# Mitogen-activated protein kinase 4 (MPK4) functions in development and resistance to biotic and abiotic stresses in *Nicotiana attenuata*

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## 1. Introduction

Plants are challenged by various environmental stresses during their whole life cycles. Accordingly, plants have evolved to cope with these stresses using sophisticated signaling systems that quickly convert the extracellular stress stimuli into intracellular responses that allow plants to promptly resist the stresses. These defense reactions are strictly controlled by elaborate regulatory networks (Jones and Dangl, 2006; Howe and Jander, 2008; Wu and Baldwin, 2010), within which, the mitogen-activated protein kinase (MAPK) cascades play essential roles, often located downstream of sensors or receptors and transducing extracellular stimuli into intracellular responses (Ichimura et al., 2000; Asai et al., 2002; Teige et al., 2004; Meszaros et al., 2006; Brader et al., 2007). Typically, a MAPK cascade consists of a three-kinase module that is conserved in all eukaryotes. MAPK, the last kinase in the cascade, is activated by the dual phosphorylation of Thr and Tyr residues in its kinase catalytic activation loop. This phosphorylation is mediated by a MAPK kinase (MAPKK or MEK), which is activated by a MAPKK kinase (MAPKKK or MEKK). Following activation, MAPKs mainly regulate gene expression by phosphorylation of DNA-binding transcription factors (Hill and Treisman, 1995; Karin and Hunter, 1995; Hazzalin and Mahadevan, 2002).

In plants, MAPKs comprise a relatively large gene family (20, 15, and 21 MAPKs in Arabidopsis, rice, and poplar respectively), suggesting that MAPKs may be important for various signaling pathways (MAPK group, 2002; Hamel et al., 2006). Consistent with this hypothesis, genetic studies have revealed that MAPKs are involved in numerous developmental processes and resistance to biotic and abiotic stresses (Zhang and Klessig, 2001; Pedley and Martin, 2005; Andreasson and Ellis, 2010; Rodriguez et al., 2010; Wu and Baldwin, 2010). MAPKs play critical roles in plant innate immunity to bacterial, oomycete, and fungal pathogens (Zhang and Klessig, 2001; Asai et al., 2002; Pedley and Martin, 2005). For example, Arabidopsis AtMPK6 and AtMPK3 are required for resistance against attack from pathogens as knock-out plants lacking AtMPK6 and AtMPK3 activity are highly sensitive to pathogen infection (Desikan et al., 2001; Asai et al., 2002; Menke et al., 2004; Ren et al., 2008; Pitzschke et al., 2009). Another MAPK, AtMPK4 was identified as a negative regulator of plant immunity to pathogens (Petersen et al., 2000). *mpk4* mutants have highly elevated salicylic acid (SA) levels, which result in greatly

increased levels of pathogenesis-related (PR) transcripts. In turn, these mutants are strongly resistant to a virulent bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), and a virulent isolate of the oomycete, *Peronospora parasitica* (Petersen et al., 2000).

In addition to MAPKs' roles in resistance to pathogens, recent research has indicated their involvement in defense against herbivores (Kandoth et al., 2007; Wu et al., 2007). Following herbivore attack, plants activate MAPK signaling and in turn alter phytohormone levels, including those of jasmonic acid (JA) and ethylene (Howe et al., 1996; Reymond and Farmer, 1998; von Dahl et al., 2007), and reshape their transcriptomes and proteomes (Hui et al., 2003; Reymond et al., 2004; Giri et al., 2006). The critical roles of JA and JA-Ile signaling in plant-herbivore interactions have been intensively studied (reviewed in Howe and Jander, 2008; Wu and Baldwin, 2010). Transgenic (or mutant) plants that are impaired in JA/JA-Ile biosynthesis or signaling have highly attenuated levels of secondary metabolites, which normally function as direct and indirect defenses, and thus plants have greatly impaired resistance against herbivores (Howe et al., 1996; Reymond et al., 2000; Kessler and Baldwin, 2001; Paschold et al., 2007).

A growing body of evidence has also indicated the involvement of MAPK signaling in plant responses to various abiotic stresses, such as unfavorable temperatures, UV-B, oxidation, and drought (Zhang and Klessig, 2001; Holley et al., 2003; Xiong and Yang, 2003; Teige et al., 2004; Jammes et al., 2009). Located on leaf epidermis, stomata are crucially important in controlling rates of $CO_2$ uptake for photosynthesis and to prevent water loss caused by transpiration. Thus plants use sophisticated regulatory systems to achieve optimum stomatal apertures, which allow them to take up $CO_2$ from the air to supply substrate for photosynthesis while minimizing water loss. ABA plays a critical role in controlling stomatal apertures. ABA-deficient plants or those with impaired ABA signaling are unable to regulate their stomatal apertures adaptively and are highly susceptible to drought stress (Iuchi et al., 2001; Desikan et al., 2004; Kim et al., 2010). As a pore through the epidermal layer into the mesophyll, stomata play essential roles in pathogen defense. Usually bacterial pathogens can penetrate into plant tissues through stomata or wounds, and ABA signaling is required for plant stomatal closure, which prevents pathogens from entering (Melotto et al., 2006). Besides its role in drought- and pathogen resistance primarily by mediating stomatal closure (Fan et al., 2004; Melotto et al., 2006; Wang and Song, 2008; Sirichandra et al., 2009; Kim et al., 2010), ABA functions in regulation of many developmental processes (Barrero et al., 2005; Fujii and Zhu, 2009; Nakashima et al., 2009).

Importantly, ABA also plays a critical role in seed dormancy and germination (Finkelstein et al., 2008).

Protein kinases are critically important in modulating ABA-induced responses, including stomatal closure (Kim et al., 2010). In Arabidopsis, SNF1-related protein kinases (SnRK2s) are activated by ABA and mutants having defects in SnRK2s are almost completely insensitive to ABA, indicating that they are key components in ABA signaling (Fujii and Zhu, 2009). Four calcium-dependent protein kinases (CPKs or CDPKs), AtCPK3, AtCPK6, AtCPK4, and AtCPK1, also control stomatal closure in an ABA-dependent manner (Mori et al., 2006; Zhu et al., 2007). Arabidopsis stomatal guard cell development and ABA-regulated stomatal closure are also regulated by MAPK signaling. Mutant lines of the MAPK kinases, AtMKK4, AtMKK5, AtMKK7, and AtMKK9, all show defects in guard cell formation and function (Lampard et al., 2009) and seedlings of the *mpk3 mpk6* double mutant have highly increased guard cells densities (Wang et al., 2007). Specifically expressing antisense *AtMPK3* in Arabidopsis partially impairs ABA- and $H_2O_2$-induced guard cell movements (Gudesblat et al., 2007). Similarly, the MAPKs AtMPK9 and AtMPK12, which are specifically localized in guard cells, redundantly and positively control transpiration rates and stomatal closure in response to ABA and $H_2O_2$ treatments (Jammes et al., 2009). Recently a MAPK phosphatase PP2C5, which directly modulates MAPK activity, was also found to be involved in ABA signaling during seeds germination and stomatal movements (Brock et al., 2010).

*Nicotiana attenuata* Torr. ex Wats. (Solanaceae) is a native tobacco species which grows in the Great Basin Desert of Southwest USA (Baldwin, 2001). This diploid, largely selfing plant exhibits great morphological and chemical phenotypic plasticity which appears to be adaptive, and therefore has been chosen as model system to study plant-herbivore interactions in a native ecosystem (Figure 1A). *N. attenuata* grows in the 'primordial agricultural' niche, the immediate post-fire environment: dormant seeds germinate synchronously into nitrogen-rich soils in response to smoke stimulants originating from fire (Baldwin and Morse, 1994; Lynds and Baldwin, 1998) (Figure 1B).

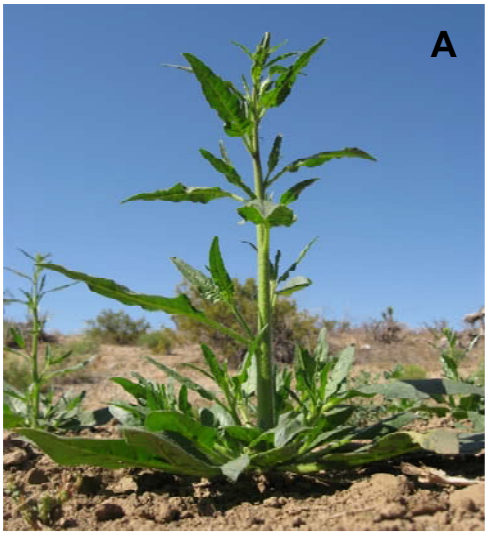



Photo courtesy: D. Kessler


**Figure 1.** *Nicotiana attenuata* in its natural habitat, the Great Basin Desert, Utah, USA.

**(A)** Bolting *N. attenuata*. **(B)** Bush fire in the Great Basin Desert.

This growth environment selected for plants with rapid, competitive growth abilities when water is available. Herbivores from more than 20 different taxa, from mammalian browsers that consume entire plants to intracellular-feeding insects, attack the plants on a variety of spatial scales. Amongst the leaf chewing insects, *Manduca sexta* (tobacco hornworm; Figure 2A), a specialist herbivore on solanaceous plants and *Spodoptera littoralis* (Figure 2B), a generalist herbivore, are major defoliators of *N. attenuata*.

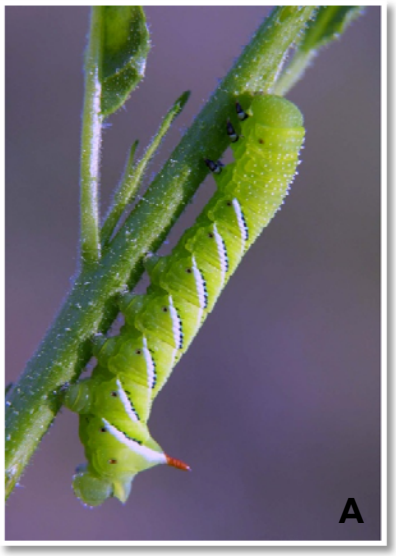




**Figure 2.** Natural herbivores of *N. attenuata*.

**(A)** *Spodoptera littoralis.*

**(B)** *Manduca sexta.*

Photo courtesy: D. Kessler; www.forestryimages.org

*N. attenuata* recognizes fatty acid-amino acid conjugates (FACs) in the oral secretions (OS) of the specialist herbivore *Manduca sexta*, which are introduced into wounds during feeding, and thereafter rapidly and highly activates MAPKs, including the salicylic acid-induced protein kinase (SIPK; an orthologue of AtMPK6) and the wound-induced protein kinase (WIPK; an orthologue of AtMPK3) (Wu et al., 2007). Silencing *SIPK* and *WIPK* in *N. attenuata* and their homologues in tomato compromises herbivory-induced JA bursts, which in turn results in attenuated levels of defensive secondary metabolites (Kandoth et al., 2007; Wu et al., 2007; Meldau et al., 2009). These metabolites include nicotine, trypsin protease inhibitor (TPI). phenolics, flavonoids, phenolic putrescine conjugates, and diterpene glycosides (Van Dam et al., 2001; Keinanen et al., 2001; Heiling et al., 2010), some of which are known to influence herbivore growth. (Glawe et al., 2003; Roda et al., 2004; Jassbi et al., 2008; Kaur et al., 2010). In addition to direct defenses, JA induces the emission of volatile organic compounds (VOCs) which function as indirect defenses by attracting predatory bugs to *M. sexta* eggs and larvae and thus dramatically increasing their mortality rate (Kessler and Baldwin, 2001). The release of some VOCs also decreases oviposition rates from adult moths (Kessler and Baldwin, 2001). These ovipositing adults may use the volatile release to identify host plants lacking predators and to avoid plants on which predators are already present.

We are starting to understand the complex mechanisms by which plants can deal with the great variety of different biotic and abiotic stresses. However, very little is known about the function of MAPKs other than SIPK and WIPK in resistance mechanisms against herbivore and pathogen attack. Moreover, although much knowledge has been obtained from genetic studies in Arabidopsis, still little is known about how other plant species regulate stomatal apertures when the plants are challenged by drought and bacterial pathogens. In *Nicotiana tabacum*, knocking down the transcript levels of *NtMPK4*, a MAPK whose sequence is highly similar to Arabidopsis *AtMPK4* and *AtMPK11*, leads to moderately reduced plant sizes, increased transpiration rates, and impaired stomatal responses to $CO_2$ and ozone treatments; however, *NtMPK4*-silenced tobacco plants showed no defect in ABA-induced closure movement of the guard cells (Gomi et al., 2005; Marten et al., 2008). Furthermore, *NtMPK4* is rapidly activated by wounding and *NtMPK4*-silenced tobacco plants show attenuated levels of *proteinase inhibitor-II* (*PI-II*) transcript levels after wounding (Gomi et al., 2005), suggesting a putative role of this MAPK in plants defense against herbivores.

In this study, the function of the orthologue of NtMPK4 in *N. attenuata*, NaMPK4, was investigated in plant growth and fitness and in defense responses against drought stress, herbivores and pathogens. *NaMPK4*-silenced *N. attenuata* plants are moderately smaller under optimal growth conditions and produce more seeds than do wild-type (WT) plants. They exhibit highly elevated photosynthetic rates, which are correlated with their enhanced transpiration rates and delayed senescence. Importantly, different from the function of NtMPK4 in tobacco, NaMPK4 is required for ABA- and $H_2O_2$-mediated stomatal closure after drought stress and NaMPK4 promotes ABA-, salt-, and methyl jasmonate (MeJA)-induced germination inhibition. Interestingly, and in contrast to SIPK and WIPK, NaMPK4 negatively mediates resistance to the specialist insect *M. sexta* in a JA-independent pathway. Furthermore, we also demonstrate that NaMPK4 plays a critical role in defending plants against bacterial pathogens (*Pseudomonas syringae* pv. *tomato* DC3000) in intercellular spaces in a salicylic acid (SA)-independent manner and very likely in stomata. These data demonstrate the multifaceted role of NaMPK4 in regulating developmental processes as well as defense responses against biotic and abiotic stresses.

## 2. Methods

### Plant growth and sample treatments

*Nicotiana attenuata* (Solanaceae) seeds were from a line maintained in our laboratory that was originally collected in Utah (USA) and inbred for 30 generations in the glasshouse. Seed germination and plant cultivation followed Krügel et al. (2002). Four- to 5-week-old plants were used for all experiments except for growth and seed number measurements.

Homozygous *mpk11* (SALK_049352C) seeds were obtained from the European Arabidopsis Stock Center (http://arabidopsis.info/) and the homozygosity was confirmed by PCR (Supplemental Table 1). Arabidopsis Col-0 and *mpk11* were directly germinated in soil and were cultivated under the long-day conditions (16 h photoperiod, 65% relative humidity, 21 $^{o}$C).

For the collection of *M. sexta* oral secretions (OS), larvae were reared on *N. attenuata* WT plants until the third to fifth instars. OS were collected on ice as described in Roda et al. (2004) and stored under nitrogen at -20 °C. To obtain FAC-free OS, 400 µL of OS were run through six ion-exchange columns containing 400 mg of the basic anion-exchange resin Amberlite IRA-400 (Sigma). FAC A (*N*-linoenoyl-L-Gln), the major FAC constituent of *M. sexta* OS (Halitschke et al., 2001), was dissolved in 0.005% (v/v) Tween 20 at a concentration of 27.6 ng/µL, which is similar to its concentration in 1/5 diluted OS (Halitschke et al., 2001). For simulated herbivory treatment, leaves were wounded with a pattern wheel and herbivore oral secretions (OS) (20 µL of 1/5 diluted OS) were immediately rubbed onto each wounded leaf (W+OS); for wounding treatment, leaves were wounded with a pattern wheel, and 20 µL of water were rubbed onto each leaf (W+W). For methyl jasmonate (MeJA) treatments, MeJA was dissolved in heat-liquefied lanolin at a concentration of 5 mg mL$^{-1}$ and 20 µL of the paste were applied to the base of a leaf using a small spatula; 20 µL of pure lanolin were applied as controls.

### Generation of transformed plants

To create *NaMPK4*-silenced plants, a 344 bp fragment of the *NaMPK4* gene was inserted into the pRESC5 transformation vector in an inverted repeat orientation (primer sequences are listed in Supplemental Table 1) to form pRESC5-MPK4. *Agrobacterium tumefaciens* harboring this vector was used for transforming *N. attenuata* (Krügel et al., 2002). The number of T-DNA

insertions was determined by Southern hybridization of genomic DNA using a PCR fragment of the *hygromycin phosphotransferase* (*hptII*) gene as a probe. Two T2 lines with single T-DNA insertions were identified and used in subsequent experiments. Crossing irCOI1, NahG, and irNPR1 with irNaMPK4 plants was done by removing anthers from flowers of irCOI1, NahG and irNPR1 plants before pollen maturation and pollinating the stigmas with pollen from irNaMPK4 plants. The transcript levels of *COI1*, *NahG*, *NPR1*, and *NaMPK4* were examined in the respective heterozygote descendents to confirm successful crossing.

**Southern blotting**

Genomic DNA samples were extracted from young leaf tissue using the CTAB method (Doyle and Doyle 1987). After overnight digestion with various endonucleases (Fermentas, Ontario, Canada), 6 μg of each digested DNA was loaded onto a 1% agarose gel and separated in TAE buffer. After being Southern blotted onto a GeneScreen Plus Hybridization Transfer membrane (PerkinElmer Life and Analytical Sciences), DNA was subsequently immobilized by UV crosslinking. Ten ng of DNA probe was labeled with α-$^{32}$P-dCTP (PerkinElmer Life and Analytical Sciences) using a random primer labeling kit (Amershambiosciences, Uppsala, Sweden), followed by purification through Probequant G-50 spin columns (Amershambiosciences). Membranes were prehybridized with ULTRAhyb hybridization buffer (Ambion, Austin, TX) for 1 h. Radioactive labeled probes were denatured at 95 °C for 5 min and quickly chilled on ice for another 5min before being added to the buffer. After overnight hybridization, membranes were washed at 62 °C once with 2 × SSC, 0.1% SDS, and three times with 0.1 × SSC, 0.1% SDS. Imaging was conducted on a FLA-3000 Phosphorimage system (Fujifilm Fuji Photo Film Europe, Düsseldorf, Germany).

**RNA extraction and quantitative real-time PCR (qPCR)**

Total RNA was extracted from ground leaf samples using TRIzol reagent (Invitrogen) following the manufacturer's instructions. For qPCR analysis, 5 replicated biological samples were used. 0.5 μg of total RNA sample was reverse-transcribed with oligo(dT) and Superscript II reverse transcriptase (Invitrogen). qPCR was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems) using qPCR Core kits (Eurogentec). An *N. attenuata actin2* gene was employed as the internal standard for normalizing cDNA concentration variations. Sequences of primers used for qPCR are listed in Supplemental Table 1.

**Measurement of photosynthetic rates, transpiration rates, stomatal conductance, chlorophyll contents, and quantification of rates of water losses in detached leaves**

Photosynthetic rates, transpiration values, and stomatal conductance were measured using a LI-COR 6400 Portable Photosynthesis System (Li-COR Biosciences). A light-responsive curve of photosynthesis was generated from at least 5 replicated plants, each at ambient $CO_2$ concentration (400 μmol $mol^{-1}$) and 6 different light intensities: 0, 200, 500, 1,000, 1500, and 2000 μmol $m^{-2}$ $s^{-1}$. Photosynthesis rates were also measured at 6 different $CO_2$ concentrations, 0, 200, 400, 600, 800, 1000 μmol $mol^{-1}$ at the light irradiance of 1200 $\mu mol^{-2}$ $s^{-1}$, using at least 5 replicated plants for each $CO_2$ concentration. Chlorophyll contents were determined using a portable chlorophyll meter (Minolta SPAD-502, Konica Minolta). Leaves at -1 position when plants were 30 days old were marked and used for all chlorophyll content measurements.

To determine darkness-induced changes in transpiration rates, rosette-stage plants were transferred to growth chambers (Snijders Scientific) and were kept for 4 h at 26 °C, 65% humidity, and under light intensity of 300 μmol $m^{-2}$ $s^{-1}$. Immediately after ights were switched off, the transpiration rates were measured with a LI-COR 6400 Portable Photosynthesis System.

For measurements of water loss rates in detached leaves, under ambient glasshouse conditions excised leaves were placed on a bench with abaxial sides facing up, and their masses were measured after various times. For ABA treatment, detached leaves were first incubated in a 20 μM ABA solution for 8 h prior the air drying treatment; leaves incubated in water (0.02% ethanol, the solvent for the 20 μM ABA) served as controls. Water losses were expressed as the percentage of initial fresh masses after excision.

**Stomatal assays in epidermal peels**

For all assays of stomatal apertures, fully expanded young leaves from 4-5-weels-old plants were used. Epidermal strips were peeled from the abaxial side of the leaves and were immediately placed in opening solution (50 mM KCl, 10 mM MES-KOH, pH 6.15) with the adaxial side upward. Stomatal opening was induced with white light illumination (300 μmol $m^{-2}$ $s^{-1}$) for 3 h. The epidermal strips were examined under a microscope (Leica LMD6000) equipped with a CCD camera and a workstation to determine the apertures of the stomatal pores at different times after application of 2 μM ABA or 100 μM $H_2O_2$ (Sigma).

**Analysis of ABA, SA, JA, and JA-Ile concentrations**

One milliliter of ethyl acetate spiked with 200 ng of $D_2$-JA and 40 ng of $D_6$-ABA, $^{13}C_6$-JA-Ile, and $D_4$-SA, the internal standards for JA, ABA, JA-Ile, and SA, respectively, was added to each briefly crushed leaf sample (~ 150 mg). Samples were then ground on a FastPrep homogenizer (Thermo Electron). After being centrifuged at 16.100 g for 10 min at 4 ºC, supernatants were transferred to fresh tubes and evaporated to dryness on a vacuum concentrator (Eppendorf). Each residue was resuspended in 0.5 mL of 70% methanol (v/v) and centrifuged to remove particles. The supernatants were analyzed on a HPLC-MS/MS (1200L LC-MS system, Varian) (Wu et al., 2007).

**Pathogen assays**

*Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) was grown at 28 °C in LB (Luria-Bertani) liquid medium with antibiotics until $OD_{600}$ was ~ 0.6. After 10 min centrifugation at 3000 g, the supernatant was discarded, and cells were resuspended to O.D. = 0.8 (~ $4 \times 10^8$ cfu/mL) in a 10 mM $MgCl_2$ solution without any detergents. To quantify stomata closure in response to *Pst* DC3000, leaves were dipped into the bacterial solution and transpiration rates were measured using a LI-COR 6400 Portable Photosynthesis System (Li-COR Biosciences). To evaluate pathogen growth, leaves were surface sterilized [70% ethanol for 1 min, sodium hypochlorite solution (3% available $Cl_2$) containing 0.005% Tween-20 for 3 min, then rinsed with sterile water] and leaf discs (4 $cm^2$) were ground in 0.3 mL of sterile water, and a series of dilutions of each leaf extract were spread on LB agar plates containing antibiotics. Plates were incubated at 28 $^oC$ until colonies in appropriate sizes appeared and colonies number were counted.

To examine the growth of intercellularly located *Pst* DC3000, leaves were inoculated with a *Pst* DC3000 suspension of O.D. 0.001 using 1 mL syringes. Mock inoculation was done by infiltrating 10 mM $MgCl_2$ solution. Pathogen growth was quantified using above mentioned method without sterilizing the surfaces.

**Salt treatment, germination and seedling root elongation assay**

To examine plant resistance to salt, rosette WT and irNaMPK4 plants were grown in 0.5-L pots. When plants reached rosette stage (30 days after germination), 80 mL of 50, 100, or 200 mM NaCl solution were given to plants each day.

To avoid other factors that affect seed germination rates, *N. attenuata* WT and irNaMPK4 plants were grown simultaneously. Mature progeny seeds were harvested at the same time and used for subsequent phenotypic comparisons. Seeds were sown on 0.6% agar media containing 1 × Gamborg's B5 (GB5) salts (Duchefa) and various chemicals as specified in the Results section, and then incubated at 26°C with a 16-hr-light photoperiod. Germination was quantified by radicle emergence from triplicates with 100 seeds each.

Seeds were germinated on 0.6% agar medium containing 1 × GB5. Five days after sowing, when roots were about 3-5 mm, seedlings were transferred to the same medium with different chemicals described in the Results section. Seedlings were grown vertically at 26°C with a 16-hr-light photoperiod.

**In-gel kinase activity assay and immune-complex kinase activity assay**

Leaf tissue pooled from 4 replicate leaves was crushed in liquid nitrogen, and 200 µL of protein extraction buffer [100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM $Na_3VO_4$, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl floride, 10% glycerol, and EDTA-free proteinase inhibitor cocktail (Roche Diagnostics)] was added to ~ 100 mg of tissue. Leaf tissue was then completely suspended by vortexing. After being centrifuged at 4°C at 16.100 g for 20 min, supernatants were transferred to fresh tubes. Protein concentrations were measured using a Bio-Rad protein assay kit with BSA as a standard. MAPK in-gel kinase activity assay were done following Zhang & Klessig (1997) using myelin basic protein (MBP) as the substrate. Gel images were obtained on an FLA-3000 phosphor imager system (Fujifilm).

For immune-complex kinase activity assays we followed Zhang & Liu (2001) with minor modifications. Protein from pooled samples of 4 replicated leaves was extracted with immunoprecipitation buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM $Na_3VO_4$, 1 mM NaF, 10 mM β-glycerophosphate, EDTA-free proteinase inhibitor cocktail (Roche Diagnostics), and 0.1% Tween 20]. Protein extracts (100 µg in 150 µL volume) were incubated with 2 µg of anti-NtMPK4 antibody at 4 °C for 1.5 h on a rocker. 20 µL (packed volume) of protein A-agarose (Sigma) were washed in immunoprecipitation buffer and was then added to the reaction. After another 2 h of incubation on a rocker at 4 °C, agarose bead-protein complexes were washed twice with 0.5 mL of immunoprecipitation buffer, and twice with 0.5 mL of kinase reaction buffer (25 mM Tris-HCl, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM

DTT, and 0.1mM $Na_3VO_4$). Kinase activity in the complex was assayed at room temperature for 20 min in 25 μL of kinase reaction buffer containing 0.1 mg $mL^{-1}$ MBP, 10 μM ATP, and 3 μCi of $\gamma$-$^{32}$P-ATP. The reaction was stopped by the addition of SDS-PAGE sample loading buffer. After electrophoresis on a 12% SDS-polyacrylamide gel, the phosphorylated MBP was visualized on an FLA-3000 phosphor imager system (Fujifilm).

**Immunoblotting analysis**

Four biologically replicated samples were pooled for protein extraction using the protein extraction buffer. Protein samples (15 μg) were separated in a 10% SDS-PAGE gel and electrotransferred to a PVDF membrane (Amersham). A WesternBreeze Chemiluminescent Immunodetection Kit (Invitrogen) was used to detect NaMPK4 protein. The primary antibody, anti-NtMPK4, was 1:2000 diluted for immunoblotting analysis. For examining equal loading, a duplicated gel was run at the same time and was subsequently stained using the GelCode Blue Safe Stain reagent (Thermo Scientific) to visualize proteins.

**Herbivore growth bioassays**

Freshly hatched *M. sexta* larvae were placed on 30 replicated plants of each genotype (1 larva/plant). To compare herbivore growth rates on WT and irNaMPK4 plants, larvae were weighed on day 4, 7, 9 and 11; for the comparison of herbivore growth on WT, irNaMPK4, irCOI1 and 119×irCOI1 plants, larval mass on day 4, 6, and 8 was recorded. In the experiment wherein *M. sexta* larvae were transferred to non-treated plants after every 24 h to minimize JA-induced defenses, 9 WT and irNaMPK4 plants were infested with 18 *M. sexta* larvae (2 larvae/plant). After each 24 h, all larvae were moved to new untreated plants of the same genotype. The larval mass was recorded every other day. The amount of leaf material consumed after the first day of neonate feeding was estimated by scanning the infested leaves to obtain digital photos and calculating the consumed areas with SigmaScan Pro software (Statcon). To measure *S. littoralis* performance, freshly hatched larvae were grown on artificial diet for 10 d and then placed on 20 replicate plants of each genotype. Larval mass gain (means ± SE) was measured 3, 5, 7, and 9 days after transfer to the plants. For the collection of OS, larvae were reared on *N. attenuata* wild-type plants until the third to fifth instar. OS was collected on ice as described in Roda et al. (2004).

**FAC Analysis**

Five microliters of *M. sexta* and *S. littoralis* OS were homogenized in 95 mL methanol spiked with 10 ng of N-cis-10-nonadecenoic acid-L-Gln (C19:1-Gln) used as internal standard. Extracts were then centrifuged to remove any particulate matter. Five-microliter aliquots of these solutions were analyzed by liquid chromatography-tandem mass spectrometry using the aforementioned LC settings. Identification of major FACs was confirmed by comparison to authentic standards, as described in Halitschke et al. (2001) and Diezel et al. (2009).

**Analyses of herbivore defense-related secondary metabolites**

Trypsin proteinase inhibitor activity was analyzed with a radial diffusion assay described by van Dam et al. (2001). The accumulation of the direct defenses, nicotine, caffeoylputrescine, and diterpene glycosides were analyzed in samples harvested 3 days after W+W, W+OS, lanolin, or MeJA treatment using a HPLC method described in Keinanen et al. (2001). To quantify W+W- and W+OS-elicited green leaf volatile emissions, treated leaves were immediately enclosed between two 50 mL food-quality plastic containers (Huhtamaki) secured with miniature claw-style hair clips. Ambient air was pulled through the collection chamber and a glass tube (ARS, Inc.) packed with glass wool and 20 mg of Super Q (Alltech). After 3 h of collection, traps were spiked with 400 ng of tetralin (Sigma-Aldrich) as an internal standard and eluted with 250 µL of dichloromethane. For collection of MeJA- and W+OS-elicited terpene emissions, 24 h after treatment emitted volatiles were collected similarly for 8 h. The eluted compounds were analyzed on a GC-MS (GC-MS 4000, Varian).

**Analysis of ethylene emissions**

Six replicated measurements were used to quantify ethylene production in WT and irNaMPK4 plants. Three leaves were treated with W+OS and immediately afterward sealed in a three-neck 250-mL flask and kept in the greenhouse for 5 h. The ethylene concentration in the headspace was measured with a photoacoustic laser spectrometer (Invivo).

**Analysis of starch levels**

Starch levels were estimated in 10 replicate fully expanded rosette leaves from WT and both irNaMPK4 lines using the Anthrone Method. Soluble sugars were removed with 80%

ethanol and after extraction with perchloric acid; samples were boiled for 8 minutes with anthrone reagent (100mg anthrone in 100mL 95% $H_2SO_4$).

**Statistical analysis**

Data were analyzed by analysis of variance (ANOVA) or unpaired *t*-test using StatView, version 5.0 (SAS Institute).

**Phylogeny analysis**

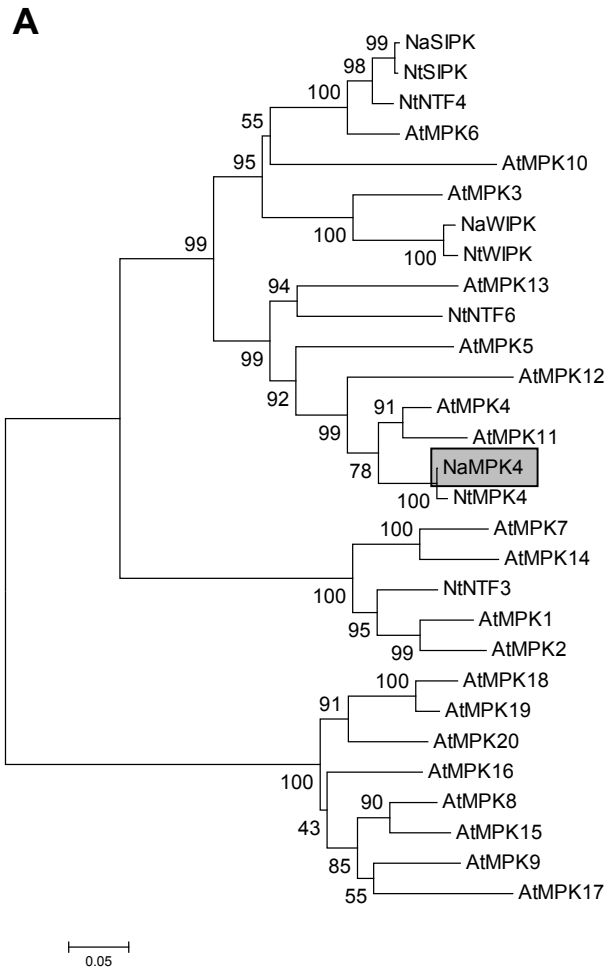
Protein sequences were retrieved from the GenBank and were aligned using the Clustal W algorithm embedded in the MegAlign software (DNASTAR Lasergene 8). The protein tree was constructed by MEGA 4 software (1000 replications) (Tamura et al., 2007).

The sequence of NaMPK4 has been deposited in the GenBank under accession number HQ236013.

# 3. Results

## 3.1. Silencing *NaMPK4* in *N. attenuata*

Using the sequence information of tobacco *NtMPK4* (Gomi et al., 2005), we cloned *NaMPK4* in *N. attenuata* (GenBank accession number: HQ236013). Phylogenetic analysis indicated that NaMPK4 clustered most closely with NtMPK4, AtMPK4, and AtMPK11 and was more distantly related to AtMPK12 (Figure 3A). It showed 98% sequence identity with *N. tabacum* NtMPK4 and 87, 85, and 77% similarity to AtMPK4, AtMPK11, and AtMPK12, respectively (Figure 3B). Furthermore, searching GenBank of *N. tabacum* EST database and analyzing a *N. attenuata* transcriptome database prepared by 454 sequencing did not reveal any further close homologue of NaMPK4 in *N. tabacum* and *N. attenuata*. Quantitative real-time PCR (qPCR) analysis indicated that NaMPK4 is expressed in all organs examined, i.e. roots, stems, flowers, and leaves (Figure 3C).



**B**

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AtMPK4    MSAESCFGSSGDQSSSKGVATHGGSYVQYNVYGNLFEVSRKYVPPLRPIGRGAYGIVCAATNSETGEEVA
AtMPK11   MSIEKPF--FGDDSN-RGVSINGGRYVQYNVYGNLFEVSKKYVPPLRPIGRGASGIVCAAWNSETGEEVA
AtMPK12   MSGESSS--GSTEHCIKVVPTHGGRYVQYNVYGQLFEVSRKYVPPIRPIGRGACGIVCAAVNSVTGEKVA
NtMPK4    MEAISGD--QGVQSNFKGVPTHGGRYAQYNVYGNLFEVSKKYVPILRPVGRGAYGIVCAAMNSETREEVA
NaMPK4    MEASSGD--QGVQSNFKGIPTHGGRYVQYNVYGNLFEVSKKYVPILRPVGRGAYGIVCAAMNSETREEVA

AtMPK4    IKKIGNAFDNIIDAKRTLREIKLLKHMDHENVIAVKDIIKPPQRENFNDVYIVYELMDTDLHQIIRSNQP
AtMPK11   IKKIGNAFGNIIDAKRTLREIKLLKHMDHDNVIAILDIIRPPQPDNFNDVHIVYELMDTDLHHIIRSNQP
AtMPK12   IKKIGNAFDNIIDAKRTLREIKLLRHMDHENVITLKDIVRPPQRDIFNDVYIVYELMDTDLQRILRSNQT
NtMPK4    IKKIGNAFDNRIDAKRTLREIKLLRHMDHDNVIATKDIIRPPQTENFNDVYIVYELMDTDLHQIIRSNQQ
NaMPK4    IKKIGNAFDNRIDAKRTLREIKLLRHMDHDNVIAIKDIIRPPQTENFNDVYIVYELMDTDLHQIIRSNQQ

AtMPK4    LTDDHCRFFLYQLLRGLKYVHSANVLHRDLKPSNLLLNANCDLKLGDFGLARTKSETDFMTEYVVTRWYR
AtMPK11   LTDDHSRFFLYQLLRGLKYVHSANVLHRDLKPSNLLLNANCDLKIGDFGLARTKSETDFMTEYVVTRWYR
AtMPK12   LTSDQCRFLVYQLLRGLKYVHSANILHRDLKPSNVLLNSKNELKIGDFGLARTTSDTDFMTEYVVTRWYR
NtMPK4    LTDDHCRYFLYQILRGLKYIHSANVLHRDLKPSNLFLNANCDLKVGDFGLARTTSETDFMTEYVVTRWYR
NaMPK4    LTDDHCRYFLYQILRGLKYIHSANVLHRDLKPSNLLLNANCDLKVGDFGLARTTSETDFMTEYVVTRWYR

AtMPK4    APELLLNCSEYTAAIDIWSVGCILGETMTREPLFPGKDYVHQLRLITELIGSPDDSSLGFLRSDNARRYV
AtMPK11   APELLLNCSEYTAAIDIWSVGCILGEIMTREPLFPGRDYVQQLRLITELIGSPDDSSLGFLRSDNARRYV
AtMPK12   APELLLNCSEYTAAIDIWSVGCILGEIMTGQPLFPGKDYVHQLRLITELVGSPDNSSLGFLRSDNARRYV
NtMPK4    APELLLNCSEYTAAIDIWSVGCILGEMMTRQPLFPGKDYVHQLKLITELIGSPDDASLGFLRSDNARRYV
NaMPK4    APELLLNCSEYTAAIDIWSVGCILGEMMTRQPLFPGKDYVHQLRLITELIGSPDDASLGFLRSDNARRYV

AtMPK4    RQLPQYPRQNFAARFPNMSAGAVDLLEKMLVFDPSRRITVDEALCHPYLAPLHDINEEPVCVRPFNFDFE
AtMPK11   RQLPQYPRQNFAARFPNMSVNAVDLLQKMLVFDPNRRITVDEALCHPYLAPLHEYNEEPVCVRPFHFDFE
AtMPK12   RQLPRYPKQQFAARFPKMPTTAIDLLERMLVFDPNRRISVDEALGHAYLSPHHDVAKEPVCSTPFSFDFE
NtMPK4    RQLPQYPRQQFAARFPNSSPGAVDLLEKMLVFDPSRRVTVDQALCHPYLAPLHDINEEPICPKPFSFDFE
NaMPK4    RQLPQYPRQQFAARFPNSSPGAVDLLEKMLVFDPSRRVTVDQALCHPYLAPLHDINEEPICPKPFSFDFE

AtMPK4    QPTLTEENIKELIYRETVKFNPQDSV.
AtMPK11   QPSLTEENIKELIYRESVKFNPL
AtMPK12   HPSCTEEHIKELIYKESVKFNPDH.
NtMPK4    QPSFTEENIKELIWRESVKFNPDPTH.
NaMPK4    QPSFTEENIKELIWRESVKFNPDPTH.
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**C**

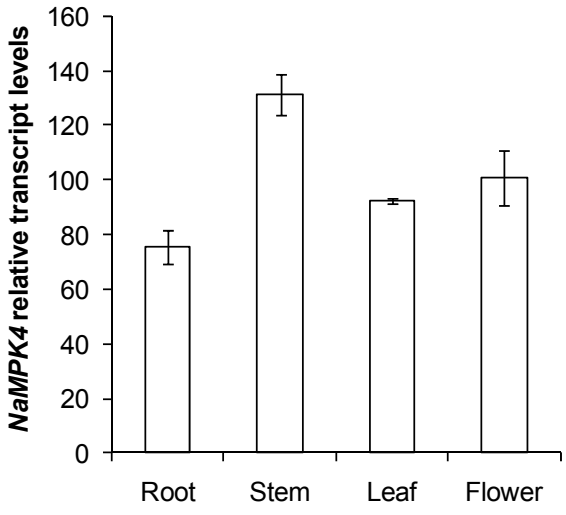



**Figure 3.** NaMPK4 is closely related to Arabidopsis AtMPK4, AtMPK11, AtMPK12, and tobacco NtMPK4 and is expressed in various organs.

**(A)** Phylogenetic relationship of MAPK proteins. Protein sequences were retrieved from GenBank and aligned using the Clustal W method. Non-rooted Neighbor-Joining tree and bootstrap values were constructed with the MEGA 4 software. NaMPK4 is highlighted in grey. **(B)** Alignment of the protein sequences of NaMPK4, NtMPK4, AtMPK4, AtMPK11, and

AtMPK12. Protein sequences were deduced from retrieved mRNA sequences of *NaMPK4*, *NtMPK4*, *AtMPK4*, *AtMPK11*, and *AtMPK12*. Sequences were aligned using the Clustal W algorithm. Black background indicates amino acid residues that are different from the consensus sequence. **(C)** *NaMPK4* transcript levels in root, stem, leaf, and flower of *N. attenuata*.

A 344 bp fragment of *NaMPK4* was cloned into a binary vector pRESC5 in an inverted repeat fashion, and *Agrobacterium* carrying this vector was further used to transform *N. attenuata* to obtain *NaMPK4*-silenced plants (irNaMPK4 plants) (Krügel et al., 2002). Several independently transformed lines of irNaMPK4 plants which harbor single transgene insertion were identified by Southern blotting (Figure 4A), and the transcript levels of *NaMPK4* in these lines were analyzed by qPCR. Two lines, line 119 and 163, whose transcript levels of *NaMPK4* were 9.8% and 5.4% of those of wild-type (WT) plants (Figure 4B), were selected for further studies.

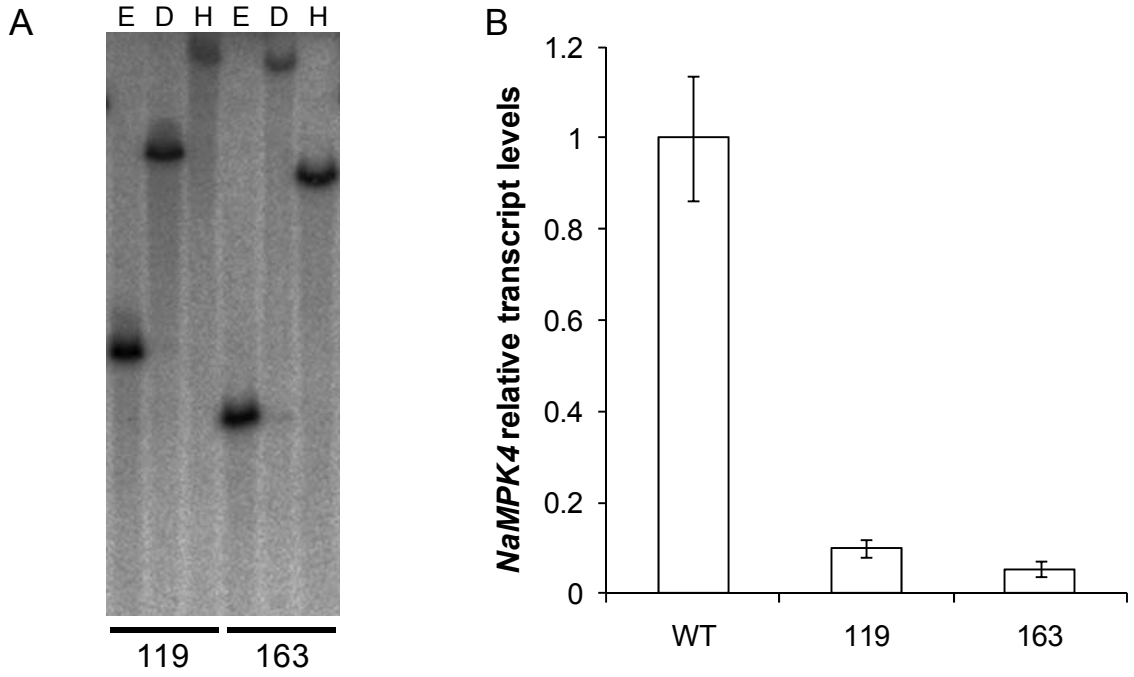



**Figure 4.** Southern blotting analysis and NaMPK4 transcript accumulation in irNaMPK4 plants. **(A)** Genomic DNA was digested with EcoRI (lane E), DraI (lane D) and HindIII (lane H) and Southern-blotted onto nylon membranes. The membrane was hybridized with a hygromycin-specific probe. **(B)** Transcript levels (mean ± SE; N = 5) of NaMPK4 were analyzed in untreated wild-type (WT) and irNaMPK4 plants (line 119 and 163) by qPCR.

## 3.2. NaMPK4 and plant development

### 3.2.1. NaMPK4 is involved in plant growth and senescence processes

In *N. tabacum*, *NtMPK4*-silenced plants have somewhat retarded growth, although not as severe as that of Arabidopsis *mpk4* mutant (Gomi et al., 2005). To examine the function of NaMPK4 in mediating *N. attenuata*'s development, we measured rosette size and stalk length until plants finished their reproductive growth. The average rosette size of irNaMPK4 plants was only slightly reduced [95% of that of WT plants ($P_{119} < 0.0005$ and $P_{163} = 0.0015$)] 38 days after germination, when plants had slightly elongated stems; greater difference appeared at later developmental stages: by day 57, rosette sizes of irNaMPK4 lines were 85% of those of WT plants (Figure 5A). Stalk lengths of irNaMPK4 plants were also reduced. At the end of reproductive growth (83 days after germination), irNaMPK4 plants were 16% shorter than WT plants ($P_{119, 163} < 0.001$) (Figure 5B). All these changes resulted in a net 27% reduction in above-ground biomass (Figure 5C). Arabidopsis knock-out mutant *mpk4*, which is a close homologue of NaMPK4, shows severely impaired growth, and over-accumulation of SA partly account for its arrested development (Petersen et al., 2000). However, the contents of SA irNaMPK4 were not higher than those of WT plants in all examined developmental stages (Figure 5D). Importantly, irNaMPK4 plants exhibited elevated chlorophyll contents and delayed senescence. In the early rosette stage (30-day old), irNaMPK4 plants had slightly more chlorophyll than did WT plants (Figure 5E). This difference became more pronounced at the later developmental stages when the decline in chlorophyll contents in WT plants was more rapid than that of irNaMPK4 plants: by day 56, irNaMPK4 plants had 30% more chlorophyll in their leaves than did WT plants and showed an obvious slower senescence (Figure 5E). Thus NaMPK4 appears to negatively regulate plant senescence.

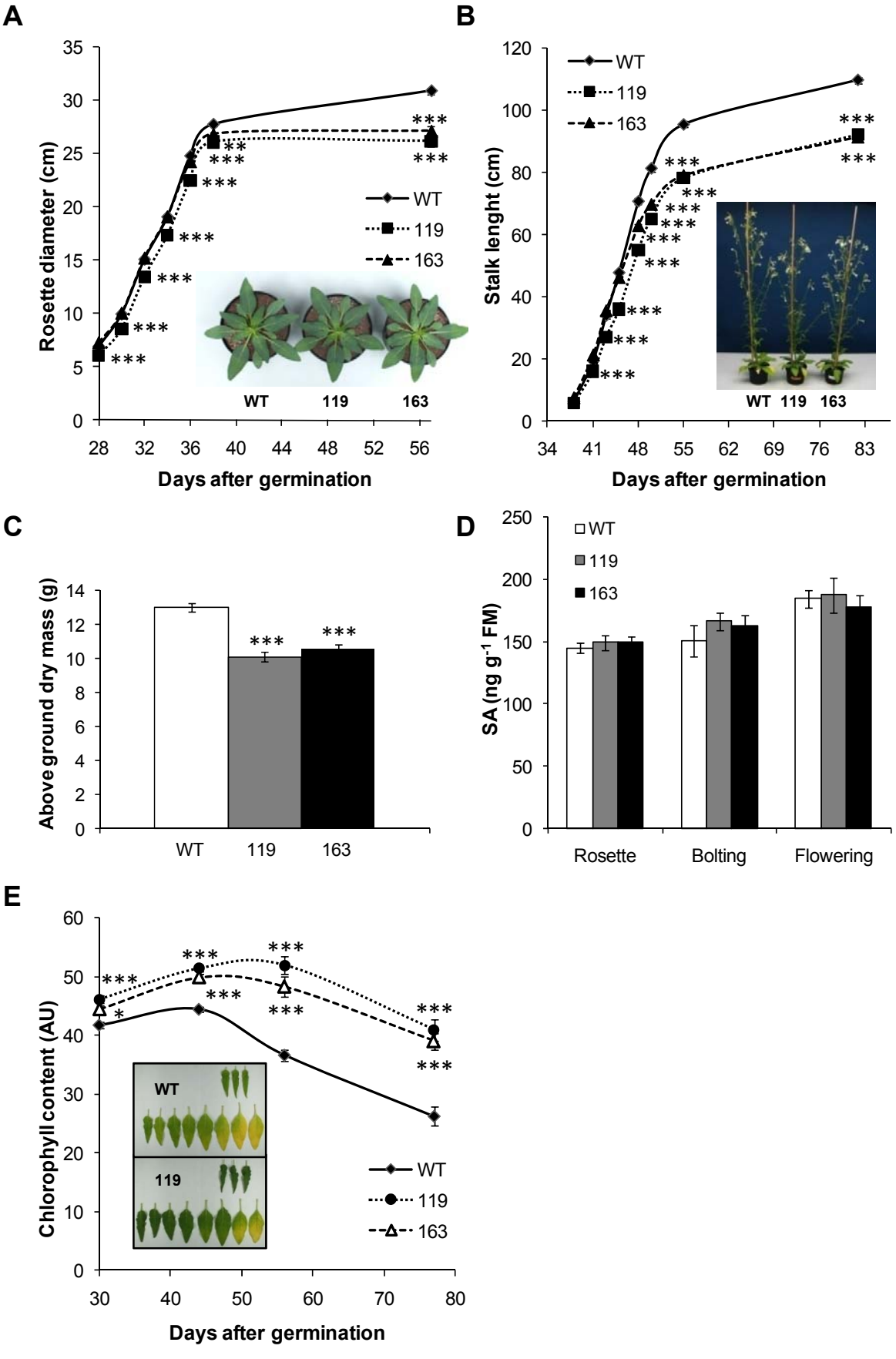

A
Rosette diameter (cm)
Days after germination
WT
119
163
B
Stalk lenght (cm)
Days after germination
C
Above ground dry mass (g)
D
SA (ng g-1 FM)
Rosette
Bolting
Flowering
E
Chlorophyll content (AU)
Days after germination

**Figure 5.** Silencing *NaMPK4* decreases plant stature and delays senescence.

All plants were germinated in Petri dishes to synchronize germination, 10 days after germination plants were transferred to soil. **(A)** Rosette sizes of wild-type (WT) and irNaMPK4 plants (line 119 and 163). Rosette diameters were measured on 30 individual plants from each genotype. Inset depicts rosette-stage WT and irNaMPK4 plants (30 days after germination). **(B)** Stalk length of WT and irNaMPK4 plants (line 119 and 163). Data were obtained from 30 individual plants from WT, 119, and 163. Inset depicts plants at flowering stage (55 days after germination). **(C)** Silencing NaMPK4 reduces plant above-ground biomass. Wild-type (WT) and irNaMPK4 plants (line 119 and 163) were grown in a glasshouse for 120 days, when they finished their reproductive stage. Plant shoots were harvested and dried for 3 days in an 80 °C oven, and the dry mass of samples was measured (N=15; mean ± SE). **(D)** SA levels in rosette leaves of 8 wild-type (WT) and irNaMPK4 plants (lines 119 and 163) at rosette stage (30 days old), bolting stage (40 days old), and flowering stage (50 days old). **(E)** Relative chlorophyll contents (AU: arbitrary units) of wild-type (WT) and irNaMPK4 (lines 119 and 163) plants. Values (mean ± SE) were obtained from the rosette leaves at an identical leaf position (+1 position) of 10 replicated plants. Inset depicts WT and irNaMPK4 rosette leaves 56 days after germination. Note that leaves of irNaMPK4 plants are greener due to their higher chlorophyll contents. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$;**, $P < 0.01$; ***, $P < 0.001$).

### 3.2.2. NaMPK4 controls photosynthetic rates and fitness under glasshouse conditions

In *N. tabacum*, silencing *NtMPK4* leads to enlarged stomata, increased stomatal conductance, and impaired stomatal closure responses to ozone and elevated $CO_2$ concentrations (Gomi et al., 2005; Marten et al., 2008). Consistently, the stomatal conductance of irNaMPK4 plants was 2 times greater than that of WT plants (Figure 6A). As expected, irNaMPK4 plants had higher photosynthetic rates than WT when plants were supplied with increasing concentrations of $CO_2$ (Figure 6B inset). To confirm that these differences were only attributed to changes in stomatal conductance and not caused by, for instance, the amount of RuBPCase and it $V_{max}$, the photosynthesis rates of WT and irNaMPK4 plants were blotted against the intracellular $CO_2$ levels ($C_i$) (Figure 6B). The obtained $A/C_i$ slopes are almost identical for WT and both silenced lines but differ at higher $CO_2$ levels, most likely because of the higher chlorophyll content and thus increased photosynthetic capacity of irNaMPK4 plants. At the rosette stage (30 days after germination), when irNaMPK4 plants had only slightly higher chlorophyll contents than did WT, irNaMPK4 plants showed greater photosynthetic rates as the light intensity increased under ambient $CO_2$ concentration (400 μmol $mol^{-1}$) (Figure 6C). Fifty-six days after germination, when plants produced their first seed capsules and the differences in chlorophyll contents between irNaMPK4 and WT plants were starker (Figure 5D), irNaMPK4 plants had

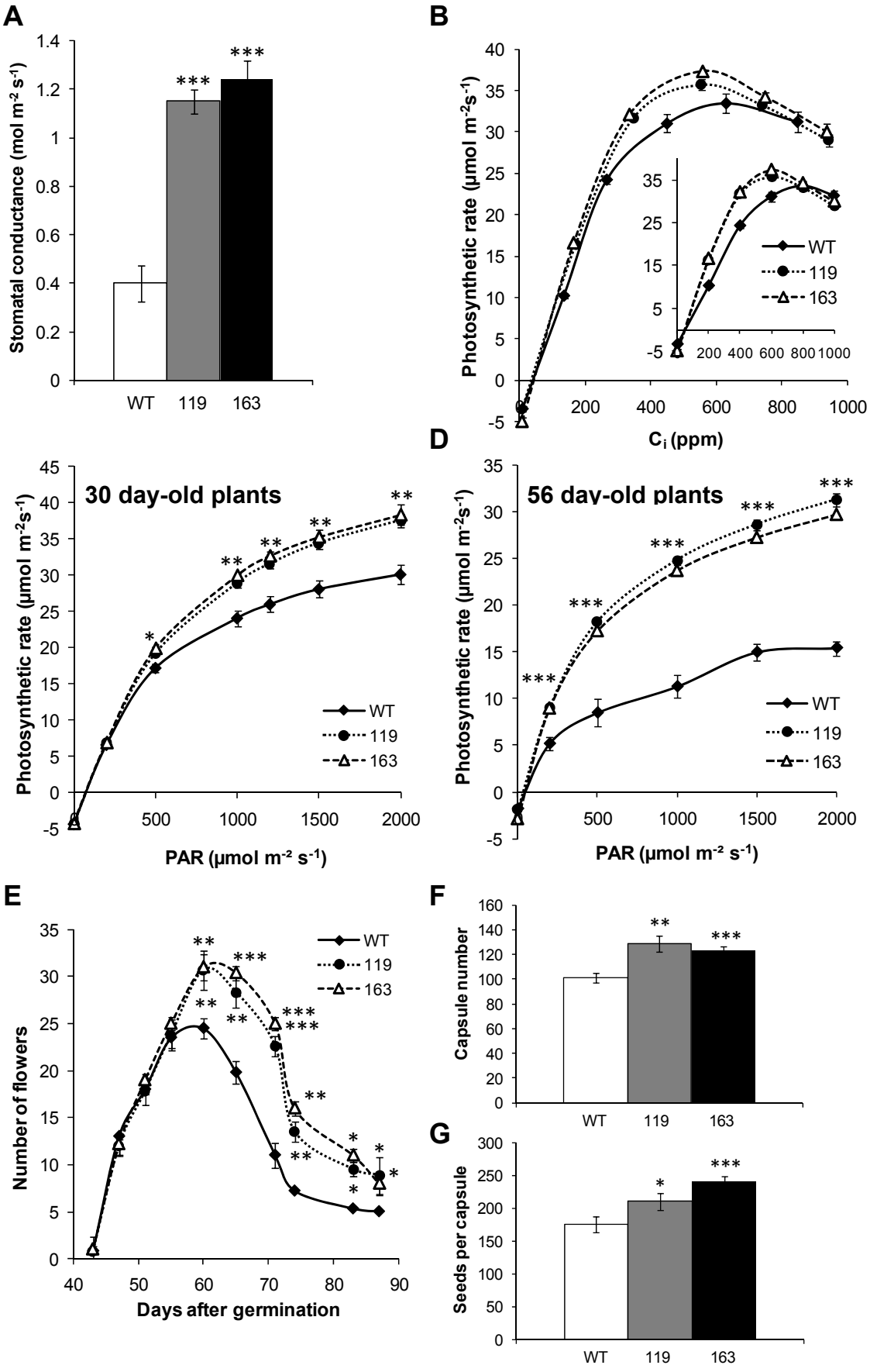

A
Stomatal conductance (mol m-2 s-1)
WT
119
163
B
Photosynthetic rate (µmol m-2s-1)
Ci (ppm)
WT
119
163
C
30 day-old plants
Photosynthetic rate (µmol m-2s-1)
PAR (µmol m-2 s-1)
D
56 day-old plants
Photosynthetic rate (µmol m-2s-1)
PAR (µmol m-2 s-1)
E
Number of flowers
Days after germination
F
Capsule number
G
Seeds per capsule

**Figure 6.** Silencing *NaMPK4* increases stomatal conductance, photosynthetic rates, and reproductive output.

Wild-type (WT) and irNaMPK4 (line 119 and 163) plants were grown concurrently. **(A)** Stomatal conductance (mean ± SE; N = 10) were measured from leaves at +1 positions of 30 day-old plants. **(B)** Photosynthetic rates (mean ± SE; N = 6) of plants supplied with different $CO_2$ concentrations (0, 200, 400, 600, 800, and 1000 μmol $mol^{-1}$) under the light irradiance of 1200 $\mu mol^{-2}$ $s^{-1}$; values are photosynthetic rates relative to intracellular $CO_2$ concentration; inset: photosynthesis rates relative to $CO_2$ concentrations in the sample cell. **(C)** and **(D)** Photosynthetic rates (mean ± SE; N = 7) are higher in irNaMPK4 plants than in WT plants under the ambient $CO_2$ concentration. Plants were supplied with indicated light intensities and with ambient $CO_2$ concentration (400 μmol $mol^{-1}$), and 7 replicate plants were used to obtain the mean photosynthetic rates (± SE) in 30 day-old (C) and 56 day-old plants **(D)**. **(E)** and **(F)** Silencing *NaMPK4* increases the number (mean ± SE; N = 15) of flowers **(E)** and seed capsules **(F)**. **(G)** Seed numbers (mean ± SE) per capsule of WT and irNaMPK4 plants. Seeds in 16 capsules of WT and irNaMPK4 lines (65 day-old) were counted. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

about 1 fold higher photosynthetic rates than did WT plants under almost all light intensities (Figure 6D). We conclude that silencing *NaMPK4* enhances the levels of photosynthesis activity in *N. attenuata*, especially at later stages in development.

Photosynthetic rates are often associated with increased biomass or seed yield (Long et al., 2006). To test whether the elevated photosynthetic rates were translated into greater reproductive output in *NaMPK4*-silenced plants, we examined the numbers of flowers and seed capsules produced in irNaMPK4 plants as well as the number of seeds per capsule. While the date of appearance of the first flower was similar and WT and irNaMPK4 plants had similar number of flowers in their early flowering stages, irNaMPK4 plants produced considerably more flowers (28% more) 60 days after germination (Figure 6E). Consistently, 20% higher total capsule numbers were observed in irNaMPK4 plants than in WT plants (Figure 6F). In addition, the number of seeds produced in the first capsules increased 20 to 35% in line 119 and 163, respectively (Figure 6G). Germination assays indicated identical viability between WT and irNaMPK4 seeds (data not shown). Therefore, silencing *NaMPK4* leads to considerably augmented photosynthetic rates and seed production under glasshouse conditions.

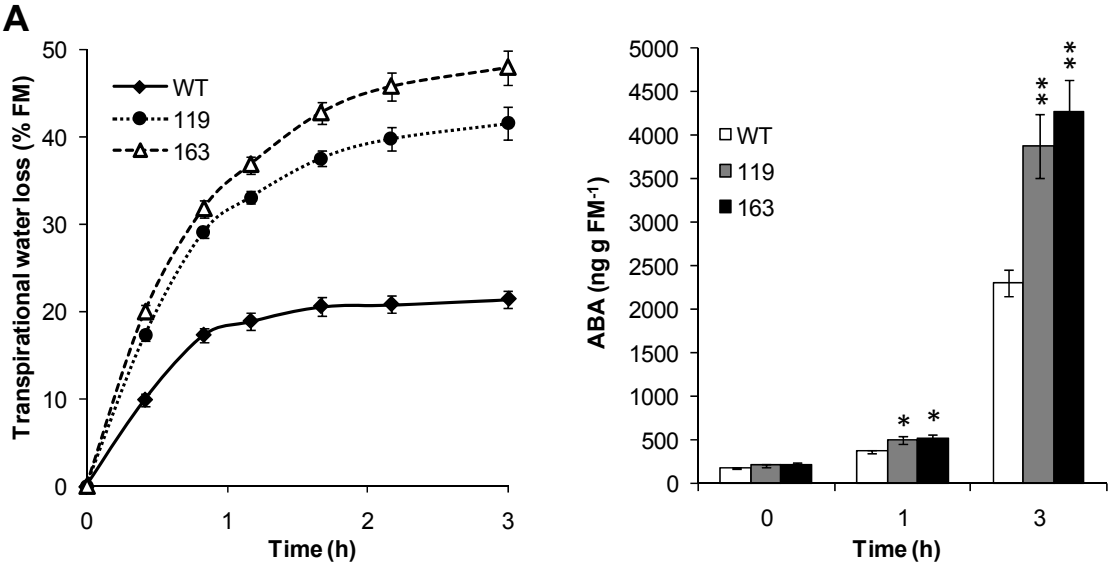
## 3.3. NaMPK4 regulates plants responses to abiotic stress

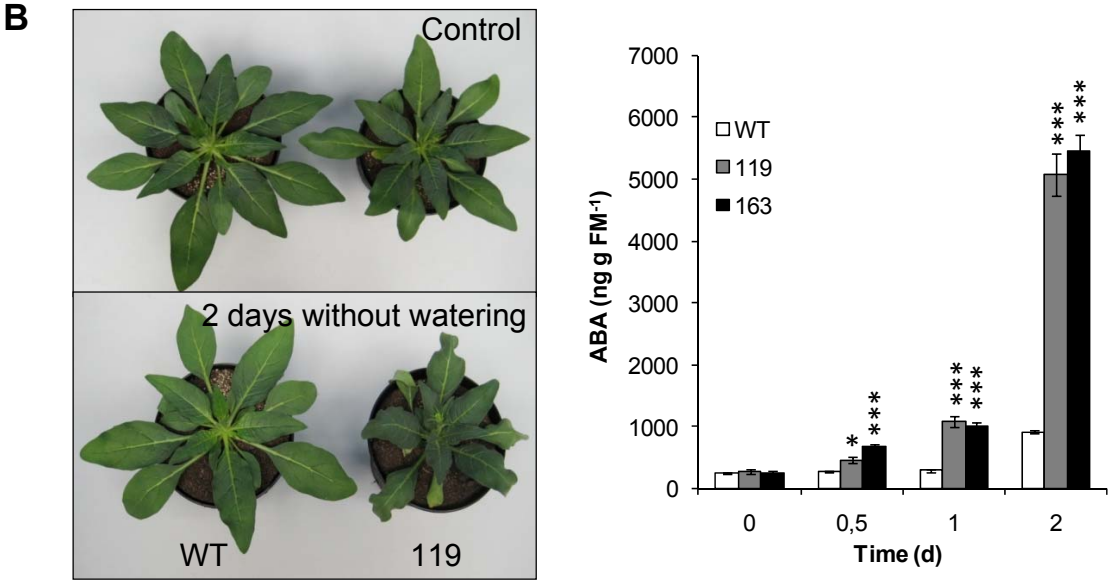
### 3.3.1. irNaMPK4 plants have highly impaired ABA-, $H_2O_2$-, and dark-induced stomatal closure responses

The highly increased stomatal conductance in irNaMPK4 suggested that NaMPK4 may be involved in the regulation of guard cell movement. To test this scenario, the response of irNaMPK4 plants to drought stress was examined.

Firstly, leaves were excised from well watered irNaMPK4 and WT and their masses were recorded over time. A rapid water loss was seen in WT leaves; however, by 50 min the masses of WT leaves were 17% reduced and similar masses were found even 3 h after excision, indicating that WT leaves closed their stomata in response to the initial water loss (Figure 7A). In contrast, irNaMPK4 leaves showed a more rapid decline of their masses: by 50 min water loss reached 30% and by 3 h these leaves had more than 40% water loss (Figure 7A). Given the critical role of ABA in stomatal closure response, the contents of ABA were measured in these detached leaves. No significant differences were found in freshly detached leaves [210 ng/g fresh mass (FM)]; however, in agreement with the degrees of water loss, after 1 h ABA contents in WT and irNaMPK4 leaves increased to 370 and 500 ng/g FM and by 3 h, 2300 ng/g FM ABA were seen in WT leaves and irNaMPK4 leaves had almost 1 fold higher amount of ABA (4000 ng/g FM) (Figure 7A). Furthermore, the ability to conserve water on a level of whole plant was examined. Plants were subjected to drought treatment by keeping plants under normal glasshouse conditions but without watering. Leaf turgor and ABA contents were monitored over time. One day after onset of drought treatment, neither WT nor irNaMPK4 showed an obvious wilting phenotype, but the ABA levels in irNaMPK4 increased 3 fold (1078 ng/g FM), whereas ABA contents in WT showed almost no changes (294 ng/g FM) (Figure 7B). By day 2, WT plants had relatively normal turgor, but irNaMPK4 were strongly wilted; consistently, ABA contents reached 920 ng/g FM in WT and 5200 ng/g FM of ABA were detected in irNaMPK4 (Figure 7B).

These data indicate that irNaMPK4 plants have normal accumulations of ABA in response to drought stress, but very likely NaMPK4 is required for ABA-induced stomatal closure. To further test this hypothesis, excised leaves of WT and irNaMPK4 were incubated in a 20 μM (a concentration that is close to the endogenous ABA levels in highly dehydrated leaves) ABA solution for 8 h and leaves were then were allowed to dry under ambient conditions. In line


A
Transpirational water loss (% FM)
WT
119
163
Time (h)
ABA (ng g FM-1)
WT
119
163
Time (h)


B
Control
2 days without watering
WT
119
ABA (ng g FM-1)
WT
119
163
Time (d)

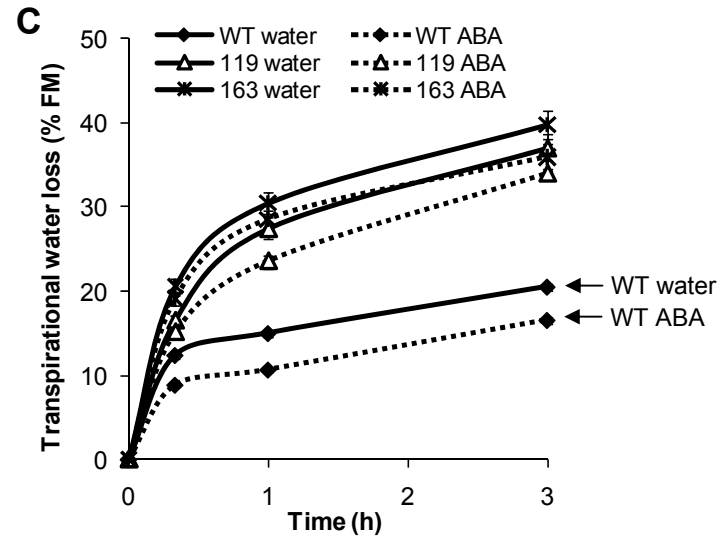

C
WT water
119 water
163 water
WT ABA
119 ABA
163 ABA
Transpirational water loss (% FM)
WT water
WT ABA
Time (h)

**Figure 7.** irNaMPK4 plants are highly susceptible to drought stress.

**(A)** Detached irNaMPK4 (lines 119 and 163) leaves loose water faster and accumulate more ABA than do detached wild-type (WT) leaves. WT and irNaMPK4 leaves were detached from plants and were kept abaxial sides up under the glasshouse conditions; the masses were recorded over time to calculate the percentages of transpirational water loss (left panel) and ABA contents (right panel) were quantified in similarly treated samples. All data represent mean ± SE (N = 8). **(B)** irNaMPK4 plants dehydrate faster and show higher ABA levels than do WT, after being deprived of water. Upper left panel: well-watered control plants; lower left panel: 2 days after plants were deprived of watering; right panel: ABA contents (mean ± SE; N = 8) in watering-deprived WT and irNaMPK4 plants. **(C)** Exogenously applying ABA to detached irNaMPK4 leaves only partially recovers its stomatal closure response to dehydration. Detached leaves of wild-type (WT) and irNaMPK4 (lines 119 and 163) plants were incubated in water containing ABA (20 μM) for 8 h. The water loss from 10 detached leaves was measured. Values are means ± SE. The experiment was repeated twice with similar results. FM = fresh mass.

with our hypothesis, exogenously applied ABA somewhat inhibited water loss from both WT and irNaMPK4 leaves; however, ABA treatment did not reduce the transpiration levels of irNaMPK4 leaves to those of WT (Figure 7C).

The closure response of stomata on WT and irNaMPK4 leaves was further measured using epidermal peels; this technique has been widely used in studying stomatal responses. Similar to the stomata in tobacco with defect in NtMPK4, irNaMPK4 have somewhat enlarged stomatal size and when not treated with ABA, the guard cells of irNaMPK4 showed greater apertures than did WT (Figure 8A and 8B). Importantly, fifteen minutes after application of 2 μM ABA, stomatal apertures of WT epidermal peels decreased 67%, while apertures of irNaMPK4 stomata only reduced 20% even after 1 h (Figure 8A and 8B). In guard cells, ABA induces the accumulation of $H_2O_2$, which is produced by guard cell-located NADPH oxidases; the increased level of $H_2O_2$ is essential in the signaling pathway that regulates the closure response of stomata (Kwak et al., 2003; Desikan et al., 2004; Wang and Song, 2008; Kim et al., 2010).

To further dissect the function of NaMPK4 in ABA signaling, the epidermal peels from WT and irNaMPK4 leaves were treated with $H_2O_2$ and the stomatal apertures were quantified (Figure 8C). WT stomata exhibited a rapid closure response after the application of $H_2O_2$: the average stomatal aperture reduced about 50% and 86% by 15 and 30 min and remained unchanged by 1 h; in contrast, irNaMPK4 stomata did not show a response by 15 min, but decreased average aperture 40% and 60%, 30 and 60 min after $H_2O_2$ application (Figure 8C).

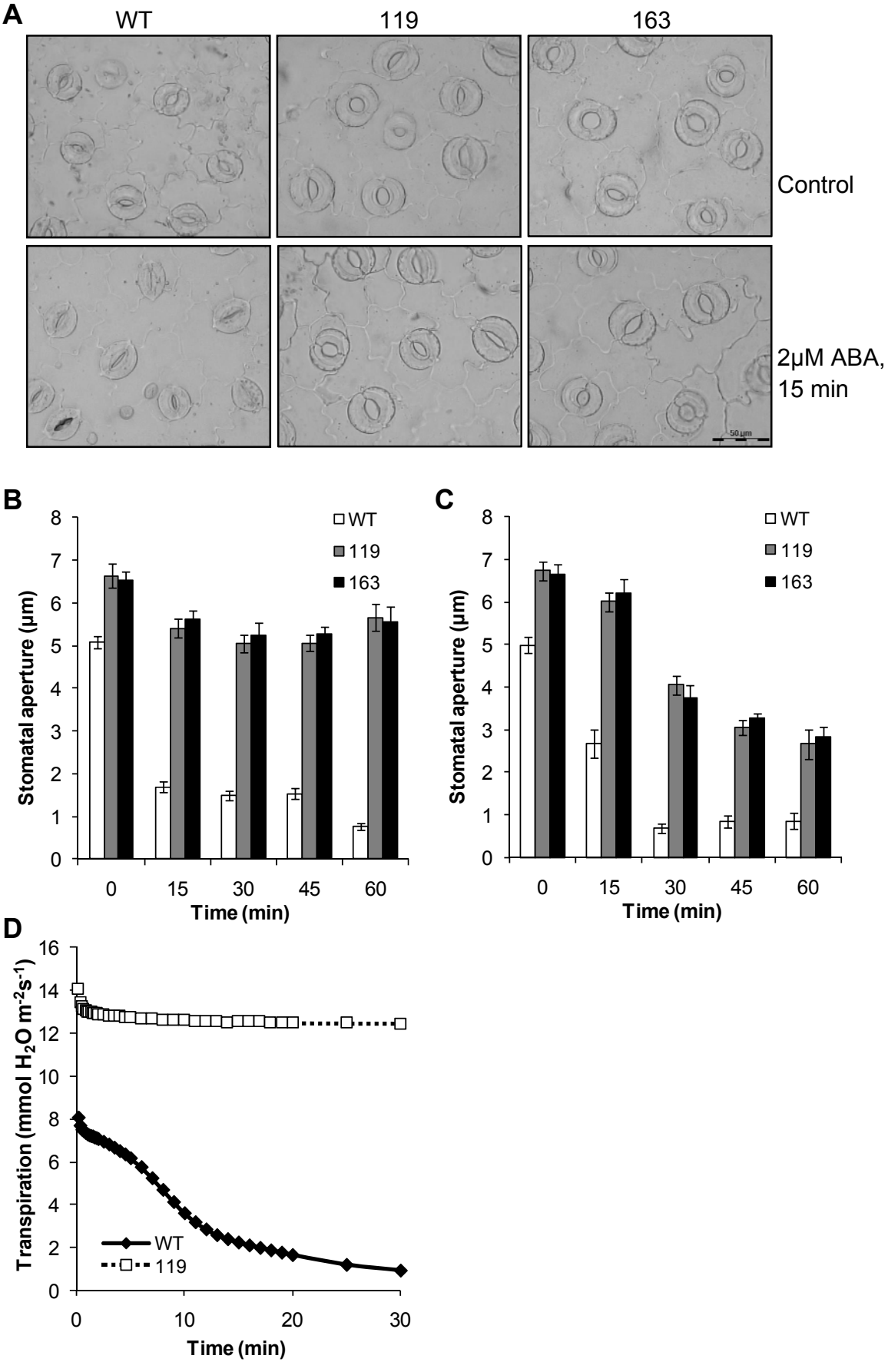

A
WT
119
163
Control
2μM ABA,
15 min
50 μm
B
Stomatal aperture (μm)
WT
119
163
Time (min)
C
Stomatal aperture (μm)
WT
119
163
Time (min)
D
Transpiration (mmol H2O m-2s-1)
WT
119
Time (min)

**Figure 8.** NaMPK4 is required for ABA-, $H_2O_2$-, and darkness-induced stomatal closure.

**(A)** Photographs of stomata in the epidermal peels from wild-type (WT) and irNaMPK4 (lines 119 and 163) 15 min after being treated with 2 μM of ABA (the same volume of the solvent of ABA were added as control). **(B)** and **(C)** Apertures (mean ± SE) of the stomata on 2 μM ABA- **(B)** and 50 μM $H_2O_2$ **(C)**-treated WT and irNaMPK4 epidermal peels. The width of at least 50 stomatal pores were measured and used to calculate the average stomatal apertures. **(D)** Stomata of irNaMPK4 plants (line 119) have almost no response to darkness-induced changes in transpiration rates. Plants were illuminated under white light (300 μmol $m^{-2}$ $s^{-1}$) for 4 h to open stomatal pores and after lights were switched off, the transpiration rates were recorded.

In the dark, C3 and C4 plants close stomata to conserve water. Stomata of *NtMPK4*-silenced tobacco do not close when light is removed, while WT tobacco shows a rapid closure phenotype (Marten et al., 2008). Similarly, we found that the transpiration rates of irNaMPK4 plants showed no responses to light-dark transitions, but stomata of WT quickly closed within 10 min, indicating that NaMPK4 is required for dark-induced stomatal closure (Figure 8D).

We concluded that NaMPK4 is located downstream of ABA-induced $H_2O_2$ to promote stomatal closure in response to drought stress and is also required for the normal closure during light-dark transitions.

### 3.3.2. NaMPK4 is not involved in salinity stress resistance but negatively controls the ABA-induced suppression of seed germination

Apart from drought tolerance, ABA signaling plays important roles in resistance to salinity, inhibition of seed germination and root elongation (Finkelstein et al., 2002; Xiong et al., 2002; Zhu, 2002; Finch-Savage and Leubner-Metzger, 2006). To examine whether NaMPK4 is also involved in salinity tolerance, rosette-staged WT and irNaMPK4 plants were watered daily with 80 mL of 200 mM NaCl solution. As early as 4 days after the onset of the salt treatment, WT and irNaMPK4 exhibited similar salt stress-induced phenotype (Figure 9A). Similar patterns were obtained from plants that were watered with 50 and 100 mM NaCl, although the stress symptoms developed somewhat more slowly (data not shown).

In the natural habitat of *N. attenuata*, ABA and MeJA exist in leachates from leaf litter of juniper, sagebrush, and blackbrush and they function as allelochemical compounds (Krock et al., 2002). Sensing the existence of ABA and MeJA is important in allowing seeds to germinate into open niches free from competitors (Preston and Baldwin, 2000). Examining the inhibitory effects

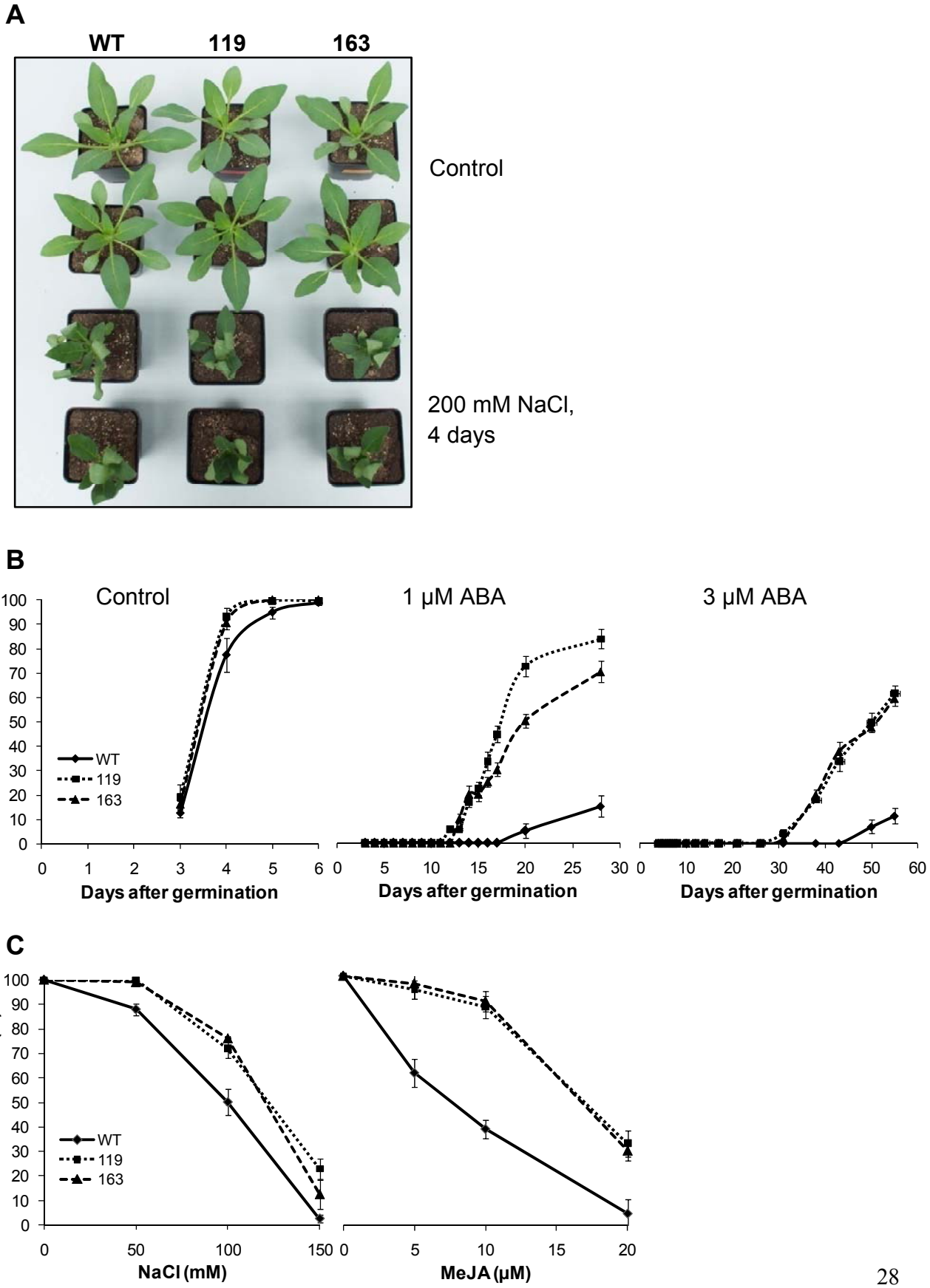

A
WT
119
163
Control
200 mM NaCl,
4 days
B
Control
1 µM ABA
3 µM ABA
Germination rate (%)
WT
119
163
Days after germination
Days after germination
Days after germination
C
Germination rate (%)
WT
119
163
NaCl (mM)
MeJA (µM)

**Figure 9.** NaMPK4 is not involved in salt resistance in established plants but is required for the inhibitory effect of ABA, salt, and MeJA during germination.

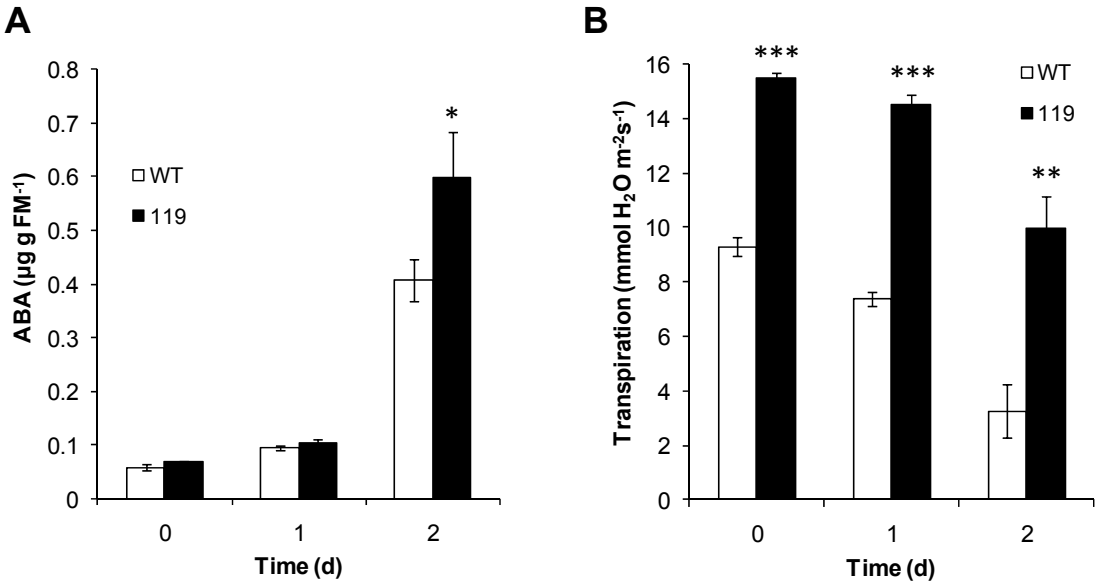
**(A)** A photograph of plants 4 days after being treated with salt. Rosette-staged wild-type (WT) and irNaMPK4 (lines 119 and 163) plants were watered with 80 mL of 200 mM NaCl in addition to normal watering; untreated plants served as control. B to D, Silencing *NaMPK4* represses the ABA- **(B)**, salt- **(C)**, and MeJA **(D)**-induced germination inhibition. Seeds were sown on media containing ABA, NaCl, or MeJA at the indicated concentrations. Note: C and D indicate germination rates 11 days after sowing.

of ABA on germination has also been widely used as a tool to identify mutants in ABA signaling. Similarly, sensing high salinity during germination is an adaptive response as well. Thus, we performed germination assays to determine whether NaMPK4 mediates ABA-, salt-, and MeJA-inhibited seed germination. Under normal conditions, irNaMPK4 seeds tend to germinate marginally earlier than do WT, whereas on germination medium containing 1 μM ABA, germination was strongly inhibited in WT seeds (only 5% germination even 20 days after sowing), while more than 50% of irNaMPK4 seeds germinated (Fig. 9B). Similar trends were seen when seeds were germinated on medium containing 3 μM ABA, although 3 μM ABA had much stronger inhibitory effect (Fig. 9B). Eleven days after sowing, irNaMPK4 and WT seeds showed 74% and 50% germination rates on 100 mM NaCl medium (Fig. 9C). A greater difference was detected between WT and irNaMPK4 seeds when they were sown on medium containing MeJA: after 11 days, 5 and 10 μM MeJA augmented media had no effect on irNaMPK4 seeds, whereas WT seeds germinated only 63 and 43%, respectively (Fig. 9C). Conversely, seedling root elongation assays on medium containing ABA, NaCl, or MeJA did not show any differences between WT and irNaMPK4 (data not shown).

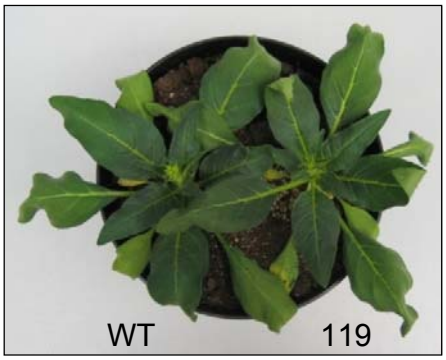
We concluded that NaMPK4 is required for ABA-, salinity-, and MeJA-induced germination inhibition, but does not play a role in the responses to these factors in established plants.

### 3.3.3. NaMPK4 is likely not involved in the transcriptional regulation of drought stress-induced genes

The majority of MAPK targets are transcription factors that regulate the transcript abundance of their downstream targets (Chang and Karin, 2001; Rodriguez et al., 2010). We first sought to examine whether ABA-induced transcriptional responses are altered in irNaMPK4 plants by spraying ABA solutions on WT and irNaMPK4 plants. However, both lines showed very little response to externally supplied ABA, unless very high and non-physiologically relevant concentrations were used, i.e. > 300 µM. This might result from the relatively thick cuticle of *N. attenuata*, which may hinder ABA from entering plant leaves. Thus exogenous application of ABA was not used. To further explore the function of NaMPK4 in mediating drought-induced responses and to minimize the different degrees of drought stress in WT and




C



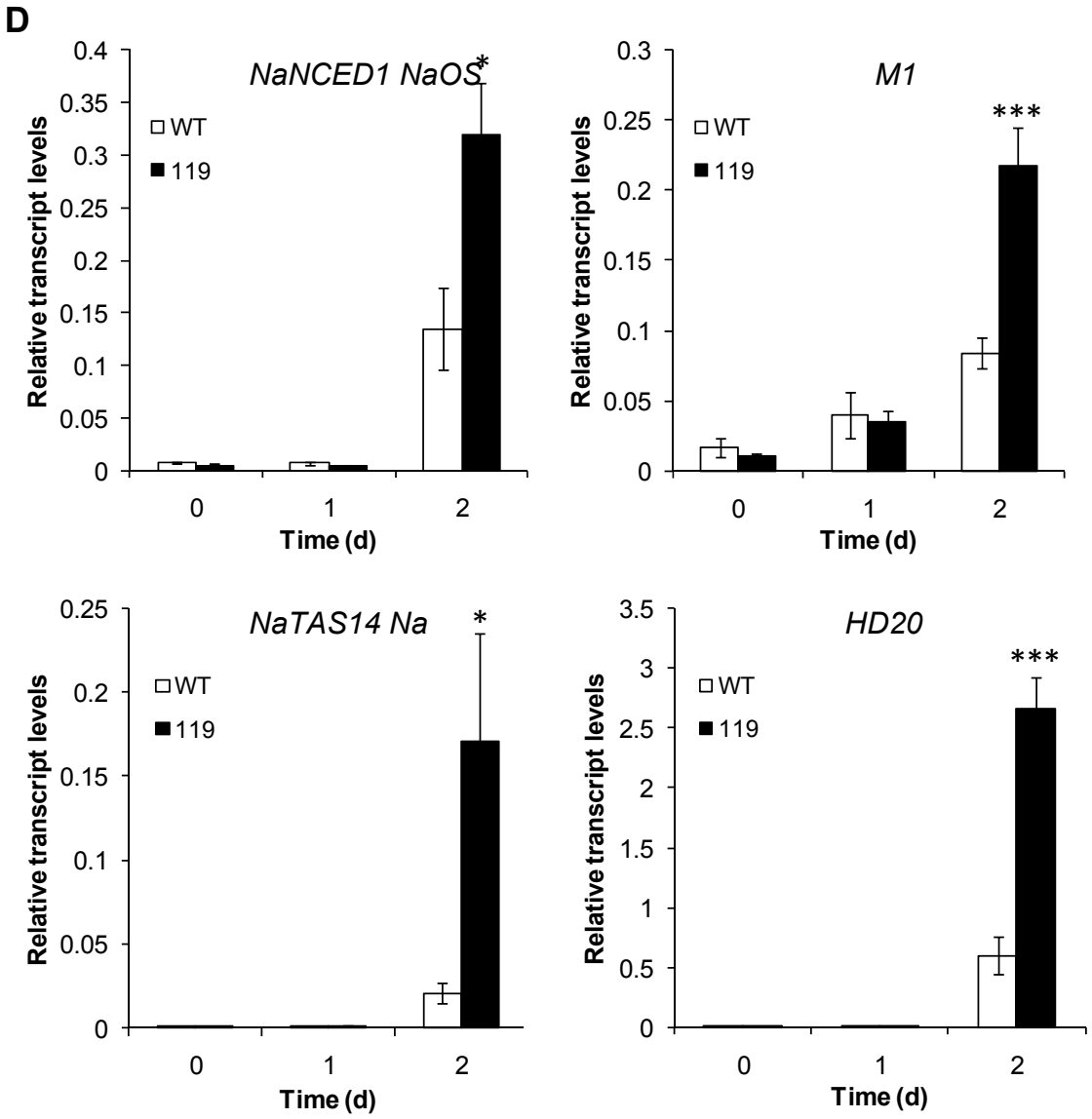



**Figure 10.** Drought-induced transcriptional regulation in wild-type and irNaMPK4 plants.

Wild type (WT) and irNaMPK4 were grown pairwise in the same 2-liter pots. When plants were 35 days old, they were exposed to drought stress by stopping watering. **(A)** ABA contents in the +1 leaves of WT and line 119 (mean ± SE; N = 5) over 2 days after being exposed to drought stress. **(B)** The transpiration rates (mean ± SE, N = 5) were measured at indicated times. **(C)** WT and irNaMPK4 (line 119) showed similar symptoms of dehydration 2 days after the initiation of drought stress. **(D)** Transcript levels (mean ± SE; N = 5) of drought-inducible genes, *NaNCED1*, *NaOSM1*, *NaTAS14*, and *NaHD20* in WT and irNaMPK4 plants after being treated with drought. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). FM = fresh mass.

irNaMPK4 plants grown in single pots, WT and irNaMPK4 (line 119) were grown side by side in 2-liter pots. After one day of drought treatment, when plants showed no phenotype of drought stress, ABA contents were similar between WT and irNaMPK4 (100 ng/g FM; Figure 10A), although transpiration of irNaMPK4 was about 1 fold higher than from WT (Figure 10B). Two days after drought treatment, as expected, similar levels of wilting were seen in both genotypes (Figure 10C) and drought stress elevated ABA contents to 407 and 598 ng/g FM in WT and irNaMPK4, respectively (Figure 10A). Consistent with the impaired stomatal closure response in irNaMPK4 plants, even when dehydrated, irNaMPK4 still transpired twice the amount of water per leaf area than did WT (Figure 10B).

In *N. attenuata*, drought stress elevates the levels of transcript of *NaNCED1*, *NaOSM1*, *NaTAS14*, and *NaHD20* (Parra et al., 1996; Re et al., 2011). One day after drought treatment, all these genes did not show augmented levels (Figure 10D). Consistent with the greater ABA contents of irNaMPK4 plants, 2 days after being deprived of water, all these genes' transcript abundance was elevated in WT, while irNaMPK4 plants exhibited even greater levels of these transcripts (Figure 10D).Therefore, NaMPK4 seems not to be required for the transcript regulation of these genes induced by drought stress. Large scale transcriptional analyses are needed to further examine its role in drought stress-induced transcriptome reconfiguration.

## 3.4. The role of NaMPK4 in plant-pathogen responses

### 3.4.1. NaMPK4 confers resistance to *Pst* DC3000 in intercellular spaces and likely in stomata

As a part of the plant's innate immune system, stomata play an important role in limiting bacteria's entry into plant tissues, since guard cells close stomata after perceiving bacterium-derived elicitors; furthermore, ABA signaling is required for this defense response (Meloto et al. 2006). We hypothesized that the reduced guard cell sensitivity to ABA in the *NaMPK4*-silenced plants may impair stomatal responses to *Pst* DC3000 and eventually result in higher infection rates.

In Arabidopsis incubating leaves or epidermal peels with *Pst* DC3000 leads to stomatal closure within the first 1 to 2 h (Meloto et al. 2006). However, guard cells on *N. attenuata* epidermal peels did not respond to *Pst* DC3000 in various densities (data not shown). Therefore, to monitor the closure response of stomata, we measured the transpiration rates at different times after dipping leaves in a *Pst* DC3000 suspension. WT decreased their transpiration rates about 42% 2.5 h after applying pathogens to the leaf surfaces (Figure 11A). irNaMPK4 showed an even stronger reduction in transpiration rates and by 2.5 h transpiration rates decreased to WT levels (57% reduced), although by 1 h the transpiration rates of irNaMPK4 plants remained 30% higher than those of WT (Figure 11A). After one day, WT transpiration rates almost completely recovered to levels of non-treated plants and those irNaMPK4 remained very low, which might be due to the large populations of bacteria that had propagated in the plants: no bacteria were detected in WT and strikingly *Pst* DC3000 amplified to $10^6$ and $10^7$ cfu/cm$^2$ by day 1 and 2, respectively (Figure 11B). Consistently, 3 days after application of *Pst* DC3000, chlorosis was observed in infected leaves of irNaMPK4, but not in WT; by day 10, irNaMPK4 exhibited necrotic lesions, while only some of the bacterium-applied WT leaves (about 30%) showed slight chlorosis (Figure 11C).

At least two steps account for the successful colonization of bacteria in plants: entry from stomata or wounds and amplification in the intercellular spaces of plant tissues. To further dissect the function of NaMPK4 in regulating stoma-mediated resistance to bacterial pathogens, *Pst* DC3000 was pressure infiltrated into WT and irNaMPK4 leaves to circumvent the requirement of entry through stomata. One day after inoculation, the bacterial population in WT was approximately 8 times smaller than that in irNaMPK4; 2 days after infiltration, bacteria density in

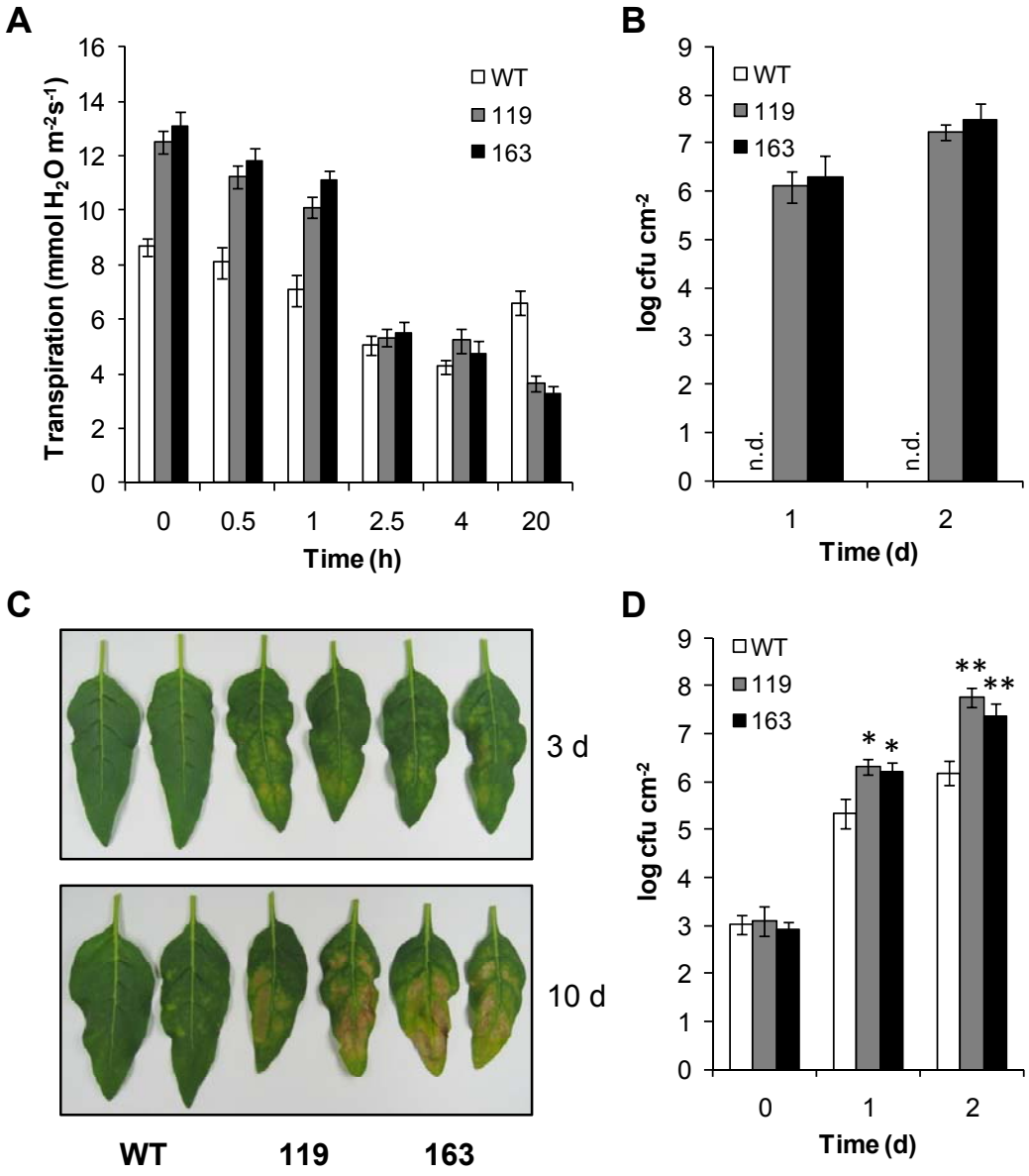



**Figure 11.** Silencing *NaMPK4* compromises resistance to the bacterial pathogen, *Pst* DC3000.

**(A)** and **(B)** Transpiration rates and bacterial populations in wild-type (WT) and irNaMPK4 (lines 119 and 163) leaves after applying *Pst* DC3000 to leaf surfaces. Leaves of WT and irNaMPK4 were dipped in a *Pst* suspension ($O.D._{600}$ = 0.8) and the transpiration rates (mean ± SE, N = 8) **(A)** and bacterial populations (mean ± SE, N = 10) were measured at indicated time **(B)**. **(C)** Photographs taken from leaves 3 and 10 days after dipping in pathogen suspension (two replicated leaves from each line are shown). Note that the second WT leaf shows slight chlorosis after 10 days. **(D)** *Pst* DC3000 ($O.D._{600}$ = 0.001) was pressure infiltrated to abaxial sides of leaves and the bacterial population (mean ± SE, N = 10) were examined in samples collected at indicated times. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$).

WT was around 25 times less (Figure 11D), indicating that silencing *NaMPK4* compromises the innate immunity of *N. attenuata* against *Pst* DC3000 bacteria that are located in plant intercellular spaces. Notably, compared with the bacterial populations in plants whose leaf surfaces were applied with bacteria, much smaller differences were found in plants in which bacteria were infiltrated, especially one day after bacterial dipping and after infiltration. These data strongly suggest that NaMPK4 functions in *N. attenuata*'s innate immunity against bacterial pathogen *Pst* DC3000 by probably limiting bacterial entry from stomata and suppressing bacterial amplification after they invade into the intercellular spaces.

Notably, the positive role of NaMPK4 in defense against *Pst* DC3000 in *N. attenuata* is not consistent with that of AtMPK4 in Arabidopsis, since *mpk4* mutant plants have highly elevated SA levels and exhibit augmented resistance to *Pst* DC3000 (Petersen et al., 2000). We next examined whether the decreased resistance to *Pst* DC3000 resulted from impaired SA defense in irNaMPK4 plants. Under normal conditions, WT and irNaMPK4 plants had the same levels of SA (145 ng/g FM); after bacterial infiltration irNaMPK4 showed much higher levels of SA than did WT: by 12 h, ~ 2300 and ~ 6000 ng/g FM SA were found in WT and irNaMPK4, respectively, and greater amounts of SA were also found in irNaMPK4 plants 1 and 2 days after infiltration (Figure 12A). Inoculation of the pathogen solvent (Mock) did not induce SA accumulation after 12 h (data not shown). Most likely, increased SA levels in irNaMPK4 plants after *Pst* DC3000 infection resulted from the rapid growth of pathogen populations in these plants. In certain plant-pathogen interactions, JA and ABA also regulate plant resistant levels (Adie et al., 2007; de Torres-Zabala et al., 2007; Robert-Seilaniantz et al., 2007; Flors et al., 2008; Zabala et al., 2009). We also determined the levels of this phytohormones after *Pst* DC3000 infection; however, no differences of JA or ABA contents were found between WT and irNaMPK4 plants (data not shown).

### 3.4.2. NaMPK4 regulates plant resistance to *Pst* DC3000 independent of SA and NPR1

Despite having high SA levels, *Pst* DC3000 amplified more in irNaMPK4; hence, SA might be dispensable in *N. attenuata*'s resistance to *Pst* DC3000. To examine this possibility, we sprayed 1 mM SA on WT and irNaMPK4 plants, and after 1 day we infiltrated these leaves with *Pst* DC3000. SA application was repeated once each day. Plants sprayed with water containing 0.1% ethanol (the solvent of 1mM SA solution) served as controls. The growth of *Pst* DC3000 was examined up to 3 days after pathogen infiltration. Neither WT nor irNaMPK4 plants showed

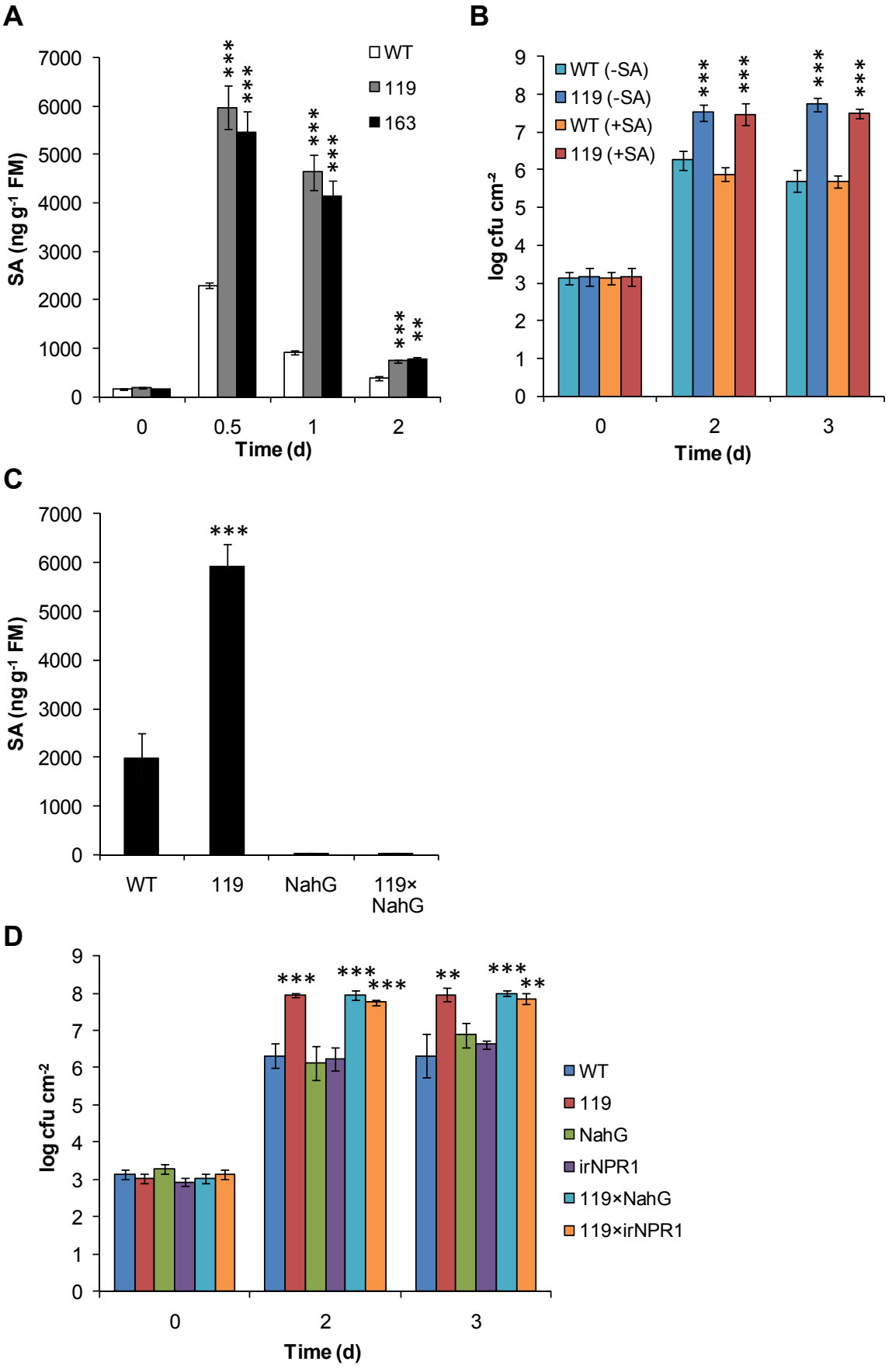

A
SA (ng g-1 FM)
7000
6000
5000
4000
3000
2000
1000
0
WT
119
163
***
0
0.5
1
2
Time (d)
B
log cfu cm-2
9
8
7
6
5
4
3
2
1
0
WT (-SA)
119 (-SA)
WT (+SA)
119 (+SA)
***
0
2
3
Time (d)
C
SA (ng g-1 FM)
7000
6000
5000
4000
3000
2000
1000
0
***
WT
119
NahG
119×
NahG
D
log cfu cm-2
9
8
7
6
5
4
3
2
1
0
***
**
0
2
3
Time (d)
WT
119
NahG
irNPR1
119×NahG
119×irNPR1

**Figure 12.** NaMPK4 mediates the resistance of *N. attenuata* to *Pseudomonas syringae* pv. *tomato* DC3000 in an SA- and NPR1-independent manner.

**(A)** SA contents in leaves of WT and irNaMPK4 after being infiltrated with *Pst* DC3000. *Pst* DC3000 ($O.D._{600}$ = 0.001) was infiltrated to abaxial sides of leaves and SA contents (mean ± SE, N = 5) were examined in samples collected at indicated times. **(B)** Exogenously applying SA does not alter the resistant levels of wild-type or irNaMPK4 plants. Leaves of wild-type (WT) and irNaMPK4 (lines 119) plants were sprayed with 1 mM SA and after one day, *Pst* DC3000 ($O.D._{600}$ = 0.001) was infiltrated into WT and line 119. Pathogen populations were determined after indicated times. SA was applied once a day until experiments finished. **(C)** NahG effectively minimizes SA accumulation in *Pst* DC3000-infected plants. Wild-type (WT), irNaMPK4 (line 119), NahG, and 119×NahG were infiltrated with *Pst* DC3000 ($O.D._{600}$ = 0.001) SA contents were measured in the infected areas 2 days after pathogen treatment. **(D)** NaMPK4 confers resistance to intercellular located *Pst* DC3000 in a SA- and NPR1-independent manner. irNaMPK4 (line 119) was crossed with NahG and irNPR1 plants to create 119×NahG and 119×irNPR1 plants and the growth of bacteria was examined in WT, line 119, NahG, irNPR1, 119×NahG, and 119×irNPR1 plants. All values are mean ± SE. Asterisks indicate significant differences between WT and irNaMPK4 (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). FM, Fresh mass.

higher resistance to *Pst* DC3000 despite the exogenously applied SA, suggesting that SA is not required in *N. attenuata*'s resistance to *Pst* DC3000 (Figure 12B). Furthermore, we generated plants transformed with a bacterial *salicylic acid hydroxylase* gene (*NahG*) downstream of a 35S promoter (NahG plants). NahG and irNaMPK4 (line 119) were crossed to obtain plants with minimal SA accumulation after pathogen infection. We noticed that 119×NahG plants were morphologically identical to irNaMPK4 plants, confirming that the decreased rosette size and stalk length of irNaMPK4 plants were not dependent on SA. NPR1 protein plays an essential role in SA-mediated gene expression and disease resistance (Shah, 2003; Dong, 2004; Loake and Grant, 2007). An irNPR1 line (Rayapuram and Baldwin, 2007), which was silenced in *NPR1* transcript levels, was also crossed with irNaMPK4 plants (119×irNPR1) to generate plants silenced in both *NPR1* and *NaMPK4*. In line with the pathogen growth data obtained after SA application, removing SA by *NahG* overexpression (Figure 12C) did not alter pathogen growth in WT and irNaMPK4 plants (Figure 12D). Similarly, *Pst* DC3000 populations increased to the same levels in irNPR1 and 119×irNPR1 plants compared with WT and irNaMPK4 plants, respectively (Figure 12D). Thus, we concluded that NaMPK4 mediates the resistance of *N. attenuata* to *Pst* DC3000 located in the intercellular spaces in a pathway that is independent of SA and NPR1.

### 3.4.3. AtMPK11 is not involved in drought or pathogen resistance in Arabidopsis

In addition to AtMPK4, NaMPK4 also has a short sequence distance to AtMPK11 (Figure 3A). It is possible that in Arabidopsis AtMPK11, but not AtMPK4, is functionally similar to NaMPK4 in drought resistance and pathogen defense.

As reported by Kosetsu et al. (2010), we found that *mpk11* mutant (SALK_049352C) is morphologically identical to WT (Col-0). Measurement of transpiration rates indicated that WT and *mpk11* mutant had similar values (Figure 13A). Furthermore, WT and *mpk11* had the same water loss rates in detached leaves and accumulated similar amount of ABA (Figure 13B and 13C). When plants were subjected to a drought treatment, no differences in either the speed of wilting or the ABA levels were found between WT and *mpk11* (data not shown). We infer that AtMPK11 is not involved in drought stress resistance.

To determine the role of AtMPK11 in defense against *Pst* DC3000, bacterial suspension was infiltrated into WT and *mpk11* leaves and *Pst* DC3000 amplification was quantified. Compared with Col-0 WT, *mpk11* was slightly more resistant to the pathogen (Figure 13D). After dipping leaves of WT and *mpk11* in a *Pst* DC3000 suspension, similar cell death phenotype was found in all plants (data not shown). Thus, AtMPK11 does not have similar function as NaMPK4 in pathogen defense.

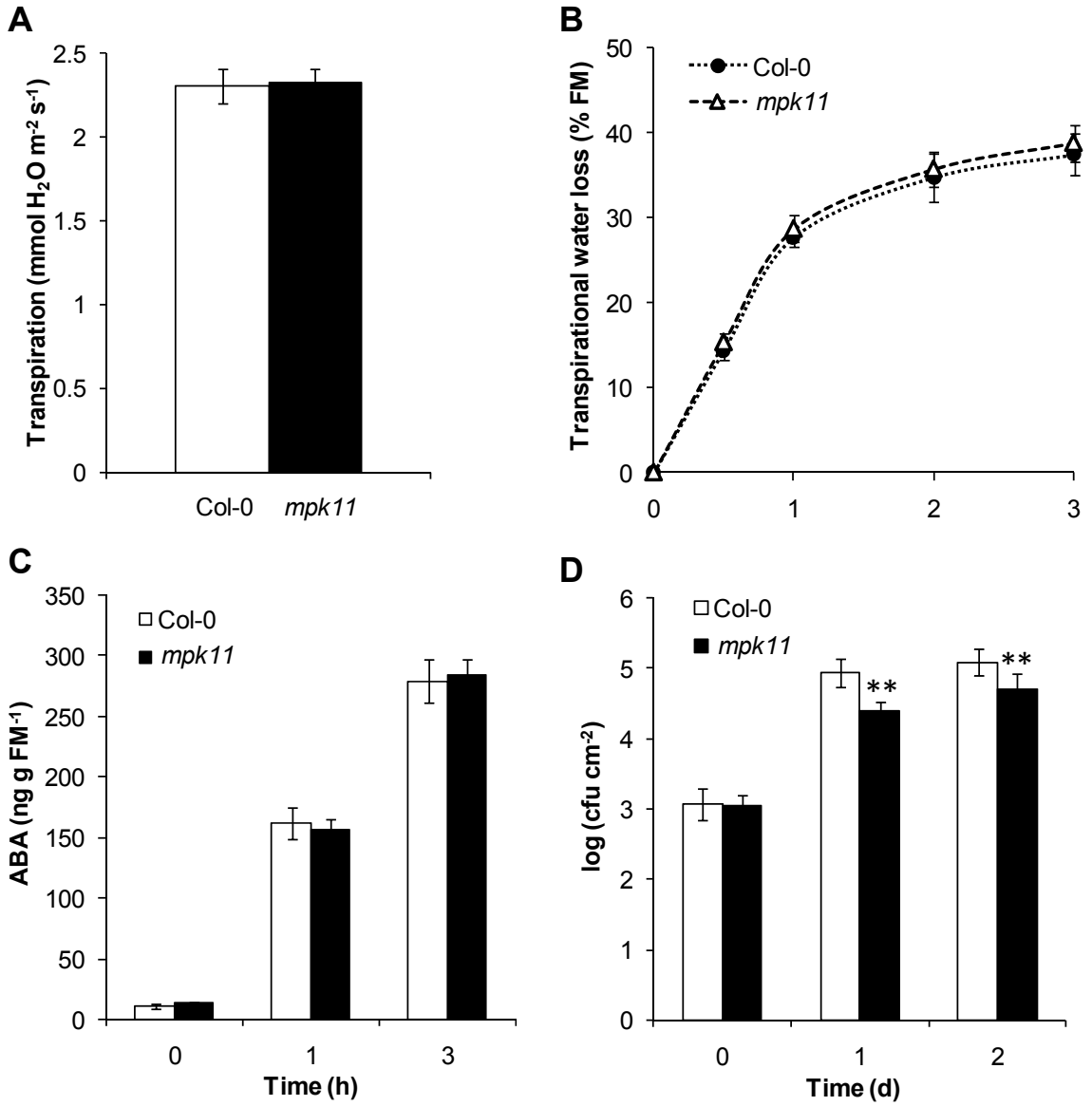



**Figure 13.** Arabidopsis AtMPK11 is not involved in the resistance to drought or pathogen *Pst* DC3000.

**(A)** Col-0 and *mpk11* were grown under the long-day conditions and transpiration rates (mean ± SE; N = 7) were measured. **(B)** Leaves of Col-0 and *mpk11* were excised and kept abaxial sides up on a bench under the long-day conditions. The masses of leaves were recorded at indicated times to calculate the percentage of transpirational water loss (mean ± SE, N = 7). **(C)** The contents of ABA (mean ± SE, N = 10) were determined by HPLC-MS/MS. **(D)** *mpk11* mutant is slightly more resistant to *Pst* DC3000. Col-0 and *mpk11* were infiltrated with *Pst* DC3000 ($O.D._{600}$ = 0.001) and the bacterial populations were determined after indicated times (N = 8). Asterisks indicate significant differences between Col-0 and *mpk11* (*t*-test; **, $P < 0.01$).

## 3.5. NaMPK4 and plant defense against herbivores

### 3.5.1. NaMPK4 is activated by simulated *M. sexta* herbivory, but not by mechanical wounding

To study the function of NaMPK4 in *N. attenuata*'s defense against herbivores, we performed bioassays on WT and irNaMPK4 plants to examine whether silencing *NaMPK4* influences the growth of *M. sexta*. Freshly hatched *M. sexta* larvae were placed on WT and irNaMPK4 plants and larval mass over time was recorded. From as early as day 4, compared with those on WT plants, larvae on irNaMPK4 plants exhibited decreased growth rates; by day 11, larvae on irNaMPK4 plants had only 1/3 of the mass of the larvae fed on WT plants (Figure 14).

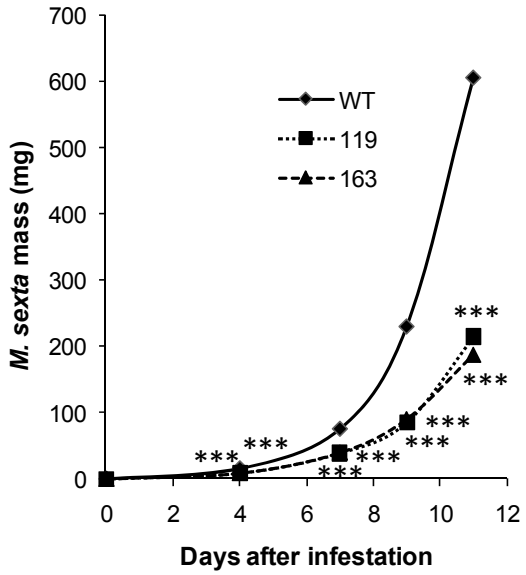



**Figure 14.** NaMPK4 negatively regulates *N. attenuata*'s resistance to the specialist insect herbivore *M. sexta*.

Wild-type (WT) and irNaMPK4 plants were infested with 30 neonate *M. sexta* larvae (1 larva/plant). Mass of these larvae (mean ± SE) on WT and irNaMPK4 plants was determined on day 4, 7, 9, and 11. Asterisks indicate significant differences between the mass of larvae reared on WT and irNaMPK4 plants (*t*-test; ***, $P < 0.001$; $N = 30$).

Wounding is known to elevate WIPK transcript levels in cultivated tobacco (Seo et al., 1995; Zhang and Klessig, 1998). Similarly, in *N. attenuata* wounding or simulated herbivore treatment increased WIPK and SIPK transcript levels (Wu et al. 2007). To determine if NaMAPK4 is involved in activating defense responses to herbivore attack, leaves of rosette-staged WT and irNaMPK4 plants were wounded with a pattern wheel and 20 µL of 1/5 diluted *M. sexta* oral secretions (OS) were applied immediately to wounds (W+OS) to mimic herbivory (Halitschke et al., 2001; Halitschke et al., 2003); for comparison with mechanical wounding, 20 µL of water were applied to wounds (W+W). Quantitative RT-PCR (q-PCR) measurement

showed that after W+W treatment, *NaMPK4* transcript levels were only slightly and not significantly elevated compared with those in untreated plants. W+OS increased *NaMPK4* transcript levels one fold after 1.5 h ($P = 0.0025$, unpaired t test) (Figure 15A).

Using an antibody raised against the N-terminus of NtMPK4 (Gomi et al., 2005), where NaMPK4 shares identical sequence with NtMPK4, we examined whether NaMPK4 is activated by wounding and herbivory. Immunoblotting analysis indicated that neither W+W nor W+OS noticeably changed the abundance of NaMPK4 protein by 1 h (Figure 15B). Moreover, NaMPK4 protein levels were about 80% decreased in irNaMPK4 plants, confirming the effectiveness of gene silencing and the specificity of the antibody. Thereafter, we measured the activity of NaMPK4 before and after W+W and W+OS treatment with an immune-complex kinase assay: NaMPK4 protein was immuno-pulled down from W+W- and W+OS-treated WT and irNaMPK4 tissue extracts using the NtMPK4/NaMPK4-specific antibody and the kinase activity was measured subsequently using myelin basic protein as a substrate. In contrast to NtMPK4 in *N. tabacum*, which is rapidly (10 min) activated by wounding (Gomi et al., 2005), the activity of NaMPK4 was not noticeably elevated after wounding. However, three independent experiments revealed that 10 min after W+OS treatment, the activity of NaMPK4 increased 1 fold, and this W+OS-induced activity rapidly vanished by 30 min (Figure 15C). These findings suggest that *M. sexta* herbivory specifically and transiently activates NaMPK4 in *N. attenuata*.

Fatty acid-amino acid conjugates (FACs) in *M. sexta* OS are necessary and sufficient to trigger *M. sexta* herbivory-specific responses in *N. attenuata*, including rapid activation of SIPK and WIPK and initiation of JA and JA-Ile biosynthesis (Halitschke et al., 2001; Halitschke et al., 2003; Wu et al., 2007). We next explored whether FACs are also elicitors that activate NaMPK4. Twenty µL of FAC A (N-linolenoyl-L-Gln, 27.6 ng/µL), one of the most abundant FACs in *M. sexta* OS (Halitschke et al., 2001), were applied to freshly wounded *N. attenuata* leaves (W+FAC); for comparison, W+W and W+OS treatments were performed in parallel. Samples were harvested 10 min after each treatment, when the kinase activity attained the highest values (Figure 15C). Immune-complex kinase assays were done to determine NaMPK4 activity in these plants (Figure 15D). Again, W+W treatment didn't noticeably elevate the levels of NaMPK4 activity; as expected, W+FAC and W+OS treatment enhanced NaMPK4 activity levels. Furthermore, W+OS-induced NaMPK4 activity was totally abolished when FACs were removed from *M. sexta* OS by ion exchange chromatography (FAC free OS; Figure 15D). All these data

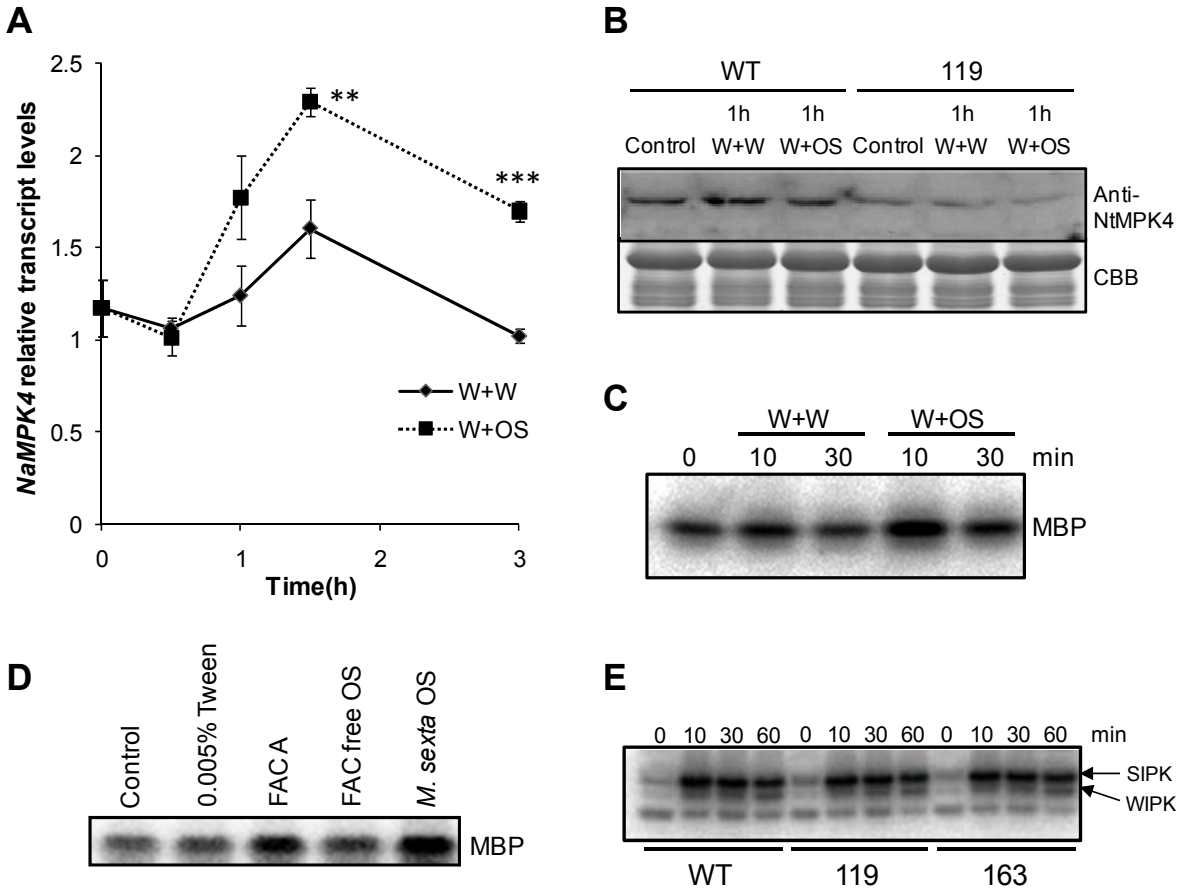



**Figure 15.** NaMPK4 is specifically activated by OS elicitation.

**(A)** Mean transcript levels (±SE) of *NaMPK4* in WT plants after W+W and W+OS treatments as measured with q-PCR. Asterisks represent significantly different transcript levels between untreated control plants and W+OS treated plants at the indicated times (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(B)** Immunoblotting analysis of NaMPK4. Wild-type (WT) and irNaMPK4 (line 119) were wounded with a pattern wheel and 20 µL of water (W+W) or *M. sexta* OS (W+OS) were immediately applied to puncture wounds. Samples were collected after 1 h; non-treated plants served as controls. Tissue from 3 replicate treatments was pooled and total soluble protein extracts were separated with SDS-PAGE and further blotted onto a PVDF membrane. An anti-NtMPK4 antibody was used for NaMPK4 detection. Top: image of immunoblotting analysis; bottom: image of a duplicated gel, which was loaded with same samples, and stained with Coomassie Brilliant Blue (CBB). **(C)** Immune-complex kinase assay of NaMPK4 activity after W+W and W+OS treatment. WT plants were treated with W+W and W+OS, and samples were collected after indicated times. NaMPK4 were immunoprecipitated with the anti-NtMPK4 antibody, and its activity levels were assayed with myelin basic protein as a substrate. **(D)** Immune-complex kinase assay of NaMPK4 activity in FAC-treated plants. *N. attenuata* WT plants were wounded with a pattern wheel, and 20 µL of 0.005% Tween 20 (solvent of FAC A), FAC A solution (27.6 ng/µL), FAC-free *M. sexta* OS, or *M. sexta* OS were immediately applied to wounds. NaMPK4 were immunoprecipitated with the anti-NtMPK4 antibody, and its activity

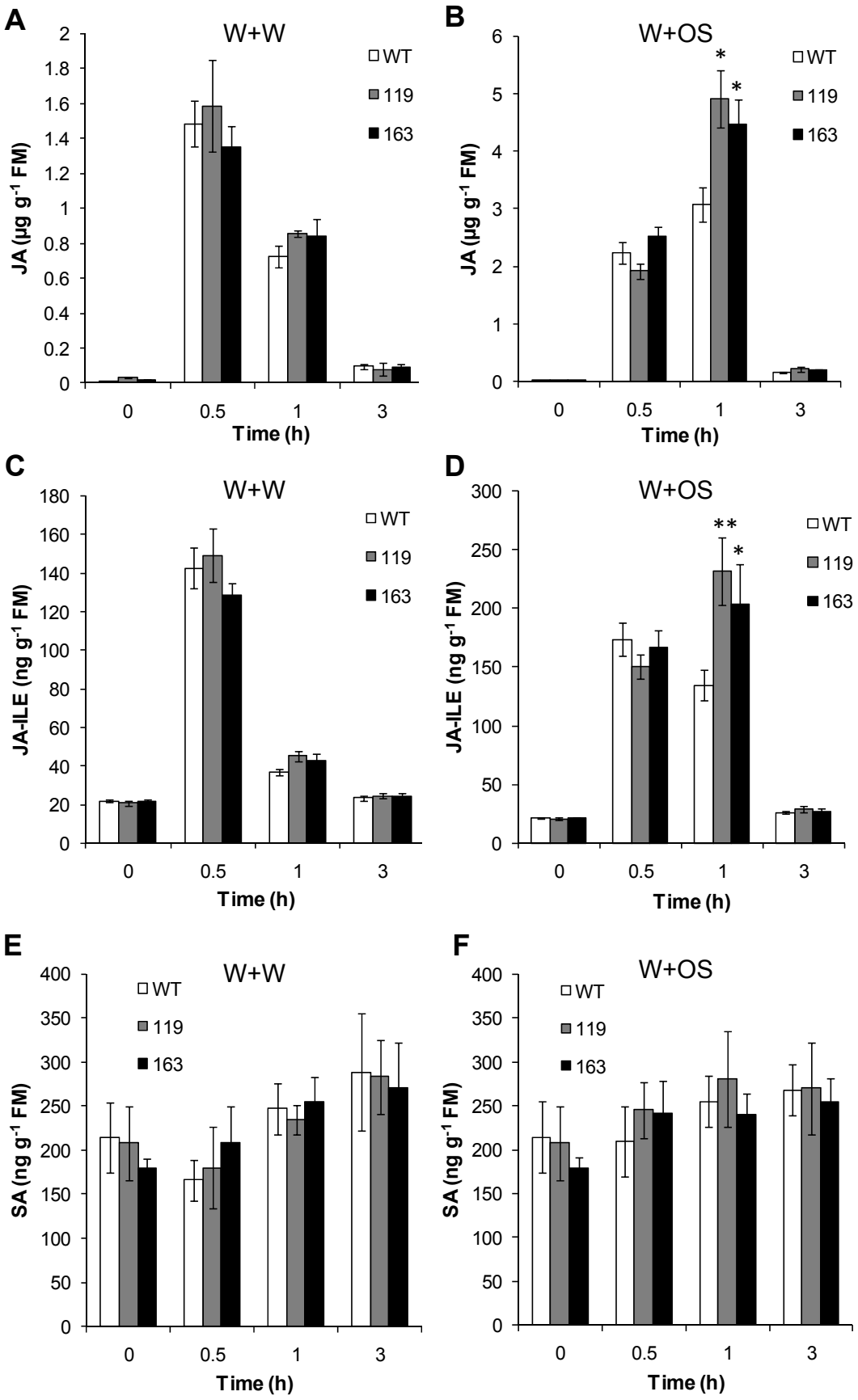
levels were assayed with myelin basic protein as a substrate. **(E)** irNaMPK4 plants have normal levels of SIPK and WIPK activity after herbivory. Wild-type and irNaMPK4 (line 119 and 163) were treated with W+OS, and samples were harvested at indicated times. SIPK and WIPK kinase activity levels were determined in these samples (each sample were pooled from 4 biological replicates) using an in-gel kinase assay.

indicate that during herbivory, FACs in *M. sexta* OS are necessary and sufficient to activate three MAPKs: SIPK, WIPK (Wu et al., 2007), and NaMPK4.To examine whether silencing *NaMPK4* altered the activity of SIPK and WIPK, an in-gel kinase assay was performed to measure the levels of SIPK and WIPK activity after W+OS treatment in WT and irNaMPK4 plants (Figure 15E). No differences of SIPK and WIPK activity between WT and irNaMPK4 plants were found.

### 3.5.2. Silencing *NaMPK4* alters phytohormone levels after simulated *M. sexta* herbivory

To investigate whether NaMPK4 modulates wounding- and herbivory-elicited JA levels and thus alters *N. attenuata*'s defense against *M. sexta*, we treated plants with W+W and W+OS, and the JA contents in WT and irNaMPK4 plants after these treatments were determined by LC-MS/MS. No differences in JA levels were detected between WT and irNaMPK4 plants before and after W+W treatment (Figure 16A). However, irNaMPK4 plants showed 65% increased levels of JA 1 h after W+OS treatment compared to those in W+OS-treated WT plants (Figure 16B). Consistently, JA-Ile, the conjugate of JA and isoleucine, which binds to the F-box protein COI1 and executes most functions of JA (Staswick and Tiryaki, 2004; Chini et al., 2007; Thines et al., 2007), was similarly increased: 1 h after W+OS treatment, JA-Ile contents were 60% higher in irNaMPK4 plants, while JA-Ile contents in W+W-induced WT and irNaMPK4 plants were similar (Figure 16C, D).

The antagonistic effect of SA on JA accumulation and signaling has been well documented (Doares et al., 1995; Niki et al., 1998; Kunkel and Brooks, 2002; Spoel et al., 2003). We quantified SA concentrations in order to determine if the elevated JA levels in W+OS-treated irNaMPK4 plants resulted from attenuated SA levels. WT and irNaMPK4 plants showed no difference in SA levels before and after either treatment (Figure 16E, F). *M. sexta* herbivory and OS elicitation, but not wounding, increase ethylene biosynthesis and emission in *N. attenuata*, which in turn, regulate herbivore-elicited nicotine accumulation (Steppuhn et al., 2004; von Dahl et al., 2007). After W+OS–elicitation, very similar levels of ethylene were emitted from WT and irNaMPK4 plants (Figure 16G).


A
W+W
WT
119
163
JA (µg g-1 FM)
Time (h)
B
W+OS
*
*
C
JA-ILE (ng g-1 FM)
D
**
*
E
SA (ng g-1 FM)
F

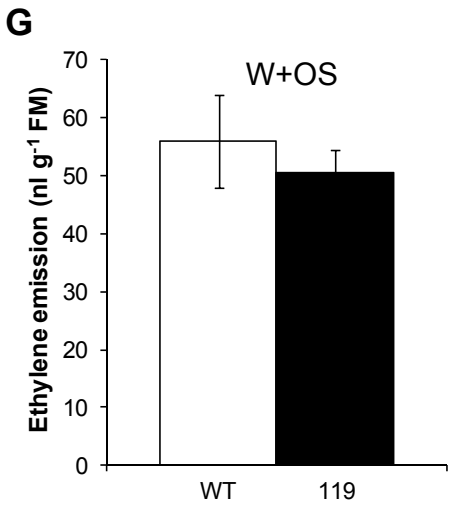



**Figure 16.** Silencing *NaMPK4* specifically elevates herbivory-induced JA and JA-Ile levels. Wild-type (WT) and irNaMPK4 plants (line 119 and 163) were wounded with a pattern wheel and 20 µL of water (W+W) or *M. sexta* OS (W+OS) were immediately applied to wounds. Samples were harvested at indicated times, and their JA (**A** and **B**), JA-Ile (**C** and **D**) and SA (**E** and **F**) contents (mean ± SE; N = 5) were analyzed with HPLC-MS/MS. (**G**) Ethylene production as quantified using a photoacoustic laser spectrometer. In 6 replicates were used for each genotype. Three leaves were treated with W+OS, immediately afterward sealed in a three-neck 250-mL flask and kept in the greenhouse for 5 h. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$).

We conclude that NaMPK4 specifically and negatively modulates *M. sexta* herbivory-induced JA accumulation and thus comprises a part of the regulatory network that is required for normal regulation of JA levels in *N. attenuata* in response to *M. sexta* attack.

### 3.5.3. Silencing *NaMPK4* increases herbivory-induced levels of TPIs and *trans*-α-bergamotene, but not of other defense metabolites

Many plant secondary metabolites function as defenses against herbivores (Hadacek, 2002). In *N. attenuata*, trypsin proteinase inhibitors (TPIs) (Van Dam et al., 2001), nicotine (Steppuhn et al., 2004), caffeoylputrescine (Kaur et al., 2010), and diterpene glucosides (DTGs) (Jassbi et al., 2008; Heiling et al., 2010) are all important direct defenses against herbivores, and the accumulations of these compounds are regulated by JA signaling (Paschold et al., 2007). We sought to determine whether these metabolites were responsible for the increased resistance of

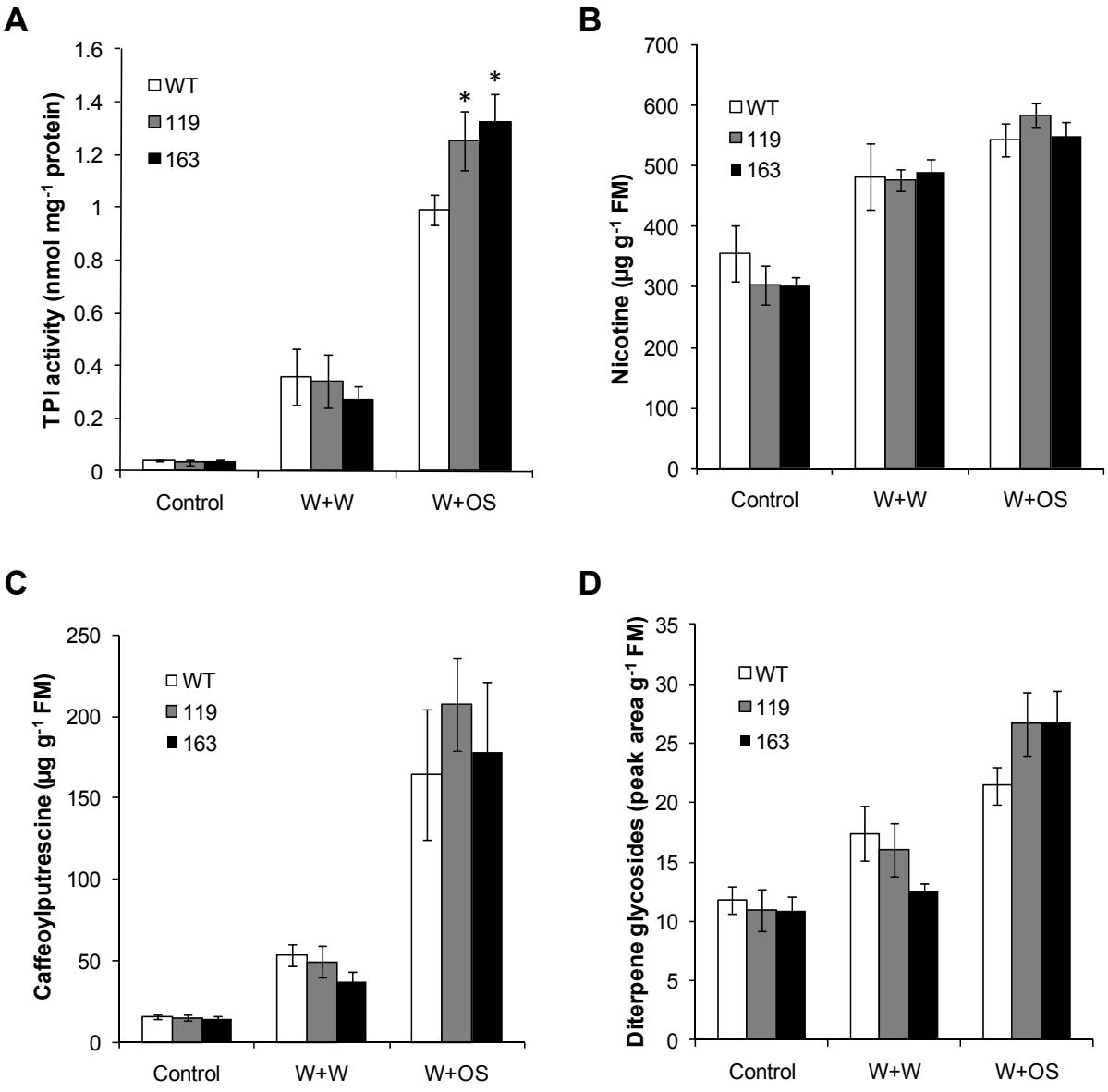


**Figure 17.** Wounding- and herbivory-induced direct defense metabolites in wild-type and irNaMPK4 plants.

Wild-type (WT) and irNaMPK4 plants (line 119 and 163) were wounded with a pattern wheel and 20 µL of water (W+W) or *M. sexta* OS (W+OS) were immediately applied to the puncture wounds. **(A)** The levels of trypsin proteinase inhibitor activity (TPI) were analyzed by radial diffusion assay; nicotine **(B)**, caffeoylputrescine **(C)**, and diterpene glucosides **(D)** (mean ± SE; N=5) were analyzed with a HPLC in samples harvested 3 days after treatment.

irNaMPK4 plants. Consistent with the increased levels of W+OS-induced JA in irNaMPK4 plants, these plants had 25% higher TPI activity than did WT plants after W+OS elicitation but not after W+W treatment (Figure 17A). In contrast, nicotine, caffeoylputrescine, and DTG contents were the same in WT and irNaMPK4 plants (Figure 17B, C, D).

Apart from these direct defenses, *M. sexta* herbivory elicits the release of volatile organic compounds (VOCs), such as *trans*-α-bergamotene (TAB), from *N. attenuata*, which attracts predators of *M. sexta* larvae and eggs and thereby functions as an indirect defense (Kessler and Baldwin, 2001). Furthermore, wounding and herbivory result in the release of green leaf volatiles (GLVs), which can attract predators of herbivores or increase herbivore loads (Halitschke et al., 2008; Meldau et al., 2009; Dicke and Baldwin, 2010). Compared with those in WT plants, TAB emissions in irNaMPK4 plants increased by about 85% in response to W+W treatment and more than 5 fold in response to W+OS treatment (Figure 18A). The quantities of GLVs released from WT and irNaMPK4 plants after W+W and W+OS treatments were similar (Figure 18B). Since GLVs also function as feeding stimulants in *N. attenuata* (Halitschke et al., 2004; Meldau et al., 2009), these GLV data excluded the possibility that the reduced *M. sexta* larval mass gain resulted from impaired GLV emission.

Herbivore growth is not only determined by the concentration of plants defense metabolites but also by the nutritional value of the diet. We measured protein concentrations and starch contents in WT and irNaMPK4 plants. WT and irNaMPK4 plants did not differ in their protein content (data not shown), however irNaMPK4 plants accumulated 57% -93% more starch than WT plants (Figure 19). These results ruled out the possibility that the highly enhanced defense against *M. sexta* resulted from decreased nutrient contents of irNaMPK4 plants.

Thus, NaMPK4 highly suppresses OS-elicited JA accumulation, TPI activity, and release of TAB, but not other known anti-herbivore secondary metabolites, and suppresses resistance of *N. attenuata* to its natural herbivore, *M. sexta*.

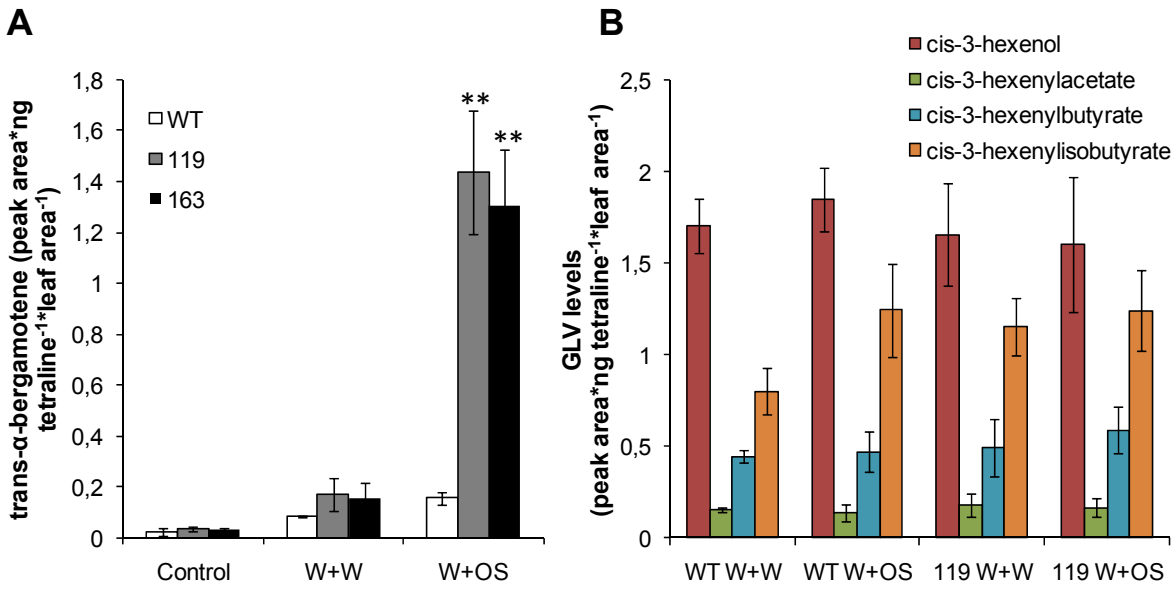



**Figure 18.** Volatile emissions in WT and *MPK4*-silenced plants.

**(A)** *trans*-*α*-bergamotene emission was determined in WT and irNaMPK4 (line 119) plants 24 h after W+W and W+OS treatment. **(B)** Immediately after W+W and W+OS treatment, green leaf volatiles (GLVs) were trapped for 3 h from treated leaves. Volatiles of 8 replicate samples per genotype and treatment were quantified with a GC-MS (mean ± SE). Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

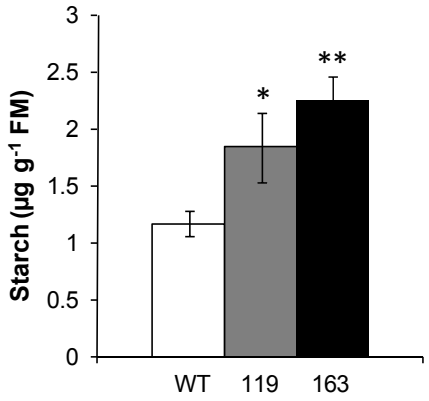



**Figure 19.** Starch accumulation in WT and *MPK4*-silenced plants.

Starch levels were estimated in 10 replicate fully expanded rosette leaves from WT and both irNaMPK4 lines using the Anthrone method. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$).

### 3.5.4. Silencing *NaMPK4* augments transcript levels of *WRKY* transcription factors

WRKY transcription factors are known to regulate plant defense responses and are potential targets of MAPKs (Eulgem et al., 2000; Kim and Zhang, 2004; Ulker and Somssich, 2004; Qiu et al., 2008). In *N. attenuata*, WRKY3 and WRKY6 have been identified as playing a role regulating JA elicitation, the accumulation of TPI, and *trans*-*α*-bergamotene emissions, as silenced lines failed to induce these defenses after herbivory and, consequently, were highly vulnerable to herbivores (Skibbe et al., 2008). To determine if NaMPK4 regulates *WRKY3* and *WRKY6* transcript levels, we measured their transcript accumulation after W+W and W+OS treatments in WT and irNaMPK4 plants. Wounding alone increased the levels of *WRKY3* in both lines transiently 30 fold after 0.5 h. Thirty min after W+OS treatment, in WT plants *WRKY3* transcript levels were about 1 fold higher than those induced by W+W and even higher levels of *WRKY3* transcript were detected in irNaMPK4 plants after 1 h (Figure 20A). Furthermore, compared with WT, irNaMPK4 plants showed higher transcript levels of *WRKY6* after both W+W and W+OS treatments (Figure 20B). It is likely that NaMPK4 modulate many more transcription factors.

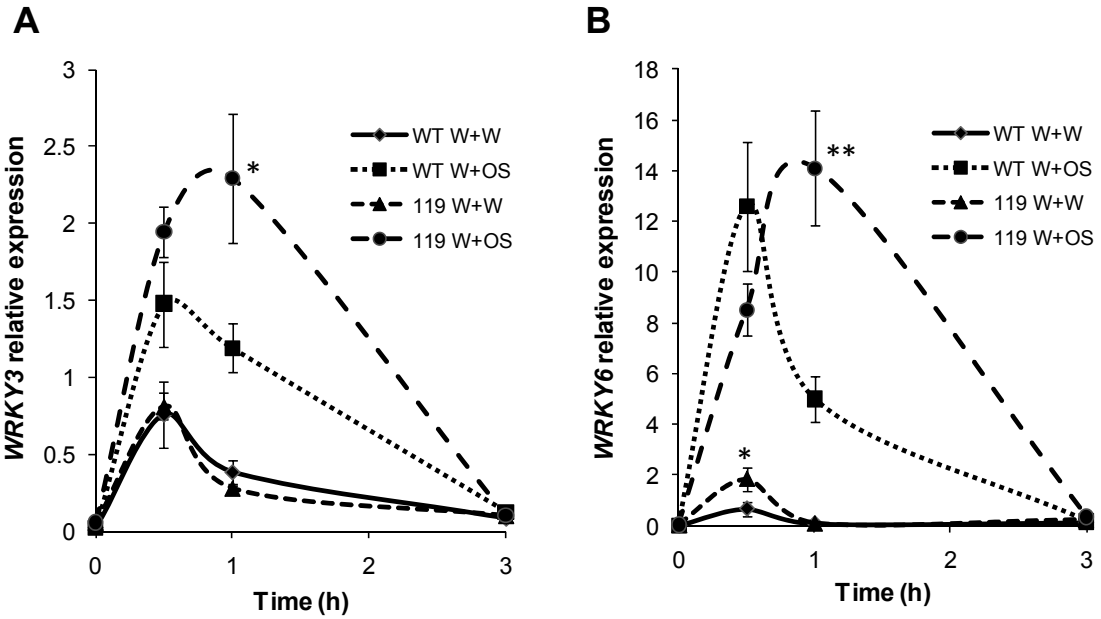



**Figure 20.** NaMPK4 mediates transcript levels of two transcription factors, *WRKY3* and *WRKY6*.

Leaves were wounded with a pattern wheel; 20 µL of water (W+W) or *M. sexta* OS (1/5-diluted) (W+OS) was applied to the wounds, and leaves from five replicate plants were harvested at the indicated times. Mean transcript levels (±SE, N = 5) of *WRKY3* **(A)** and *WRKY6* **(B)** were analyzed with q-PCR. Asterisks indicate significant differences (*t*-test; *, $P < 0.05$; **, $P < 0.01$).

### 3.5.5. NaMPK4 regulates specific JA-induced responses

JA-induced responses play a major role in development and interactions with herbivores and pathogens (Shah, 2005; Wasternack, 2007; Howe and Jander, 2008; Wu and Baldwin, 2010). In Arabidopsis, AtMPK4 is required for the transcript accumulation of JA-responsive genes (Petersen et al., 2000; Brodersen et al., 2006), and in tobacco plants NtMPK4 regulates methyl jasmonate (MeJA)-induced TPI levels (Gomi et al., 2005).

To examine the function of NaMPK4 in mediating the accumulation of JA-inducible transcripts and defense-related secondary metabolites, WT and irNaMPK4 plants were treated with 20 μL of lanolin that contained methyl jasmonate (MeJA) at a concentration of 5 mg $mL^{-1}$, and plants treated with pure lanolin served as controls. qPCR was used to examine the transcript levels of several JA-inducible genes. Lanolin application did not alter the levels of any genes or secondary metabolites we investigated (data not show). Compared with those in WT plants, 2.4- and 5.4-fold higher levels of *LOX3* (*lipoxygenase 3*), a gene involved in JA biosynthesis, were seen in irNaMPK4 plants 6 h and 10 h after MeJA treatment (Figure 21A). Similarly, *AOC* (*allene oxide cyclase*) and *OPR3* (*OPDA reductase 3*), two other JA biosynthesis genes, also showed about doubled mRNA levels in irNaMPK4 plants (Figure 21B, C). However, *TD* (*threonine deaminase*), a key enzyme involved in the formation of isoleucine, showed similar levels of transcript accumulation (Figure 21D). Ten hours after MeJA treatment, 75% higher levels of *TPI* transcript were detected in irNaMPK4 plants (Figure 21E).

Moreover, important secondary metabolites were quantified 3 days after MeJA treatment. Consistent with transcript data, after MeJA treatment irNaMPK4 plants showed about 30% higher levels of TPI activity than did WT plants (Figure 22A). However, TAB emission and contents of nicotine, caffeoylputrescine, and DTGs increased to the same levels in both WT and irNaMPK4 plants (Figure 22B, C, D, and E).

From these results we conclude that NaMPK4 is specifically involved in regulating some but not all of the MeJA-induced responses in *N. attenuata*.

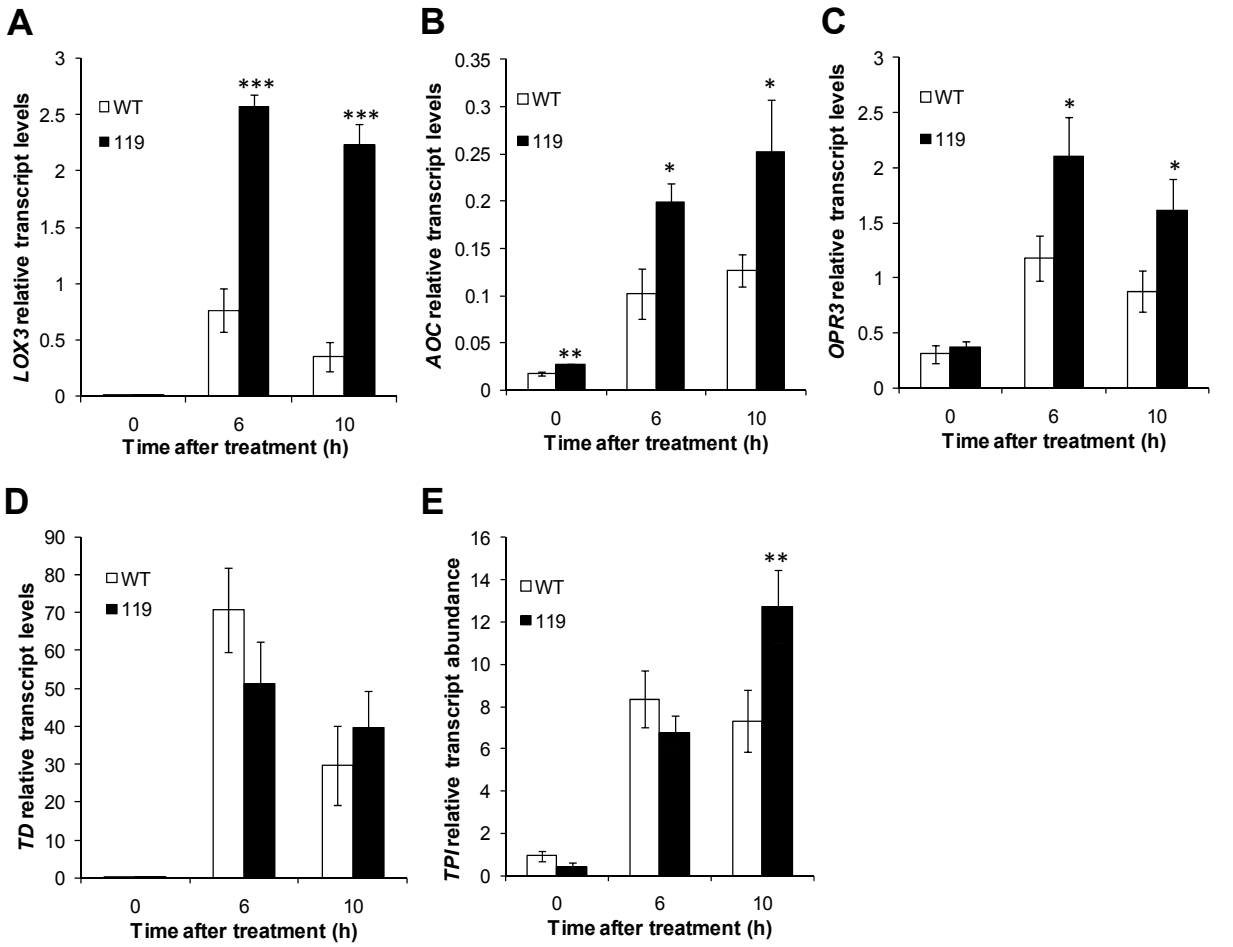



**Figure 21.** NaMPK4 modulates MeJA-induced transcript accumulation of specific genes in *N. attenuata*.

Wild-type (WT) and irNaMPK4 plants (line 119) were treated with 20 µL of lanolin containing 100 µg of MeJA. Samples were harvested at indicated times and the transcript levels of *lipoxygenase 3* (*LOX3*) **(A)**, *allene oxide cyclase* (*AOC*) **(B)**, *OPDA reductase 3* (*OPR3*) **(C)**, and *threonine deaminase* (*TD*) **(D)**, and *trypsin proteinase inhibitor* (*TPI*) **(E)** were analyzed with qPCR. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $N = 5$).

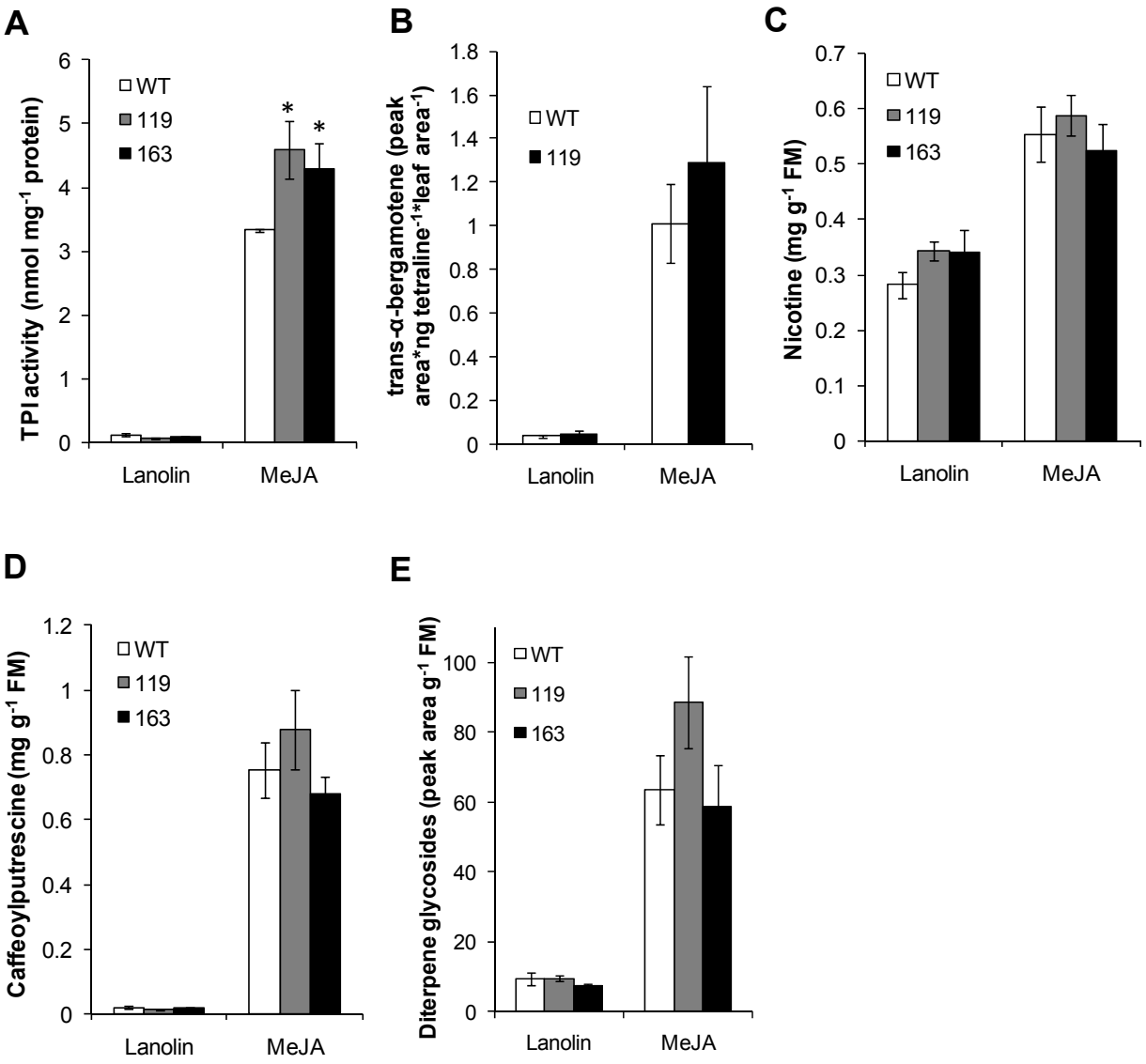
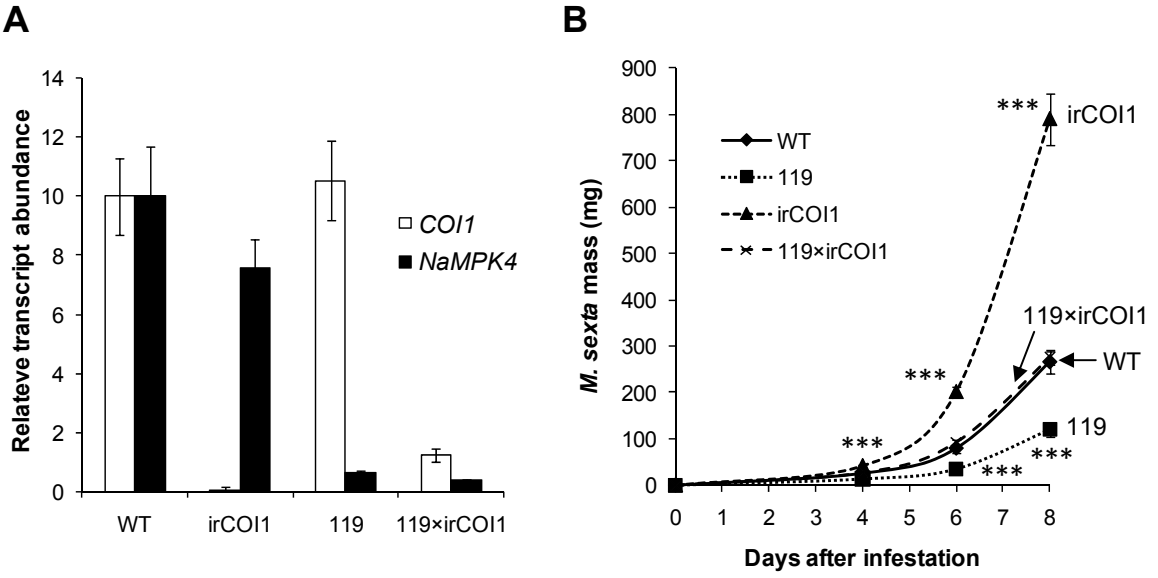



**Figure 22.** Levels of defense-related plant secondary metabolites in wild-type and irNaMPK4 plants after MeJA treatment.

Wild-type (WT) and irNaMPK4 plants (line 119 and 163) were treated with 20 µL of lanolin containing 100 µg of MeJA. Plants treated with lanolin were used for comparisons. **(A)** Trypsin proteinase inhibitor (TPI) activity 3 days after treatments. **(B)** *trans*-*α*-bergamotene emission 1 day after treatments. Contents of nicotine **(C)**, caffeoylputrescine **(D)**, and diterpene glycosides (DTGs) **(E)** (mean ± SE) in WT and irNaMPK4 plants 3 days after treatments. Asterisks indicate significant differences between WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; $N = 5$).

### 3.5.6. NaMPK4's effects on *M. sexta* resistance are largely independent of JA signaling

In *N. attenuata*, the deployment of all known inducible direct and indirect defenses is dependent on JA signaling (Paschold et al., 2007). Although irNaMPK4 plants have increased JA levels after OS elicitation, only one of the known direct defensive compounds, TPI, showed moderately elevated levels (Figure 17A). Given the dramatically enhanced resistance levels of irNaMPK4 plants against *M. sexta* attack, we speculated that NaMPK4 may also regulate a defense pathway that is independent of JA signaling. To test this hypothesis, a *N. attenuata* line silenced in *COI1* (irCOI1 plants) (Paschold et al., 2007) was crossed with irNaMPK4 (line 119) to obtain 119×irCOI1 plants, which were silenced in both *NaMPK4* and *COI1* (Figure 23A). *M. sexta* neonates were placed on WT, irCOI1, irNaMPK4 (line 119), and 119×irCOI1 plants and herbivore growth was recorded over 8 days (Figure 23B). After 8 days, compared with larvae fed on WT plants, *M. sexta* larvae on irNaCOI1 plants gained around 2 times more mass, whereas the mass of larvae fed on irNaMPK4 plants were 50% less than on WT plants. Importantly, although silencing *COI1* in irNaMPK4 plants clearly increased the mass of *M. sexta* allowing them to attain the mass of those on WT plants, these larvae still only weighed 1/3 of the mass of the larvae reared on irCOI1 plants (Figure 23B). We further analyzed the secondary metabolites involved in direct and indirect defenses against *M. sexta*, and confirmed that 119×irCOI1 plants had substantially diminished levels of TPI, nicotine, caffeoylputrescine, DTGs, α-duprezianene and TAB owing to silencing of *COI1* (Figure 23C).



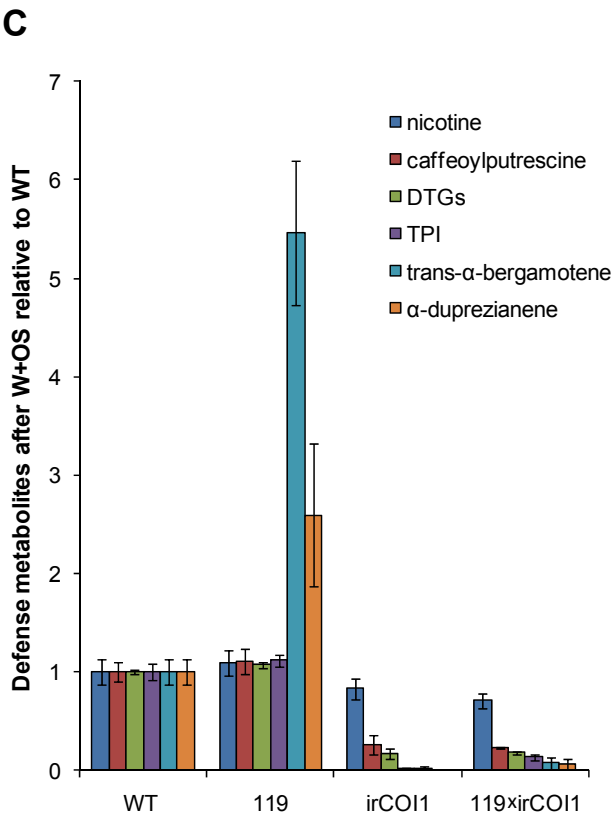



**Figure 23.** Silencing *NaMPK4* enhances *N. attenuata*'s resistance to the specialist herbivore *M. sexta* in a JA signaling-independent manner.

**(A)** Transcript levels (mean ± SE) of *COI1* and *NaMPK4* in wild-type (WT), irCOI1, irNaMPK4 (line 119), and 119×irCOI1 plants. Samples were collected from 5 replicate rosette-stage plants, and the transcript levels were measured with qPCR. Transcript levels of *COI1* and *NaMPK4* were normalized with the levels of these genes in WT plants, which were designated as 10. **(B)** Wild-type (WT), irNaMPK4 (line 119), irCOI1, and 119×irCOI1 plants were infested with 30 neonate *M. sexta* larvae (1 larva/plant). Mass of these larvae (mean ± SE) was recorded on day 4, 6, and 8. Asterisks indicate significant differences between the mass of larvae reared on WT and irNaMPK4, irCOI1, or 119×irCOI1 plants (*t*-test; ***, $P < 0.001$, $N = 30$). **(C)** Levels (mean ± SE) of nicotine, caffeoyl-putrescine, diterpene glucosides (DTGs), trypsin proteinase inhibitor (TPI), *α*-duprezianene and *tans-α*-bergamotene in 5 replicated WT, irCOI1, irNaMPK4 (line 119), and 119×irCOI1 plants. Plants were treated with W+OS, and the levels of these compounds were quantified. Their levels in WT plants were designated as 1, and their levels in other types of plants were normalized those in WT plants.

To examine whether the resistance of irNaMPK4 plants to *M. sexta* is largely JA signaling-independent, WT and irNaMPK4 plants were infested with 18 *M. sexta* neonates (2 larvae/plant), and after each 24 h all the larvae were moved to new uninduced plants. Since all JA-induced direct defenses require more than 24 h for their biosynthesis and accumulation to attain effective levels (Baldwin et al., 1998; Wu et al., 2006; Heiling et al., 2010; Kaur et al., 2010), relocating larvae to new uninduced plants reduces the intake of defensive compounds and results in elevated larval mass (Paschold et al., 2007). Consistent with the observation that silencing *NaMPK4* enhances defense in *N. attenuata* plants in a JA-independent fashion, larvae on irNaMPK4 plants still consumed less leaf tissue and grew slower than those reared on WT plants (Figure 24A, B).

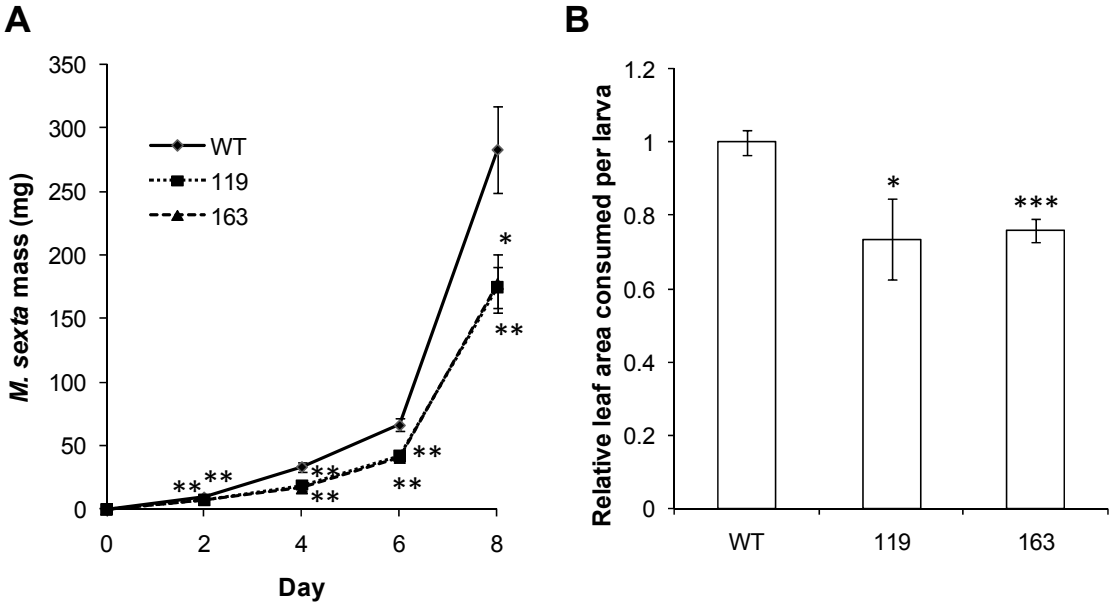
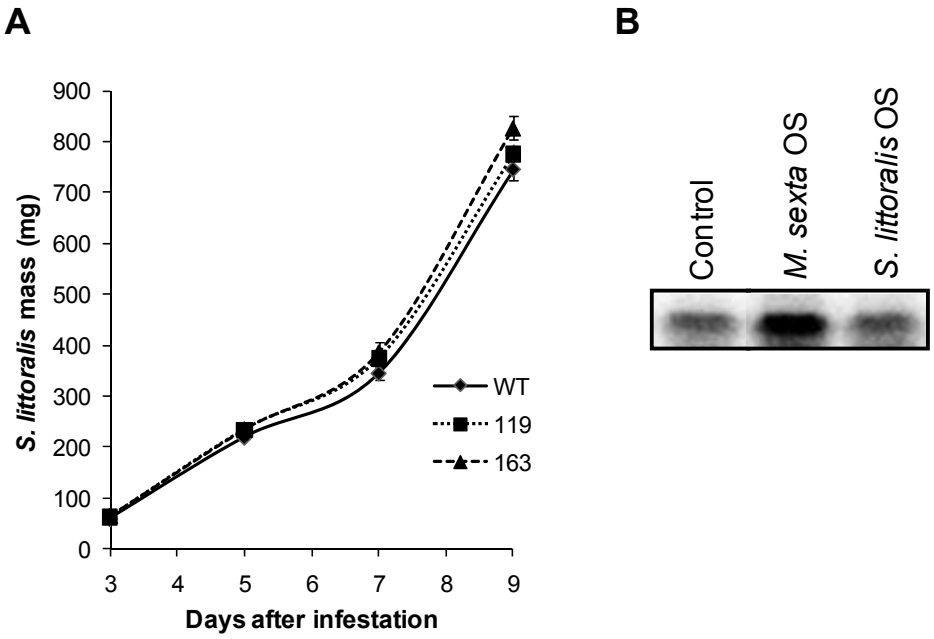



**Figure 24.** irNaMPK4 plants exhibit higher resistance to *M. sexta* than do wild-type plants even when larvae are relocated to unelicited plants every 24 h.

*N. attenuata* wild-type (WT) and irNaMPK4 plants (line 119 and 163) were infested with 18 *M. sexta* neonates (2 larvae/plant). These larvae were relocated to non-treated plants of the same genotype after every 24 h. **(A)** The mass of larvae (mean ± SE) was recorded on day 2, 4, 6 and 8. **(B)** Relative comparison of the leaf areas (mean ± SE) consumed by *M. sexta* on WT and irNaMPK4 plants after the first 24 h of *M. sexta* feeding. Asterisks indicate significant differences between values obtained from WT and irNaMPK4 plants (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N=18).

### 3.5.7. Silencing *NaMPK4* does not affect *N. attenuata*'s resistance to the generalist herbivore *Spodoptera littoralis*

To determine whether the NaMPK4 is also involved in the interactions between *N. attenuata* and other chewing herbivores, we performed feeding experiments with the polyphagous pest insect *Spodoptera littoralis* (Lepidoptera, Noctuidae). Larvae were grown on artificial diet for 10 d and afterwards placed on 20 replicate plants of each genotype. Larval mass was measured after 3, 5, 7 and 9 days. *S. littoralis* feeding on WT and irNaMPK4 plants performed equally well (Figure 25A). Thus, silencing *NaMPK4* does not enhance the defense levels of *N. attenuata* against generalist *S. littoralis*. We collected *S. littoralis* OS ($OS_{SL}$) from insects that were fed on *N. attenuata* WT plants for several days. NaMPK4 activity induced by W+$OS_{SL}$ treatment was analyzed with an immune-complex kinase assay (Figure 25B). In contrast to leaves treated with *M. sexta* OS, after W+$OS_{SL}$ treatment *N. attenuata* didn't elevate NaMPK4 activity. Because FACs in the OS of *M. sexta* is important for activation of NaMPK4, we measured the composition of these elicitors in *M. sexta* and *S. littoralis* OS. Levels of the most abundant FACs in *S. littoralis* OS were about 500-times lower than in *M. sexta* OS (Figure 25C).



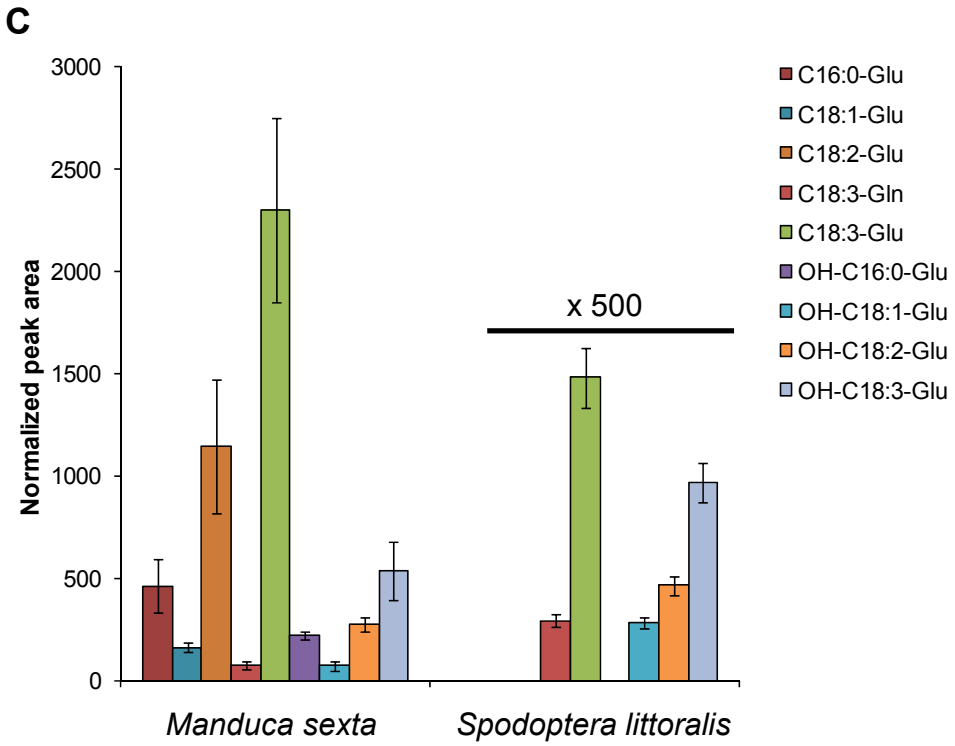



**Figure 25.** Silencing *NaMPK4* does not change plant resistance to generalist herbivore *Spodoptera littoralis*.

**(A)** Larvae were grown on artificial diet for 10 d and then placed on 20 replicate plants of each genotype (1 larva each). Larval mass gain (mean ± SE) was measured 3, 5, 7, and 9 days after being transferred to the plants. **(B)** Immune-complex kinase assay of NaMPK4 activity after treatment with *M. sexta* or *S. littoralis* OS. WT plants were treated with W+OS, and samples were collected after 10 min. NaMPK4 were immunoprecipitated with the anti-NtMPK4 antibody, and its activity levels were assayed with myelin basic protein as a substrate. **(C)** FAC profiles in *M. sexta* and *S. littoralis* OS. Values are relative concentrations of the most abundant FACs of three pooled samples from 10 third- to fifth-instar larvae of each species that were feeding on wild-type *N. attenuata* plants. N-palmitoyl-L-Glu (C16:0-Glu), N-olyl-L-Glu (C18:1 Glu), N-linoleoyl-L-Glu (C18:2 Glu), N-linolenoyl-L-Gln (C18:3-Gln), N-linolenoyl-LGlu (C18:3-Glu), N-hydroxypalmitoyl-L-Glu (OH-C16:0-Glu), N-hydroxyolyl-L-Glu (OH-C18:1 Glu), N-hydroxylinoleoyl-L-Glu (OH-C18:2 Glu), N-hydroxylinolenoyl-LGlu (OH-C18:3-Glu). Note the changes in y-axis scale (500x) to better visualize less abundant FACs in *S. littoralis* OS.

# 4. Discussion

MAPK signal transduction cascades have long been recognized as critical regulators in various aspects of cellular processes in all eukaryotes. However, their roles in plant development and stress responses remain largely unknown. Using a reverse genetic approach, we examined the functions of a MAPK, NaMPK4, in regulating various ecologically important traits in *N. attenuata*. Silencing *NaMPK4* decreases plant size and biomass, but enhances photosynthetic rate and fecundity. We also show that NaMPK4 controls at least two aspects of ABA-induced responses: plant drought tolerance mediated by ABA-induced stomatal closure and ABA-mediated seed germination inhibition. Moreover, NaMPK4 functions in pathogen defense likely by preventing bacteria from entering through stomata and suppressing bacterial amplification after they invade intercellular spaces. Furthermore, we show that NaMPK4 plays important roles in plant resistance to herbivores.

**NaMPK4 and plant fitness**

Although NaMPK4 is phylogenetically related to AtMPK4, Arabidopsis *mpk4* mutant and irNaMPK4 have distinct growth phenotypes. The Arabidopsis *mpk4* mutant is severely dwarfed and this results partly from their highly elevated SA levels (Petersen et al., 2000); however under glasshouse conditions, which were optimized for *N. attenuata* growth (sufficient fertilization and lightning, automated watering, and minimized pest and pathogen stress), *NaMPK4*-silenced plants exhibit only slightly smaller rosette sizes and moderately shorter stalk lengths than do WT plants. NaMPK4 appears to modulate plant development in a SA-independent manner, since irNaMPK4 plants have the same levels of SA as WT plants and minimizing SA contents of irNaMPK4 plants by overexpressing *NahG* does not restore WT growth rates in irNaMPK4 plants. This is consistent with the dwarf phenotype of *NtMPK4*-silenced *N. tabacum* plants, which also does not have altered basal SA levels, although the rosette sizes of *NtMPK4*-silenced *N. tabacum* plants are more severely reduced (Gomi et al., 2005), which might result from species-specific differences. However, given that irNaMPK4 transpires water more rapidly than does WT, the possibility that irNaMPK4 is always suffering from low levels of drought stress even under well-watered glasshouse condition, which leads to its reduced size, cannot be ruled out.

Importantly, silencing *NaMPK4* considerably enhances photosynthetic rates. *NtMPK4*-silenced tobacco has enlarged guard cells and decreased stomatal closure in response to ozone and $CO_2$ (Gomi et al., 2005; Marten et al., 2008). Similarly, irNaMPK4 plants have increased stomatal sizes and greatly elevated stomatal conductance. In addition, the high chlorophyll contents of irNaMPK4 plants apparently also contributes to the increased photosynthetic rates especially at the later stage of development when substantial degradation of chlorophyll happens in WT plants. Chlorophyll content is one of the most important markers for plant senescence (Lim et al., 2007). The high chlorophyll contents in irNaMPK4 plants in their late stage of development suggest that irNaMPK4 plants have delayed senescence. The mechanism by which NaMPK4 negatively regulates senescence is unknown. Biosynthesis and degradation both control the levels of chlorophyll in plants. irNaMPK4 plants have marginally higher levels of chlorophyll contents when they are in the rosette stage; however, over time the levels of chlorophyll contents in irNaMPK4 plants become increasingly higher than in WT plants. Examining the rates of chlorophyll synthesis and degradation especially at later developmental stages will clarify the function of NaMPK4 in modulating the accumulation of chlorophyll.

When soil nitrogen content is favorable, photosynthetic rate is usually positively correlated with plant biomass (Fichtner et al., 1993; Richards, 2000; Mitra and Baldwin, 2008). However, despite having elevated photosynthetic rate, the above-ground biomass of irNaMPK4 plants is 27% decreased. Seed production is a complex trait controlled at least by photosynthetic rate, sink strength, duration of photosynthesis, and plant architecture (Richards, 2000; Zhu et al., 2010). Silencing *NaMPK4* greatly increases the number of flowers which enlarges the reproductive sink capacity of irNaMPK4 plants. We speculate that this results from the apparent delayed senescence of these plants. Genetically modifying crop architecture, in particular, shortening stalk lengths was central to the improved seed yields during the “green revolution” (Khush, 2001; Zhu et al., 2010). It is conceivable that the semidwarf stature of irNaMPK4 plants also contributes to the increased yield of irNaMPK4 plants. Hence given their highly enhanced photosynthetic rate, sink capacity, and shortened stalks, it is not surprising that irNaMPK4 plants have greater yield of seeds than do WT plants.

**NaMPK4 functions in ABA signaling**

Guard cells play critical roles in controlling water conservation in response to drought stress, rates of $CO_2$ assimilation, and pathogen infections. Drought-treated irNaMPK4 plants accumulate high levels of ABA but maintain elevated transpiration rates and in epidermal peels guard cell have strongly impaired closure responses after ABA treatment, suggesting NaMPK4 acts downstream of ABA in regulating drought stress-activated stomatal closure in *Nicotiana attenuata*. In contrast, silencing the orthologue of NaMPK4 in tobacco, NtMPK4, does not affect ABA-induced stomatal closure response (Gomi et al., 2005; Marten et al., 2008). The discrepancy might result from species-specific differences.

Many proteins and small molecules are involved in ABA-induced stomatal closure (Schroeder et al., 2001; Desikan et al., 2004; Fan et al., 2004; Kim et al., 2010). ABA is perceived by a small family of receptor proteins, PYR/PYL/RCARs (Ma et al., 2009; Park et al., 2009). Binding of these ABA-bound receptors to PP2Cs (a small group of kinase phosphatases) inhibits their phosphatase activity. This leads to increased activity of SnRK2s (targets of PP2C2) and thereby enhances the production of reactive oxygen species (ROS), which is important for activating downstream reactions and finally leading to stomatal closure (Kim et al., 2010). Several studies have demonstrated that in Arabidopsis MAPKs are involved in ABA signaling. *AtMPK3* is important in various stress responses (Tena et al., 2001; Zhang and Klessig, 2001; Rodriguez et al., 2010); specifically silencing *AtMPK3* in Arabidopsis guard cells also reduces $H_2O_2$-induced inhibition of stomatal opening or promotion of stomatal closure (Gudesblat et al., 2007). Importantly, *AtMPK9* and *AtMPK12* are mainly localized in guard cells and plants silenced in both MAPKs have strong defects in ABA-induced stomatal closure, since AtMPK9 and AtMPK12 act downstream of ROS to regulate the activity of anion channels (Jammes et al., 2009). Similar to these MAPKs in Arabidopsis, NaMPK4 appears to be also located downstream of ROS to mediate ABA-induced stomatal closure. Notably, silencing *NtMPK4* in tobacco compromises stomatal closure when plants are exposed to ozone (Gomi et al., 2005). Ozone exposure quickly induces ROS and several proteins that are important for ABA signaling are also required for ozone-induced stomatal closure (Vahisalu et al., 2010). This supports the hypothesis that in *Nicotiana* MPK4 is located downstream of $H_2O_2$ in promoting stomatal closure. Moreover, both knocking down *NtMPK4* in *N. tabacum* and *NaMPK4* in *N. attenuata* abolishes dark-induced stomatal closure response (Marten et al., 2008). The S-type anion channel SLAC1 is

important for stomatal closure in response to ABA, $H_2O_2$, nitric oxide, $Ca^{2+}$, and light/dark transitions (Vahisalu et al., 2008; Kim et al., 2010). Given that NtMPK4 is required for the activation of S-type anion channels during light-dark transitions (Marten et al., 2008), it is likely that both ABA/$H_2O_2$- and darkness-induced signaling pathways converge on NaMPK4 which modulates the activity of S-type anion channels and thus stomatal closure response.

Apparently, NaMPK4 is not located in all the ABA-regulated pathways. NaMPK4 functions in ABA-induced stomatal closure and germination, but ABA does not inhibit irNaMPK4 root growth. Similarly, compared with WT, irNaMPK4 seeds are less sensitive to salinity and MeJA during germination, but do not have a root growth phenotype in response to salt and MeJA. Since ABA signaling is largely overlapped with salt- and MeJA-activated pathways (Zhu, 2002; Acharya and Assmann, 2009), it is not surprising that irNaMPK4 shows similar phenotype when being treated with ABA, salt, and MeJA. Organ-specific analysis of NaMPK4 expression ruled out the possibility that NaMPK4 is not expressed in roots and therefore not involved in mediating ABA-inhibited root growth. Moreover, by growing WT and irNaMPK4 in the same pots, we minimized the differences in levels of drought stress resulting from differential stomatal control and examined whether NaMPK4 is involved in transcriptional regulation of drought-responsive genes. It seems that NaMPK4 is not involved in certain, if not all, the regulatory pathways that control ABA-induced transcriptional responses. Since whole leaves were used for transcriptional analyses, it is feasible that NaMPK4 regulates transcriptional changes specifically in guard cells but not in mesophyll cells.

**NaMPK4 and defense against bacterial pathogens**

The important role of guard cell in resistance to the bacterial pathogen *Pst* DC3000 has been demonstrated in Arabidopsis: plants sense *Pst* DC3000 by perceiving flagellin and lipopolysaccharides and produce NO in guard cells, which leads to stomatal closure and blocks bacterium entry (Melotto et al., 2006). A striking difference was found between the *Pst* DC3000 population in WT and irNaMPK4 after dipping leaves in bacterial suspensions. We detected almost no bacteria in WT but found more than $10^6$ cfu/$cm^2$ in irNaMPK4. In contrast, when bacteria enter plant tissue without the barrier of stomata (directly by infiltration), by 24 h *Pst* DC3000 amplified similarly in WT and irNaMPK4, whereas by 48 h the number of bacteria in irNaMPK4 plants was only about 25 fold higher than in WT. It is very likely that the stoma-mediated defense is strongly compromised in irNaMPK4 plants.

Strikingly, silencing *NaMPK4* does not influence bacterium-induced stomatal closure and even irNaMPK4 plants exhibit slightly faster closure speeds. These data strongly suggest that irNaMPK4 still possesses intact components of the signaling pathway which controls pathogen-induced stomatal closure. In Arabidopsis, ROS is important for both ABA- and bacterial pathogen-induced stomatal closure (Kwak et al., 2003; Mersmann et al., 2010). The stomata of irNaMPK4 have highly decreased sensitivity to ABA but normal (or even slightly stronger) response to *Pst* DC3000. Given that the stomata closure of irNaMPK4 is partially abolished after $H_2O_2$ treatment, it is possible that the guard cells of *N. attenuata* respond to *Pst* DC3000 in a ROS-independent pathway. The species-specific difference between *N. attenuata* and Arabidopsis in response to surface-landed bacteria also includes that guard cells in both the epidermis and intact leaves of Arabidopsis react to pathogens, but only *N. attenuata* leaves and not epidermal peels close stomata after *Pst* DC3000 treatments. It seems that the *N. attenuata* guard cells require a signal derived from mesophyll cells to complete closure after bacterial elicitation.

Although the greater transpiration rates of irNaMPK4 indicated that after applying bacteria on their leaf surfaces, the stomatal openings of irNaMPK4 remain larger than those of WT and this difference is maintained for least 1 h, it is unlikely that the greater stomatal apertures of irNaMPK4 solely account for the highly compromised defense against surface-landed *Pst* DC3000. Using an unknown mechanism, *Pst* DC3000 specifically move to open but not closed stomata of Arabidopsis and invade plants (Melotto et al., 2006). We speculate that the decreased levels of resistance to intercellular pathogens and probably more importantly, an unknown form of guard cell-mediated defense are abolished in irNaMPK4, which enhances the attraction of stomata to bacteria and therefore results in greater number of invading pathogens. Additionally or alternatively, highly increased survival rates of *Pst* DC3000 after entering the stomata of irNaMPK4 but before their amplify intercellular amplification can also not be ruled out.

In Arabidopsis, AtMPK4 plays a negative role in resistance against *Pst* DC3000 in a SA-dependent manner (Petersen et al., 2000). In contrast, silencing *NaMPK4* reduces *N. attenuata*'s resistance to *Pst* DC3000. Analysis of *Pst* DC3000 population growth in WT and irNaMPK4 plants with different SA levels (exogenously SA application and endogenously removal of SA by *NahG* expression) indicated that the resistance of *N. attenuata* to *Pst* DC3000 is not mediated by SA. In line with this, we found that NPR1, a protein required for many (but not all) SA-induced

defense responses (Durrant and Dong, 2004), is also not involved in *N. attenuata*'s resistance to *Pst* DC3000. Although SA and its signaling have long been known to be required for plant resistance to many biotrophic pathogens, SA-independent pathways have been uncovered in certain plant-pathogen interactions (Hauck et al., 2003; Zhang et al., 2003; Bartsch et al., 2006). The molecular basis of NaMPK4's function in *N. attenuata*'s interaction with *Pst* DC3000 deserves further study.

**NaMPK4 and plant resistance to herbivores**

Thus far, only two MAPKs, SIPK and WIPK, were known to play important roles in plant-herbivore interactions (Kandoth et al., 2007; Wu et al., 2007). Silencing *SIPK* and *WIPK* greatly compromises *M. sexta* herbivory-induced JA and ethylene accumulation, which in turn reduces direct and indirect defenses (Kandoth et al., 2007; Wu et al., 2007; Meldau et al., 2009). Using bioassays, we demonstrate a surprising role of NaMPK4 in plant-herbivore interactions: silencing *NaMPK4* greatly increases the defense levels of *N. attenuata* against *M. sexta* in a JA-independent manner.

It is likely that similar to SIPK and WIPK, NaMPK4 is also located downstream of the putative FAC receptors (Wu and Baldwin, 2010) and the binding of FACs to the FAC receptors triggers the activation of SIPK, WIPK, and NaMPK4, although these MAPKs appear to be located downstream of different MAPKKs: MEK2 phosphorylates SIPK and WIPK (Zhang and Liu, 2001), and SIPKK seems likely to be responsible for the phosphorylation of NaMPK4 (Gomi et al., 2005) (Figure 26). Unlike SIPK and WIPK, which are activated by both herbivory and wounding, NaMPK4 is only activated by herbivory. Furthermore, the activation of NaMPK4 after W+OS is very transient – 10 min after W+OS treatment the activity level of NaMPK4 is rapidly elevated 1 fold and this quickly drops to basal levels within 30 min. In comparison, after W+OS treatment SIPK activity increases many times and these elevated levels are maintained for more than 1 h (Wu et al., 2007). SIPK mediates W+OS-induced JA levels for longer than does NaMPK4 and this is consistent with the longer duration of the activation of SIPK (Wu et al., 2007). Both SIPK and WIPK positively regulate the levels of JA after wounding or herbivory challenge (Wu et al., 2007); in contrast, NaMPK4 is a negative regulator of herbivory-induced JA accumulation (Figure 26). How these MAPKs alter the activity and/or substrate availability of JA biosynthetic enzymes merit further attention. Moreover, the biosynthesis of chlorophyll and the precursors of JA all take place in the chloroplasts. The altered chlorophyll contents and

herbivory-induced JA levels in irNaMPK4 plants suggest a connection between NaMPK4 and chloroplast function.

Only one known direct defensive compound, TPI (a direct defense), had somewhat elevated levels in W+OS-induced irNaMPK4 plants and this is consistent with the relatively transient and moderate elevation of the W+OS-induced JA burst in irNaMPK4 plants. Furthermore, using irNaMPK4×irCOI1 plants, which are deficient in both *NaMPK4* and *COI1*, we examined the contribution of JA signaling in irNaMPK4's defense against *M. sexta*. Strikingly, in spite of highly compromised levels of all known anti-herbivore compounds, irNaMPK4×irCOI1 plants still retained their elevated resistance levels. Clearly, silencing *NaMPK4* enhances certain direct defense(s) that is (are) independent of JA signaling (Figure 26). Given that silencing *COI1* only reduces the resistance levels of irNaMPK4 plants to those of WT plants, this JA-independent defense in irNaMPK4 plants is remarkably effective against *M. sexta*. Moving *M. sexta* larvae to non-treated plants every 24 h, which avoids the full accumulation of JA-inducible defensive compounds, still resulted in reduced growth rates of *M. sexta* on irNaMPK4 plants, which suggests that the resistance is rapidly activated in irNaMPK4 plants, i.e. in less than 24 h, or is constitutively expressed. The identity of this JA-independent defense is still unclear. In *N. attenuata*, the biosynthesis of this (these) unknown defensive compound(s) must be highly suppressed by NaMPK4. Elucidating the biosynthesis of this defensive compound and ectopically expressing the components of its biosynthetic pathway in crops may be used for plant protection.

In contrast, *S. littoralis* larvae feeding on *NaMPK4*-silenced plants gained similar weight to those on WT plants. If the NaMPK4-regulated defensive metabolite is constitutively expressed in *NaMPK4*-silenced plants, *S. littoralis* must be highly resistant to this compound. Alternatively, this defensive metabolite might not be biosynthesized after *S. littoralis* feeding. Analysis of the FAC contents in the oral secretions of *S. littoralis* larvae revealed that the FAC levels are about 500-times lower than in *M. sexta* OS. The immunoblotting analysis showed that NaMPK4 is activated by FACs in the OS of *M. sexta* but not by the OS of *S. littoralis*. Given the critical role of FACs in eliciting herbivory-induced responses, including MAPK activation, phytohormone accumulation, and biosynthesis of defensive compounds, it is likely that the low contents of FACs in *S. littoralis* OS result in lack of recognition of herbivory in *N. attenuata* and thus *S. littoralis* larvae perform equally well on WT and irNaMPK4 plants.

**Regulation of transcript accumulation and secondary metabolites in irNaMPK4 plants**

Silencing *NtMPK4* in tobacco leads to decreased levels of wounding-induced *PI-II* transcripts, but *AOS* transcript levels were not altered (Gomi et al., 2005). irNaMPK4 plants exhibit increased levels of W+OS-induced TPI activity and TAB emission, while W+OS-induced levels of nicotine, caffeoylputrescine, and DTGs, which are also important herbivore resistance-related compounds, are not mediated by NaMPK4. In Arabidopsis, AtMPK4 is required for the upregulation of JA-responsive genes, *PDF1.2* and *THI2.1*, after MeJA treatment (Petersen et al., 2000). Silencing *NaMPK4* alters the transcript accumulation of some but not all genes that were examined after MeJA treatment. Similarly, after MeJA induction, among herbivore defense-related compounds, only TPI showed increased levels in irNaMPK4 plants compared to WT plants. These findings demonstrate that NaMPK4 specifically modulates the levels of certain but not all herbivory- and jasmonate-induced transcripts and defensive secondary metabolites.

The regulation of NaMPK4 on TAB, an indirect defense, is particularly intriguing. After wounding, irNaMPK4 plants emit about 1 fold higher amount of TAB. However, after W+OS treatment, the amount of TAB produced in irNaMPK4 plants is about 5 fold higher than in WT plants, which is similar to that produced in MeJA-induced WT and irNaMPK4 plants (Figure 18A and 22B). Given that these remarkably elevated levels of TAB in W+OS-treated irNaMPK4 plants are abolished when *COI1* is also silenced, NaMPK4 appears to suppress herbivory-induced TAB biosynthesis in a JA signaling-dependent manner. We speculate that NaMPK4 is a suppressor of certain JA signaling-regulated transcription factors which activate the biosynthesis of TAB, and this suppression function of NaMPK4 is probably induced by W+OS, presumably the FAC components in *M. sexta* OS (Figure 26).

Several WRKY transcription factors are known to be involved in MAPK signaling. They are direct phosphorylation targets of MAPKs or are transcriptionally regulated by MAPKs (Asai et al., 2002; Kim and Zhang, 2004; Menke et al., 2005). In *N. attenuata*, JA elicitation, the accumulation of TPI and α-bergamotene are regulated by two WRKY transcription factors, WRKY3 and WRKY6, as silenced lines do not induce these defenses after herbivory and consequently were highly vulnerable to herbivores (Skibbe et al., 2008). *NaMPK4*-silenced plants accumulate higher *WRKY3* and *WRKY6* transcript levels after W+OS; they elicit 50% more JA and have higher levels of TPI and 5-fold more α-bergamotene than WT plants. It is tempting to speculate that NaMPK4 controls the expression and/or activity of WRKY3 and WRKY6 or other

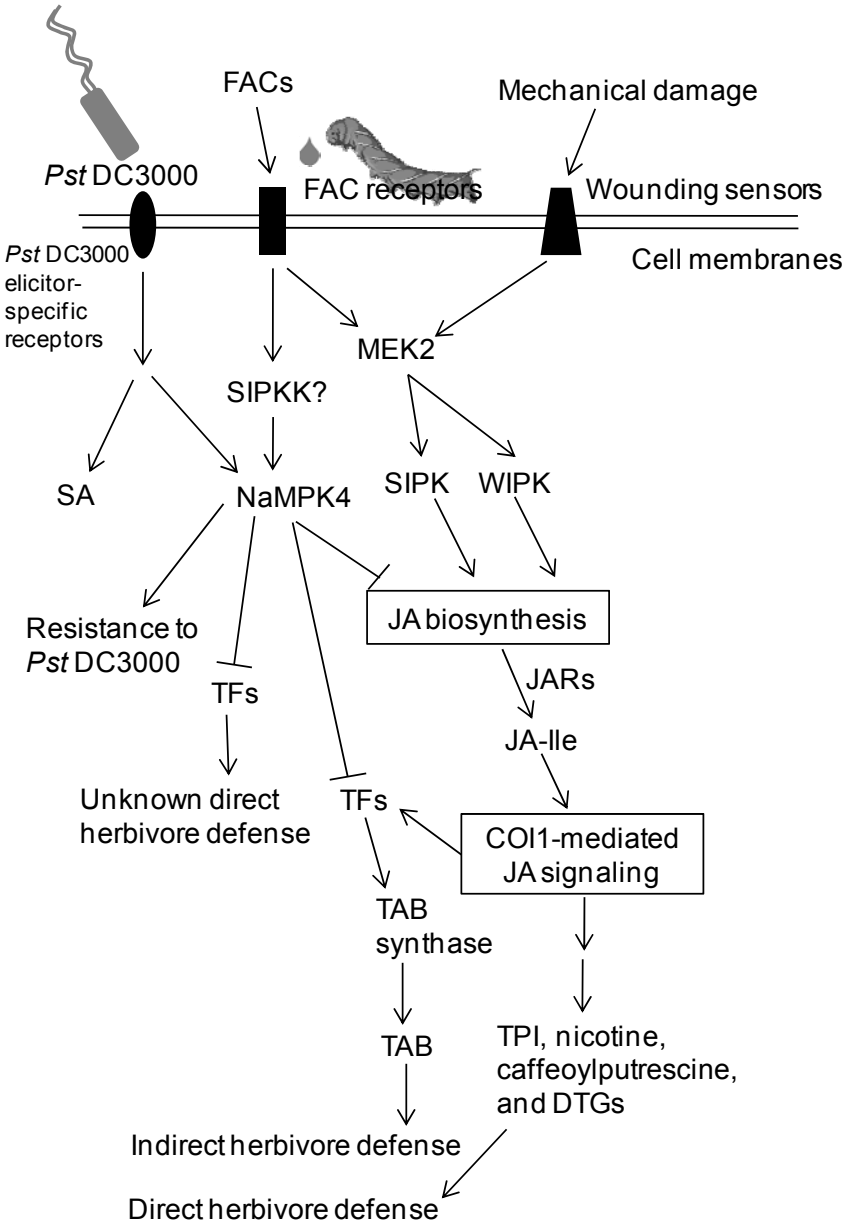



**Figure 26.** Summary of the function of NaMPK4 in *N. attenuata*'s resistance to herbivores and pathogens.

After *N. attenuata* perceives FACs in *M. sexta* oral secretions, NaMPK4, SIPK, and WIPK are rapidly activated by upstream MAPKKs, which play negative and positive roles in regulating herbivory-induced accumulation of JA. Through the COI1-mediated JA signaling, levels of trypsin proteinase inhibitor (TPI), *trans*-α-bergamotene (TAB), nicotine, caffeoylputrescine, and diterpene glycosides (DTGs) are elevated. Importantly, NaMPK4 suppresses an unknown form of potent defense against *M. sexta*. Furthermore, NaMPK4 is required for *N. attenuata*'s resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) through a pathway that is independent of SA signaling. (TFs = transcription factors)

transcription factors in *N. attenuata* and thereby plays a major role in plants defense against herbivores. Finding the direct substrates of NaMPK4, which are most probably transcription factors, will give further insight in to the mechanisms how NaMPK4 suppresses *N. attenuata*'s direct and indirect defense against *M. sexta* and strengthens plant resistance to *Pst* DC3000.

**Evolution of MPK4**

Both, phylogenetic analysis and their similar function in mediating plant development and stomatal aperture indicated that NaMPK4 and NtMPK4 are orthologues. Tobacco NtMPK4 is activated after wounding; however, NaMPK4 is not activated by wounding but only by OS-elicitation. It seems likely that *N. tabacum* and *N. attenuata* have evolved different responses to wounding which result in different patterns of regulation of MPK4 activity. Studying the function of the orthologues of NaMPK4 and NtMPK4 in other *Nicotiana* species will provide valuable insight into the evolution of MPK4 and its regulatory networks in *Nicotiana*.

Compared with Arabidopsis MAPKs, NaMPK4 has the highest similarities to AtMPK4 and AtMPK11. Although AtMPK4 is mainly localized in guard cells (Petersen et al., 2000), the drastically different phenotypes of the Arabidopsis *mpk4* mutant from those of irNaMPK4 strongly suggest that NaMPK4 has a distinct function in *N. attenuata*. Recent evidence indicates that AtMPK4 is important for cytokinesis and microtubule organization in Arabidopsis, which at least accounts for the highly retarded growth phenotype of *mpk4* mutant (Beck et al., 2010; Kosetsu et al., 2010). Given the very mild growth phenotype of irNaMPK4, it is very unlikely that NaMPK4 also functions in cytokinesis and microtubule organization. The phenotypic differences between irNaMPK4 and *mpk4* mutant seem not to result from differences in degrees of gene silencing (knock-down and knock-out), since silencing *AtMPK4* in Arabidopsis using an RNAi vector also generated extremely dwarf T1 plants which were morphologically very similar to *mpk4* and were mostly sterile like *mpk4* (data now shown). NaMPK4 is not functionally similar to AtMPK11 either: *mpk11* mutant has no detectable growth abnormalities compared with WT and our data indicate that AtMPK11 is not involved in drought and pathogen resistance. Although double mutant *mpk4 mpk11* has more severe growth phenotype than does single mutant *mpk4*, the exact function of AtMPK11 is unknown (Kosetsu et al., 2010).

Notably, although having somewhat lower identity to NaMPK4 (77%) (Figure 3A), AtMPK12 is also important for ABA- and ROS-induced stomatal closure response (Jammes et

al., 2009). Similar inconsistency between functions and sequence similarities can also be seen between AtMPK9 and AtMPK12: AtMPK9 belongs to MAPK subgroup D, while AtMPK12 is in subgroup B; however, these two proteins show functional redundancy in controlling guard cells (MAPK group, 2002; Jammes et al., 2009). It is tempting to hypothesize that the real homologues of NaMPK4 in Arabidopsis are AtMPK9 and AtMPK12. This hypothesis should be tested at least by measuring the defense levels of *mpk9*, *mpk12*, and *mpk9 mpk12* double mutant against *Pst* DC3000 and by examining whether NaMPK4 complement the function of AtMPK9 and AtMPK12 in ABA- and $H_2O_2$-induced stomatal closure and pathogen defense (if *mpk9*, *mpk12*, or *mpk9 mpk12* has decreased resistance to pathogens). Sequence analyses and functional studies of the homologues of *MPK4* in other plant species are needed to further understand the driving force underlying the evolution of *MPK4*.

## 5. Summary

MAPKs have been intensively studied in yeast and mammals. However, the functions of MAPKs in plants remain largely unknown. In this study, we show silencing a MAPK, *NaMPK4*, in a wild tobacco species, *N. attenuata*, enhances plant fitness and elevates defense levels against herbivores, but compromises resistance to pathogens and drought stress. NaMPK4 regulates certain (but not all) ABA-, salt-, and MeJA-induced responses: it is required for the inhibitory effect of these compounds in germination, but is not involved these compounds' suppression of root elongation or in the regulation of gene expression after drought or MeJA treatment. Importantly, NaMPK4 acts downstream of ABA-induced $H_2O_2$ to promote stomatal closure and is also very likely to be required for stoma-mediated pathogen defense. NaMPK4 is also specifically activated by FACs and negatively mediates a JA-independent defense pathway against insect herbivore, *M. sexta*. These data highlight the important function of NaMPK4 in mediating various ecologically important traits in *N. attenuata*. Large scale transcriptome analysis and phosphorylation target identification will shed light on the molecular basis of NaMPK4's function in plant development and resistance to biotic and abiotic stresses. Studying the performance of irNaMPK4 plants in *N. attenuata*'s natural habitat, where these plants are challenged with a full spectrum of biotic and abiotic factors, will provide further insight into the ecological and evolutionary significance of NaMPK4. Furthermore, we propose that homologues of MPK4 in agriculturally important seed crops may be potential targets for genetically modification to increase seed yield and resistance to herbivores and pathogens.

## 6. Zusammenfassung

Mitogen-aktivierte Protein Kinasen (MAPK) sind essentielle Regulatoren vieler Wachstumsvorgänge und stressinduzierter Reaktionen in fast allen Eukaryoten. Trotzdem ist, speziell in Pflanzen, die Regulation der MAPK und nachgeschalteter Signalwege noch größtenteils unerforscht. In dieser Arbeit habe ich die Funktion einer MAPK, genannt NaMPK4, in wildem Tabak (*Nicotiana attenuata*) untersucht. Dazu wurden transgene Pflanzen hergestellt, in denen die Transkription des *NaMPK4* Gens durch einen RNA-Interferenzmechanismus unterdrückt ist. Anhand dieser Pflanzen habe ich den Einfluss des MPK4 Gens auf verschiedene Charakteristika wie beispielsweise Wachstum, Samenproduktion und die Verteidigung der Pflanzen gegen Herbivoren (*Manduca sexta* und *Spodoptera littoralis*) und Pathogene (*Pseudomonas syringae* pv. *tomato* DC3000) sowie die Resistenz der Pflanzen gegen Trockenstress untersucht. Unter Gewächshausbedingungen haben Pflanzen mit ausgeschaltetem *NaMPK4* Gen kleinere Rosettendurchmesser und eine verringerte Wuchshöhe, dennoch aber höhere Photosynthesewerte und gesteigerte Samenproduktion. Die gentechnisch veränderten Pflanzen sind weniger resistent gegen Trockenstress und unsere Ergebnisse legen die Vermutung nahe, dass NaMPK4 in der Stress-induzierten Signalkaskade dem Hormon Abscisinsäure und dem Signalmolekül Wasserstoffperoxid nachgeschaltet ist und die Schließung der Spaltöffnungen der Blätter bei Trockenheit und auch bei Pathogenbefall kontrolliert. Das NaMPK4 auch Bestandteil der Verteidigung der Pflanze gegen Pathogene ist, zeigt sich insbesondere nach Pathogen Infiltration direkt in die Blätter. *Pseudomonas syringae* pv. *tomato* DC3000 vermehrt sich signifikant schneller in transgenen Pflanzen und diese im Vergleich zum Wildtyp veränderte Immunreaktion ist unabhängig von Salicylsäure und NPR1, zweier Hauptregulatoren der Pflanzenverteidigung in Arabidopsis.

NaMPK4 spielt auch eine wichtige Rolle in der Verteidigung der Pflanzen gegen Herbivorenbefall. Dabei wird NaMPK4 nicht durch die bloße mechanische Verwundung der Blätter aktiviert sondern durch Fettsäure-Aminosäure Verbindungen (FAC) im oralen Raupensekret und ist deshalb Teil der spezifischen Verteidigung der Pflanze gegen ihre Fraßfeinde. Pflanzen mit ausgeschaltetem *NaMPK4* Gen sind resistenter gegen *Manduca sexta* Raupen und diese Resistenz ist zum großen Teil unabhängig von der Jasmonsäure-induzierten Immunantwort der Pflanze,

welche normalerweise die Verteidigung gegen Herbivoren reguliert. Damit ist NaMPK4 die erste bekannte MAPK die die Verteidigung gegen Herbivoren negativ reguliert. Im Gegensatz zu den Reaktionen auf *M. sexta* ist NaMPK4 nicht in die Verteidigung gegen den Generalisten *S. littoralis* involviert, vermutlich aufgrund der geringen FAC Konzentration in deren Oralsekret. Die in dieser Arbeit gewonnenen Erkenntnisse zeigen die zentrale Rolle die NaMPK4 in Wachstum und Verteidigung des wilden Tabaks spielt. *NaMPK4*-homologe Gene in landwirtschaftlich bedeutsamen Pflanzen könnten zukünftige Ziele der Gentechnik sein um zum Beispiel deren Ertrag zu steigern und die Resistenz gegen Schädlinge zu verbessern.

## 7. Supplemental Data

**Supplemental Table 1** Sequences of primers

| Primers | Sequences (5'-3')* | Purposes |
|---|---|---|
| NaMPK4-1 | GCGGCGCTGCAGGATCATGACAATGTGATTGCC | Preparation of pRESC5-MPK4 |
| NaMPK4-2 | GCGGCGCCATGGGGTGCCCGATACCACCG | Preparation of pRESC5-MPK4 |
| NaMPK4-3 | GCGGCGGAGCTCGATCATGACAATGTGATTGCC | Preparation of pRESC5-MPK4 |
| NaMPK4-4 | GCGGCGCTCGAGGGTGCCCGATACCACCG | Preparation of pRESC5-MPK4 |
| NaMPK4-5 | TAGGAGCAACTCCGGTGCC | q-PCR of *NaMPK4* |
| NaMPK4-6 | GCAAGGACAACATCTGAGACAGAT | q-PCR of *NaMPK4* |
| NCED1-1 | ACAGCCGACCCACGTGTCCA | q-PCR of *NaNCED1* |
| NCED1-2 | CGACAAGCGTAACTTGCGGAGC | q-PCR of *NaNCED1* |
| OSM1-1 | CTGCGACTATCGAGGTCCGAAAC | q-PCR of *NaOSM1* |
| OSM1-2 | GTACCTCGTGGTGCATTGATCAC | q-PCR of *NaOSM1* |
| TAS14-1 | TGGGTGGAGAGTATGGAACC | q-PCR of *NaTAS14* |
| TAS14-2 | CCACCTTCACCATCATCCTC | q-PCR of *NaTAS14* |
| HD20-1 | CCGAGAAAGAAGGTGGACAGTATTG | q-PCR of *NaHD20* |
| HD20-2 | AGCCGAATAATCAGCCTTTATGC | q-PCR of *NaHD20* |
| Actin2-1 | GGTCGTACCACCGGTATTGTG | q-PCR of *Actin2* |
| Actin2-2 | GTCAAGACGGAGAATGGCATG | q-PCR of *Actin2* |
| AtMPK11-1-LP | TGCTCGAAATCAAAATGGAAC | *mpk11* homozygosity (border sequence) |
| AtMPK11-1-RP | AATAAGACCACCTCAGCCAGAC | *mpk11* homozygosity (border sequence) |
| Salk_LBb1.3 | ATTTTGCCGATTTCGGAAC | *mpk11* homozygosity (T-DNA sequence) |
| NaWRKY3-1 | CAGGATATGCAAATTCAGAGGATTC | q-PCR of *NaWRKY3* |
| NaWRKY3-2 | ATTCAATTCAGCAGAGCAATGTG | q-PCR of *NaWRKY3* |
| NaWRKY6-1 | ACAAAACAAAGATGAAGTTCCAAAG | q-PCR of *NaWRKY6* |
| NaWRKY6-2 | GGAGAAGCTGGTGATGAAGATG | q-PCR of *NaWRKY6* |
| TPI-1 | TCAGGAGATAGTAAATATGGCTGTTCA | q-PCR of *TPI* |
| TPI-2 | ATCTGCATGTTCCACATTGCTTA | q-PCR of *TPI* |
| LOX3-1 | GGCAGTGAAATTCAAAGTAAGAGC | q-PCR of *LOX3* |
| LOX3-2 | CCCAAAATTTGAATCCACAACA | q-PCR of *LOX3* |
| TD-1 | TAAGGCATTTGATGGGAGGC | q-PCR of *TD* |
| TD-2 | TCTCCCTGTTCACGATAATGGAA | q-PCR of *TD* |
| OPR3-1 | ATGCCAGATGGAACTCATGCTATTT | q-PCR of *OPR3* |
| OPR3-2 | TATGAATTTGCAACGGTTGGCTAGT | q-PCR of *OPR3* |
| AOC-1 | ATCGTACTTGACTTACGAGGATACT | q-PCR of *AOC* |
| AOC-2 | TCACAAGCTTTAGCTTCAGGTGCTT | q-PCR of *AOC* |

* Nucleotides underlined in primers NaMPK4-1, NaMPK4-2, NaMPK4-3, and NaMPK4-4 are *Pst* I, *Nco* I, *Sac* I, and *Xho* I sites, respectively.

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## 10. Curriculum vitae

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## 11. Selbstständigkeitserklärung

Ich erkläre, entsprechend § 5 Abs. 3 der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena, dass mir die geltende Promotionsordnung der Fakultät bekannt ist. Ich habe die Dissertation selbständig und nur unter Zuhilfenahme der im Text angegebenen Quellen und Hilfsmittel angefertigt. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Diese Dissertation wurde von mir niemals zuvor als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Desweiteren habe ich keine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht.

Christian Hettenhausen

Jena, am 05. Juli 2011