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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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Abbreviations: CHX, cycloheximide; GDA, geldanamycin; HIR, hypersensitive induced reaction, HR, hypersensitive response; HSP90, heat shock protein90; 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; TMV, *Tobacco mosaic virus*.

Footnotes

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Abstract

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 or more. 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 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

Introduction

Plants are exposed to various forms of environmental stress such as infections by pathogenic microbes and wounding caused by tissue damage or herbivore. 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 defence mechanisms, some unique to individual forms of stress and others more broadly applicable.

Plants have at least two systems to detect pathogens and induce defence 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 defence. The second system uses Resistance (R) proteins that sense specific pathogen effectors, also known as avirulence proteins, and induces strong defence responses, which often leads to a rapid localized cell death, called the hypersensitive response (HR). The induction of these defence 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 defence responses which are effective against distinct types of pathogens. For example, a 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 herbivore (Koo and Howe 2009). Since unnecessary defence 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 MKK2, 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 express 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 (SAG) in the discs were determined. The increase in the levels of SA and SAG was first observed between 12 and 15 hours (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 of *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. 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 or more 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 or more, 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). And it 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 synthesized in the wounded margins of the discs (Fig. 4A, upper). In clear contrast, most of 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 oligo nucleotides probes are set. As a result, 71 probes targeting 60 genes showed more than 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 (Czernic et al. 1996). *NG1* and pepper homologs of *HIR1* and *PR10a* have been reported to induce HR-like cell death when

over-expressed (Karrer et al. 1998, Jung and Hwang 2007, Choi et al. 2012). The last group contained typical defence 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 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, *Grp E*; 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 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 defence genes and 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 under 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 (*pre-N*) were investigated by qRT-PCR with primers which anneal the 4th exon and 4th 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 *pre-N* transcript showed a pattern very similar to that of the *N* mRNA, indicating 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 protein90 (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, 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 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 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 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 over-expression or the over-expression 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 defence-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. Especially, 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 over-expression of *R* genes often results in the constitutive activation of defence 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*, defence 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 defence responses including accumulation of SA in WIPK/SIPK-suppressed plants. This hypothesis was supported by the results that wound-induced SA accumulation was partially suppressed by GDA and high temperature (Fig. 8). However, effect of GDA and high temperature is not specific to R proteins; rather both would affect wide range of cellular functions. To obtain better insight into the involvement of R proteins in wound-induced SA accumulation, we will test whether over-expression 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 or more (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 leaf discs), but also in the distal unwounded part (center of 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 is decreased in WIPK/SIPK-suppressed plants (Seo et al. 2007). In fact, SA accumulation was partially suppressed by exogenously applied methyl JA (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 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, very high concentration of MeJA was required to suppress wound-induced SA accumulation (Supplementary Fig. S5B). Suppression of SA accumulation by high concentration of MeJA would be 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 ~10,000 ng/g fresh weight (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 defence. When pathogen effectors target host components and suppress basal defence, R proteins are activated and induce strong defence responses. Since WIPK and SIPK play important roles in the production of JA, a major phytohormone involved in the resistance to herbivore (Seo et al. 2007), they would be targeted by effectors of herbivore (Hogenhout and Bos 2011). Plants might activate a second layer of defence by inducing expression of the *R* gene and *R* gene-like genes when they recognized that they were attacked by herbivore, but activation of WIPK and SIPK was not induced. This hypothesis is consistent with the results 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 (*Nicotiana tabacum*) cultivars Samsun NN containing the *N* gene and Samsun nn 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% 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, Carlsbad, CA) according to the manufacturer's instructions. The microarray analysis was performed using the Tobacco Oligo Microarray (021113, Agilent Technologies, Inc., Palo Alto, CA, USA). 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 PrimeScript RT-PCR Kit II (Takara Bio, Inc., Shiga, Japan). 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.

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Legends to figures

Fig. 1 Accumulation of SA in WIPK/SIPK-suppressed plants after wounding. Leaf discs were excised from the leaves of WIPK/SIPK-suppressed plants, and floated on water for the periods indicated. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3 to 8).

Fig. 2 Wound-induced accumulation of SA in WIPK/SIPK-suppressed plants requires *de novo* protein synthesis. Leaf discs of WIPK/SIPK-suppressed plants were floated on CHX at the indicated concentrations or water as a control for 24 h. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3).

Fig. 3 Effect of CHX on wound-induced accumulation of SA in WIPK/SIPK-suppressed plants depends on the timing of treatment. (A) Leaf discs of WIPK/SIPK-suppressed (WS3) plants were floated on water for the periods indicated. The discs were then transferred to 300 µM CHX, and further incubated for a total of 24 h. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3 to 4). (B, C) Leaf discs of vector control (V1), another line of WIPK/SIPK-suppressed (WS2), WIPK-suppressed (W2), SIPK-suppressed (S3) and WS3 plants were floated on water for 9 h. The discs were then transferred to 300 µM CHX, and further incubated for a total of 24 h. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3).

Fig. 4 Spatial distribution of wound-induced accumulation of SA and SAG in WIPK/SIPK-suppressed plants. (A) Leaf discs of WIPK/SIPK-suppressed plants were floated on water for 24 h (upper) or on water for 9 h and then on 300 µM CHX for a total of 24 h (lower). The center and margins of the discs were separated by punching out the center with a cork borer. (B) Leaf discs of WIPK/SIPK-suppressed plants were floated on water for the periods indicated, infiltrated with water or 300 µM CHX, and further floated on the same solution for a total of 24 h (lower). The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3). Schematic representations of the experimental design are shown at the top.

Fig. 5 Categorization of genes up-regulated in WIPK/SIPK-suppressed plants. The number of genes in each class is shown in parentheses.

Fig. 6 Transcript levels of genes up-regulated in WIPK/SIPK-suppressed plants over the time course after wounding. Leaf discs of the vector control (V1) and WIPK/SIPK-suppressed (WS3) plants were floated on water for 9 h, then transferred to 300 μM CHX and further incubated for the periods indicated. Transcript levels of the genes were quantified by qRT-PCR and normalized to the level of *Actin2* as an internal standard. Values are means with SD (n=3).

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 (WS) 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).

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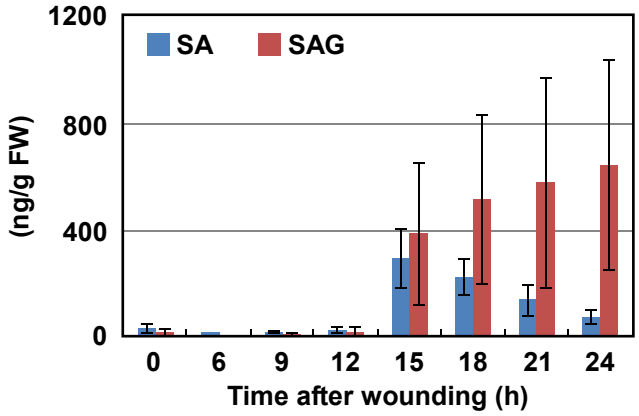
Supplementary Fig. S1 Wound-induced and CHX-enhanced accumulation of SA in WIPK/SIPK-suppressed plants depends on the developmental stage of the plants. Leaf discs were excised from the leaves of WIPK/SIPK-suppressed plants at the indicated time points after sowing. The discs were floated on water for 9 h, then transferred on 300 μM CHX, and further incubated for a total of 24 h. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3).

Supplementary Fig. S2 Wound-induced and CHX-enhanced accumulation of SA in WIPK/SIPK-suppressed plants is more prominent in the dark. Leaf discs of WIPK/SIPK-suppressed plants were floated on water for 9 h in the dark (D) or light (L). The discs were then transferred to 300 µM CHX and further incubated in the dark (D) or light (L) for a total of 24 h. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3).

Supplementary Fig. S3 Accumulation pattern of wound-induced and CHX-enhanced SA in WIPK/SIPK-suppressed plants. Leaf discs of WIPK/SIPK-suppressed plants were floated on water for 9 h, then transferred to 300 µM CHX and further incubated for the periods indicated. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3 to 5).

Supplementary Fig. S4 Wound-induced accumulation of the *N* transcript in WIPK/SIPK-suppressed plants results from *de novo* transcription. (A) Specificity determination of primers for the *N* gene. Total RNA was extracted from the tobacco cultivars Samsun nn lacking the *N* gene and NN containing the *N* gene, and used to quantify the transcript levels of the *N* gene by qRT-PCR. (B) Accumulation of the *N* gene transcript results from *de novo* transcription. Leaf discs of the vector control (V1) and WIPK/SIPK-suppressed (WS3) plants were floated on water for 9 h, then transferred to 300 uM CHX and further incubated for the periods indicated. Levels of the *N* precursor mRNA (pre-N) were quantified by qRT-PCR with primers which anneal the 4th exon and 4th intron of the N gene, respectively. Transcript levels of the *N* gene (A) and *pre-N* (B) were normalized to the level of *Actin2* as an internal standard. Values are means with SD (n=3).

Supplementary Fig. S5 Effect of methyl jasmonate (MeJA) on wound-induced accumulation of SA and the transcript levels of *PI-II* in WIPK/SIPK-suppressed plants. Leaf discs of WIPK/SIPK-suppressed (WS3) plants were floated on MeJA at the indicated concentrations or 0.1% DMSO as a control for 24 h. (A) The levels of total SA (SA + SAG) were determined. Values are means with SD (n=3). (B) Transcript levels of *PI-II* were quantified by qRT-PCR and normalized to the level of *Actin2* as an internal standard (n=2).



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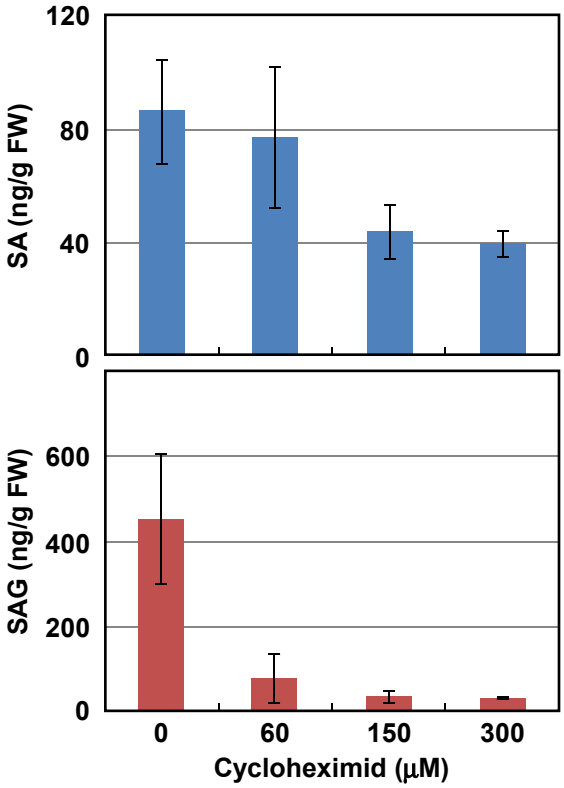

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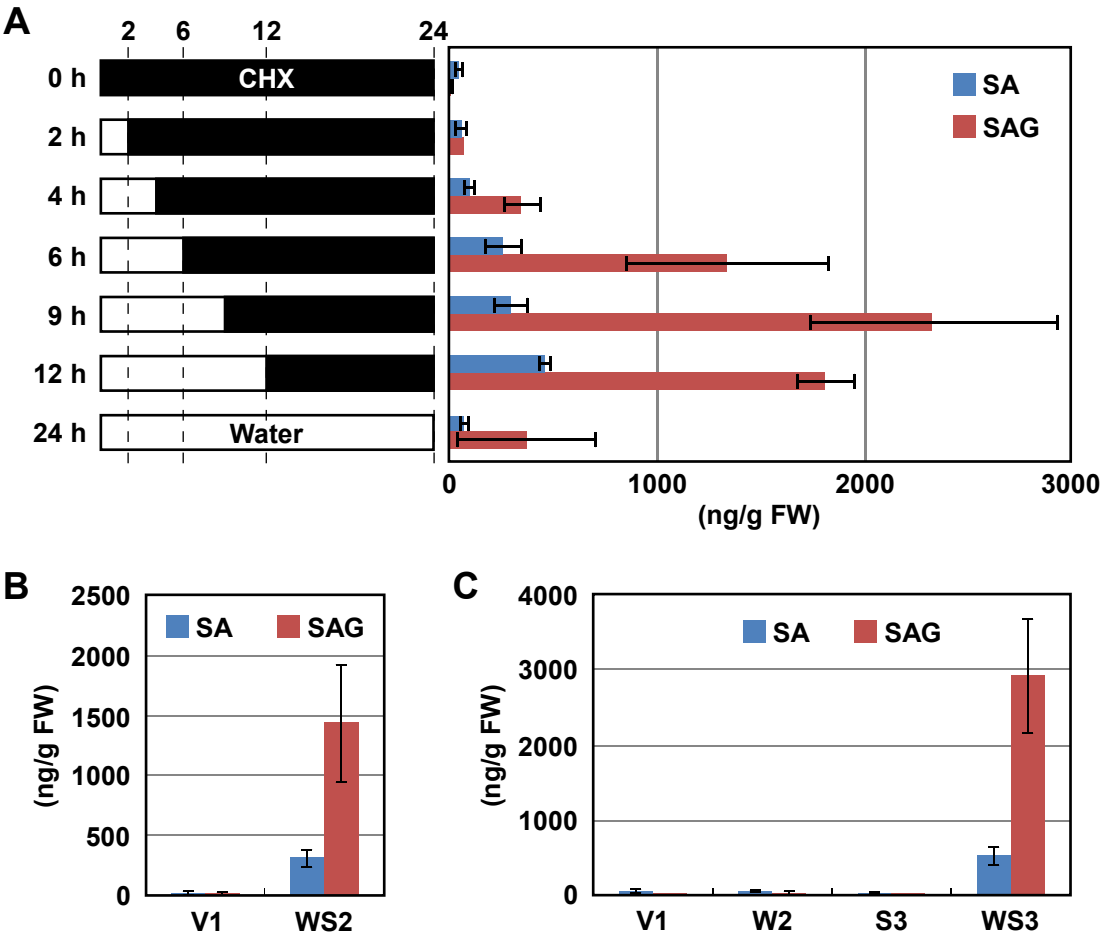

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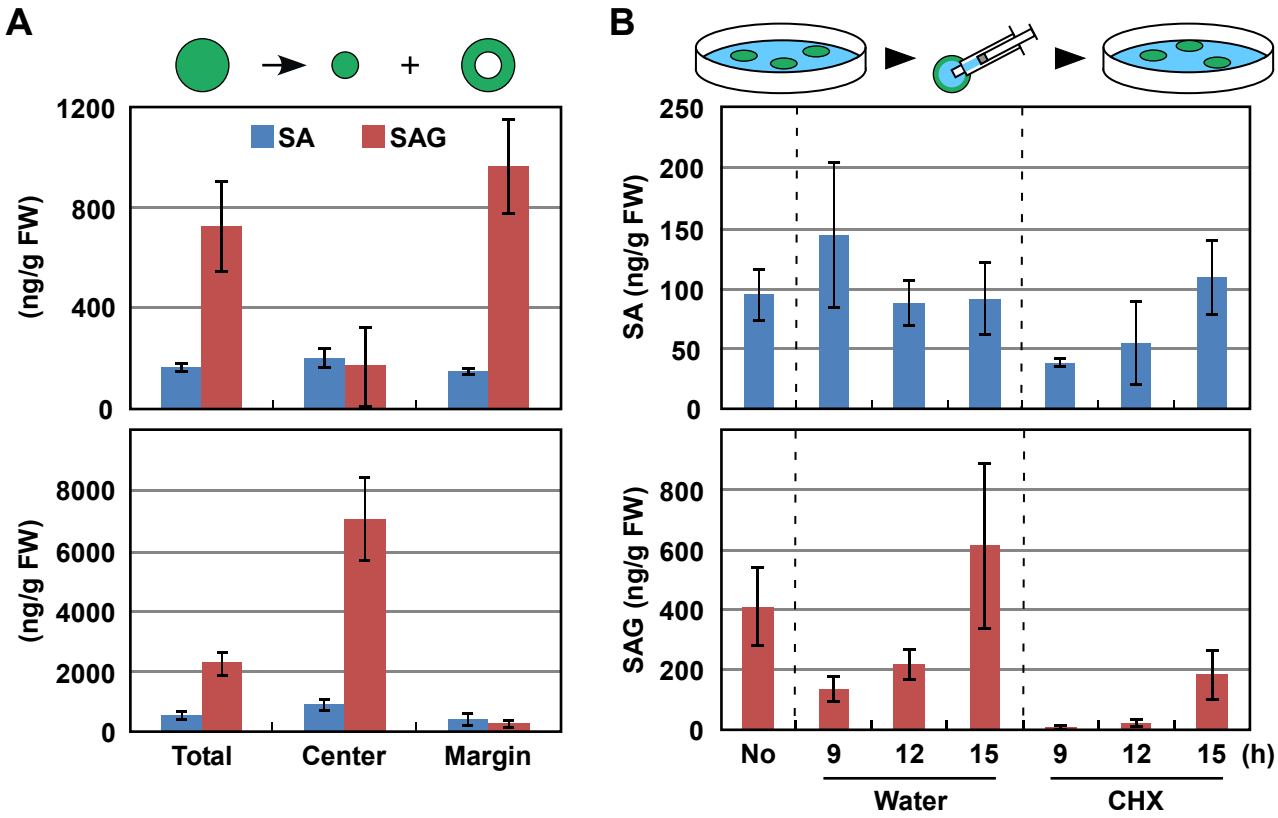

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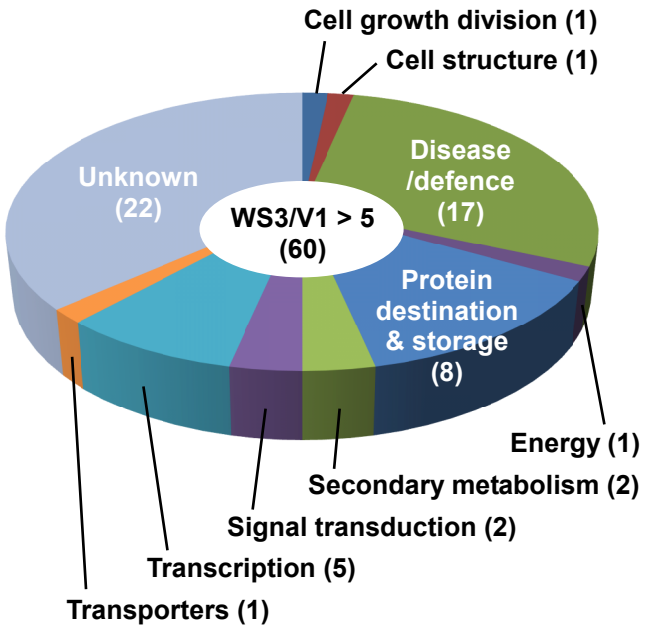

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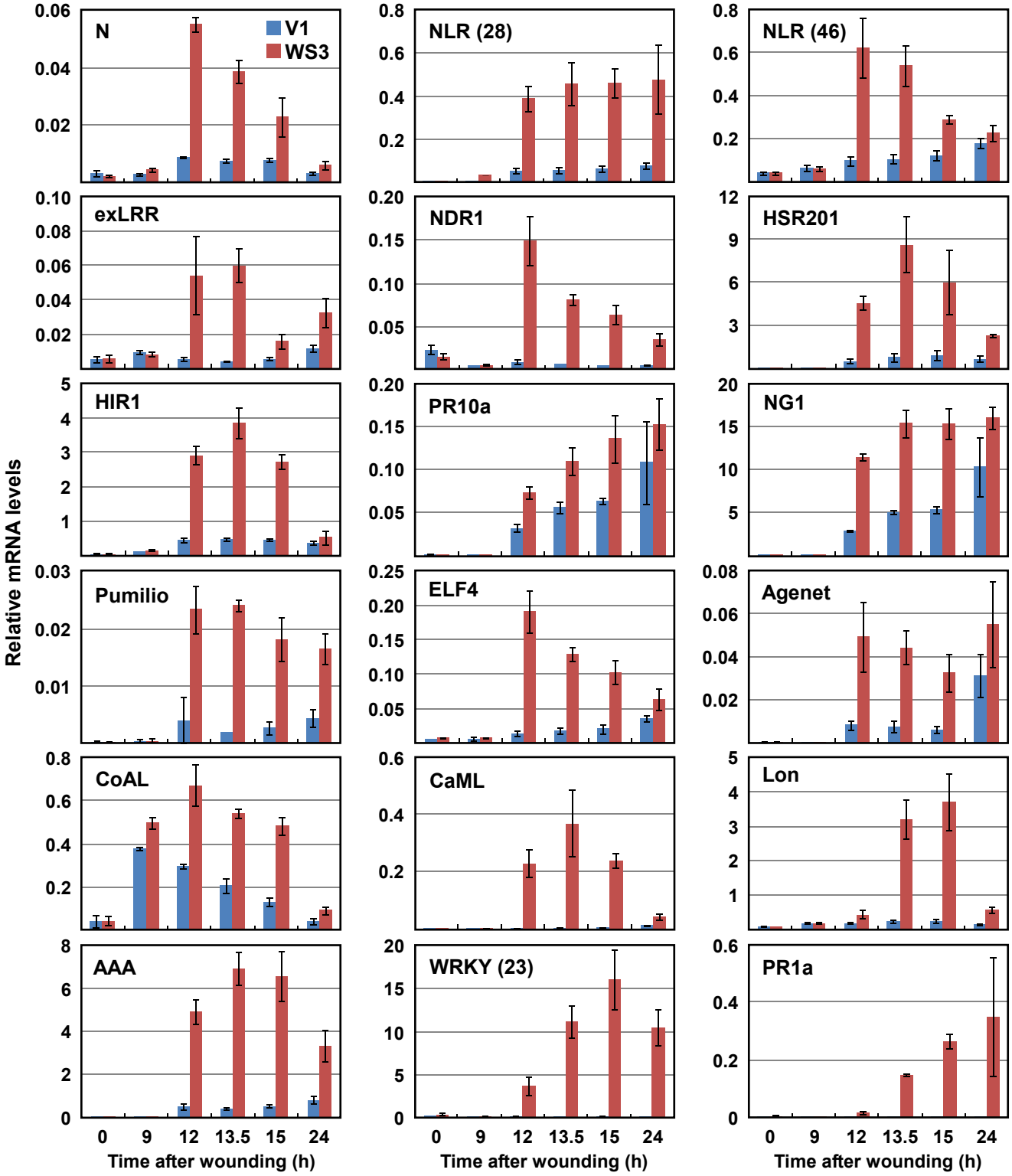

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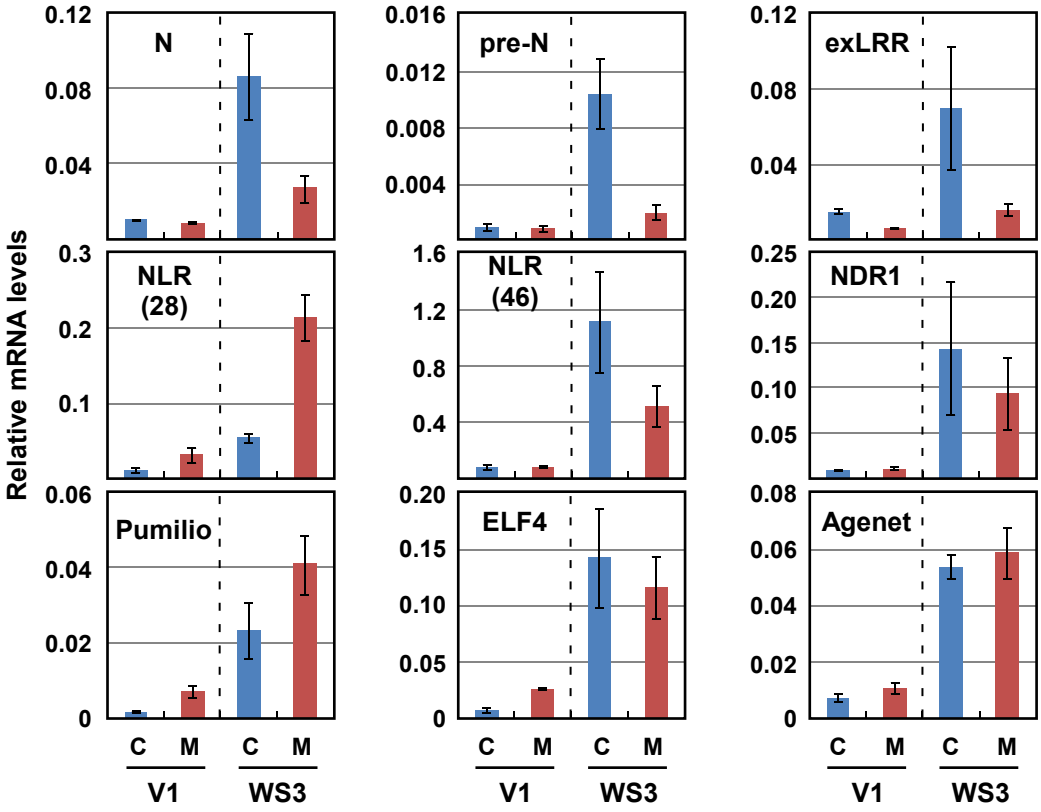

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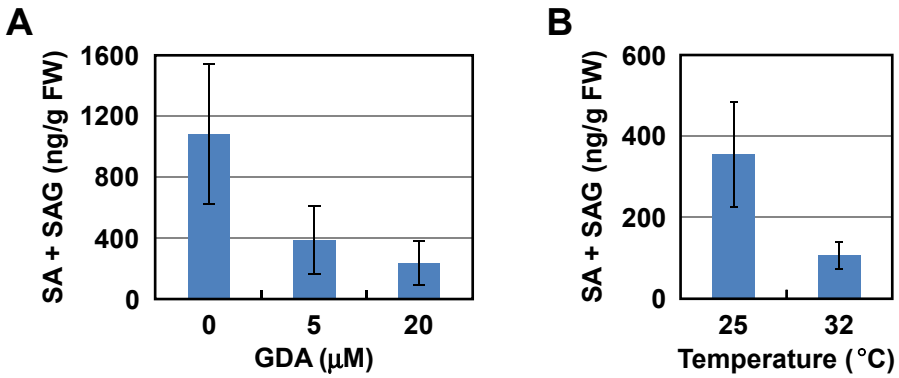

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Supplementary Table S1 List of transcripts up-regulated in WS3

No.	ProbeID	Target transcript				Ratio	V1	WS3
		TargetID	Length (bp)	UniGeneID	Description			
1	A_95_P164808	ta|EH624149	575	Nta.3703	Rep: Pathogenesis-related protein 1B precursor - Nicotiana tabacum (Common tobacco), complete [TC94867]	120.9	6	760
2	A_95_P101693	ta|CN498894	568		Rep: Chromosome chr1 scaffold_84, whole genome shotgun sequence - Vitis vinifera (Grape), partial (30%) [TC115041]	78.2	22	1,743
3	A_95_P129412	ta|EB429294	1,545	Nta.8190	Rep: Chromosome chr8 scaffold_68, whole genome shotgun sequence - Vitis vinifera (Grape), partial (54%) [TC82033]	66.1	34	2,227
4	A_95_P147042	ta|EB451291	947		Unknown	52.0	6	289
5	A_95_P161287	ta|EH620031	440	Nta.3548	Rep: Hsr201 protein - Nicotiana tabacum (Common tobacco), complete [TC77367]	45.0	1,530	68,828
6	A_95_P006951	gb|U66272	615	Nta.3633	Nicotiana tabacum ORF mRNA, complete cds [U66272]	40.7	63	2,556
7	A_95_P021401	gb|EH621793	817	Nta.3897	CHO_SL025xp12f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH621793]	37.3	7	279
8	A_95_P007481	gb|X95343	1,563	Nta.3548	N.tabacum mRNA for HSR201 protein [X95343]	36.2	2,190	79,285
9	A_95_P002421	gb|AJ291738	1,047	Nta.6389	Nicotiana tabacum mRNA for putative alpha7 proteasome subunit (a7 gene) [AJ291738]	33.4	64	2,130
10	A_95_P283533	gb|EF091690	3,426	Nta.14804	Nicotiana tabacum TMV resistance protein N (CN) mRNA, complete cds [EF091690]	32.7	6	207
11	A_95_P027141	ta|TA15716_4097	1,448		Unknown	28.1	8	226
12	A_95_P032341	gb|AB024510	1,346	Nta.3699	Nicotiana tabacum mRNA, complete cds, 1346 bp sequence [AB024510]	27.9	174	4,843
13	A_95_P099008	ta|BP534728	437		Unknown	26.5	1,312	34,735
14	A_95_P306398	gb|FG635140	558	Nta.19433	TT-08_A23 tobacco leaf library Nicotiana tabacum cDNA, mRNA sequence [FG635140]	23.5	6	130
15	A_95_P053051	ta|BP133078	358		Unknown	20.9	7	141
16	A_95_P028456	gb|X83852	817	Nta.2645	N.tabacum mRNA for PAR-1c [X83852]	19.1	87	1,660
17	A_95_P155552	ta|EG650123	374		Rep: Receptor-like protein kinase - Nicotiana tabacum (Common tobacco), partial (17%) [TC111691]	16.4	111	1,813
18	A_95_P087703	ta|BP529861	549		Rep: APUM7 (ARABIDOPSIS PUMILIO 7); RNA binding - Arabidopsis thaliana, partial (40%) [TC122616]	15.4	13	203
19	A_95_P217537	ta|TA20955_4097	310		Unknown	15.3	2,290	34,945
20	A_95_P238859	ta|CV016103	415	Nta.3897	Unknown	15.1	30	451
21	A_95_P139527	gb|EB443295	641	Nta.22871	KR2B.001N20F.050815T7 KR2B Nicotiana tabacum cDNA clone KR2B.001N20, mRNA sequence [EB443295]	15.1	67	1,017
22	A_95_P073055	ta|BP526113	618		Unknown	15.1	9	128
23	A_95_P032896	gb|AF193771	864	Nta.3714	Nicotiana tabacum DNA-binding protein 4 (WRKY4) mRNA, partial cds [AF193771]	14.8	2,757	40,832
24	A_95_P255359	gb|EH614541	747	Nta.8761	EST_CSP001xg06f1.ab1 EST_CSP Nicotiana tabacum cDNA, mRNA sequence [EH614541]	13.6	1,394	18,956
25	A_95_P156662	ta|EH614463	732	Nta.8761	Rep: 52 kDa PDI - Leishmania amazonensis, partial (4%) [TC86617]	12.3	410	5,043
26	A_95_P057686	gb|BP134278	743	Nta.21775	BP134278 MAT001 Nicotiana tabacum cDNA clone BY6638, mRNA sequence [BP134278]	12.2	10	120
27	A_95_P164472	ta|EH623710	971		Unknown	11.7	225	2,620
28	A_95_P085485	ta|BP529298	515		Unknown	11.6	36	420
29	A_95_P222837	ta|TA22119_4097	762		Rep: DNA-binding protein 4 - Nicotiana tabacum (Common tobacco), partial (98%) [TC90035]	11.5	1,260	14,525
30	A_95_P036223	ta|BP128676	1,134		Rep: Chromosome chr18 scaffold_137, whole genome shotgun sequence - Vitis vinifera (Grape), partial (46%) [TC120098]	11.5	27	306
31	A_95_P313583		1,162		Unknown	11.3	10	112
32	A_95_P258031	gb|EH618950	867	Nta.9442	CHO_SL015xn14f2.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH618950]	11.1	2,120	23,625
33	A_95_P188912	gb|EH614541	747	Nta.8761	EST_CSP001xg06f1.ab1 EST_CSP Nicotiana tabacum cDNA, mRNA sequence [EH614541]	10.1	2,342	23,749
34	A_95_P032526	gb|AB125232	1,292	Nta.4022	Nicotiana tabacum GA2ox1 mRNA for gibberellin 2-oxidase 1, complete cds [AB125232]	9.3	71	660
35	A_95_P125857	ta|EB424950	973		Unknown	9.2	94	866

36	A_95_P296443	gb|EB452161	881	Nta.17428	KT7C.113L02F.051221T7 KT7 Nicotiana tabacum cDNA clone KT7C.113L02, mRNA sequence [EB452161]	8.5	22	190
37	A_95_P041161	ta|BP130022	742		Unknown	8.4	246	2,068
38	A_95_P240449	gb|AB174847	1,491	Nta.4338	Nicotiana tabacum NtAAA1 mRNA for putative ATPase, complete cds [AB174847]	8.2	5,331	43,964
39	A_95_P304183	gb|FG641123	732	Nta.18989	TT-23_M13 K326 trichome library Nicotiana tabacum cDNA, mRNA sequence [FG641123]	7.9	160	1,260
40	A_95_P004116	ta|EB681315	720	Nta.1088	Unknown	7.6	306	2,327
41	A_95_P162662	ta|EH621663	1,070	Nta.15271	Rep: Hypersensitive-induced reaction protein - Capsicum annuum (Bell pepper), complete [TC85053]	7.5	4,678	35,015
42	A_95_P109317	ta|TA11922_4097	656	Nta.5628	Unknown	7.4	15	112
43	A_95_P217182	ta|TA20874_4097	798		Unknown	7.4	148	1,098
44	A_95_P273666	gb|EH617861	797	Nta.12804	CHO_SL009xg14f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH617861]	7.3	159	1,166
45	A_95_P160082	gb|EH618720	302	Nta.8993	CHO_SL015xc04f2.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH618720]	7.1	374	2,671
46	A_95_P149962	ta|EB679985	789		Unknown	7.0	822	5,754
47	A_95_P104662	ta|CV017122	172		Unknown	7.0	42	291
48	A_95_P102562	ta|CV016080	504	Nta.9442	Rep: WRKY transcription factor 6 - Solanum tuberosum (Potato), partial (24%) [TC118217]	6.8	1,511	10,261
49	A_95_P159222	ta|EH617180	836	Nta.3548	Unknown	6.7	2,621	17,455
50	A_95_P273501	gb|AM781872	487	Nta.12769	AM781872 seedling library, SL Nicotiana tabacum cDNA clone nt002236041, mRNA sequence [AM781872]	6.6	132	870
51	A_95_P162787	ta|EH621769	1,013	Nta.15051	Rep: Lon protease homolog - Vitis vinifera (Grape), partial (24%) [TC109254]	6.5	25	162
52	A_95_P162632	ta|EH621622	729	Nta.15051	Rep: Lon protease homolog - Vitis vinifera (Grape), partial (24%) [TC109254]	6.2	58	357
53	A_95_P237794	gb|U66273	409	Nta.3634	Nicotiana tabacum ORF mRNA, complete cds [U66273]	6.2	148	915
54	A_95_P147922	gb|AB032529	2,311	Nta.3905	Nicotiana tabacum B57 mRNA for hypothetical protein, complete cds [AB032529]	6.1	33	201
55	A_95_P280223	gb|AM822217	498	Nta.14132	AM822217 seedling library, SL Nicotiana tabacum cDNA clone nt002133027, mRNA sequence [AM822217]	6.0	75	453
56	A_95_P163152	gb|AM806848	504	Nta.9203	AM806848 seedling library, SL Nicotiana tabacum cDNA clone nt002059013, mRNA sequence [AM806848]	5.8	608	3,512
57	A_95_P158027	ta|EH616092	788		Unknown	5.6	56	314
58	A_95_P231129	gb|FG146917	912	Nta.1855	AGN_RPC007xc14f1.ab1 AGN_RPC Nicotiana tabacum cDNA 5', mRNA sequence [FG146917]	5.6	44	248
59	A_95_P209112	ta|TA19118_4097	152		Unknown	5.6	103	577
60	A_95_P101648	ta|CN498883	416		Unknown	5.5	2,956	16,328
61	A_95_P001276	gb|EH623494	235	Nta.17863	CHO_SL014xn09f1.ab1 CHO_SL Nicotiana tabacum cDNA, mRNA sequence [EH623494]	5.5	3,078	16,996
62	A_95_P101663	ta|CN498886	426		Unknown	5.5	27	150
63	A_95_P101378	ta|CN498814	329		Unknown	5.5	261	1,424
64	A_95_P034648	gb|AY055111	453	Nta.3801	Nicotiana tabacum pathogenesis-related protein PR10a mRNA, partial cds [AY055111]	5.4	22	119
65	A_95_P012481	gb|DV160348	834	Nta.2727	KP1B.102P18F.050722T7 KP1B Nicotiana tabacum cDNA clone KP1B.102P18, mRNA sequence [DV160348]	5.4	774	4,145
66	A_95_P149377	ta|EB679209	871		Rep: Chromosome chr18 scaffold_61, whole genome shotgun sequence - Vitis vinifera (Grape), partial (40%) [TC111367]	5.2	25	132
67	A_95_P107407	ta|CV018355	255	Nta.2531	Unknown	5.2	37	191
68	A_95_P143522	gb|FS417300	557	Nta.12456	FS417300 normalized full-length tobacco cDNA library Nicotiana tabacum cDNA clone TBK02GR0045_4_E07 5', mRNA sequence [FS417300]	5.1	363	1,869
69	A_95_P148372	gb|EB677664	903	Nta.23048	KG9B.102G09F.051126T7 KG9B Nicotiana tabacum cDNA clone KG9B.102G09, mRNA sequence [EB677664]	5.1	260	1,324
70	A_95_P203382	gb|FG644812	746	Nta.8043	TT-33_F01 K326 late senescent leaf library Nicotiana tabacum cDNA, mRNA sequence [FG644812]	5.1	364	1,853
71	A_95_P037018	ta|BP128888	576		Unknown	5.0	20	100

Supplementary Table S2 BLASTX analysis of transcripts up-regulated in WS3

	Microarray			BLASTX		
No.	Ratio	V1	WS3	Accession	Description	Score
1	120.9	6	760	CAA31009.1	PR1b preprotein [Nicotiana tabacum]	Score = 286 bits (731), Expect = 7e-96
2	78.2	22	1,743	XP_002269392.1	probable calcium-binding protein CML30 [Vitis vinifera]	Score = 98.2 bits (243), Expect = 2e-22
3	66.1	34	2,227	XP_002530954.1	Protein grpE, putative [Ricinus communis]	Score = 150 bits (379), Expect = 1e-37
4	52.0	6	289		Unknown	
5	45.0	1,530	68,828	X95343	N.tabacum mRNA for HSR201 protein [X95343]	
6	40.7	63	2,556	AAR21296.1	NDR1-like protein [Nicotiana benthamiana]	Score = 130 bits (326), Expect = 4e-34
7	37.3	7	279	BAA14220.1	PR1a protein precursor [Nicotiana tabacum]	Score = 324 bits (830), Expect = 2e-109
8	36.2	2,190	79,285	X95343	N.tabacum mRNA for HSR201 protein [X95343]	
9	33.4	64	2,130	AJ291738	Nicotiana tabacum mRNA for putative alpha7 proteasome subunit (a7 gene) [AJ291738]	
10	32.7	6	207	EF091690	Nicotiana tabacum TMV resistance protein N (CN) mRNA, complete cds [EF091690]	
11	28.1	8	226	XP_002523681.1	ubiquitin-conjugating enzyme e2S, putative [Ricinus communis]	Score = 415 bits (1067), Expect = 2e-140
12	27.9	174	4,843	XP_003543703.1	probable WRKY transcription factor 40-like isoform 2 [Glycine max]	Score = 228 bits (581), Expect = 8e-68
13	26.5	1,312	34,735	XP_002270721.1	probable E3 ubiquitin-protein ligase RNF144A-B [Vitis vinifera]	Score = 56.6 bits (135), Expect = 3e-07
14	23.5	6	130		Unknown	
15	20.9	7	141		Unknown	
16	19.1	87	1,660		Unknown	
17	16.4	111	1,813	BAC07504.2	receptor-like protein kinase [Nicotiana tabacum]	Score = 108 bits (269), Expect = 4e-25
18	15.4	13	203	XP_003524740.1	putative pumilio homolog 7, chloroplastic-like [Glycine max]	Score = 193 bits (491), Expect = 3e-56
19	15.3	2,290	34,945	ABU49725.1	WRKY transcription factor 6 [Solanum tuberosum]	Score = 68.9 bits (167), Expect = 2e-12
20	15.1	30	451	BAA14220.1	PR1a protein precursor [Nicotiana tabacum]	Score = 324 bits (830), Expect = 2e-109
21	15.1	67	1,017		Unknown	
22	15.1	9	128		Unknown	
23	14.8	2,757	40,832	AF193771	Nicotiana tabacum DNA-binding protein 4 (WRKY4) mRNA, partial cds [AF193771]	
24	13.6	1,394	18,956	XP_003548379.1	probable E3 ubiquitin-protein ligase RNF144A-like [Glycine max]	Score = 169 bits (427), Expect = 2e-47
25	12.3	410	5,043	XP_003548379.1	probable E3 ubiquitin-protein ligase RNF144A-like [Glycine max]	Score = 169 bits (427), Expect = 2e-47
26	12.2	10	120		Unknown	
27	11.7	225	2,620	AAR21296.1	NDR1-like protein [Nicotiana benthamiana]	Score = 363 bits (933), Expect = 1e-123
28	11.6	36	420	XP_002326566.1	nbs-lrr resistance protein [Populus trichocarpa]	Score = 88.2 bits (217), Expect = 2e-17
29	11.5	1,260	14,525	TC90035	Rep: DNA-binding protein 4 - Nicotiana tabacum (Common tobacco), partial (98%) [TC90035]	
30	11.5	27	306	ACP19338.1	phosphate transporter 12 [Glycine max]	Score = 371 bits (953), Expect = 2e-121
31	11.3	10	112	NP_001234471.1	transcription factor IIIA [Solanum lycopersicum]	Score = 469 bits (1206), Expect = 2e-161
32	11.1	2,120	23,625	ABU49725.1	WRKY transcription factor 6 [Solanum tuberosum]	Score = 304 bits (778), Expect = 2e-99
33	10.1	2,342	23,749	XP_003548379.1	probable E3 ubiquitin-protein ligase RNF144A-like [Glycine max]	Score = 169 bits (427), Expect = 2e-47
34	9.3	71	660	AB125232	Nicotiana tabacum GA2ox1 mRNA for gibberellin 2-oxidase 1, complete cds [AB125232]	
35	9.2	94	866	BAG80546.1	UDP-glucose:glucosyltransferase [Lycium barbarum]	Score = 593 bits (1529), Expect = 0.0

36	8.5	22	190	ABQ65859.1	serine carboxypeptidase III [Nicotiana tabacum]	Score = 473 bits (1218), Expect = 8e-163
37	8.4	246	2,068	XP_002263877.2	medium-chain-fatty-acid--CoA ligase [Vitis vinifera]	Score = 136 bits (343), Expect = 7e-34
38	8.2	5,331	43,964	AB174847	Nicotiana tabacum NtAAA1 mRNA for putative ATPase, complete cds [AB174847]	
39	7.9	160	1,260	ACK56123.1	ELF4-like protein [Solanum tuberosum]	Score = 151 bits (381), Expect = 6e-43
40	7.6	306	2,327	ABM05953.1	proline-rich protein [Gossypium hirsutum]	Score = 125 bits (313), Expect = 5e-32
41	7.5	4,678	35,015	TC85053	Rep: Hypersensitive-induced reaction protein - Capsicum annuum (Bell pepper), complete [TC85053]	
42	7.4	15	112		Unknown	
43	7.4	148	1,098	BAA88636.1	elicitor-inducible LRR receptor-like protein EILP [Nicotiana tabacum]	Score = 237 bits (604), Expect = 1e-68
44	7.3	159	1,166	BAA88636.1	elicitor-inducible LRR receptor-like protein EILP [Nicotiana tabacum]	Score = 237 bits (604), Expect = 1e-68
45	7.1	374	2,671		Unknown	
46	7.0	822	5,754	AAC72977.1	disease resistance protein RPP1-WsA [Arabidopsis thaliana]	Score = 53.9 bits (128), Expect = 2e-05
47	7.0	42	291		Unknown	
48	6.8	1,511	10,261	ABU49725.1	WRKY transcription factor 6 [Solanum tuberosum]	Score = 304 bits (778), Expect = 2e-99
49	6.7	2,621	17,455	X95343	N.tabacum mRNA for HSR201 protein [X95343]	
50	6.6	132	870	P10978.1|POLX_TOBAC	Retrovirus-related Pol polyprotein from transposon Tnt1 [Nicotiana tabacum]	Score = 277 bits (708), Expect = 1e-83
51	6.5	25	162	TC109254	Rep: Lon protease homolog - Vitis vinifera (Grape), partial (24%) [TC109254]	
52	6.2	58	357	TC109254	Rep: Lon protease homolog - Vitis vinifera (Grape), partial (24%) [TC109254]	
53	6.2	148	915	U66273	Nicotiana tabacum ORF mRNA, complete cds (NG1) [U66273]	
54	6.1	33	201		Unknown	
55	6.0	75	453		Unknown	
56	5.8	608	3,512		Unknown	
57	5.6	56	314	XP_002892335.1	agenet domain-containing protein [Arabidopsis lyrata subsp. lyrata]	Score = 103 bits (256), Expect = 5e-24
58	5.6	44	248	ABI98682.2	chloroplast NADP-malic enzyme precursor [Nicotiana tabacum]	Score = 261 bits (666), Expect = 2e-78
59	5.6	103	577		Unknown	
60	5.5	2,956	16,328		Unknown	
61	5.5	3,078	16,996		Unknown	
62	5.5	27	150		Unknown	
63	5.5	261	1,424	XP_002530431.1	mutt domain protein, putative [Ricinus communis]	Score = 133 bits (335), Expect = 5e-36
64	5.4	22	119	AY055111	Nicotiana tabacum pathogenesis-related protein PR10a mRNA, partial cds [AY055111]	
65	5.4	774	4,145	ADY38663.1	calmodulin-related protein CAM53 [Wolffia arrhiza], calmodulin 7 [Arabidopsis thaliana]	Score = 295 bits (754), Expect = 5e-98
66	5.2	25	132		Unknown	
67	5.2	37	191		Unknown	
68	5.1	363	1,869	NP_173391.2	defensin-like protein 19 [Arabidopsis thaliana]	Score = 58.9 bits (141), Expect = 4e-09
69	5.1	260	1,324		Unknown	
70	5.1	364	1,853		Unknown	
71	5.0	20	100		Unknown	

Supplementary Table S3 qRT-PCR analysis of transcripts up-regulated in WS3

Probe No.	BLASTX Accession	BLASTX Description	Abbreviation	Category[a]	WS3/V1[b,c,d] AVG ± SD
1	CAA31009.1	PR1b preprotein [Nicotiana tabacum]	PR1b	3-3	PR/SA-inducible
2	XP_002269392.1	probable calcium-binding protein CML30 [Vitis vinifera]	CaML	10	319.8 ± 81.5 **
3	XP_002530954.1	Protein grpE, putative [Ricinus communis]	GrpE	7	2.8 ± 0.4 **
4		Unknown	Unknown	13	
5, 8, 49	X95343	N.tabacum mRNA for HSR201 protein [X95343]	HSR201	3-2	12.5 ± 1.4 **
6, 27	AAR21296.1	NDR1-like protein [Nicotiana benthamiana]	NDR1	3-1	20.3 ± 5.5 **
7, 20	BAA14220.1	PR1a protein precursor [Nicotiana tabacum]	PR1a	3-3	276.5 ± 93.9 **
9	AJ291738	Nicotiana tabacum mRNA for putative alpha7 proteasome subunit (a7 gene) [AJ291738]	Pa7	7	1.4 ± 0.2
10	EF091690	Nicotiana tabacum TMV resistance protein N (CN) mRNA, complete cds [EF091690]	N	3-1	6.2 ± 0.3 **
11	XP_002523681.1	ubiquitin-conjugating enzyme e2S, putative [Ricinus communis]	UBQ-E2	7	2.4 ± 0.3 **
12	XP_003543703.1	probable WRKY transcription factor 40-like isoform 2 [Glycine max]	WRKY (12)	3-3	288.8 ± 54.4 **
13	XP_002270721.1	probable E3 ubiquitin-protein ligase RNF144A-B [Vitis vinifera]	UBQ-E3 (13)	7	30.0 ± 1.4 **
14		Unknown	Unknown	13	
15		Unknown	Unknown	13	
16		Unknown	Unknown	13	
17	BAC07504.2	receptor-like protein kinase [Nicotiana tabacum]	RLK	3	1.1 ± 0.1
18	XP_003524740.1	putative pumilio homolog 7, chloroplastic-like [Glycine max]	Pumilio	11	14.8 ± 1.8 **
19, 32, 48	ABU49725.1	WRKY transcription factor 6 [Solanum tuberosum]	WRKY (19)	3-3	12.8 ± 2.7 **
21		Unknown	Unknown	13	
22		Unknown	Unknown	13	
23, 29	AF193771	Nicotiana tabacum DNA-binding protein 4 (WRKY4) mRNA, partial cds [AF193771]	WRKY (23)	3-3	17.0 ± 5.7 **
24, 25, 33	XP_003548379.1	probable E3 ubiquitin-protein ligase RNF144A-like [Glycine max]	UBQ-E3 (24)	7	17.9 ± 4.1 **
26		Unknown	Unknown	13	
28	XP_002326566.1	nbs-lrr resistance protein [Populus trichocarpa]	NLR (28)	3-1	7.2 ± 0.4 **
30	ACP19338.1	phosphate transporter 12 [Glycine max]	PiTP	12	1.2 ± 0.1
31	NP_001234471.1	transcription factor IIIA [Solanum lycopersicum]	TFIIIA	11	1.4 ± 0.1 *
34	AB125232	Nicotiana tabacum GA2ox1 mRNA for gibberellin 2-oxidase 1, complete cds [AB125232]	GA2O	1	1.8 ± 0.2 **
35	BAG80546.1	UDP-glucose:glucosyltransferase [Lycium barbarum]	UDP-GT	9	PR/SA-inducible
36	ABQ65859.1	serine carboxypeptidase III [Nicotiana tabacum]	SCPL	7	2.5 ± 0.3 **
37	XP_002263877.2	medium-chain-fatty-acid--CoA ligase [Vitis vinifera]	CoAL	9	3.2 ± 0.4 **
38	AB174847	Nicotiana tabacum NtAAA1 mRNA for putative ATPase, complete cds [AB174847]	AAA	7	10.9 ± 0.4 **
39	ACK56123.1	ELF4-like protein [Solanum tuberosum]	ELF4	11	11.0 ± 0.9 **
40	ABM05953.1	proline-rich protein [Gossypium hirsutum]	Pro-rich	2	PR/SA-inducible
41	TC85053	Rep: Hypersensitive-induced reaction protein - Capsicum annuum (Bell pepper), complete [TC85053]	HIR1	3-2	8.1 ± 0.5 **
42		Unknown	Unknown	13	

43, 44	BAA88636.1	elicitor-inducible LRR receptor-like protein EILP [Nicotiana tabacum]	exLRR	3-1	12.5 ± 3.9	**
45		Unknown	Unknown	13		
46	AAC72977.1	disease resistance protein RPP1-WsA [Arabidopsis thaliana]	NLR (46)	3-1	6.8 ± 2.1	**
47		Unknown	Unknown	13		
50	P10978.1|POLX_TOBAC	Retrovirus-related Pol polyprotein from transposon Tnt1 [Nicotiana tabacum]	Retro	11	1.0 ± 0.2	
51, 52	TC109254	Rep: Lon protease homolog - Vitis vinifera (Grape), partial (24%) [TC109254]	Lon	7	5.2 ± 2.1	*
53	U66273	Nicotiana tabacum ORF mRNA, complete cds (NG1) [U66273]	NG1	3-2	4.7 ± 0.8	**
54		Unknown	Unknown	13		
55		Unknown	Unknown	13		
56		Unknown	Unknown	13		
57	XP_002892335.1	agenet domain-containing protein [Arabidopsis lyrata subsp. lyrata]	Agenet	11	4.8 ± 1.7	*
58	ABI98682.2	chloroplast NADP-malic enzyme precursor [Nicotiana tabacum]	ME	4	2.9 ± 0.4	**
59		Unknown	Unknown	13		
60		Unknown	Unknown	13		
61		Unknown	Unknown	13		
62		Unknown	Unknown	13		
63	XP_002530431.1	mutt domain protein, putative [Ricinus communis]	Mutt	3-3	PR/SA-inducible	
64	AY055111	Nicotiana tabacum pathogenesis-related protein PR10a mRNA, partial cds [AY055111]	PR10a	3-2	2.7 ± 0.4	**
65	ADY38663.1	calmodulin-related protein CAM53 [Wolffia arrhiza], calmodulin 7 [Arabidopsis thaliana]	NtCaM3/4	10	2.4 ± 0.1	**
66		Unknown	Unknown	13		
67		Unknown	Unknown	13		
68	NP_173391.2	defensin-like protein 19 [Arabidopsis thaliana]	Defensin	3-3	PR/SA-inducible	
69		Unknown	Unknown	13		
70		Unknown	Unknown	13		
71		Unknown	Unknown	13		

[a]Categorized according to Bevan et al. (1998). 1, cell growth division; 2, cell structure; 3, disease/defence; 4, energy; 5, intracellular traffic; 6, metabolism; 7, protein destination and storage; 8, protein synthesis; 9, secondary metabolism; 10, signal transduction; 11, transcription; 12, transporters; 13, unknown

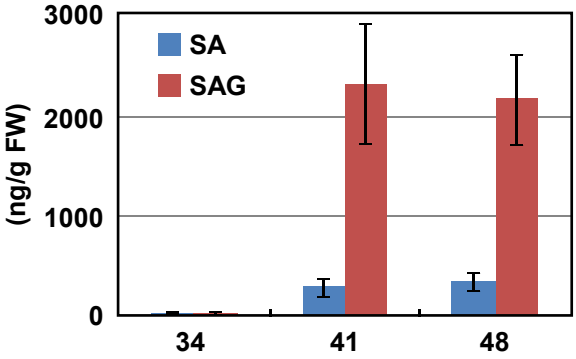
[b]The data represent the means and SD of qRT-PCR analysis with three biological replicates

[c]Asterisks indicate a significant difference between V1 and WS3: **$p < 0.01$, *$p < 0.05$

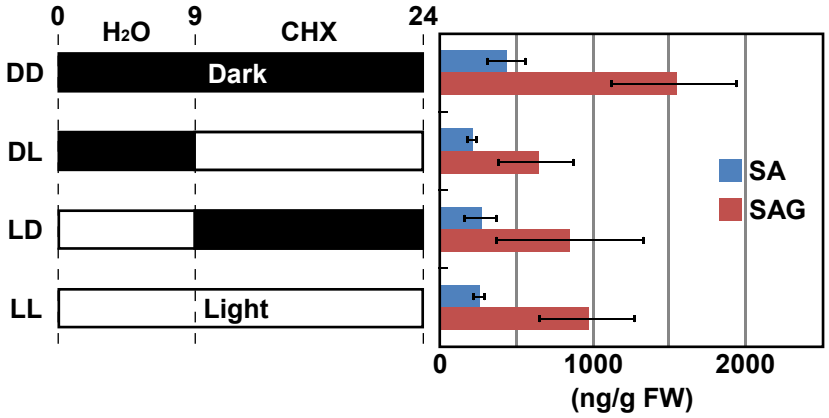
[d]The data which do not coincide with the microarray analysis are indicated in red

Supplementary Table S4 List of primers used for qRT-PCR analysis

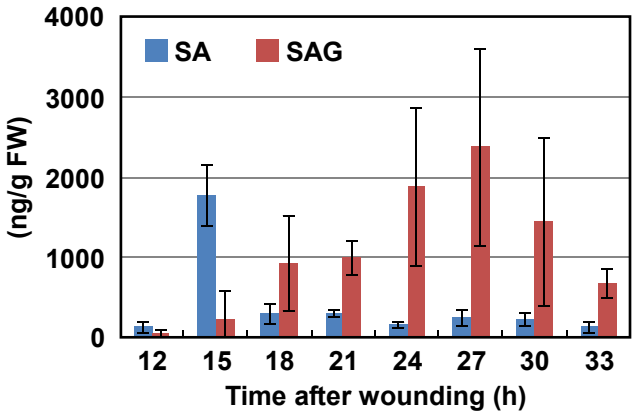
Probe No.	Abbreviation	Forward	Reverse	Reference
2	CaML	TGCTAGATGAGAACCCTAGTTTGG	AGAACTCTTTGCAGCTCAAAAGC	This study
3	GrpE	GTCTTTGCTTCAAATGGGGATGCTG	TCTACATTCTCCTCCTGCTCGGA	This study
7	PR1a	GTCCATACTAATTGAAACGACCTAC	CCACTTCAGAGGATTACATATATAGTAC	Takabatake et al. (2007b)
8	HSR201	TATCCATTTGCTGGCCGTCTCC	CCGCTTCGACAAACATAATCCCCT	This study
9	Pa7	CCGGGATCTAATCGTCGGATTCAC	TTGGTTGCTTCAGACTTCGTGC	This study
10	N	AGAAGGACCAGAGGTTAATGCCT	TGAGTCCTCCTTATCCCAGTGGA	This study
11	UBQ-E2	CGACATGTTCTCATGGTGGTAAGGT	CAAGCAGCATTTTACCAGCTTGCTC	This study
12	WRKY (12)	CCTCCGATCAAAGTGCAGTGGT	TCTTGACTGGGCATGATGGTGC	This study
13	UBQ-E3 (13)	AGTGAAGAGCATTGGAGTTGCCA	TGGTGGACAACAAACATCCAGC	This study
17	RLK	GGTATGAGGAGCAAATGCTTTTGGT	AACATCCTTACTGTTAGGCCTCTCC	This study
18	Pumilio	GCAGAGAATGCAGTTTGTGCTCG	AACTTTTGCACAACACGGGTCC	This study
23	WRKY (23)	CTTCGTCACTGAAAGATCGACGAGG	GGCATTGAGAATGTGCTTCTGACC	This study
24	UBQ-E3 (24)	CGACATGAAGAGATCGTTCAGTGCA	TGAAACTTGTCACAATCACGCCCA	This study
27	NDR1	AGCTACTAGAGTCAGGTACAAGGT	TTACCTGAACCATCCACTTTCACTG	This study
28	NLR (28)	GGTATAGGGAAAACTGCCCTAGTC	TGTCCGTAACATCAGAAAACCTTGG	This study
30	PiTP	TGTGTCAGCCTAAGGTTTGCCTG	TCCTTTAGCCTGCTTGTGGCAC	This study
31	TFIIIA	ACTCCAAAATGCAAGAGGCAGG	TGGCATACAAATGGCCTCACCA	This study
32	WRKY (19)	CCGGAAAAGTCATCAGCGGATCTG	TCCTCAGCCATAATGGTTGCAAGAG	This study
34	GA2O	GAGTGACTCTGTTTTCAGGCT	TGATTTGTGGGTCAGTATGCT	This study
36	SCPL	TGGCTGTGTTTTACGAAAACGGACC	AGGTTTGAGACCTTGTCCCAGC	This study
37	CoAL	ACCTGTGTATTTGTGGACATTGCCA	TTAGTTCCACCAACAGCAGCCA	This study
38	AAA	AGTGGACAAGAAGAATCAGGAGCT	GCATAGGCATCATGTGGCTTGAG	This study
39	ELF4	CATATTGGACCAGAACAGGCTGC	ACATTCCTGCTCAAATTGTCAGGGA	This study
41	HIR1	ATCAGGATGTGCTTGAGCCAGG	TTGTCTTGGTTTCACAGCGCAC	This study
44	exLRR	TGGGAGATCTTATTGCACTTCGGGT	CTCCCAGTGACTGTGGTATGCGA	This study
46	NLR (46)	TGGTGCAGGAATCAAGTAACAGCT	CCTGAAGCATGGATTATGCGGGAC	This study
50	Retro	GGTTCTTTGACTTCCCACGAGC	GCCTGAAAATCAAGGACATGCTCTC	This study
51	Lon	TCCGAGTCGTTTAGCGATTCTTCC	ACACTGCTAGGAGAAGTGCAACG	This study
53	NG1	TCTTTCGCGACTTCGTTCAGTACG	CAGAATGTAGCACAAGCCGTGG	This study
57	Agenet	GATAATTCTGCGCCATTAGAGG	CCATCATTATCAAGTGCATCAACC	This study
58	ME	AAGATCTACGCGTCCGGTCAGG	TCATCGTGGACACGAATTGCACC	This study
64	PR10a	CAGTCAGCAGATGGTGGTTCCA	ACAACTTTGAACATAGCCGAGACCT	This study
65	NtCaM3/4	GGTCATGATGGCCAAGTAATTTCA	CAGGTCCAATACATCCAGATGG	Takabatake et al. (2007b)
	Actin2	TGGTCCTGCAATTGTTCACAGGAA	GCACGTAAACTGGAACAAGGTCAC	This study
	pre-N	GGATAAGGAGGACTCAATATAACAAC	TCACATAAACTGGAACGGATGG	This study
	PI-II	ATGCTAAACTCTGTAATGTGGGC	CATTAAATTCTTGGGCATGTCCTTG	Takabatake et al. (2007b)
	WIPK	CCAAGTATCGTCCTCCTATTATG	TCACGGAGAGTCCTCTTAGC	Takabatake et al. (2007a)
	SIPK	CCACGGTGGCAGGTTCATTC	CAGAACAAACGATGCCGTAAGC	Takabatake et al. (2007a)



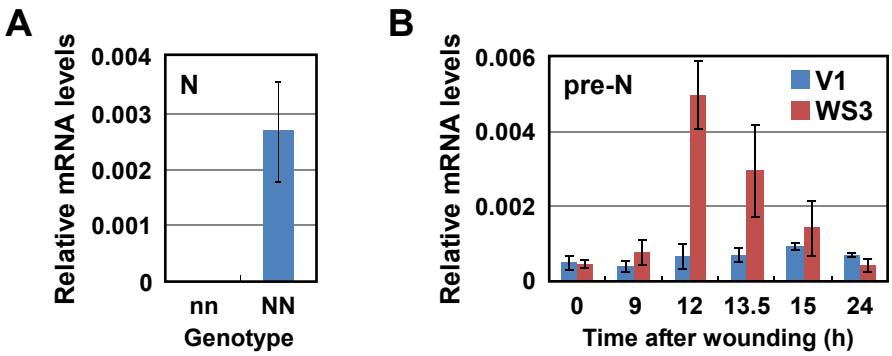
Supplementary Fig. S1 Wound-induced and CHX-enhanced accumulation of SA in WIPK/SIPK-suppressed plants depends on the developmental stage of the plants. Leaf discs were excised from the leaves of WIPK/SIPK-suppressed plants at the indicated time points after sowing. The discs were floated on water for 9 h, then transferred on 300 μM CHX, and further incubated for a total of 24 h. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3).



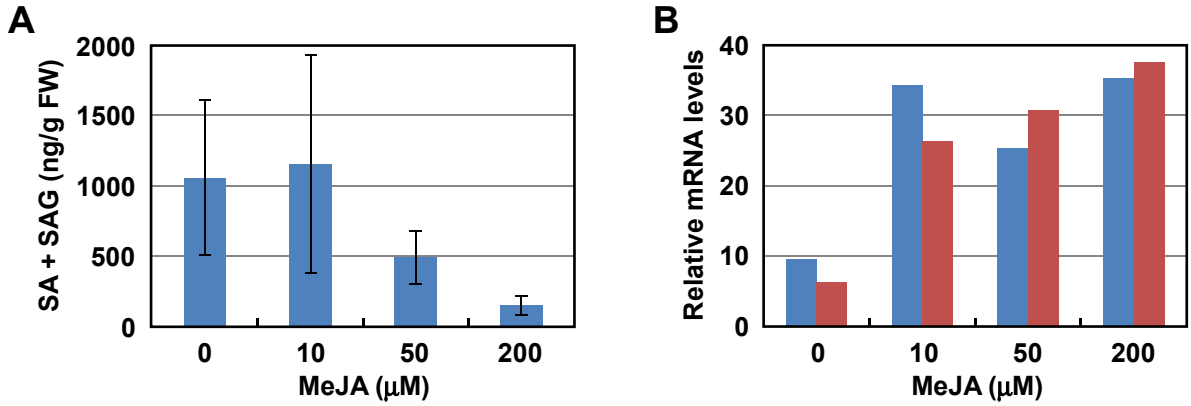
Supplementary Fig. S2 Wound-induced and CHX-enhanced accumulation of SA in WIPK/SIPK-suppressed plants is more prominent in the dark. Leaf discs of WIPK/SIPK-suppressed plants were floated on water for 9 h in the dark (D) or light (L). The discs were then transferred to 300 μM CHX and further incubated in the dark (D) or light (L) for a total of 24 h. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3).



Supplementary Fig. S3 Accumulation pattern of wound-induced and CHX-enhanced SA in WIPK/SIPK-suppressed plants. Leaf discs of WIPK/SIPK-suppressed plants were floated on water for 9 h, then transferred to 300 μM CHX and further incubated for the periods indicated. The levels of free (blue bars) and conjugated (red bars) SA were determined. Values are means with SD (n=3 to 5).



Supplementary Fig. S4 Wound-induced accumulation of the *N* transcript in WIPK/SIPK-suppressed plants results from *de novo* transcription. (A) Specificity determination of primers for the *N* gene. Total RNA was extracted from the tobacco cultivars Samsun nn lacking the *N* gene and NN containing the *N* gene, and used to quantify the transcript levels of the *N* gene by qRT-PCR. (B) Accumulation of the *N* gene transcript results from *de novo* transcription. Leaf discs of the vector control (V1) and WIPK/SIPK-suppressed (WS3) plants were floated on water for 9 h, then transferred to 300 μM CHX and further incubated for the periods indicated. Levels of the *N* precursor mRNA (pre-N) were quantified by qRT-PCR with primers which anneal the 4th exon and 4th intron of the *N* gene, respectively. Transcript levels of the *N* gene (A) and *pre-N* (B) were normalized to the level of *Actin2* as an internal standard. Values are means with SD (n=3).



Supplementary Fig. S5 Effect of methyl jasmonate (MeJA) on wound-induced accumulation of SA and the transcript levels of *PI-II* in WIPK/SIPK-suppressed plants. Leaf discs of WIPK/SIPK-suppressed (WS3) plants were floated on MeJA at the indicated concentrations or 0.1% DMSO as a control for 24 h. (A) The levels of total SA (SA + SAG) were determined. Values are means with SD (n=3). (B) Transcript levels of *PI-II* were quantified by qRT-PCR and normalized to the level of *Actin2* as an internal standard (n=2).