with that of known defense response response elicitors. We tested chitosan, a component of fungal cell wall (Felix and Boller, 1995); flg22, a 22-amino acid conserved peptide found in flagellin from bacteria (Felix et al., 1999); and pep-13, a 13-amino acid peptide derived from a larger fungal elicitor protein (Nürnberger et al., 1994). Alkalinization activity was performed by measuring

Figure 4.2. MALDI-MS analysis of the isolated peptides, RALF1 (A), RALF2 (B), and RALF3 (C). C18 HPLC-isolated RALF1, RALF2, and RALF3 were analyzed by MALDI-MS using the linear mode. A. RALF1; the spectrum shows the signals of the singly charged RALF1 peptide, $(M+H)^+$ at the 5487.1 m/z and the doubly charged RALF1 peptide, $(M+2H)^{2+}$ at the 2744.24 m/z. B. RALF2; the marks indicate the signals of the singly charged RALF2 peptide, $(M+H)^+$ at the 5403.73 m/z and the doubly-charged RALF2 peptide, $(M+2H)^{2+}$ at the 2702.90 m/z. C. RALF3; the MALDI-MS analysis of RALF3 generates three signals at the 5515.41, 4980.78, and 4967.04 m/z, indicating RALF3 is likely a mixture of three molecules.

A


5487.1
2744.24
Counts
Mass (m/z)

B


2702.396
5403.727
Mass (m/z)

C

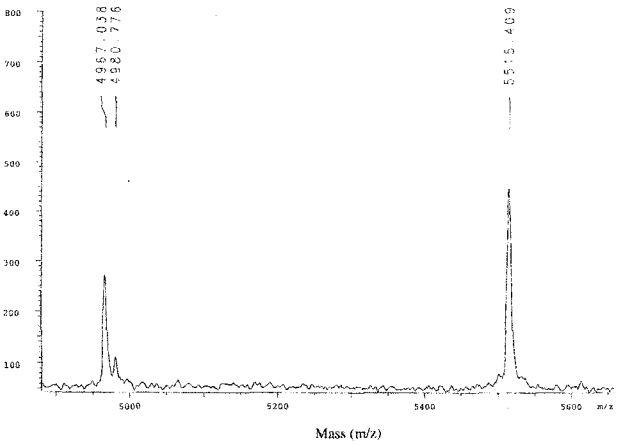

4967.038
4980.776
5515.409
Mass (m/z)

Table 4.1. Characteristics of RALF peptides identified in hybrid poplar. Three RALF peaks were separated by SCX HPLC and further purified using C18 HPLC as described in "Materials and Methods." Molecular masses of peptides were calculated by subtracting 1 from values of m/z observed in MALDI-MS analysis. MALDI-MS analysis indicated that RALF1 and RALF2 are pure compounds, whereas RALF3 is a mixture of three related peptides. cDNAs encoding RALF3-1, RALF3-2, and RALF3-3 were not obtained.

Peptide	Retention Time (SCX-HPLC)	Mass	cDNA encoding peptide
RALF1	23.5 min	5488.7	PtdRALF1
RALF2	26.1 min	5402.7	PtdRALF2
RALF3-1	36.8 min	5514.4	-
RALF3-2	36.8 min	4979.8	-
RALF3-3	36.8 min	4966.0	-

cDNAs encoding RALF3-1, RALF3-2, and RALF3-3 were not obtained (shown as -).

culture medium pH every 5 min after elicitor addition. The alkalinization by poplar RALF1 we observed was clearly stronger and more rapid than any of the other elicitors tested, reaching its maximum within 30 min of addition and then declining (Fig. 4.3). In contrast, the pH change caused by chitosan and flg 22 was slower and less dramatic, despite higher concentrations applied, and both induced increases of approximately 0.5 pH units over a 60-min period (Fig. 4.3). This pH increase is comparable with the one reported in tomato cells (Felix and Boller, 1995; Felix et al., 1999). RALF-induced alkalinization was consistently faster and dropped sooner than alkalinization triggered by the other elicitors, where it continued to rise until at least 60 min before declining. RALF2 and RALF3 fractions showed induction kinetics identical to that RALF1 (Fig. 4.1B). Therefore, we suspected that the RALF-induced response is distinct from the elicitor- induced responses, and speculate that RALF does not trigger a defense-related reaction. Induction of PAL was detected in poplar cultures after treatments with chitosan, MeJa, and *Phytophthora* culture extracts (pmg) ; however RALF peptides did not induce PAL (Fig. 4.4). Pep-13, although active in parsley cells as an elicitor of alkalinization and phytoalexin synthesis (Nürnberger et al., 1994), did not induce alkalinization in the poplar cell culture.

Cloning and Characterization of RALF cDNAs from poplar

To further characterize poplar RALFs at the molecular level, we isolated poplar RALF cDNAs. The high DNA sequence similarity between poplars and aspens (Constabel et al., 2000; Haruta et al., 2001b) allowed us to make use of the aspen EST clone, identified as highly homologous to the RALF peptide, to design PCR primers. Using a poplar cDNA library as a template, we amplified a 327-bp fragment, which was sequenced and found to contain a partial RALF sequence. After screening the cDNA library (5 X 10^5 plaques) with this fragment, we isolated 10 plaques with positive signals. Seven clones were sequenced, classified into two different groups based on the nucleotide sequences. Among these, five had identical coding sequences encoding a protein of 120 amino acids. These cDNAs all contained the exact N-terminal sequence of the purified RALF1 peptide obtained by Edman degradation (Fig. 4.5); thus, we conclude that these cDNAs encode RALF1. The N-terminal sequence of the RALF was found at

Figure 4.3. Medium alkalinization in poplar suspension culture in response to RALF and elicitors. Cell culture medium was monitored every 5 min. RALF1, flg22, and pep13 peptides were assayed at concentrations of 1 nM, 1 μM, and 5 μM, respectively. Chitosan was assayed at a final concentration of 1 μg /ml.

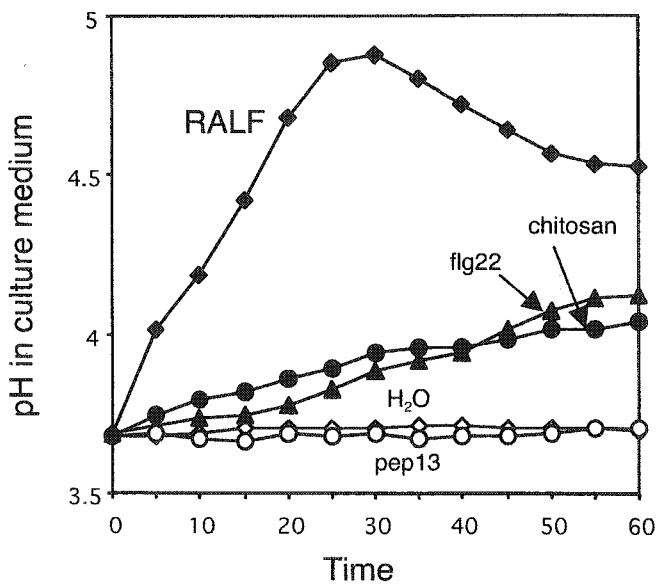

5
4.5
4
3.5
pH in culture medium
RALF
chitosan
flg22
H2O
pep13
0
10
20
30
40
50
60
Time

Figure 4.4. Expression analysis of PAL gene in poplar suspension cultures after treatment with RALFs and elicitors. Cultures (40 ml) were treated for 3 h with compounds known to induce pH alkalinization in poplar suspension cultures. The cells were harvested for RNA extraction and used for analysis of PAL gene expression. RALF1 + RALF2; a mixture of RALF1 and RALF2, pmg; *P. megasperma* elicitor. Wounded leaf extract is shown as a positive control for the size of PAL transcript. The ethidium-stained gel is shown on the lower panel as an RNA loading control.

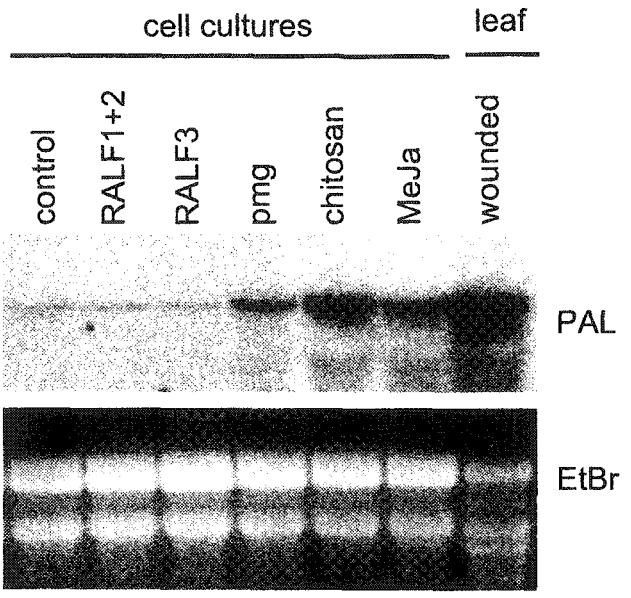

cell cultures
leaf
control
RALF1+2
RALF3
pmg
chitosan
MeJa
wounded
PAL
EtBr

Figure 4.5. Deduced amino acid sequences of two PtdRALF cDNAs compared with the aspen EST sequence (accession no. AI163551) and the tobacco RALF sequence (accession no. AF407278). The N-terminal sequence determined by Edman sequencing of RALF1 is underlined. The dots indicate conserved Cys residues. Regions underscored with a dashed line correspond to sequences used for PCR primers. The GenBank accession numbers are AY172330 and AY172331 for PtdRALF1 and PtdRALF2, respectively.

```
PtdRALF1    1:MAKWSSWFLISATILIL-MAMGLSSTVQGS 29
PtdRALF2    1:MARLNSFFLISATVLIL-MVMGWPSTVQGN 29
AI163551    1:------------------MVMGLPSTVQGN 12
TobRALF     1:---------MGVPSGLILCVLIGAFFISMAA 22
                                 *

PtdRALF1   30:GD---HHLGW1P---ATRSSVC-KGSIAEC 52
PtdRALF2   30:GDHHHHHLGWIPTTATTRSSICDKGSLAEC 59
AI163551   13:GD-HHHHLGWIPTTTTTRSSICDKGSLAEC 41
tobRALF    23:AGDSGAY-DWV-MPARS-GGGC-KGSIGEC 48
                     *           * ***  **

PtdRALF1   53:MA-EDGEEFEMDTEINRRILATTKYVSYGA 81
PtdRALF2   60:MAEEDGEEFGMDTEINRRILATSRYVSYGA 89
AI163551   42:MAEEDGEEFGMDTEINRRILATSSYVSYGA 71
tobRALF    49:IAEE--EEFELDSESNRRILATKKYISYGA 76
               * *  ***  * * *******  * ****
                        .         .
PtdRALF1   82:LQRNNVPCSRRGASYYNCQRGAQANPYSRG 111
PtdRALF2   90:LQKNNVPCSRRGASYYNCKNGAQANPYSRG 119
AI163551   72:LQKNNVPCSRRGASYYNCKNGAQANPYSRG 101
tobRALF    77:LQKNSVPCSRRGASYYNCKPGAQANPYSRG 106
              ** * *************  **********
              .     .
PtdRALF1  112:CSRITRCRS                      120
PtdRALF2  120:CSRITRCRG                      128
AI163551  102:CSRITRCRG                      110
tobRALF   107:CSAITRCRS                      115
              ** *****
```

position 72 in the predicted protein sequence, indicating that, like tobacco RALF, the poplar RALF1 peptide is synthesized as a 120-amino acid precursor protein and processed into a biologically active mature form of 49 amino acid residues (Fig. 4.5). The molecular weight of the poplar RALF1 peptide as predicted from the nucleotide sequence is 5,493.1. Active tobacco RALF contained two disulfide bridges (Pearce et al., 2001b); accounting for the loss of the four protons from the cystein during disulfide bridge formation, the expected molecular weight of active RALF1 wound be 5,489.1. This is very close to the mass of 5,488.7 we obtained from the MS analysis, further confirming that these cDNAs encode the RALF1 peptide. We chose an 838-bp cDNA as a representative of these cDNAs and named it PtRALF1, and used this for subsequent experiments. Surprisingly, despite identical nucleotide sequences in the coding region, we observed that all five of these cDNA clones had variable 3'-untranslated region (UTR) sequences and size from each other (data not shown). This is suggestive of posttranscriptional modifications or alternative polyadenylation sites. In other eukaryotic organisms, transcript stability or translatability can be regulated by signals in the 3'-UTR (Hunt and Messing, 1998).

Two additional cDNA clones obtained were distinct from PtdRALF1. They both contained the identical nucleotide sequence as the PCR product used to screen the cDNA library. We designated one clone as PtdRALF2, and used this cDNA for the further experiments. PtdRALF1 and PtdRALF2 showed a nucleotide identity of 88.4 % over the coding sequences, and 84.2 % similarity at the amino acid sequence levels, suggesting they represent different genes (Fig. 4.5; Table 4.2). PtdRALF2 encodes a predicted protein of 128 amino acid residues, which by analogy to PtdRALF1 is predicted to be processed into a mature peptide of 49 amino acid residues. The molecular weight of this predicted peptide is 5,406.99. Again, if the active form of the mature Group 2 RALF peptide contains two disulfide bridges as expected, the measurable mass should be 5,402.99, very close to the mass of RALF2 peptide isolated (Table 4.1). Therefore, we concluded that PtdRALF2 likely encodes the RALF2 peptide. Again, both PtdRALF2 cDNA had variable 3'-UTRs; interestingly, there are several possible polyadenylation sites in this region (data not shown).

Table 4.2. Comparison of PtdRALF1 and PtdRALF2 with *P. tremula* and *P. tremuloides* EST sequences. Nucleotide sequences of EST clones homologous to PtdRALF were obtained from PopulusDB (http://poppel.fysbot.umu.se/blastsearch.html). Sequence similarities were analyzed by GeneTool and PepTool Analysis Software (Biotools, Inc., Edmonton, AB, Canada). Percent amino acid similarity is shown in bold, and nucleotide identity is shown in normal type.

	PtdRALF1	UB62BP.E02	PtdRALF2	UB31BPC05	A044P29U	UB33PBE02	G058P65Y
PtdRALF1		**95.9**	**88.4**	**87.9**	**84.1**	**65.3**	**57.8**
UB62BP.E02	94.7		**86.6**	**87.5**	**77.8**	**65.3**	**53.4**
PtdRALF2	84.2	84.2		**97.1**	**97.9**	**62.8**	**61.7**
UB31BPC05	85.8	86.0	95.3		**99.1**	**63.7**	**61.0**
A044P29U	81.8	76.4	97.3	99.1		**64.0**	**62.1**
UB33PBE02	40.8	41.2	38.3	38.6	41.8		**69.7**
G058P65Y	45.1	39.6	47.3	47.3	47.3	75.8	

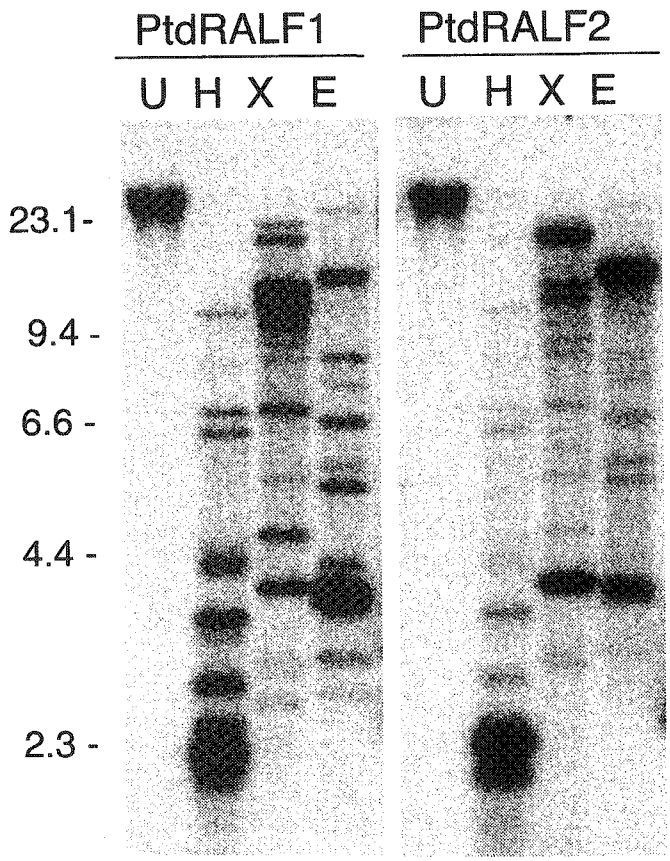
The PtdRALF1 and PtdRALF2 coding sequences both have 62 % over all amino acid similarity with the previously described tobacco RALF cDNA (Pearce et al., 2001b). Over the 49 amino acids of both predicted mature peptides, however, the amino acid similarities with tobacco RALF are 86 %, demonstrating that the mature RALF peptide sequence is highly conserved. As noted by Pearce et al. (2001b), the sequence database searches contain numerous unannotated RALF-like ESTs from a diversity of species. Outside of the poplar ESTs (see below), the highest score was with an EST from *Medicago trunculata* (accession no. AJ501009), with amino acid similarities of 62 % and 57 % with PtdRALF1 and PtdRALF2, respectively.

Using both PtdRALF1 and PtdRALF2 cDNAs as probes, we performed Southern analysis on hybrid poplar genomic DNA to estimate the size of the RALF gene family in poplar. We first hybridized Southern-blotted membrane with the full-length PtdRALF1 cDNA, and subsequently stripped the membrane and rehybridized with PtdRALF2. With either probe, the same set of approximately 10 to 12 bands was detected in the restricted genomic DNAs (Fig. 4.6). However, for each enzyme digest, the most strongly hybridizing bands were different for PtdRALF1 and PtdRALF2; that is, the relative strengths of the signals differed between the probes. Therefore, we conclude that both probes are recognizing essentially the same members of the PtdRALF gene family, and that the strongest one or two bands in each case represent the specific PtdRALF1 and PtdRALF2 genes. Because the experimental material is an interspecific hybrid, this Southern hybridization is likely detecting both alleles of each PtdRALF gene as distinct bands. Thus, it appears that the poplar genome contains a small family of RALF-like genes. This is consistent with the observation of five potential RALF peptides in poplar extract.

Additional Homologs of RALF Genes in Populus

Although there was evidence for five distinct RALF peptides from the biochemical purification and detected a number of RALF-like genes in the H11-11 hybrid poplar genome by Southern analysis, we could only isolate two types of cDNA by library screening. The Populus DB database (http://poppel.fysbot.umu..se/blastsearch.html), which contains *P. tremula* and *P. tremuloides* ESTs, provided us with an additional tool

Figure 4.6. Southern analysis of the RALF gene family in poplar. Ten micrograms of restricted genomic DNA was probed with the full-length PtdRALF1 cDNA (left). The same membrane was stripped and rehybridized with the PtdRALF2 cDNA (right). U, Undigested; H, *Hin* dIII; X, *Xba* I; E, *Eco* RV.


PtdRALF1
PtdRALF2
U H X E
U H X E
23.1-
9.4 -
6.6 -
4.4 -
2.3 -

to investigate RALF homologs in poplar. Using poplar RALF cDNA sequences for homology searches, we identified five unique ESTs (UB62BP.E02, UB31BPC05, A044P29U, UB33BPE02, and G058P65Y) in the Populus DB. The EST A044P29U is the previously identified RALF EST (AI163551; see above). Multiple sequence alignments indicated that four Cys residues are conserved in all sequences (not shown). As noted by Pearce et al. (2001b), the C-terminal portions of the predicted proteins (corresponding to the mature RALF peptides) were most conserved, whereas the N-terminal regions were more divergent. Pair wise analyses of the sequences showed that these RALF homologs were 38.3 % to 99.1 % identical at the amino acid sequence level (Table 4.2). Based on their high scores, it appears that the aspen EST UB62BP.E02 is an orthologs of PtdRALF1 (95.9 % nucleotide identity) and that aspen EST clones UB31BPC05 and A044P29U are orthologs of PtdRALF2 (97.1 and 97.9 % nucleotide identity, respectively). Therefore, all sequences identified to date appear to represent a total of four distinct poplar RALF genes, represented by PtdRALF1, PtdRALF2, UB33PBE02, and G058P65Y (Table 4.2).

We also considered if the most divergent RALF ESTs (UB33BPE02 and G058P65Y) might encode the peptides corresponding to the RALF3 MS peaks we found in poplar leaf extracts. Calculation of molecular weights of their predicted RALF peptides, however, gave molecular weights significantly different from the measured masses of RALF3 peaks (Table 4.1). Therefore, ESTsUB33BPE02 and G058P65Y likely represent distinct members of the poplar RALF gene family, corresponding to peptides not yet identified in poplar leaf extracts. Analysis of the complete set of poplar RALF peptides and genes will require the availability of additional ESTs or genomic sequences.

Expression Analysis of PtdRALF mRNA in Poplar Saplings and Cell Culture

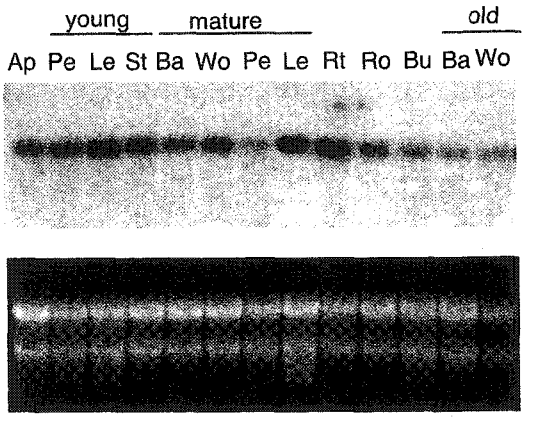
To obtain clues for understanding the biological roles of RALF peptides in planta, we conducted northern analysis to study the expression of PtdRALF. The availability of two different RALF cDNA sequences allowed us to investigate the differential expression of PtdRALF mRNAs in plants and cell cultures. For this purpose, gene-specific probes for PtdRALF1 and PtdRALF2 were generated from the sequence of 5'-UTRs of both cDNAs, where the sequence was least conserved. The specific probes

shared 60.2 % nucleotide sequence identity and their cross hybridization was negligible (data not shown). We first analyzed expression of PtdRALF1 in a variety of poplar sapling tissues. PtdRALF1 was expressed in all tissues tested, including shoot apex, petiole, leaf, stem, root, bud, bark, and wood (Fig. 4.7A). In contrast, expression of PtdRALF2 was observed in most tissues but absent or expressed at a very low level in young and mature leaves (Fig. 4.7B). A general comparison of Figure 4.7A and B, shows that the expression of PtdRALF2 varied more between tissues than did PtdRALF1. We also detected constitutive expression of both PtdRALF genes in poplar suspension culture (Fig. 4.8, control).

This almost ubiquitous pattern of expression led us to hypothesize that RALF gene products are likely involved in fundamental cellular processes, such as in hormone responses or primary metabolism. Therefore, we tested the effects of manipulating culture conditions on PtdRALF gene expression. Specifically, we asked whether the level of PtdRALF transcripts in the cells could be altered by the application of phytohormones (cytokinin, auxin, or methyl jasmonate), by modulation of the nutrient concentration (increasing sucrose 3-fold and increasing nitrogen 10-fold), or by treatment with pathogen elicitors. We also tested the effect of culture medium acidification on PtdRALF expression by reducing the pH to 2.5 using HCl. After the addition of the test substances, the cultures were incubated for 5 hr, and then harvested for northern analysis. Relative to control cells, the expression of PtdRALF1 and PtdRALF2 did not change significantly in the cells after most of the treatments. However, MeJa almost completely suppressed PtdRALF2 mRNA, and greatly reduced the abundance of PtdRALF1 mRNA (Fig. 4.8). We investigated the effect of MeJa on PtdRALF2 expression in more detail in time course experiments. The level of PtdRALF2 transcript decreased dramatically by 5 hr after MeJa treatment, and then recovered again by 48 hr (Fig. 4.9). In parallel, the pH of medium increased moderately, peaking 5 hr after MeJa addition and then decreasing by 24 hr. At 48 hr, the medium pH increased again, but control experiments indicated that this was due to the normal rise in medium pH due to aging of the cells through the culture cycle (data not shown). The inverse correlation of medium pH with PtdRALF2 transcript was observed consistently in repeated experiments. However, the kinetics of this extracellular pH increase by MeJa were much slower than the alkalinization observed

Figure 4.7. Northern analysis of PtdRALF expression in poplar saplings. RNA was extracted from tissues of different ages from a 3-month-old sapling. Total RNA (20 μg/ lane) was blotted onto membranes and hybridized with gene-specific probes for PtdRALF1 or PtdRALF2 (see "Materials and Methods"). EtBr, Ethidium bromide-stained gel; Ap, apical tissue; Pe, petiole; Le, leaf; St, stem; Ba, bark; Wo, wood; Rt, root tip, Ro, root; Bu, bud.

A. PtdRALF1



B. PtdRALF2

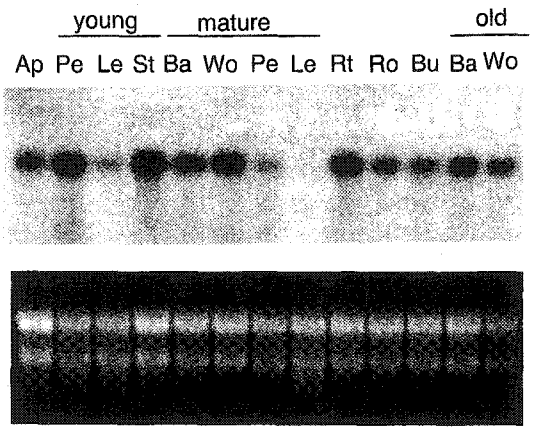
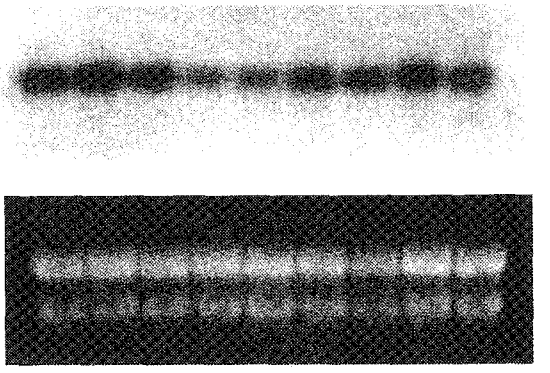


Figure 4.8. Northern analysis of PtdRALF in poplar cells after various treatments. Three-day-old cultures were treated for 5 h with compounds at concentrations as described in "Materials and Methods," and analyzed for PtdRALF1 and PtdRALF2 gene expression. EtBr, Ethidium bromide-stained gel; Co, control; BA, benzyl adenine; NA, naphthalene acetic acid; MJ, MeJa; Suc, Suc; N, ammonium nitrate; Ch, chitosan; Pm, *P. megasperma* elicitor; HCl; hydrochloric acid.

A. PtdRALF1

Co BA NA MJ Suc N Ch Pm HCl



B. PtdRALF2

Co BA NA MJ Suc N Ch Pm HCl

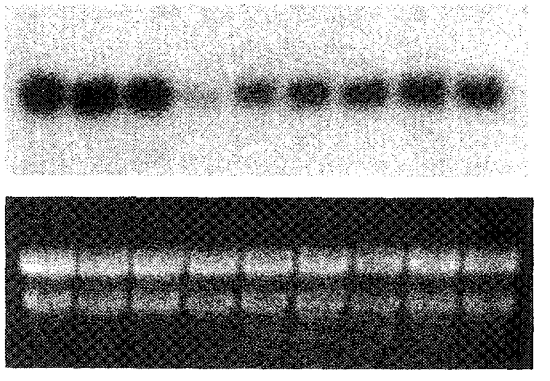
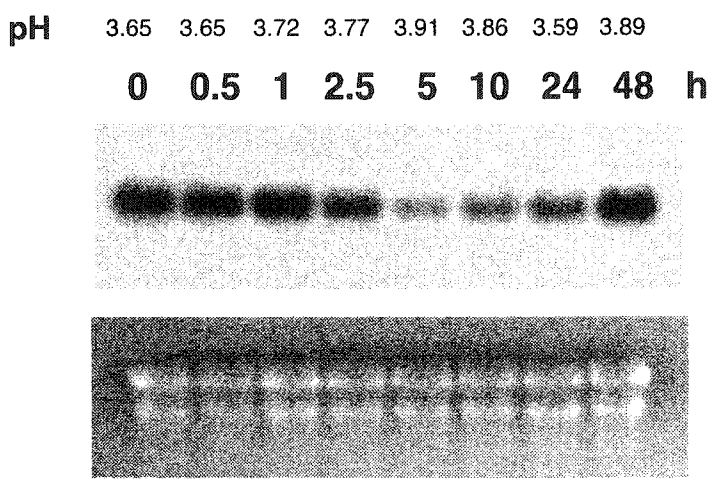


Figure 4.9. Northern analysis showing the effect of MeJa and PtdRALF2 expression in poplar cell cultures. Cultures (40 ml) were treated with 50 μM MeJa, and at the times indicated the cells were harvested for RNA extraction and the medium pH was measured. The ethidium-stained gel is shown on the lower panel as a loading control.


pH
3.65 3.65 3.72 3.77 3.91 3.86 3.59 3.89
0 0.5 1 2.5 5 10 24 48 h

with RALF itself (compare with Fig. 4.3). In additional experiments, we treated cells with KOH and NaOH to artificially increase the pH of the culture medium. However, these treatments did not result in any change in the level of PtdRALF2 transcripts (data not shown), suggesting that the reduction of PtdRALF2 transcript level by MeJa is not simply a consequence of MeJa-induced pH alkalinization.

4.4 Discussion

A Family of Poplar RALF Peptides and Genes

The first peptide with hormone-like properties in plants was reported more than 10 years ago with the discovery of the tomato wound signal systemin (Pearce et al., 1991), yet the widespread importance of peptide hormones in the plant kingdom is only beginning to be recognized (Ryan et al., 2002). RALF, a novel peptide causing rapid alkalinization of cell culture media, was very recently discovered in tobacco, alfalfa (Medicago sativa), and tomato, and similar ESTs from a number of plant species were identified in sequence databases (Pearce et al., 2001b). In this study, we isolated a series of RALF peptides from poplar, cloned two distinct poplar RALF cDNAs, and identified additional genes from databases of Populus ESTs. Using MS analysis, we identified five individual compounds from three HPLC peaks of poplar leaf extracts (Fig. 4.1; Table 4.1). All three peaks induced the identical alkalinization response and exhibited very similar chromatographic profiles on both reverse phase and SCX HPLC, indicating they all contain similar active constituents. Direct peptide sequencing of the purified compound in peak 1 identified it as a RALF peptide, which we later named RALF1 (encoded by PtdRALF1 gene). The mass of the alkalinizing compound in HPLC peak 2 corresponded exactly with the predicted molecular weight of the PtdRALF2 encoded peptide, strongly suggesting that this compound is the RALF2 peptide. Although there is no sequence information on the alkalinizing compounds in HPLC peak 3, based on the MS analysis, chromatographic behavior, and alkalinization kinetics, these constituents are very likely to be additional RALF peptides. Thus, this study extends the work of Pearce et al. (2001b) by demonstrating the presence of multiple distinct RALF peptides within the same leaf extract. This could represent functional redundancy or specialization based on differential expression.

We isolated two distinct cDNAs representing two RALF genes (PtdRALF1 and PtdRALF2) from a poplar cDNA library, encoding the RALF1 and RALF2 peptides. Given the number of RALF peptides that appear to be present in leaf extracts, additional RALF genes are likely expressed; presumably, the sequence similarity of the probe with other RALF genes was insufficient for these to hybridize during the cDNA library screening. Southern analysis of hybrid poplar genomic DNA identified 10 to 12 bands, which given the hybrid nature of the plant material, could represent up to five or six individual genes. In the Populus DB database, which contains ESTs from a number of different libraries, we identified five RALF homologs with nucleotide identities ranging from 53.4 % to 99.1 % (Table 4.2). It should be noted that the C-terminal portions of the predicted proteins, which contain the mature RALF peptide, are more conserved than the N-terminal portions (not shown; Pearce et al., 2001b). Analyses of those sequences indicates there are at least four genes within the poplar genome (Table 4.2); therefore, the ESTs provide evidence for the presence of a small gene family in poplar. In addition, there are likely to be other, more distantly related genes in poplar; a recent search for small peptides in Arabidopsis identified 34 genes with similarity to RALF (Olsen et al., 2002).

Expression of PtdRALF Genes

Northern blot analyses indicated that RALF genes were expressed in all tissues tested (Fig. 4.7). This widespread pattern of expression is consistent with the presence of RALF-like sequences in plant EST databases constructed from a wide variety of tissues and organs, including roots, flowers, conducting tissues, and fruit (Pearce et al., 2001b). PtdRALF2 transcript levels appeared to be more variable than PtdRALF1; specifically, PtdRALF2 mRNA was barely detectable in old leaves, and expressed at lower levels in younger leaves than any other tissues (Fig. 4.7). Nonetheless, we were able to isolate the RALF2 peptide during the original peptide purification; it was likely present in petioles or other tissue types that were included in the large-scale extraction.

Both PtdRALF genes were highly expressed in suspension cells (Fig. 4.8). Their expression levels showed no detectable variation after several environmental and hormonal stimuli, including changing the medium pH, addition of auxin, cytokinin,

fungal elicitors, or nitrogen. We did, however, observe a strong reduction in RALF expression after MeJa treatment, especially for PtdRALF2 (Fig. 4.8B). The decrease in PtdRALF2 expression over time was tightly correlated with a very slow increase in cell medium pH, which may suggest a link between these phenomena. The alkalinization was measured in hours rather than minutes; therefore, it appeared to be unrelated to the previous rapid alkalinization responses we had observed. Simply increasing the pH of the medium with NaOH or KOH did not influence PtdRALF2 expression (data not shown), which suggests that it is not alkalinization itself that represses PtdRALF2 expression in cells. Therefore, there appears to be no causal connection between the shift in pH and the expression of RALF gene expression, but rather both are manifestations of the cells' response to MeJa. MeJa is known to repress a number of housekeeping genes, such as Rubisco, chlorophyll a/b binding protein, carbonic anhydrase, and α-tubulin (Wasternach et al., 1998; Schenk et al, 2000). Thus, MeJa may be inducing developmental changes and cellular differentiation (see below). Since we hybridized northern blots with only PrdRALF1 and PtdRALF2 probes, we have little information on the expression of other members of the PtdRALF gene family. It is possible that other RALF gene show a much greater degree of tissue-specific gene expression, or that they vary more dramatically in response to environmental stimuli in suspension cells.

Sequence analysis using PSORT software (http://psort.nibb.ac.jp) predicts that RALF is most likely to be secreted to the extracellular space. Because in normally dividing cultures RALF genes are highly expressed, it is surprising that these cells still respond to additional, exogenous RALF peptide. This may indicate that RALF in the medium is rapidly degraded. Other posttranscriptional regulatory mechanisms, such as processing or sequestration, could also account for this. Elucidating these aspects of RALF will require more in-depth studies.

Toward Possible Functions for RALF

Alkalinization of culture medium has been most commonly reported in response to microbially or plant-derived defense signals, for example flagellin, chitin, xylanase, pep-13, and systemin, where it appears to be a necessary part of the signal transduction pathway (Felix et al., 1993, 1999; Nürnberger et al., 1994; Felix and Boller, 1995;

Enkerli et al., 1999). In contrast, RALF appears not to be a signal for defenses because it does not induce the antiherbivore proteinase inhibitors in tomato plantlets, but, rather causes inhibition of root growth (Pearce et al., 2001b). Our experiments with poplar cell cultures support the idea that RALFs unlikely to be involved in plant defense for the following reasons: (a) there was no induction of either RALF gene by these elicitor treatments (Fig. 4.4); (b) unlike flg22 or chitosan, RALF did not induce expression of the defense marker PAL in poplar cell cultures (Fig. 4.4); and alkalinization triggered by RALF is much more rapid than that induced by flg22 or chitosan, elicitors of the defense response (Fig. 4.3).

A non-defensive role for RALF could imply a housekeeping or developmental function, consistent with the broad pattern of expression of both poplar RALF genes (Fig. 4.7). Although we tested a series of treatments, the only significant change in RALF gene expression that we observed was after the addition of MeJa to the cells. Although MeJa plays a key role in inducing pest and pathogen defense responses, it can also stimulate or inhibit developmental processes such as senescence, flower formation, and pollen maturation, and is important for tendril curling and internode elongation (Sembdner and Parthier, 1993; Weiler, 1997). In poplar cell culture, it is conceivable that the MeJa-induced changes are more of a developmental rather than a stress-related nature because in these cultures MeJa induces a developmentally regulated isoform of polyphenol oxidase, but not the wound-induced form (data not shown). In other systems, extracellular alkalinzation has also been linked to processes other than defense. For example, Felle et al. (2000) observed extracellular alkalinization of root in response to nod factors, and Felix et al. (2000) documented the alkalinization response in cell culture following changes in osmotic pressure. Other groups have demonstrated that extracellular alkalinization correlates with gravitropic responses (Johannes et al., 2001), and medium pH was found to be important for tracheary element differentiation in cultures of *Zinnia elegans* (Robert and Haigler, 1994). Ultimately, the search for functions of RALF in growth and development will have to relay on more direct tests using transgenic plants or mutants.

In summary, we have identified a family of RALF genes and peptides in poplar. Our analysis of RALF gene expression in poplar indicates that they are expressed in most

plant tissues and organs, as well as suspension cell cultures. Significantly, we observed reduced RALF transcript abundance in cell after MeJa treatment, which may be a first clue in working toward a function for these novel peptides.

Chapter 5

Search for Extracellular pH Alkalinization Peptides from Poplar II. Slow Alkalinization Factor (SALF) Derived from Photosystem I Complex Protein Subunit D (PSI-D)

5.1. Introduction

Upon tissue damage such as herbivory or pathogen infection, plants undergo dynamic metabolic changes. Induction of defense-related genes and following accumulation of defense-related proteins play major roles on plants' adaptation to the stress environments. In addition to dynamic changes in transcriptional status (Cheong et al., 2002; Hui et al., 2003), a growing body of evidence suggests the importance of proteolysis during induction of defenses. It has been demonstrated that proteasome activity is necessary for induction of defenses (Ito et al., 1999; Becker et al., 2000; Takai et al., 2002). It has been shown that inhibition of proteasome activity causes the accumulation of ubiquitinated high molecular weight proteins and suppression of wound-induction of defense-related genes (Ito et al., 1999). The involvement of proteolysis is also implicated during cell death associated with hypersensitive responses (Solomon et al., 1999; Lam and del Pozo, 2000). Moreover, proteolysis is known to be important for removal of damaged proteins during oxidative stress (Desimone et al., 1998). It has also been suggested that proteolysis of regulatory proteins is involved in the defense signaling pathway (Schaller and Ryan, 1994). Furthermore, coincidental degradation of the disease resistance (R) gene products during defense responses was also observed (Boyes et al., 1998). These observations all indicate the presence of regulated proteolysis mechanisms for degradation of regulatory proteins and proteolytic turnover of damaged or unnecessary proteins during induced defenses.

Induction of defenses in plant tissues is partially reproducible in plant suspension cultures. Suspension cultures have been often utilized to investigate cellular signal transduction during defense responses. Tomato cultures treated with systemin show rapid ion fluxes, the activation of protein kinases, and the accumulation of ethylene (Felix and Boller, 1999; Pearce et al., 2001a; see also Introduction of Chapter 4). Likewise, a bacterial elicitor, flagellin, and a fungal elicitor, chitin, induce rapid ion fluxes, and activation of protein kinase, which are followed by induction of defense-related genes in cultured cells of tomato (Felix et al., 1999; Felix et al., 1993). Among the cellular responses to these defense-related signals, alkalinization of the culture medium was observed as one of earliest events, occurring within a few minutes. Since the rapid alkalinization of culture medium often correlates with the subsequent induction of

defenses in suspension cells, the pH of culture medium has been often measured as a convenient indicator to detect defense responses (Felix et al., 1999; Felix and Boller, 2003).

Based on our interests in inducers of defenses in this study, endogenous molecules causing alkalinization were sought with the use of poplar cell cultures. In the previous chapter, the investigation of alkalinizing molecules from poplar resulted in the isolation of 5 kD peptides called rapid alkalinization factors (RALFs), that do not appear to be defense-related signals (Chapter 4). Therefore, the search to identify systemin-like peptides that regulate the induction of defenses was continued, and this chapter describes the isolation and characterization of other peptide-based alkalinization factors from poplar leaf extract.

The alkalinization assay-directed purification of the active fractions from wounded leaf extract resulted in the isolation of a small peptide, slow alkalinization factor (SALF). Structural analysis of SALF suggested that SALF is likely derived from the 20 kD photosystem I complex subunit protein (PSI-D). Though the processes that generate SALF from the PSI-D protein are unknown, the isolation of this peptide with alkalinization activity may indicate a link of a proteolyzed product in leaf extracts with a signal-like role during wound responses.

5.2. Materials and Methods

Plant Material

Poplar hybrid H11-11 (*Populus trichocarpa x Populus deltoides*) was propagated from green cuttings in peat (Terra-Lite Redi-Earth, WR Grace, Ajax, ON, Canada) in 15-cm-diameter pots as described (Constabel et al., 2000). Plants were maintained in environmental chambers under 16-h days at 18 C and 75 % relative humidity. Light intensity was 300 mEm^{-2} s^{-1} at pot height, composed of approximately 20 % incandescent (2,700-W) and 80 % cool-white (11,880-W) light. Plants were watered daily with solution containing 1g L^{-1} 20-20-20 Plant-Prod complete fertilizer (Plant Products, Brampton, ON).

Cell cultures, alkalinization assays, and elicitor treatments

Hybrid poplar (H11-11) suspension cells were obtained from Dr. Carl Douglas (University of British Columbia) (de Sá et al., 1992) and maintained in Murashige Skoog medium (Sigma, St. Louis, MO) adjusted to pH 5.5-5.6 with KOH. For routine maintenance, 5 ml of a 1-week-old culture was transferred into 40 ml of medium in 200 ml flasks and maintained on an orbital shaker at 100 rpm in dark at room temperature. A 2-ml aliquot of cells was transferred into each well of 12-well tissue culture plates (Corning, Corning, NY) and allowed to equilibrate on an orbital shaker at 120 rpm for 50 min. Fractions (2 to 5 µl) were added to the cells and the change in pH of the medium was measured every 15 min for 90 min using an Accumet pH meter with an Accuphast pH electrode (Fisher Scientific, Nepean, ON, Canada).

Isolation of SALF

Hybrid poplar leaf and petiole tissue (800g) was collected from 2-month-old poplar saplings 3-10 h after mechanical wounding. The tissues were homogenized in 200-g batches in a blender with 1 L of 1 % (v/v) trifluoroacetic acid (TFA), filtered through two layers of Miracloth (Calbiochem, La Jolla, CA), and the homogenate centrifuged at 10,000 g for 15 min. The supernatant was treated with polyvinylpolypyrrolidone (PVPP; 5% g/original tissue weight) by stirring the mixture for 15 min and the mixture was centrifuged at 10,000g for 15 min. The recovered supernatant was separated on C18 media (J.T. Baker, Phillipsburg, NJ) using open column reverse-phase chromatography (2.5 x 20 cm). The 20 % (v/v) methanol-eluting fraction showing medium pH-alkalinizing activity was lyophilized, the active material was redissolved in 0.1 % (v/v) TFA, and separated on a Sephadex G-25 (Pharmacia, Uppsala) gel chromatography column (2.5 x 72 cm). A portion of the active fraction was lyophilized and further purified on an anion exchange HPLC (polyWAXLP, 4.6 X 200 mm, The Nest Group, Southboro, MA) running a 0 to 500 mM KCl gradient in 5 % (v/v) acetonitrile/15 mM potassium phosphate buffer (pH6.0). Fractions containing alkalinization activity were concentrated with the use of Speed Vac concentrator. The active material was further separated using C18 HPLC (Bondpack C18, 3.9 X 300 mm, Waters, Milford, MA) running a 0 to 30 % acetonitrile gradient in 0.1 % (v/v) TFA. The active fraction was

concentrated and further purified by applying on SCX HPLC (PolySulfoethyl A, 200 X 4.6 mm, The Nest Group) running a 0 to 400 mM KCl gradient in 25 % (v/v) acetonitrile/5mM potassium phosphate buffer (pH3.0). An activity peak was applied to a C18 HPLC column and isolated with a 0 to 20 % acetonitrile gradient in 0.1 % (v/v) TFA. The purified active material was analyzed by electrospray ionization (ESI)-MS at the University of Victoria's Proteomics Facility. N-terminal sequencing and MALDI-MS analysis were carried out using Edman chemistry at Washington State University (Pullman, WA).

Database Searches Genes Encoding SALF and Bioinformatic Analysis

Populus genomic DNA sequences were searched on the US Department of Energy, Joint Genome Institute Populus website (http://genome.jgi-psf.org/poplar0/poplar0.home.html). cDNA sequences were searched on EST database available at the web site of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). PSORT analysis for computational prediction of cellular localization of proteins was conducted at http://psort.nibb.ac.jp/. Multiple sequence alignments of PSI-D proteins were carried out with the Clustal program at http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html.

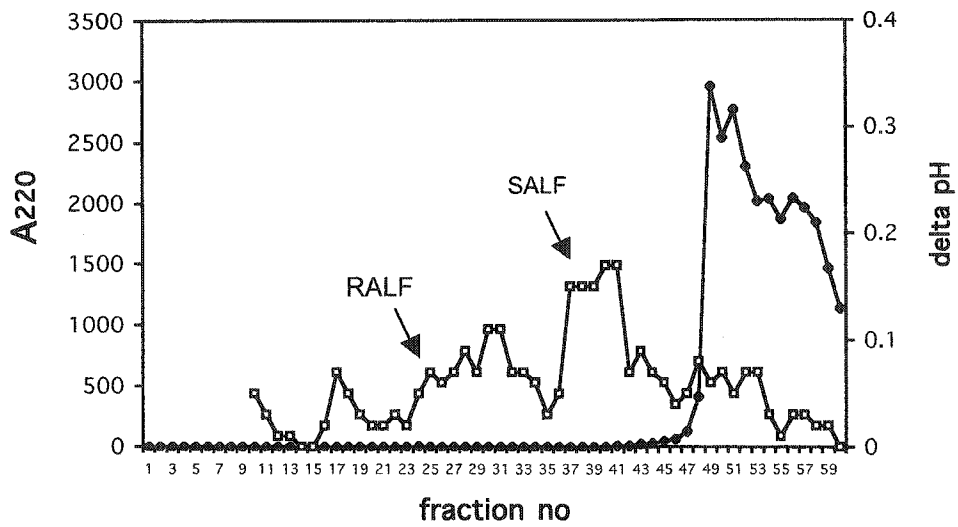
5.3. Results

Isolation of the Slow Alkalinization Factor

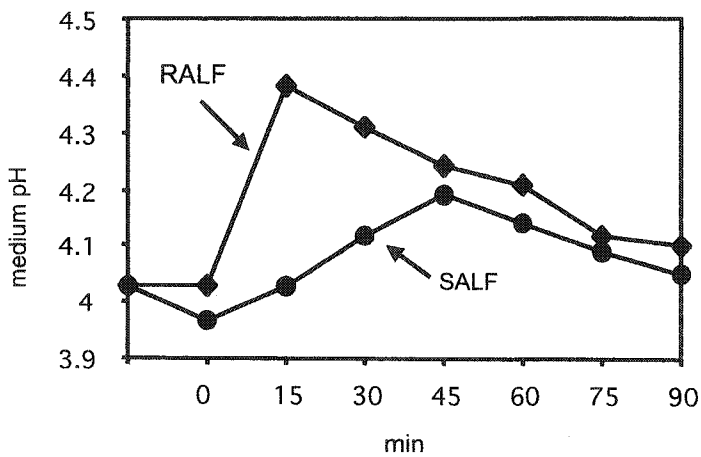
In order to search for molecules that cause alkalinization in the medium of poplar suspension cultures, fractions causing alkalinization activity were purified from poplar leaf extract. Preliminary experiments showed that poplar leaf extract contained several alkalinization activities that were separated with C18 open column chromatography (Chapter 4). In this study, an active fraction eluted by 20 % MeOH from C18 open column chromatography was chosen for further purification. The 20 % MeOH fraction showing alkalinization activities was subjected to Sephadex G25 gel filtration chromatography (Fig. 5.1A). The activities which eluted in the very early fractions showed similar alkalinization kinetics as the previously isolated RALF (Haruta and

Fig. 5.1. Alkalinization activities found in fractions from Sephadex G-25 chromatography (A) and their alkalinization kinetics (B). A. Five microliters of each 6 ml fraction was tested for alkalinization in cell cultures. Alkalinization activity was shown with open squares. UV absorbance detected at 220 nm is shown with solid diamonds. The activity peaks containing RALF and SALF are indicated with arrows. B. Kinetics of alkalinization caused by crude RALF and SALF fractions isolated above. The fractions were added at 0 min after equilibrating cultures for 50 min. pH of the medium was measured prior to the addition of fractions, at 0 min, and every 15 min for 90 min.

A


3500
3000
2500
2000
1500
1000
500
0
A220
0.4
0.3
0.2
0.1
0
delta pH
SALF
RALF
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
fraction no

B


4.5
4.4
4.3
4.2
4.1
4
3.9
medium pH
RALF
SALF
0
15
30
45
60
75
90
min

Constabel, 2003; Fig. 5.1B). Here, the late-eluting fractions containing slow alkalinization factors were further purified. In order to characterize the chemical properties of the slow alkalinization factors, the active materials eluting from the Sephadex G25 column were subjected to a heat stability test and protease digestion. The treatment of the active materials with trypsin reduced alkalinization activity by approximately 50 % (data not shown). Heating the active materials at 100 °C for 5 min did not affect alkalinization activity (data not shown). These results suggest that a peptide moiety in the active compounds was likely important for the alkalinization activity.

Based on the assumption that the active compounds were peptides, the semi-purified materials were further purified using weak anion exchange (Fig. 5.2), strong cation exchange (Fig. 5.3A), and C18 HPLC (Fig. 5.3B). Pepsin treatment of the active materials that eluted from the final C18 HPLC completely lost alkalinization activity, confirming that the active materials were proteinaceous molecules. The isolated active material caused weak but repeatable alkalinization in the medium of suspension cells (Fig. 5.4). Therefore, it was named the slow alkalinization factor, SALF. In order to further characterize SALF, structural analyses were carried out.

Characterization of SALF by Mass Spectrometry and Peptide Sequencing

Analyses of purified SALF using electrospray ionization (ESI)-mass spectrometry and MALDI-mass spectrometry indicated the presence of a peptide-like molecule with the molecular mass 1330 as a major compound. Attempting peptide sequencing by ESI-tandem mass spectrometry provided tentative peptide sequences that were predicted by the fragmentation pattern of amino acids from the peptide. In order to unambiguously determine amino acid sequence of the peptide, SALF was subject to Edman sequencing. The N-terminal sequence of SALF was determined to be $^{+}$AEEKTATKEAPVG^{-}. The theoretical molecular weight of SALF based on this sequence was 1330, in good agreement with the molecular mass determined by both ESI- and MALDI-mass spectrometry. By comparing the peak height of the first N-terminal amino acid during Edman degradation to the amino acid standard, the yield of SALF peptide was estimated approximately 260 pmol from 800g wounded poplar leaf tissue. From the yield of SALF

Fig. 5.2. Weak anion exchange (WAX) HPLC of SALF. Active compounds semi-purified by Sephadex G25 were separated using a 0 - 500 mM KCl gradient starting at 10 min after an isocratic run with 0 mM KCl in a solution consisting of 15 mM KPO_4 (pH6.0) and 5 % acetonitrile. Alkalinization activity was assayed by adding 5 μl of each fraction to 2 ml of cells and measuring the pH every 15 min for 90 min. The maximum pH increase (ΔpH) was calculated and plotted with the elution profile monitored at 215 nm. The HPLC peak shown with an arrow was recovered and further purified with C18 HPLC.

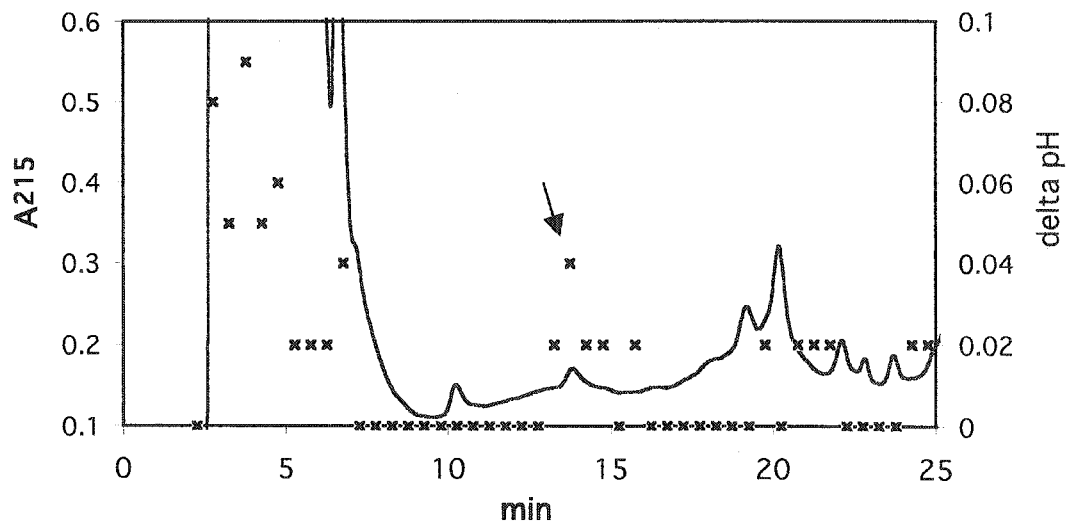

0.6
0.5
0.4
0.3
0.2
0.1
A215
0.1
0.08
0.06
0.04
0.02
0
delta pH
0
5
10
15
20
25
min

Fig. 5.3. Strong cation exchange (SCX) HPLC of SALF (A) and the subsequent, second C18 HPLC of SALF (B). A. Active compounds from the first C18 HPLC were separated by SCX HPLC using a 0 - 400 mM KCl gradient in a solution consisting of 5 mM KPO_4 (pH3.0) and 25 % acetonitrile. Alkalinization activity was assayed by adding 2 μl of each fraction to 2 ml of cells and measuring the pH every 15 min for 90 min. The maximum pH increase (ΔpH) was calculated and plotted with the elution profile monitored at 215 nm. B. The activity peak obtained from the SCX-HPLC was further purified with C18 HPLC running with a gradient 0 – 20 % acetonitrile in 0.1 % TFA. Alkalinization activity was determined as described above.

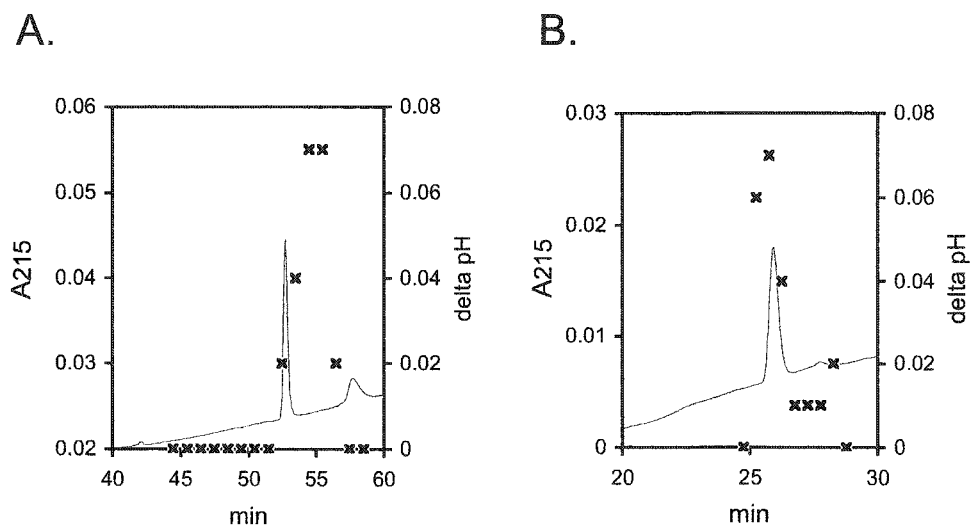
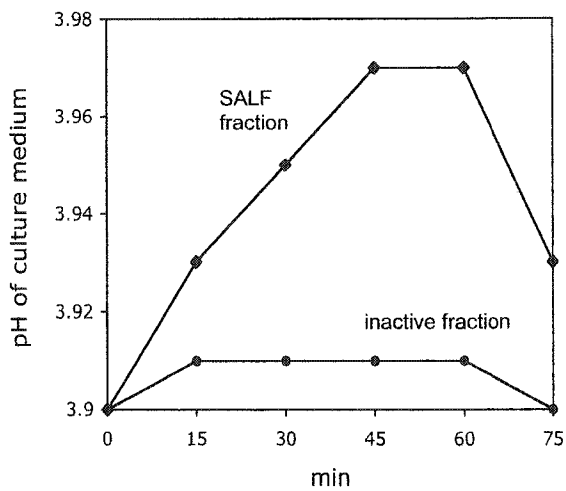

A.
B.
A215
delta pH
min
0.06
0.05
0.04
0.03
0.02
0.08
0.06
0.04
0.02
0
40
45
50
55
60
0.03
0.02
0.01
0
20
25
30

Fig. 5.4. Alkalinization activity of isolated SALF peptide from the final C18 HPLC. Changes in medium pH by SALF-containing fraction and an inactive fraction as control are shown. The SALF fraction was assayed at approximately 340pM of SALF peptide.


3.98
3.96
3.94
3.92
3.9
pH of culture medium
SALF
fraction
inactive fraction
0
15
30
45
60
75
min

peptide obtained from leaf tissues, the concentration of the peptide used for the assay during the last C18 HPLC was calculated to be as approximately 340 pM. The alkalinization activity of SALF at this concentration is at most 0.1 pH unit and much weaker than RALF, which causes alkalinization by 1.0 pH unit at a similar concentration (see chapter 4, Fig. 4.3).

Further Characterization of SALF Using Sequence Databases

In order to identify genes encoding SALF and possible precursor proteins, the peptide sequence obtained from Edman sequencing was used to conduct DNA and protein database searches. First, BLAST searches at the NCBI site were carried out in the datasets consisting non-redundant sequences, with settings for a short peptide sequence. Searching similar sequences to SALF with the use of such a short peptide sequence resulted in finding several moderately matched sequences from the databases. The highest scoring hit was a photosystem I reaction center subunit II precursor-related protein from *Arabidopsis*. This match has a relatively high expect value (E=18) because of querying with a short peptide sequence. Additionally, other less similar sequences that are derived from non-plant organisms were identified (see Discussion). In order to refine our searches, analyses were focused on surveying sequence databases including a large number of sequences from *Populus*. Availability of draft sequences of the poplar (*P. trichocarpa*) genome DNA facilitated a search of genomic DNA sequences encoding SALF. The *Populus* genome database available at the web site of the US Department of Energy contained a very similar sequence (clone ID XXI207113.y1, 12 residues out of 13 residues are identical) to SALF. Further surveys of ESTs from GenBank NCBI identified many unannotated sequences found from various tissues of *Populus* including poplar (*P. trichocarpa*) and hybrid aspen (*P. tremula x P. tremuloides*). Using the sequence of a matching full-length EST found from hybrid aspen (accession no. BU866695), homologous peptide sequences were retrieved from the SwissProt protein database. The analyses revealed that highly conserved SALF-like sequences are found in many plants, and annotated as a 20 kD photosystem I center subunit protein (PSI-D) (Iwasaki et al., 1990; Yamamoto et al., 1991; Fig. 5.5). In order to increase confidence in this unexpected result, cellular localization of the deduced protein encoded by the aspen EST

(BU866695), was analyzed by a target prediction program, PSORT. Based on the amino acid composition and sequence at the N-terminus of the protein, PSORT predicted that protein encoded by the EST, BU866695, is likely targeted to the stroma of chloroplasts (data not shown). The peptide sequence corresponding to SALF is located at the 50th amino acid position of ~ 200 amino-acid PSI-D precursor protein and matches with the N-terminus of mature PSI-D protein (Fig. 5.5).

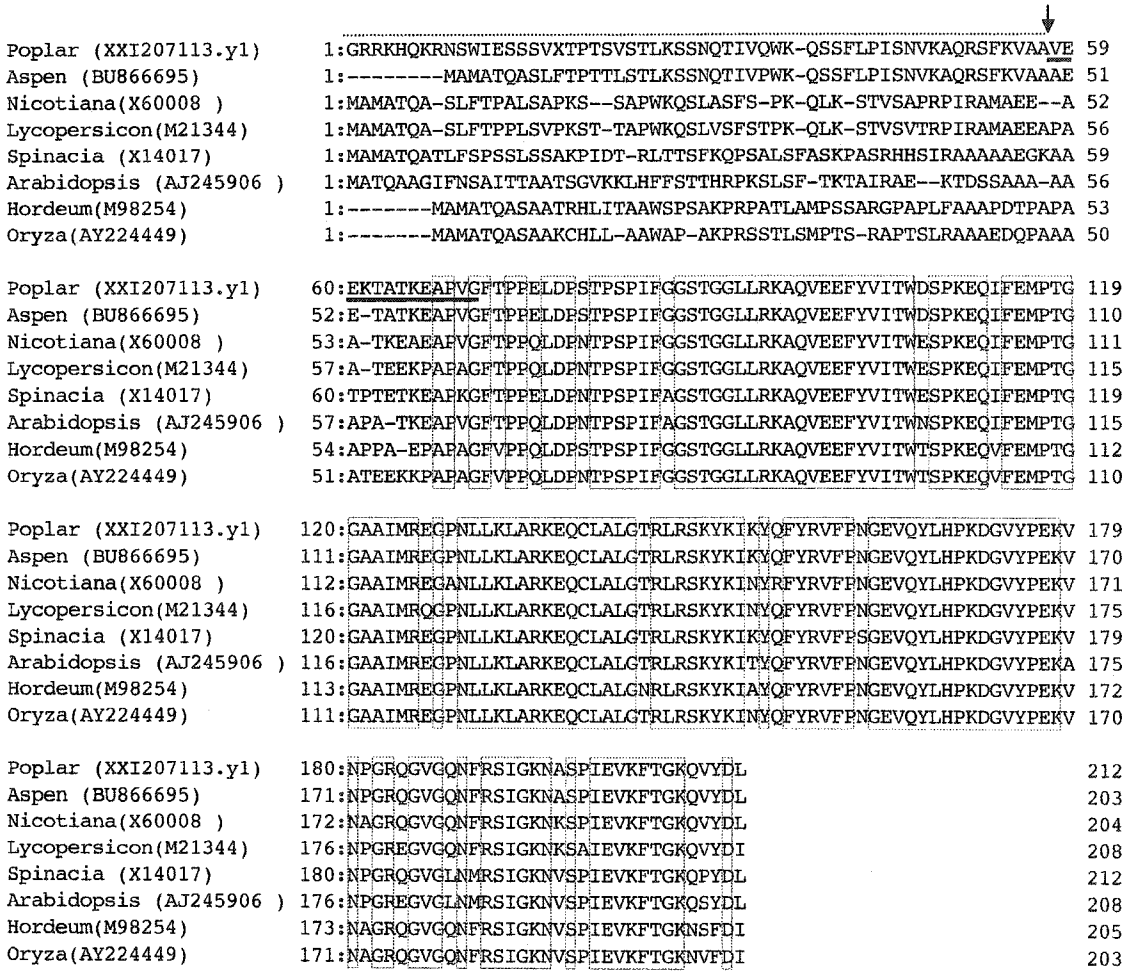
In multiple sequence alignments of PSI-D proteins, conservation of peptide sequence was found throughout the mature PSI-D proteins but not the SALF region (Fig. 5.5). Neither the deduced peptide sequences of poplar or aspen PSI-D ESTs completely matched the SALF peptide sequence determined by Edman sequencing (Fig. 5.5). Specifically, the Ala residue, the first amino acid residue of SALF, is found in the aspen EST (BU866695), but not in the poplar genomic sequence (XXI207113.y1). However, the aspen EST has a deletion of Lys residue at the 4th amino acid of the SALF peptide sequence (Fig. 5.5, compare the underlined sequence). Since the plant material used for peptide isolation in this study was *P. trichocarpa x P. deltoides* hybrid, SALF may be a gene product of *P. deltoides*. Since peptide sequences corresponding to SALF are variable among other plant families, it is possible that different genotypes of *P. trichocarpa* have minor amino acid substitutions.

5.4. Discussion

Characterization of SALF as a Small Peptide Found in a PSI Subunit Protein

Alkalinization in the medium of cultures is observed as one of earliest cellular responses following elicitation. A search for molecules causing alkalinization enabled the isolation of elicitor molecules from microorganisms and wound hormones from tobacco leaf extracts (Felix et al., 1999; Pearce et al., 2001a; Felix and Boller, 2003). In a previous study on poplar extracts, peptides causing very rapid alkalinization, RALFs, were isolated with the use of pH alkalinization test (Chapter 4; Haruta and Constabel, 2003). In addition to RALF, poplar leaf extract contained other minor factors causing alkalinization in the medium of poplar cultures (Fig. 5.1A, Fig. 5.2). In this chapter, an extensive search for alkalinization factors led to the isolation of SALF, which causes slower and weaker alkalinization than RALF. Structural characterization of SALF with

Fig. 5.5. Multiple sequence alignments of higher plant PSI-D proteins. Conserved amino acid residues are boxed. The chloroplast transit peptide for the poplar PSI-D sequence is shown with a dotted line, and the cleavage site for the N-terminus of mature proteins is indicated with an arrow. The SALF peptide sequence obtained from N-terminal sequencing is underlined. The plant sources for the deduced peptide sequences are shown by common or genus names, together with accession numbers for GenBank or the clone ID for the poplar genomic sequence).


Poplar (XXI207113.y1) 1:GRRKHQKRNSWIESSSVXTPTSVSTLKSSNQTIVQWK-QSSFLPISNVKAQRSFKVAAVE 59
Aspen (BU866695) 1:--------MAMATQASLFTPTTLSTLKSSNQTIVPWK-QSSFLPISNVKAQRSFKVAAAE 51
Nicotiana(X60008) 1:MAMATQA-SLFTPALSAPKS--SAPWKQSLASFS-PK-QLK-STVSAPRPIRAMAEE--A 52
Lycopersicon(M21344) 1:MAMATQA-SLFTPPLSVPKST-TAPWKQSLVSFSTPK-QLK-STVSVTRPIRAMAEEAPA 56
Spinacia (X14017) 1:MAMATQATLFSPSSLSSAKPIDT-RLTTSFKQPSALSFASKPASRHHSIRAAAAAEGKAA 59
Arabidopsis (AJ245906) 1:MATQAAGIFNSAITTAATSGVKKLHFFSTTHRPKSLSF-TKTAIRAE--KTDSSAAA-AA 56
Hordeum(M98254) 1:--------MAMATQASAATRHLITAAWSPSAKPRPATLAMPSSARGPAPLFAAAPDTPAPA 53
Oryza(AY224449) 1:--------MAMATQASAAKCHLL-AAWAP-AKPRSSTLSMPTS-RAPTSLRAAAEDQPAAA 50
Poplar (XXI207113.y1) 60:EKTATKEAPVGFTPPELDPSTPSPIFGGSTGGLLRKAQVEEFYVITWDSPKEQIFEMPTG 119
Aspen (BU866695) 52:E-TATKEAPVGFTPPELDPSTPSPIFGGSTGGLLRKAQVEEFYVITWDSPKEQIFEMPTG 110
Nicotiana(X60008) 53:A-TKEAEAPVGFTPPQLDPNTPSPIFGGSTGGLLRKAQVEEFYVITWESPKEQIFEMPTG 111
Lycopersicon(M21344) 57:A-TEEKPAPAGFTPPQLDPNTPSPIFGGSTGGLLRKAQVEEFYVITWESPKEQIFEMPTG 115
Spinacia (X14017) 60:TPTETKEAPKGFTPPELDPNTPSPIFAGSTGGLLRKAQVEEFYVITWESPKEQIFEMPTG 119
Arabidopsis (AJ245906) 57:APA-TKEAPVGFTPPQLDPNTPSPIFAGSTGGLLRKAQVEEFYVITWNSPKEQIFEMPTG 115
Hordeum(M98254) 54:APPA-EPAPAGFVPPQLDPSTPSPIFGGSTGGLLRKAQVEEFYVITWTSPKEQVFEMPTG 112
Oryza(AY224449) 51:ATEEKKPAPAGFVPPQLDPNTPSPIFGGSTGGLLRKAQVEEFYVITWTSPKEQVFEMPTG 110
Poplar (XXI207113.y1) 120:GAAIMREGPNLLKLARKEQCLALGTRLRSKYKIKYQFYRVFFNGEVQYLHPKDGVYPEKV 179
Aspen (BU866695) 111:GAAIMREGPNLLKLARKEQCLALGTRLRSKYKIKYQFYRVFFNGEVQYLHPKDGVYPEKV 170
Nicotiana(X60008) 112:GAAIMREGANLLKLARKEQCLALGTRLRSKYKINYRFYRVFFNGEVQYLHPKDGVYPEKV 171
Lycopersicon(M21344) 116:GAAIMRQGPNLLKLARKEQCLALGTRLRSKYKINYQFYRVFFNGEVQYLHPKDGVYPEKV 175
Spinacia (X14017) 120:GAAIMREGPNLLKLARKEQCLALGTRLRSKYKIKYQFYRVFFSGEVQYLHPKDGVYPEKV 179
Arabidopsis (AJ245906) 116:GAAIMREGPNLLKLARKEQCLALGTRLRSKYKITYQFYRVFFNGEVQYLHPKDGVYPEKA 175
Hordeum(M98254) 113:GAAIMREGPNLLKLARKEQCLALGNRLRSKYKIAYQFYRVFFNGEVQYLHPKDGVYPEKV 172
Oryza(AY224449) 111:GAAIMREGPNLLKLARKEQCLALGTRLRSKYKINYQFYRVFFNGEVQYLHPKDGVYPEKV 170
Poplar (XXI207113.y1) 180:NPGRQGVGQNFRSIGKNASPIEVKFTGKQVYDL 212
Aspen (BU866695) 171:NPGRQGVGQNFRSIGKNASPIEVKFTGKQVYDL 203
Nicotiana(X60008) 172:NAGRQGVGQNFRSIGKNKSPIEVKFTGKQVYDL 204
Lycopersicon(M21344) 176:NPGREGVGQNFRSIGKNKSAIEVKFTGKQVYDI 208
Spinacia (X14017) 180:NPGRQGVGLNMRSIGKNVSPIEVKFTGKQPYDL 212
Arabidopsis (AJ245906) 176:NPGREGVGLNMRSIGKNVSPIEVKFTGKQSYDL 208
Hordeum(M98254) 173:NAGRQGVGQNFRSIGKNVSPIEVKFTGKNSFDI 205
Oryza(AY224449) 171:NAGRQGVGQNFRSIGKNVSPIEVKFTGKNVFDI 203

mass spectrometry and N-terminal peptide sequencing indicated that SALF is a 13 amino-acid peptide with a molecular weight of 1330. Further, careful database searches using the peptide sequence suggested that SALF peptide was likely derived from phtotosystem I (PSI) subunit protein D, PSI-D. Although there were minor differences in amino acid sequences among SALF peptide and sequences from *Populus* species found in the databases, the peptide sequences identified here were the highest hits from the database containing the whole poplar genome, or from over 94,000 EST clones from *Populus*. The single amino acid discrepancy is likely due to the *P. deltoides* allele in our hybrid, for which little sequence data is available. Therefore, it is possible that the SALF peptide isolated here is encoded by a gene that is orthologous to PSI-D proteins characterized in other plant species.

The PSI protein complex catalyzes the transport of electrons from plastocyanin to ferredoxin in the electron transfer chain during photosynthesis (Golbeck, 1987). PSI-D is encoded in the nucleus and the precursor PSI-D protein is synthesized in the cytoplasm and transported into the chloroplast (Scheller and Møller, 1990 and reference therein). The 20 kD mature PSI-D is extrinsic but firmly bound to the PSI complex and located on the stromal side of thylakoid membrane, and functions as a ferredoxin-docking protein (Scheller and Møller, 1990). Highly conserved PSI-D proteins are found in higher plants and photosynthetic microorganims, indicating the essential function of the protein (Scheller and Møller, 1990; Fig. 5.5). The poplar SALF peptide sequence is located at the very N-terminus of the mature PSI-D protein (Fig. 5.5). Although conservation of peptide sequences is observed throughout the mature peptide sequences of PSI-D protein from various species, the region corresponding to the SALF peptide shows significant variation among PSI-D protein sequences. These variable sequences in the SALF region may suggest that this domain is not directly involved in the maintenance of the secondary structure of PSI-D protein or tertiary structure of PSI complex. In a study of spinach PSI-D protein, it was shown that a peptide motif corresponding to SALF in spinach PSI-D is exposed to the media and readily accessible by proteases (Lagoutte and Vallon, 1992). This would be consistent with this peptide possibly being released in wound-damaged leaves (see below).

Origin of SALF via Partial Proteolysis of PSI-D Protein

The alkalinization-assay guided fractionation of poplar leaf extracts resulted in the isolation of a small peptide that is likely derived from the 20 kD chloroplast protein, PSI-D. It is not clear whether proteolysis of PSI-D occurred *in vivo* prior to extracting leaf, or *in vitro* during the extraction process. Since alkalinization factors were extracted from wounded leaf tissue under very acidic conditions which are not optimal for the majority of proteases (with 1% TFA, pH 1.0), proteolysis of PSI could have occurred *in vivo* when leaves were wounded. Furthermore, limited proteolysis would be more likely to occur *in vivo* while degradation of proteins *in vitro* during extraction processes would be unregulated and more chaotic. In particular, uniform proteolysis of each PSI-D protein molecule is unlikely to occur *in vitro* during the extraction process. Therefore, it is possible that SALF peptide fragments were already present in the wounded leaf material, and were subsequently extracted and isolated in this experiment. This hypothesis could be tested by comparing the amount of SALF peptides which are present in both unwounded and wounded leaf extracts. During the purification of the slow alkalinization fraction, the activity was recovered by a relatively hydrophilic solvent from reverse phase chromatography and by a medium salt concentration from ion exchange chromatography (Fig. 5.2, 5.3A). SALF is a small peptide that is rich in charged amino acids and hydrophilic. These biochemical features of SALF explain its chromatographic behavior during the isolation procedure.

PSI-D is one of the major proteins found in chloroplasts, a protein-rich organelle which in photosynthetic tissue contains up to 50 % of cellular protein (Viersta, 1996). Protein turnover in chloroplasts, therefore, could be an important process for maintenance of the organelle (Andersson and Aro, 1997). Controlled proteolysis of chloroplast proteins has been observed when tissues undergo senescence and are exposed to heat, light, or oxidative stresses (Wilson et al., 2002; Yang et al., 1998; Desimone et al., 1996). Stress-damaged proteins such as photosystem II D1 protein are degraded by a stepwise regulated proteolysis that requires energy including GTP and ATP, in a manner similar to proteolysis in bacteria (Shanklin et al., 1995; Spetea et al., 1999; Viersta, 1996). Given that several stresses lead to denaturing and proteolysis of chloroplast proteins, it is possible that the mechanical wounding conducted on leaves prior to extraction caused

damage to cells and chloroplast, which resulted in proteolysis of chloroplast proteins including PSI-D. This hypothesis is consistent with the evidence that proteasome activity is required for induction of defenses in response to wounding and elicitor treatment (Ito et al., 1999; Takai et al., 2002; Becker et al., 2000). Degradation of suppressive regulatory proteins is thought to switch on the expression of defense-related genes. It could be also speculated that amino acids derived from degradation of proteins are mobilized for the synthesis of defense-related compounds and proteins during wound responses. Additionally, it is possible that some breakdown products of these proteins could serve as signals in wounded plants (see below).

Possible Signal-like Function of SALF for Wound Responses

Using cultured cells of plants, Boller's group has studied early cellular responses against biotic stresses intensively (Boller, 1995). Suspension cultures of tomato, tobacco, and *Arabidopsis* show both alkalinization response that are followed by the induction of defenses when they perceive bacterial flagellin (Felix et al., 1999). A comparative analysis of flagellin peptide sequences revealed that the 15 amino-acid peptide fragment (flg15; $^{+}$RINSAKDDAAGLQIA^{-}) from flagellin was sufficient for induction of alkalinization and defense responses in plant cultures. Similarly, parsley cell cultures recognize the 13 amino-acid peptide (pep13; $^{+}$VWNQPVRGFKVYE^{-}) residing in transglutaminase of *Phytophthora* species, and show alkalinization response (Nürnberger et al., 1994). Tobacco cell cultures show the alkalinization response upon perception of a 15 amino-acid peptide (CSP15; $^{+}$VKWFNAEKGFGFITP^{-}), which forms the core of a conserved domain of CSP, a bacterial cold shock protein (Felix and Boller, 2003). These small peptides serve as an "epitope-like" motif during plants' recognition of pathogenic microorganisms. In addition to these exogenous signals, plant-derived small peptides are also known to be important signals for induction of defenses. In suspension cultures of tomato and tobacco, the 18 amino-acid endogenous peptides, systemin, trigger both alkalinization and defense responses (Felix and Boller, 1995; Pearce et al., 2001a). Binding of these peptide signals to plants cells is specific, saturable, and reversible, indicating that perception of these signals is mediated by specific receptors (Baureithel et al., 1994; Meindl et al., 2000; Nürnberger et al., 1994; Scheer and Ryan, 1999).

In this study, a 13 amino-acid peptide fragment from the PSI-D protein, was found to cause alkalinization in poplar suspension cultures. It was surprising that this small peptide derived from PSI-D is active in the pH alkalinization assay, since the purification was intended to identify systemin-like peptides in poplar. One possible explanation is that the SALF peptide may have sequence similarity to as yet unknown peptide elicitors. Searching related sequences using the 13 amino acid sequence using BLAST at Genbank identified the presence of several moderately similar sequences (9 to 11 amino acids out of 13 amino acids identical) including surface protein of *Staphylococcus aureus* (accession no. P80544, E=32) and HMG-CoA reductase from *Penicillium citrinum* (BAC20567.1, E=186).

A more likely explanation could be that PSI-D protein undergoes limited proteolysis upon wounding and that the proteolyzed product of PSI-D may be perceived by the plant as a cue of tissue damage. Other studies have shown degradation products from plant tissues to be active in the alkalinization assay and to induce defenses in cell cultures. Breakdown products of the plant cuticle induce alkalinization and serve as signals for induction of defenses (Schweizer et al., 1996). Likewise, oligogalacturonides derived from plant cell walls induce alkalinization responses that are followed by the induction of defense-related genes (Mathieu et al., 1991).

To date, it is not known if proteolycally-released peptide fragments from known proteins have signal-like roles for the induction of plant defenses, but there are parallels in other systems. In human granulocytes, it has been reported that proteolyzed fragments of histone possess antibacterial activity (Wang et al., 2002). In prokaryotes, small pheromone peptides involved in mating for plasmid transfer are produced from a portion of the signal sequence of membrane lipoproteins (Clewell et al., 2000 and references therein). In addition to these examples, it was reported that several biologically active small peptides are produced from a part of known proteins in animals (Weihofen and Martoglio, 2003 and references therein).

Therefore, it is conceivable that the chloroplast PSI-D protein found here has a dual function in plants. Recent discoveries of chloroplast proteins involved in defense mechanisms also support this hypothesis. Silencing of a gene for a photosystem II component protein, which shows binding activity to elicitors from tobacco mosaic virus,

enhances virus replication (Abbink et al., 2002). Furthermore, carbonic anhydrase, known to function in the equilibration of CO_2 to HCO_3^- in chloroplasts, has a salicylic-acid binding activity and plays a role in hypersensitive responses during pathogen defense (Slaymaker et al., 2002). Seo et al. (2000) also reported that reduced levels of the chloroplast DS9 metalloprotease accelerates the hypersensitive response upon virus infection.

Although SALF was isolated during the searche for systemin-like peptides in poplar, it could not be determined if SALF induces defenses in poplar. In order to verify that this peptide is a biologically active signal-like compound, it will be necessary to carry out further characterization such as the dose-response curve in alkalinization assays and additional study of induction of defenses genes using the pure peptide.

It should be noted that during the isolation steps of SALF in this study, the several additional fractions containing minor alkalinization activity were observed. Conceivably, the wounded leaf extract contained other alkalinization factors not pursued in this experiments but which may have signal-like functions (Fig. 5.2; see Chapter 6).

Chapter 6

General Discussion

In this thesis, two different research projects were undertaken; i) molecular analyses of inducible defense genes in trembling aspen (Chapters 2 and 3) and ii) the biochemical isolation of alkalinization peptides from poplar (Chapters 4 and 5). This chapter aims to discuss results in a broader context and present their significance.

6.1. Molecular Analyses of Inducible Anti-Herbivore Defense Genes in Trembling Aspen (*Populus tremuloides* Michx.)

Trembling aspen is an ecologically important tree species that is often subject to severe defoliation by herbivores. Extensive studies by Lindroth *et al.* have been shown that phytochemicals such as phenolics play important roles in trembling aspen anti-herbivore defenses (Lindroth and Hwang, 1996). Whereas there are many reports indicating the presence of protein-based defenses in other plant species including poplar (Constabel, 1999; Carlini and Grossi de Sá, 2002), no prior studies had investigated protein-based defenses in trembling aspen. Here, genes encoding the defense proteins, polyphenol oxidase (PPO) and trypsin inhibitor (TI), were isolated and characterized as the first step of a molecular analysis of protein-based induced defenses in trembling aspen.

6.1.1. Characterization of Wound-, Herbivory-, and Methyl Jasmonate- Induction of Anti-Herbivore Genes in Trembling Aspen (*Populus tremuloides* Michx.)

In order to initiate the investigation of protein-based defenses in trembling aspen, cDNAs encoding wound-inducible PPO and TI were first isolated as molecular tools. Using a poplar PPO cDNA as a probe (Constabel et al., 2000), a full-length cDNA encoding PPO was isolated from an aspen cDNA library, constructed from wounded leaf as part of this study. This aspen cDNA clone isolated here was confirmed to encode PPO by sequence analysis. In Southern analysis, it was revealed that aspen PPO is encoded by a small gene family with two members (Fig. 2.3). Using PtPPO as a probe, the expression of PPO mRNA was further characterized in aspen foliage. The expression of

PPO in trembling aspen was induced by mechanical wounding, herbivory, and MeJa in a similar manner to tomato in which induction of defense-related genes are well characterized (Constabel et al., 1995; Bergey et al., 1996). Furthermore, the wound-induction of PPO mRNA was followed by the increase in PPO enzymatic activity, further confirming the biological significance of PPO as a part of induced defenses trembling aspen. Overall the expression pattern of PPO suggests the defensive role of PPO in anti-herbivore defense responses in trembling aspen.

In addition to PPO, TI cDNAs were isolated and characterized from trembling aspen. Three different TI genes were identified (Fig. 3.1). In contrast to PPO, TI appears to be encoded by a larger gene family, as observed by Southern blot analysis (Fig. 3.2). Similar to PPO, TI mRNA accumulated after mechanical wounding, FTC herbivory, and MeJa treatment, indicating that the same signaling mechanisms (i.e. the octadecanoid pathway) is likely involved in the regulation of PPO- and TI-gene expression. The biological activity of TI was later confirmed by measuring trypsin-inhibitory activity of recombinant TI2 protein, heterogeneously expressed in *Escherichia coli* (achieved by Ian Major and Mary Christopher in Constabel laboratory; Haruta et al., 2001a). Based on the expression pattern of TI genes and the biological activity of heterogeneously expressed TI proteins, it was concluded that TI likely plays an anti-herbivore defensive role in trembling aspen.

Isolation and characterization of aspen PPO and TI cDNAs facilitated a general comparison of these two genes from an evolutionary perspective. In Southern analysis, aspen PPO showed no restriction fragment polymorphisms detected between different genotypes (Fig. 2.3). In contrast, Southern analyses of TI indicated the presence of several polymorphisms between genotypes (Fig. 3.2). Thus, the genetic organization of aspen TIs seems to be more variable than that of PPO in aspen. Furthermore, the high conservation of deduced peptide sequences from other plant families was observed in PPO sequences, but not in TI sequences (data not shown). In general, one can speculate that TI evolves more rapidly compared to PPO. This may be explained by the biological functions of PPO and TI in defenses. TI as a serine protease inhibitor inhibits activities of insect trypsin and related serine proteases during defense responses. On the other hand, PPO would generate various types of reactive quinones that modify various

proteins in both damaged plant tissues and insect gut. Hence, TI may be specialized in target specificities and subject to co-evolution with structures of overcoming insect proteases, while PPO may have more general effects during defenses and tend to be conserved due to common structures of endogenous phenolic substrates. Ultimately, it will be interesting to study the detailed mechanisms of action of these defense proteins during defense, and their effects on different types of insect herbivores (see 6.1.3).

6.1.2. Significance of the Studies

The studies conducted here are, to my knowledge, the first demonstration of wound-inducible genes in trembling aspen. Characterization of several of inducible genes here establish the potential role of protein-based defenses in trembling aspen, in addition to the phytochemical defenses which have been well documented previously (Lindroth and Hwang, 1996). Understanding two different defense mechanisms (i.e. phytochemical-and protein-based) in the same plant provides a useful experimental system to further investigate how plants use different defense mechanisms in different situations, such as various environmental conditions and infestation by different insects (see 6.1.3.).

The characterization of induced defenses here provides marker genes for the induction of defense in trembling aspen. For example, one could use PPO and TI induction as detection systems for investigating signaling events during the induction of defenses. Furthermore, TI and PPO would be useful to select aspen clones with strong induction of defense genes and possibly resistant genotypes against herbivory.

Additionally, obtaining aspen TI and PPO cDNA provide tools to genetically manipulate insect resistances in aspen. Although the productions of transgenic *Populus* species that have increased insect resistances have been proposed (Ellis and Raffa, 1997; Heuchelin et al., 1997; Kang et al., 1997; McCown et al., 1991; Leplé et al., 1995; Gill et al., 2003), the industrial application of those studies could be issues because of the uses of foreign genetic materials such as Bt toxin and proteinase inhibitors from other plant species. Genetic engineering with genes that are derived from the same plant species or even genotype would be less problematic in obtaining public acceptance and better in concerning environmental protection.

Overall, molecular analyses of aspen PPO and TI conducted here are the first step for understanding molecular mechanisms underlying protein-based defenses in trembling aspen. Trembling aspen has high genetic diversity across a wide range of North America, and is subject to herbivory by diverse arrays of insects. Understanding the interaction of aspen and insects will be a useful model system for a study of tree defenses against insects and provide insights into the evolution of tree defense mechanisms and insect feeding (see below).

6.1.3. Suggestions for Future Study

This study focused primarily on the characterization of wound-inducible genes as protein-based defense mechanisms. The next step will be more in-depth investigations of PPO and TI in terms of their biological functions. For instance, their target herbivores may be determined. Trembling aspen is a host of several insects such as leaf-feeding caterpillars, sap-feeding aphids, and woodborers (Mattson et al., 2001). It may be informative to understand the detailed tissue specificity of PPO and TI localization *in planta*, providing hints as to their target insects. It will also be important to understand the interaction of plant-derived defensive proteins with target insects, with regards to the co-evolution of plant defense mechanisms and insect feeding strategies.

Another research direction arising from this study is analyzing interactions of the induced defenses and the environmental conditions. Plants used in the studies carried out here were grown under the optimum growth conditions for induction of defense responses, which may not reflect actual situations of herbivory. In order to understand plant defense responses in the larger context of forestry, environmental factors affecting defense responses should be investigated. In fact, we have observed that individual aspen saplings of the identical genotype often show varying inducibilities of defense-related genes including PPO and dihydroflavonol reductase. This may indicate that minor differences in growth conditions such as light intensity affects plant defense responses. Variations in the level of aspen foliar phytochemicals (phenolics and tannin) were also observed by Lindroth et al. (Lindroth and Hwang, 1996). Therefore, it will be essential to elucidate the relationship of the inducibility of protein-based defenses and environmental factors.

Additional points to be further investigated are comparative analyses of defense mechanisms in various *Populus* species (such as aspen versus poplar). It has been observed that PPO and TI genes are more strongly induced in hybrid poplar than trembling aspen (Mary Christopher and Peter Constabel, unpublished data). On the contrary, trembling aspen accumulates significant of amounts of the defensive chemicals, condensed tannin upon wounding, but hybrid poplar much less so (Peters and Constabel, 2002). Thus, understanding the balance of protein-based and phytochemical defense in induced defenses of various *Populus* species would be helpful for understanding specificity in various *Populus*-insect interactions.

6.2. Biochemical Surveys of pH Alkalinization Peptides from Poplar

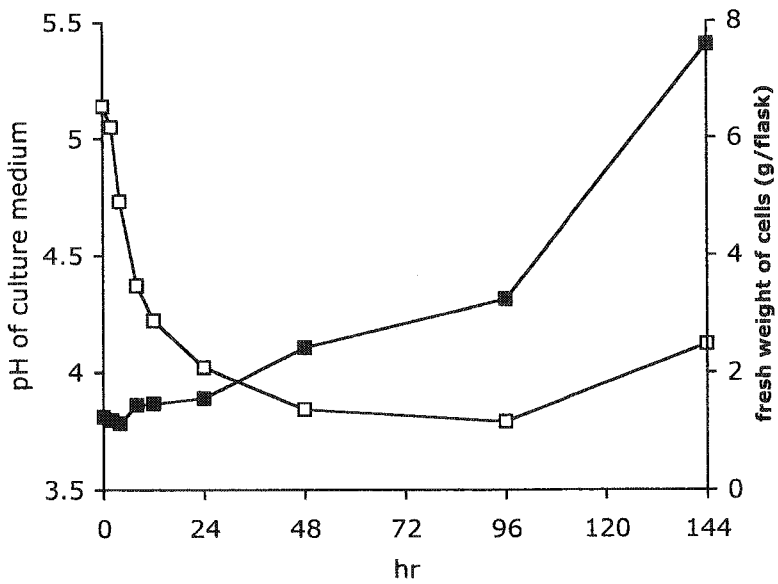
Cellular recognition of wound signals and subsequent signal transduction are important processes during the induction of defenses. Suspension cultures of plant cells partially reflect signaling events occurring in plant tissues, and many studies of defense signal transduction have been conducted using suspension cultures (Boller, 1995 and reference therein). In order to survey signal molecules for the induction of defenses from *Populus* (Constabel et al., 2000, Chapters 2 and 3 in this thesis), putative defense-related signals were sought from poplar leaf extracts using the pH test as described in Chapters 4 and 5. Hybrid poplar was chosen for the experimental system for several reasons including the stronger induction of defenses in hybrid poplar (Mary Christopher and Peter Constabel, unpublished data) and the availability of a suspension culture genetically identical to whole plant materials used for molecular analyses of defense. The initial objective of the experiments conducted here was to purify peptide-based signals, systemin-like peptides, from poplar. The assay-guided fractionation of the alkalinization factors present in poplar leaf extracts resulted in the isolation of novel 5 kD peptides, RALFs (Chapter 4), and a 13-amino acid peptide, SALF, that is likely derived from a photosystem I centre subunit protein (Chapter 5). The peptide sequences of these two peptides indicate that they are unrelated to systemin, demonstrating the presence of alkalinization peptides other than systemin-like peptides in poplar leaf extracts.

6.2.1. Isolation and Characterization of Culture Medium Alkalinization Factors from Poplar

In order to look for peptide-based endogenous signals for induction of defenses, a novel assay to detect cellular responses to test substances was established using poplar suspension cultures. In general, it was found that poplar suspension cells normally change the medium pH during growth (Fig. 6.1), suggesting the presence and the importance of cellular mechanisms regulating extracellular pH. In the experiment testing cellular responses to known elicitors, 2-day-old cultures, which have stable extracellular pH, showed alkalinization responses to a peptide derived from flagellin and to chitosan, presumably indicating the competence of our poplar suspension cells to recognize and transmit signals (Chapter 4, Fig. 4.3). Furthermore, poplar leaf extract tested with the pH assay also induced alkalinization responses, indicating the presence of endogenous alkalinization factors in poplar leaf extracts. The crude leaf extracts were fractionated and separated alkalinization activities into two different fractions. The fraction causing rapid and intense alkalinization was further purified and isolated (Chapter 4). Structural analysis of the alkalinization factors, a group of 5 kD peptides, revealed that they are very similar to the rapid alkalinization factor (RALF) that was very recently isolated from tobacco leaf extract during the purification procedure of systemin-like peptides (Pearce et al., 2001b).

Poplar RALF peptides were further characterized at the molecular level. Two poplar RALF cDNAs corresponding to isolated RALF peptides were cloned and their expression pattern was characterized. The deduced peptide sequence showed that RALF is synthesized as a 120 amino acid precursor protein with a signal sequence, and processed into a smaller mature form. Conserved RALF-like sequences were found from many plant species including *Arabidopsis* in sequence database. In order to determine whether or not RALF is a signal for the induction of defenses, the expression of PAL gene was analyzed in suspension cultures after treatments with RALF. In contrast to treatment with other pathogen-derived elicitors, RALF did not induce the expression of PAL (Chapter 4, Fig. 4.4). Pearce et al. (2001b) reported that RALF does not induce the accumulation of PIs in excised tomato leaves. Together with the results, it was concluded that the RALF found here is unlikely to be a signal for defense. However, specific

Figure 6.1. Changes in medium pH during growth of poplar cells. Changes in cell culture mass is shown using solid squares. pH of the culture medium is shown with empty squares.


5.5
5
4.5
4
3.5
pH of culture medium
8
6
4
2
0
fresh weight of cells (g/flask)
0
24
48
72
96
120
144
hr

biological function of RALF *in planta* has not yet been determined. The function of RALF could be analyzed by observing the phenotype of plants that have increased or reduced level of RALF peptides. The genetic resources of *Arabidopsis* may also be useful to conduct analyses of RALF-mutant plants.

Further investigations were conducted to look for alkalinizing peptides which may act as defense-related signals. The fraction that showed weaker and slower alkalinization was purified using a series of chromatography steps. The isolated peptide, named SALF, was subjected to structural analyses and determined to be 13 amino-acid peptide fragment of the photosystem I centre protein subunit, PSI-D. The peptide sequence of SALF is located at the N-terminus of PSI-D mature protein (Fig. 5.5). Although it is not clear how SALF peptide is produced, it is likely that the wounded leaf contained SALF peptide prior to extracting leaf. One possible scenario is that mechanical wounding caused the limited proteolysis of PSI-D protein and generated the small peptide. During the isolation procedure of SALF, the effects of SALF on the accumulation of PI or PAL proteins were analyzed in the poplar suspension cultures and leaf tissue. However, SALF did not induce the accumulation of defense proteins in the detection system tested here (data not shown). At this point, it is not clear whether SALF is a biologically active molecule that act as a signal *in planta*, or a simple peptide fragment generated as a consequence of protein turnover.

The two different alkalinizing peptides isolated here do not relate to systemin, but the discovery of novel alkalinizing peptides provide opportunities to understand peptide-based signal molecules in plants in general. Thus, the alkalinization assay may be an interesting assay system to detect and look for novel molecules regulating cellular activity and for further investigation of their signaling pathways. Changes in extracellular pH have been observed when plant cells or tissues are exposed to various stimuli and chemical substances. The presence of NADH in the bathing medium of sugarcane cells induces an extracellular pH increase within minutes that is followed by cellular O_2 consumption (Komor et al., 1987). In oat coleoptiles, uptake of amino acids across the plasma membrane causes a transient depolarization of the plasma membrane and alkalinization of cell-free space (Kinraide et al., 1984). Extracellular pH alkalinization was also observed in the pulvinus of *Phaseous vulgaris* that was exposed to

blue light (Okazaki et al., 1995). Moreover, in cultured cells of rose, UV exposure induces an increase in extracellular pH (Huerta and Murphy, 1989). Although the mechanisms causing alkalinization are not known in these cases, they imply that extracellular alkalinization is related to a broad range of cellular activities. pH changes in the culture medium was also observed during the growth of poplar cells (Fig. 6.1). Thus, alkalinization of extracellular pH occurring in response to various environmental stimuli might be a common cellular event, as is the case for activation of protein kinases and increases in cellular calcium levels common during cellular signaling (reviewed in Jonak et al., 1999; reviewed in Rudd and Franklin-Tong, 1999).

6.2.2. Significance of the Studies

Although it has long been recognized that peptide hormones are important in various physiological processes in animals, the involvement of peptide-based signals in plant intercellular communication has emerged only recently (Ryan et al., 2002). Since the discovery of tomato systemin, the first plant peptide hormone, in 1991 (Pearce et al., 1991), two other peptide hormones were isolated and characterized from plants; phytosulfokine and RALF (Matsubayashi et al., 1996; Pearce et al., 2001b; Haruta and Constabel, 2003). Phytosulfokine promotes proliferation of cells at nanomolar concentrations and was biochemically isolated from the culture medium of asparagus cells using an assay for mitogenic activity (Matsubayashi et al., 1996). The isolation of systemin, phytosulfokine, and RALF all resulted from the investigation of molecules with biological activities of interests, emphasizing the importance of developing assay systems for the discovery of biologically active signal molecules.

The isolation of biologically active peptides also directly confirms the involvement of peptide hormones in physiological processes of plants. Genetic analyses of mutant plants also led to discovery of genes encoding small peptides that are expected to be involved in intercellular communication (Fetcher et al., 1999; Schopfer et al., 1999). Although the importance of the genes encoding small peptides was proposed in these cases, the biochemical structures of the putative active peptides are not yet clear. Since all peptide hormones known to date are biosynthesized as precursor proteins and processed into smaller forms with post-translational modifications including

glycosylation (Pearce et al., 2001a), sulfation of tyrosine residues (Matsubayashi et al., 1996), hydroxylation of proline residues (Pearce et al., 2001a), and formation of disulfide bridges (Pearce et al., 2001b; Haruta and Constabel, 2003), it may be very difficult to predict the structures of mature peptides without having actual peptide molecules. Therefore, the isolation and characterization of peptide molecules will be necessary to find novel peptides with hormone-like properties in plant biology.

In addition to RALF peptides that cause rapid alkalinization of the culture medium, SALF, causing slower and weaker alkalinization, was isolated and characterized from poplar leaf extracts. Although it is not clear whether the SALF peptide, which is likely derived from the PSI-D protein, is present as a biologically active signal *in planta*, the isolation of the peptide with the pH assay has given rise to the hypothesis that a proteolyzed product may act as a signal for induction of defenses (see Chapter 5 Discussion).

6.2.3. Suggestions for Future Study

The original objective of the study was to find systemin-like peptides from poplar, as this plant has similar inducible defenses to tomato (Constabel et al., 2000). It is still unknown whether *Populus* has systemin or not. If systemin-like peptides are present in poplar, there may be several possible explanations why they could not be detected in the study conducted here. One possibility is that our poplar cell cultures did not respond to it with alkalinization due to the lack of systemin signaling components. The ability of cells to show alkalinization responses is entirely dependent on the presence of the systemin receptor and the mechanisms causing alkalinization. It has been reported that tomato cultures of *Lycopersicon peruvianum* show alkalinization in response to systemin, but not that of *L. esculentum* (Felix et al., 1999). Since systemin was originally isolated from leaves of *L. esculentum*, the nurture of the cultured cells may have a strong effect on alkalinization response than the genetic background of cells. Therefore, the use of different poplar cell lines may be helpful for finding systemin-like peptides from poplar.

Another possibility is that systemin-like peptides present in poplar are at an extremely low abundance compared to high concentrations of foliar secondary metabolites, which may make it difficult to detect alkalinization activity. In order to

solve this problem, plant materials could be prepared from vascular tissues or phloem sap that may be enriched in systemin, as it was reported that the prosystemin promoter is active in vascular bundles (Jacinto et al., 1997).

In addition to the current biochemical approaches for finding systemin, recent progress in poplar genomics could also contribute to surveying systemin-like peptides from poplar. Using the bioinformatics approach as described by (MacIntosh et al., 2001), genes encoding small open reading frames (~ 200 amino acids) with a pair of the motif of Pro-Pro-Ser or Pro rich domains could be computationally screened. Because of the nature of peptide hormones that are generally biosynthesized as precursor proteins, genetic or molecular analyses would be helpful to see if candidate genes encoding systemin-like peptides are present, rather than a biochemical approach. The genes encoding candidate peptides for systemin may be overexpressed in poplar and their effects on the expression of defense-related genes could be analyzed. Alternatively, peptides having pH alkalinization activity and TI-inducing activity could be purified from a mixture of synthetic peptides (synthetic peptide libraries consisting ~18-mer random peptides). Sequences of candidate peptides which are obtained by the assay-guided purification could be surveyed from poplar genomic sequences. The biological functions of the systemin candidates could be tested by transgenic experiments as well.

Note

As of August 20, 2003, systemin-like peptides modified with glycosylation and hydroxylation of proline residues were also isolated from tomato and should be added on the list of systemin homologs in Section 1.2.3 (Pearce and Ryan, 2003). Regarding to Section 1.1.4. E stating that brassinolide has a positive effect on pathogen resistance, a recent study has shown that brassinolide acts as a competitor of systemin signaling in tobacco (Scheer et al., 2003).

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