MOLECULAR MECHANISM UNDERLYING THE WOUND-INDUCED EXPRESSION OF A HORSERADISH PEROXIDASE GENE

（西洋ワサビのペルオキシダーゼ遺伝子における傷害誘導発現の分子機構）

Pulla Kaothien

奈良先端科学技術大学院大学
バイオサイエンス研究科　植物代謝調節学講座
（新名　惇彦　教授）

平成 13 年 2 月 2 日提出

CONTENTS

INTRODUCTION

Being sessile, plants are forced by nature to encounter several kinds of biological and environmental stresses. Wounding caused by animals and some mechanical stresses is obviously one of the major stresses for plants. When plants are wounded, the cells adjacent to the wound site are exposed to the environment and are very prone to infection by bacteria, fungi, and viruses. In order to heal the wound and prevent further damage, plants arm themselves with various defense-response mechanisms. These mechanisms involve the actions of several kinds of proteins that function in the wound healing and/or defense processes. The accumulations of these proteins are usually preceded by the accumulation of the corresponding mRNAs indicating that the expression of the genes encoding these proteins is regulated at transcription step.

The DNA sequences determining the timing and the level of transcription are generically referred to as "promoters". While the minimal (basal) level of transcription can be supported by a basal transcription machinery assembled on the core promoter elements, the enhanced transcription activity of a gene in responses to environmental factors — in this case, wound signals — requires the specific interaction(s) between regulatory transcription factor (*trans* factor) and its target recognition site (*cis* element) on the promoter region. Therefore, the interaction between the wound-responsive *trans* factor and its targeted *cis* element is one of the most important steps that control the "on/off switch" of the defense-response mechanisms.

In attempts to understand the mechanisms used by plants to activate the transcription of their defense genes, promoters of several wound-inducible genes were studied. Although some wound-responsive *cis* elements were isolated (see Chapter I: Literature Review), the functional

relationship between these elements and their specific *trans* factor *in planta* remains ambiguous.

With their abilities to generate singlet oxygen, remove hydrogen peroxide, and regulate biosynthesis/degradation of lignin, plant peroxidases (EC. 1.11.1.7) are among the proteins that play important roles in the wound-healing and defense processes. Although the expression of peroxidase genes from several plant species are known to be induced by wounding, little is known about the molecular mechanisms underlying the wound-induced expression of plant peroxidase genes. To gain insight into this matter, we set our aim to isolate and characterize the wound-responsive *cis* element and *trans* factor of a *prxC2* gene, which encodes a wound-inducible horseradish peroxidase.

CHAPTER I

LITERATURE REVIEW

SIGNAL TRANSDUCTION PATHWAY LEADING TO EXPRESSION OF WOUND-RESPONSIVE GENES

In response to mechanical wounding, plants activate a set of wound-healing and defense proteins by a complex signaling network triggered by wound signals generated locally at wound site or systemically at distance. Currently, some components involved in the transduction of wound signals in this network have been identified in tomato, potato, tobacco, and *Arabidopsis*. Evidences accumulated until now suggest that the signal-transduction pathways leading to the wound-induced expression of defense genes in these plants are different.

In solanaceae plants (tomato and potato), oligosaccharides, systemin, jasmonic acid (JA), abscisic acid (ABA), ethylene, and electric signals are the components of the complex signal transduction pathways leading to the expression of genes encoding proteinase inhibitors (PIs) and other systemic wound response proteins (SWRPs) (Bishop *et al.*, 1984; Doares *et al.*, 1995; O'Donnell *et al.*, 1996; Pearce *et al.*, 1991; Pena-Corte *et al.*, 1995; Weiss and Bevan, 1991; Wildon *et al.*, 1992). Both oligosaccharides (local signal) and systemin (systemic signal) produced in response to wounding activate the octadecanoid pathway leading to the synthesis of JA, which in turn activate the production/activation of the PIs and the SWRPs in the plants (Bergey *et al.*, 1996; Doares *et al.*, 1995; Howe *et al.*, 1996). In addition, ethylene amplify the effect of JA leading to the maximum level of wound responses (O'Donnell *et al.*, 1996). These evidences suggest that tomato and

tomato use a unified wound-signaling pathway, which operate through JA to activate the expression of the same set of genes in both local and systemic tissues. However, O'Donell *et al.* (O'Donnell *et al.*, 1998) argued that JA-independent wound-signaling pathway might also exist in tomato becuse this unified wound-signaling pathway model fails to explain the fact that different set of proteins are accumulated in local and systemic tissues in tomato plants (Dalkin and Bowles, 1989; Lightner *et al.*, 1993). Based on recent evidences and a review by Ryan (Ryan, 2000), a model representing the wound-induced expression of the *pin2* gene is shown in Figure 1-1. Similar to potato and tomato, the pathway leading to the expression of defense genes in tobacco — another member of the solanaceae subfamily — is also regulated by ethylene (Niki *et al.*, 1998) and JA (Seo *et al.*, 1995). However, there is no evidence for the existence of systemin or other peptide hormone in tobacco.

In contrast to the solanaceous plants, Rojo *et al.* (1999) found that JA and oligosaccharides in *Arabidopsis thaliana* antagonistically trigger different wound-signaling pathways, which in turn activate different sets of genes in local and systemic tissues. In local tissues, they found that oligosaccharides induce the expression of a specific set of wound-responsive genes while repress the expression of several JA responsive genes that are activated in the systemic tissues (Figure 1-2). They also reported that this oligosaccharide-mediated repression of the JA-dependent signaling pathway is exerted through the production and the perception of ethylene in local tissues.

MECHANISMS UNDERLYING TRANSCRIPTIONAL REGULATION OF WOUND-RESPONSIVE GENES

Stress-induced metabolic changes are often caused by altered patterns of gene expression and this is also true in the case of wound-

induced metabolic changes in plants. The accumulations of majority of the proteins that function in the wound-healing and/or defense processes are preceded by the accumulations of the corresponding mRNAs. This phenomenon indicates that the expressions of the genes encoding these wound-responsive proteins are regulated at transcription step. Examples of the genes that are transcriptionally activated under wound stress are tomato *tap1/tap2* encoding peroxidase (Mohan *et al.*, 1993), *Arabidopsis PAL1* encoding phenylalanine ammonia-lyase (Ohl *et al.*, 1990), tobacco *gPAL1* encoding phenylalanine ammonia-lyase (Fukasawa-Akada *et al.*, 1996), tomato *AFR* encoding ascorbate free-radical reductase (Grantz *et al.*, 1995), maize *COMT* encoding caffeic acid O-methyltransferase (Capellades *et al.*, 1996), potato *pinIIK* encoding proteinase inhibitor (Palm *et al.*, 1990), and tomato *pin2* encoding proteinase inhibitor-2 (Stankovic and Davies, 1996). Since transcriptional regulation of gene expression involves interaction between specific nuclear *trans* factor and its target *cis* element on the corresponding promoter, promoters of several wound-responsive genes were analyzed.

As early as in 1989, Siebertz *et al.* (1989) have studied a promoter of a potato wound-inducible gene, *wun1*, and reported that *cis* element required for the wound-induced expression of *wun1* might located at the position between -571 to -1022 bp from transcriptional start site; however, no detail study has been done in order to specify tlocation or sequence of the *cis* element. In 1990, a study of a promoter of *Arabidopsis thaliana PAL1* gene has located the wound-responsive *cis* element of this promoter to the position between –290 to –540 bp from the transciption start site (Ohl *et al.*, 1990). Again, the precise position and the sequence of the *cis* element were not described. In 1994, Clarke *et al.* reported that promoter of the *win6* gene, which encodes a poplar chitinase, contains regulatory elements that are responsive to wound signals in heterologous host since the expression of *win6* promoter::*β-glucuronidase (GUS)* chimeric gene was increased in

response to wounding locally and systemically in transgenic tobacco plants (Clarke *et al.*, 1994).

The hunt for *trans* factor working as an "on switch" for the expression of wound-inducible genes was started in 1990 when Palm *et al.* reported the interaction between nuclear protein(s) from wounded tomato leaves and a 10-bp sequence within the promoter region of the wound-inducible potato proteinase inhibitor IIK (*pin-IIK*) gene and proposed that this binding could regulate the wound responsiveness of the *pin-IIK* promoter. The only report on the finding of a wound-responsive *trans* factor between then (1990) and now (2001) is the report on the finding that interaction between a *trans* factor in WRKY family (reviewed by Eulgem *et al.*, 2000) and its targeted W-box element regulates the expression of a barley *HVLox1* gene in response to JA (Rouster *et al.*, 1997).

Interestingly, the expression of several transcription factors are regulated by wound stress, for example, the expression of *WIZZ*, which encodes a tobacco transcription factor in the WRKY family (Hara *et al.*, 2000), the expression of ethylene-responsive transcription factors in *Arabidopsis* (Fujimoto *et al.*, 2000), the expression of *Pti4*, which encodes a tomato transcription factor that bind to ethylene-responsive element (Thara *et al.*, 1999), and the expression of *ZPT2-2*, which encodes a petunia zinc-finger transcription factor (van Der Krol *et al.*, 1999). However, there is no direct evidence for the function of these transcription factors in the wound-induced expression of their target genes. In fact, it is not known whether the target genes of some of these transcription factors are transcriptionally activated by wound stress. Isolation of the targeted *cis* elements of these *trans* factors and characterization of the wound-signaling pathways leading to their expression will give us a better view of the signal-transduction pathway leading to the wound-induced expression of defense genes in plants.

WOUND-INDUCED EXPRESSION OF PLANT PEROXIDASES

Plant peroxidases (EC. 1.11.1.7) are among the proteins working in the wound-healing and/or defense processes due to their abilities to generate singlet oxygen (Kanofsky, 1988), remove hydrogen peroxide (Baker *et al.*, 2000), and regulate lignin biosynthesis/degradation (Mäder and Füssl, 1982; Quiroga *et al.*, 2000). Although the expression of peroxidase genes from several plant species are known to be induced by wounding (Curtis *et al.*, 1997; Hiraga *et al.*, 2000; Huh *et al.*, 1997; Ishige *et al.*, 1993; Ito *et al.*, 1994; Mohan *et al.*, 1993; Roberts and Kolattukudy, 1989), little is know about the molecular mechanism underlying the wound-induced expression of plant peroxidase genes.

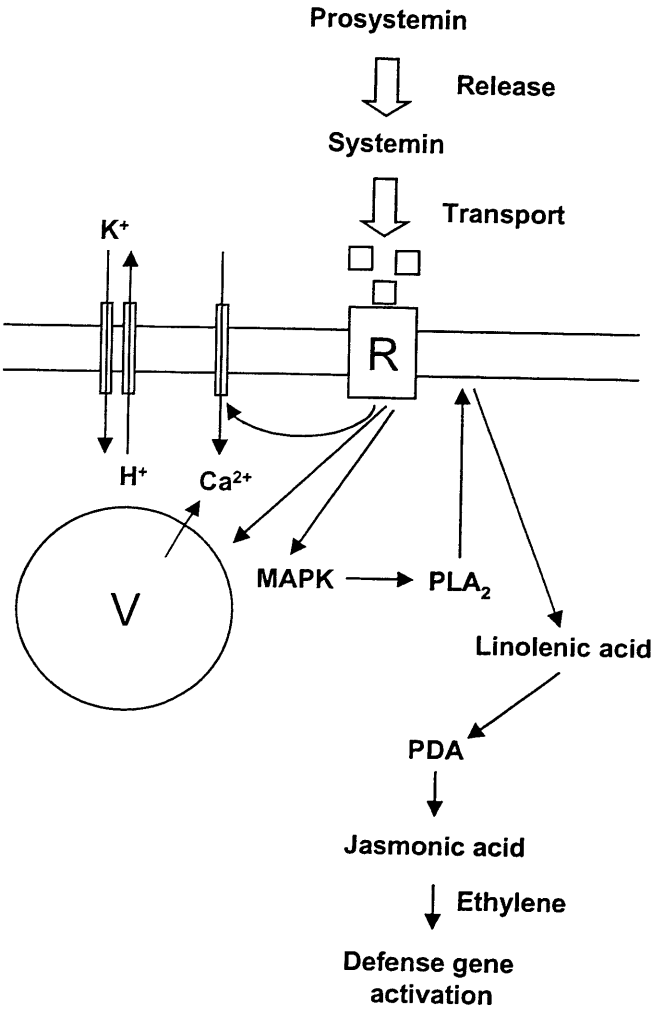


Figure 1-1. A current model for systemin signaling pathway leading to wound-induced expression of defense genes in tomato plants. The interaction between systemin (white cubes) with its membrane receptor (R) initiates intracellular events that activate a PLA_2 phospholipase to release linolenic acid from cell membrane. Linolenic acid is converted to jasmonic acid that work together with ethylene to regulate the expression of defense genes (Ryan, 2000).

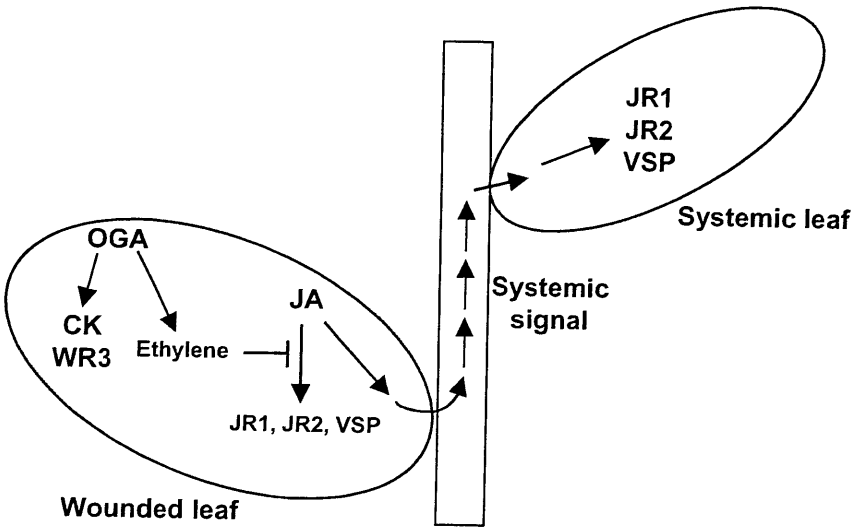


Figure 1-2. A model for wound signal transduction in *Arabidopsis thaliana*. The putative roles of endogenous oligosaccharides (OGA), jasmonic acid (JA), and ethylene in the activation of defense gene expression in wounded leaf and systemic leaf of *Arabidopsis thaliana* are shown. Arrows indicate positive regulation and blunt-ended line indicates negative regulation. In this model, expressions of choline kinase (*CK*) and *WR3* are activated in wounded leaf. Expressions of *JR1*, *JR2*, and vegetative storage protein (*VSP*) are suppressed in wounded leaf but activated in systemic leaf (Rojo *et al.* 1999).

CHAPTER II

ISOLATION OF A WOUND-RESPONSIVE *CIS* ELEMENT OF *prxC2* PROMOTER

INTRODUCTION

Previously, we isolated a peroxidase gene named *prxC2* (Fujiyama *et al.*, 1990) from horseradish and found that, like most of the plant peroxidase genes, the transcription of the *prxC2* gene is activated by wound treatment (Figure 2-1) (Kawaoka *et al.*, 1994a). Moreover, we found that promoter of the *prxC2* gene retains its wound-responsive activity even in tobacco plants implying the existence of analogous regulatory mechanisms in the 2 plant species (Figure 2-2). To understand molecular mechanism underlying the wound-induced expression of plant peroxidase genes, we studied the molecular mechanisms underlying the wound responsiveness of the *prxC2* promoter in detail.

As a result from a 5′-deletion analysis, a positive *cis* element of the *prxC2* promoter was previously found at a position between -296 and -283 bp from the initiation site of translation (Kawaoka *et al.*, 1994b). This *cis* element contains a CACGTG sequence, which is a core motif of G box, a common *cis* element involves in responses to environmental stresses such as wounding and UV light (Staiger *et al.*, 1989). The existence of a G-box binding protein in nuclear protein extracted from wounded tobacco leaf was also confirmed by gel shift assay; and consequently, a cDNA encoding a G-box binding protein, TFHP-1 (*trans*-acting Factor of Horseradish Peroxidase 1), was isolated (Kawaoka *et al.*, 1994b). The functions of TFHP-1 and G

box in the wound responsiveness of the *prxC2* promoter, however, remain unclear. In this chapter, results from further internal-deletion analysis of the *prxC2* promoter in order to identify a wound-responsive *cis* element of the *prxC2* promoter are reported.

MATERIALS AND METHODS

Plasmid constructions

Construction of chimeric genes was performed following the method of Maniatis *et al.* (Maniatis *et al.*, 1982). A 1000-bp fragment of the *prxC2* promoter (Figure 2-3) was inserted in front of the *GUS* reporter gene in pBI101 plasmid and the resulting plasmid (-1000 *prxC2*::*GUS* in pBI101) was used as a template for PCR-assisted constructions of the constructs used in the transformations of tobacco BY2 protoplasts and tobacco plants. For constructs used in transient expression assay in protoplasts of tobacco BY2 cells (pG123::GUS, pG12::GUS, pG1::GUS, pG::GUS, pG13::GUS, pG3::GUS, and p3::GUS), the oligonucleotide primers shown in Figure 2-4 were used to generate various PCR fragments, which were reconstituted into the internal-deletion constructs following the steps shown in Figure 2-5. The p::GUS construct was obtained by sequential digestion of the *prxC2* promoter with exonuclease III and mung bean nuclease (Kawaoka, 1992). For the constructs used to transform tobacco plants (F, ΔG, ΔPOP, ΔPAL, and rPAL; Figure 2-11), the -1000 *prxC2*::*GUS* in pBI101 was use as a template for the oligonucleotide primers shown in Figure 2-6 to generate various PCR fragments, which were reconstituted to generate internal-deletion constructs following the steps shown in Figure 2-7. The mPAL construct was generated by recombinant PCR technique shown in Figure 2-8.

Plant materials

Tobacco suspension culture cells, *Nicotiana tobacum* L. cv Bright Yellow 2 (BY2), were grown in a modified liquid Linmaier and Skoog (LS) medium pH 5.8 (Nagata *et al.*, 1981). The cells were cultivated in this

medium at 27°C in the dark orbital shaker (BR-3000, Taitec, saitama, Japan) at 130 rpm. To maintain the cells, 2 ml of a 7-day-old culture was transferred to a 95-ml fresh LS medium.

Tobacco plants, *Nicotiana tabacum* L. cv Petit Havana SR-1, were grown in 25°C greenhouse with 16-h light and 8-h dark cycle.

Transient expression assay in tobacco BY2 protoplasts

A suspension culture of *Nicotiana tobacum* L. cv BY2 cells was grown at 28°C for 4 d in LS medium containing 0.2 mg/l of 2,4-dichlorophenoxy acetic acid, 3% sucrose, 100 mg/l of *myo*-inositol, and 1 mg/l of thiamine-HCl, pH 5.8 (Nagata, 1987). Protoplasts were prepared by incubating the BY2 cells with 0.1% Pectolyase Y23 (Seishin, Tokyo, Japan) and 1% Cellulase Onozuka RS (Yakult, Tokyo, Japan) in a 0.4 M mannitol solution (pH 5.5) for 2 h at 30°C. The 3×10^6 protoplasts were mixed with 20 μg of plasmid DNA in 1 ml of electroporation buffer (5 mM MES, 70 mM KCl, 0.3 M mannitol, pH 5.8). The electroporation was carried out with two rectangular pulses of 200 μs at 300 V with a 0.1-s interval across a 0.4 cm path, using the Electro Gene Transfer System (Shimazu, Tokyo, Japan). The resulting protoplasts were incubated in the LS medium containing 0.4 M mannitol for 24 h at 25°C in darkness. The protoplasts were harvested and the GUS activity in crude protein extracted from the protoplasts was measured.

Plant transformation

Tobacco plants were transformed with *Agrobacterium tumefaciens* EHA105 using leaf disk transformation method (Rogers *et al.*, 1986). Tobacco plants transformed with the constructs in pBI121 plasmids (Clontech, Palo Alto, CA, USA) were selected in MS medium (Murashige

and Skoog, 1962) containing 100 µg/ml kanamycin. The present of the *GUS* transgene in all transgenic plants was confirmed by genomic PCR using primers that amplify the *GUS* coding region.

RNA extraction: ATA method

Tobacco leaves were ground in liquid nitrogen and extracted with 1 ml of ATA extraction buffer (50 mM Tris-HCL pH 8.0, 300 mM NaCl, 5 mM EDTA, 2% w/v SDS, 2% w/v Na-triisopropylnaphthalene sulfonate, 2 mM aurintricarboxylic acid (ATA), and 12.6 mM 2-mercaptoethnol). After the addition of 140 µl of 3 M KCL, the mixture was incubated on ice for 15 min, and was centrifuged at 9,000*g* for 5 min at 4°C. Total RNA was precipitated from the supernatant by addition of 440 µl of 10 M LiCl, incubation on ice for 4 h, and centrifugation at 12,000*g* for 20 min at 4°C. The pellet of total RNAs was resuspended in 400 µl of sterile distilled water and was extracted twice with 1×volume of phenol:chloroform:isoamyl alcohol (25:24:1). 0.1×volume of 5 M NaCl and 2.5×volume of absolute ethanol were added to the aqueous phase and the RNA was precipitated by centrifugation at 12,000*g* for 20 min at 4°C. The pellet was washed with 70% v/v ethanol, vacuum dried, and resuspended in 30 µl of sterile distilled water.

Caution: ATA is a potential carcinogen and RNA isolated by ATA method cannot be used in the experiments that required enzyme (including Rnase) reaction.

Northern blot analysis

Total RNA (20 µg) isolated by ATA method was fractionated on a 1.5% agarose-16% formaldehyde gel and blotted onto a nylon membrane (Hybond N, Amersham Pharmacia Biotech, Bucks, UK) using 20×SSC

buffer (3M NaCl, 0.3 M $C_6H_5Na_3O_7 \cdot 2H_2O$, pH 7.0) as transfer solution. After UV cross-linking, the membrane was hybridized overnight at 43°C with ^{32}P-labeled probe in Church hybridization buffer (7% w/v SDS, 0.5 M $NaPO_4$, 0.25 M NaCl, 1 mM EDTA; Church and Gilbert, 1984) containing 1% w/v bovine serum albumin (BSA) and 50% v/v formamide. The membrane was washed with 1×SSC-0.1%SDS buffer at 43°C and exposed overnight at -80°C to X-ray film for autoradiograph.

Assay of GUS activity

Crude proteins were extracted from tobacco BY2 protoplasts or tobacco leaves using GUS lysis buffer (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA and 10 mM 2-mercaptoethanol). In the case of tobacco BY2 protoplasts, the protoplasts were harvested from the medium by centrifugation at 3,000 rpm for 5 min at 4°C. The protoplast pellet was dissolved in GUS lysis buffer and sonicated on ice. In the case of tobacco leaves, the leaves were ground to fine powder in liquid nitrogen and the crude protein was extracted with GUS lysis buffer. After centrifugation at 10,000 rpm for 10 min at 4°C, GUS activity in the supernatant (crude soluble-protein extract) was measured by fluorescence assay method using 4-methyl-umbelliferyl glucuronide as a substrate (Jefferson *et al.*, 1987). The GUS activity was described as nmol of methyl-umbelliferone per minute per mg protein. Protein concentration was quantified by Bradford method (Bradford, 1976).

Nuclear protein extraction

Nuclear proteins were extracted from healthy and wounded tobacco leaves by Staiger method (Staiger *et al.*, 1989). Tobacco leaves were homogenized in Ni buffer (25 mM MES, 0.25 M sucrose, 5 mM EDTA, 10

mM KCl, 0.5 mM DTT, 0.5 mM PMSF) containing 0.3% v/v NP-40 and 0.5 mM spermidine. The mixture was re-homogenized with polytron homoginizer and filtrated through MIRA cloth. The filtrate was centrifuged at 5,000*g* for 10 min at 4°C and the pellet was washed once with Ni buffer containing 0.3% v/v NP-40 and twice with Ni buffer. The pellet was dissolved in 3×volume of NE buffer (25 mM HEPES, 5% v/v glycerol, 0.1 mM EDTA, 420 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 μM leupeptin, 1 μM pepstatin) and the resulting mixture was sonicated and incubated on ice for 45 min. After the incubation, the mixture was centrifuge at 42,500 rpm for 45 min at 4°C and the supernatant was dialyzed at 4°C overnight in dialysis buffer (25 mM HEPES pH 7.8, 20% v/v glycerol, 0.1 mM EDTA, 50 mM KCl, 14 mM 2-mercaptoethanol). Protein concentration was quantified by Bradford method (Bradford, 1976). The nuclear protein extract was stocked at -20°C.

Wound treatment

For nuclear protein extraction, leaves of greenhouse-grown tobacco plants were cut by scissors and soaked in 50 mM sodium phosphate buffer (pH 7.0) for 4 h at 25°C under continuous light. For northern blot analysis and assay of GUS activity, leaves of greenhouse-grown tobacco plants were cut by surgical blade into small pieces (~ 3 mm^2) and floated on 50 mM sodium phosphate buffer (pH 7.0) at 25°C under continuous light for 12 or 30 h.

Gel shift assay

The DNA-protein binding reaction was carried out in a 20-μl reaction mixture containing 2 ng of radio-labeled DNA probe, 2 μg of nuclear protein extract, and 1 μg of poly (dI-dC) (Pharmacia Biotech Bucks,

UK) in binding buffer (10 mM Tris-HCl pH 8.0, 80 mM HCl, 0.016% NP40, 7.5% glycerol, and 1 mM DTT). The reaction mixtures were pre-incubated at 23°C for 15 min, mixed with radio-labeled DNA probe, and incubated at 23°C for 30 min. Each sample was applied without dye onto a 5% native polyacrylamide gel in 0.5×TBE buffer and the gel was subjected to electrophoresis for 70 min at 15 V/cm in 0.5×TBE buffer. Following the electrophoresis, the gel was dried and exposed overnight to X-ray film at -80°C for autoradiograph. For the competitive binding assay, 50 ng or 100 ng of non-labeled probe was added to the reaction mixture at the pre-incubation step.

RESULTS

Cis element containing a sequence similar to PAL-box and Box-P consensus sequences is required for activity of *prxC2* promoter in tobacco protoplasts

In search of a novel *cis* element of the *prxC2* promoter, further functional analysis of the promoter was performed. Previously, we found that a *cis* element containing G-box consensus sequence is necessary for the activity of *prxC2* promoter irrespective of wound stress (Kawaoka *et al.*, 1994b); therefore, here, a series of promoter deletion was made between the G box and the CAAT box (Figure 2-9). Internal deletion series of the *prxC2* promoter fused with the *GUS* reporter gene were introduced into protoplasts of tobacco BY2 cells by electroporation and the GUS activity of crude protein extracted from the protoplasts was measured after 24 h of incubation. Considering the stress imposed to the BY2 cells by the protoplast preparation and the electroporation processes, we assumed that the GUS activity detected in this experiment represents the level of *GUS* expression driven by the *prxC2* promoter under wound condition. The relative values of the GUS activity obtained from each construct are summarized in Figure 2-9. As expected, high level of GUS activity was obtained in protoplasts transformed with the *GUS* reporter gene under the control of the intact *prxC2* promoter (pG123::GUS). We found that the deletion of region 3 alone (pG12::GUS) gave a significant decrease in the GUS activity comparable to that detected in the triple deletion of regions 1, 2, and 3 (pG::GUS). Moreover, the construct with a double deletion of regions 1 and 2, which resulted in the presence of region 3 with the G-box (pG3::GUS), gave high GUS activity comparable to that of the positive control (pG123::GUS) construct. Therefore, we conclude that a new positive *cis*

element of the *prxC2* promoter, which functions in combination with the previously found G box, locates within region 3 at a position between -177 and -135 bp from the translation start site. The sequence of this *cis* element is shown in Figure 2-10(A). This sequence shares some similarity with the consensus sequence of PAL-box and Box-P elements, which are elicitor responsive *cis* elements found in the promoter of a parsley (*Petroselinum crispum*) phenylalanine ammonia-lyase gene (Logemann *et al.*, 1995; Lois *et al.*, 1989) (Figure 2-10(B)). We named this *cis*-element POP for PAL-box related *cis* element of *prxC2* promoter.

PAL-box related *cis* element of *prxC2* promoter (POP) functions as a wound-responsive *cis* element *in planta*

In order to investigate the functional relationship between the POP element and the wound-induced expression of the *prxC2* gene, we examined whether deletion of the POP element from the *prxC2* promoter could hinder its wound-responsive promoter activity. Six kinds of promoter constructs shown in Figure 2-11 were used to direct the expression of the *GUS* reporter gene in tobacco plants and the transgenic tobacco plants were named after the construct used to transform them – in F construct, the *GUS* reporter gene was put under the control of an intact *prxC2* promoter. Northern blot analysis was used to determine the level of *GUS* expression in healthy and wounded leaves of the transgenic plants. As shown in Figure 2-12, high level of wound-induced expression of the *GUS* gene was detected in F plants, but not in ΔG, ΔPOP, ΔPAL, mPAL, and rPAL plants. In consistent with the expression level, high level of wound-induced GUS activity was detected only in the crude proteins extracted from wounded leaves of the F plants (Figure 2-13). These results indicate that the POP element functions as a wound-responsive *cis* element of the *prxC2* promoter *in planta*.

Nuclear protein(s) from tobacco leaves specifically interacts with POP element

To confirm the existence of nuclear *trans* factor binding to the POP element, we performed gel shift assay to investigate the interaction between nuclear proteins extracted from healthy or wounded tobacco leaves and the POP element (Figure 2-14). In our assay, in order to confirm the specificity of DNA binding activity, non-labeled probe P (POP element) and non-labeled probe mP (POP element with a 2-base substitution in the PAL-box region) were also used as competitors. As shown in Figure 2-14, we found that nuclear proteins from both healthy and wounded tobacco leaves could bind to the POP element in this assay with different binding pattern — the nuclear proteins extracted from wounded tobacco leaves gave higher intensity of the slow-moving shift band, which represents a higher order of complexity of the protein(s)-DNA interaction, while the nuclear protein extracted from healthy tobacco leaves gave higher intensity of the fast-moving shift band, which represents a lower order of complexity of the protein(s)-DNA interaction. However, in both cases, 2 shift bands observed in the absence of the competitors (Figure 2-14, lane 1 and 6) were competed out with an excess amount of non-labeled probe P (Figure 2-14, lane 2, 3, 5, and 6) but not with the non-labeled probe mP (Figure 2-14, lane 4, 5, 9, and 10) suggesting that nuclear protein(s) of both healthy and wounded tobacco leaves could bind to the PAL-box region of the POP element in a sequence specific manner.

DISCUSSION

Using transient expression of *GUS* reporter gene under the control of deletion series of *prxC2* promoter in tobacco protoplasts, a novel positive *cis* element of the *prxC2* promoter was identified. This *cis* element was found at the position between –177 and –134 bp from the translation start site of the *prxC2* transcript and homology search revealed that this *cis* element contains a sequence similar to the consensus sequence of PAL-box and Box-P elements, which are elicitor responsive *cis* elements found in the promoter region of a parsley (*Petroselinum crispum*) phenylalanine ammonia-lyase gene (Lois *et al.*, 1989; Logemann *et al.*, 1995). Therefore, we named the newly isolated *cis* element "PAL-box related *cis* element of *prxC2* promoter (POP)". By deletion analysis of the *prxC2* promoter in stably-transformed tobacco plants, we have shown that deletion of the POP element from the *prxC2* promoter abolished the wound-responsive activity of the *prxC2* promoter *in planta*. The same result was obtained with the deletion of the G box and the deletion/mutation of the PAL-box region of the POP element. These results indicate that the G box and the POP element are necessary for the *prxC2* promoter to drive the expression of the *GUS* reporter gene in response to wound stress; however, neither the G-box nor the POP element alone is sufficient to confer the wound-induced expression of the *GUS* reporter gene. Therefore, we conclude that the G box and the POP element function in concert to control wound responsiveness of the *prxC2* promoter *in planta*. This conclusion has not come as a surprise since evidence for the interactive activity between the G box and other *cis* elements exist: in a functional model for the tissue-specific expression of a *PAL2* gene (Hatton *et al.*, 1995), the G box function in concert with different AC elements of the *PAL2* promoter to direct a complex pattern of tissue-

specific expression of the *PAL2* gene.

The existence of nuclear protein(s) that bind in a sequence-specific manner to the PAL-box region of the POP element was also confirmed by gel shift assay using the radio-labeled POP element and nuclear proteins extracted from tobacco leaves. By using gel shift assay, Sanchez-Serrano *et al.* (1990) reported that nuclear proteins extracted from potato leaves could specifically bind to a DNA sequence on the promoter of a potato wound-inducible proteinase inhibitor II gene and that mechanical wounding of the leaves gave no effect on the amount of specific protein-DNA complexes formed. However, result from our gel shift assay shows that the nuclear proteins extracted from wounded tobacco leaves gave higher intensity of the slow-moving shift band, which represents a higher order of complexity of the protein(s)-DNA interaction, while the nuclear protein extracted from healthy tobacco leaves gave higher intensity of the fast-moving shift band, which represents a lower order of complexity of the protein(s)-DNA interaction. This result implies that several proteins might be incorporated to the protein-POP element complex in response to wound stress. We suggest that some of the nuclear proteins in this complex constitutively bind to the POP element because they are constitutively produced/activated irrespective of wound stress, while some of them bind to the POP element only after plants are wounded because they are produced/activated only after plants sense wound signals.

In 1992, Hildmann *et.al.* reported that jasmonic acid strongly induces the mRNA accumulation of all of the isolated ABA-responsive/wound-induced genes in potato leaves ,and that jasmonic acid could bypass the initial recognition events requiring ABA and triggers the induction of the expression of the ABA-responsive/wound-induced genes in ABA-deficient plants. They also suggested the role of jasmonic acid as an intermediate in the signaling pathway that leads from ABA accumulation in response to wounding to the transcriptional activation of the wound-

inducible genes. Methyl jasmonate, a methyl ester of jasmonic acid, however, gave no effect on the wound-induced expression of the *Arabidopsis* glutathione *S*-transferase gene (McConn *et al.*, 1997). Therefore, evidences existing thus far suggest that wound-induced expression of defense genes in plants could be regulated through the methyl jasmonate-dependent or the methyl jasmonate-independent pathways. Interestingly, as shown in Figure 2-15, a part of the POP element including the PAL-box region itself contains a sequence similar to that of methyl jasmonate responsive element (MeJA-RE) of tomato lipoxygenase (*LOX*) gene (Beaudoin and Rothstein, 1997). Moreover, the expression of legume peroxidase isogenes, *Shpx6a* and *Shpx6b*, were induced at transcription level by both wound stress and methyl jasmonate treatment (Curtis *et al.*, 1997). Therefore, we are interested in finding out whether the expression of *prxC2* gene is also induced by methyl jasmonate and the experiment to test the effect of methyl jasmonate on the wound responsiveness of the *prxC2* promoter is in progress. For now, the pathway to which the wound-induced expression of *prxC2* gene utilizes remains unknown; however, it is obvious that isolation of the protein(s) binding to the POP element will be an important step to help us understand the mechanisms that control the wound-induced expression of the *prxC2* gene.

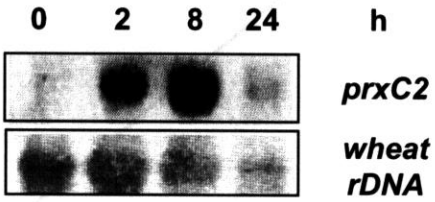


Figure 2-1. Wound-induced expression of *prxC2*. Total RNAs derived from healthy (0 h) and wounded (2, 8, and 24 h) leaves of horseradish were probed with radio-labeled exon 4 of *prxC2* gene. Same blot was reprobed with radio-labeled wheat ribosomal DNA for loading control (Kawaoka *et al*. 1994).

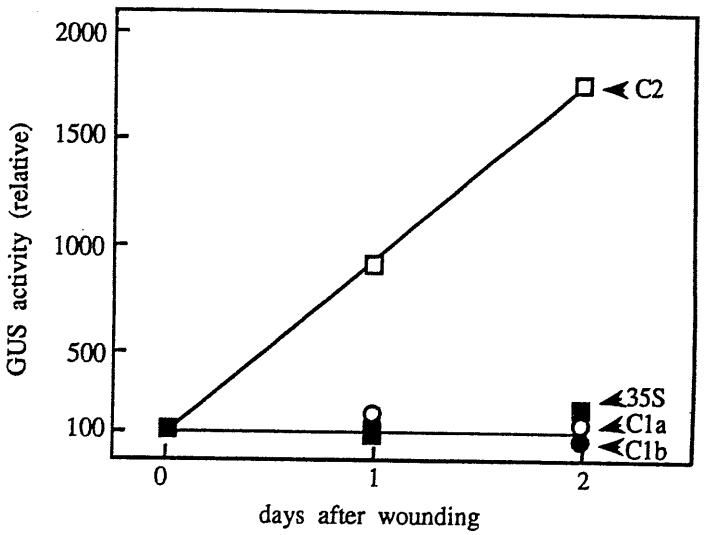


Figure 2-2. Induction of GUS activity by wounding in transgenic tobacco plants harboring *GUS* reporter gene under the control of *prxC2* promoter (C2), 35S promoter (35S), *prxC1a* promoter (C1a), and *prxC1b* promoter (C1b). Data shown here represent the mean value of relative GUS activity of 5 independent transgenic tobacco plants (Kawaoka *et al.* 1994).

ATGCATGATTACATTTAATGCAAATACATGAATTTATCTGCTTATTCAATATTCACTCTT -979

TTCCTTTCTCGACAATAACCATTGAGAAAAATCAATGTGCAATAAGTGCATCAACTTCGT -919

ATGAATATGTAAACAGTAAAATCTCTATAAATTAATATTTTATAAATTATTAAACACTAT -859

ATGGTAATAAAATCTTCCAGATCCGATTCGAACTAGTGTAAAAATTAACAAAATTTAATA -799

AAATGATAAAATTATATTTTTTTTTTGAAATTTCTATTTAAATATATGGTCCTATTTACTA -739

TCATAAGTTAATAATCACACATATATATAAGATAACATATTAATGTATGGTTTTAGTGTT -679

TTTCTAAAAAAATAAATATTATTATAGTTCGTCTCTAATTTTTATTATATCAAATAATTA -619

TTATATTGTGTTCCAAACTAGTGAGTTGCAGTATCGCCTATAACTTCATTTCAAAGTAGA -559

TAACATTTTTTGTAAGTTTCCAAATTATGAATATGCATCATTTTCATTTTTATAATCTTA -499

TAGTTTATGATAGATCATAAATTTGAATTTATAATATATGTGTTTGCAAATTTGCATAAG -439

AAATTCATTAGTACCTTTTATTTATCGTGTAAATATTTTTTTTCTAAATATTAAATATTT -379

AAGTTGAAACTACAAATTTATAAAAATACAAATTGAATTTATAAATGTATTCAAAGTACA -319

TAAAAGAAAAGATTCTATATTATAAACACGTGATATTCTTTAAATTTTAATCCAATTTGC -259

CGAAAGAAAACAGAAACTTTTAAACCACTTTGTACAGAATTATTAAACACATTGTCGTAT -199

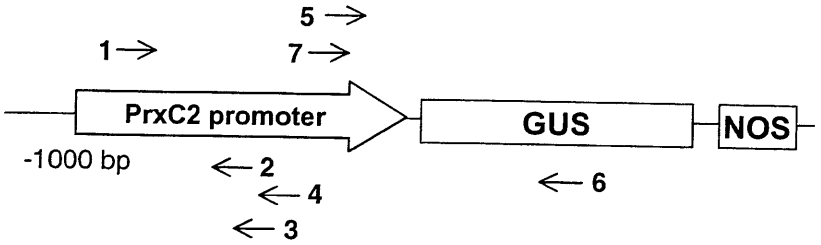
TATCTTCCACATGTAATAAGTCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGT -139

ACAAATATCTCCAATAATTCACAAAAAGTAAAAGCCGCCGAGCTTCCTTTTTTGCTTATA -79

AAAGGATTTGTTAAGACGAAACTTTATTCACTCAACTTCAAACCTAACCAAAGAATTTTA -19

TCTTAGAGAGCAAAGAAAatg 3

Figure 2-3. Nucleotide sequence of *prxC2* promoter. The G box (CACGTG) and the putative CAAT and TATA sequences are underlined. Lower case letters indicate the translation start site of *prxC2* gene.



Primer 1 $^{-563}$TAGATAAGCTTTTTTGTAAGTTTCC^{-539}
*Hin*dIII

Primer 2 $^{-268}$ATTAAAATCTGCAGAATATCACGTG^{-292}
*Pst*I

Primer 3 $^{-218}$AATTCTGCAGAAAGTGGTTTAAAAG^{-242}
*Pst*I

Primer 4 $^{-169}$CAATACCTGCAGTATTACATGTGGA^{-193}
*Pst*I

Primer 5 $^{-142}$GAGTACTGCAGTCTCCAATAATTCA^{-118}
*Pst*I

Primer 6 ^{394}CGGTGATACGTACACTTTTCCCGGC^{369}
*Sna*BI

Primer 7 $^{-186}$GTAATACTGCAGGTATTGGATAAGA^{-162}
*Pst*I

Figure 2-4. PCR primers used in PCR-assisted construction of an internal-deletion series of *prxC2* promoter joined to *GUS* reporter gene for transient expression assay in protoplasts of tobacco BY2 cells. The approximate annealing position of the PCR primers (arrows) on the –1 kb *prxC2* promoter::*GUS* template and the sequence of the primers are shown. The designed restriction enzyme sites are indicated and underlined. Numbers indicate the base position relative to the translation start site (+1) of the *prxC2* gene.

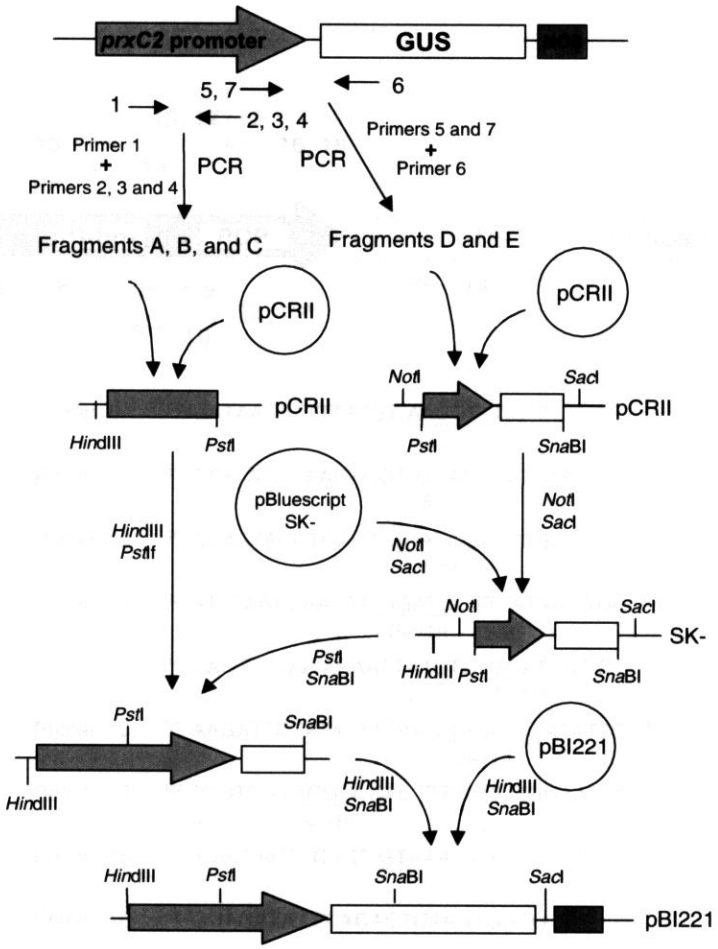
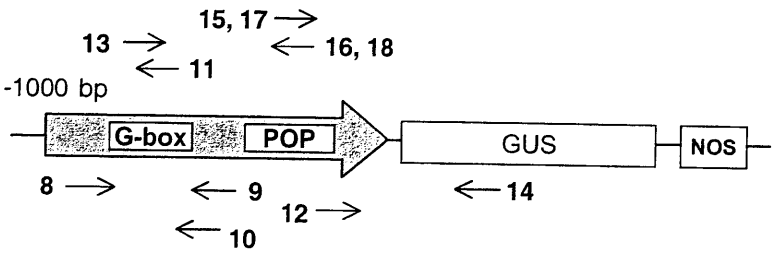


Figure 2-5. PCR-assisted construction of an internal-deletion series of *prxC2* promoter joined to *GUS* reporter gene for transient expression assay in protoplasts of tobacco BY2 cells. Fragment A (G-box), fragment B (G-box + region-1), and fragment C (G-box + region-1 and-2) were obtained by PCR using forward primer 1 in pair with reverse primers 2, 3, and 4, respectively. Fragment D (CAAT + TATA + part of *GUS*) and fragment E (region-3 + CAAT + TATA+ part of *GUS*) were obtained by PCR using forward primers 5 and 7 in pair with reverse primer 6, respectively. Each fragment was cloned into pCRII plasmid. As indicated in the diagram, fragments D and E were transferred to SK^- plasmid and fragments A, B, and C, were inserted in front of fragment D and the reconstituted fragments (AD, BD, and CD) were transferred to pBI221 plasmid to generate pG::GUS, pG1::GUS, and pG12::GUS constructs, respectively. In the same way, fragments A, B, and C, were inserted in front of fragment E and the reconstituted fragments (AE, BE, and CE) were transferred to pBI221 plasmid to generate pG3::GUS, pG13::GUS, and pG123::GUS constructs, respectively. For p3::GUS construct the *Pst*I and *Sna*BI digested fragment E was directly clone into pBI221 plasmid.



Primer 8 $^{-560}$GATAACATTTTTTGT AAGCTT CC^{-538}
*Hin*dIII

Primer 9 $^{-135}$TTGTACTCAAGTGGT GGATCC ATATAC $^{-161}$
*Bam*HI

Primer 10 $^{-168}$CCAATACCTGACT GGATCC ATGTG $^{-191}$
*Bam*HI

Primer 11 $^{-124}$ATTTAAAGAATAT GGATCC TTTATAATATA $^{-153}$
*Bam*HI

Primer 12 $^{-147}$CACTTGAGTACAAATATCTC GGATCC TTC $^{-119}$
*Bam*HI

Primer 13 $^{-310}$AAGATTCTATATTATAAA GGATCC ATATTC $^{-281}$
*Bam*HI

Primer 14 ^{394}CGGTGA TACGTA CACTTTTCCCGGCAAT ^{367}
*Sna*BI

Primer 15 $^{-150}$CACCACTTGAGT **GT**AAATATCTCC $^{-127}$

Primer 16 $^{-127}$GGAGATATTT **AC**ACTCAAGTGGTG $^{-150}$

Primer 17 5'- GATCAATCTGTCGACCTGCAG AAGCTT T-3'
*Hin*dIII

Primer 18 5'- GATCA AAGCTT CTGCAGGTCGACAGATT-3 '
*Hin*dIII

Figure 2-6. PCR primers used in PCR-assisted construction of internal-deletion series of *prxC2* promoter joined to *GUS* reporter gene for functional analysis of the *prxC2* promoter in tobacco plants. The approximate annealing position of the primers (arrows) on the –1 kb *prxC2* promoter::*GUS* template and the sequence of the primers are shown. The designed restriction enzyme sites are indicated and underlined. Numbers indicate the base position relative to the translation start site of the *prxC2* gene. In the sequence of primers 15 and 16, the 2-base substitution in the PAL-box region is shown in bold font with underline. Primers 17 and 18 are random sequences, which were annealed and used to substitute the PAL-box region of the POP element.

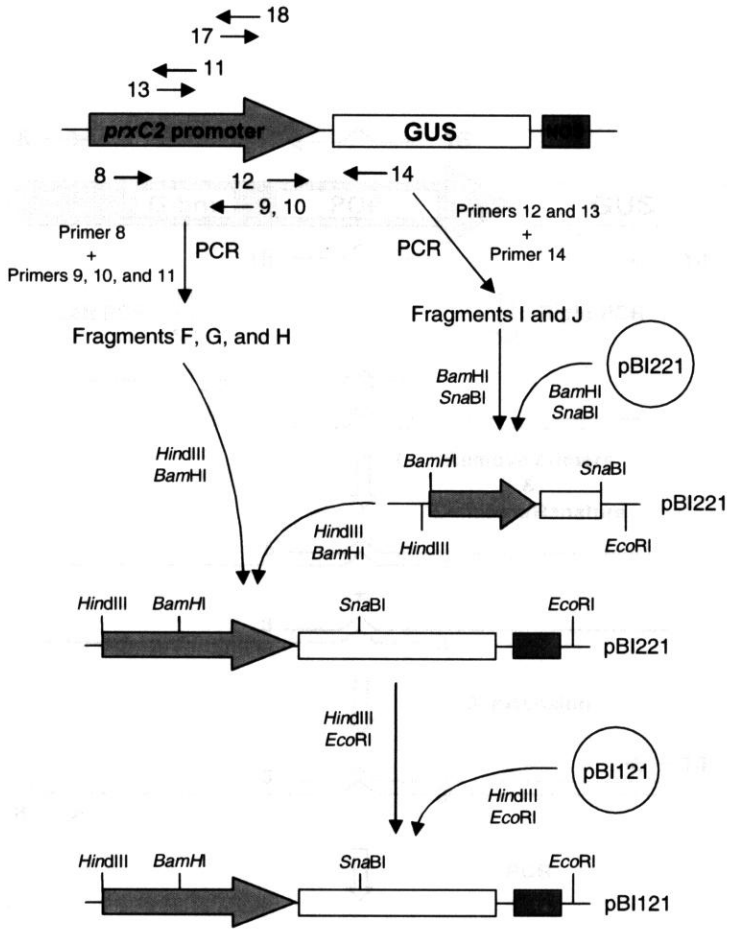


Figure 2-7. PCR-assisted construction of internal-deletion series of *prxC2* promoter joined to *GUS* reporter gene for functional analysis of the *prxC2* promoter in tobacco plants. Fragments F, G, and H, were generated by PCR using forward primer 8 in pair with reverse primers 9, 10, and 11, respectively. Fragment I and J were generated by PCR using forward primers 12 and 13 in pair with reverse primer 14, respectively. Each fragment was cloned into pBI221 plasmid as indicated in the diagram. For ΔG construct, fragment H was cloned in front of fragment J and the reconstituted fragment was cloned into pBI121 plasmid. For ΔPOP construct, fragment G was cloned in front of fragment I and the reconstituted fragment was cloned into pBI121 plasmid. For ΔPAL construct, fragment F was cloned in front of fragment I and the reconstituted fragment was cloned into pBI121 plasmid. For rPAL construct, primers 17 and 18 were annealed and the annealing product was directly inserted into the *Bam*HI-digested ΔPAL construct. For F construct, the PCR fragment amplified by using forward primer 8 and reverse primer 14 was digested with *Hin*dIII and *Sna*BI and inserted into the *Hin*dIII and *Sna*BI digested pBI121 plasmid.

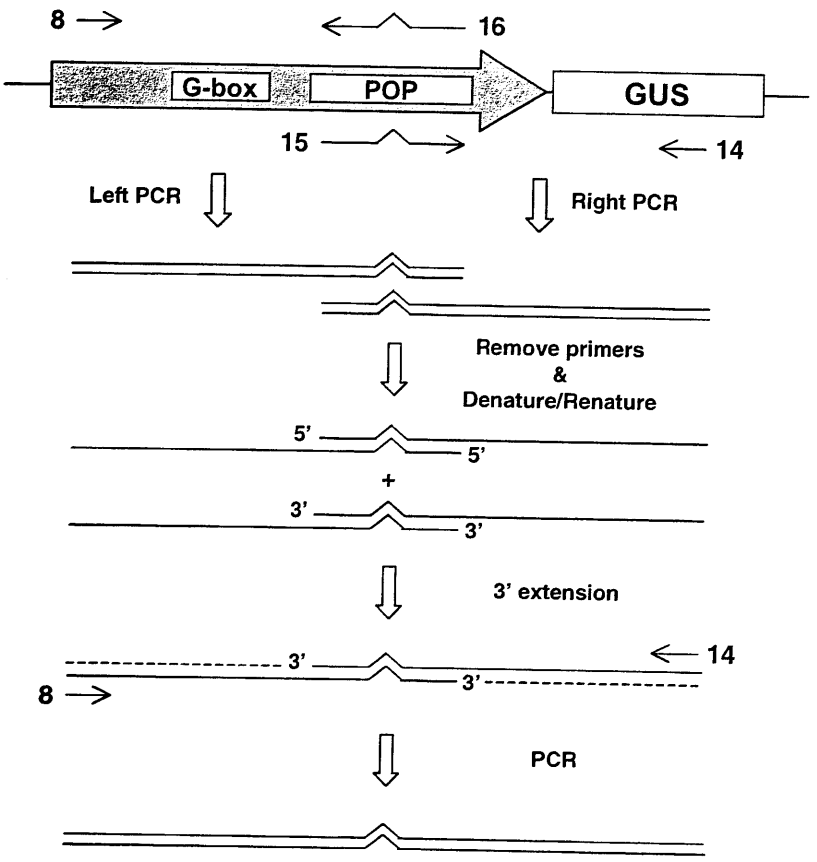


Figure 2-8. Recombinant PCR technique used in construction of mPAL construct. The F construct in pBI121 was used as a template for the "Left PCR" and the "Right PCR" using primer 8 in pair with primer 16, and primer 15 in pair with primer 14, respectively. The products of these PCR reactions, which are partly overlapped (complementary to each others) and contain the same mutation introduced into the PAL-box region of the POP element, were denatured and allowed to re-anneal together. The re-annealed products with recessive 3′ends were extended by DNA polymerase to produce fragments that are the sum of the left and the right PCR products. These fragments were used as template for the second round of PCR using primers 8 and 14. The resulting PCR products were digested with *Hin*dIII and *Sna*BI and was introduced into a *Hin*dIII and *Sna*BI-digested pBI121 plasmid to generate mPAL construct.

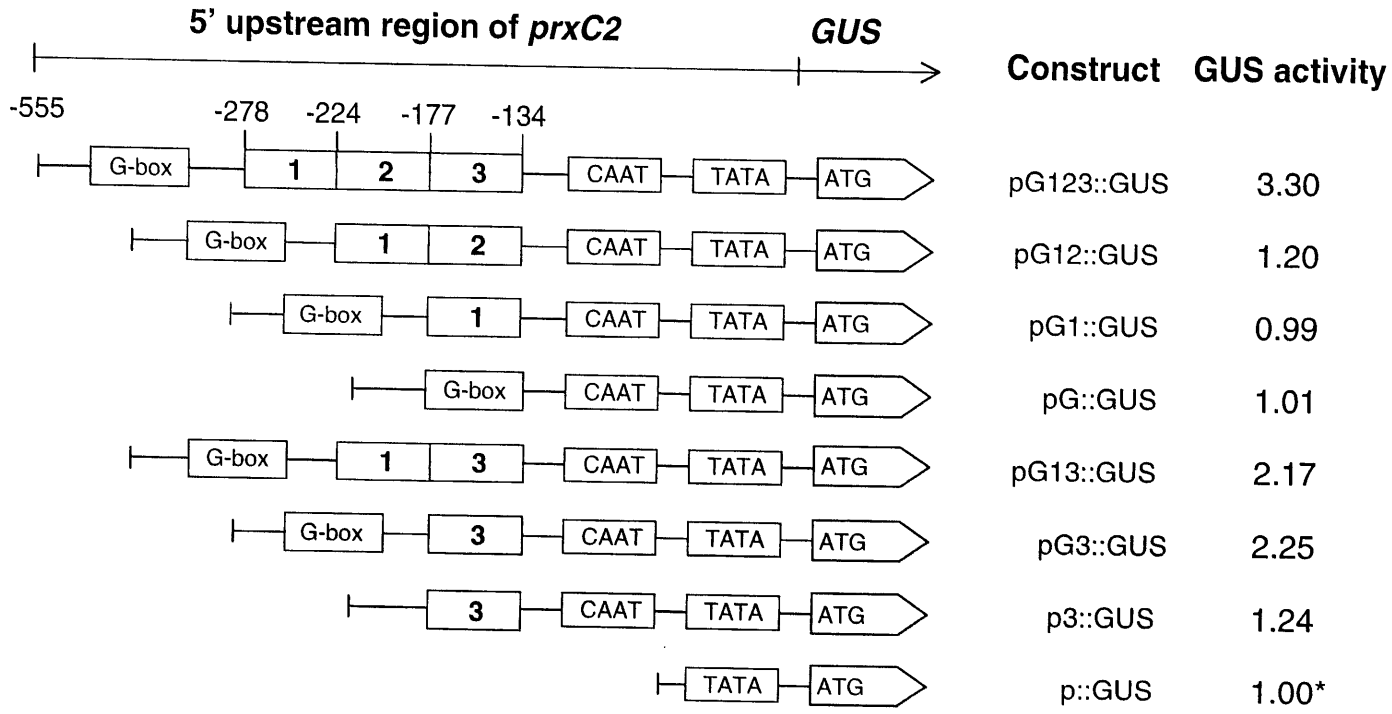


Figure 2-9. Internal deletion analysis of the *prxC2* promoter in protoplasts of tobacco BY2 cells. The *GUS* reporter gene was put under the control of an internal deletion series of the *prxC2* promoter and the resulting *prxC2* promoter::*GUS* chimeric constructs were used to transform BY2 protoplasts. GUS activity in crude protein extracted from each set of the transformed BY2 protoplasts was measured after incubation for 24 h at 25°C. Numbers indicate the position in bases from the translation start site. The GUS activity is presented here as fold difference relative to the GUS activity detected in the BY2 protoplasts transformed with the negative control construct, which contains only the TATA-box (*1.00 = 23.6 pmol/min/mg-protein). Each value represents mean value of the GUS activity obtained from 3 independent experiments.

(A)

-178 TCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGTACAAT -133

(B)

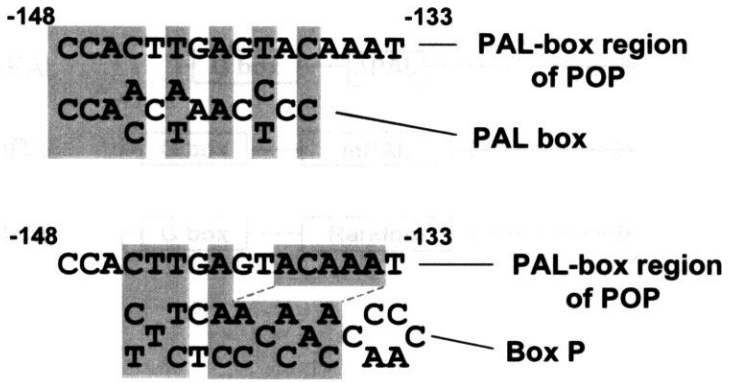


Figure 2-10. (A) Nucleotide sequence of POP (PAL-box related *cis* element of *prxC2* promoter). The PAL-box like region is underlined. (B) Nucleotide sequence alignments: PAL-box region of POP *vs.* PAL box consensus sequence and PAL-box region of POP *vs.* Box P consensus sequence.

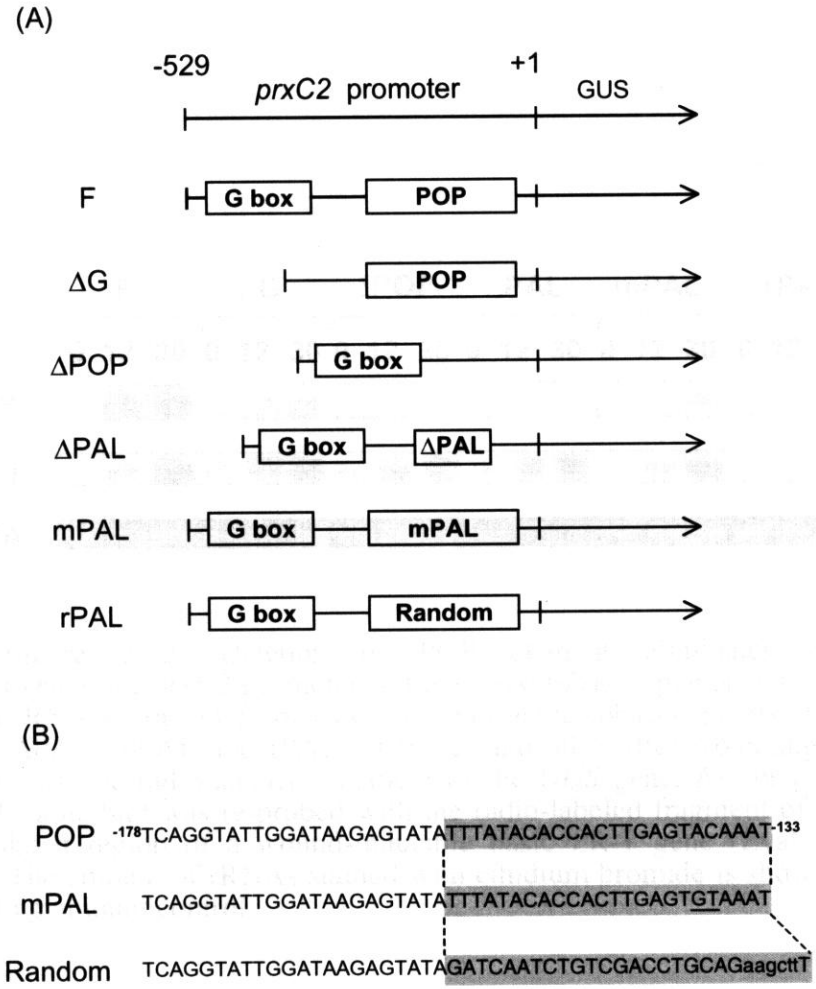


Figure 2-11. (A) Constructs used for tobacco plant transformation. F construct contains an intact –529 *prxC2* promoter, and was used as a positive control. ΔG, ΔPOP, ΔPAL, mPAL, and rPAL construct contain the -529*prxC2* promoter without the G box, without the POP element, without the PAL-box region, with 2-base substitution in the PAL-box region, and with random sequence in place of the PAL box region, respectively. (B) Nucleotide sequence of the mPAL and the Random fragments in the mPAL and the rPAL constructs. Position of 2-base substitution in the PAL-box region is underlined.

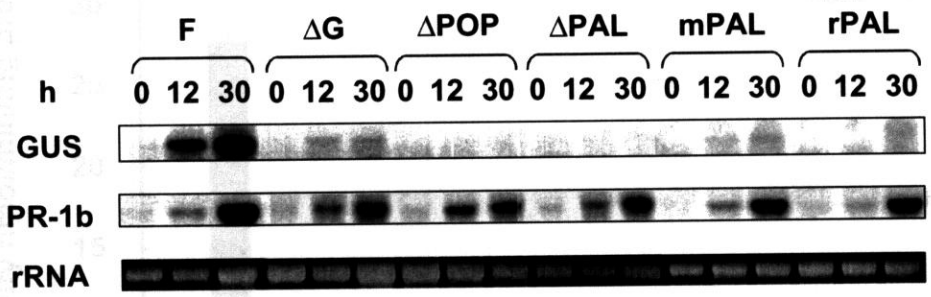


Figure 2-12. Deletion of POP element eliminates wound responsiveness of *prxC2* promoter in transgenic tobacco plants. Twenty µg of total RNA extracted from leaves of transgenic tobacco plants (F, ΔG, ΔPOP, ΔPAL, mPAL and rPAL) at 0, 12, and 30 h after wounding were probed with the radio-labeled fragment of the *GUS* gene. As for positive control, same blot was re-probed with the radio-labeled fragment of the 3′ untranslated region of a wound-inducible basic PR-1 gene (Eyal *et al.*, 1992). The amount of rRNAs stained with ethidium bromide is shown here as loading amount control.

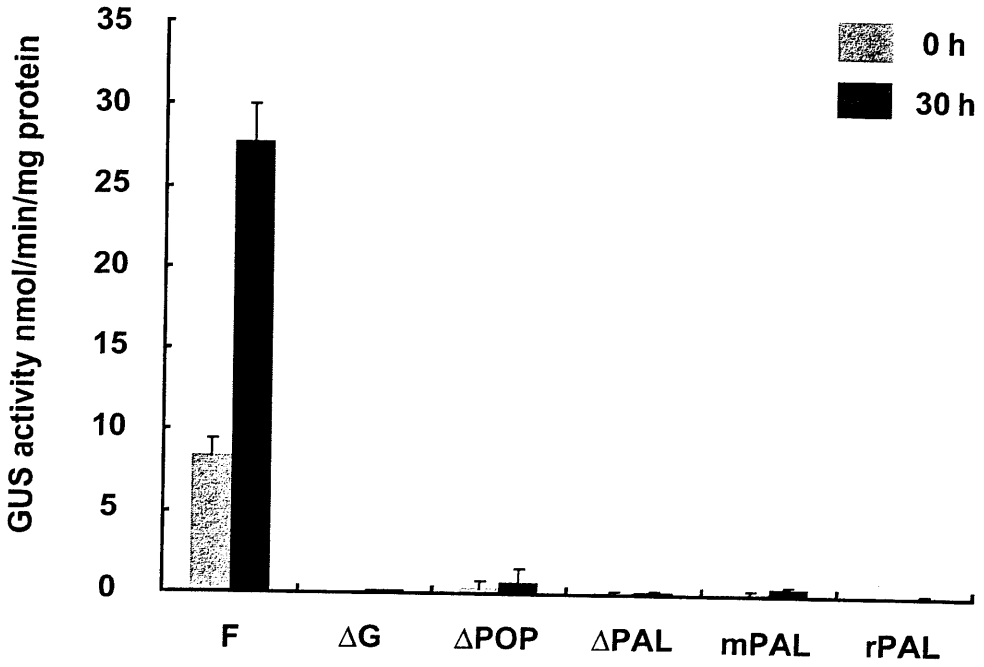
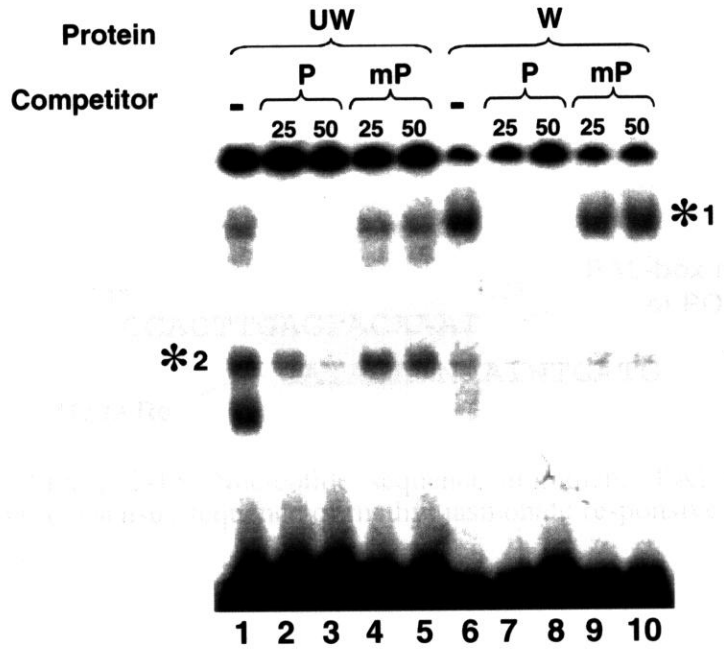


Figure 2-13. Deletion of POP element eliminates wound-induced GUS activity detected in transgenic tobacco plants transformed with the *prxC2* promoter::*GUS* chimeric construct. The specific GUS activity (nmol/min/mg protein) in leaves of transgenic tobacco plants (F, ΔG, ΔPOP, ΔPAL, mPAL, and rPAL) was detected at 0 and 30 h after wounding. Error bars indicate standard deviation from mean value of GUS activity detected in 3 plants of each line.



Probe P agct^{-178}TCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGTACAA^{-135}

Probe mP $^{-178}$TCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGTGTAA^{-135}

Figure 2-14. Specific interaction between POP element of *prxC2* promoter and nuclear proteins extracted from unwounded or wounded tobacco leaves. Gel shift assay was performed using the POP element as probe. Each binding reaction contains 2 ng of radio-labeled probe P and 2 mg of nuclear proteins extracted from unwounded (lanes 1-5) or wounded tobacco leaves (lanes 6-10). Competition assay was carried out by addition of 25- or 50-fold molar excess of non-labeled probe P (lane 2, 3, 7, and 8) or non-labeled probe mP (lane 4, 5, 9, and 10) to the reaction mixtures. Oligonucleotide sequences of probe P (POP element) and probe mP (POP element with a 2-base substitution in the PAL-box region) are shown. Lower case letters in the sequence of probe P indicate additional sequence for restriction enzyme (*Hin*dIII) digestion and bold letters indicate the PAL-box region of the POP element. Position of the 2-base substitution in the PAL-box region is underlined. Asterisks indicate specific DNA-protein complexes.

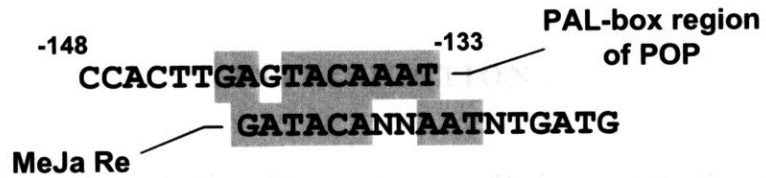


Figure 2-15. Nucleotide sequence alignment: PAL-box region of POP *vs.* consensus sequence of methyl jasmonate responsive element (MeJa Re).

CHAPTER III

ISOLATION AND CHARACTERIZATION OF A *TRANS* FACTOR BINDING TO POP ELEMENT

INTRODUCTION

Since the data described in Chapter II demonstrate that the POP element functions as a wound-responsive *cis* element of the *prxC2* promoter, it is possible that tobacco nuclear protein(s) binding to the POP element in gel shift assay is a wound-responsive *trans* factor that conveys wound signals to the *prxC2* promoter. In this chapter, we report the isolation and characterization of a tobacco nuclear protein that binds specifically to the PAL-box region of POP element and function as a wound-responsive *trans* factor *in planta*.

MATERIALS AND METHODS

RNA extraction: GTC method

For construction of tobacco cDNA libraries, total RNA was extracted from tobacco leaves and stems with guanidinium thiocyanate followed by centrifugation in cesium chloride solution. In brief, tobacco leaves or stems were ground to fine powder in liquid nitrogen and transferred to 5×volume of GTC solution (4 M guanidinium isothiocyanate, 0.5% sodium lauryl sarcosinate, 25 mM sodium acetate, adjust pH to 7.0 by NaOH, filter through a Whatman No. 1 filter paper, add β-mercaptoethanol to a final concentration of 0.14 M before use). After the tissue powder was

concentration of 0.14 M before use). After the tissue powder was mixed thoroughly with the solution, the mixture was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and the step was repeated once. The supernatant from the second round of extraction was transferred to centrifuge tube containing CsCl solution (5.7 M CsCl, 0.1 M EDTA, adjust pH to 7.0 by NaOH, 0.1% v/v diethyl pyrocarbonate (DEPC), mix well, allow to stand for 30 min at room temperature, and autoclave at 121°C for 40 min). After centrifuge at 28,000 rpm for 24 h at 20°C, the GTC solution was removed and the pellet was dissolved in DEPC water (0.1% v/v DEPC in distilled H_2O, mix well, autoclave at 121°C for 40 min). The solution was extracted twice with same volume of phenol:chloroform:isoamyl alcohol (25:24:1). 0.25×volume of 10 M LiCl was added to the aqueous phase and the solution was incubated on ice for 90 min. The solution was centrifuge at 15,000 rpm for 20 min at 4°C. The RNA pellet was washed with 70% ethanol, dried, and dissolved in DEPC water (0.1% diethyl pyrocarbonate 0.1% in water).

Caution: Diethyl pyrocarbonate is suspected to be a carcinogen and should be handle with care.

Selection of poly(A)$^+$ mRNA

Total RNA was mixed to the same volume of 2×loading buffer (20 mM Tris, imM NaCl, 10 mM EDTA, adjust pH to 7.4, autoclave at 121°C for 40 min) and the solution was loaded onto an oligo(dT)-cellulose column pre-equilibrated with 1×loading buffer. The column was washed twice with 1×loading buffer and poly(A)$^+$ mRNA was eluted from the column with elution buffer (10 mM Tris, 5 mM EDTA, adjust pH to 7.4, autoclave at 121°C for 40 min). The poly(A)$^+$ mRNA was precipitated overnight at -20°C in 0.1×volume of 2 M potassium acetate pH 5.2, 0.1×volume of 2 M NaCl, and 2×volume of absolute ethanol. Poly(A)$^+$ mRNA was pelleted

and 2×volume of absolute ethanol. Poly(A)$^+$ mRNA was pelleted from this solution by centrifugation at 15,000 rpm for 20 min at 4°C. The pellet was rinse with 70% ethanol, dried, and dissolved in DEPC water (0.1% diethyl pyrocarbonate 0.1% in water).

Caution: Diethyl pyrocarbonate is suspected to be a carcinogen and should be handle with care..

Construction and screening of λgt11 expression library

Poly(A)$^+$ mRNA was used as template for cDNAs synthesis. Using oligo(dT) in pair with random primers, a tobacco-leaf and a tobacco-stem cDNA libraries were constructed in λgt11 vector by the method described in the user manual (Stratagene, CA, USA). The amplified tobacco leaf and tobacco stem libraries (each contains approximately 1×10^6 independent clones) were screened by the method described by Singh *et al.* (Singh *et al.*, 1988) with minor modifications. Approximately 5×10^4 PFU of λ phage was co-cultured with 600 µl of a log phase culture of *E. coli* host cells strain Y1090 at 37°C for 15 min. The co-cultured solution was mixed with 7.5 ml of 50°C top agarose (NZYM with 0.7% w/v agarose instead of agar) and the mixture was used to overlay an NZYM plate (per liter: 10 g NZ amine, 5g NaCl, 2 g $MgSO_4\cdot7H_2O$, 15 agar). After 8 h of incubation at 42°C, an isopropyl β-D-thiogalactoside (IPTG)-saturated nitrocellulose filter was placed on the surface of the plate and the plate was incubated for 8 h at 37°C. After a brief incubation at 4°C to facilitate the removal of the filter, the filter was transferred immediately to BLOTTO buffer (5% skim milk, 50 mM Tris pH 7.5, 50 mMNaCl, 1 mM EDTA, 1mM DTT) and was incubated for 60 min at 25°C with gentle agitation. The filter was washed twice with TNE-50 buffer (10 mM Tris pH 7.5, 50mM NaCl, 1 mM EDTA, 1 mM DTT). After the washes, the filter was hybridized with a digoxigenin-labeled

triple repeat of $^{5'}$CCACTTGAGTAC$^{3'}$ (PAL-box region of the POP element) in TNE-50 buffer containing 10 μg/ml poly(dI-dC) for 1 h at 25°C. The filter was washed 4 times with TNE-50 buffer, dried, and subjected to immunological detection using anti-digoxiginin antibody conjugated to alkaline phosphatase. A CDP-*star* ready-to-use solution (Roche Molecular Biochemicals, Germany) was used as substrate and the filter was expose to X-ray film at room temperature for 25 min. Plagues at the position corresponding to the positive signals detected on the filter were recovered and stored in SM buffer (200 mM NaCl, 10 mM Tris-HCL pH 7.4, 5 mM $MgSO_4$, 0.1% w/v gelatin) After the second and the third round of screening, The inserted DNA fragment of a positive phage was amplified by PCR using forward and reverse primers of λgt11 vector. The amplified fragment was subcloned into pNoTA/T7 vector by PCR Cloner (5 Prime, 3 Prime Inc., CO, USA). Sequence analysis was performed using DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin Elmer, CA, USA).

Genomic DNA extraction: CTAB method

Genomic DNA was isolated from tobacco leaves by CTAB method. Tobacco leaves were ground to fine powder in liquid nitrogen and the powder was resuspended in 65°C 2×CTAB buffer (2% w/v CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP). The solution was extracted twice with 1×volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was mixed with 0.1×volume of 10% CTAB solution (10% w/v CTAB, 0.7 M NaCl) and the solution mixture was re-extracted with 1×volume of chloroform:isoamyl alcohol (24:1). To precipitate the DNA, 1×volume of CTAB precipitation buffer (1% w/v CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) was added to the aqueous phase. The solution was incubated for 30 min at 25°C and was centrifuged at 2,800 rpm for 20 min at 25°C. The pellet was suspended in high-salt TE buffer (10 mM Tris-HCl

pH 8.0, 1 mM EDTA, 1 M NaCl) and the DNA was precipitated with 1×volume of isopropanol. After centrifugation at 2,800 rpm for 15 min at 25°C, the DNA pellet was dissolved in TE buffer and treated with DNase-free RNaseA prior to Southern blot analysis.

Southern blot analysis

Twenty µg of tobacco genomic DNA was digested with restriction enzymes (*Eco*RV or *Dra*I) and electrophoretically separated through 1% w/v agarose gel in 1×TAE buffer. To denature the DNA, the gel was soaked in acid denature solution (0.25 M HCl) for 10 min with gentle agitation, rinsed with distilled water, and soaked in basic denature solution (1.5 M NaCl, 0.5 M NaOH) for 30 min with gentle agitation. After denaturation, the gel was washed twice, 15 min each, with neutral solution (1.5M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) and was rinsed 3 times with distilled water. The DNA was blotted overnight onto a nylon membrane (Hybond N, Amersham Pharmacia Biotech, Bucks, UK) using 20×SSC buffer (3M NaCl, 0.3 M $C_6H_5Na_3O_7 \cdot 2H_2O$, pH 7.0) as transfer solution and the DNA was cross-linked to the membrane by UV irradiation. Before hybidization, the membrane was pre-incubated at 65°C for 10 min in Denhardt solution (freshly prepare from 100×stock solution: 2% w/v BSA, 2% w/v Ficoll 400, 2% w/v PVP, filtered and stored at -20°C) containing denatured salmon sperm DNA. The membrane was hybridized overnight at 65°C in Denhardt hybridization solution (5×SSPE, 5×Denhardt solution, 0.5% w/v SDS, 10% sodium dextran sulfate) containing radio-labeled full-length cDNA of *Ntlim1* as probe. The membrane was wash at 65°C with 1×SSC-0.1%SDS buffer and exposed overnight at -80°C to X-ray film for autoradiograph.

Nuclear localization analysis of Ntlim1

The open reading frame of *Ntlim1* was amplified by PCR and inserted into pTH-2 plasmid (a gift from Dr. Yasuo Niwa, University of Shizuoka, Japan) at position 5′and in-frame to the open reading frame of *sGFP* (Niwa *et al.*, 1999), which was put under the control of the CaMV 35S promoter. The resulting construct and the pTH2 plasmid alone were introduced separately into onion epidermal cells by particle bombardment. The bombardment was performed using BIOLISTIC® PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA). The size of gold particles was 1 μm in diameter, the distance between the onion scaly leaf and the stopping screen was 9 cm, and the chamber was evacuated to 28 inch of mercury. The GFP fluorescence was visualized under fluorescence microscopy and the position of nuclei was visualize by DAPI staining.

Production and purification of recombinant GST-fusion proteins

For production of Ntlim1::GST fusion protein, the 600-bp *Bam*HI fragment of *Ntlim1* open reading frame was inserted into the *Bam*HI site of pGEX-2TK plasmid in-frame at the end of the open reading frame of glutathion-S-transferase (GST) as shown in Figure 3-1(A). For production of LIM1::GST fusion protein, the 282-bp *Bam*HI-EcoRI fragment encoding the LIM1 proximal region (amino acid number 1-88) was amplified by PCR using the forward primer $^{5'}$CACGGATCCATGGCTTTTGCA$^{3'}$ in pair with the reverse primer $^{5'}$GACGAATTCGGGTTTCTGTGG$^{3'}$ and the amplified fragment was inserted in-frame into the *Bam*HI-EcoRI site of pGEX-4T-1 (Amersham Pharmacia Biotech, Bucks, UK) at the end of the open reading frame of glutathion-S-transferase (GST) as shown in Figure 3-1(B). For production of LIM2::GST fusion protein, the 205-bp *Bam*HI-EcoRI fragment encoding the LIM2 proximal region (amino acid number 104-172) was amplified by PCR using the forward primer $^{5'}$-

GGTGGATCCAGAGAGAAATGTTTTGG$^{-3'}$ in pair with the reverse primer $^{5'}$CGTTGCCCTTCTCCTTGATGAATTCG$^{-3'}$ and the amplified fragment was inserted into the *Bam*HI-EcoRI site of pGEX-4T-1 in the same way as the LIM1 fragment. The resulting plasmids were used to transformed *E. coli* strain DH5α. The production of the fusion proteins was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to the final concentration of 2 mM to the mid-log phase bacterial culture. The GST-fusion proteins were purified by glutathione Sepharose®4B column (Amersham Pharmacia Biotech, Bucks, UK) and the GST parts were excised by digestion with thrombin protease following the procedure in user manual. The fusion and the purified proteins were denatured at 100°C for 2 min in the same volume of 2×SDS sample buffer (0.125 M Tris-HCl pH 6.8, 4% w/v SDS, 20% glycerol, 0.3 M β-mercaptoethanol, 0.05% w/v Bromophenol blue) and subjected to SDS-PAGE in 10 to 15% acrylamide gel. The gel was run under constant current (20-30 mA). The protein bands were stained with Coomassie Brilliant Blue (CBB) staining solution (50% v/v methanol, 0.05% w/v Coomassie brilliant blue R-250, 10% v/v acetic acid) and washed with de-staining solution (5% v/v methanol, 7% v/v acetic acid).

Transient *trans*-activation assay in tobacco BY2 protoplasts

Protoplasts of tobacco suspension culture BY2 cells were produced and transformed as described in Chapter I (Materials and methods: Transient expression assay in tobacco BY2 protoplasts). After incubation in the LS medium containing 0.4 M mannitol for 24 h at 25 °C in darkness, the protoplasts were harvested and the GUS/LUC activity in the crude proteins extracted from the protoplasts were measured. Protein concentration was quantified by Bradford method (Bradford, 1967).

Assay of GUS/LUC activity

The BY2 protoplasts were harvested from the medium by centrifugation at 3,000 rpm for 5 min at 4°C. The pellet was dissolved in GUS/LUC lysis buffer (0.1 M KPO4, 2 mM EDTA, 5% v/v glycerol, 20 mM DTT, pH 7.8) and sonicated on ice. The crude protein extract were obtained by centrifugation at 10,000 rpm for 10 min at 4°C. The GUS activity was measured as described in Chapter I (Materials and methods: Assay of GUS activity). LUC activity was measured using luciferase assay system (PicaGene, Toyo Ink, Tokyo).

Plant transformation

Tobacco plants were transformed with *Agrobacterium tumefaciens* EHA105 by leaf disk transformation method (Rogers *et al.*, 1986). The tobacco plants double transformed with the constructs in pBI121 and pBI101Hm2 plasmids were selected in MS medium containing 100 µg/ml kanamycin and 20 µg/ml hygromycin. The present of the *GUS* transgene in all transgenic plants was confirmed by genomic PCR using primers that amplify the *GUS* coding region.

Note: For gel shift assay, transient expression in tobacco protoplasts, assay of GUS activity, plant material, RNA extraction by ATA method, northern blot analysis, and wound treatment, see Materials and Methods, Chapter II: Isolation of a wound-responsive *cis* element of the *prxC2* promoter.

RESULTS

Tobacco nuclear protein binding to PAL-box region of POP element has a unique structure of two N-terminal LIM domains and one C-terminal acidic region

To isolate cDNA that encodes a nuclear protein binding to the PAL-box region of the POP element, South-western screening was performed. In this screening, a digoxigenin-labeled triple repeat of the PAL-box region of POP element was used as probe to screen λgt11 expression libraries constructed with mRNAs derived from leaves or stems of tobacco plants. From approximate 1×10^6 recombinant phages of tobacco stem library, one positive cDNA clone was isolated. A 988-bp insert of this cDNA clone was amplified by PCR, subcloned into pNoTA/T7 plasmid, and sequenced. The insert was found to encode a protein of 200 amino-acid residues with an expected molecular mass of 24 kDa (Figure 3-2). This protein contains one acidic region and two double zinc-finger domains called LIM domain (Figure 3-3); thus, we named this protein Ntlim1. By searching GenBank database, we found that the nucleotide sequence of *Ntlim1* is very similar to that of *HaPLIM1* (Accession No. AF187104, Baltz *et al.*, 1996B), a putative transcription factor required for the expression of late pollen genes in sunflower. The deduced amino acid sequence of the Ntlim1 protein is aligned to that of HaPLIM1 protein in Figure 3-4. The two sequences share 55% identity and all of the putative metal-chelating residues are conserved.

Expression profile and copy number of *Ntlim1* in tobacco plants

Organ specificity and wound-responsive expression of the *Ntlim1* gene in tobacco plants was determined by northern blot analysis using the

full-length cDNA of *Ntlim1* as probe. As shown in Figure 3-8(A), the *Ntlim1* mRNA was abundant in stem and was presented in a detectable amount in leaf and root.

To determine whether the expression of *Ntlim1* itself is also regulated by wound stress, we performed northern blot analysis using the full-length cDNA of *Ntlim1* to probe total RNA extracted from tobacco leaves wounded for 0, 12, and 30 h. As shown in Figure 3-8(B), the *Ntlim1* transcripts were detected even at 0 h, which represent the expression of the *Ntlim1* transcript in healthy (unwounded) leaf, and remains almost stable thereafter. The result suggests that *Ntlim1* is constitutively transcribed irrespective of wound stress.

Copy number of genes related to *Ntlim1* in the tobacco genome was also determined by Southern blot analysis (Figure 3-8(C)). Tobacco genomic DNA (20 μg/lane) was digested with restriction enzymes *Eco*RI or *Dra*I and hybridized with radio-labeled fragment of the full-length *Ntlim1* cDNA. Pattern of the band detected in this experiment suggests that few copies of *Ntlim1* are present in tobacco genome.

Nuclear localization of Ntlim1

To confirm nuclear localization of the Ntlim1 protein, we observed the subcellular localization of the Ntlim1::GFP fusion protein by transient expression assay of the *Ntlim1::GFP* fusion construct in onion epidermal cells. Using particle bombardment procedure, we introduced plasmids expressing the Ntlim1::GFP fusion protein or the GFP protein alone into onion epidermal cells and GFP signals within the cells was checked 24 h after the introduction. As shown in Figure 3-9, the Ntlim1::GFP fusion protein was specifically localized to the nuclear compartment of the transformed cell in contrast to the random subcellular localization of the control GFP protein. This result indicates that Ntlim1 is a nuclear protein.

Ntlim1 is a PAL-box binding protein

In order to determine whether the Ntlim1 protein does indeed bind to the PAL-box region of the POP element, we produced the Ntlim1 protein in *E. coli* as a GST-fusion protein. The GST::Ntlim1 fussion protein was purified and the GST part was excised by thrombin protease digestion. The band of the purified Ntlim1 protein (molecular mass = 24 kDa) was observed by SDS-PAGE (Figure 3-10).

The PAL-box binding activity of the purified Ntlim1 protein was tested by gel shift assay using the radio-labeled POP element (probe P) as probe. As shown in Figure 3-11, the purified Ntlim1 protein bound specifically to the PAL-box region because the shift band detected in lane 6 was competed out with cold POP element (cold probe P; lane 7) and cold PAL box (cold probe sP; lane 9) but not with cold POP element that contains a 2-base substitution in its PAL-box region (cold probe mP; lane 8). Interestingly, the cold PAL box failed to compete out the shift band given by the nuclear protein extracted from wounded-tobacco leaves and the mobility of the Ntlim1-POP complex was different from that of the nuclear protein-POP complex. These results imply that Ntlim1 protein is only one of a number of proteins that form an oligomeric DNA-binding complex on the POP element.

LIM domains of Ntlim1 function as DNA binding domain

Although the LIM domain almost always functions as a protein-dimerization domain in animals, the Ntlim1 protein contains no other functional domain than the two LIM domains; therefore, we have no other choice but to speculate that LIM domains could function as DNA binding domain in the interaction between the Ntlim1 protein and the PAL-box region of the POP element. In order to prove this hypothesis, the system to

produce GST-fusion protein in *E. coli* was used again to produce LIM1 and LIM2 proteins, which are the truncated derivatives containing only the LIM1 domain and the LIM2 domain of the Ntlim1 protein, respectively. The LIM1::GST and the LIM2::GST fusion proteins were purified and the GST part of the proteins was excised by thrombin protease digestion. The bands of the purified LIM1 (molecular mass = 11 kDa) and the purified LIM2 (molecular mass = 8 kDa) proteins were detected by SDS-PAGE as shown in Figure 3-12. The PAL-box binding activity of the purified LIM1 and LIM2 proteins was tested by gel shift assay using radio-labeled POP element (probe P) as probe. As shown in Figure 3-10, both purified LIM1 and LIM2 proteins could bind to the POP element in the same manner as the purified Ntlim1 indicating that LIM domains of Ntlim1 protein function as DNA binding domains.

Ntlim1 has *trans*-activation activity *in vivo*

To determine whether the Ntlim1 could function as a transcription factor *in vivo*, we examined *trans*-activation activity of the Ntlim1 protein using transient *trans*-activation assay in protoplasts of tobacco BY2 cells. In this assay, the effector (35S::*Ntlim1* or 35S::*Ntlim1ΔAC*), the reporter (*prxC2* promoter::*GUS*), and the internal control (35S::*LUC*) constructs were introduced simultaneously into protoplasts of tobacco BY2 cells, and after 24 h of incubation, the GUS/LUC activity in the crude proteins extracted from the BY2 protoplasts transformed with the effector, the reporter, and the internal control constructs, was measured and reported as percent increased from the activity detected in the crude proteins extracted from the BY2 protoplasts transformed with the reporter and the internal control constructs without the effector construct. As shown in Figure 3-14, introduction of 35S::*Ntlim1* effector gave a three-fold increase in the relative GUS/LUC activity suggesting that Ntlim1 has *trans*-activation activity *in*

vivo. Moreover, the *trans*-activation domain of Ntlim1 was mapped to the C-terminal acidic region (amino acids 164-200) because a sharp decrease in the GUS/LUC activity was detected in the BY2 protoplasts transformed with the 35S::*Ntlim1ΔAC* effector. By computer analysis with the Genetyx-win program (Software Development, Tokyo, Japan) the isoelectric point (pI) of this C-terminal acidic region is 3.92, though the pI of the full-length Ntlim1 protein is 8.39.

Ntlim1 is a *trans* factor regulating wound responsiveness of *prxC2* promoter

The data described in Chapter II demonstrated that the POP element is the wound-responsive *cis* element of *prxC2* promoter; thus, it is possible that the Ntlim1, proven to be the *trans* factor binding to the PAL-box region of the POP element, could function as a *trans* factor responsible for the wound-inducible activity of the *prxC2* promoter. In order to confirm this hypothesis, we investigated whether antisense expression of *Ntlim1* give any effect on the wound-induced expression of the *prxC2* promoter::*GUS* chimeric construct in transgenic tobacco plants. Transgenic tobacco plant harboring the 35S::antisense *Ntlim1* construct were double transformed with the F construct (*prxC2* promoter::*GUS*; Figure 3-15(A)) and the double-transformed transgenic plants (AF plants) with a low expression level of *Ntlim1* were selected (Figure 3-15(B)). As shown in Figure 3-16, the wound-induced expression of the *GUS* reporter gene detected in the F plant was strongly suppressed in the AF plants. Likewise, the increased GUS activity detected in the crude proteins extracted from wounded leaves of the F plant was absent in the crude proteins extracted from wounded leaves of the AF plants (Figure 3-17). These results indicate that Ntlim1 functions as a *trans* factor that conveys wound signals to the *prxC2* promoter *in planta*.

DISCUSSION

A cDNA clone encoding a PAL-box binding factor was isolated from a tobacco stem cDNA library using the PAL-box region of the POP element as probe. The inserted cDNA fragment of this clone encodes a 200-amino acid protein, of which the amino-acid sequence shares 55% identity with that of the HaPLIM1 (formerly known as SF3 or PLIM1), a transcription factor required for the expression of late pollen genes in sunflower (Baltz *et al.*, 1996). Similar to PLIM1, the protein encoded by this clone contains 2 conserved motifs called LIM domain; therefore, we named this clone Ntlim1 (GenBamk accession number AB023479). Currently, Eliasson *et al* (2000) have isolated cDNA clones that encode LIM proteins from several kinds of flowering plants (see phylogenetic tree, Figure 3-5). Among these clones, an NtWLIM1 cDNA clone (GenBank accession number AF184109) isolated by PCR of reverse-transcribed tobacco leaf poly A^+ RNA contains a sequence almost identical to our Ntlim1 cDNA clone (Figure 3-6). The deduced amino acid sequence of the NtWLIM1 protein is also very similar to that of Ntlim1 protein (Figure 3-7). The two sequences share 94% identity and all of the putative metal-chelating residues are conserved.

LIM domain composes of 2 zinc-fingers that are joined by a 2 amino-acid linker (Dawid *et al.*, 1995). Proteins with LIM domain are found in animals and plants and are grouped into LIM-homeodomain protein, LIM-only protein, cytoskeleton associated LIM protein, LIM-GAP (GTPase activating protein) protein, and LIM kinase protein (Dawid *et al.*, 1995). The Ntlim1 protein contains two LIM domains (LIM1 and LIM2) without any other putative domain; thus, Ntlim1 is classified as a LIM-only protein. Although LIM domain binds zinc ions and shows structural similarity to the DNA-binding zinc fingers of GATA-1 transcription factor (Perez-Alvarado

et al., 1994), scientific evidences have favored the role of the LIM domain in protein-protein interaction (Dawid *et al.*, 1998; Feuerstein *et al.*, 1994; Sanchez-Garcia and Rabbitts, 1994). However, a recent study of a mouse LIM protein hic-5 has shown that each of the four LIM domains of hic-5 protein could bind to DNA in a distinctive manner (Nishiya *et al.*, 1998).

Our results from gel shift assay show that the Ntlim1 protein bind to the PAL-box region of the POP element in the same manner as that of nuclear proteins extracted from wounded tobacco leaves, with only one exception — we found that the sP competitor failed to compete with the radio-labeled full-length POP element in binding with the tobacco nuclear factors but not in binding with the purified recombinant Ntlim1 protein. The explanation for this exception could be the formation of a DNA-binding protein complex. Protein(s) other than Ntlim1 possibly binds to another region of the POP element and belittles the effect of the sP competitor. The slow mobility of the protein-DNA complex observed in gel shift assay with nuclear proteins extracted from wounded tobacco leaves also supports our hypothesis that a number of proteins form an oligomeric DNA-binding complex on the POP element. Although the result from gel shift assay indicate that nuclear proteins from healthy and wounded tobacco leaves gave different pattern of shift bands, we still not know whether the Ntlim1 is one of the proteins that bind constitutively to the POP element or one of those bound to the POP element only after plants are wounded. We also give the first report on a sequence-specific DNA-binding activity of plant LIM domain. Under the condition used in our gel shift assay, each of the 2 LIM domains of Ntlim1 was able to bind with sequence specificity to the PAL-box region of the POP element. Therefore, we conclude that Ntlim1 binds to the POP element through its LIM domains, or in other words, LIM domains of Ntlim1 function as DNA binding domain.

However, we should not rule out the possibility that the LIM domains of Ntlim1 protein could also function as protein-dimerization

domain. Result from gel shift assay indicate that a total DNA binding activity could be reached in the presence of only 1 of the 2 LIM domains; thus, one of the LIM domains might functions as a DNA-binding domain while another one functions as a protein-dimerization domain. Evidence for the function of LIM domain as a protein-dimerization domain in a DNA-binding protein complex is also exist. A LIM-only protein, Lmo2, was found to be an obligatory component of an oligomeric DNA-binding protein complex — though the Lmo2 protein itself does not bind to DNA (Wadman *et al.*, 1997). In this complex, the DNA-binding modules within the oligomeric complex are provided by an E47-TAL1 and GATA-1 heterodimer, which binds to a region covering an E-box sequence (CANNTG) and a GATA sequence. The consensus of the target DNA sequence of this oligomeric DNA binding complex is E-box-$(N)_9$-GATA. Interestingly, the POP element also contains GATA sequence 17 bp downstream and 15 bp upstream of the E-box sequence giving the pattern of E-box-$(N)_{17}$-GATA-$(N)_{15}$-E-box. Nevertheless, the importance of the two E-boxes and the GATA sequence within the POP element is still unclear.

By transient *trans*-activation assay in tobacco protoplasts, we confirmed that the Ntlim1 protein functions as a transcription factor activating the expression of the *prxC2* promoter::*GUS* reporter gene *in vivo*. A domain responsible for the *trans*-activation activity of the Ntlim1 protein was also mapped to the C-terminal acidic region. Approximately 24% of the amino acids in this region are acidic (negatively charged) and the pI of this region is 3.92. No homology to any known *trans*-activation domains was observed and no distinctive features found in this C-terminal acidic region; however, plants transcription factors including maize VP1 (McCarty *et al.*, 1991) and Dof1 (Yanagisawa and Sheen, 1998) have acidic domain that functions as a *trans*-activation domain supporting the hypothesis that the C-terminal acidic domain of Ntlim1 functions as a *trans*-activation domain.

By following the changes in the level of wound-induced expression of the *GUS* reporter gene in transgenic tobacco plants double transformed with the 35S::antisense *Ntlim1* and the *prxC2* promoter::*GUS* chimeric constructs, we confirmed that the Ntlim1 protein functions as a wound-responsive *trans* factor *in planta*. This finding is also in consistent with the data obtained from transgenic plants harboring the *GUS* reporter gene under the control of the *prxC2* promoter with deletion or mutation in the PAL-box region of the POP element. Therefore, we conclude that the POP element and the Ntlim1 *trans* factor work in concert to control the wound-responsiveness of the *prxC2* promoter ***in planta.***

The results from northern blot analysis indicate that *Ntlim1* is constitutively transcribed regardless of wounding and some evidences are available for activation of the nuclear factor by post-translational modification, for example, activity of GBF-1, an *Arabidopsis* G-box binding factor, is activated by phosphorylation (Klimczak *et al.*, 1992); therefore, post-translational modification, e.g. phosphorylation, dephosphorylation, ligand binding, or change of partner, may responsible for the activation of the Ntlim1 protein.

Although the result from transient expression of the Ntlim1::GFP fusion protein in onion epidermal cells indicates that the Ntlim1 protein is localized to nucleus, the question remains whether the Ntlim1 protein is constitutively located inside the nucleus or transiently translocated from cytoplasm to the nucleus only after plants sense signals from wound stress.

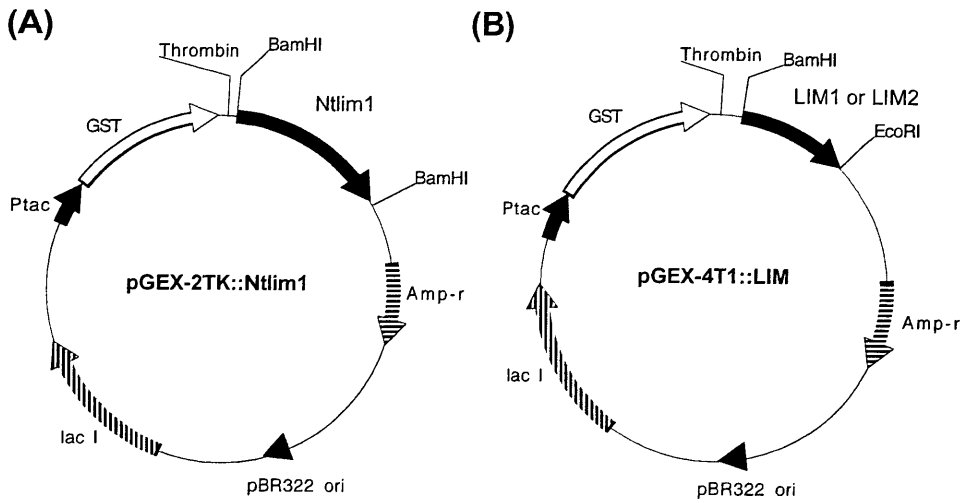
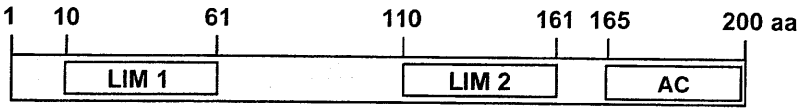


Figure 3-1. Plasmid constructs for production of GST-Ntlim1, GST-LIM1, and GST-LIM2 recombinant proteins.

```
GAATTCGCGGCCGTTCCAAAAACCAAGTGCTAACACAAAGAAAGGGAAAGAGCCACAAAG      60

ACCATTTTTGTTTTCTGTAAAACTTGCTCGTATATAGCCATGGCTTTTGCAGGAACCACA     120
                                        M  A  F  A  G  T  T
CAGAAATGCATGGCATGTGACAAGACTGTCTATCTGGTTGACAAATTAACTGCAGATAAC     180
Q  K  C  M  A  C  D  K  T  V  Y  L  V  D  K  L  T  A  D  N
AGAATCTATCACAAAGCTTGTTTCAGATGCCATCACTGCAAGGGCACTGTCAAGCTTGGC     240
R  I  Y  H  K  A  C  F  R  C  H  H  C  K  G  T  V  K  L  G
AACTACAATTCCTTTGAGGGAGTTCTATACTGTAGACCACACTTTGATCAGCTCTTCAAA     300
N  Y  N  S  F  E  G  V  L  Y  C  R  P  H  F  D  Q  L  F  K
CAAACTGGCAGTTTGGATAAAAGCTTTGAAGGTACACCAAAAATTGTGAAGCCACAGAAA     360
Q  T  G  S  L  D  K  S  F  E  G  T  P  K  I  V  K  P  Q  K
CCCATTGACAGTGAGAAACCACAGGTAGCCAAAGTGACAAGCATGTTTGGTGGAACAAGA     420
P  I  D  S  E  K  P  Q  V  A  K  V  T  S  M  F  G  G  T  R
GAGAAATGTTTTGGCTGCAAGAAAACTGTCTACCCAACAGAAAAGGTATCAGCCAATGGC     480
E  K  C  F  G  C  K  K  T  V  Y  P  T  E  K  V  S  A  N  G
ACGCCATACCATAAGAGCTGCTTCCAATGCAGCCACGGAGGCTGTGTAATAAGCCCTTCC     540
T  P  Y  H  K  S  C  F  Q  C  S  H  G  G  C  V  I  S  P  S
AACTATACCGCACATGAGGGGCGCTTATATTGTAAACATCACCATATTCAACTTATCAAG     600
N  Y  T  A  H  E  G  R  L  Y  C  K  H  H  H  I  Q  L  I  K
GAGAAGGGCAACTTAAGCAAGCTTGAGGGTGACCATGAAATGAATTCCACGACAACAACA     660
E  K  G  N  L  S  K  L  E  G  D  H  E  M  N  S  T  T  T  T
GAAGTTACTGCAGAGTCATACACAGCCGACCAAGTTGATTGATCCTTATCTTTACCGCGA     720
E  V  T  A  E  S  Y  T  A  D  Q  V  D

TCATGTATTACGTATCTGCTGTTAGTTGTAAGAATCGAAGGCGTTCAGCAGCTTCCATGA     780

ATGCACTTGCCTTGCCCCAGCGTATGTTTTACTCTAATCTAGCTTCAATTAATTTGATGT     840

TGAACTATATATTGTCTAGCTTTTGTGTGTAGATTTTTGACCTTTGTTTGCTTGTGCTTC     900

ACTTGTATTATGTGAATGTTGAATGAGATTGAATATAACATGGTTTTGCTGTCCCAGTGC     960

ATGCAAATCTTTGAGCGGCCGCGAATTC                                      988
```

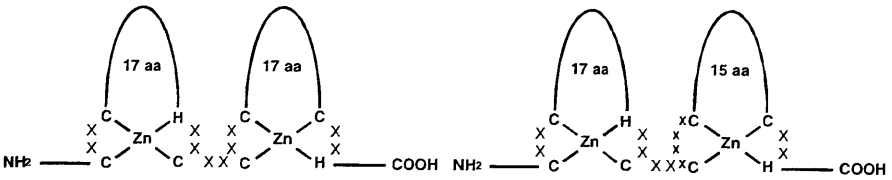
Figure 3-2. Nucleotide and deduced amino-acid sequences of *Ntlim1*. For the deduced amino acid sequence, Underlines indicate the 2 LIM domains, red fonts indicate the potential metal-chelating residues (C and H), and blue fonts indicate amino acid in the C-terminal acidic region.

(A)



Ntlim1 protein

(B)



LIM 1 domain

LIM 2 domain

(C)

LIM domain consensus = $CX_2CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_2(C/H/D)$

Figure 3-3. (A) Graphic representation of the Ntlim1 protein structure. Positions of the two LIM domains (LIM1 and LIM2) and the carboxyl-terminal acidic region are indicated. (B) Double zinc-finger structures of LIM1 and LIM2 domains of Ntlim1. (C) LIM domain consensus.

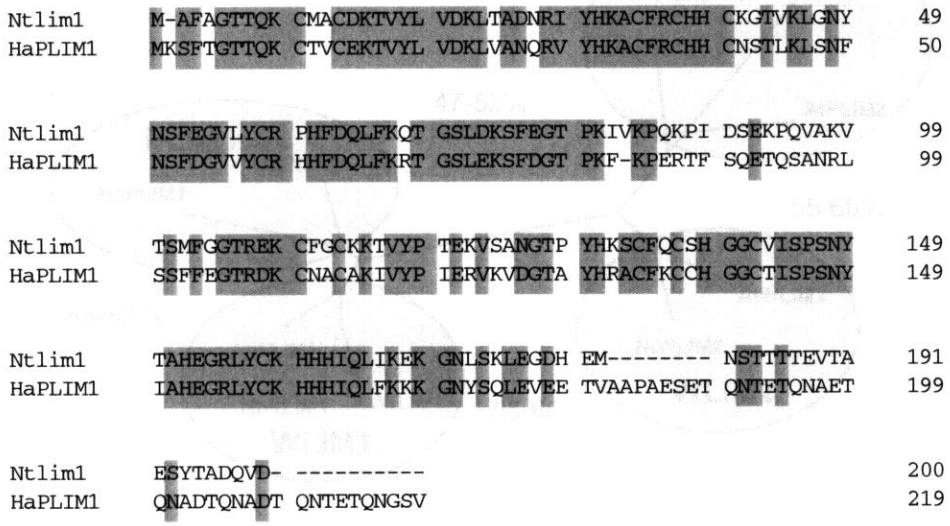


Figure 3-4. Alignment of deduced amino acid sequences: Ntlim1 *vs.* HaPLIM1 proteins. The two sequences share 55% identity and all of the putative metal-chelating residues are conserved.

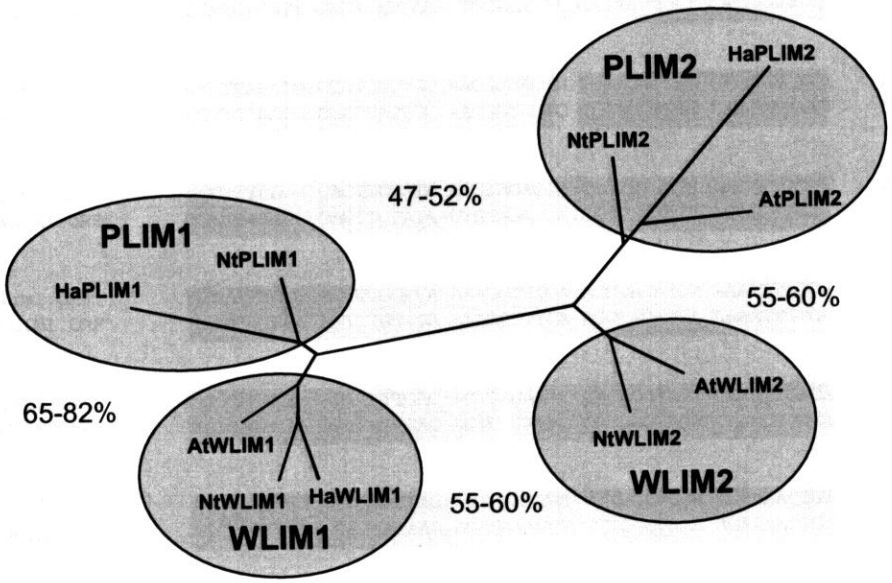


Figure 3-5. Phylogenetic relationships of plant LIM proteins. Four groups of LIM proteins are shown here, PLIM1 and PLIM2 groups are specifically expressed in pollen and WLIM1 and WLIM2 groups are expressed in almost all sporophytic tissues. Ha = *Helianthus annuus* (sunflower), Nt = *Nicotiana tabacum* (tobacco), At = *Arabidopsis thaliana* (thale cress). Numbers in percentage between groups show the range of sequence identities observed between the proteins in the groups (Eliasson *et al.*, 2000).

```
Ntlim1.ORF      ATGGCTTTTG CAGGAACCAC ACAGAAATGC ATGGCATGTG ACAAGACTGT   50
NtWLIM1.ORF     ATGGCTTTTG CAGGCACTAC GCAGAAATGC ATGGCATGTG ACAAGACTGC   50

Ntlim1.ORF      CTATCTGGTT GACAAATTAA CTGCAGATAA CAGAATCTAT CACAAAGCTT  100
NtWLIM1.ORF     CTATCTGGTT GACAAATTAA CTGCAGATAA CAGAATCTAT CACAAAGCTT  100

Ntlim1.ORF      GTTTCAGATG CCATCACTGC AAGGGCACTG TCAAGCTTGG CAACTACAAT  150
NtWLIM1.ORF     GTTTCAGATG CCATCACTGC AAGGGCACTC TCAAGCTTGG CAACTACAAT  150

Ntlim1.ORF      TCCTTTGAGG GAGTTCTATA CTGTAGACCA CACTTTGATC AGCTCTTCAA  200
NtWLIM1.ORF     TCCTTTGAGG GAGTTCTATA CTGTAGACCA CACTTTGATC AGCTCTTCAA  200

Ntlim1.ORF      ACAAACTGGC AGTTTGGATA AAAGCTTTGA AGGTACACCA AAAATTGTGA  250
NtWLIM1.ORF     ACAAACTGGC AGTTTGGATA AAAGCTTTGA AGGTACACCA AAAATTGTGA  250

Ntlim1.ORF      AGCCACAGAA ACCCATTGAC AGTGAGAAAC CACAGGTAGC CAAAGTGACA  300
NtWLIM1.ORF     AGCCACAGAA ACCCATTGAC AGTGGGAAAC CACAGGTAGC CAAAGTGACA  300

Ntlim1.ORF      AGCATGTTTG GTGGAACAAG AGAGAAATGT TTTGGCTGCA AGAAAACTGT  350
NtWLIM1.ORF     AGCATGTTTG GTGGAACAAG AGAGAAATGT TTTGGCTGCA AGAAAACTGT  350

Ntlim1.ORF      CTACCCAACA GAAAAGGTAT CAGCCAATGG CACGCCATAC CATAAGAGCT  400
NtWLIM1.ORF     CTACCCAACA GAAAAGGTAT CAGTTAATGG CACGCCATAC CATAAGAGCT  400

Ntlim1.ORF      GCTTCCAATG CAGCCACGGA GGCTGTGTAA TAAGCCCTTC CAACTATACC  450
NtWLIM1.ORF     GCTTCAAATG CAGCCACGGA GGCTGTGTAA TAAGCCCTTC CAACTATATC  450

Ntlim1.ORF      GCACATGAGG GGCGCTTATA TTGTAAACAT CACCATATTC AACTTATCAA  500
NtWLIM1.ORF     GCACATGAGG GGCGCCTATA TTGTAAACAT CACCATATTC AACTTATCAA  500

Ntlim1.ORF      GGAGAAGGGC AACTTAAGCA AGCTTGAGGG TGACCATGAA ATGAATTCCA  550
NtWLIM1.ORF     GGAGAAGGGC AACTTAAGCA AACTTGAGGG TGACCATGAA ATGAATTCCA  550

Ntlim1.ORF      CGACAACAAC AGAAGTTACT GCAGAGTCAT ACACAGCCGA CCAAGTTGAT  600
NtWLIM1.ORF     CGACAACAAC AGAAGTTACT GCAGAGTCA- ---------- ----------  579

Ntlim1.ORF      TGA                                                    603
NtWLIM1.ORF     ---                                                    579
```

Figure 3-6. Alignment of open reading frames: *Ntlim1 vs. NtWLIM1*. The two sequences share 94% identity.

```
Ntlim1    MAFAGTTQKC MACDKTVYLV DKLTADNRIY HKACFRCHHC KGTVKLGNYN   50
NtWLIM    MAFAGTTQKC MACDKTAYLV DKLTADNRIY HKACFRCHHC KGTLKLGNYN   50

Ntlim1    SFEGVLYCRP HFDQLFKQTG SLDKSFEGTP KIVKPQKPID SEKPQVAKVT  100
NtWLIM    SFEGVLYCRP HFDQLFKQTG SLDKSFEGTP KIVKPQKPID SGKPQVAKVT  100

Ntlim1    SMFGGTREKC FGCKKTVYPT EKVSANGTPY HKSCFQCSHG GCVISPSNYT  150
NtWLIM    SMFGGTREKC FGCKKTVYPT EKVSVNGTPY HKSCFKCSHG GCVISPSNYI  150

Ntlim1    AHEGRLYCKH HHIQLIKEKG NLSKLEGDHE MNSTTTTEVT AESYTADQVD  200
NtWLIM    AHEGRLYCKH HHIQLIKEKG NLSKLEGDHE MNSTTTTEVT AES-------  193
```

Figure 3-7. Alignment of deduced amino acid sequences: Ntlim1 *vs.* NtWLIM1 proteins. The two sequences share 94% identity and all of the putative metal-chelating residues are conserved.

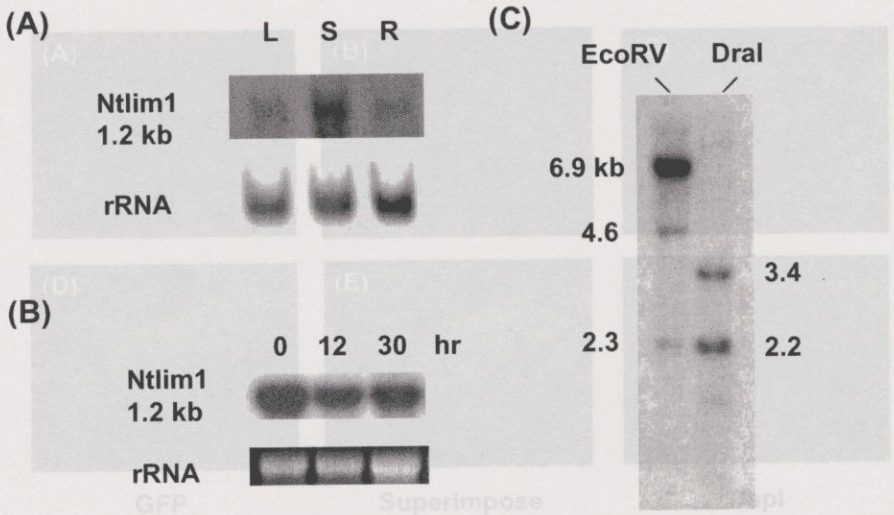


Figure 3-8. (A) Tissue specific expression of *Ntlim1*. Total RNAs derived from leaf (L), stem (S), and root (R) of tobacco plants were probed with radio-labeled cDNA fragment of *Ntlim1*. (B) *Ntlim1* is a constitutively expressed gene. Total RNAs (10-20 μg/lane) extracted from tobacco leaves 0-30 h after wounding were probed with radio-labeled cDNA fragment of *Ntlim1*. (C) Southern blot analysis of the *Ntlim1* gene. Tobacco genomic DNA (20 μg/lane) was digested with restriction enzymes *Eco*RV or *Dra*I and probed with radio-labeled cDNA fragment of *Ntlim1* under high stringency condition.

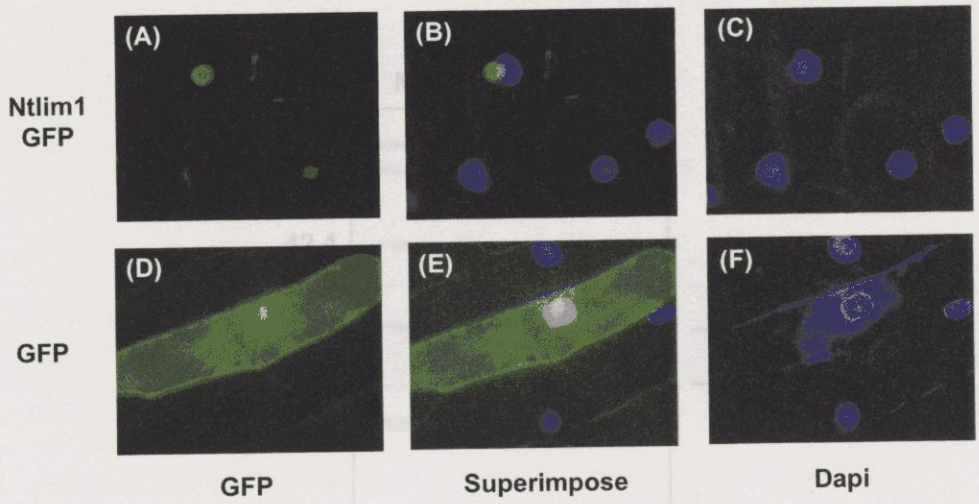


Figure 3-9. Nuclear localization of Ntlim1::GFP fusion proteins. Constructs encoding the Ntlim1::GFP fusion protein and the GFP protein were introduced into onion epidermal cells by particle bombardment. After 24 h of incubation, subcellular localizations of the Ntlim1::GFP and the GFP proteins were visualized under fluorescence microscope (A and D, respectively). The nucleus of each cell was visualized by DAPI staining (C and F). B is the superimpose picture of A and C and E is the superimpose picture of D and F, respectively.

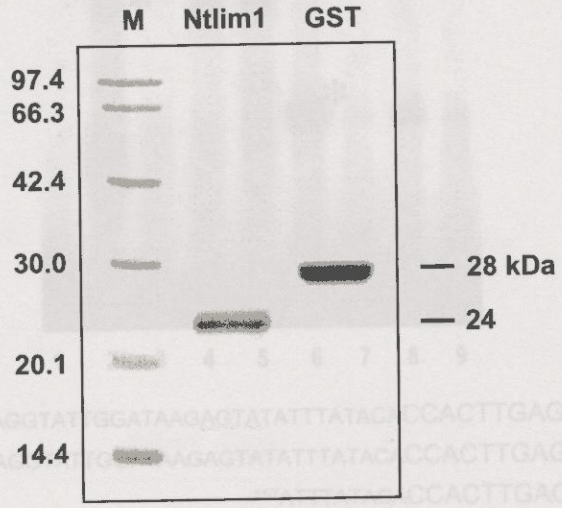
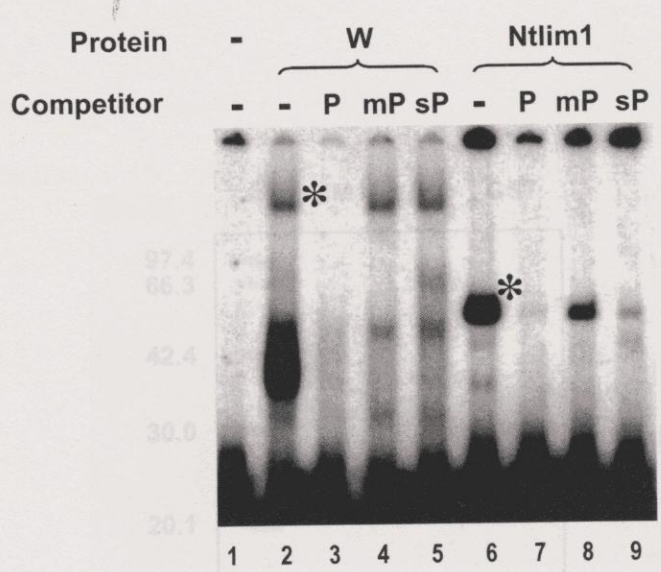


Figure 3-10. SDS-PAGE of Ntlim1 (24 kDa) and GST (28 kDa) proteins. M indicates molecular mass marker in kDa.



Probe P agct^{-178}TCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGTACAA^{-135}

Probe mP $^{-178}$TCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGTGTAA^{-135}

Probe sP $^{-157}$ATTTATACACCACTTGAGTACAAAT^{-133}

Figure 3-11. Ntlim1 specifically binds to PAL box region of POP element in gel shift assay. Binding reactions contain 2 ng of radio-labeled probe P (POP element) and 2 μg of nuclear protein extracted from wounded tobacco leaves (lanes 2-5), or 500 ng of purified Ntlim1 (lanes 6-9), in the absence or in the presence of 50 fold molar excess of the competitors cold probe P, cold probe mP (POP element with 2-base substitution in the PAL-box region), or cold probe sP (PAL-box region) as indicated. Asterisks indicate specific DNA-protein complexes. Oligonucleotide sequences of probe P, probe mP, and probe sP are shown. Lower case letters indicate additional sequence for restriction enzyme (*Hin*dIII) digestion. Bold letters indicate the PAL-box region and the position of 2-base substitution in the PAL-box region is underlined.

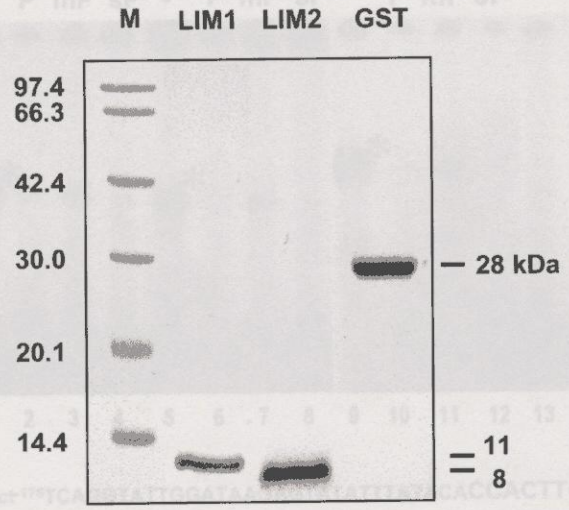
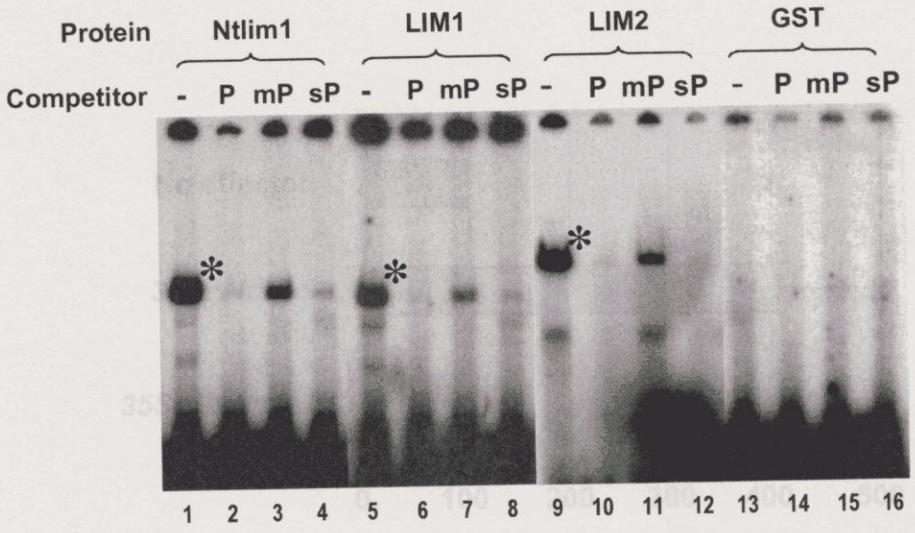


Figure 3-12. SDS-PAGE of LIM1 (11 kDa), LIM2 (8 kDa), and GST (28 kDa) proteins. M indicates molecular mass marker in kDa.



Probe P agct^{-178}TCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGTACAA^{-135}

Probe mP $^{-178}$TCAGGTATTGGATAAGAGTATATTTATACACCACTTGAGTGTAA^{-135}

Probe sP $^{-157}$ATTTATACACCACTTGAGTACAAAT^{-133}

Figure 3-13. Ntlim1 proteins bind to PAL box region of POP element through its LIM domains in gel shift assay. Each reaction contains 2 ng of the radio-labeled probe P (POP element) and 500-600 ng of Ntlim1 (lanes1-4), LIM1 (lanes 5-8), LIM2 (lanes 9-12), or GST (lanes 13-16) proteins. The binding reactions were carried out in the absence or in the presence of 50 fold molar excess of cold probe P, probe mP (POP element with 2-base substitution in the PAL-box region), or probe sP (PAL-box region) as indicated on the top of each lane. Asterisks indicate specific DNA-protein complexes. Oligonucleotide sequences of probe P, probe mP, and probe sP are shown. Lower case letters represent the additional sequence for restriction enzyme (*Hin*dIII) digestion. Bold letter indicate the PAL-box region and the position of 2-base substitution in the PAL-box region is underlined.

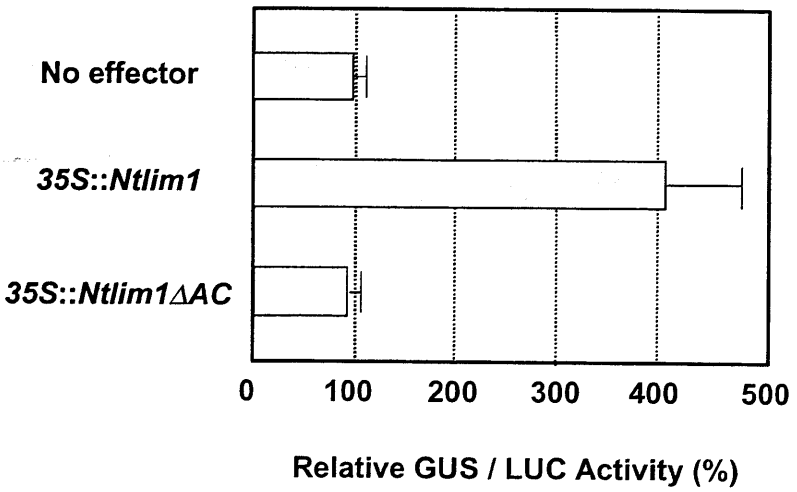


Figure 3-14. Ntlim1 activates expression of *prxC2* promoter::*GUS* reporter construct in transient expression assay in protoplasts of tobacco BY2 cells. In this assay, the *prxC2* promoter::*GUS* reporter construct, the *35S::LUC* internal control construct, and the *35S::Ntlim1* or the *35S::Ntlim1ΔAC* effector constructs were introduced together into the BY2 protoplasts and the GUS/LUC activity in the protoplast extracts was measured 24 h after the introduction. Bars indicate standard error of 3 independent experiments.

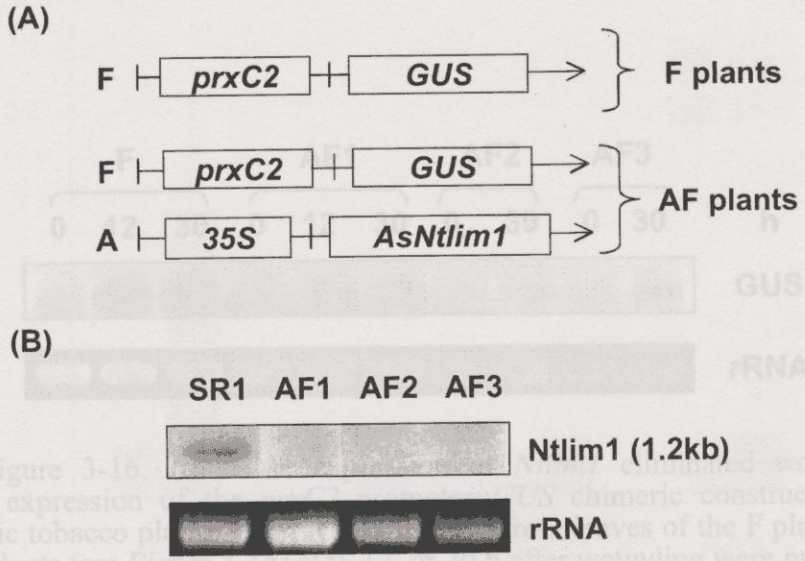


Figure 3-15. (A) Constructs used to transform tobacco plants. *PrxC2* = intact *prxC2* promoter, *GUS* = open reading frame (ORF) of *GUS* reporter gene, *35S* = Cauliflower mosaic virus 35S promoter, *AsNtlim1*= Antisense sequence of *Ntlim1* ORF (1 kb). (B) Double transformed tobacco plants (AF plants) have lower level of *Ntlim1* expression than wild-type tobacco plant (SR1). Total RNAs derived from leaves of the wild-type tobacco plant (SR1) or the double transformed tobacco plants (AF1, AF3, & AF4) were probed with radio-labeled cDNA fragment of *Ntlim1* (1.2 kb). The amount of rRNAs stained with ethidium bromide is shown here as loading amount control.

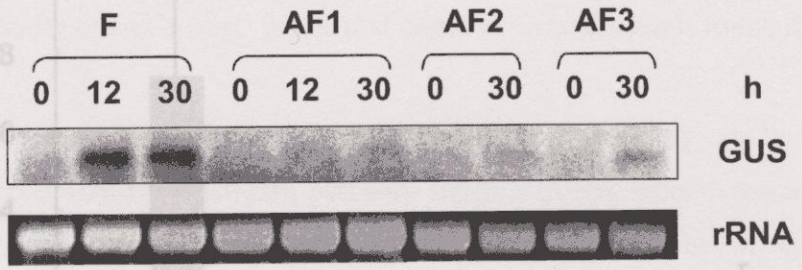


Figure 3-16. Antisense expression of *Ntlim1* eliminated wound-induced expression of the *prxC2* promoter::*GUS* chimeric constructs in transgenic tobacco plants. Total RNAs derived from leaves of the F plant or the AF plants (see Figure 3-15) at 0, 12, or 30 h after wounding were probed with radio-labeled cDNA fragment of *Ntlim1*. The amount of rRNAs stained with ethidium bromide is shown here as loading amount control.

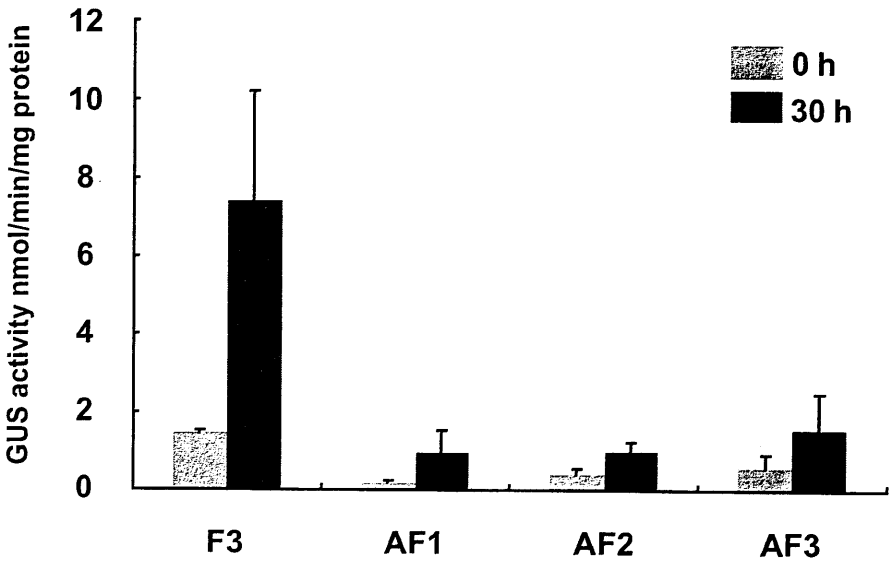


Figure 3-17. Antisense expression of *Ntlim1* eliminated wound-induced GUS activity detected in transgenic tobacco plants transformed with the *prxC2* promoter::*GUS* chimeric construct. Specific GUS activity (nmol/min/mg protein) in leaves of F plants or AF plants (see Figure 3-15) was measured at 0 and 30 h after wounding. Error bars indicate standard deviation from mean value of GUS activity detected in 3 plants of each line.

CONCLUSIONS

Given the data obtained from the present and the previous work, we propose a working model for wound-induced transcriptional regulation of the *prxC2* gene (Figure I). In this model, the G box and the POP element work in concert to control the wound-induced expression of the *prxC2* gene and the Ntlim1, which binds specifically to the PAL-box region of the POP element, functions as a *trans* factor that conveys wound signals to the *prxC2* promoter.

Based on the information presented here, further study can be done in order to define the upstream pathway leading to this wound-responsive transcriptional regulation. For example, the use of yeast two-hybrid system to screen for protein partner(s) of Ntlim1 might help us find other components of the oligomeric POP-element binding complex. Moreover, since at least 2 tobacco MAP kinases, WIPK (Seo *et al.*, 1999) and SIPK (Zhang and Klessig, 1998), are activated in response to wound stress, it is of our interest to investigate the relationship between the MAPK cascade and the wound-responsiveness of the *prxC2* promoter. We plan to introduce the *prxC2* promoter::*GUS* chimeric construct into transgenic plant with low level of *WIPK* expression (a gift from Dr. Yuko Ohashi, Department of Molecular Genetics, National Institute of Agrobiological Resources, Tsukuba, Japan). The difference in the level of wound-induced expression of the *GUS* reporter gene in the double transformed tobacco plants and in the tobacco plants single-transformed with the *prxC2* promoter::*GUS* construct will be observed.

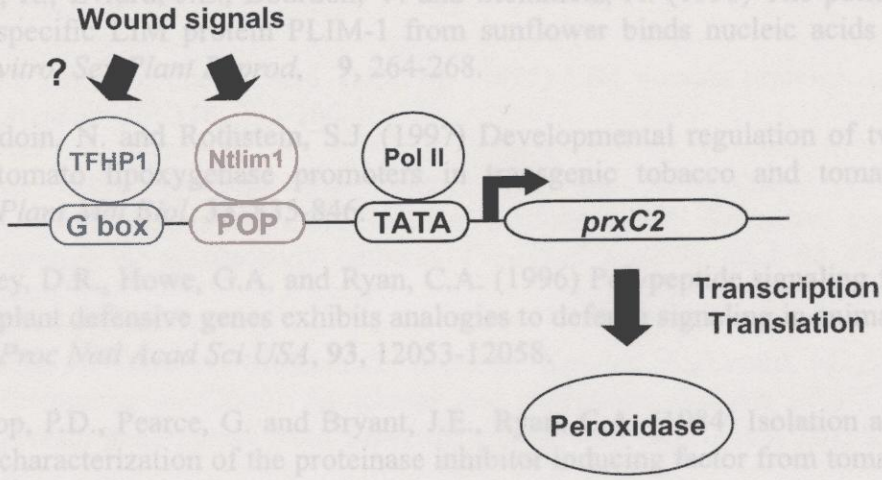


Figure I. Molecular mechanism underlying the wound-induced expression of the *prxC2* gene.

REFFERENCES

Baker, C.J., Deahl, K., Domek, J. and Orlandi, E.W. (2000) Scavenging of H_2O_2 and production of oxygen by horseradish peroxidase. *Arch Biochem Biophys*, **382**, 232-237.

Baltz, R., Evrard, J.L., Bourdon, V. and Steinmetz, A. (1996) The pollen-specific LIM protein PLIM-1 from sunflower binds nucleic acids *in vitro*. *Sex Plant Reprod*, **9**, 264-268.

Beaudoin, N. and Rothstein, S.J. (1997) Developmental regulation of two tomato lipoxygenase promoters in transgenic tobacco and tomato. *Plant Mol Biol*, **33**, 835-846.

Bergey, D.R., Howe, G.A. and Ryan, C.A. (1996) Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proc Natl Acad Sci USA*, **93**, 12053-12058.

Bishop, P.D., Pearce, G. and Bryant, J.E., Ryan, C.A. (1984) Isolation and characterization of the proteinase inhibitor inducing factor from tomato leaves: identification and activity of poly- and oligo-galacturonide fragments. *J. Biol. Chem.*, **259**, 13172-13177.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-254.

Capellades, M., Torres, M.A., Bastisch, I., Stiefel, V., Vignols, F., Bruce, W.B., Peterson, D., Puigdomenech, P. and Rigau, J. (1996) The maize caffeic acid *O*-methyltransferase gene promoter is active in transgenic tobacco and maize plant tissues. *Plant Mol Biol*, **31**, 307-322.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc Natl Acad Sci U S A*, **81**, 1991-1995.

Clarke, H.R., Davis, J.M., Wilbert, S.M., Bradshaw, H.D., Jr. and Gordon, M.P. (1994) Wound-induced and developmental activation of a poplar tree chitinase gene promoter in transgenic tobacco. *Plant Mol Biol*, **25**, 799-815.

Curtis, M.D., Rae, A.L., Rusu, A.G., Harrison, S.J. and Manners, J.M. (1997) A peroxidase gene promoter induced by phytopathogens and

methyl jasmonate in transgenic plants. *Mol Plant Microbe Interact*, **10**, 326-338.

Dalkin, K. and Bowles, D.J. (1989) Local and systemic changes in gene experssion induced in tomato plants by wounding and by elicitor treatment. *Planta*, **179**, 367-375.

Dawid, I.B., Breen, J.J. and Toyama, R. (1998) LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet*, **14**, 156-162.

Dawid, I.B., Toyama, R. and Taira, M. (1995) LIM domain proteins. *C R Acad Sci III*, **318**, 295-306.

Doares, S.H., Syrovets, T., Weiler, E.W. and Ryan, C.A. (1995) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc Natl Acad Sci USA*, **92**, 4095-4098.

Eliasson, A., Gass, N., Mundel, C., Baltz, R., Krauter, R., Evrard, J.L., and Steinmetz, A. (2000). Molecular and expression analysis of a LIM protein gene family from flowering plants. *Mol Gen Genet*, **264**, 257-67.

Eulgem, T., Rushton, P.J., Robatzek, S. and Somssich, I.E. (2000) The WRKY superfamily of plant transcription factors. *Trends Plant Sci*, **5**, 199-206.

Eyal, Y., Sagee, O. and Fluhr, R. (1992) Dark-induced accumulation of a basic pathogenesis-related (PR-1) transcript and a light requirement for its induction by ethylene. *Plant Mol Biol*, **19**, 589-599.

Feuerstein, R., Wang, X., Song, D., Cooke, N.E. and Liebhaber, S.A. (1994) The LIM/double zinc-finger motif functions as a protein dimerization domain. *Proc Natl Acad Sci USA*, **91**, 10655-10659.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, **12**, 393-404.

Fujiyama, K., Takemura, H., Shinmyo, A., Okada, H. and Takano, M. (1990) Genomic DNA structure of two new horseradish-peroxidase-encoding genes. *Gene*, **89**, 163-169.

Fukasawa-Akada, T., Kung, S.D. and Watson, J.C. (1996) Phenylalanine ammonia-lyase gene structure, expression, and evolution in Nicotiana. *Plant Mol Biol*, **30**, 711-722.

Grantz, A.A., Brummell, D.A. and Bennett, A.B. (1995) Ascorbate free radical reductase mRNA levels are induced by wounding. *Plant Physiol*, **108**, 411-418.

Hara, K., Yagi, M., Kusano, T. and Sano, H. (2000) Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor upon wounding. *Mol Gen Genet*, **263**, 30-37.

Hatton, D., Sablowski, R., Yung, M.H., Smith, C., Schuch, W. and Bevan, M. (1995) Two classes of cis sequences contribute to tissue-specific expression of a PAL2 promoter in transgenic tobacco. *Plant J*, **7**, 859-876.

Higuchi, R. (1990) *PCR protocol: A guide to methods and applications*. Acadamic Press, CA.

Hildmann, T., Ebneth, M., Pena-Cortes, H., Sanchez-Serrano, J.J., Willmitzer, L. and Prat, S. (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. *Plant Cell*, **4**, 1157-1170.

Hiraga, S., Ito, H., Sasaki, K., Yamakawa, H., Mitsuhara, I., Toshima, H., Matsui, H., Honma, M. and Ohashi, Y. (2000) Wound-induced expression of a tobacco peroxidase is not enhanced by ethephon and suppressed by methyl jasmonate and coronatine. *Plant Cell Physiol*, **41**, 165-170.

Howe, G.A., Lightner, J., Browse, J. and Ryan, C.A. (1996) An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell*, **8**, 2067-2077.

Huh, G.H., Lee, S.J., Bae, Y.S., Liu, J.R. and Kwak, S.S. (1997) Molecular cloning and characterization of cDNAs for anionic and neutral peroxidases from suspension-cultured-cells of sweet potato and their differential expression in response to stress. *Mol Gen Genet*, **255**, 382-391.

Ishige, F., Mori, H., Yamazaki, K. and Imaseki, H. (1993) Identification of a basic glycoprotein induced by ethylene in primary leaves of azuki bean as a cationic peroxidase. *Plant Physiol*, **101**, 193-199.

Ito, H., Kimizuka, F., Ohbayashi, A., Matsui, H., Honma, M., Shinmyo, A., Ohashi, Y., Caplan, A.B. and Rodrigues, R.L. (1994) Molecular cloning and characterization of two complementary DNAs encoding putative peroxidases from rice (*Oryza sativa* L.) shoots. *Plant Cell Reports*, **13**, 361-366.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *Embo J*, **6**, 3901-3907.

Kanofsky, J.R. (1988) Singlet oxygen production from the peroxidase-catalyzed oxidation of indole-3-acetic acid. *J Biol Chem*, **263**, 14171-14175.

Kawaoka, A., Kaothien, P., Yoshida, K., Endo, S., Yamada, K. and Ebinuma, H. (2000) Functional analysis of tobacco LIM protein Ntlim1 involved in lignin biosynthesis. *Plant J*, **22**, 289-301.

Kawaoka, A., Kawamoto, T., Ohta, H., Sekine, M., Takano, M. and Shinmyo, A. (1994a) Wound-induced expression of horseradish peroxidase. *Plant Cell Reports*, **13**, 149-154.

Kawaoka, A., Kawamoto, T., Sekine, M., Yoshida, K., Takano, M. and Shinmyo, A. (1994b) A *cis*-acting element and a *trans*-acting factor involved in the wound- induced expression of a horseradish peroxidase gene. *Plant J*, **6**, 87-97.

Kawaoka, A., Shinichi, S., Nakahara, K., Matsushima, N., Okada, N., Sekine, M., Shinmyo, A., Takano, M. (1992) Expression and promoter activity of genes for isozymes of horseradish peroxidase. *Plant cell Physiology*, **33**, 1143-1150.

Klimczak, L.J., Schindler, U. and Cashmore, A.R. (1992) DNA binding activity of the Arabidopsis G-box binding factor GBF1 is stimulated by phosphorylation by casein kinase II from broccoli. *Plant Cell*, **4**, 87-98.

Lightner, J., Pearce, G., Ryan, C.A. and Browse, J. (1993) Isolation of signaling mutants of tomato (Lycopersicon esculentum). *Mol Gen Genet*, **241**, 595-601.

Logemann, E., Parniske, M. and Hahlbrock, K. (1995) Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. *Proc Natl Acad Sci USA*, **92**, 5905-5909.

Lois, R., Dietrich, A., Hahlbrock, K. and Schulz, W. (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. *Embo J*, **8**, 1641-1648.

Mäder, M. and Füssl, R. (1982) Role of peroxidase in lignification of tobacco cells. *Plant Physiol.*, **70**, 1132-1134.

Maniatis, t., Fritsch, E.F. and Sambrook, J. (1982) *Molecular cloning: A laboratory Manual.* Cold Spring Harbor Laboratory Press., Cold Spring Harbor, NY.

McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. and Vasil, I.K. (1991) The Viviparous-1 developmental gene of maize encodes a novel transcriptional activator. *Cell*, **66**, 895-905.

McConn, M., Creelman, R.A., Bell, E., Mullet, J.E. and Browse, J. (1997) Jasmonate is estential for insect defense in Arabidopsis. *Proc Natl Acad Sci USA*, **94**, 5473-5477.

Mohan, R., Bajar, A.M. and Kolattukudy, P.E. (1993) Induction of a tomato anionic peroxidase gene (*tap1*) by wounding in transgenic tobacco and activation of *tap1/GUS* and *tap2/GUS* chimeric gene fusions in transgenic tobacco by wounding and pathogen attack. *Plant Mol Biol*, **21**, 341-354.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plants*, **18**, 473-479

Nagata, T., Takabe, I., and Matsui, C. (1981) Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (liposomes). *Mol Gen Gener*, **184**, 161-165.

Nagata, Y. (1987) Interaction of plant protoplast and liposome. *Meth Enzymol*, **148**, 34-39.

Nakayama, H., Yoshida, K., Ono, H., Murooka, Y. and Shinmyo, A. (2000) Ectoine, the compatible solute of Halomonas elongata, confers hyperosmotic tolerance in cultured tobacco cells. *Plant Physiol*, **122**, 1239-1247.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N. and Ohashi, Y. (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogen-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol*, **39**, 500-507.

Nishiya, N., Sabe, H., Nose, K. and Shibanuma, M. (1998) The LIM domains of hic-5 protein recognize specific DNA fragments in a zinc-dependent manner in vitro. *Nucleic Acids Res*, **26**, 4267-4273.

Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. and Kobayashi, H. (1999) Non-invasive quantitative detection and applications of non-toxic, S65T- type green fluorescent protein in living plants. *Plant J*, **18**, 455-463.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. (1996) Ethylene as a Signal Mediating the Wound Response of Tomato Plants. *Science*, **274**, 1914-1917.

O'Donnell, P.J., Truesdale, M.R., Calvert, C.M., Dorans, A., Roberts, M.R. and Bowles, D.J. (1998) A novel tomato gene that rapidly responds to wound- and pathogen-related signals. *Plant J.*, **14**, 137-142.

Ohl, S., Hedrick, S.A., Chory, J. and Lamb, C.J. (1990) Functional properties of a phenylalanine ammonia-lyase promoter from Arabidopsis. *Plant Cell*, **2**, 837-848.

Palm, C.J., Costa, M.A., An, G. and Ryan, C.A. (1990) Wound-inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato. *Proc Natl Acad Sci USA*, **87**, 603-607.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science*, **253**, 895-898.

Pena-Corte, H., Fisahn, J. and Willmitzer, L. (1995) Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. *Proc Natl Acad Sci USA.*, **92**, 4106-4113.

Perez-Alvarado, G.C., Miles, C., Michelsen, J.W., Louis, H.A., Winge, D.R., Beckerle, M.C. and Summers, M.F. (1994) Structure of the carboxy-terminal LIM domain from the cysteine rich protein. *Nat Struct Biol*, **1**, 388-398.

Quiroga, M., Guerrero, C., Botella, M.A., Barcelo, A., Amaya, I., Medina, M.I., Alonso, F.J., de Forchetti, S.M., Tigier, H. and Valpuesta, V. (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. *Plant Physiol*, **122**, 1119-1127.

Roberts, E. and Kolattukudy, P.E. (1989) Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberization-associated

highly anionic peroxidase. *Mol Gen Genet*, **217**, 223-232.

Rogers, S.G., Hoesch, R.B. and Fraley, R.T. (1986) Gene transfer in plant: production of transformed plant using Ti plasmid vectors. *Meth Enzyml*, **118**, 627-640.

Rojo, E., Leon, J. and Sanchez-Serrano, J.J. (1999) Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. *Plant J*, **20**, 135-142.

Rouster, J., Leah, R., Mundy, J. and Cameron-Mills, V. (1997) Identification of a methyl jasmonate-responsive region in the promoter of a *lipoxygenase 1* gene expressed in barley grain. *Plant J*, **11**, 513-523.

Ryan, C.A. (2000) The systemin signaling pathway: differential activation of plant defensive genes. *Biochim Biophys Acta*, **1477**, 112-121.

Sanchez-Garcia, I. and Rabbitts, T.H. (1994) The LIM domain: a new structural motif found in zinc-finger-like proteins. *Trends Genet*, **10**, 315-320.

Sanchez-Serrano, J.J., Pena-Cortes, H., Willmitzer, L. and Prat, S. (1990) Identification of potato nuclear proteins binding to the distal promoter region of the proteinase inhibitor II gene. *Proc Natl Acad Sci USA*, **87**, 7205-7209.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H. and Ohashi, Y. (1995) Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. *Science*, **270**, 1988-1992.

Seo, S., Sano, H. and Ohashi, Y. (1999) Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. *Plant Cell*, **11**, 289-298.

Siebertz, B., Logemann, J., Willmitzer, L. and Schell, J. (1989) *cis*-analysis of the wound-inducible promoter wun1 in transgenic tobacco plants and histochemical localization of its expression. *Plant Cell*, **1**, 961-968.

Singh, H., LeBowitz, J.H., Baldwin, A.S., Jr. and Sharp, P.A. (1988) Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. *Cell*, **52**, 415-423.

Staiger, D., Kaulen, H. and Schell, J. (1989) A CACGTG motif of the *Antirrhinum majus* chalcone synthase promoter is recognized by an

evolutionarily conserved nuclear protein. *Proc Natl Acad Sci USA*, **86**, 6930-6934.

Stankovic, B. and Davies, E. (1996) Both action potentials and variation potentials induce proteinase inhibitor gene expression in tomato. *FEBS Lett*, **390**, 275-279.

Thara, V.K., Tang, X., Gu, Y.Q., Martin, G.B. and Zhou, J.M. (1999) Pseudomonas syringae pv tomato induces the expression of tomato EREBP- like genes pti4 and pti5 independent of ethylene, salicylate and jasmonate. *Plant J*, **20**, 475-483.

van Der Krol, A.R., van Poecke, R.M., Vorst, O.F., Voogt, C., van Leeuwen, W., Borst-Vrensen, T.W., Takatsuji, H. and van Der Plas, L.H. (1999) Developmental and wound-, cold-, desiccation-, ultraviolet-B-stress-induced modulations in the expression of the petunia zinc finger transcription factor gene ZPT2-2 [In Process Citation]. *Plant Physiol*, **121**, 1153-1162.

Wadman, I.A., Osada, H., Grutz, G.G., Agulnick, A.D., Westphal, H., Forster, A. and Rabbitts, T.H. (1997) The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *Embo J*, **16**, 3145-3157.

Weiss, C. and Bevan, M. (1991) Ethylene and a wound signal modulate local and systemic transcription of *win2* genes in transgenic potato plants. *Plant Physiol.*, **96**, 943-951.

Wildon, D.C., Thain, J.F., Minchin, P., Gubb, I., Reilley, A., Skipper, Y., Doherty, H., O'Donnell, P. and Bowles, D.J. (1992) Electrical signalling and systemic proteinase inhibitor induction in the wounded plant. *Nature*, **360**, 62-65.

Yanagisawa, S. and Sheen, J. (1998) Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. *Plant Cell*, **10**, 75-89.

Zhang, S. and Klessig, D.F. (1998) The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. *Proc Natl Acad Sci USA*, **95**, 7225-7230.

LIST OF PUBLICATIONS

1. Kaothien P., Shimokawatoko, Y., Kawaoka, A., Yoshida, K., Shinmyo, A. (2000) A *cis* element containing PAL-box functions in the expression of the wound-inducible peroxidase gene of horseradish. *Plant Cell Reports*, **19**, 558-562.

2. Kawaoka, A., Kaothien, P., Yoshida, K., Endo, S., Tamada, K., Ebinuma, H. (2000) Functional analysis of tobacco LIM protein Ntlim1 involved in lignin biosynthesis. *Plant Journal*, **22**, 289-301.

3. Kaothien P., Kawaoka, A., Ebinuma, H, Yoshida, K., Shinmyo, A. (2000) Tobacco PAL-box binding factor, Ntlim1, regulates the expression of genes encoding the enzymes of phenylpropanoid pathway. *Proceeding of the 5th International Symposium on Environmental Biotechnology.*

4. Kaothien, P., Kawaoka, A., Yoshida, K., Shinmyo, A. *Cis*-element and *trans*-factor controlling the wound responsiveness of a horseradish peroxidase promoter. *In preparation.*

ACKNOWLEDGMENTS

"Now, the only part of this thesis that can never go wrong."

I would like to give my sincerest thanks to the most wonderful *senseis*, Professor Atsuhiko Shinmyo and Associate Professor Kazuya Yoshida. Thank you very much for helping me bringing this work from an idea to a finished piece of research. Working under your supervision was a terrific experience.

I would like to give a special thank to my committee members, Professor Hiroshi Sano, Professor Akiho Yokota, and Professor Takashi Hashimoto. This work would not come to completion without you. I truly appreciate your time and attention. Thank you very much.

I would like to give my very special thanks to Professor Dee Worman. Thank you very much for teaching me how to write scientific paper. I would take centuries to write this thesis without your advices.

I would like to thank Dr. Masami Sekine and Dr. Ko Kato for keeping this laboratory so well organized and for giving us a very "sparkling" research atmosphere. Working here would never be so smooth and entertaining without you.

I would like to give my sincere thanks to Dr. Akiyoshi kawaoka and Mr. Yasutaka Shimokawatoko. Our papers would never come to completion without your invaluable data and your collaboration.

I would like to thank Professor Amaret Bhumiratana, Proffessor Watanalai Panbangread, and Professor Gunjana Theeragool for giving me chances and motivation to work in the field of Sciences. You have sent me down the right path. I have found my dream and my future here.

I would like to thank Ministry of Education, Culture and Science of Japan for giving me 6 years of financial support, and more importantly, this precious education opportunity.

I would like to give my very special thanks to Dr. Hideki Nakayama. You might have worked as hard as me on this thesis. Thank you for teaching me everything from the experiment stuffs to the computer stuffs and a lot of thanks for staying up with me as I ran back and forth getting this thesis done. And... sorry for your car, it was not my intention!

I would like to thank members of Shinmyo laboratory: Thank you for making my years in Japan so special. Ohashi *san*, and Chieko *san*, you are among the very first people I met here. Thank you very much for taking care of me in the "transition stage". Asao *san*, thank you not only for teaching me from scratch but also for the most delicious strawberries... I was just wondering, are they transgenics? Yamanaka *san*, thank you very much for your help. You are the best secretary ever! Dan *tchan* thank you very much for your friendship and for the *sugoku oishii sukiyaki*. Hori, what a wonderful trip we had! 13 flights and a lift in cop's car. Extraordinary trip!

Special thanks also go to my family and friends: Thank you Papa, Mama, Paula, grandpa, and grandmas for your support and your loves. Eiji, thank you for everything. I just hope I can thank you enough for what you have done for me. It is your turn now to write a thesis. I know you are a very good writer and you are so smart, so, there is only one advice left for me to give to you – wear the right soccer shoes! Beer, thank you for all the wonderful dinners. Pom, Son, Noi, Aor, Kae, and Jane, thank you for your friendships and for keeping in touch.

It is my wish to thank everyone who has contributed at any rate to this work; however, I will have to stop here to avoid making this already-very-long "acknowledgements" the longest part of this thesis.

"*Minna san, nagai aida osewa ni narimashita.*"

Cold morning of spring, 2001

Pulla Kaothien