Transcriptome Analysis of WIPK/SIPK-Suppressed Plants Reveals Induction by Wounding of Disease Resistance-Related Genes Prior to the Accumulation of Salicylic Acid

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(Received November 7, 2012; Accepted April 3, 2013)

Salicylic acid (SA) plays a key role in plant resistance to pathogens. Accumulation of SA is induced by wounding in tobacco plants in which the expression of WIPK and SIPK, two mitogen-activated protein kinases, is suppressed. Here, the mechanisms underlying the abnormal accumulation of SA in WIPK/SIPK-suppressed plants have been characterized. SA accumulation started around 12 h after wounding and was inhibited by cycloheximide (CHX), a protein synthesis inhibitor. SA accumulation, however, was enhanced several fold when leaf discs were transferred onto CHX after floating on water for ≥6 h. Temporal and spatial analyses of wound-induced and CHX-enhanced SA accumulation suggested that wounding induces activators for SA accumulation followed by the generation of repressors, and late CHX treatment inhibits the production of repressors more efficiently than that of activators. Microarray analysis revealed that the expression of many disease resistance-related genes, including N, a Resistance (R) gene for Tobacco mosaic virus and R gene-like genes, was up-regulated in wounded WIPK/SIPK-suppressed plants. Expression of the N gene and R gene-like genes peaked earlier than that of most other genes as well as SA accumulation, and was mainly induced in those parts of leaf discs where SA was highly accumulated. Moreover, wound-induced SA accumulation was decreased by the treatments which compromise the function of R proteins. These results indicate that signaling leading to the expression of disease resistance-related genes is activated by wounding in WIPK/SIPK-suppressed plants, and induction of R gene and R gene-like genes might lead to the biosynthesis of SA.

Keywords: MAPK • Nicotiana tabacum • R gene • Salicylate • Wound.

Abbreviations: CHX, cycloheximide; GDA, geldanamycin; HIR, hypersensitive induced reaction; HR, hypersensitive response; HSP90, heat shock protein 90; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; MeJA, methyl jasmonate; NLR, nucleotide-binding leucine-rich repeat; PR, pathogenesis-related; qRT–PCR, quantitative reverse transcription–PCR; R, Resistance; SA, salicylic acid; SAG, salicylic acid glucoside; TMV, Tobacco mosaic virus.

Introduction

Plants are exposed to various forms of environmental stress such as infections by pathogenic microbes and wounding caused by tissue damage or herbivores. Penetration by pathogenic microbes is one of the most serious environmental insults and can result in the death of infected plants. Wounding also affects the growth and reproduction of plants. To cope with environmental stress, plants have evolved sophisticated defense mechanisms, some unique to individual forms of stress and others more broadly applicable.

Plants have at least two systems to detect pathogens and induce defense responses (Jones and Dangl 2006). The first system uses transmembrane pattern recognition receptors that recognize conserved microbial molecules, called pathogen-associated molecular patterns, and induces basal defense. The second system uses Resistance (R) proteins that sense specific pathogen effectors, also known as avirulence proteins, and induces strong defense responses, which often leads to a rapid localized cell death, called the hypersensitive response (HR). The induction of these defense responses is partially mediated by phytohormones and signaling molecules such as salicylic acid (SA) and jasmonic acid (JA) whose accumulation is rapidly induced after the recognition of pathogens. SA and JA regulate separate sets of defense responses which are effective against distinct types of pathogens. For example, an SA-responsive pathway is involved in resistance to the biotrophic pathogen Hyaloperonospora arabidopsidis but not to the necrotrophic pathogens Alternaria brassicicola and Botrytis cinerea, and vice versa for the JA-responsive pathway in Arabidopsis (Arabidopsis thaliana) (Thomma et al. 1998). In contrast to the recognition of pathogens, the accumulation of JA, but not...
SA, is induced by wounding, leading to specific JA-regulated responses which are effective against herbivores (Koo and Howe 2009). Since unnecessary defense responses are detrimental to plant growth and development, production of SA and JA must be tightly controlled. However, the molecular mechanisms controlling the production of SA and JA are largely unknown.

A growing body of evidence indicates that mitogen-activated protein kinase (MAPK) cascades play an important role in controlling the production of SA and JA. MAPK cascades are components of signal transduction pathways which transduce various extracellular stimuli into intracellular responses, and consist of three interacting kinases, MAPK, MAPK kinase and MAPK kinase kinase (Widmann et al. 1999, MAPK Group 2002). The activation of components of MAPK cascades in response to various forms of environmental stress including pathogen attack and wounding has been widely reported. Genetic evidence has indicated that MAPK cascades are involved in controlling the production of SA and JA. In Arabidopsis, a MAPK cascade consisting of MEKK1, MKK1 and M KK2, and MPK4 has been reported to regulate the accumulation of SA negatively (Petersen et al. 2000, Ichimura et al. 2006, Nakagami et al. 2006, Suarez-Rodriguez et al. 2007, Gao et al. 2008, Qiu et al. 2008). The Nicotiana attenuata MAPKs NaWIPK and NaSIPK were reported to be required for wound- and herbivore-induced JA accumulation (Wu et al. 2007). We have shown that suppression of both WIPK and SIPK, pathogen- and wound-responsive MAPKs, results in not only reduced accumulation of JA, but also the abnormal accumulation of SA by wounding in tobacco (Nicotiana tabacum) (Seo et al. 2007). Importantly, SA accumulation is not observed in WIPK/SIPK-suppressed plants without wounding, suggesting that an activator of SA biosynthesis is induced to be expressed by wounding in WIPK/SIPK-suppressed plants. Here, we characterized the molecular mechanisms underlying the abnormal accumulation of SA caused by wounding in WIPK/SIPK-suppressed plants. We show that the expression of many disease resistance-related genes including the R gene and R gene-like genes is induced by wounding prior to the accumulation of SA in WIPK/SIPK-suppressed plants, and induction of the R gene and R gene-like genes might activate the biosynthesis of SA.

**Results**

**Wound-induced accumulation of SA requires de novo protein synthesis**

To clarify the mechanisms underlying the wound-induced accumulation of SA in WIPK/SIPK-suppressed plants, first of all, the time course of SA accumulation was investigated. Leaf discs were prepared from the leaves of WIPK/SIPK-suppressed plants and floated on water. Levels of free and conjugated SA [salicylic acid glucoside (SAG)] in the discs were determined. The increase in the levels of SA and SAG was first observed between 12 and 15 h after wounding (Fig. 1). Then the levels of SA gradually decreased whereas those of SAG increased, suggesting that SA is rapidly converted to SAG.

As SA accumulated slowly, the requirement for de novo protein synthesis for wound-induced SA accumulation was investigated. As expected, the accumulation of SA was inhibited by cycloheximide (CHX), a potent protein synthesis inhibitor, in a dose-dependent manner (Fig. 2), indicating that de novo protein synthesis is required for SA accumulation. A wound-induced increase in the levels of SA was nearly completely suppressed by CHX at a concentration of 300 μM and, as the rate of protein synthesis in leaf discs was reported to be reduced by about 90% within 30 min in the presence of 300 μM CHX (Usami et al. 1995), 300 μM CHX was used in the subsequent experiments.

**Wound-induced accumulation of SA is regulated by complex mechanisms**

To clarify the amount of time required for SA to accumulate, leaf discs were floated on water for specific periods and then transferred to CHX (Fig. 3A). A 4 h incubation on water was found to be sufficient to induce the accumulation of SA. Unexpectedly, when leaf discs were transferred onto CHX after ≥6 h on water, SA accumulation was enhanced several fold. Since levels of SA peaked when leaf discs were transferred to CHX after floating on water for ≥9 h, in the subsequent experiments, leaf discs were transferred onto CHX after floating on water for 9 h. Wound-induced and CHX-enhanced accumulation of SA was observed in another line of WIPK/SIPK-suppressed plants (Fig. 3B; WS2), but not in vector control (V1), WIPK-suppressed (W2) or SIPK-suppressed (S3) plants (Fig. 3C). Also, this accumulation was dependent on the developmental stage of plants (Supplementary Fig. S1), and affected by light conditions (Supplementary Fig. S2). These results ruled out the possibility that it was caused by the introduction of the transformation vector or a toxic effect of CHX.

Temporal and spatial patterns of wound-induced and CHX-enhanced accumulation of SA were investigated next. The temporal pattern of SA accumulation was not significantly affected...
by CHX; SA accumulation started between 12 and 15 h after wounding, and SA was rapidly converted to SAG, although levels of SA were greatly increased (Supplementary Fig. S3). The spatial pattern of SA accumulation was investigated by separating the center and margins of the leaf discs. When the discs were floated on water throughout, the majority of SA was separated in the wounded center (Fig. 4A, upper). In contrast, when the SA was observed in the undamaged center when the leaf discs were transferred to CHX after floating on water for 9 h (Fig. 4A, lower). These results suggested that the activator of SA biosynthesis was primarily generated at the wounded site and transferred to the undamaged center, especially when leaf discs were transferred onto CHX after floating on water, and that protein synthesis was inhibited by CHX only at the margins, probably because CHX did not infiltrate the center. To test this notion directly, CHX inhibited by CHX only at the margins, probably because CHX after floating on water, and that protein synthesis was inhibited by CHX; SA accumulation started between 12 and 15 h after wounding, and SA was rapidly converted to SAG, although levels of SA were greatly increased (Supplementary Fig. S3). The spatial pattern of SA accumulation was investigated by separating the center and margins of the leaf discs. When the discs were floated on water throughout, the majority of SA was separated in the wounded center (Fig. 4A, upper). In contrast, when the SA was observed in the undamaged center when the leaf discs were transferred to CHX after floating on water for 9 h (Fig. 4A, lower). These results suggested that the activator of SA biosynthesis was primarily generated at the wounded site and transferred to the undamaged center, especially when leaf discs were transferred onto CHX after floating on water, and that protein synthesis was inhibited by CHX only at the margins, probably because CHX did not infiltrate the center. To test this notion directly, CHX was introduced into the center of leaf discs by syringe when they were transferred from water to CHX. As expected, SA accumulation was no longer enhanced by CHX; rather it was completely suppressed, suggesting that the genes involved in SA accumulation are highly expressed in the center of leaf discs (Fig. 4B). In subsequent experiments, leaf discs transferred to CHX after floating on water for 9 h were used, because very high levels of SA and the strong expression of genes involved in SA biosynthesis were expected under these conditions.

Identification of the genes up-regulated in WIPK/SIPK-suppressed plants by microarray analysis

To identify genes involved in SA accumulation, transcripts which are up-regulated in WIPK/SIPK-suppressed plants were searched for using a microarray. As SA-responsive genes would account for the majority of transcripts up-regulated in WIPK/SIPK-suppressed plants after the induction of SA, total RNA was extracted from leaf discs which were floated on water for 9 h and then on CHX for a total of 12 h, at which time the amount of SA is close to the basal level (Supplementary Fig. S3). The analysis was performed using an Agilent Tobacco Oligo Microarray (021113) on which 43,759 oligonucleotide probes are set. As a result, 71 probes targeting 60 genes showed a >5-fold increase in WIPK/SIPK-suppressed plants compared with control plants (Supplementary Table S1). Functions of the target genes were predicted based on BLASTX searches of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Supplementary Table S2), and were categorized into 13 classes according to Bevan et al. (1998) (Fig. 5: Supplementary Table S3). Approximately one-third of the target genes were of unknown function, and were excluded from the subsequent analyses. Among the remaining genes, about half were those involved in disease resistance and separated into three groups (Supplementary Table S3). The first group consisted of R gene-related genes including the N gene, an R gene conferring resistance to Tobacco mosaic virus (TMV) (Whitham et al. 1994), three R gene-like genes, of which two were of the nucleotide-binding leucine-rich repeat (NLR)-type and one was of the extracellular leucine-rich repeat type, and NDR1, a critical component of R gene signaling (Century et al. 1995). The second group consisted of HR-related genes (HIR1, PR10a, NG1 and HSR201) whose transcripts are expressed in cells undergoing HR (Czenic et al. 1996). NG1 and pepper homologs of HIR1 and PR10a have been reported to induce HR-like cell death when overexpressed (Karrer et al. 1998, Jung and Hwang 2007, Choi et al. 2012). The last group contained typical defense genes, six of which were pathogenesis-related (PR) genes and SA-inducible genes such as PR1a. Since PR1a is the best known SA-inducible PR gene, it was chosen as a representative, and the remaining five genes were excluded from the subsequent analyses. The second largest category was ‘protein destination and storage’ including four genes related to the ubiquitin–proteasome system which is involved in numerous cellular processes. Interestingly, NtAAA1 (AAA), encoding the ATPase associated with various cellular activities domain, was classified in this category, but its transcription is known to be induced by pathogen inoculation (Sugimoto et al. 2004). The remaining categories contained only one or two genes, except for ‘transcription’ which contained five genes, but their predicted functions varied.

Excluding genes of unknown function and PR/SA-inducible genes, transcript levels of the remaining 33 genes were quantified by quantitative reverse transcription–PCR (qRT–PCR) to...
check the reproducibility of the microarray analysis. Transcript levels of four genes (Pa7, RLK, PiTP and Retro) showed no significant difference between WIPK/SIPK-suppressed and control plants (Supplementary Table S3). Because these genes, except for Pa7, have many homologs, primers would not be specific to the target genes. Another four genes (TFIIIA, GA2O, SCPL and ME) showed up-regulation in WIPK/SIPK-suppressed plants, but the difference was small. Also a preliminary analysis revealed that three genes (GrpE, UBQ-E2 and NtCaM3/4) were up-regulated in WIPK/SIPK-suppressed plants before wounding (WS3/V1 = 3.1 ± 0.7, GrpE; 1.9 ± 0.4, UBQ-E2; 5.1 ± 0.9, NtCaM3/4). Therefore, these 11 genes were excluded from the subsequent analyses.

Transcript accumulation of the N gene and R gene-like genes is induced by wounding prior to the accumulation of SA in WIPK/SIPK-suppressed plants

Transcript levels of the remaining 22 genes over the time course after wounding were analyzed by qRT–PCR. As expression patterns of three WRKY genes and two UBQ-E3 genes were similar to each other, the result for WRKY (23) is shown as a representative. Transcript levels of all R gene-related genes (N gene, three R gene-like genes and NDR1) peaked at 12 h after wounding, earlier than those of most other genes (Fig. 6). In addition to R gene-related genes, transcript levels of a putative RNA-binding protein (Pumilio), early flowering 4-like (ELF4), Agenet domain-containing protein (Agenet) and coenzymeA ligase-like (CoAL) also peaked at 12 h after wounding, although the expression of CoAL was induced not only in WIPK/SIPK-suppressed plants, but also in the control plants (Fig. 6). Transcript levels of the HR-related genes, typical defense genes and the remaining genes peaked at 13.5 h or later after wounding. The accumulation of the PR1a transcript showed a pattern very similar to the accumulation of total SA (Supplementary Fig. S3, and data not shown).

Since there are many N gene-like genes in tobacco (Whitham et al. 1994, Gao et al. 2007), the specificity of the primers for the N gene was confirmed with RNA extracted from tobacco cultivar Samsun nn which lacks the N gene. The primers were highly specific to the N gene; signal levels with RNA from...
Samsun nn were below the detectable limit (Supplementary Fig. S4A). Recently, it was reported that transcript levels of the $N$ gene and other $R$ genes are post-transcriptionally regulated by microRNAs (Li et al. 2012, Shivaprasad et al. 2012). To determine whether the increase in the transcript levels of the $N$ gene results from de novo transcription or release from negative regulation by microRNA, levels of $N$ precursor mRNA ($\text{pre-N}$) were investigated by qRT–PCR with primers which anneal the fourth exon and fourth intron of the $N$ gene, respectively. If the increase in the transcript level is caused by de novo transcription, levels of precursor mRNA would increase in a pattern similar to those of the transcript, whereas if it is caused by release from negative regulation by microRNA, levels of precursor mRNA would not increase. As shown in Supplementary Fig. S4B, the accumulation of the $\text{pre-N}$ transcript showed a pattern very similar to that of the $N$ mRNA, indicating that accumulation of the $N$ mRNA results from de novo transcription.

Expression of the $N$ gene and $R$ gene-like genes is predominantly induced in the unwounded part of leaf discs where SA is highly accumulated

SA mainly accumulated in the center of leaf discs when the discs were transferred onto CHX after floating on water (Fig. 4A). To check the correlation between SA levels and transcript levels, transcript levels in the center and margins of leaf discs were quantified by qRT–PCR. The earliest genes, whose expression peaked at 12 h after wounding (Fig. 6), were preferentially analyzed. As shown in Fig. 7, transcript levels of the $N$
gene and R gene-like genes except for NLR (28) correlated with SA levels, which were higher in the center, but lower in the margins of leaf discs from WIPK/SIPK-suppressed plants. In contrast, transcript levels of the remaining earliest genes and some of the late induced genes showed no clear correlation with SA levels (Fig. 7, and data not shown), indicating specific correlation of the SA levels with the transcript levels of the N gene and R gene-like genes.
Wound-induced SA accumulation was inhibited by geldanamycin and high temperature

The specific correlation between SA levels and the transcript levels of the N gene and R gene-like genes suggested their involvement in wound-induced SA accumulation in WIPK/SIPK-suppressed plants. Many NLR-type R proteins including N require heat shock protein 90 (HSP90) for function (Kadota and Shirasu 2012) and are sensitive to high temperature (Whitham et al. 1996, Zhu et al. 2010). To investigate the possible involvement of R proteins in wound-induced SA accumulation, the effect of geldanamycin (GDA), a specific inhibitor of HSP90, and high temperature on the levels of SA induced by wounding was tested. As shown in Fig. 8A, GDA suppressed SA accumulation in a dose-dependent manner. To check the effect of high temperature, leaf discs were incubated at 32°C because the function of many R proteins is compromised around 30°C (Zhu et al. 2010). Consistent with the effect of GDA, wound-induced SA accumulation was decreased at 32°C (Fig. 8B), supporting the hypothesis that the function of R proteins is required for wound-induced SA accumulation in WIPK/SIPK-suppressed plants.

Discussion

Here, we showed that expression of disease resistance-related genes is induced by wounding prior to the accumulation of SA in WIPK/SIPK-suppressed plants (Figs. 5, 6). These results indicate that signaling leading to the expression of disease resistance-related genes is activated by wounding in WIPK/SIPK-suppressed plants. The resistance-related genes up-regulated in WIPK/SIPK-suppressed plants were classified into three groups (Supplementary Table S3). The first group consisted

Fig. 7 N gene and R gene-like genes are preferentially expressed in the center of leaf discs where SA is highly accumulated. Leaf discs of the vector control (V1) and WIPK/SIPK-suppressed (WS3) plants were floated on water for 9 h, and then on 300 μM CHX for a total of 12 h. The discs were separated into a center (C) and margins (M) by punching out the center with a cork borer. Transcript levels of genes up-regulated in WIPK/SIPK-suppressed plants were quantified by qRT–PCR and normalized to the level of Actin2 as an internal standard. Values are means with SD (n = 3).

Fig. 8 Effect of geldanamycin (GDA) and high temperature on wound-induced accumulation of SA in WIPK/SIPK-suppressed plants. (A) Leaf discs of WIPK/SIPK-suppressed plants were infiltrated with GDA at the indicated concentrations or 0.2% DMSO as a control, and floated on the same solution for 24 h. (B) Leaf discs of WIPK/SIPK-suppressed plants were floated on water and incubated at the indicated temperature for 24 h. The levels of total SA (SA + SAG) were determined. Values are means with SD (n = 5).
of R gene-related genes including the N gene, R gene-like genes and NDR1. The second group comprised HR-related genes including NG1, HIR1 and PR10a. Their overexpression or the overexpression of their homologs has been reported to induce cell death (Karrer et al. 1998, Jung and Hwang 2007, Choi et al. 2012). NG1 and HIR1 are members of the hypersensitive induced reaction (HIR) gene family (Nadimpalli et al. 2000), and pepper homologs of both PR10a and HIR1 interact with the same LRR protein (Choi et al. 2012), suggesting that genes belonging to the second group are functionally related. Moreover, it was reported that Arabidopsis HIR1 homologs form complexes with RPS2, an R protein recognizing the bacterial effector protein AvrRpt2, and are required for RPS2-mediated resistance to *Pseudomonas* pathogens (Qi et al. 2011). These results suggest that genes belonging to the first and second groups are involved in the R gene-mediated resistance. The last group contains typical defense-related genes such as the genes for WRKY-type transcription factors and PR genes. Because the expression of genes belonging to the first and second groups clearly preceded the accumulation of SA (Fig. 6), they are unlikely to be SA-inducible genes; rather their expression might lead to the activation of SA biosynthesis. In particular, transcriptional induction of the N gene and R gene-like genes by wounding is of interest. Primarily, the function of R proteins is regulated by the interaction with their corresponding effector proteins. Many R genes including the N gene, however, have been reported to be increased at the transcript level (Yoshimura et al. 1998, Levy et al. 2004, Zipfel et al. 2004), and overexpression of R genes often results in the constitutive activation of defense responses including accumulation of SA (Oldroyd and Staskawicz 1998, Tang et al. 1999). Moreover, the Arabidopsis R gene-like gene *SNC1* encodes an auto-active NLR protein. When its transcript or protein levels are increased by the introduction of a genomic fragment of *SNC1* or mutations in regulators such as *SRFR1*, defense reactions including accumulation of SA are strongly induced (Gou and Hua 2012). We showed that expression of the N gene and two R gene-like genes peaked earliest after wounding and was mostly induced at sites where SA was highly accumulated (Figs. 6, 7). Therefore, expression of the N gene and R gene-like genes due to wounding might lead to the activation of defense responses including accumulation of SA in WIPK/SIPK-suppressed plants. This hypothesis was supported by the findings that wound-induced SA accumulation was partially suppressed by GDA and high temperature (Fig. 8). However, the effect of GDA and high temperature is not specific to R proteins; rather both would affect a wide range of cellular functions. To obtain better insight into the involvement of R proteins in wound-induced SA accumulation, we will test whether overexpression of any of the R gene-like genes up-regulated in WIPK/SIPK-suppressed plants induces SA accumulation.

Wound-induced SA accumulation in WIPK/SIPK-suppressed plants was completely inhibited by CHX when leaf discs were directly floated on CHX, but greatly enhanced when leaf discs were transferred to CHX after floating on water for ≥6 h (Figs. 2, 3). Many wound responses are induced in leaves distal to the site of injury (Green and Ryan 1972). Wound-induced SA accumulation was also observed not only at the wounded site (margin of the leaf discs), but also in the distal unwounded part (center of the leaf discs), especially when leaf discs were transferred onto CHX after floating on water (Fig. 4A). Collectively, these results suggest that activators of SA biosynthesis are primarily generated in wounded areas and then transferred to the unwounded parts in leaf discs. The accumulation of SA, however, is unwanted and so plants repress the production and movement of activators once they have recognized the induction of SA biosynthesis. When the leaf discs were transferred to CHX after floating on water, the production of repressors would have been inhibited by CHX more efficiently than that of activators, leading to the production of a large amount of SA in the central unwounded area of the discs. One candidate for such repressors was JA, because SA and JA often act antagonistically (Loake and Grant 2007), and levels of JA induced by wounding are decreased in WIPK/SIPK-suppressed plants (Seo et al. 2007). In fact, SA accumulation was partially suppressed by exogenously applied methyl jasmonate (MeJA), a methyl ester form of JA, in a dose-dependent manner (Supplementary Fig. S5A). However, several lines of evidence suggest that endogenous JA is unlikely to be a repressor of SA accumulation. First, the levels of wound-induced JA are decreased not only in WIPK/SIPK-suppressed plants, but also in WIPK-suppressed and SIPK-suppressed plants; however, SA accumulation is induced by wounding only in WIPK/SIPK-suppressed plants (Seo et al. 2007). Secondly, compared with the induction of PI-II, a marker gene of JA response, a very high concentration of MeJA was required to suppress wound-induced SA accumulation (Supplementary Fig. S5B). Suppression of SA accumulation by a high concentration of MeJA would be an indirect effect, and identification of repressors as well as activators should be a subject of future analysis. Identifying such activators and repressors will help us to understand the complex regulatory mechanisms of SA production.

Levels of SA induced by wounding in the absence of CHX were much lower than those induced by the recognition of TMV by the N gene, which reached approximately 10,000 ng g⁻¹ FW (Figs. 1, 2). On the other hand, levels of wound-induced and CHX-enhanced SA production were high, especially in the undamaged parts of leaf discs, values being close to levels induced by the recognition of TMV by the N protein (Fig. 4A). Moreover, the temporal pattern of wound-induced and CHX-enhanced SA accumulation was tightly controlled, with a sharp peak at 15 h after wounding (Supplementary Fig. S3). This experimental system enabled us to identify genes up-regulated in WIPK/SIPK-suppressed plants. When leaf discs were floated throughout on water, increases in the transcript levels of the N gene were small and it was difficult to see constantly a significant difference between the control and WIPK/SIPK-suppressed plants (data not shown). Recognition of effector proteins by R proteins induces the biosynthesis of many phytohormones and signaling
molecules such as JA and ethylene in addition to SA, and often leads to localized HR cell death, which makes it difficult to analyze what is happening in the cells. In contrast, production of SA is specifically induced by wounding without cell death in WIPK/SIPK-suppressed plants. Therefore, this experimental system would be useful to analyze mechanisms of SA biosynthesis and its regulation.

In this study, we showed that transcription of the N gene and R gene-like genes is induced by wounding in WIPK/SIPK-suppressed plants (Fig. 6; Supplementary Fig. S4), but their physiological roles are unclear. One possible role is explained by a variation of the so-called guard hypothesis (Jones and Dangl 2006). In the guard hypothesis, R proteins monitor host components that are important for basal defense. When pathogen effectors target host components and suppress basal defense, R proteins are activated and induce strong defense responses. Since WIPK and SIPK play important roles in the production of JA, a major phytohormone involved in the resistance to herbivores (Seo et al. 2007), they would be targeted by effectors of herbivores (Hogenhout and Bos 2011). Plants might activate a second layer of defense by inducing expression of the R gene and R gene-like genes when they recognized that they were attacked by herbivores, but activation of WIPK and SIPK was not induced. This hypothesis is consistent with the results showing that expression of the N gene and R gene-like genes is not up-regulated without wounding in WIPK/SIPK-suppressed plants (Fig. 6), and is supported by reports that components of MAPK cascades are targeted by pathogen effectors in both mammals and plants (Orth et al. 1999, Zhang et al. 2007, Wang et al. 2010). Very recently, it was reported that Arabidopsis MPK4 is a target of Pseudomonas syringae effector HopAI1, and inactivation of MPK4 by HopAI1 results in activation of defense responses mediated by NLR protein SUMM2 (Zhang et al. 2012).

Transcriptional induction of the R genes including the N gene has been suggested to be important for their functions, but the mechanisms involved are largely unknown. Haque et al. (2009) and ourselves identified neighboring cis-regulatory elements which are involved in transcriptional activation of the N gene by TMV (Kobayashi et al. 2010). It will be of interest to test whether these cis-regulatory elements are also required for wound-induced expression of the N gene and R gene-like genes in WIPK/SIPK-suppressed plants. By elucidating the mechanisms underlying transcriptional induction by wounding of the N gene and R gene-like genes in WIPK/SIPK-suppressed plants, we will understand how WIPK and SIPK regulate their transcription.

Materials and Methods

Plant materials and plant growth conditions
The tobacco (N. tabacum) cultivars Samsun NN containing the N gene and Samsun nna lacking the N gene were used. The generation of SIPK-, WIPK-, and WIPK- and SIPK-silenced Samsun NN plants has been described previously (Seo et al. 2007). Plants were grown in pots containing vermiculite in a chamber maintained at 25°C with 16 h of light. Unless otherwise stated, the upper and middle, fully expanded leaves of 6- to 7-week-old plants were used for experiments.

Wounding and chemical treatments
Discs were excised from the leaves using a cork borer (diameter 10 mm). The leaf discs were floated on water or CHX at the concentrations indicated, and incubated at 25°C in the dark, unless otherwise stated. To separate the center and margins of the leaf discs, the center was punched out with a cork borer (diameter 5.5 mm). For infiltration, the center of the leaf discs was infiltrated with water or CHX using a syringe without a needle.

For GDA treatment, excised discs were infiltrated with GDA at the concentrations indicated or 0.2% dimethylsulfoxide (DMSO) as a control, floated on the same solution, and incubated at 25°C in the dark.

For MeJA treatment, excised discs were floated on MeJA at the concentrations indicated or 0.1% DMSO as a control, and incubated at 25°C in the dark.

SA measurement
The extraction and quantification of SA and SAG were performed as described previously (Seo et al. 1995).

RNA extraction, microarray analysis and qRT–PCR analysis
Total RNA was extracted using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. The microarray analysis was performed using the Tobacco Oligo Microarray (021113, Agilent Technologies). Total RNA from the control (V1) and WIPK/SIPK-suppressed (WS3) plants was labeled with Cy3 and Cy5, respectively, and competitively hybridized according to the manufacturer’s instructions. The putative functions of the transcripts up-regulated in WIPK/SIPK-suppressed plants were predicted based on BLASTX searches of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and categorized into 13 classes according to Bevan et al. (1998).

The qRT–PCR analysis was performed using a SYBR Prime-Script RT–PCR Kit II (TAKARA BIO INC.). All data were normalized to the expression of Actin2 as an internal control. Actin2 was chosen from among three candidate genes of which two encode actin and one encodes glyceraldehyde 3-phosphate dehydrogenase. Primer pairs are listed in Supplementary Table S4.

Supplementary data
Supplementary data are available at PCP online.

Funding
This work was supported by the Japan Society for the Promotion of Science KAKENHI (grant Nos. 21880020, 23688005 to
We thank Takeshi Hosaka and Takeshi Shimosato (Shinshu University) for their technical assistance.

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