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要旨

Plants cope with environmental stress by transducing external signals into cells through interlinked signaling pathways, which ultimately activate defense responses by up-regulating a series of defense-related genes. Among those signaling pathways, MAPK (mitogen-activated protein kinase) cascade is known to be one of the most conserved and representative pathways in eukaryotes.

In tobacco plants, wound induced protein kinase (WIPK) has been known as a typical MAPK which plays a central role in stress responses, including wounding and pathogen infection. In our previous studies for searching down-stream elements of WIPK, a novel transcription factor was identified and designated as NtWIF (*N. tabacum* WIPK interacting factor). However, its molecular function and physiological roles in tobacco plants have remained to be elucidated.

The first chapter of this study discusses the relationship between NtWIF and WIPK. The full-length NtWIF was shown to interact with WIPK, and especially its C-terminus was found to be essential for interaction with WIPK. NtWIF was also shown to be specially was phosphorylated at the N-terminus but not the C-terminus by WIPK. Dual-luciferase assay indicated that co-expression of NtWIF with WIPK increased transcriptional activity of NtWIF. The phosphorylation site was found to be Thr-Pro at positions 109 and 110 in the N- terminus, being essential for WIPK-mediated transcriptional activation. These results suggested that NtWIF is a down-stream substrate of WIPK, which is directly phosphorylated by WIPK, thereby being activated for transcription of target gene(s) involved in defense.

In the second and third chapter of this study, transgenic tobacco plants were constructed, in which

NtWIF was over-produced (sense-transgenic lines) or suppressed (RNAi lines) in order to examine physiological functions of NtWIF, and also to identify genes that are potentially controlled by NtWIF. The results showed that the presence both WIPK and NtWIF and their activation under stress environments were necessary for resistance in tobacco plants. NtWIF contains a specific motif similar to the B3 DNA binding domain, which recognizes the core TGTCTC motif called the auxin-responsive element (ARE). Using synthetic ARE sequences, NtWIF was shown to recognize degenerated core ARE motifs and to transactivate the Luciferase (Luc)-reporter gene driven by such AREs. In order to identify direct target genes of NtWIF, up-regulated genes by NtWIF were screened using the microarray, and 178 genes were initially identified. Among these genes, pathogenesis-related (PR)-Q and glucanase were selected as representative genes and analyzed in detail. PR-Q promoter-driven Luc activity was actually increased by co-expression with NtWIF and WIPK. Moreover, WIPK itself, which contained the ARE motif on its promoter, was also shown to be activated by NtWIF and WIPK. These results suggest that first, direct down-stream cis-elements of NtWIF was the core ARE (TGTCTC)-like motif, and second, expression of WIPK might be regulated by NtWIF in a feedback manner, thereby forming a self-control-led MAPK cascade.

In the fourth chapter, NtWIF was shown to be involved not only in defense response but also in plant development, particularly, in seed and root development. Data analysis of the microarray screening indicated that NtWIF was involved in up-regulation of multifunctional genes, which play roles in not only defense responses but also growth and development. This was also confirmed in NtWIF RNAi lines, which exhibited a decrease of seed pod size, incomplete development of embryo and endosperm and defective development of primary and lateral roots of seedlings.

Overall, the present study revealed that WIPK directly phosphorylates a transcription factor, which broadly functions in plant metabolic pathways, including not only pathogen/wound response but in developmental processes. These findings would broaden our knowledge on MAPK cascade, which is believed to be one of the most important and common signaling systems in eukaryotic organisms.

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Down-stream Components of MAP Kinase Cascade in Tobacco Plants

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INTRODUCTION

Plants are constantly exposed to diverse environmental stresses which are divided into **abiotic factors** and **biotic factors. Abiotic factors include** nutrients, light, salinity, high or low temperature and touch, and **biotic factors** include challenge by other organisms. Under those inescapable environmental stresses, plants have adapted, minimized damages and also protected themselves through their specific defense mechanisms. These abilities of plants are considered to be essential for normal growth and development. Among diverse stress factors, wounding and pathogen attack are known to be the severest stress and plants` responses to them have been intensively studied.

Wounding and its signaling

Plants are commonly wounded by abiotic factors such as wind, rain, mechanical damage and by biotic factors such as insect feeding (Cheong et al., 2002). Wounding causes decompartmentalization, release of stored materials and excessive loss of water in plant cells (Pearce et al., 1991). In response to wounding stress, a set of genes which are involved in healing and defense reaction are locally and systemically induced. Typical genes include those encoding extensin for reinforcement of cell wall, proteinase inhibitor (PI) for interfering with digest in insect gut, and basic pathogenesis-related (PR) proteins for anti-microbial activities (Green and Ryan, 1972; Terras et al., 1995;

Epple et al., 1997; Kende, 1993). However, mechanism of **wounding signal perception and transduction** from wounded leaf (site) to unwounded leaf (site) are not completely understood. Participations of fatty acid (octadecanoid), peptide, oligosaccharides, Ca^{2+}/camodulin and protein kinases-mediated signaling have been reported. In addition to these signals, plant hormones including ethylene, abscisic acid, auxin and reactive oxygen species (ROS), together with their cross-talking have also been implicated in wound signaling (Kernan and Thornburg, 1989; Leon et al., 2001).

In response to pathogens, plants also induce defense responses through diverse combination of these components.

Pathogen and its signaling

Prior to initiation of defense responses, pathogen recognition is prerequisite, which is mediated by two major mechanisms. In non-host resistance, elicitors which are derived from fungal or plant cell wall fragments are recognized by non-specific receptor(s) at plasmamembrane. In race specific resistance, gene product encoded by avirulence (*Avr*) gene of the pathogen is recognized by resistance (R) gene product of plant (gene-for-gene model) (Flor, 1971). After perception of signals, local defense response called **hypersensitive response** (HR) is induced, which plays a role in restricting pathogens to small regions by lesion formation through programmed cell

death (Ueda et al., 2006; Kombrink and Somssich, 1995). In addition to these local defense response, uninfected leaf develops `**systemic acquired resistance** (SAR)` which contributes resistance to the initial or even unrelated pathogens (Yang et al., 1997) During both HR and SAR, plants induce expression of diverse defense genes including *PR-1a* (encoding a protein of unknown function), *PR2* (β-1,3-glucanase) and PAL (Phenylalanine ammonia-lyase) (related to cell-wall associated defense compounds) (Rodrigo et al., 1989) and the accumulate of phytoalexins and cell wall components such as hydroxyproline-rich glycol protein. The HR and SAR also accompany changes in flux of calcium ion, protein kinases activation, diverse hormones and signaling compounds including salicylic acid (SA), nitric oxide (NO), ethylene (ETH) and jasmonic acid which activate downstream signaling.

Prior to induction of defense events and terminal gene expression, multiple signal transduction networks are switched on, leading to activation of transcriptional machinery (Reymond et al., 2000 ; Ryan, 2000).

MAP kinase cascade

Among signal transduction pathways for wounding and pathogen, **mitogen-activated protein kinase (MAPK) cascade** is one of the conserved and representative pathways in eukaryotes, by transducing signals into cells. (Hirt, 1997 ;

Kultz, 1998). Its importance has been evidenced in several studies using protein kinase inhibitors or phosphatases. For example, harpin induces rapid and transient activation of 49-kD MAPK (Ádám et al., 1997) and harpin-induced HRs including oxidative burst (Baker et al., 1993), alkalization of the extracellular medium (He et al., 1994), ion flux and membrane depolarization (Popham et al., 1995) are blocked by protein kinase phosphatase.

MAPK cascade is composed of three-tier kinases, including MAPK extracelluar regulated kinase kinase (**MEKK**), MAPK-extracellular regulated kinase (**MEK**) and **MAPK**, which phosphorylate and activate each other in a linear pathway. (Morris, 2001). After external signals are recognized, MEKK is activated. Its activation mechanism is known to be diverse, ranging from the phosphorylation by MEKKK or protein kinase C, to activation via interaction with G proteins, or two-component receptor systems (Fanger et al., 1997; Wurgler-Murphy and Saito, 1997). The activated MEKK also activates MEK via phosphorylates on two serine(S)/ threonine(T) residues of MEK, which in turn activates MAPK. Activation of MAPK is mediated by the dual phosphorylation of threonine(T) and tyrosine(Y) residues which are located between subdomain VII and VIII of the kinase catalytic domain (Gartner et al., 1992). Generally, based on the kind of one amino acid which is located between these T and Y and which

can be Glutamine (E), Proline (P) or Glycine (G), MAPK has been divided into three groups. As the first groups, there are ERK (extracellular signal-regulated kinase) 1 and 2 (Seger and Krebs, 1995). They have TEY motif as a phosphorylation site and it is known that they are activated by diverse mitogen and they are involved in differentiation and reentry into the cell cycle. As the second group which has TPY motif for its phosphorylation, there is the Jun N-terminal kinase/ stress activated protein kinases (JNK/SAPK). Lastly, the p38 and Hog1-homologous kinases belong to the third group which has TGY motif as its phosphorylation motif. Both the second and the third group kinases are involved in the response to stress signals (Canman and Kastan, 1996). In the case of plant MAPKs, D (asparagine) can also be located between T and Y, and this TDY typed MAPK of plants is known to be close to these p38 and Hog1-homologous kinases. Moreover, all MAPKs of plants have only either a TEY or TDY as their phosphorylation site (Mishra et al., 2006).

Recent genome projects focused on mammals and plants have identified a number of genes encoding MAP Kinase and other components of MAP kinase signal transduction pathways, as exemplified by the Arabidopsis genome, now shown to possess 20 kinds of MAPK, 10 MEKs and 60 MEKKs. These kinases are considered to contribute not only to defense against biotic and abiotic stresses (Yang et al., 2001;

Jonak et al., 1996; Seo et al., 1995; Zhang and Klessig, 1998), but also to other physiological processes, such as regulation of cell growth and cell cycle (Soyano et al., 2002; 2006; Bogre et al., 1998; Hirt, 2000; Ligterink, 2000; Ligterink and Hirt 2001; Morris, 2001).

MAP kinase cascades which are related to stress responses

Plant MAPK cascades have been studied through correlation with biotic and abiotic stresses. For example, in parsely, after *Phytophthora*-derived 13 amino acids peptide fragment (Pep-13) was recognized by a receptor, PcMKK5 was activated with Pc MPK3 and 6, which are required for PR gene promoter activation (Lee et al., 2004).

AtMPK3, AtMPK4, AtMPK6, and AtMEKK1 from Arabidopsis, SAMK, SIMK and SIMKK from alfalfa, and WIPK and SIPK from tobacco have been shown to be involved in not only biotic stress such as pathogen stresses but also abiotic stress such as drought, low or high temperature and osmotic stress (Jonak et al., 1996; Mizoguchi et al., 1996, Droillard et al., 2000; Iajczyk et al., 2000; Kiegerl et al. 2000). Specially, WIPK (wound induced protein kinase) in tobacco vs. AtMPK3 in Arabidopsis and SIPK (Salicyslic induced protein kinase) in tobacco vs. AtMPK6 in Arabidopsis have been attracted interests of researchers as representative orthologs between two plant species.

WIPK is known as one of representative MAPKs in tobacco plant which was first

isolated by differential screening of cDNA library for identifying genes which are involved in wounding and lesion formation after TMV infection. Its transcripts were induced as early as 1 min after mechanical wounding in both wounded and adjacent unwounded leaves and it is involved in JA and MeJA biosynthesis during wounding stress showing induction of acidic PR transcripts and suppression of JA biosynthesis in response of WIPK co-suppressed transgenic lines to wounding (Seo et al., 1995). This speculation was then supported by another report showing that high level of JA biosynthesis and basic PR transcripts in WIPK over-expressing lines (Seo et al. 1999).

As another representative MAPK of tobacco plants, SIPK was identified by Zhang and Klessig (1997). They suggested that wounding activated protein kinase is encoded by SIPK rather than WIPK. Moreover, many other studies showed that SIPK is responsive to a number of biotic and abiotic stresses, including pathogen or elicitor, ozone, wounding, salt and osmolarity stresses (Romeis et al., 1999; Hoyos and Zhang, 2000; Mikolajczyk et al., 2000; Samuel et al., 2000; Zhang and Klessig, 2000).

Although both WIPK and SIPK are activated in gene-for-gene specific manner and they share common upstream kinase, NtMEK2, many studies have been interested in that which MAPK between them is more involved in defense response. Their activation mechanism has been known to be different; WIPK is regulated in the level of both

mRNA and protein, while SIPK is only activated post translationally. Indeed, Liu et al. (2003) suggested that SIPK is involved in regulating WIPK gene expression in N-gene mediated resistance showing that the induction of WIPK protein by TMV was suppressed in SIPK-silenced plants. While, it were evidenced that both kinases are required for leading to HR-like cell death, showing that SIPK alone is sufficient to induce HR-like cell death whereas WIPK plays a role in accelerating the cell death process.

MAPK cascades which are related to cell growth and differentiation

In addition to involvement of MAPKs in defense response, they have also been proposed to function in growth and development of plant and cells. For instance, it was reported that NPK1 gene from tobacco which encodes MAPKKK and its ortholog ANP1 of Arabidopsis are involved in cell division showing their transcription in proliferating and division cells (Nishihama et al., 1997; Nakashima et al., 1998). In arabidopsis, it was showed that a MAPK was activated by auxin in its roots (Mockaitis and Howell, 2000), and that *Arabidopsis* MKK7 negatively regulates polar auxin transport, which in turn affected growth and development (Dai et al. 2006). Bögre et al (1999) reported that although alfalfa MAPK, Medicago MAP kinase 3 is expressed during all stages of the cell cycle, the changes in its activity are involved in the

regulation of plant cytokinesis.

Downstream of MAP kinase cascade

Although 800 MAPKs have so far been reported from eukaryotes, the identity of targets that are phosphorylated by them has been limited. In mammalian cells, transcription factors, including MADS box, zinc finger, bZIP, and bHLH proteins were shown to be targeted by MAPKs (Yang et al., 2003). In plants, few examples have so far been reported; Liu and Zhang (2004) reported that activated MPK6, the Arabidopsis ortholog of tobacco SIPK is involved in ethylene biosynthesis through direct phosphorylation of ACS (1-aminocyclopropane-1-carboxylic acid synthase)2 and ACS6, and Kim et al. (2004) evidenced that activation of SIPK in $MEK2^{DD}$ plants which carrying an active mutant of the upstream of both SIPK and WIPK induced ethylene production, which then activated ethylene response factor genes. In rice, phosphorylation of OsEREBP1 by a MAPK (BWMK1) was found to enhance its binding to a GCC box (AGCCGCC), resulting in expression of various PR genes and elevated resistance to pathogen attack (Cheong et al., 2003). In tobacco (*Nicotiana tabacum*) plants, NtWRKY1 was identified as a substrate of SIPK and its binding activity to the W-box (TGAC) of class I basic chitinase gene, CHN50, was shown to increase on SIPK-mediated phosphorylation (Menke et al., 2005). Moreover,

Co-expression of SIPK and NtWRKY1 in *N. benthamiana* leads to more rapid cell death in comparison with SIPK alone, suggesting a close relationship between the two (Menke et al., 2005). Such studies indicate that identification of MAPK-targeted transcription factors and their target genes is critical to understanding the biological significance of MAPK cascades.

NtWIF as a downstream factor of WIPK

In previous study of my laboratory, **NtWIF (*Nicotiana tabacum* WIPK interacting factor)** was identified as a target protein of WIPK (wound-induced protein kinase) by yeast two hybrid screening. As shown in under Figure, NtWIF is a protein of 648 amino acids, with three domains: 250 amino acids at the N-terminal containing a DNA binding motif with a high similarity to auxin response factors (ARF); a 200 amino acid stretch in the middle region showing trans-activation activity; and 200 amino acids at the C-terminus constituting the WIPK-interacting region

Homology search indicated that overall sequence of NtWIF best matched with a potato (Solanum tuberosum) expressed sequence tag contig (TC104941) and the C terminus with a tomato (Lycopersicon esculentum) expressed sequence tag derived from nutrient-deficient root tissue (BF097731).

NtWIF was suggested to play some common roles in both wounding and pathogen signal transduction pathways together with WIPK. However, its relationship to WIPK and its physiological roles in tobacco plants have remained to be elucidated.

The aim and organization of this study

Identification of MAPK-targeted transcription factors and their target genes is critical to understanding biological significance of MAPK cascades. In this study, I analyzed molecular characteristics and physiological function of NtWIF. I also identified its downstream target genes. In the first chapter, I showed that NtWIF is a down-stream substrate of WIPK, being activated for transcription of target gene(s) involved in defense. In the second chapter, I examined physiological function of NtWIF under stressed environments such as wounding and pathogen attack using *NtWIF* over-produced (sense-transgenic lines) or suppressed (RNAi lines). Transgenic tobacco plants suggested that NtWIF directly contributes to enhance resistance. In the third chapter, I proved that NtWIF is directly or indirectly involved in up-regulation of diverse genes by microarray analysis. Moreover, I found that the target genes of NtWIF contain TGTCTC motif called the auxin-response element on their promoters. Examples are *PR-Q, ACS2 and WIPK*. In the fourth chapter, I showed that NtWIF possess dual functions in defense and differentiation and/or development through analyses of genes

which are up-regulated in NtWIF sense-transgenic lines and that of phenotypes of *NtWIF*-transgenic plants.

Chapter I. Molecular Characteristics of NtWIF

I.1 Introduction

In previous study of my laboratory, NtWIF (*Nicotiana tabacum* WIPK interacting factor) was identified as a target protein of WIPK (wound-induced protein kinase) by yeast two hybrid screening and it's structural characteristics were analyzed showing that ; 250 amino acids at the N-terminus containing a DNA binding motif with a high similarity to auxin response factors (ARF); a 200 amino acid stretch in the middle region showing trans-activation activity; and 200 amino acids at the C-terminus constituting the WIPK-interacting region. However, it has been remained to clarify what relationship exists between NtWIF and WIPK.

In this chapter, I described that NtWIF is a down-stream substrate of WIPK, which is directly phsophorylated by WIPK and that WIPK-mediated phosphorylation is essential for an increase in the transcriptional activity of NtWIF.

I.2 Materials and Methods

Fusion Proteins

A His-tagged WIPK construct was obtained by cloning a 1.2-kb *WIPK* full length cDNA fragment in the pET32 vector. The resulting plasmids were transformed into *E. coli* BL21, and expression of fusion proteins was induced with 0.5 mM

isopropyl-β-D-thiogalactoside at 25ºC for 3 h. A GST-tagged NtWIF was obtained by cloning corresponding *NtWIF* cDNA fragments into the GATEWAY cloning system (Invitrogen) according to the manufacturer's instructions. The final fragments were cloned into the pDEST20 vector plasmid and transformed into *E. coli* together with bacumid (genome of a baculovirus) to allow *in vivo* recombination, and resulting single plaques were picked up and further propagated. Sf9 cells (6×10^9), maintained in Grace's insect medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 500 μg ml^{-1} gentamycin, were infected with the recombinant baculovirus (500 μL) and incubated at 27°C for 4 d (Wada et al., 2003). Cells from one dish were suspended in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 25% [v/v] glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 100 μg mL^{-1} aprotinin) and sonicated for 10 s twice. Fusion proteins were purified through a glutathione-Sepharose column (Amersham Biosciences) or Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. Protein concentrations were estimated by the Bradford method.

Pull-Down Binding Assay

Full length (NtWIF) and truncated (ΔNtWIF/N or ΔNtWIF/C) proteins were expressed in Sf9 cells as the GST fusion as described above, and His-tagged WIPK was

expressed in E. coli. Approximately 5 μg of GST fusion protein was bound to a glutathione Sepharose 4B column (MicroSpin GST Purification Module; Amersham Pharmacia Biotech). The binding reaction was performed with 0.5 μg of His-tagged WIPK at 4 ºC for 1 h (Swaffield and Johnston, 1996). After three washes with PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3), GST fusion proteins bound to the columns were eluted with a buffer containing 10 mM reduced glutathione in 50 mM Tris-HCl at pH 8. Eluted proteins were fractionated on 7.5% SDS-PAGE and subjected to western-blot hybridization using rabbit anti-His tag antibodies (Santa Cruz Biotechnology) and anti-rabbit HRP conjugate antibodies (Bio-Rad).

Assays of Transcriptional Activity

Mutated NtWIF constructs were prepared by substituting Thr^{109} with Ala (Ala-Pro) and Thr^{109} and Pro^{110} with Ala and Ala, respectively, as described previously (Ueda et al., 2006). GBD-NtWIF and GBD–mutated NtWIF were constructed by fusing each cDNA fragment with the GAL4 DNA binding domain in yy64 vector, a derivative of pMA560 (Yamamoto and Deng, 1988). The reporter plasmid contained a luciferase gene placed under the control of the GAL4 binding site (Yamamoto and Deng, 1998). An internal control (reference) plasmid containing an R-luciferase gene placed under the

control of a CaMV 35S-promoter was used to normalize for bombardment efficiency. For effector plasmids, each PCR product of *NtWIF* or nucleus localized sequence-attached *NtWIF* or WIPK was cloned into pBI221 driven by the 35S-CaMV promoter. Five- or six-day-old BY2 suspension cells (2 ml) maintained in liquid medium were plated on 3% agar plates and briefly allowed to dry to remove excess liquid. They were then bombarded with plasmids (effector: reporter: reference = 2:2:1) coated on a 1.0-μm microcarrier in a vacuum of 28 inches of mercury using a helium pressure of 1,100 psi (PDS 1000; Bio-Rad). Cells were placed 6 cm from the stopping screen. After bombardment, cells were harvested with 20 ml of liquid medium, transferred into a flask, and then incubated in the dark at 25°C with gentle agitation. Luciferase and R-luciferase activities were assayed using a dual-luciferase assay kit (Promega) according to the manufacturer's instructions. Chemical luminescence was measured using a luminometer (Lumat LB9507; EG & G Berthold). The presented results are the means of 6-repeats with standard deviations.

Phosphorylation Assay

In vitro kinase assays were performed with 5 μg of purified GST-WIPK fusion protein in a buffer containing 20 mM HEPES-KOH, pH 7, 0.5 mM EGTA, 1 mM DTT, 20 mM $MgCl_2$, and 5 mM [γ-^{32}P] ATP (New England Nuclear). 5 μg of myelin basic

protein or GST fusion proteins (full-length NtWIF, ΔNtWIF/C, or ΔNtWIF/N) were added as substrates in separate reactions. Mixtures were incubated at 30ºC for 30 min, and an appropriate volume of SDS-PAGE dye was added to terminate the reactions. Proteins were separated on SDS-polyacrylamide gels (7.5%), stained with 0.25% Coomassie Brilliant Blue, and subjected to autoradiography for kinase activity, which was detected by applying BioMax film (Kodak) to the gels at 28ºC for 72 h.

I.3 Results

Interactions between WIPK and NtWIF

In previous study, interaction of WIPK and NtWIF was confirmed by a yeast two-hybrid interaction assay. The results indicated that the region containing 196 amino acids at the C terminus to be sufficient for WIPK interaction, and this was thus designated as the WIPK interacting domain. However, it was showed that the full-length NtWIF (amino acids 1–648) interacted with WIPK rather inefficiently, showing the binding activity at one-sixth that of ΔNtWIF/C.

In this study, interaction of WIPK and NtWIF was confirmed in an *in vitro* pull-down assay (Fig. 1A). His-tagged WIPK was applied to a glutathione-Sepharose column containing full or truncated NtWIF-glutathione S-transferase (GST) proteins, eluted with a buffer containing reduced glutathione, separated by SDS-PAGE, and

subjected to western blot hybridization with anti-His tag antibodies. Results clearly showed that WIPK bound to ΔNtWIF/C but not to ΔNtWIF/N (Fig. 1A) consistent with results from yeast two-hybrid assays. However, in contrast to the yeast assay, WIPK was found to bind to the full-length NtWIF at equal efficiency with that of ΔNtWIF/C (Fig.1A). This discrepancy might due to different assay methods: an *in vivo* system using yeast cells and an *in vitro* system using large amounts of purified proteins.

Phosphorylation of NtWIF by WIPK

The observed preferential interaction suggested that NtWIF can possibly be phosphorylated by WIPK. In order to examine this possibility, *in vitro* phosphorylation assay was performed using recombinant proteins. As substrates, three constructs, full-length NtWIF, ΔNtWIF/C, and ΔNtWIF/N, were prepared in insect cells as the expression system. Using these protein preparations, kinase reactions with bacterially expressed WIPK were performed. The results clearly showed the full-length NtWIF protein to be phosphorylated (Fig. 1B, lane 2). The N terminus (ΔNtWIF/N) was also phosphorylated, but the C terminus (ΔNtWIF/C) was not at all (Fig. 1B, lanes 3 and 4, respectively). Under these experimental conditions, autophosphorylation of WIPK was strongly apparent (Fig. 1B, lane 1). The results thus indicated that differential phosphorylation activity toward NtWIF is an inherent property of WIPK.

WIPK-Mediated Activation of NtWIF

Interaction between NtWIF and WIPK was further examined by Luc assay in BY2 cells. First, I generated a kinase-deficient WIPK by PCR mutagenesis as the reference. In this mutant protein, the essential Lys at position 73, which is critical for ATP binding, was substituted with a non-active Arg residue (WIPK/ K73R). In previous *in vitro* kinase assays, it has been already confirmed that the kinase activity of this mutant was abolished showing the inability to phosphorylate itself and also a synthetic MAPK substrate myelin basic protein. Second, I generated several constructs for transformation, which could be temporarily expressed in BY2 cells (Fig 2A). Using these constructs, I determined whether transcriptional activity of NtWIF is activated by WIPK in planta. Upon introduction into cells, NtWIF alone could induce transcription of the reporter gene to a level 10-fold that of the control (Fig. 2B). However, with WIPK, the transcriptional activity increased up to 50-fold. This increase was clearly due to phosphorylation, since kinase-deficient WIPK failed to exhibit such activation, leaving the activity at the same level as NtWIF alone. It was concluded that, in BY2 cells, both WIPK and NtWIF become imported into the same cell and that the former actively phosphorylates the latter, thereby enhancing the transcriptional activity.

Determination of Phosphorylation Sites

As I described above, NtWIF was phosphorylated by WIPK in its N-terminal region. Since the minimal consensus site for phosphorylation by MAPK is proposed to be Ser/Thr-Pro (Yang et al., 2003), I suggested the target site in NtWIF to be the threonine at position 109. In order to verify this, I constructed two mutants in which the Thr^{109}-Pro^{110} (TP) site was changed to Ala-Pro (AP) and Ala-Ala (AA), respectively, and examined their effects on trans-activation using a GAL-4 transient expression system in BY2 cells (Fig. 3). Mutated or wild type NtWIF effector constructs were co-bombarded with plasmids containing a reporter *Luc* gene, or *WIPK* driven by the CaMV 35*S* promoter together with a reference plasmid, and assayed for Luc expression (Fig. 3A). While NtWIF alone showed a low level of transcription activity, this was increased 3- to 5-fold in the presence of WIPK. In contrast, with mutant NtWIF in which the TP was destroyed, activity was completely lost, even in the presence of WIPK (Fig. 3B). These results clearly indicated that T^{109} and P^{110} are essential amino acids conferring WIPK-mediated transcriptional activity on NtWIF.

I.4 Discussion

In this chapter, it was confirmed that NtWIF interacts with WIPK and it is phosphorylated by WIPK. Moreover, since an *in vitro* kinase assay showed efficient phosphorylation of not only full-length NtWIF but also just N terminus (ΔNtWIF/N) by

WIPK, tight binding between these two may not be a prerequisite for the phosphorylation reaction. Perhaps WIPK is able to recognize the C terminus of NtWIF despite its higher conformation and catalyze phosphorylation of the N terminus. This is consistent with suggestions that MAPKs phosphorylate many proteins without forming tight complexes (Sharrocks et al., 2000).

Specificity of MAPKs has been suggested to be determined by the docking domain and phosphoacceptor sites. However, the former does not appear to be directly involved in phosphorylation but rather to increase phosphorylation efficiency by recruiting target proteins (Kallunki et al., 1996; Holland and Cooper, 1999; Sharrocks et al., 2000). My results are consistent with this idea, showing that the N terminus fragment was phosphorylated to a certain extent, despite the lack of docking domains. I thus speculated that a conformational change of the full-length NtWIF is induced by the N terminus phosphorylation, which is catalyzed by WIPK, preferentially interacting with the C-terminal docking domain. If this is indeed the case, the active form of WIPK appears to be critical. It has been established that phosphorylation of the Thr-Glu-Tyr (TEY) site in MAPK is required for enzymatic activation (Pelech and Sanghera, 1992). Usually this phosphorylation is catalyzed by MAPKK (MEK) but also can be accomplished by MAPK itself (Craig et al., 1991). Bacterially expressed MAPK (ERK)

exhibits distinct autophosphorylation at the TEY site and concomitant enzymatic activity (Boulton et al., 1991; Huang et al., 2000). Analysis of AtMPK4 in Arabidopsis has also indicated that phosphorylation of the TEY site by MEK might be required for full activation (Huang et al., 2000). Since my bacterially expressed WIPK showed clear autophosphorylation activity, it is evident that NtWIF/WIPK interactions are dependent on the active form of WIPK itself.

In vivo trans-activation assays clearly indicated NtWIF to be a novel type of transcription factor. This was confirmed by the *in planta* transcription activity of NtWIF. When expressed in BY2 cells, NtWIF activated the reporter gene 10-fold over the control, this probably being mediated through endogenous WIPK activated by the bombardment. However, this activity increased up to 50-fold with additional expression of WIPK. Since inactive mutant WIPK failed to activate NtWIF, phosphorylation evidently was responsible for the enhanced activity.

All MAPKs phosphorylate similar motifs conforming with the minimal consensus sequence Ser/Thr-Pro (Sharrocks et al., 2000). Sequence analysis of NtWIF has revealed two Thr-Pro sites, which possibly serve as the targets for MAPK (Yang et al., 2003): one at position 109 and the other at position 382, located in the N- and C-terminal regions, respectively. Since the C-terminal region was not phosphorylated in

our *in vitro* assay, the N terminus is likely the correct phosphorylation site of WIPK. The present study confirmed this to be the case, transcriptional activation of NtWIF by WIPK being totally abolished when Thr^{109}-Pro^{110} was mutated to Ala^{109}-Pro^{110} or Ala^{109}-Ala^{110}.

Taken all the available evidence together, I propose the following model for WIPK/NtWIF interaction. NtWIF is a transcription factor, which upon external stresses, WIPK is activated through phosphorylation by MEK and also by itself and preferentially interacts with the C-terminal region of NtWIF. WIPK then phosphorylates the Thr-Pro target site located in the N-terminal region, resulting in activation of NtWIF to function as a transcriptional activator.

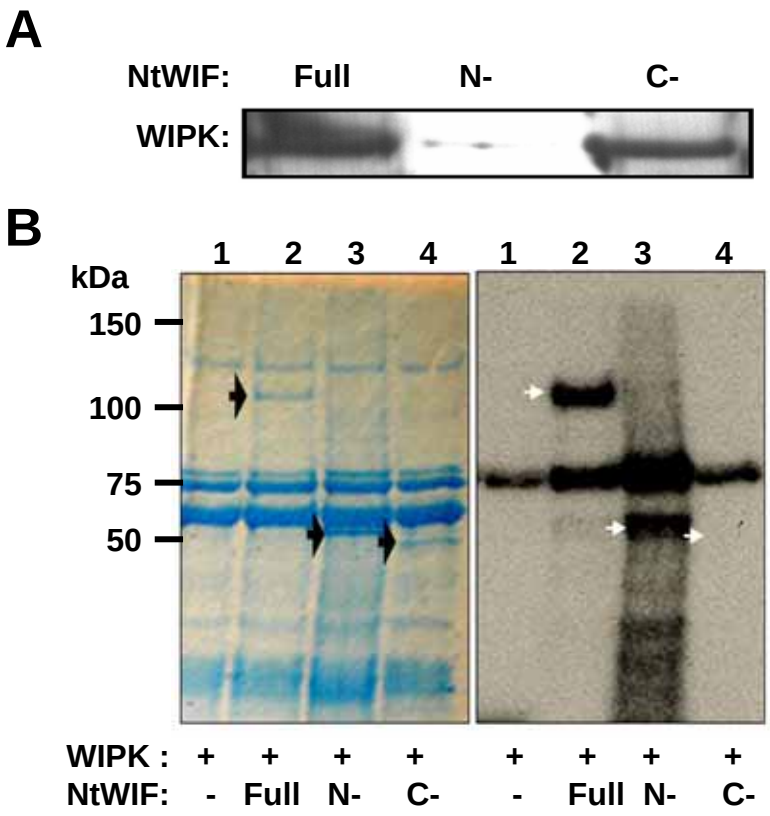

Figure 1. Interaction between NtWIF and WIPK and phosphorylation of NtWIF by WIPK .

A. Pull-down assay of recombinant WIPK and NtWIF.

Interaction was tested using GST-fused full-length NtWIF (Full), ΔNtWIF/C (C) and ΔNtWIF/N (N) proteins expressed in Sf9 cells and bacterially expressed His-tagged WIPK protein. NtWIF-GST proteins were bound to a glutathione column, and His-tagged WIPK was added. After elution with reduced glutathione, proteins were separated by SDS-PAGE, and WIPK was detected by anti-His-tag antibodies.

B. In vitro kinase assays.

GST-tagged full-length (NtWIF) or truncated (ΔNtWIF/C or ΔNtWIF/N) proteins expressed in Sf9 cells were subjected to phosphorylation reaction with bacterially expressed GST-WIPK (75 kD) protein. After electrophoresis on 7.5% polyacrylamide, the gel was stained by Coomassie Brilliant Blue (CBB; left), dried, and used for exposure to x-ray film (right). Samples are GST-WIPK (lane 1), full-length NtWIF (110 kD; lane 2, arrowhead), ΔNtWIF/ N (55 kD; lane 3, arrowhead), and ΔNtWIF/C (43 kD; lane 4, arrowhead).The sizes of proteins are indicated on the left.

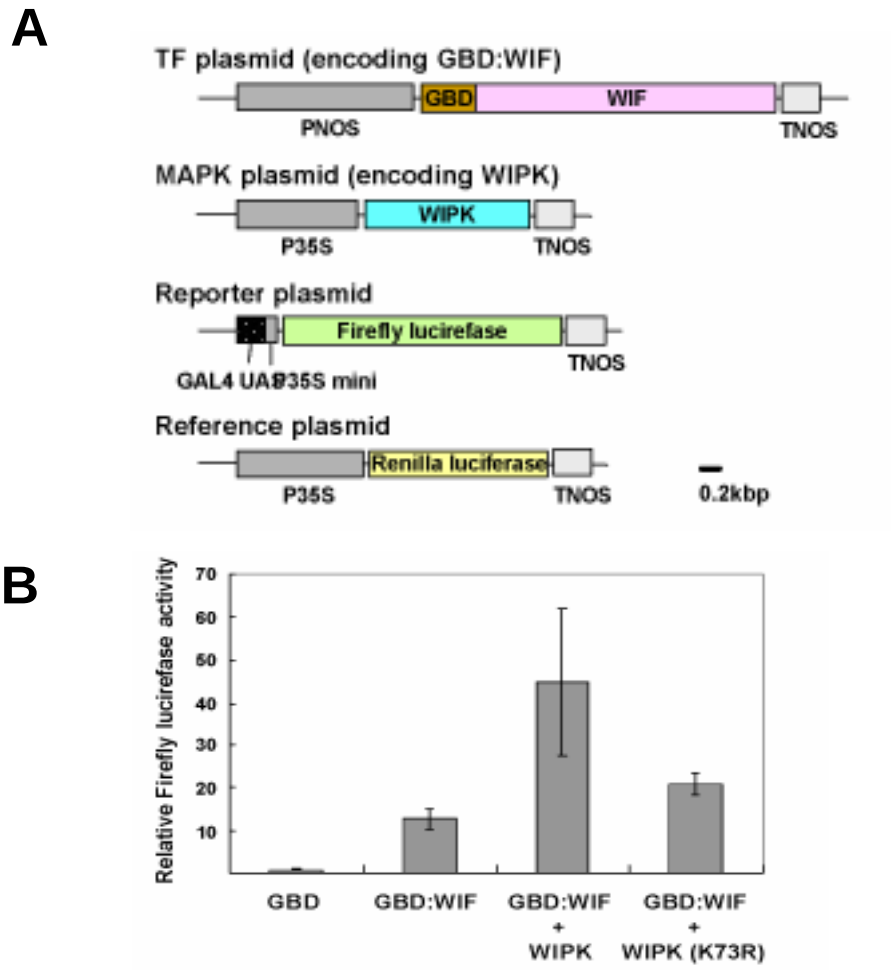

Figure 2. Transcriptional activation by WIPK in planta.

A. Schematic illustration of plasmids used in the WIPK-mediated trans-activation assay.

PNOS, nopaline synthase promoter; TNOS, nopaline synthase terminator; P35S, 35S promoter of cauliflower mosaic virus (CaMV); GAL4UAS, GAL4 binding sequence; P35Smini,CaMV35S minimal promoter.

B. WIPK-mediated trans-activation assay.

Trans-activation assay with BY2 cells was performed with plasmids containing indicated constructs. Indicated plasmids were mixed and used for coating gold particles, which were introduced into BY2 cells using particle bombardment. Dual-luciferase activity was assayed in nine independent experiments by a luminometer (Lumat LB9507; EG & G Berthold)

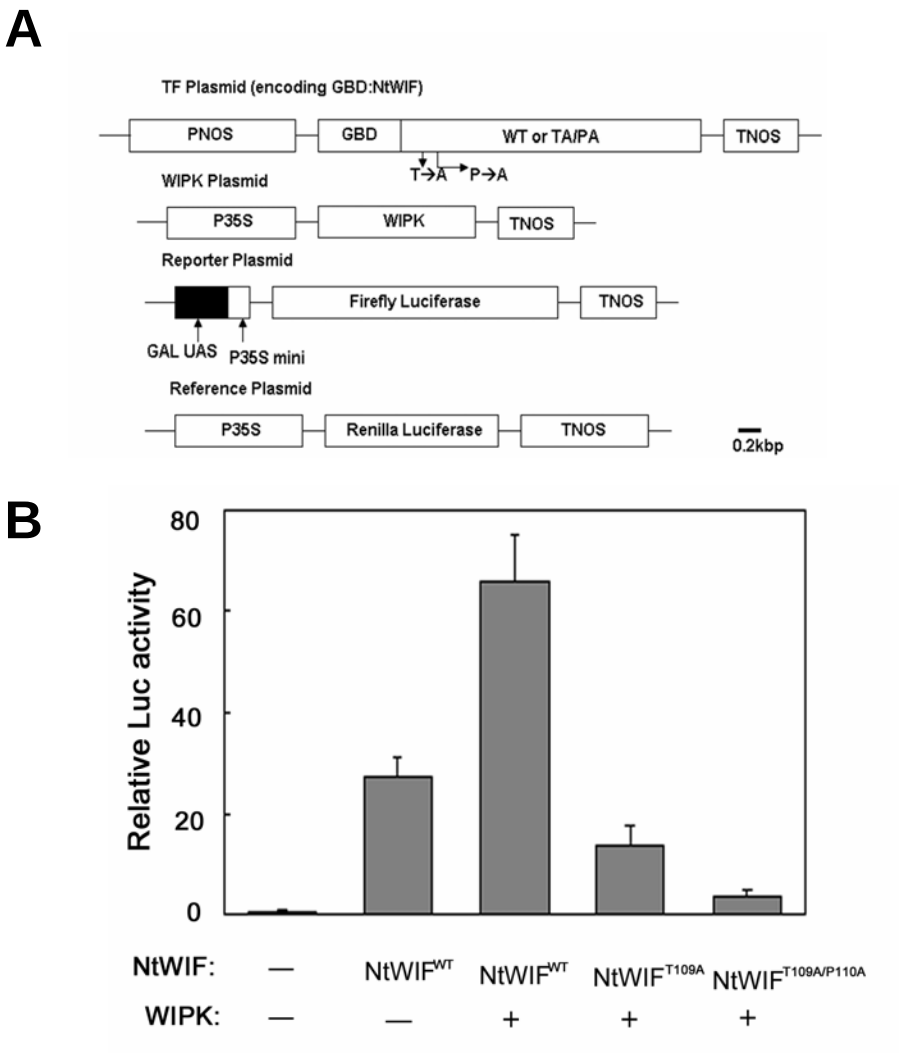

Figure 3. Determination of NtWIF-Phosphorylation site by WIPK

A. Schematic illustration of plasmids used in the NtWIF-mediated trans-activation assay.

PNOS, nopaline synthase promoter; TNOS, nopaline synthase terminator; P35S, cauliflower mosaic virus (CaMV) 35S promoter; GAL4UAS, GAL4 binding sequence; P35S mini, CaMV35S minimal promoter.

B. Trans-activation of the Luc-reporter gene.

Assays with BY2 cells were performed with plasmids containing the indicated constructs in the presence (+) or absence (-) of WIPK. Plasmids were mixed, coated onto gold particles and introduced into BY2 cells by particle bombardment. Luciferase activity of each type of transformant was normalized to the respective R-luciferase activity. Relative luciferase activity was calculated by dividing the luciferase activity of each clone by that of the clone containing the GAD4 DBD effector construct (six independent experiments).

Chapter II. Physiological Functions of NtWIF in Stress Responses of Tobacco Plants

II.1 Introduction

In previous work, it was suggested that NtWIF might play some common role in both wounding and pathogen signal transduction pathways together with WIPK since its expression was identical to that of WIPK. Moreover, my observations in the first chapter strongly suggested that NtWIF is a transcription factor which is activated by WIPK-mediated phosphorylation. However, it was remained to be elucidated what physiological roles NtWIF plays with WIPK in the stress responses.

In this chapter, I reported production and characterization of transgenic tobacco plants including *NtWIF* sense-transgenic lines and RNAi lines under wounding and pathogen-stressed environments. The results suggested that NtWIF directly contributes to the elevation of resistance in the response to the tobacco mosaic virus (TMV) infection and wounding, showing that presence of NtWIF was involved in the limitation of TMV-spreading through fast formation of limited size of lesions and in the up-regulation of diverse defense-related genes.

II.2 Materials and Methods

Plant Materials and Treatments

All tobacco plants (*Nicotiana tabacum* cv Xanthi nc) were grown in a growth cabinet at 23ºC under a 14/10-h light/dark cycle. For wounding, intact leaves of 3 month-old plants were punched with a paper puncher, floated in water for 15, 30 and 60 min and then immediately frozen in liquid nitrogen. The controls were sampled at time 0. Tobacco mosaic virus (TMV) inoculation was performed by mechanically inoculating detached leaves by rubbing with Carborundum, keeping them for 48 h at 30°C, at which temperature the resistance *N* gene does not function, allowing viruses to multiply, and then transferring to 20°C, to initiate the hypersensitive response (Yap et al. 2002). Development of lesions was assessed 8, 24 and 48 h from the time of the temperature shift (t=0). For determination of the percentage of fully developed lesions, between 200 and 1000 (from at least 5 different leaves each from wild type, #S7) were evaluated. Lesion size was determined 48 h after temperature shift.

Transgenic plants

Transgenic tobacco plants (*N. tabacum* cv. Xanthi nc) overexpressing *NtWIF* were produced by replacing the GUS (β-glucuronidase) gene in the binary vector pBI121 (Clonetech) with a full-length cDNA of *NtWIF,* ligated into the *Bam*H1-*Sac*I-digested pBI121 vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter followed by the NOS terminator sequence (Yap et al. 2002). For RNAi plants, the

pKANNIBAL vector designed for producing hairpin RNA with a loop was used. For sense orientation, the coding region of *NtWIF1* cDNA was amplified by PCR using forward (5'-TATCTAGATGTCATTTCCTCAGGGTCAAC-3') and reverse (5'-TAGG ATCCTTCCAAAACAGGGTGGAATCTC-3') primers. For anti-sense orientation, the same region was amplified using forward (5'-TAGTCGACGATGTCATTTCCTCAGG GTCAAC-3') and reverse (5'-TACAATTGCTTCCAAAACAGGGTGGAATCTC-3') primers. All PCR products were introduced into the pGEM-T Easy vector and sequenced. Digested fragments were ligated to corresponding sites of the pKANNIBAL vector. Resulting plasmid was digested by *Not*I and ligated to the corresponding site of pART27, which was introduced into *A. tumefaciens* strain LBA4404 cells. Tobacco transformation was performed as described previously (Yap et al., 2002).

Histochemical Detection of Hydrogen Peroxide

Hydrogen peroxide was visually detected in the leaves of plants using 3,3-diamino-benzidine (DAB) as the substrate (Yoda et al., 2003). Briefly, sampled plants were immersed in 1 mg mL^{-1} solution of DAB (pH 3.8) for 8 h under darkness at $25^{o}C$, and then were washed in boiling ethanol (96%) for 10 min. After cooling, the leaves were photographed.

Determination of plant hormone contents

Tobacco plants were wounded as described above, and leaves were harvested at 0, 1, 3, 6 and12 h. Each was split along the middle vein and one half was weighed and immediately soaked in 5 ml of 99% ethanol (p.a.) containing 30 pmol· ml^{-1} of each of the hormone standards ($[_2H]^4$-salicylic acid, $[_{13}C]^2$-jasmonic acid, $[_2H]^2$-indoleacetic acid, $[_2H]^6$-abscisic acid, $[_2H]^5$-OPDA; for details, see Müller et al. 2002). Samples were incubated at 50°C for 30 min and at 22°C for 16 h. The supernatants were transferred into reaction tubes and the solvent was removed in a vacuum concentrator (SpeedVac). Dried extract fractions were prepared for GC-MS analysis as described previously (Müller et al. 2002).

RNA blot hybridization

Total RNA was isolated by the hot-phenol method and 10-µg aliquots were fractionated by formaldehyde/ agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N, Amersham). After crosslinking using a UV crosslinker (RPN 2501, Amersham), the membrane was subjected to hybridization with appropriate ^{32}P-labeled probes at 42ºC for 16 h. After washing several times, the membrane was used to expose either BAS or x-ray film (Kodak)

II.3 Results

Transgenic Tobacco Plants

In order to examine physiological functions of NtWIF, transgenic tobacco plants were constructed in which NtWIF was over-produced (sense-transgenic lines) or suppressed (RNAi lines). Among kanamycin-resistant shoots which were initially generated, four sense-transgenic lines (S4–S7) were selected on the basis of its highly constitutive expression under non-stress condition (Fig. 4A upper) and two RNAi lines (R20 and R4) were finally selected on the basis of complete suppression of *NtWIF* transcripts in leaves 30 min after wounding (Fig. 4A lower). Features of both transgenic lines did not differ from wild type plants under standard culture conditions (Fig. 4B)

Properties of *NtWIF* sense transgenic lines

1) Expression profile in *NtWIF*-sense transgenic plants

To examine the correlation with wound and/or pathogen response, transcript accumulation for defense-related genes was initially examined by reverse transcription-PCR (RT-PCR). As representative genes, *PR-1a* and *PR-2* were selected, and their transcripts were found to accumulate at constitutively high steady-state levels, with or without wounding in lines S4, S6 and S7 (Fig. 5A). This was in marked contrast to control plants, in which levels of both *PR-1a* and *PR-2* transcripts were undetectable without stress, and temporarily accumulated to a lower level after wounding in comparison with transgenic lines (Fig. 5A). This result results suggested that the

defense-related signaling pathway was constitutively activated in transgenic plants.

2) Measurement of hormone amount

Subsequently, since it has been known that salicylic acid (SA) is a powerful inducer of *PR-1a*, endogenous levels were estimated using the line S7 as a representative of transgenic plants. The amounts of SA were approximately 0.1 and 6.8 nmol g FW^{-1} of leaf tissues from unchallenged wild-type and S7 transgenic plants, respectively (Fig. 5B SA). This indicates the transgenic line constitutively to contain 50-fold higher SA levels than the control. When wounded, SA contents dynamically increased in the S7 plants up to 13 nmol 1 h later. This increase was transient, showing a gradual decline to the basal level of about 4 nmol 3 h after wounding and thereafter (Fig. 5B SA). An increase of SA was also observed in wild-type plants, showing 0.5 nmol 1 h after wounding (Fig. 5B SA), although the absolute amounts were only 1/30 to 1/50 of those of the transgenic line. The increased level remained at about 0.4 nmol 3 h after wounding and later. In contrast to SA, the level of jasmonic acid (JA) was similarly 1 pmol g FW^{-1} in both unchallenged wild-type and transgenic plants (Fig. 5B JA). Upon wounding, JA increased to 1 nmol in both cases after 1 h, and gradually declined to a level below 0.2 nmol by 12 h (Fig. 5B JA). A notable feature was that the decline was faster in the transgenic line than in the control, showing already the basal level of 0.3 nmol at 3 h in

the former, as compared with 40.7 nmol in the latter at the same time point (Fig. 5B JA). The level of 3- indolylacetic acid (IAA) did not significantly change after wounding in both wild-type and transgenic plants, with a basal concentration of approximately 30 pmol g FW^{-1} (Fig. 5B IAA). In the case of abscisic Acid (ABA), the basal level was about 0.32 nmol g FW^{-1} in both plants without wounding (Fig. 5B ABA). In wild-type plants, the level gradually increased up to 0.63 nmol g FW^{-1} 6 h after wounding, and then declined to 0.46 nmol by 12 h. In transgenic plants, the level slightly declined at 3 h after wounding, and then increased in parallel with the control, continuing to increase up to 0.76 nmol by 12 h (Fig. 5B ABA). This could be the result of a 3 h lag period at the onset of ABA increase in the transgenic plants.

Comparison of the responses of *NtWIF*- sense transgenic, -RNAi and WT plants to TMV infection

The high SA level in the *NtWIF*-sense transgenic line suggested an altered response to pathogen attack. In order to assess this possibility, lesion development was evaluated upon TMV infection. Healthy leaves from *NtWIF*-sense transgenic, -RNAi and WT plants were mechanically inoculated with TMV, and lesion formation was monitored 30 h after temperature shift. Results showed that, in sense-transgenic line S7, their formation was faster and lesion size was smaller, while in the RNAi line R20, lesions

were much larger than in the controls (Fig. 6A). Since amounts of virus particles did not vary among samples (Fig. 6A), the difference in pathogen response was clearly due to NtWIF. Since RNAi line R20 was significantly different from WT and *NtWIF*-sense transgenic plants, lesion development was studied in detail in WT and *NtWIF*-sense transgenic plants. In wild-type plants, the first lesions were visible about 6–8 h after temperature shift. They gradually developed from the 'early stage', in which a dark ring occurs and tissue inside this ring is still green, to the 'late stage', in which the tissue inside the lesion consists of dead cells and is brownish in color (Fig. 6B, upper panel). Based on this measuring method, lesions in S7 plants and wild-type plants after TMV infection were observed (Fig. 6B, lower panel). In wild-type leaves, lesions were at the 'early stage' at 24 h, and developed to the 'late stage' 48 h later. After 72 h, all lesions completely matured, showing brown, large necrotic spots. In S7 leaves, a high percentage of lesions already developed to the 'late stage' at 24 h. Finally, lesion size at 72 h after temperature shift, when all lesions were at the 'late stage', was determined by measuring the average lesion diameter. In wild-type plants, for average lesions, this was 3.93±0.14 mm, whereas it was 1.67±0.39mm in S7 transgenic plants. These results indicated that, in sense transgenic lines, lesions developed much faster and were smaller than in wild-type plants.

Comparison of responses of *NtWIF*-sense trnasgenic, -RNAi and WT plants to cryptogein infiltration

During the HR, hydrogen peroxide is generated, functioning as the signaling molecule and also as the physical barrier against the pathogen (Thordal-Christensen et al., 1997). To confirm that the observed differences in lesion formation were due to variation in HR among transgenic lines, hydrogen peroxide production in leaves was examined by infiltrating cryptogein (Fig. 7), a protein of fungus which induces HR-like necrosis (Viard et al., 1994). After infiltration, phenotypic changes were not observed at least until 6 h later in wild type, transgenic S7 and R20 cases (Fig. 7). However, amounts of hydrogen peroxide clearly differed among plants, showing much higher accumulation in the S7 than in the control, but almost none in the R20 plants (Fig. 7).

II.4 Discussion

This chapter describes the construction and physiological analysis of transgenic tobacco plants including *NtWIF*-sense transgenic and -RNAi plants.

Although transgenic plants were not apparently different from wild-type plants, especially *NtWIF*-sense transgenic plants possessed about 50-fold higher amount of SA than in the wild-type controls. Because SA levels do not significantly change with wound stress in wild-type tobacco plants (Seo et al. 1995), the constitutively high SA

content and its increase upon wounding in transgenic plants is unusual. Indeed, the observed high levels are similar to those induced by pathogen infection in wild-type plants, reaching up to 10 nmol (~1.4 μg) $gFW^{-1}$of leaf tissues (Yoda and Sano 2003). This implies that wound signaling mimicked pathogen signaling in transgenic plants. In the responses of transgenic plants and WT plants to pathogen attack, they showed exactly opposite aspects. These provide a critical evidence to predict the physiological function. Against TMV infection, *NtWIF*-sense transgenic plants developed smaller necrotic lesions at a faster rate than *NtWIF* RNAi and wild-type plants. Moreover, in the formation of HR lesions, *NtWIF*-sense transgenic plants showed much higher accumulation of H_2O_2, while *NtWIF*-RNAi plants did not. It has been known that HR functions in limitation of infected pathogens to small region and that during HR-lesion formation, H_2O_2 is known to function as the physical barrier against the pathogen to prevent its spreading from infected site to other site (Sangpen Chamnongpol et al. 1996). This indicates that small size of HR lesion means the strong HR and resistance ability. Therefore, my results suggest that the presence of NtWIF as a downstream factor of WIPK positively regulates the resistance of plants through fast HR lesion formation by strong H_2O_2- production. This idea is compatible with the finding that HR-like cell death is accelerated when WIPK is activated by its upstream kinase, $NtMEK2^{DD}$ (Liu et

al. 2003).

Another notable finding is that the increase of the ABA concentration after wounding is delayed by 3 h in the transgenic lines, and a higher ABA content is measured 12 h after wounding, possibly reflecting a shift in the transient ABA increase. The significance of these differences is currently not clear, but involvement of ABA in wound and pathogen response has repeatedly been documented. For example, β-aminobutyric acid, which induces resistance against necrotrophic pathogens, was shown to depend on ABA signaling (Ton and Mauch-Mani 2004). In TMV-inoculated tobacco leaves, the ABA content was reported to increase 4-fold compared with untreated leaves 16 and 26 d after inoculation (Whenham et al. 1986). A cDNA microarray study in Arabidopsis showed many wound-responsive genes to be induced by ABA, and to be related to water stress (Delessert et al. 2004). Considering these observations, it is conceivable that the timing of the transient increase and the level of accumulation of ABA might be important to control the pathogen response properly through the MAPK cascade. Overall, the present study suggested that NtWIF is involved in a process in which SA production is controlled, and that its overexpression might disturb crosstalk between wound and pathogen signaling pathways. Currently I have no clear explanation for the increase in SA of sense transgenic plants, but recent

studies on Arabidopsis MPK4 are suggestive. When MPK4 was inactivated by transposon insertion, endogenous levels of SA were elevated, with a simultaneous increase in pathogen resistance (Petersen et al. 2000). A subsequent study identified an MPK4-interacting protein, MKS1, the overexpression of which again resulted in an increase of SA and pathogen resistance (Andreasson et al. 2005). It was speculated that accumulation of unphosphorylated MKS1 exhibits a phenotype similar to that of plants lacking MPK4 (Andreasson et al. 2005). These observations are consistent with our results, although the phosphorylation status of overexpressed *NtWIF* in planta is currently not known. Whatever the mechanism may be, the MAPK cascade appears to be closely involved in adjusting the SA levels in both Arabidopsis and tobacco plants. In contrast, considering that SA levels sensitively change upon introduction of foreign genes, such as one encoding a small GTP binding protein (Yoda and Sano 2003), the possibility remains that NtWIF indirectly regulates SA levels by, for example, modulating the network of wound/ pathogen response pathways through activating other protein factor(s).

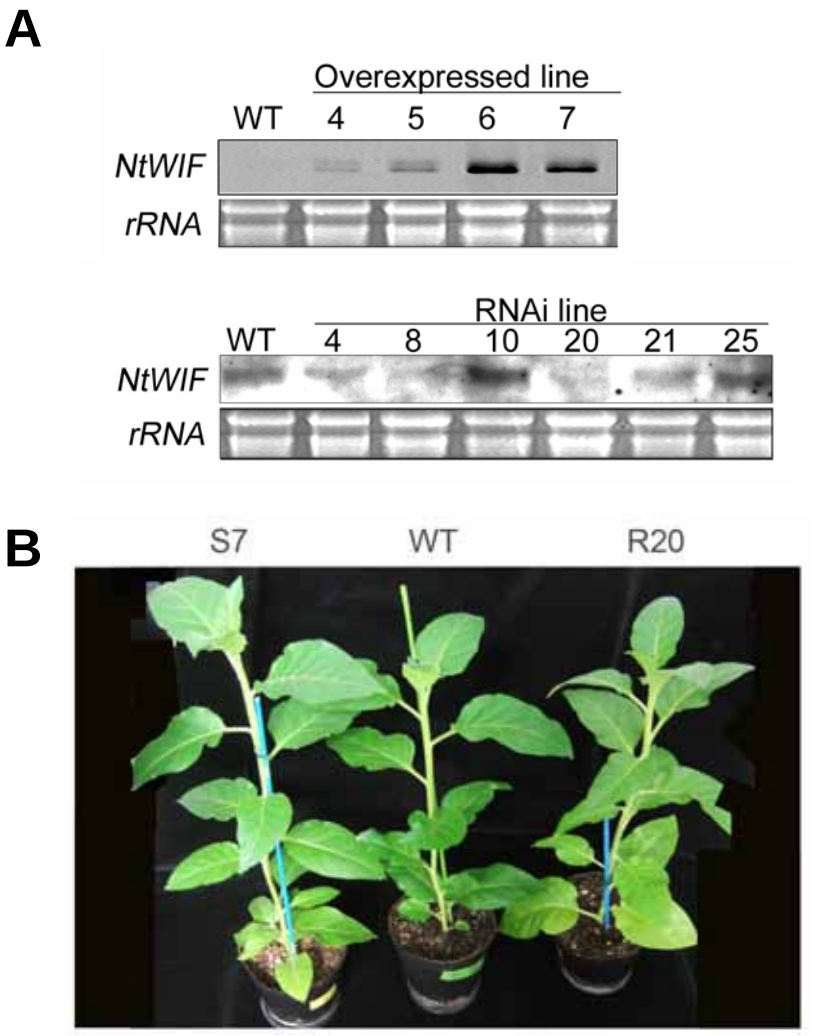

Figure 4. Transgenic plants.

A. RNA blot hybridization.

Total RNA was isolated from healthy leaves of wild type (WT) and *NtWIF*-sense-transgenic lines (S4,S5,S6 and S7) (upper panel) and from wounded leaves of wild type and NtWIF-RNAi lines (R4,R8, R10, R20, R21 and R25)(lower panel). For wounding, leaves were punched with paper puncher, floated in water for 30 min and then they were collected. Total RNA was isolated from leaves, and a 10-μg aliquot per lane was fractionated by gel electrophoresis and transferred onto a nylon membrane, which was subjected to hybridization using ^{32}P-labeled *NtWIF* cDNA as a probe. Equal loading of RNA was monitored with rRNA.

. **B. Phenotypes of transgenic *NtWIF*-sense transgenic lines (S7), -RNAi line (R20) and wild type plants.**

Photographs were taken 6 weeks after vegetative propagation.

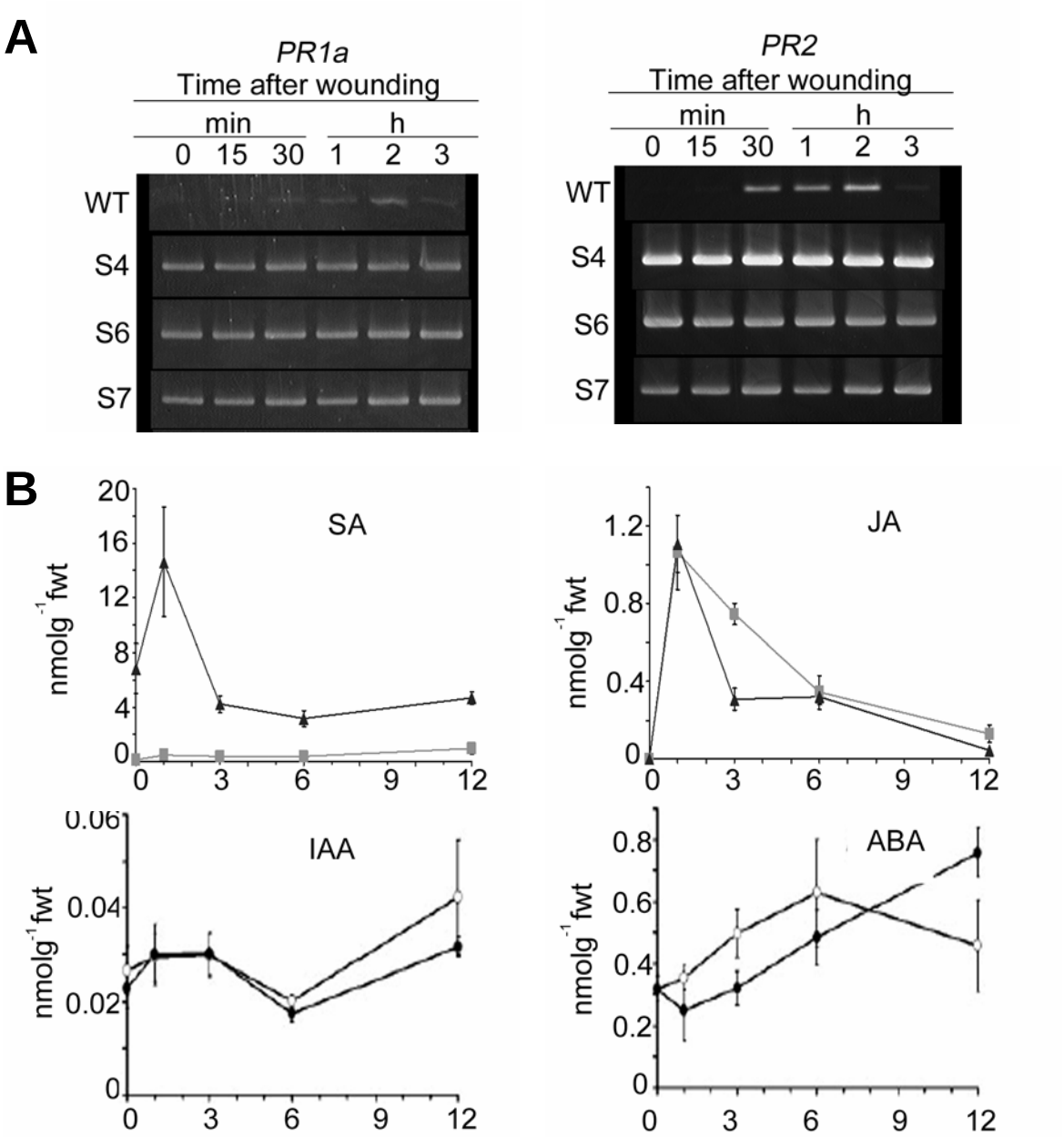

Figure 5. Expression profile.

A. Constitutive expression of PR genes.

Leaves from the WT or the indicated transgenic lines (S4, S6 and S7) were harvested before (0) and the indicated time period after wounding, and total RNA was isolated.RT–PCR was performed with specific primers for PR-1a and PR-2.

B. Phytohormone levels.

Leaves of wild-type (open circle) and transgenic S7 plants (filled circle) were mechanically wounded and sampled at the indicated time points for quantification of salicylic acid (SA), jasmonic acid (JA), 3- indolylacetic acid (IAA) and abscisic Acid (ABA). Amounts are given as nmol g FW^{-1}. Values are means of 6–9 plants each with standard deviations.

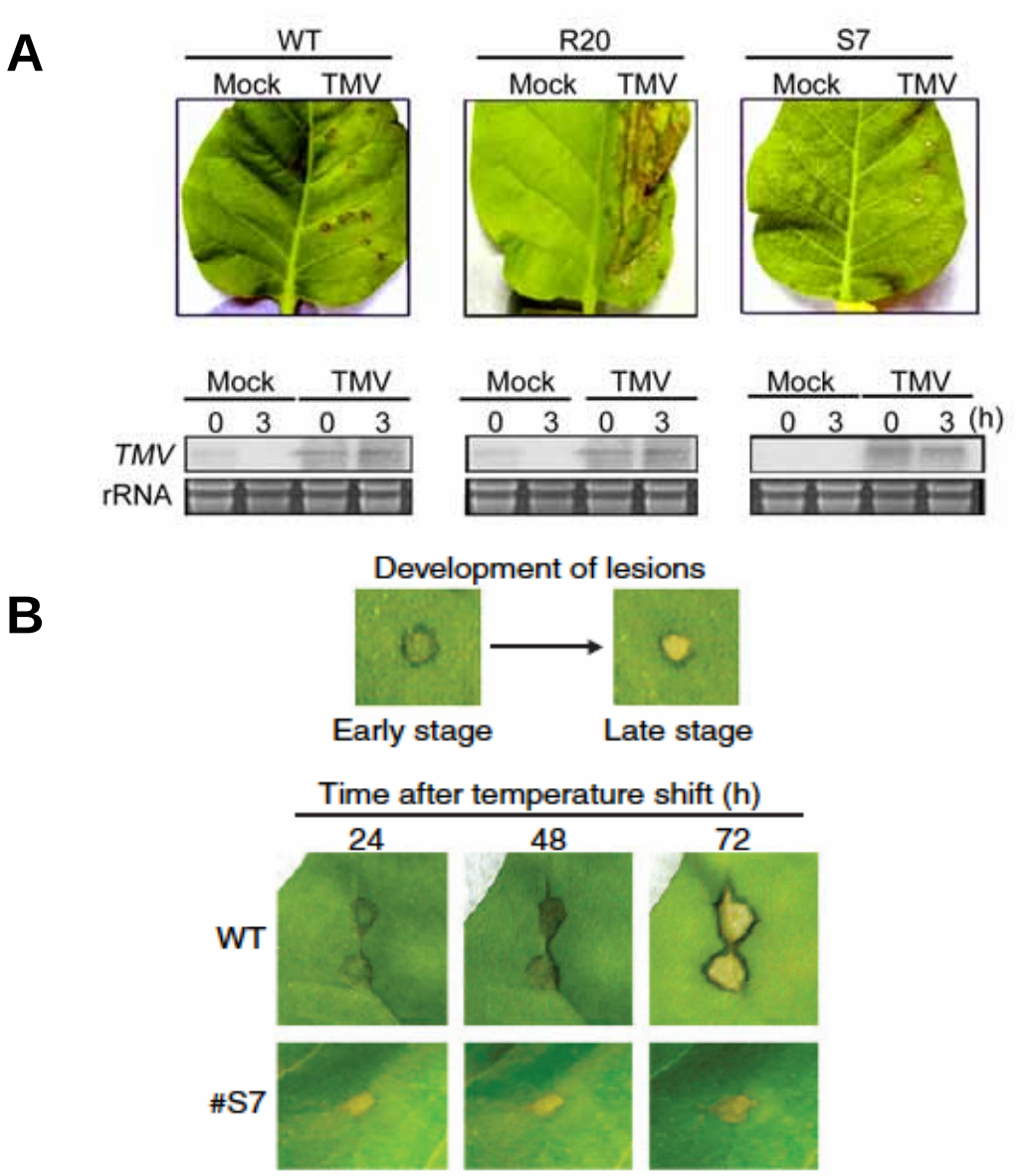

Figure 6. Response to tobacco mosaic virus infection.

A. Healthy leaves from wild type, RNAi (R20) and sense-transgenic (S7) lines were detached, inoculated with tobacco mosaic virus (TMV), kept at 30ºC for 48 h and then transferred to 20ºC to induce the HR (by temperature shift). Necroticlesion development was then assessed. Propagation of virus particles wasmonitored at 0 and 3 h after temperature shift by RNA blot hybridization with a ^{32}P-labeled cDNA for TMV coat protein.

B. Development of lesions.

Lesions of the early stage are characterized by dark ring-like structures with green tissue in the center, and lesions of the late stage are characterized by uniformbrown circles consisting of necrotic tissue (upper panel). Examples of lesion development at the indicated time points upon TMV infection are shown in WT, S7(lower panel).

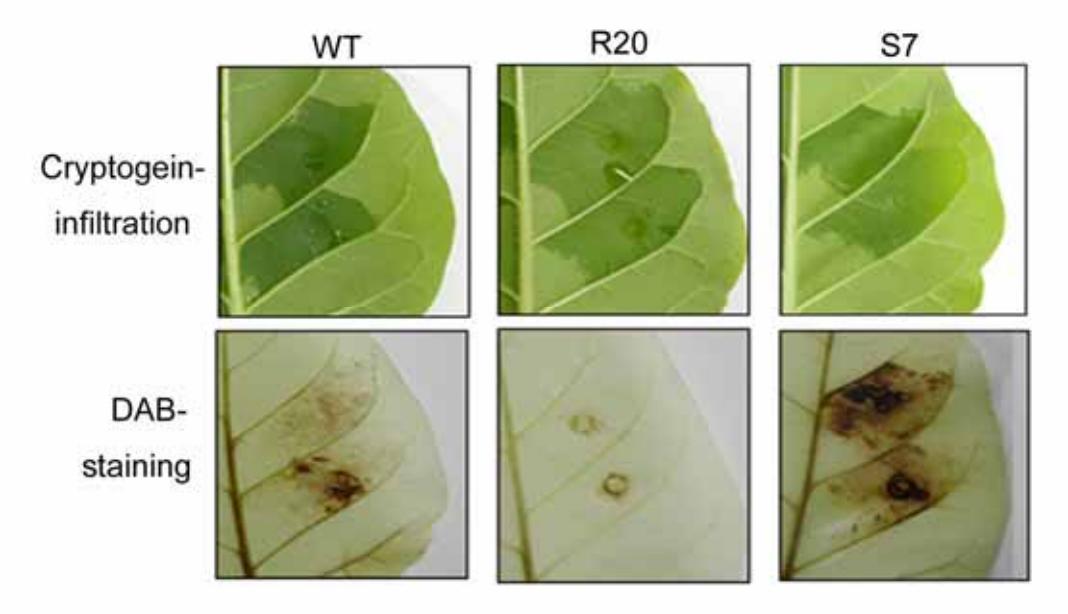

Figure 7. Production of hydrogen peroxide upon cryptogein elicitation.

Healthy leaves from wild type, transgenic R20 and S7 plants were detached and infiltrated with cryptogein solution. Each leaf was sampled 6 h after infiltration (upper panel) and hydrogen peroxide accumulation was visualized by staining with 1 mg mL^{-1} DAB (diaminobenzidine, pH3.8) solution (lower panel).

Chapter III. Genes which are Directly Targeted by NtWIF

III.1 Introduction

Recently, several studies have showed that MAPKs target transcription factors, which are involved in the regulation of their target genes depending on upstream MAPK-mediated phosphorylation. For example, it was evidenced that NtWRKY1 is targeted by SIPK whose binding activity to the W-box (TGAC) of class I basic chitinase gene, CHN50, was shown to increase on SIPK-mediated phosphorylation (Menke et al., 2005). This indicates that identification of MAPK-targeted transcription factors and their target genes is critical to understanding the biological significance of MAPK cascades.

In above chapters, it was clarified that NtWIF is a downstream target transcription factor of WIPK. However, it has been remained to be proved what genes are targeted by NtWIF in WIPK-NtWIF cascade. In this chapter, I identified the target genes of NtWIF. A clue for that is offered by the high homology with the auxin response factor (ARF), allowing me to speculate that the targets of NtWIF and ARFs might share common recognition motifs. Intensive analyses have revealed that ARF proteins recognize and bind to a common sequence containing the core TGTCTC, which is called the auxin responsive element (ARE) (Guilfoyle et al., 1998; Ulmasov et al., 1995; 1997; 1999). In

this chapter, I also found that NtWIF is able to recognize and activate genes containing the core ARE in their promoter regions.

III.2 Materials and Methods

Plant Materials and Treatments

All tobacco plants (*Nicotiana tabacum* cv Xanthi nc) including wild type and *NtWIF*-transgenic plants were grown in a growth cabinet at 23ºC under a 14/10-h light/dark cycle. They were wounded by punching, floated in water for 15, 30 and 60 min and immediately frozen in liquid nitrogen. The controls were sampled at time 0.

RNA Isolation and RNA Blot Hybridization

Total RNA was isolated by the hot-phenol method and 10-μg aliquots were fractionated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N, Amersham). After crosslinking using a UV crosslinker (RPN 2501, Amersham), the membrane was subjected to hybridization with appropriate ^{32}P-labeled probes at 42ºC for 16 h. After washing several times, the membrane was used to expose either BAS or x-ray film (Kodak)

Microarray screening

Differential hybridization of tobacco microarrays (Katoh et al. 2003) was performed

using a mixed probe of Cy3-labeled cDNA from wild type and Cy5-labeled cDNA from *NtWIF*-sense transgenic plants (S7), or Cy5-labeled cDNA from wild type and Cy3-labeled cDNA from S7 line. Total leaf RNA was extracted from each three independent plants which were grown under non-stressed conditions, and poly(A)$^+$ mRNA was prepared using GeneElute mRNA miniprep kit (Sigma, St. Lois, MO) according to the manufacturer`s instruction. Each mRNA sample was transcribed in the presence of Cy3-dCTP or Cy5-dCTP (Amersham Phamacia biotech, Dübendorf, Switzerland) by Label Star Array kit (Qiagen). Duplicated hybridization was performed three times for each mixture to confirm reproducibility.

Fusion Proteins

A GST-tagged NtWIF was obtained by cloning corresponding *NtWIF* cDNA fragments into the GATEWAY cloning system (Invitrogen) according to the manufacturer's instructions. The final fragments were cloned into the pDEST20 vector plasmid and transformed into *E. coli* together with bacumid (genome of a baculovirus) to allow *in vivo* recombination, and resulting single plaques were picked up and further propagated. Sf9 cells (6 x 10^9), maintained in Grace's insect medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 500 µg mL^{-1} gentamycin, were infected with the recombinant baculovirus (500 µl) and incubated at 27°C for 4 d

(Wada et al., 2003). Cells from one dish were suspended in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 25% [v/v] glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 100 μg mL^{-1} aprotinin) and sonicated for 10 s twice. Fusion proteins were purified through a glutathione-Sepharose column (Amersham Biosciences) or Ni-NTA agarose (Qiagen) according to the manufacturer's instructions. Protein concentrations were estimated by the Bradford method.

Assays of Transcriptional Activity

The β-glucuronidase (GUS) gene in pBI221 (Clontech) was replaced with the firefly luciferase gene derived from pGL3-Basic (Promega) to yield a plasmid pBI221-Luc. Approximately a 1 kb of *PR-Q* promoter was amplified by PCR using primers (forward: 5'-AAGCTTTCCTGGGAGGAAATAATCCACAA-3'; and reverse: 5'-ATTTCCTCTC TTCTTTCTTATGCT-3'), and a 1.2 kb of WIPK promoter was similarly amplified with primers forward: 5'-GCTAATTCTCTTTGGCCAGCGATGT-3'; and reverse: 5'-AGA GGGTGTAGGGGTCAATGAAGAA-3'). PCR products were substituted for the CaMV 35S promoter of pBI221-Luc. DR5 hexamer of 5'-TGTCTCCCTTT TGTCTC-3' was amplified by PCR, digested with *Hin*dIII and *Sal*I, and successively fused to the CaMV35S-46 minimal promoter and firefly luciferase gene to generate DR5-min-Luc. An internal control (reference) plasmid, containing an R-luciferase gene placed under

the control of a CaMV 35*S*-promoter was used to normalize for bombardment efficiency. For effector plasmids, each PCR product of *NtWIF* or nucleus localized sequence-attached *NtWIF* or WIPK was cloned into pBI221 driven by the 35*S*-CaMV promoter. Five- or 6-day-old BY2 suspension cells (2 ml) maintained in liquid medium were plated on 3% agar plates and briefly allowed to dry to remove excess liquid. They were then bombarded with plasmids (effector: reporter : reference = 2:2:1) coated on a 1.0-μm microcarrier in a vacuum of 28 inches of mercury using a helium pressure of 1,100 psi (PDS 1000; Bio-Rad). Cells were placed 6 cm from the stopping screen. After bombardment, cells were harvested with 20 ml of liquid medium, transferred into a flask, and then incubated in the dark at 25°C with gentle agitation. Luciferase and R-luciferase activities were assayed using a dual-luciferase assay kit (Promega) according to the manufacturer's instructions. Chemical luminescence was measured using a luminometer (Lumat LB9507; EG & G Berthold). The presented results are the means of 6-repeats with standard deviations.

Electrophoretic Mobility Shift Assays

DNA fragments containing DR5 (5'-TGTCTCCCAAATGTCTC-3') and DR7 (5'-TGTCTCCTCAATATGTCTC-3') were obtained from constructs used for transcriptional activity assays by PCR amplification and gel purification. Electrophoretic mobility shift

assays were performed essentially as described (Ito et al., 2003). The probes were radioactively labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase (Takara) by incubation for 30 min at 37ºC, and purified by phenol and ethanol extraction. A 1-μl aliquot of the probe containing approximately 10^4 cpm was mixed with approximately 0.1 μg of purified GST-NtWIF protein in a 20 μl reaction mixture containing 20 mM HEPES (pH7.9), 50 mM KCl, 5% glycerol, 1 mM EDTA (pH8.0), 10 mM DTT, 1 μg BSA and 2 μg poly(dI-dC). After incubation at room temperature for 30 min, the reaction mixture was loaded on a 4% non-denaturing polyacrylamide gel (30%; 29 : 1 acrylamide : bisacrylamide) in 0.5 × TBE after pre-run for 1 h at 100V, and subjected to electrophoresis at 100V. Gels were dried at 80ºC under vacuum for 30 min and autoradiographed using x-ray film. Competition experiments were carried out by adding unlabelled competitors to the binding reaction at a molar ratio to the probe of 25 : 1.

III.3 Results

Differential microarray screening

A microarray screening was performed using the array containing 3766 cDNA clones prepared from root and leaf tissues of *Nicotiana sylvestris,* and 48 cDNA clones prepared from wounded or TMV-treated *N. tabacum* (Katoh et al. 2004). Probes were prepared from non-stressed wild type or *NtWIF* sense transgenic line 7 (S7) plants.

Initial screening brought out 178 clones, which showed over 3-fold higher expression in S7 than in wild type plants. The average false positive rate was calculated to be less than 1% (Appendix Table 1). To verify the accuracy of microarray results, representative genes were selected and subjected to RNA blot hybridization (Figure 8). RNA samples were prepared not only from wild type and S7 plants, but also from *NtWIF*-RNAi line (R20), so that the accuracy of gene identification can be confirmed by the opposite feature of transcript accumulation to the overexpressing plants. During the course of transgenic plants analyses, it was implied that more highly up-regulated genes in *NtWIF*-sense transgenic plants than in WT plants are not necessarily regulated directly by NtWIF, but possibly indirectly through other factors such as SA-induced transcription factor(s). Thus, expression profiles of several representative clones were examined upon wounding treatment which is considered to be independent on SA levels (Lei et al., 2002; Niki et al., 1998) in WT and RNAi plants. Results showed that genes for *PR-Q* (accession no. M29868), *acidic* (Z11563) and *basic chitinase III* (Z11564), *acidic β-1,3-glucanase* (X69794), *auxin-induced mRNA* (pCNT115, X56267) and *PR-1b* were markedly activated in wild type, while they were totally silent in the R20 line (Fig. 8). It is clear that these identified genes were indeed under the control of NtWIF. These were constitutively expressed in the S7 line. The results confirmed that

the microarray results were reliable for identification of differentially regulated genes.

Functional diversity

When identified genes were classified according to their putative functions, 49 (27.5%) were found to be related to defense, suggesting NtWIF to play a critical role in general defense reaction (Figure 9). In addition, 19 (10.7%) were shown to be involved in metabolism, 16 (9%) were putative transcription factors and 26 (14.6%) were related to cell wall and cytoskeleton architectures (Figure 9). Functional classification of array-identified genes indicated NtWIF to be involved in a broad range of cellular activity (Appendix Table 1). First, defense-related genes appeared to be the main target, as a high level of up-regulation was evident in both biotic- and abiotic-stress responsive genes, including *PR-1b* and *PR-Q* for the former, and *TAS14* and *osmotin* for the latter. Genes involved in ROS production, in stress hormone biosynthesis such as ethylene, and in secondary metabolism such as putresine methyltransferase and cytochrom p450 were also up-regulated by NtWIF. These results are consistent with upper my findings showing an enhanced resistance to tobacco mosaic virus-infection in *NtWIF* sense transgenic plants. Second, genes involved in cell wall and cytoskeleton formation were greatly affected. Encoded proteins included cell wall degradation enzymes such as β-galactosidase, β-D-xylosidase, β-1,3-glucanase and chitinase, cell wall

expansion-related enzyme, and cell wall synthesis enzyme such as cellulose synthase and putative pectinacetylesterase. They regulate plant growth, development and morphology in coordination, as exemplified by expansin LeEXP1 for fruit softening (Chen et al. 2001) and cell-wall invertase for phloem unloading during seed development (Miller and Chourey 1992). In addition, they are also involved in protection against environmental stress and in releasing signal molecules, as seen from β-1,3-glucanase and chitinase functioning not only in defense responses against fungal infection (Mauch et al. 1988) but also in physiological and developmental processes including embryogenesis, microsporogenesis and flowering (Neale et al. 1990; Grosset et al. 1990; Balandin and Castresana 1997). Third, many genes encoding transcription factors were also up-regulated. They included WRKY-type proteins such as NtWRKY3 and WRKY2, MYB family proteins such as LBM1, LBM3 and LBM4, EREBP/AP2-type transcription factors such as Tsi 1, and basic leucine zipper transcription factors such as TGA2.2 and TGA1b. They are reported to play important roles in regulation of many genes involved in diverse physiological function such as MYB proteins for stress and defense response (Daniel et al. 1999; Sugimoto et al. 2000) and for regulation of meristem formation and seed development (Schmitz et al. 2002; Penfield et al. 2001); TGA family proteins for defense response by interacting with

NPR1 to activate SA-inducible genes (Zhang et al. 2003), and for flower organ formation (Corinna et al. 2005); and WRKY-type transcription factors as downstream factors of stress-related MAPK (Kim and Zhang 2004). The array data thus suggested that NtWIF may pleiotrophically contribute to diverse physiological functions, among which defense response and developmental control appeared to be unique. Whether its effect is direct or indirect remain to be determined.

DNA Binding Motifs

The N-terminus of NtWIF shows high similarity to the B3 type DNA binding domain of Arabidopsis auxin response factor (ARF) (Yap et al., 2005). Since ARF proteins generally recognize the auxin response element (ARE) consisting of TGTCTC (Guilfoyle et al., 1993), I examined interactions between NtWIF and ARE. Experimentally, gel-mobility shift assays were performed using GST-fused full length NtWIF protein and a synthetic ARE, named DR5, containing a direct repeat of TGTCTC sequence spaced with CCTTT (Guilfoyle et al., 1998). The GST-NtWIF was found to form a complex with the DR5 probe, dependent on the protein concentration (Fig. 10A, left panel). The specificity of binding was confirmed by a competition test, showing a 25-fold excess of unlabeled DR5 competitor to effectively interfere. Further assays revealed that NtWIF equally binds to the DR5 motif spaced by seven bases (Fig.

10, right panel), being consistent with previous observation on ARF protein (Guilfoyle et al., 1998). These results suggested that NtWIF recognizes the core ARE, and therefore that its target genes would contain the ARE core motif in their promoter regions.

DR5-Driven *Luc* Gene Expression

The DR5 motif was fused to the 35*S* minimal promoter-*Luc* and assayed for transcriptional activation by NtWIF in BY2 cells. The validity of DR5 in this experimental system was first confirmed by its response to auxin, with a 4-fold increase in Luc activity in the presence of naphthalene acetic acid (NAA) (Fig. 10B). In the presence of NtWIF, a similar increase up to 4-fold was noted. However, this activity was not enhanced, but rather suppressed by WIPK, in clear contrast with the GAL 4 assay case (Fig. 2B). One conceivable reason is that, in contrast to the GAL4 system in which the test protein is forced to become incorporated into the nucleus, NtWIF is not efficiently incorporated into nucleus under standard experimental conditions. Subsequently, a nucleus localization sequence (NLS) was attached to the C-terminus of NtWIF, thereby causing the protein to migrate into the nucleus. Transactivation assays then revealed NLS-attached NtWIF to be more effective than wild type NtWIF in the absence of WIPK (Fig. 10B). When WIPK was present, the activity significantly

increased up to 3-fold that in its absence. Effects of NLS in the presence of WIPK were also evident as seen from the nearly 5-fold higher activity than with the wild type control (Fig. 10B). The results suggest that, upon phosphorylation by WIPK, NtWIF is able to transactivate genes equipped with the ARE motif. An apparent suppression of transactivation activity of wild type NtWIF by WIPK was possibly due to formation of inactive complexes between over-produced WIPK and NtWIF in the cytoplasm, thereby interfering with the latter to migrate into nucleus.

Identification of NtWIF-target Genes

To determine whether or not identified genes might be directly controlled by NtWIF, the *PR-Q* gene was selected and its promoter region was isolated. Initially, a 1.5-kb promoter segment was fused to the 35*S* minimal promoter-*Luc* and examined for transcriptional activation by NtWIF in BY2 cells. The 1.5-fold increase indicated that NtWIF alone is able to activate the *PR-Q* gene. The level of activation remained the same even if NtWIF was equipped with NLS (Fig. 11A). When WIPK was added to the reaction system, the activity did not apparently change with wild type NtWIF, while it increased over 2-fold with NtWIF containing NLS (Fig. 11A). These results were essentially consistent with the case of the DR5 synthetic promoter (Fig. 10B), indicating *PR-Q* to be a possible direct target of NtWIF. However, the tested promoter region

contained no typical TGTCTC core sequence, but rather two degenerated TGTCCT sequences separated by 78 spacer bases. These results indicate that NtWIF is able to recognize genes containing degenerated ARE motifs, including TGTCTC and TGTCCT. Consequently we screened microarray-identified genes (Appendix Table I), for which promoter sequences were available, to identify common motifs. Alignment of genes encoding acidic β-1,3-glucanase, aminocyclopropane carboxylic acid synthase 2 (ACS2) and P-450 revealed all to possess a TGTC with variable proximal nucleotides (Fig. 11B). In addition, the promoter region of *WIPK* was found to contain this motif. Sequence comparison showed that three out of six examined sequences are TGTCCT (two from *PR-Q* and one from *ACS2*), and the remaining are typical TGTCTC (*WIPK*, β-*1,3-glucanase* and *P-450*) (Fig. 11B). Sequence comparison also showed that the nearest neighbor of the 5' end of the core is thymine (T) or adenine (A), and that of the 3' is predominantly T, and that all promoters examined are enriched with A and/or T at both 5' and 3' proximal regions of the core TGTC (Fig. 11B).

WIPK Activation

In my laboratory, promoter of WIPK has been identified and precisely studied (Yap et al., 2002). I found that promoter sequences of WIPK possess the ARE motif. In this study, to establish whether these genes are transcriptionally activated by NtWIF, a

1.2-kb region of the *WIPK* gene was selected (accession no. AB052964) and tested for transactivation (Fig. 12A). Results were essentially the same as for the *PR-Q* promoter, exhibiting a 2-fold increase in the presence of WIPK (Fig. 12A). When NLS-NtWIF was used, activity was increased 2.5-fold over the control value in the presence of WIPK (Fig. 12A). In the absence of WIPK, wild type NtWIF was not effective, and NLS-NtWIF was equivalent to wild type NtWIF with WIPK (Fig. 12A). The upregulation of *WIPK* by NtWIF was further examined by *in planta* analysis. Healthy leaves from wild type and *NtWIF*-sense transgenic tobacco plants were mechanically wounded, and transcript accumulation of *WIPK* was periodically monitored (Fig. 12B). In wild type leaves, WIPK transcripts began to accumulate 30 min after wounding, being consistent with previous observation (Yap et al., 2002). In transgenic lines, they were constitutively accumulated regardless the wound stress (Fig. 12B). These experiments clearly showed that the *WIPK* gene is directly upregulated by NtWIF.

III.4 Discussion

Since NtWIF exhibits transcription activation activity upon phosphorylation by WIPK, the question arises of target sequences or genes. I set two working hypotheses. First, I assumed that NtWIF can recognize the auxin responsive element (ARE) which contains the core TGTCTC sequence, and is recognized by auxin responsive factors

(ARFs) with N-terminus resembling that of NtWIF (Yap et al., 2005), all having a plant-specific DNA binding domain, B3. Second, I proposed that genes showing altered expression in NtWIF transgenic lines would have ARE motifs in their promoter regions. *In vitro* binding assays revealed that NtWIF specifically and efficiently binds to synthetic AREs, DR5 and its derivative, and that it activates the DR5-driven *Luc*-reporter gene *in planta*. Based on these observations, I next screened specific genes differentially regulated in transgenic plants: one over-expressing *NtWIF*, and the other with suppression of *NtWIF* by RNAi. Differential micro-array screening finally identified 49 defense-related genes, among which genes for PR-Q, acidic β-1,3-glucanases, ACS2, P-450 and WIPK were found to possess the core TGTC sequence, although proximal nucleotides were variable. Due to its distinct expression profile, *PR-Q* was further subjected to transactivation analyses. *Luc*-reporter assays showed that NtWIF might recognize TGTCCT sequence, a degenerated TGTCTC core motif. Similar activation was also observed with a 1.2-kb WIPK promoter, which contains one core TGTCTC sequence. Subsequent sequence comparison among identified genes suggested that the core might be constituted of TGTCTC and TGTCCT, and that the proximal regions are rich in A and T. It was thus speculated that NtWIF may commonly recognize and regulate genes which are equipped with the degenerated

ARE motif, (T/A)TGTC(TC/CT)(T/C) surrounded by AT clusters in their promoter regions. The B3 domain has been identified in maize VP1 (McCarty et al., 1991), Arabidopsis ABI3 (Giraudat et al., 1992) and ARFs (Ulmasov et al., 1999), with suggestions of involvement in regulation of auxin- and abscisic acid-responsive genes (Suzuki et al., 2001). My observation that the B3 domain of NtWIF activates genes possessing AREs suggests its novel function in wound and pathogen signaling pathways.

Two novel aspects were here revealed with regard to the MAPK cascade in plants: first, that direct down-stream factor of WIPK, NtWIF and its downstream element exist; and second, that NtWIF contributes to feedback regulation of WIPK. I found here that at least *PR-Q* is a direct target of NtWIF, and genes for acidic β-1,3-glucanase and ACS2 are also highly probable targets, as judged from the presence of the same ARE motif in their promoters. PR-Q is an acidic chitinase with an apparent molecular mass of 25 kDa, and localized to apoplastic fraction (Payne et al., 1990). Its biological role is not completely clear, but the enzyme is considered to play a direct role in defense against fungal infection in combination with β-1,3-glucanase, both hydrolyzing chitin, a major cell wall component of pathogenic fungi (Mauch et al., 1988; Hahn et al., 1988). Notably, β-1,3-glucanase could also be a potential target of NtWIF. ACS plays a key

role in ethylene production, catalyzing conversion of *S*-adenosyl-methionine into 1-aminocyclopropane-1-carboxylic acid (Wang et al., 2002). Recent studies showed that an Arabidopsis MAPK (AtMPK6) directly phosphorylates ACS2, and leads to ethylene production (Liu and Zhang, 2004). Since AtMPK6 is an ortholog of tobacco MAPK (salicylic acid-induced protein kinase; SIPK), which coordinately functions with WIPK during pathogen responses (Zhang and Klessig, 2001), it is possible that ethylene production by ACS is regulated at the protein level by SIPK, and at the transcriptional level by WIPK through NtWIF. Overall, within the limits of promoter sequence information for tobacco plants, the presently available data suggest that NtWIF has a broad range of target genes which are involved in production of actual defense substances, such as chitinases and glucanases, and also of signal molecules such as ethylene.

An unexpected finding was that *WIPK* is a direct target of NtWIF. *WIPK* is transcriptionally activated 30 min after mechanical wounding, and 1 h after onset of the HR (Yap et al., 2005). However, WIPK itself is phosphorylated by its upstream MEK within a few min after wounding (Seo et al., 1995), suggesting that transcriptional activation is to compensate for deficiency of WIPK protein. If this is the case, a well-balanced regulation system would be expected between transcription and protein

production. It appears to be reasonable that WIPK-activated NtWIF promotes WIPK transcription, thereby forming a kind of self-amplifying system within the MAPK cascade, although the opposing system that attenuates this circuit remains to be identified.

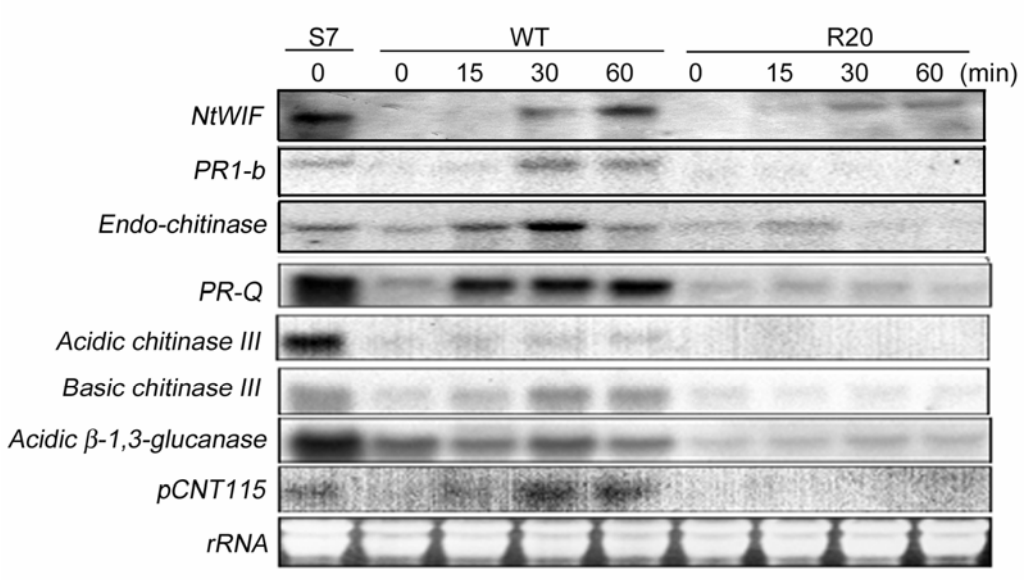

Figure 8. Verification of differential expression profiles.

Among genes which showed over 3-fold higher expression in the NtWIF sense-transgenic S7 in comparison with the wild type control by micro-array analyses, those for PR-1b (accession no. D90197), endo-chitinase(X16938), PR-Q (M29868), acidic (Z11563), basic chitinase III (Z11564), acidic-β-1,3-glucanase (X69794) and auxin induced mRNA (pCNT115, X56267) were subjected to RNA blot hybridization analysis to confirm their differential expression patterns among untreated S7, wound-treated wild type and R20 lines. Healthy wild type and R20 leaves were wounded by punching, and floated in water for the indicated time periods. Leaves from the S7 line were not treated. Total RNA was isolated and a 10-μg aliquot per lane was fractionated by agarose gel electrophoresis and subjected to RNA blot hybridization with the indicated ^{32}P-labeled cDNA probes. rRNA was employed as the loading control.

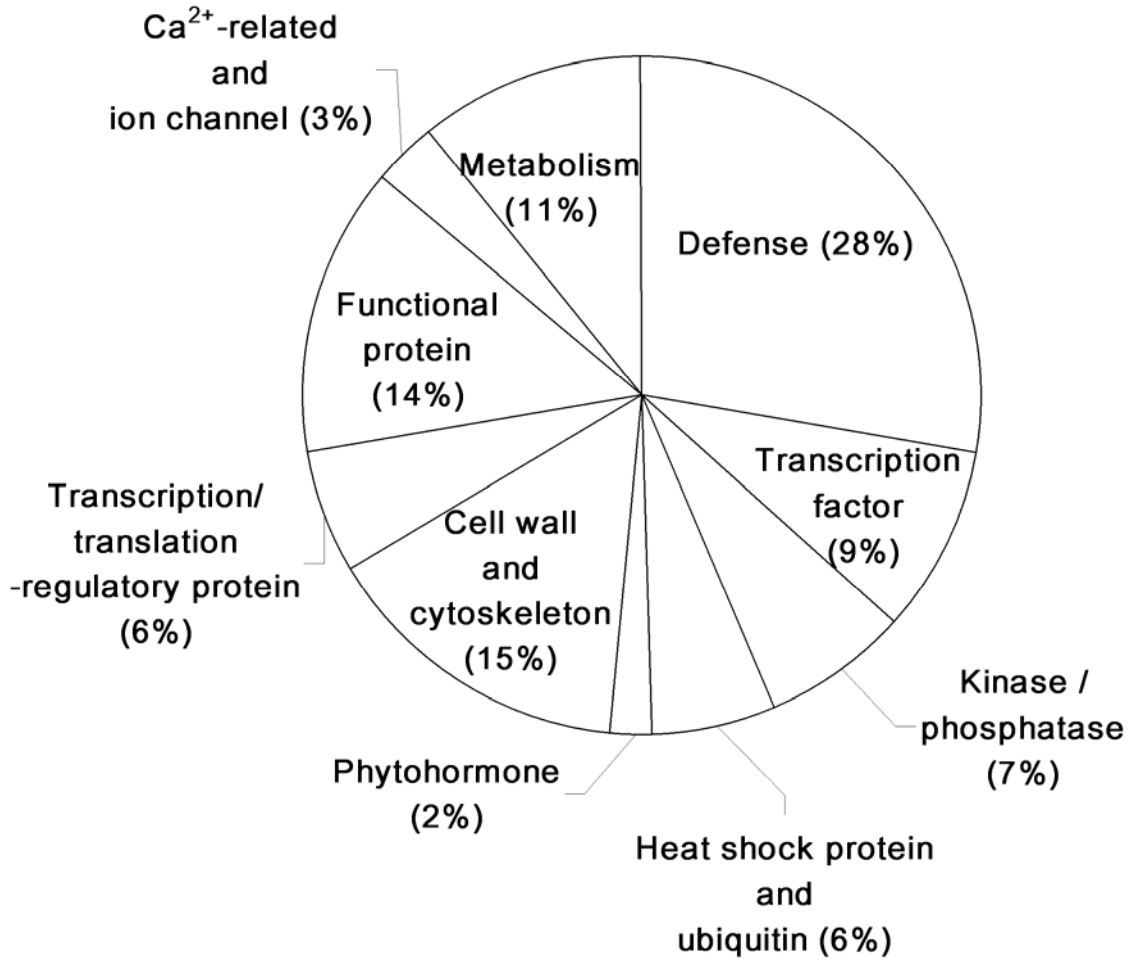

Figure 9. Functional classification of microarray-identified genes.

Circle chart shows classification of genes which showed over 3-fold higher expression in S7 than in wild type plants. Among totally 178 identified genes, 49 (27.5%) were for defense, 26 (14.6%) for cell wall and cytoskeleton, 19 (10.7%) for metabolism, 16 (9%) for putative transcription factors, 13 (7%) for kinase/ phosphatase, 10 (5.6%) for heat shock protein and ubiquitin, 3 (2%) for phytohormone, 11 (6.1%) for transcriptional/translational regulatory protein, 25 (14%) for functional protein, 6 (3%) for Ca2+-related and ion channel.

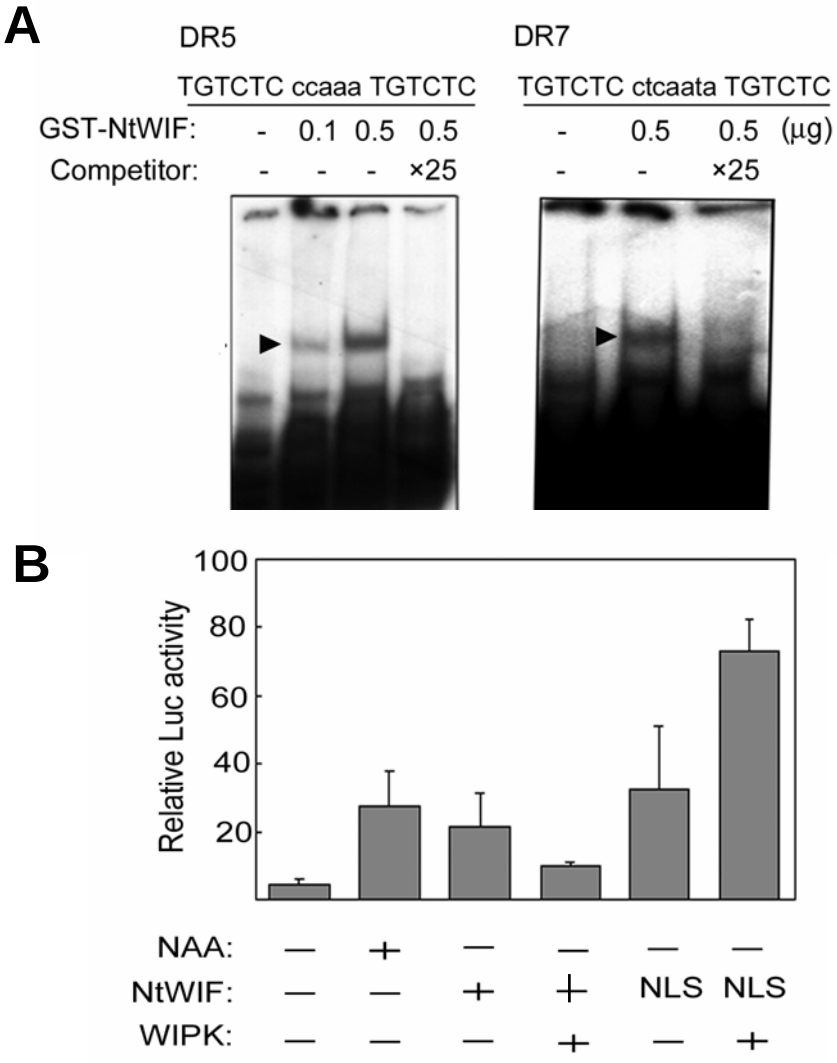

Figure 10. Activation of synthetic ARE.

A. Gel-shift assays were performed using GST-fused full length NtWIF protein and synthetic AREs; DR5 containing a direct repeat of TGTCTC sequence spaced by a 5-bp of CCTTT; and DR7 containing different spacing sequences (CTCAATA) from DR5. The specificity of binding (indicated by an arrowhead) was confirmed by competition tests with a 25-fold excess of each unlabeled competitor.

B. Transactivation by DR5: 35Smin*Luc*.

DR5-35S minimal promoter-driven luciferase activity was measured in BY2 cells in the presence (+) or absence (-) of NtWIF, with or without a nucleus localized sequence (NLS), in combination of the presence (+) or absence (-) of WIPK (indicated by plus and minus). Plasmids were mixed, coated onto gold particles, and introduced into BY2 cells by particle bombardment. Luciferase activity of each type of transformant was normalized to the respective R-luciferase activity. For the validity of DR5 in this experimental system, 25 μM naphthalene acetic acid (NAA) was added into culture medium after bombardment. Data are from six experiments with standard deviations.

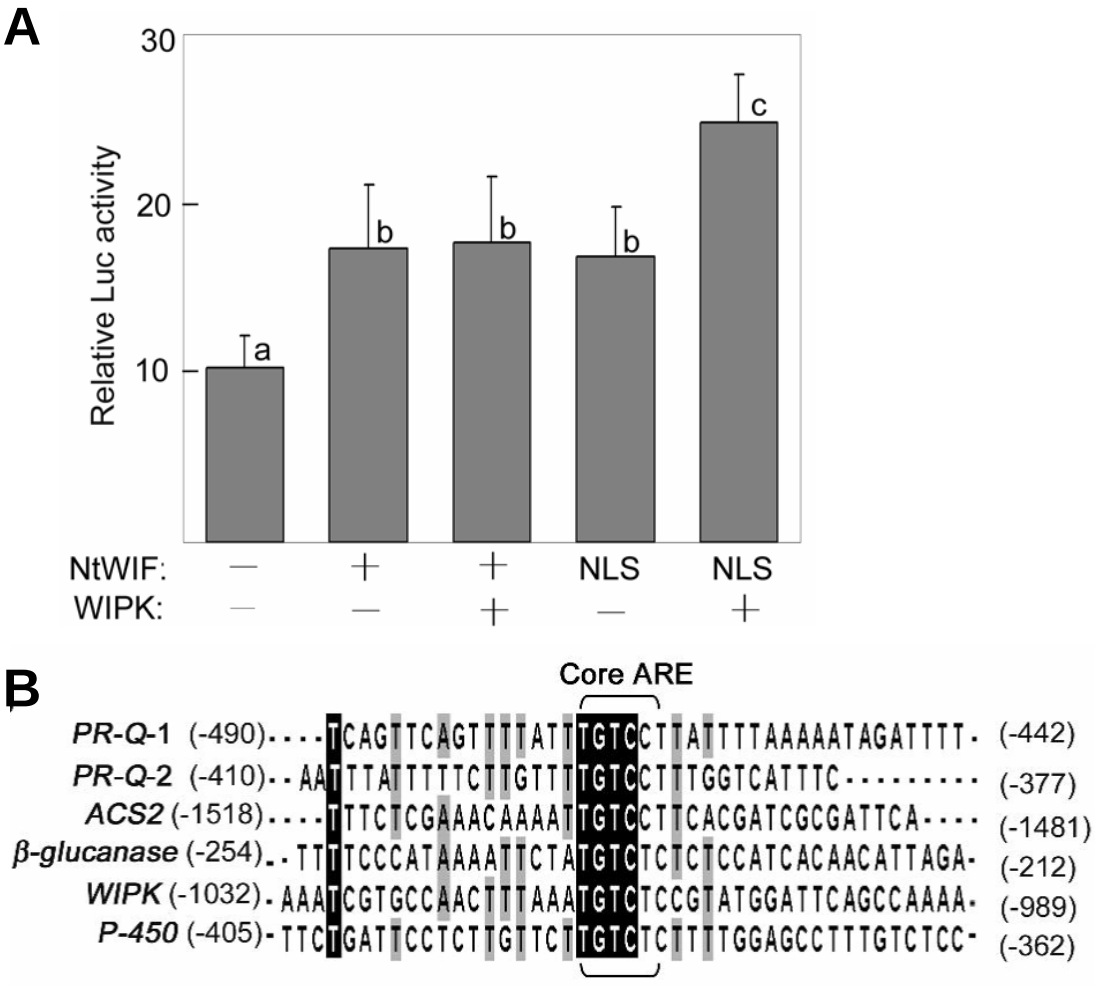

Figure 11. Transactivation of *PR-Q* and deduced binding sequence.

A. Transactivation of the *PR-Q* promoter.

The 1.5 kb promoter region was fused to the *Luc*-reporter gene, and luciferase activity was monitored in the presence (+) or absence (-) of NtWIF, with or without a nucleus localized sequence (NLS) in combination with the presence (+) or absence (-) of WIPK. Plasmids were mixed, coated onto gold particles, and introduced into BY2 cells by particle bombardment. Luciferase activity of each type of transformant was normalized to the respective R-luciferase activity. Data are from six independent experiments with standard deviations. Means with the same letter are not significantly different at $P<0.05$ by the Tukey's studentized range test.

B. Common core sequence.

Promoter sequences of indicated genes were screened for the core TGTC segment and compared on the basis of core segment (bracketed). Common bases among six sequences are boxed and those among four are shaded. Numbers indicate nucleotide positions from the ATG codon in each gene.

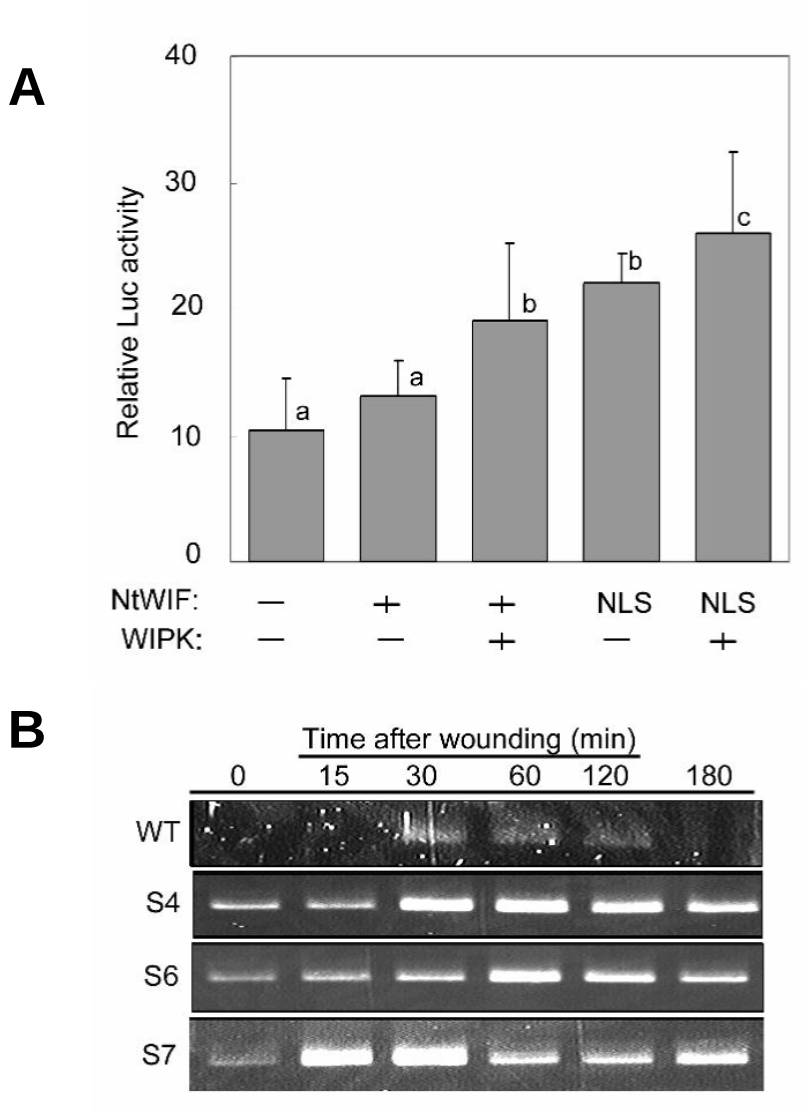

Figure 12. Upregulation of *WIPK*.

A. Transactivation of the WIPK 1.2-kb promoter.

WIPK containing the core TGTC within 1.2-kb region was examined for transativation of luciferase activity in BY2 cells as described in the legend for Figure 11B. Assays were performed in the presence (+) or absence (-) of NtWIF with or without NLS in combination with the presence (+) or absence (-) of WIPK. Means with the same letter are not significantly different at $P<0.05$ by the Tukey's studentized range test.

B. Transcript accumulation.

Leaves from wild-type (WT) or indicated transgenic lines (S4, S6 and S7) were harvested before (0) and indicated time period after wounding, and total RNA was isolated. RT-PCR was performed with specific primers for *WIPK*.

Chapter IV. Pleiotropic Functions of NtWIF

IV.1 Introduction

Growth and development of plants are regulated by diverse factors, including phytohormones (specially, in other words, growth regulators), light, oxygen, water as a kind of external environmental signals, or nutrients. Among them, specially, phytohormones which consist of auxin, ethylene, gibberellins, (+)-abscisic acid (ABA) and cytokinine have been considered to play as a major factor (Kende and Zeevaart, 1997). In the case of root development, auxin is known to influence root morphology, promoting the lateral and adventitious roots formation by stimulation of cell elongation and division whereas cytokinin inhibits root formation (Malamy and Benfey, 1997; Lmin et al. 1997). However, in recent studies, it has been noticed that these hormone mediated-signaling pathways or -phenotypes are affected not only by direct hormone treatment but also by other environmental stress factors-mediated signalings. And also, it has been pointed that latter are able to be affected by former. These mean that diverse signaling pathways are connected one another, namely they are cross-talking. Recently, involvement of MAPK, especially stress-activated MAPK in plant growth and development regulation has been evidenced by several studies. For instance, Kotvun et al. (1998) clarified that NPK1, a plant specific MAPKKK, activates a MAPK cascade

that leads to suppression of auxin signaling. As another report on NPK1-mediated cascade, it was clarified that NPK1-MEK1-NTF6 pathway had dual functions which were involved not only in N-mediated resistance but also in regulation of cytokinesis. Nakagami et al. (1996) reported that MEKK1 and its downstream target, MPK4 which are activated by H_2O_2 functioned in regulation of multiple vegetative development processes through control of ROS homeostasis in *Arabidopsis*.

In the above chapter, I attempted to identify genes which are under the control of NtWIF. Among them, the genes which are involved in cell differentiation were notable. These micro-array and transgenic approaches suggested NtWIF to dual function in defense and in differentiation and/or development although it has been identified as a stress-induced WIPK-activated transcription factors.

IV.2 Materials and Methods

Plant materials

Wild type and transgenic tobacco plants (*Nicotiana tabacum* cv Xanthi nc) were grown in a growth cabinet at 23ºC under a 14/10-h light/dark cycle.

Phenotypic observation

Seeds of transgenic or wild type plants were sterilized in 70% ethanol for 1 min. and then in 1.6% hypochloride supplemented with 0.1% Tween-20 for 15 min under

vigorous shaking. After being rinsed in sterile distilled water, approximately 100 seeds were placed on a plate containing germination medium (Murashige and Skoog MS basal medium supplemented with 3% sucrose and 8 g l^{-} agar, adjusted to pH 5.8) and allowed to grow at 23^{o}C under a 16/8 h light/dark cycle. Germination rate was examined 10 days after germination, and the means values were calculated from triplicate samples. Phenotype was observed 3 weeks later to determine root growth parameters (length of primary root and numbers of lateral roots). For histological analysis, root tips (about 0.5 cm) were fixed in 1.6% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 50 mM phosphate buffer (pH 6.8) for 24 h at 4^{o}C. After fixation, root tips were dehydrated by ethanol and embedded in Technovit 7100 (Kulzer, Wehrheinm, Germany) (Yeung 1999). Serial sections (10 μm) were prepared by cutting samples with a Histoknife H (Kulzer, Wehrheinm, Germany) on rotary microtome (Leica RM2145, Wetzlar, Germany), stained with 0.05% toluidine blue, and photographed using a light microscope (Nikon E600, Japan).

IV.3 Results

Seed development

Phenotypic features of transgenic lines (S7 and R20) apparently did not differ from those of wild type plants during vegetative growth (Fig. 4B). However, a distinct

difference was observed in pod development and seed germination. Pod development appeared to be retarded in RNAi lines showing an average size at maturation to be significantly smaller than that of S7 and wild type plants (Fig. 13A). This was especially distinct in R20 line, showing less than half size to the control. Pod size of S7 line was similar to that of wild type (Fig. 13A). When allowed to germinate on MS agar plates, wild type and S7 seeds normally germinated, whereas R4 and R20 germinated at a reduced frequency with 80% and 22% that of the control, respectively (Fig. 13B). This inability of germination was due to defective and/or incomplete development of embryo and endosperm as seen from vacant seed coats (Fig.13B). These results indicated that NtWIF plays a critical role in seed development, perhaps independently of defense response.

Root growth

Another distinct phenotypic differences between wild type and transgenic plants was root development, as a clear retardation of growth was observed 3 weeks after germination (Fig.14 A-C). Average length of primary roots in wild type was 67.2 mm, while it was 53.5 mm in S7 and 31.4 mm in R20, showing respective reduction of 20.4% and 53.3% (Fig.14A and B). Average number of lateral roots was also reduced; 5.6 in wild type, while 2.4 in R20 and less than 0.8 in S7 transgenic plants (Figure 14C).

When root meristem was visually observed with microscope, the cell number was apparently increased in S7 but decreased in R20 lines in comparison with wild type samples (Fig. 15A and B). This was evident by quantification of cell numbers per 250 μm, showing 22 in wild type, 43 in S7 and 11 in R20, each being double and half of the control (Fig. 15B). These features indicated that retarded root growth might be due to abnormal cell numbers, either excess (S7) or deficiency (R20). Since auxin plays a critical role in root formation (Casimiro et al. 2001; Guo et al. 2005), the results suggested that auxin function was disturbed in transgenic lines.

IV.4 Discussion

Recent studies have revealed that MAPK cascade mediates a variety of signal transduction pathways, which are not always simply liner but complicatedly connected with each other (Jonak et al. 2002; Zwerger and Hirt 2001). For example, a single MAPK-mediated cascade was suggested to function in diverse pathways depending on physiological conditions (Nakagami et al. 2005). NPK1-MEK1-NTF6 pathway showed dual functions, which were involved not only in R gene-mediated resistance but also in regulation of cytokinesis (Jin et al. 2002). Although NtWIF was identified as a downstream factor of stress signaling, structurally it exhibited a unique feature, having a region with a high homology to the B3-type DNA binding domain at the N-terminus.

The B3 domain was identified in maize VP1 (McCarty et al. 1991), Arabidopsis ABI3 (Giraudat et al. 1992) and auxin response factors (Ulmasov et al. 1999), and suggested to be involved in regulation of auxin- and abscisic acid-responsive genes (Suzuki et al. 2001). Such structural properties allowed me to speculate that NtWIF might possess other physiological functions such as auxin and ABA responses in addition to defense responses. Subsequent microarray screening and functional classification of identified genes strongly suggested that NtWIF is indeed involved not only in defense response but also in a broad range of cellular activity including cytokinesis and organogenesis. In this context, NtWIF may be assigned as one of the fundamental proteins that pleiotrophically contribute to diverse physiological functions, among which defense response and developmental control appears to be representative. This idea was substantiated by transgenic approach, which showed that disturbance of NtWIF and its downstream genes clearly induced phenotypic abnormalities in seed and root development. The absence of NtWIF caused a decrease of seed pod size, incomplete development of embryo and endosperm, and defective development of primary and lateral roots of seedlings. These morphological abnormalities have been known to be the results of imbalance of hormonal and/or signaling molecules (Feldman 1984; McCarty 1995; Osmont et al. 2007) For instance, initiation and emergency of lateral roots were

reported to be closely associated with not only auxin but also cell cycle phase, nutrients such as sucrose and nitrogen, and ABA-mediated signaling (Casimiro et al. 2003). Seed development was also shown to be affected by cross talking among diverse signaling molecules such as sugars, light and phytohormones including ABA and GA (Smeekens 2000). These observations together with my present findings suggest that a set of diverse genes could be simultaneously controlled by NtWIF during development, and that WIPK-NtWIF cascade possesses at least dual functions, defense response under stressed condition and developmental control during ordinary growth.

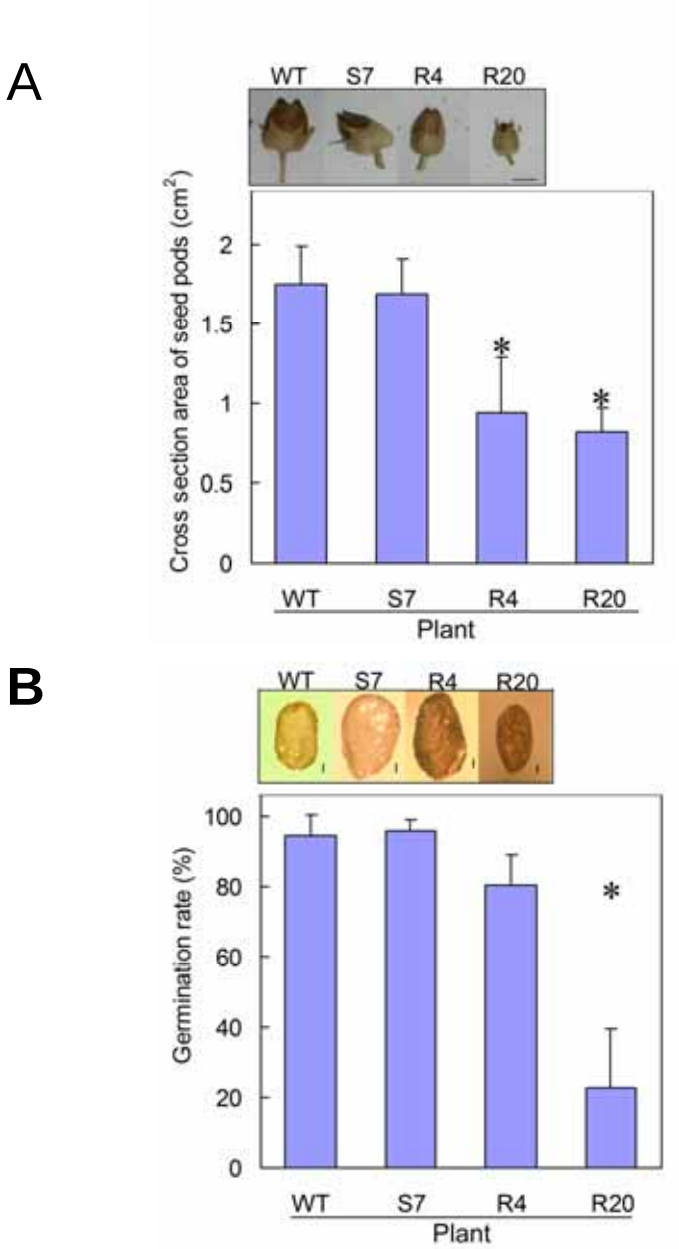

Figure 13. Size comparison of seed pods and germination rate.

A. Size of seed pod.

Pod size is represented as a mean of values estimated from width and height lengths from transgenic (S7, R4 and R20) and wild type (WT) plants. Photographs (upper panel) depict corresponding pods to each sample. Bar indicates 0.5 cm.

B. Germination rate.

Seeds were placed on germination medium, and germination rate was calculated 10 days after germination as a mean of triplicate samples with standard deviation. Asterisks indicate a significant difference from wild type (t-test; *P<0.05). Photographs (upper panel) depict corresponding seed to each sample taken after cutting in half. Bar indicates 0.1 mm.

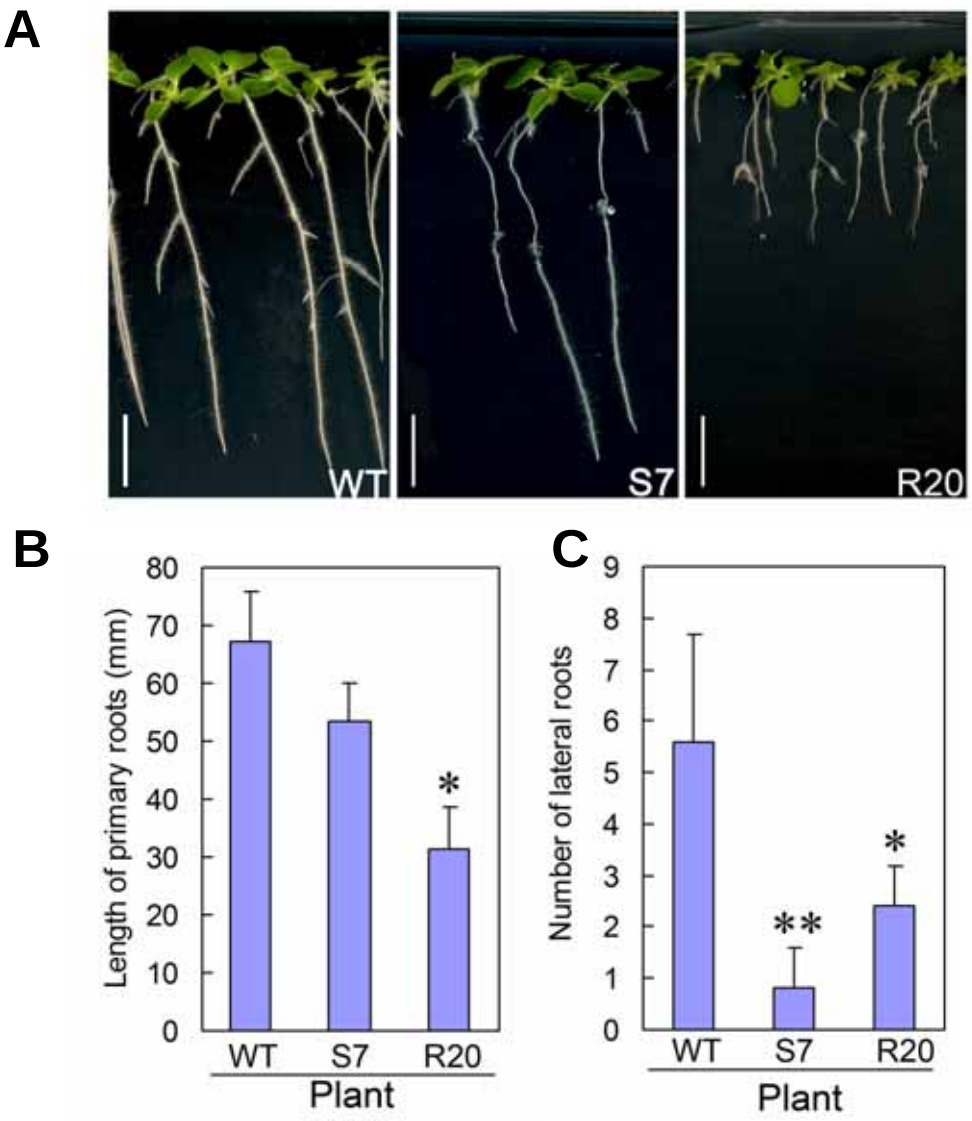

Figure 14. Root growth and development.

A. Seedlings.

Seeds of wild type (WT), S7 or R20 were plated on vertical agar plates, incubated for 3 weeks and photographed. Vertical bar stands for 1 cm.

B. Length of primary roots.

Root length of indicated plants was measured from more than 15 samples and mean values presented with standard deviation from triplicate experiments.

C. Number of lateral roots.

Lateral root number of indicated plants was measured from more than 15 samples and mean values presented with standard deviation from triplicate experiments. Asterisks indicate a significant difference from wild type (t-test; *P<0.05, **P<0.01).

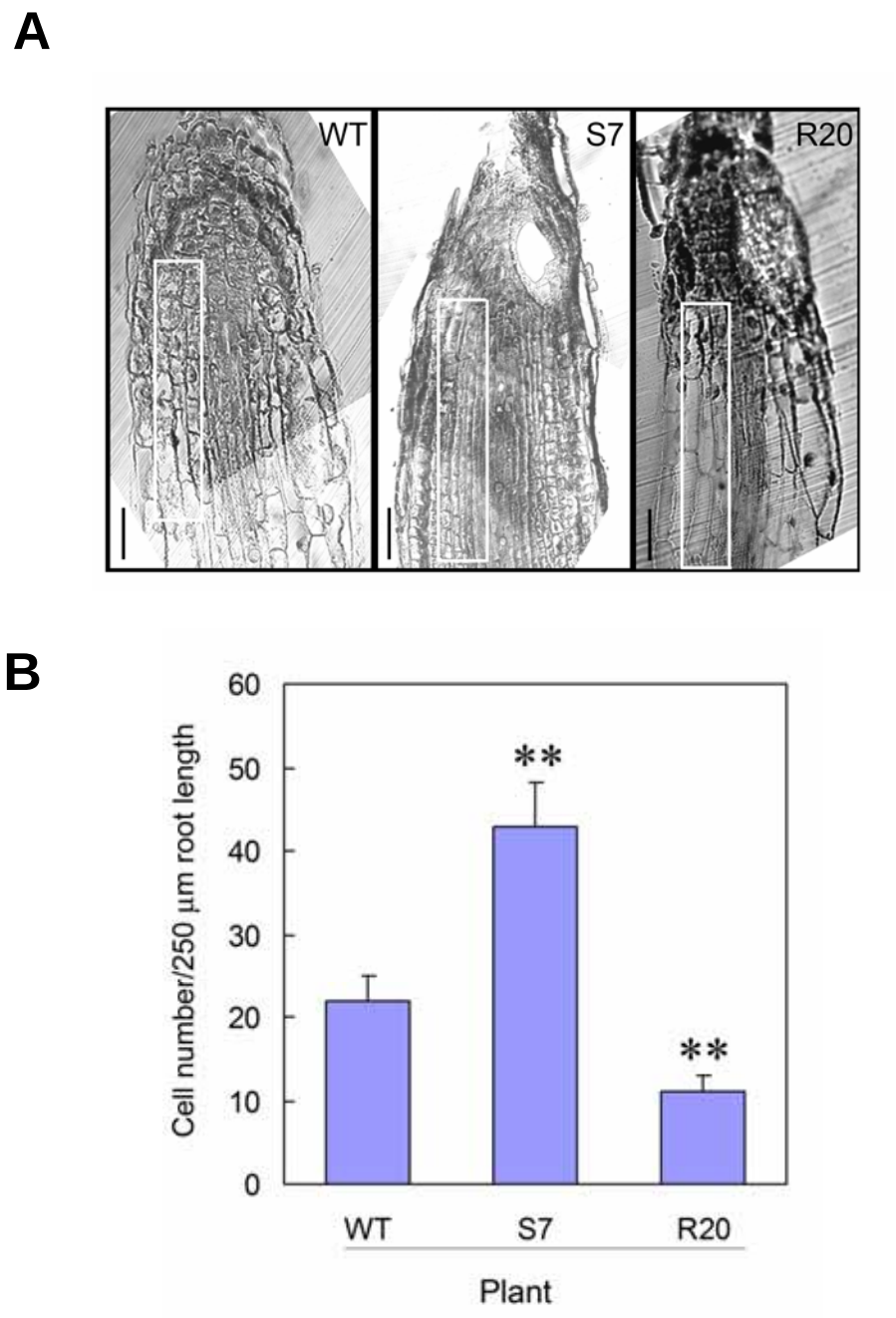

Figure 15. Phenotype of root meristem.

A. Microscopic observation.

Root tip from indicated plant was sectioned and observed under a microscope. Bar stands for 50 μm.

B. Comparison of cell numbers.

Cell number of primary roots at meristem zones of 250 μm (indicated by boxes in A) was counted and plotted by mean values with standard deviation from triplicate experiments. Asterisks indicate a significant difference from wild type (t-test; $^{**}P<0.01$).

CONCLUSION REMARKS

In eukaryotes, MAPK cascades have been known to be as representative signal transduction pathways, which are involved not only in defense response but also in other physiological processes, such as regulation of cell growth and cell cycle. Although recent many studies have identified a number of genes encoding MAP Kinase and other components of MAP kinase signal transduction pathways, information about their downstream has been limited.

WIPK is known as one of representative MAPKs in tobacco plant, which is involved in defense response against to wounding and pathogen stress. NtWIF was identified as a WIPK-interacting protein, and in this thesis, I presented data that NtWIF is a direct WIPK-downstream transcription factor and contributes to elevation of resistance in the response to stress through transcriptional activation of defense genes containing the ARE- cis element on their promoter regions. Moreover, I showed that NtWIF is involved not only in defense response but also in plant development, particularly, in seed and root development through up-regulation of multifunctional genes. Based on these results, I depict a model illustrating that WIPK-NtWIF cascade in both defense response and the process of growth and development (Fig 16). Characterization of NtWIF is meaningful because it broaden our general knowledge on

function of MAPK cascade, and particularly, of WIPK. My finding that NtWIF functions not only in defense response but also in physiological process of plants suggests that WIPK-NtWIF cascade is one of the key pathways for plant survival.

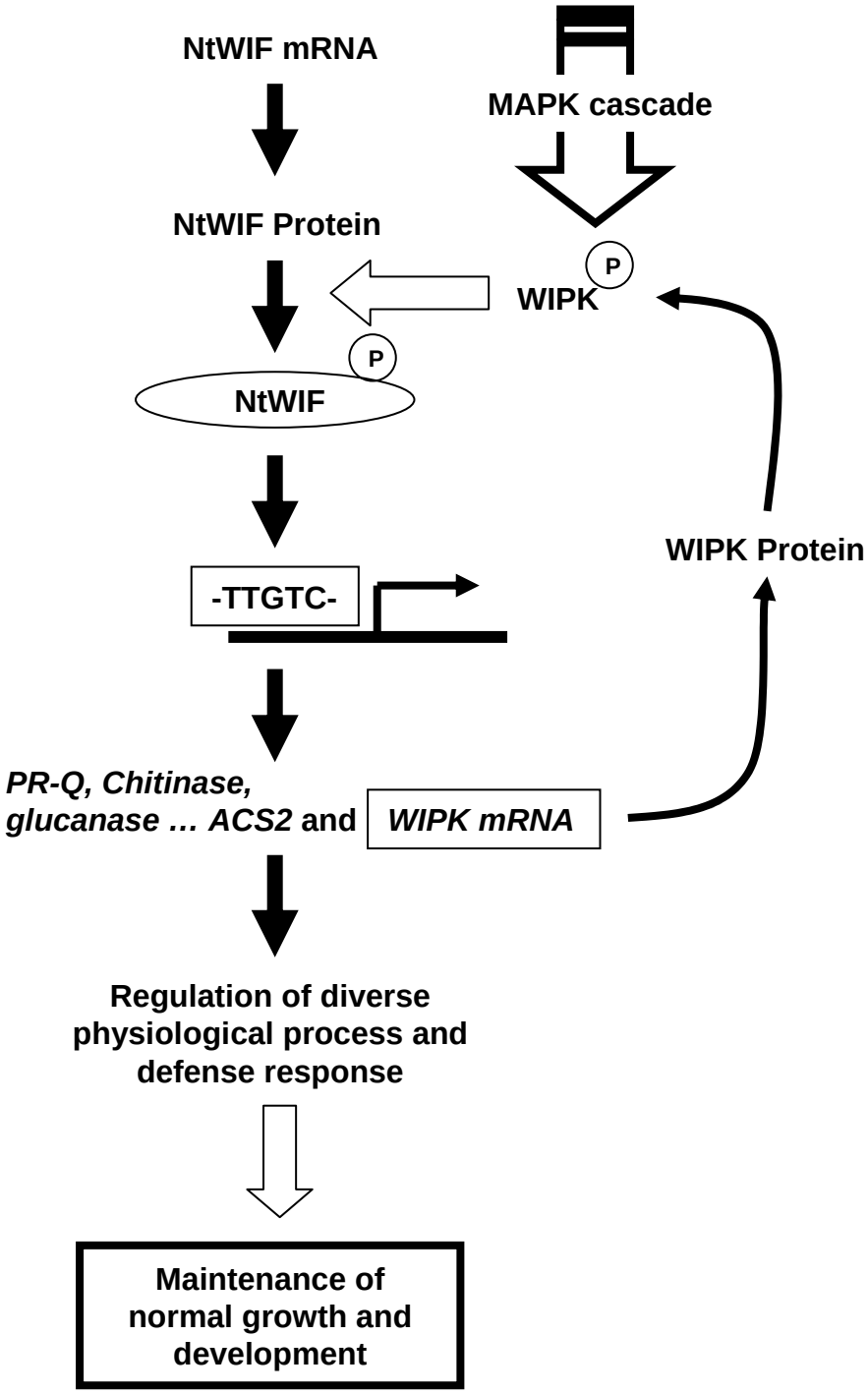

Figure 16. Model depicts the functions of WIPK-NtWIF cascade for normal growth and development of plants.
Under stressed environments, WIPK-NtWIF cascade and its target genes are involved in defense responses whereas under normal ondition, they are involved in diverse physiological processes such as roots or seeds growth and development. These suggest that WIPK-NtWIF cascade is one of the key pathways for plant survival.

Appendix Table 1.List of differentially expressed genes between NtWIF-overexpressing transgenic line, S7, and wild type tobacco plants.

Description	S7/WT (fold)	P value	Origin
Defense			
PMT	10.78	0.01033	*N. tabacum*
PR1b	8.00	0.00386	*N. tabacum*
cystathionine gamma-synthase isoform 1	5.45	0.00303	*S. tuberosum*
PR-Q	5.25	0.00002	*N. tabacum*
acidic chitinase III	5.23	0.00061	*N. tabacum*
CYP73A27	4.82	0.00364	*A.thaliana*
acidic beta-1,3-glucanase	4.76	0.00014	*N. tabacum*
ornithine decarboxylase	4.65	0.00798	*N. glutinosa*
cytochrome oxidase subunit 1	4.45	0.00159	*Sabal palmetto*
ACCS2	4.23	0.00130	*N. tabacum*
phenylalanine ammonia-lyase	4.19	0.00277	*N. tabacum*
putative Cytochrome P450 protein	4.17	0.00140	*S. melongena*
THT1(tyramine hydroxycinnamoyltransferase)	4.17	0.00373	*N. tabacum*
MRP4	4.15	0.00056	*A. thaliana*
osmotin-like protein	4.10	0.00491	*N. tabacum*
P450 hydroxylase	3.98	0.00106	*N. tabacum*
TAS14 (dehydrin)	3.97	0.00175	*N. tabacum*
IVR-like protein	3.94	0.00004	*N. tabacum*
HMGR1	3.92	0.00233	*N. tabacum*
acetyl-CoA synthetase	3.88	0.00885	*S. tuberosum*
glucosyltransferase	3.86	0.00042	*N. tabacum*
putative chloroplast terpene synthase	3.84	0.00323	*Quercus ilex*
endo-1,4-beta-glucanase	3.83	0.00990	*L. esculentum*
TMV resistance protein N - tobacco	3.82	0.00358	*N. glutinosa*
rbohF	3.78	0.00245	*N. tabacum*
NADPH cytochrome P450	3.70	0.00037	*Vigna radiata*
superoxide dismutase [Fe]	3.68	0.00828	*L. esculentum*
probable cinnamyl-alcohol dehydrogenase	3.67	0.00252	*L. esculentum*
putative AtMlo-h1 protein	3.67	0.00037	*A. thaliana*
Avr9 elicitor response protein	3.66	0.00053	*N. tabacum*
carboxylase/oxygenase,RBP	3.58	0.00648	*N. tabacum*
anionic peroxidase	3.56	0.00111	*N. tabacum*

cytochrome c oxidase	3.54	0.00440	*N. tabacum*
endo-1,4-beta-glucanase	3.54	0.00089	*N. tabacum*
wound-inducible carboxypeptidase	3.50	0.00084	*L. esculentum*
PPO	3.46	0.00196	*N. tabacum*
protoporphyrinogen oxidase PX-1	3.45	0.00098	*N. tabacum*
CYP71D21	3.43	0.00961	*N. tabacum*
WI9	3.34	0.00195	*N. tabacum*
cytosolic cysteine synthase	3.31	0.00662	*S. tuberosum*
nitrate reductase	3.31	0.00307	*N. tabacum*
Al-induced protein	3.31	0.01005	*N. tabacum*
putative geranyl diphosphate synthase (GPPS) (dimethylallyltransferase)	3.28	0.00034	*A. thaliana*
cytochrome P450 like_TBP	3.25	0.00112	*N. tabacum*
endochitinase	3.16	0.00213	*N. sylvestris*
THLP	3.16	0.00301	*N. tabacum*
VAP27-1	3.15	0.00347	*A. thaliana*
putative amine oxidase 1	3.02	0.00292	*A. thaliana*
F-box protein family	3.00	0.00057	*A. thaliana*
Transcription factor			
NtHSF1	4.68	0.00256	*N. tabacum*
LBM4	4.55	0.00539	*N. tabacum*
TOM (target of myb1)-like protein	4.45	0.01026	*A. thaliana*
TIZZ	4.26	0.00031	*N. tabacum*
BAC98495.1| AG-motif binding protein-5	4.18	0.00150	*N. tabacum*
WRKY2	4.07	0.00908	*N. tabacum*
NtWRKY3	3.99	0.01054	*N. tabacum*
LBM1	3.82	0.00043	*N. tabacum*
LBM3	3.74	0.00092	*N. tabacum*
Tsi1	3.59	0.00607	*N. tabacum*
TGA2.2	3.47	0.00146	*N. tabacum*
putative CCAAT-binding transcription factor subunit	3.42	0.00562	*A. thaliana*
DNA-binding protein NtWRKY3	3.41	0.00150	*N. tabacum*
DNA binding protein TGA1b	3.39	0.00488	*N. tabacum*
DNA-binding WRKY-like protein	3.32	0.00408	*A. thaliana*
heat shock transcription factor 34	3.18	0.00196	*Glycine max*
Kinase/Phosphatase			

NPK1	4.38	0.00312	*N. tabacum*
NQK1	4.38	0.00157	*N. tabacum*
NRK1	4.20	0.00052	*N. tabacum*
NtMEK1	4.16	0.00138	*N. tabacum*
Ser/Thr specific protein phosphatase 2A	4.09	0.00220	*M. sativa subsp. varia*
NtCDPK3	3.87	0.00330	*N. tabacum*
calcium/calmodulin-dependent protein kinase CaMK1	3.60	0.00296	*N. tabacum*
WAPK	3.59	0.00267	*N. tabacum*
calcium-dependent protein kinase	3.38	0.00106	*Fragaria ananassa*
purple acid phosphatase	3.21	0.00191	*N. tabacum*
NtCDPK2	3.20	0.01048	*N. tabacum*
putative protein kinase	3.18	0.00100	*A. thaliana*
shaggy-like kinase	3.17	0.00192	*Ricinus communis*
Heat shock protein and ubiquitin			
chaperonin 21 precursor	4.63	0.00902	*L.esculentum*
heat shock protein 70	4.37	0.00067	*Oryza sativa (indica cultivar)*
putative heat-shock protein	3.98	0.00934	*A. thaliana*
non-cell-autonomous heat shock cognate protein 70	3.58	0.00546	*Cucurbita maxima*
chaperonin 21 precursor	3.54	0.00983	*L.esculentum*
molecular chaperone Hsp90-2	3.39	0.00360	*N. benthamiana*
polyubiquitin	3.81	0.00135	*Pinus sylvestris*
SUMO protein	3.49	0.00316	*Lycopersicon esculentum*
putative ubiquitin-specific protease	3.45	0.00112	*Oryza sativa (japonica cultivar)*
ubiquitin-activating enzyme E1	3.15	0.00244	*N. tabacum*
Phytohormone			
GAST-like gene product	4.38	0.00560	*Fragaria ananassa*
putative auxin response factor protein	3.28	0.00185	*Arabidopsis thaliana*
putative IAA amidohydrolase	3.02	0.00526	*Oryza sativa (japonica cultivar)*
Cell wall and cytoskeleton			
putative pectinacetylesterase	12.17	0.00167	*A. thaliana*
GDP-D-mannose pyrophosphorylase	5.24	0.00680	*N. tabacum*
fructose-1,6-bisphosphate aldolase	4.72	0.00076	*Pisum sativum*

similar to dTDP-D-glucose 4,6-dehydratase	4.66	0.00001	*A. thaliana*
putative actin-binding protein	4.65	0.00095	*Malus domestica*
putative microfibril-associated protein	4.00	0.00110	*A. thaliana*
actin-depolymerizing factor 2	4.00	0.00095	*Petunia hybrida*
glucose-6-phosphate isomerase	3.96	0.00167	*Spinacia oleracea*
kinesin-like protein	3.82	0.00054	*N. tabacum*
pectinesterase-like protein	3.77	0.00066	*A. thaliana*
Ntbfruc1	3.74	0.00880	*N. tabacum*
beta-galactosidase	3.67	0.00207	*Prunus armeniaca*
putative pyrophosphate--fructose-6-phosphate 1-phosphotransferase	3.54	0.00280	*A. thaliana*
putative syntaxin protein	3.50	0.00353	*Oryza sativa (japonica cultivar)*
LOB DOMAIN 15 [Arabidopsis thaliana]	3.50	0.00416	*N. tabacum*
putative expansin	3.41	0.00184	*C. annuum*
alpha-tubulin	3.36	0.00320	*N. tabacum*
xyloglucan endotransglycosylase	3.22	0.00599	*Malus domestica*
cellulose synthase catalytic subunit	3.05	0.01331	*N. alata*
alpha-galactosidase-like protein	3.29	0.00103	*Arabidopsis thaliana*
fructose-biphosphate aldolase	3.25	0.00281	*Mesembryanthemum crystallinum*
fructose-bisphosphate aldolase	3.23	0.00504	*Persea americana*
putative 6-phosphogluconolactonase	3.22	0.00071	*Elaeis guineensis*
beta-galactosidase like protein	3.14	0.00219	*A. thaliana*
beta-D-xylosidase	3.06	0.00194	*Prunus persica*
putative nucleotide sugar epimerase	3.02	0.00038	*A. thaliana*
Transcription/ Translation-regulatory protein			
translation initiation factor eIF3-like protein	6.63	0.00369	*A. thaliana*
translation initiation factor	4.85	0.00204	*Pisum sativum*
putative cleavage and polyadenylation specificity factor 73 kDa subunit	4.25	0.01415	*A. thaliana*
elongation factor 1B gamma	4.01	0.00278	*Oryza sativa*
putative replication factor C	3.82	0.00800	*A. thaliana*
putative NifU-like metallocluster assembly factor	3.81	0.00853	*A. thaliana*
RNA-directed RNA polymerase	3.76	0.00393	*N. tabacum*
putative arginine/serine-rich splicing factor	3.68	0.00296	*M. sativa*
replication factor, putative; 74998-73295	3.44	0.01085	*A. thaliana*
putative RNA-binding protein MEI2	3.37	0.00330	*A. thaliana*
chloroplast elongation factor TuB (EF-TuB)	3.17	0.00602	*N. sylvestris*

Functional Protein			
Putative integral membrane protein	8.64	0.00004	*A. thaliana*
putative germin-like protein (GLP2a) copy2	5.84	0.01272	*A. thaliana*
Moco containing protein	5.20	0.00042	*A. thaliana*
putative translationally controlled tumor protein	4.18	0.00305	*N. tabacum*
putative dim1p	4.09	0.00343	*Oryza sativa (japonica cultivar*
14-3-3 protein	4.06	0.00242	*N. tabacum*
RAD23-like protein	3.85	0.00777	*A. thaliana*
P69C protein	3.84	0.00273	*L. esculentum*
urease accessory protein G	3.80	0.00053	*S. tuberosum*
putative signal recognition particle protein	3.57	0.00207	*A. thaliana*
putative urease accessory protein F	3.50	0.00464	*L.esculentum*
RNA-binding protein AKIP1	3.47	0.00833	*Vicia faba*
transmembrane protein, putative	3.44	0.00093	*A. thaliana*
putative Ran binding protein	3.41	0.00014	*Oryza sativa (japonica cultivar)*
germin-like protein	3.40	0.00147	*Ananas comosus*
putative cytochrome b5	3.36	0.00225	*A. thaliana*
proline-rich protein family	3.35	0.00047	*A. thaliana*
stomatin-like protein	3.34	0.00076	*A. thaliana*
oligouridylate binding protein, putative	3.26	0.01545	*A. thaliana*
54-kD signal recognition particle (SRP) specific protein	3.24	0.00150	*L. esculentum*
Putative integral membrane protein	3.22	0.00047	*A. thaliana*
luminal binding protein (BiP)	3.19	0.00066	*N. tabacum*
putative transformer serine/arginine-rich ribonucleoprotein	3.15	0.00012	*Oryza sativa (japonica cultivar)*
Nonclathrin coat protein gamma-like protein	3.04	0.00259	*A. thaliana*
putative multispanning membrane protein	3.03	0.00056	*A. thaliana*
Ca^{2+}-related and Ion Channel			
MRS2-5	4.31	0.00707	*A. thaliana*
vacular H+-ATPase	3.71	0.01252	*N. tabacum*
putative potassium channel regulatory factor	3.46	0.00310	*Oryza sativa (japonica cultivar)*
MRS2-1	3.44	0.00261	*A. thaliana*
putative nitrate transporter	3.20	0.00038	*A. thaliana*
NtCam13	3.07	0.00726	*N. tabacum*

Metabolism			
long-chain-fatty-acid CoA ligase [Arabidopsis thaliana]	4.65	0.01264	*A. thaliana*
putative adenosine deaminase	4.65	0.00798	*Oryza sativa (japonica cultivar)*
catechol O-methyltransferase	4.06	0.00187	*A. thaliana*
phosphoinositide-specific phospholipase C P12	3.65	0.00090	*N. tabacum*
aspartic endopeptidase	3.57	0.01297	*Cucurbita pepo*
putative steroid dehydrogenase [Arabidopsis thaliana]	3.53	0.00462	*A. thaliana*
glycoprotein endopeptidase-like protein	3.48	0.00538	*A. thaliana*
phosphoenolpyruvate carboxylase	3.44	0.00107	*N. sylvestris*
ATP-diphosphohydrolase	3.40	0.00321	*N. tabacum*
3-hydroxyisobutyryl-coenzyme A hydrolase-like protein	3.39	0.00379	*A. thaliana*
CDP-diacylglycerol synthetase	3.36	0.00034	*S. tuberosum*
PLC2	3.27	0.00258	*N. tabacum*
glycine hydroxymethyltransferase	3.30	0.00813	*Flaveria pringlei*
S-adenosyl-methionine-sterol-C- methyltransferase	3.20	0.00245	*N. tabacum*
putative serine carboxypeptidase	3.19	0.00131	*Oryza sativa (japonica cultivar)*
alpha-rhamnosidase-like protein	3.16	0.00487	*Oryza sativa (japonica cultivar)*
putrescine N-methyltransferase	3.14	0.00153	*N. tabacum*
putative subtilisin serine proteinase	3.08	0.01321	*A. thaliana*
putative mercaptopyruvate sulfurtransferase	3.00	0.00497	*A. thaliana*

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LIST of PUBLICATIONS

1) Control of seed and root development by WIPK-activated transcription factor, NtWIF in tobacco plants. **Kwi-Mi Chung**, Yun-Soo Kim and Hiroshi Sano (2007) *Plant Biotechnology* (2007.1.22 accepted)

2) Transactivation of wound-responsive genes containing the core sequence of the auxin-responsive element by a wound-induced protein kinase-activated transcription factor in tobacco plants. **Kwi-Mi Chung** and Hiroshi Sano Plant Mol. Bio. (submitted)

3) Activation of a novel transcription factor through phosphorylation by WIPK, a wound-induced mitogen-activated protein kinase in tobacco plants. Yun-kiam Yap, Yutaka Kodama, Frank Waller, **Kwi-Mi Chung**, Hirokazu Ueda, Kimiyo Nakamura, Maren Oldsen, Hiroshi Yoda, Yube Yamaguchi, and Hiroshi Sano (Underlined authors contributed equally to the paper) 2005 *Plant Physiology* 139:127-137

4) Expression of a WIPK-activated transcription factor results in increase of endogenous salicylic acid and pathogen resistance in tobacco plants. Frank Waller, Axel Müller, **Kwi-Mi Chung**, Yun-Kiam Yap, Kimiyo Nakamura, Elmer Weiler and Hiroshi Sano 2006. *Plant Cell Physiol*. 47:1169-1174