Silencing MPK4 in *Nicotiana attenuata* Enhances Photosynthesis and Seed Production But Compromises Abscisic Acid-Induced Stomatal Closure and Guard Cell-Mediated Resistance to *Pseudomonas syringae* pv *tomato* DC3000

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Mitogen-activated protein kinases (MAPKs) play pivotal roles in development and environmental interactions in eukaryotes. Here, we studied the function of a MAPK, NaMPK4, in the wild tobacco species *Nicotiana attenuata*. The NaMPK4-silenced *N. attenuata* (irNaMPK4) attained somewhat smaller stature, delayed senescence, and greatly enhanced stomatal conductance and photosynthetic rate, especially during late developmental stages. All of these changes were associated with highly increased seed production. Using leaf epidermal peels, we demonstrate that guard cell closure in irNaMPK4 was strongly impaired in response to abscisic acid and hydrogen peroxide, and consistently, irNaMPK4 plants transpired more water and wilted sooner than did wild-type plants when they were deprived of water. We show that NaMPK4 plays an important role in the guard cell-mediated defense against a surface-deposited bacterial pathogen, *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000; in contrast, when bacteria directly entered leaves by pressure infiltration, NaMPK4 was found to be less important in the resistance to apoplast-located *Pst* DC3000. Moreover, we show that salicylic acid was not involved in the defense against *Pst* DC3000 in wild-type and irNaMPK4 plants once it had entered leaf tissue. Finally, we provide evidence that NaMPK4 functions differently from AtMPK4 and AAMPK11 in Arabidopsis (*Arabidopsis thaliana*), despite their sequence similarities, suggesting a complex functional divergence of MAPKs in different plant lineages. This work highlights the multifaceted functions of NaMPK4 in guard cells and underscores its role in mediating various ecologically important traits.

Mitogen-activated protein kinases (MAPKs) are a class of important proteins that are ubiquitous among all eukaryotes. MAPK cascades are usually located downstream of sensors or receptors and transduce extracellular stimuli into intracellular responses. In plants, MAPKs constitute a relatively large gene family (with 20, 15, and 21 family members in Arabidopsis *[Arabidopsis thaliana]*, rice *[Oryza sativa]*, and poplar *[Populus spp.*], 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 plant MAPKs function in numerous developmental processes and mediate 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). In *Nicotiana attenuata*, the salicylic acid-induced protein kinase and wound-induced protein kinase (the homologs of Arabidopsis MPK6 and MPK3) are important in defense against insect herbivores (Wu et al., 2007). A growing body of evidence has also revealed the involvement of MAPK signaling in plant responses to various abiotic stresses, such as unfavorable temperatures, UV-B exposure, oxidation, and drought (Zhang and Klessig, 2001; Holley et al., 2003; Xiong and Yang, 2003; Teige et al., 2004; Jammes et al., 2009).

The plant hormone abscisic acid (ABA) functions in the regulation of many developmental processes and in abiotic and biotic stress resistance. Plants impaired in ABA biosynthesis or signaling have stunted growth and produce few seeds (Barrero et al., 2005; Fujii and Zhu, 2009; Nakashima et al., 2009). ABA also plays positive and negative roles in plant interactions with pathogens (Asselbergh et al., 2008; Ton et al., 2009). Arabidopsis needs ABA signaling to defend against the oomycete pathogen *Pythium irregular* (Adie et al., 2007); however, ABA signaling also attenuates callose deposition caused by infection and thus reduces resistance to *Pseudomonas syringae* pv *tomato* (*Pst*)

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DC3000 (de Torres-Zabala et al., 2007). ABA is also implicated in resistance to herbivores (Bodenhausen and Reymond, 2007). Importantly, ABA plays a critical role in seed dormancy and germination (Finkelstein et al., 2008), and it mediates responses to drought mainly by controlling stomatal closure (Fan et al., 2004; Wang and Song, 2008; Sirichandra et al., 2009; Kim et al., 2010).

Stomata are pores formed by pairs of guard cells located in the epidermis. Stomata are the major entry points for atmospheric CO₂ to diffuse into plants, which is subsequently converted to sugars by photosynthesis. The stomatal aperture is usually positively correlated with photosynthetic rates, but also with water loss caused by transpiration. Thus, plants use sophisticated regulatory systems to achieve optimum stomatal apertures, which allow them to take up CO₂ 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). Moreover, as a pore through the epidermal layer into the mesophyll, stomata play essential roles in pathogen defense. Usually, bacterial pathogens penetrate into plant tissues through stomata or wounds, and ABA and salicylic acid (SA) signaling are required for stomatal closure, which prevents pathogens from entering (Melotto et al., 2006). Protein kinases are important in modulating ABA-induced responses, including stomatal closure (Kim et al., 2010).

In Arabidopsis, SnRK2s (for SNF1-related protein kinase 2) are activated by ABA, and mutants defective in SnRK2s are almost completely insensitive to ABA, indicating that they are key components in ABA signaling (Fujii and Zhu, 2009). Five calcium-dependent protein kinases (CPKs), AtCPK3 and AtCPK6 (Mori et al., 2006), AtCPK4 and AtCPK11 (Zhu et al., 2007), and AtCPK10 (Zou et al., 2010), also control stomatal closure in an ABA-dependent manner. Recent evidence also implicates MAPK signaling in Arabidopsis guard cell development and ABA-regulated stomatal closure. The MAPK kinases AtMKK4, AtMKK5, AtMKK7, and AtMKK9 are all involved in guard cell development regulation (Lampard et al., 2009), and seedlings of the mpk3 mpk6 double mutant have densely clustered guard cells (Wang et al., 2007). AtMPK9 and AtMPK12 are specifically localized in guard cells, and they redundantly and positively control transpiration rates and stomatal closure in response to ABA and hydrogen peroxide (H₂O₂) treatment (Jammes et al., 2009). Expressing antisense AtMPK3 in Arabidopsis partially impairs ABA- and H₂O₂-induced guard cell movements (Gudesblat et al., 2007). Recently, a MAPK phosphatase, protein phosphatase 2C5, that directly modulates MAPK activity was also found to be involved in ABA signaling (Brock et al., 2010).

Although much knowledge has been obtained from genetic studies in Arabidopsis, little is known about how other plant species regulate stomatal apertures when they are challenged by drought and bacterial pathogens. In tobacco (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 CO₂ and ozone treatments; however, NtMPK4-silenced tobacco plants had normal ABA-induced closure movements of the guard cells (Gomi et al., 2005; Marten et al., 2008). Here, we studied the functions of NaMPK4 in Nicotiana attenuata, a wild tobacco species native to northwestern North America, in plant growth and in resistance to drought and pathogen stress. NaMPK4-silenced N. attenuata plants are moderately smaller under optimal growth conditions and produce more seeds than do wild-type plants. They exhibit highly elevated photosynthetic rates, which is correlated with enhanced transpiration rates and delayed senescence. Importantly, different from the function of NtMPK4 in tobacco, NaMPK4 is required for ABA- and H₂O₂-mediated stomatal closure after drought stress. We further demonstrate that NaMPK4 plays a critical role in guard cell-mediated defense against the invasion of Pst DC3000 and also confers resistance to apoplast-located Pst DC3000. Furthermore, SA is not involved in pathogen defense in wild-type and NaMPK4-silenced (irNaMPK4) N. attenuata plants after Pst DC3000 enters intercellular spaces.

RESULTS

Silencing NaMPK4 in N. attenuata

Using the sequence information of tobacco NtMPK4 (Gomi et al., 2005), we cloned NaMPK4 in N. attenuata (GenBank accession no. HQ236013). Phylogenetic analysis indicated that NaMPK4 clustered most closely with NtMPK4, AtMPK4, AtMPK11, and four MPK4 proteins in soybean (Glycine max; Liu et al., 2011) and was more distantly related to AtMPK12 (Fig. 1A). It showed 98% sequence identity with N. tabacum NtMPK4, 87%, 85%, and 77% similarity to AtMPK4, AtMPK11, and AtMPK12, respectively, and 86% to 87% similarity to GmMPK4 (Supplemental Fig. S1). Quantitative real-time PCR (qPCR) analysis indicated that NaMPK4 is expressed in all organs examined (i.e. roots, stems, flowers, and leaves; Fig. 1B). We transiently overexpressed a fusion protein, NaMPK4-EGFP, in N. attenuata and found that the fusion protein is located in both cytoplasm and nucleus (Fig. 1C). This is consistent with the function of MAPKs: MAPKs generally phosphorylate enzymes or transcription factors that are mostly located in the cytoplasm and nucleus.

A 344-bp fragment of NaMPK4 was cloned into the binary RNA interference vector pRESC5 in an inverted-repeat fashion, and Agrobacterium tumefaciens carrying this vector was further used to transform N. attenuata to obtain irNaMPK4 plants. This fragment did
not show identity with any other transcripts in an N. attenuata transcriptome database prepared by 454 deep transcriptome sequencing. Several independently transformed lines of irNaMPK4 plants that harbor single transgene insertions were identified by Southern blotting, and the transcript levels of NaMPK4 in these lines were analyzed by qPCR. Two lines, lines 119 and 163, whose transcript levels of NaMPK4 were 9.8% and 5.4% of those of wild-type plants (Supplemental Fig. S2), were selected for further studies.  

NaMPK4 Controls Photosynthetic Rates, Senescence, and Fitness under Glasshouse Conditions  

In N. tabacum, NtMPK4-silenced plants have retarded growth, although not as severe as that of the Arabidopsis mpk4 mutant (Gomi et al., 2005). To examine the function of NaMPK4 in mediating N. attenuata’s development, we cultivated wild-type and irNaMPK4 plants concurrently under glasshouse conditions and 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 wild-type plants [P119 = 0.0005 and P163 = 0.0015]) 38 d after germination, when plants had slightly elongated stems. Greater differences appeared at later developmental stages: by day 57, rosette sizes of irNaMPK4 plants were 85% of those of wild-type plants (Fig. 2A). Stalk lengths of irNaMPK4 plants were also reduced. At the end of reproductive growth (83 d after germination), irNaMPK4 plants were 16% shorter than wild-type plants (P119, 163 < 0.001; Fig. 2B). All these
changes resulted in a net 27% reduction in the average aboveground biomass (Supplemental Fig. S3). Plants grown in the natural habitat of *N. attenuata*, the Great Basin desert of southwest Utah, also exhibited decreased plant sizes compared with wild-type plants (Supplemental Fig. S4). Arabidopsis knockout mutant *mpk4*, which is a close homolog of NaMPK4, shows severely impaired growth, and overaccumulation of SA partly accounts for its arrested development (Petersen et al., 2000). However, the contents of SA irNaMPK4 were similar to those of wild-type plants in all examined developmental stages (Supplemental Fig. S5). Importantly, irNaMPK4 plants exhibited elevated chlorophyll contents and delayed senescence. In the early rosette stage (30 d old), irNaMPK4 plants had only slightly more chlorophyll than did wild-type plants (Fig. 2C). This difference became more pronounced at the later developmental stages, when the decline in chlorophyll contents in wild-type plants was more rapid than that of irNaMPK4 plants: by day 56, irNaMPK4 plants had 30% more chlorophyll in their leaves than did wild-type plants and showed an obvious slower senescence (Fig. 2C; photographs are shown in Supplemental Fig. S6).

In *N. tabacum*, silencing *NtMPK4* leads to enlarged stomata, increased stomatal conductance, and impaired stomatal closure responses to ozone and elevated CO₂ concentrations (Gomi et al., 2005; Marten et al., 2008). Consistently, the stomatal conductance of irNaMPK4 plants was two times greater than that of wild-type plants (Fig. 3A). As expected, the higher stomatal conductances of irNaMPK4 plants were associated with greater photosynthetic rates when wild-type and irNaMPK4 plants were supplied with various concentrations of CO₂ (200–600 μL L⁻¹; note that CO₂ in concentrations greater than 600 μL L⁻¹ close stomata and thus decrease photosynthetic rates; Fig. 3B). Since wild-type and irNaMPK4 plants have different stomatal conductance, we plotted the photosynthetic rates against intercellular CO₂ concentration values (Supplemental Fig. S7). Wild-type and irNaMPK4 plants had similar slopes in the linear parts of their curves, indicating that these plants do not have differences in the activity of Rubisco, and the higher stomatal conductance (better access to CO₂) in irNaMPK4 was the main factor contributing to its greater photosynthetic rates. We further examined whether the increased chlorophyll contents and stomatal conductance in irNaMPK4 plants were associated with elevated photosynthetic rates. At the rosette stage (30 d after germination), when irNaMPK4 plants had only slightly higher chlorophyll contents than wild-type plants, irNaMPK4 plants showed greater photosynthetic rates as the light intensity increased.

**Figure 2.** Silencing NaMPK4 decreases plant size and delays senescence. Wild-type (WT) and irNaMPK4 (lines 119 and 163) plants were grown concurrently. A, Rosette diameters (mean ± se; n = 30). The inset depicts 30-d-old wild-type and irNaMPK4 plants. B, Stalk lengths (mean ± se; n = 30). The inset depicts plants at the flowering stage (55 d old). C, Chlorophyll contents. Relative chlorophyll contents (mean ± se) were obtained from 10 replicate plants and expressed as arbitrary units (AU). Asterisks indicate significant differences between wild-type and irNaMPK4 plants (*t* test; * P < 0.05, ** P < 0.01, *** P < 0.001).
under ambient CO\(_2\) concentration (400 \(\mu\)mol mol\(^{-1}\); Fig. 3C). Fifty-six days after germination, when plants produced their first seed capsules and the differences in chlorophyll contents between irNaMPK4 and wild-type plants were starker (Fig. 2C), irNaMPK4 plants had about twice as high photosynthetic rates than did wild-type plants under almost all light intensities (Fig. 3D). We conclude that silencing NaMPK4 enhances the levels of photosynthesis activity in \(N.\) attenuata, especially at later stages of development.

Photosynthetic rates are often associated with increased biomass or seed yield (Long et al., 2006). To test whether the elevated photosynthetic rates 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 the appearance of the first flower was similar, and wild-type and irNaMPK4 plants produced the same number of flowers in their early flowering stages, irNaMPK4 plants produced considerably more flowers (28% more) 60 d after germination (Fig. 3E). The larger number of flowers resulted in a 20% increase in total capsule numbers for irNaMPK4 plants (Fig. 3F). In addition, the number of seeds produced in the first capsules increased 20% and 35% in lines 119 and 163,
respectively (Fig. 3G). Germination assays indicated identical viability between wild-type and irNaMPK4 seeds. Therefore, silencing NaMPK4 leads to considerably augmented photosynthetic rates and seed production under glasshouse conditions.

irNaMPK4 Plants Have Highly Impaired ABA-, H₂O₂-, 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 hypothesis, the response of irNaMPK4 plants to drought stress was examined.

First, leaves were excised from well-watered irNaMPK4 and wild-type plants, and their masses were recorded over time. Wild-type leaves had a rapid water loss in the first 50 min (17% loss) but showed almost no further decrease, at least until 3 h after excision, indicating that wild-type leaves closed their stomata in response to the initial water loss (Fig. 4A). In contrast, the masses of irNaMPK4 leaves declined more rapidly: by 50 min, water loss reached 30%, and by 3 h, it reached more than 40% (Fig. 4A). Given the critical role of ABA in stomatal closure responses, the contents of ABA were measured in these detached leaves. No significant differences were found in freshly detached leaves (approximately 210 ng g⁻¹ fresh mass); however, in agreement with the degrees of water loss, at 1 h after excision, ABA contents in wild-type and irNaMPK4 leaves increased to 370 and 500 ng g⁻¹ fresh mass, respectively. By 3 h, 2,300 ng g⁻¹ fresh mass ABA was found in the wild type, while irNaMPK4 had almost double the amount of ABA (4,000 ng g⁻¹ fresh mass; Fig. 4A). Furthermore, the ability to conserve water on the whole-plant level was examined. Plants were subjected to drought treatment by keeping plants under normal glasshouse conditions but without watering. Leaf turgor and ABA content were monitored over time. One day after the cessation of watering, neither wild-type nor irNaMPK4 plants showed an obvious wilting phenotype, but the ABA levels in irNaMPK4 increased 3-fold (1,078 ng g⁻¹ fresh mass), whereas ABA contents in the wild type showed almost no changes (294 ng g⁻¹ fresh mass; Fig. 4B). By day 2, wild-type plants had relatively normal turgor but irNaMPK4 plants were strongly wilted; consistently, ABA contents reached 920 ng g⁻¹ fresh mass in the wild type and 5,200 ng g⁻¹ fresh mass in irNaMPK4 (Fig. 4B).

These data indicate that irNaMPK4 plants likely have normal accumulations of ABA in response to drought stress and that NaMPK4 is required for ABA-induced stomatal closure. To further test this hypothesis, excised leaves of wild-type and irNaMPK4 plants 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 allowed to dry under ambient conditions. In line with our hypothesis, exogenously applied ABA somewhat inhibited water loss from both wild-type and irNaMPK4 leaves; however, ABA treatment did not reduce the transpiration levels of irNaMPK4 leaves to those of the wild type (Supplemental Fig. S8). The closure response of stomata on wild-type and irNaMPK4 leaves was further measured using epidermal peels, a technique that has been widely used in studying stomatal physiology. Similar to the stomata in tobacco with a defect in NtMPK4 (Gomi et al., 2005), irNaMPK4 also had somewhat enlarged stomatal size (Fig. 5A). When not treated with ABA, the guard cells of irNaMPK4 showed greater apertures than did the wild type (Fig. 5, A and B). Importantly, 15 min after the application of 2 μM ABA, stomatal apertures of wild-type epidermal peels rapidly decreased 67%, while those of irNaMPK4 stomata were only reduced 20% even after 1 h (Fig. 5, A and B). In guard cells, ABA induces the accumulation of H2O2, which is produced by guard cell-located NADPH oxidases; the increased levels of H2O2 are 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). Attempting to quantify H2O2 levels in N. attenuata failed due to its relatively high fluorescence background (autofluorescence from certain secondary metabolites), although we successfully detected elevated levels of ABA-induced H2O2 in positive controls, the Arabidopsis epidermal peels. To further dissect the function of NaMPK4 in ABA signaling, the epidermal peels from wild-type and irNaMPK4 leaves were treated with H2O2 and the stomatal apertures were quantified (Fig. 5C). Wild-type stomata exhibited a rapid closure response after the application of H2O2; the average stomatal aperture was reduced about 50% and 86% by 15 and 30 min, respectively, and it remained unchanged by 1 h; in contrast, irNaMPK4 stomata did not show a response by 15 min, but average aperture decreased 40% and 60% at 30 and 60 min after H2O2 application (Fig. 5C). Nitric oxide (NO) acts as a secondary messenger in ABA-induced stomatal closure (Desikan et al., 2002; Garcia-Mata et al., 2003). When epidermal peels were supplied with 100 μM sodium nitroprusside (SNP), neither wild-type nor irNaMPK4 plants showed any guard cell movement (Fig. 5D), although this solution effectively closed Arabidopsis stomata, as reported previously (Supplemental Fig. S9; Bright et al., 2006). Lower or higher concentrations of SNP failed to induce stomatal closure in the wild type and irNaMPK4. The increase of cytosolic Ca2+ is very important for the closure response in guard cells (Kim et al., 2010), and exogenously supplying Ca2+ has also been used to study the function of Ca2+ in guard cell physiology (Mori et al., 2006; Zou et al., 2010). We found that applying 1 mM Ca2+ led to normal closure of irNaMPK4 stomata within 10 min, similar to the response in wild-type plants (Fig. 5E).

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 wild-type 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 those of wild-type plants quickly declined within 10 min, indicating that NaMPK4 is required for dark-induced stomatal closure (Fig. 5F).

We concluded that NaMPK4 is located downstream of ABA-induced H2O2 to promote stomatal closure in response to drought stress and is also required for the normal closure during light-dark transitions.

NaMPK4 Is Likely Not Important for 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 wild-type and irNaMPK4 plants. However, both lines showed very little response to externally supplied ABA unless very high and nonphysiologically relevant concentrations were used (i.e. more than 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 individually grown wild-type and irNaMPK4 plants, wild-type and irNaMPK4 plants were grown side by side in 2-L pots. One day after the cessation of watering, plants showed no obvious phenotype of drought stress; by day 2, wild-type and irNaMPK4 plants appeared to be similarly wilted (Fig. 6A). Notably, ABA contents were elevated to 407 and 598 ng g fresh mass in wild-type and irNaMPK4 plants, respectively, indicating that irNaMPK4 suffered from greater levels of drought stress than did the wild type (Fig. 6B). This was probably due to the rapid water loss of irNaMPK4 and the relatively slow water redistribution in the soil: even when dehydrated, irNaMPK4 still transpired twice the amount of water per leaf area as did the wild type (Supplemental Fig. S10).

In N. attenuata, drought stress elevates the levels of NaNCED1, NaOSM1, NaTAS14, and NaHD20 transcripts (Parra et al., 1996; Ré et al., 2011). One day after drought treatment, all these genes did not show augmented levels (Fig. 6C). Consistent with the greater ABA contents of irNaMPK4 plants, 2 d after being deprived of water, the transcript abundance of all these genes was elevated in the wild type, while irNaMPK4 plants exhibited even greater levels of these transcripts (Fig. 6C). Therefore, NaMPK4 seems
Figure 5. NaMPK4 is required for ABA-, H$_2$O$_2$-, and darkness-induced stomatal closure. A, Photographs of stomata in the epidermal peels from wild-type (WT) and irNaMPK4 (lines 119 and 163) plants 15 min after being treated with 2 µM ABA (the same volume of the solvent of ABA was added as a control). Bar = 50 µm. B to E, Apertures (mean ± st) of the stomata on 2 µM ABA-treated (B), 50 µM H$_2$O$_2$-treated (C), 100 µM SNP-treated (D), and 1 mM Ca$^{2+}$-treated (E) wild-type and irNaMPK4 epidermal peels. The width of at least 50 stomatal pores was measured and used to calculate the average stomatal apertures. F, Stomata of irNaMPK4 plants (line 119) have almost no response to light-dark transitions. Plants were illuminated under light (300 µmol m$^{-2}$ s$^{-1}$) for 4 h, and after lights were switched off, the transpiration rates were recorded.
not to be required for the transcriptional regulation of these genes induced by drought stress.

**NaMPK4 Confers Resistance to Surface-Deposited *Pst* DC3000 in Guard Cells and to Apoplast-Located *Pst* DC3000 in Mesophyll Cells**

As part of the plant innate immune system, stomata play an important role in limiting bacterial entry into plant tissues, since guard cells close stomata after perceiving bacterium-derived elicitors; furthermore, ABA signaling is required for this defense response (Melotto 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, applying *Pst* DC3000 to leaf surfaces leads to stomatal closure within the first 1 to 2 h (Melotto et al., 2006). To examine the closure response of stomata, we measured stomatal conductance at different times after dipping leaves in a *Pst* DC3000 suspension. Probably due to some physical difference between the leaf surfaces of Arabidopsis and *N. attenuata*, we could apply bacterial suspensions evenly to the leaf surface without adding any detergents. The wild type decreased its stomatal conductance about

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**Figure 6.** Drought-induced transcriptional regulation in wild-type and *irNaMPK4* plants. Wild-type (WT) and *irNaMPK4* (line 119) plants were grown in the same 2-L pots. When plants were 35 d old, they were exposed to drought stress by stopping watering. A, Photographs of wild-type and *irNaMPK4* plants before and after the initiation of drought stress. B, ABA contents (mean ± se; n = 5) of drought-treated wild-type and *irNaMPK4* plants. C, Transcript levels (mean ± se; n = 5) of drought-inducible genes *NaNCED1*, *NaOSM1*, *NaTAS14*, and *NaHD20* in wild-type and *irNaMPK4* plants after being treated with drought stress. Asterisks indicate significant differences between wild-type and *irNaMPK4* plants (t test; * P < 0.05, ** P < 0.001). FM, Fresh mass.
45% at 2.5 h after applying pathogens to the leaf surfaces (Fig. 7A). irNaMPK4 showed an even stronger reduction in transpiration rates: by 2.5 h, stomatal conductance decreased 64%, although the average value of irNaMPK4 plants remained 1-fold higher than that of the wild type (Fig. 7A). The decrease of stomatal conductance was due to bacterial treatment, since no changes of stomatal conductance were found in any plants after they were dipped in the suspension solution without bacteria. To further study the role of NaMPK4 in guard cell-based pathogen resistance, the bacterial population density values were quantified in these infected leaves. No bacteria were detected in the wild type, but strikingly, *Pst* DC3000 was amplified in irNaMPK4 plants to 10^6 and 10^7 colony-forming units (cfu) cm^{-2} by days 1 and 2, respectively (Fig. 7B). Consistently, 3 d after application of *Pst* DC3000, chlorosis was observed in infected leaves of irNaMPK4 but not in the wild type; by day 10, irNaMPK4 exhibited necrotic lesions, while only some of the bacterium-applied wild-type leaves (about 30%) showed slight chlorosis (Supplemental Fig. S11). To gain further insight into the mechanism by which *N. attenuata* defends against surface-deposited bacteria, the bacterial population was determined in wild-type and irNaMPK4 plants shortly after dipping to minimize the effect of the multiplication of bacteria in plants: no detectable number of *Pst* DC3000 was found in the wild type, but approximately 100 and 300 cfu cm^{-2} was detected in

**Figure 7.** 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 wild-type and irNaMPK4 plants were dipped in a *Pst* suspension (OD_{600} = 0.8), and the transpiration rates (mean ± s.e.; n = 8; A) and bacterial populations (mean ± s.e.; n = 10; B) were measured at the indicated times. C and D, Bacterial populations and SA contents in leaves of wild-type and irNaMPK4 plants after being infiltrated with *Pst* DC3000. *Pst* DC3000 (OD_{600} = 0.001) was infiltrated to leaves, and the bacterial population (mean ± s.e.; n = 10; C) and SA contents (mean ± s.e.; n = 5; D) were examined in samples collected at the indicated times. E, NaMPK4 confers resistance to apoplastic *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 the infiltrated bacteria was examined in wild-type, line 119, NahG, irNPR1, 119×NahG, and 119×irNPR1 plants. All values are means ± s.e. Asterisks indicate significant differences between wild-type and irNaMPK4 plants (t test; * P < 0.05, ** P < 0.01, *** P < 0.001). FM, Fresh mass; n.d., not detected.
irNaMPK4 lines at 2.5 and 6 h after treatment (Supplemental Fig. S12). These data indicate that NaMPK4 is an important part of guard cell-based defense against surface-deposited bacteria.

At least two steps account for the successful colonization of bacteria in plants: entry from stomata or wounds, and multiplication in the apoplast. To further study the function of NaMPK4 in regulating resistance to bacterial pathogens, Pst DC3000 was pressure infiltrated into wild-type and irNaMPK4 leaves to circumvent the requirement of entry through stomata. One day after inoculation, the bacterial population in the wild type was approximately eight times smaller than that in irNaMPK4; 2 d after infiltration, bacterial density in the wild type was around 25 times less (Fig. 7C), indicating that silencing NaMPK4 compromises the innate immunity of N. attenuata against apoplastic Pst DC3000 bacteria. 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 at 1 d after treatment. These data strongly suggest that NaMPK4 has dual functions in N. attenuata’s innate immunity against the bacterial pathogen Pst DC3000: NaMPK4 limits bacterial entry via stomata and suppresses bacterial multiplication after bacteria invade 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 the mpk4 mutant has highly elevated SA levels and exhibits 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, wild-type and irNaMPK4 plants had the same levels of SA (145 ng g⁻¹ fresh mass); after bacterial infiltration, irNaMPK4 showed much higher levels of SA than did the wild type: by 12 h, approximately 2,300 and approximately 6,000 ng g⁻¹ fresh mass SA were found in the wild type and irNaMPK4, respectively, and greater amounts of SA were also found in irNaMPK4 plants at 1 and 2 d after infiltration (Fig. 7D). Mock inoculation did not induce SA accumulation. 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 wild-type and irNaMPK4 plants, and after 1 d, we infiltrated these leaves with Pst DC3000. SA application was repeated once each day. The growth of Pst DC3000 was examined up to 3 d after pathogen infiltration. Neither wild-type nor irNaMPK4 plants showed higher resistance to Pst DC3000 despite the exogenously applied SA, suggesting that SA is not required in N. attenuata’s resistance to Pst DC3000 (Supplemental Fig. S13).

We generated plants transformed with a bacterial SA 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 (Supplemental Fig. S14) did not alter pathogen growth in wild-type and irNaMPK4 plants (Fig. 7E). Similarly, Pst DC3000 populations increased to the same levels in irNPR1 and 119×irNPR1 plants compared with wild-type and irNaMPK4 plants, respectively (Fig. 7E). Thus, 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.

AtMPK11 Is Not Involved in Drought or Pathogen Resistance in Arabidopsis

In addition to AtMPK4, NaMPK4 also has a high sequence similarity to AtMPK11 (Fig. 1A). 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 the mpk11 mutant (SALK_049352C) is morphologically identical to the wild type (ecotype Columbia [Col-0]). Measurement of transpiration rates indicated that the wild type and the mpk11 mutant had similar values (Supplemental Fig. S16). Furthermore, the wild type and mpk11 had the same water loss rates in detached leaves and accumulated similar amounts of ABA (Fig. 8, A and B). When plants were subjected to a drought treatment, no differences in either the speed of wilting or the ABA levels were found between the wild type and mpk11. We inferred 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 wild-type and mpk11 leaves and the Pst DC3000 population was quantified. In contrast to irNaMPK4 plants, compared with the Col-0 wild type, mpk11 was slightly more resistant to the pathogen (Fig. 8C). After dipping the leaves of wild-type and mpk11 plants in a Pst DC3000 suspension, a similar cell death phenotype was found in the wild type and mpk11. Thus, AtMPK11 does not have a similar function as NaMPK4 in pathogen defense.

DISCUSSION

Using a reverse genetic approach, we examined the function of NaMPK4 in regulating plant photosynthesis, development, and fitness under greenhouse conditions.
and show that NaMPK4 controls at least two stomatal traits, ABA-induced stomatal closure in response to drought stress and pathogen defense, likely by preventing bacteria from entering through the open pores. We also show that NaMPK4 suppresses bacterial multiplication after they invade intercellular spaces.

NaMPK4 and Plant Fitness

Although NaMPK4 is phylogenetically related to AtMPK4, the Arabidopsis mpk4 mutant and irNaMPK4 have distinct growth phenotypes. The Arabidopsis mpk4 mutant is severely dwarfed, and this results partly from the 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 wild-type plants. NaMPK4 appears to modulate plant development in a SA-independent manner, since irNaMPK4 plants have the same levels of SA as wild-type plants and minimizing SA contents of irNaMPK4 plants by overexpressing NahG does not restore wild-type 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 the wild type, the possibility cannot be ruled out that irNaMPK4 is always suffering from low levels of drought stress even under well-watered glasshouse conditions, which leads to its reduced size.

Importantly, silencing NaMPK4 considerably enhances photosynthetic rates. irNaMPK4 plants have increased stomatal sizes and greatly elevated stomatal conductance. In addition, the high chlorophyll content 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 wild-type plants. Chlorophyll content is one of the most important markers of plant senescence (Lim et al., 2007). The high chlorophyll content in irNaMPK4 plants in their late stage of development indicates that irNaMPK4 plants have delayed senescence. The mechanism by which NaMPK4 negatively regulates senescence is unknown. Biosynthesis and degradation both control the level of chlorophyll in plants. irNaMPK4 plants have marginally higher levels of chlorophyll when they are in the rosette stage; however, over time, the levels of chlorophyll in irNaMPK4 plants become increasingly higher than in wild-type plants. Examining the rates of chlorophyll synthesis and degradation, especially at later develop-
mental stages, will clarify the function of NaMPK4 in modulating the accumulation of chlorophyll.

When soil nitrogen content is favorable, the photosynthetic rate is usually positively correlated with plant biomass (Fichtner et al., 1993; Richards, 2000; Mitra and Baldwin, 2008). However, despite having elevated photosynthetic rates, the aboveground 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 produce more seeds than do wild-type plants.

**NaMPK4 Functions in ABA Signaling in Guard Cells**

Guard cells play critical roles in controlling water conservation, rates of CO₂ assimilation, and pathogen infection. In *N. attenuata*, NaMPK4 appears to act downstream of ABA in regulating drought stress-activated stomatal closure, given that drought-treated irNaMPK4 plants accumulate high levels of ABA but maintain elevated transpiration rates and that, in epidermal peels, guard cells have strongly impaired closure responses after ABA treatment. In contrast, silencing the ortholog of NaMPK4 in tobacco, NtMPK4, does not affect the ABA-induced stomatal closure response (Gomi et al., 2005; Marten et al., 2008). The discrepancy might result from species-specific differences.

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 the H₂O₂-induced inhibition of stomatal opening or the 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 reactive oxygen species (ROS) to regulate the activity of anion channels (Jammes et al., 2009). Although we could not determine the production of ABA-induced ROS in *N. attenuata* guard cells, knocking down NaMPK4 compromises the stomatal closure response after supplying H₂O₂. It is unlikely that NaMPK4 mediates both drought/ABA-induced H₂O₂ production and the H₂O₂-elicited downstream stomatal response. Thus, we speculated that, similar to AtMPK9 and AtMPK12, NaMPK4 might also function downstream of ROS to mediate stomatal closure. Consistent with this hypothesis, silencing tobacco *NtMPK4* compromises ozone-elicited stomatal closure (Gomi et al., 2005), and ozone exposure is known to quickly induce ROS (Vahisalu et al., 2010). Research in Arabidopsis indicated that NO is elicited by H₂O₂ and is another important messenger that triggers the drought/ABA-induced stomatal closure response (Bright et al., 2006). However, NO signaling seems not to be required in *N. attenuata*, given that SNP, a potent NO donor that induces stomatal closure in Arabidopsis, did not decrease stomatal apertures in the various concentrations tested. Moreover, knocking down *NtMPK4* in *N. tabacum* and *NaMPK4* in *N. attenuata* abolishes the dark-induced stomatal closure response (Marten et al., 2008). The S-type anion channel slow anion channel-associated1 is important for stomatal closure in response to ABA, H₂O₂, NO, Ca²⁺, and light/dark transitions (Vahisalu et al., 2008; Kim et al., 2010). Given that *NtMPK4* is also required for the activation of S-type anion channels during light-dark transitions (Marten et al., 2008), we propose that both ABA/H₂O₂- and darkness-induced signaling pathways converge on NaMPK4, which modulates the activity of S-type anion channels and thus the stomatal closure response. Importantly, supplying irNaMPK4 guard cells with Ca²⁺ induced the normal closure response. We hypothesize that the impaired activity of S-type anion channels (if it is the case) is not due to any changes in S-type anion channels per se but perhaps is a result of altered ABA- and H₂O₂-induced cytosolic [Ca²⁺], which is important for activating the anion channels (Schroeder and Hagiwara, 1989). Additional genetic and electrophysiology work is needed to further dissect the function of NaMPK4 in regulating the closure response of guard cells. Notably, although the Ca²⁺-induced stomatal closure response was not examined in *mpk9*-1/12-1 double mutants, calcium failed to activate anion channels in guard cells of *mpk9*-1/12-1 (Jammes et al., 2009), implying that NaMPK4 might be functionally different from AtMPK9 and AtMPK12.

Apparently, NaMPK4 is not located in all ABA-regulated pathways. By growing wild-type and irNaMPK4 plants in the same pots, we minimized the differences in levels of drought stress resulting from different stomatal controls and examined whether NaMPK4 is involved in the transcriptional regulation of drought-responsive genes. It seems that NaMPK4 is not important in regulating drought- and ABA-induced transcriptional responses. Since whole leaves were used for transcriptional analyses, the possibility that NaMPK4 regulates transcriptional changes specifically in guard cells but not in mesophyll cells cannot be ruled out. These data also revealed the specific and important functions of NaMPK4 in drought stress responses in guard cells but not in mesophyll cells.
NaMPK4 and Defense against Bacterial Pathogens in Guard Cells and in Mesophyll Cells

The important role of guard cells 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 populations in the wild type and irNaMPK4 after dipping leaves in bacterial suspensions: 1 d after treatment, we detected no bacteria in the wild type but found that the *Pst* DC3000 population was more than 10⁶ cfu cm⁻² in irNaMPK4. In contrast, when bacteria enter leaf tissues without the barrier of stomata (directly by infiltration), by 24 h, *Pst* DC3000 was only amplified a few fold higher in irNaMPK4 than in the wild type, and by 48 h, the difference between bacterial densities in irNaMPK4 and the wild type was also less than 30-fold. As early as 2.5 h after bacteria were applied to leaf surface, when bacteria did not have enough time to highly amplify, irNaMPK4 was already infected. All these data strongly suggest that the stomata of *N. attenuata* have a very effective defense system against surface-deposited bacterial pathogens, which is partly mediated by NaMPK4.

However, 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 that 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). Given that the stomatal closure of irNaMPK4 is partially abolished after H₂O₂ treatment, it is possible that the guard cells of *N. attenuata* respond to *Pst* DC3000 in a ROS-independent pathway. This scenario needs to be examined in genetically modified plants whose pathogen-induced ROS production is altered.

Although the greater transpiration rates of irNaMPK4 indicated that, after applying bacteria on their leaf surfaces, the stomatal openings of irNaMPK4 remained larger than those of the wild type and this difference was maintained for at least 1 h, it is unlikely that the greater stomatal apertures of irNaMPK4 solely accounted for the highly compromised defense against surface-deposited *Pst* DC3000. We speculate that the decreased levels of resistance to intercellular pathogens and, probably more importantly, an unknown form of guard cell-mediated defense that is abolished in irNaMPK4 enhance the attraction of bacteria to stomata and therefore result in greater numbers of invading bacteria. Kroupitski et al. (2009) demonstrated that the directional movement of bacteria to stomata is dependent on chemotaxis, and pathogens are likely attracted to nutrients produced by photosynthetically active cells. It needs to be confirmed whether, after applying pathogens, compared with those in the wild type, the larger stomatal apertures and the consequently greater photosynthetic activity of irNaMPK4 may have resulted in higher amounts of photosynthates that leaked out of guard cells and attracted more bacteria. Additionally, or alternatively, highly increased survival rates of *Pst* DC3000 after entering the stomata of irNaMPK4 but before they are amplified intercellularly also cannot be ruled out. Furthermore, *Pst* DC3000 reopens the stomata of Arabidopsis epidermal peels within 3 h after the application of bacteria (Melotto et al., 2006). However, in our experimental setup, stomatal reopening seems not to be an important mechanism by which *Pst* DC3000 invades irNaMPK4: (1) *Pst* DC3000 was not able to reopen *N. attenuata* stomata, given that neither the wild type nor irNaMPK4 recovered from their decreased stomatal conductance over 7 h after the application of bacteria; (2) after dipping in the bacterial suspension, leaf surfaces dried within 0.5 h, and this should have limited any further movement of bacteria, even if the stomata could be reopened.

It has been proposed that in Arabidopsis, SA signaling acts upstream of ABA signaling in bacterium-triggered stomatal closure (Zeng and He, 2010). Whether in *N. attenuata* guard cells SA and ABA signaling converge on stomatal closure and defense against pathogens needs to be studied further. 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 wild-type and irNaMPK4 plants with different SA levels (exogenous SA application and endogenous 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). Unlike Arabidopsis, in which SA is required for defense against *Pst* DC3000 in both guard cells and mesophyll cells (Melotto et al., 2006), *N. attenuata* uses SA-mediated immunity against *Pst* DC3000 only in guard cells. The hypothesis should be examined that NaMPK4 regulates SA contents or SA signaling specifically in guard cells and thus controls defense against *Pst* DC3000 invasion.

NaMPK4 Is Not a Functional Homolog of Either AtMPK4 or AtMPK11

NaMPK4 has the highest similarities to Arabidopsis AtMPK4 and AtMPK11. Although AtMPK4 is mainly localized in guard cells (Petersen et al., 2000), the dramatically different phenotypes of the Arabidopsis
**mpk4** mutant from those of irNaMPK4 strongly suggest that NaMPK4 has a distinct function in *N. attenuata*. Recent evidence indicated that AtMPK4 is important for cytokinesis and microtubule organization in Arabidopsis, which at least accounts for the highly retarded growth phenotype of the **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 the **mpk4** mutant seem not to result from differences in degrees of gene silencing (knockdown and knockout), since silencing AtMPK4 in Arabidopsis using an RNA interference vector also generated extremely dwarf T1 plants that were morphologically very similar to **mpk4** and were mostly sterile, like **mpk4**. NaMPK4 is not functionally similar to AtMPK11 either: the **mpk11** mutant has no detectable growth abnormalities compared with the wild type, and our data indicate that AtMPK11 is not involved in drought and pathogen resistance. Although the double mutant **mpk4** **mpk11** has a more severe growth phenotype than does **mpk4**, the exact function of AtMPK11 is unknown (Kosetsu et al., 2010). Notably, although having somewhat lower identity to NaMPK4 (77%, Supplemental Fig. S1), AtMPK12 is also important for ABA- and ROS-induced stomatal closure responses (Jammes et al., 2009). A 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).

A recent study indicated that soybean GmMPK4 functions similarly to Arabidopsis AtMPK4. Silencing GmMPK4s highly increases SA and H2O2 levels and thus provides elevated resistance to downy mildew and *Soybean mosaic virus* (Liu et al., 2011). Therefore, the function of MPK4 seems to be conserved in Arabidopsis and soybean but not in *Nicotiana*, underlining the functional divergence of MPK4 in different plant lineages. How MPK4 evolved in different species would be interesting to explore.

**CONCLUSION**

Taken together, silencing NaMPK4 delays the senescence process of *N. attenuata* and increases photosynthetic rates. NaMPK4 also functions in carbon allocation; namely, NaMPK4 positively influences vegetative growth but negatively affects reproductive growth. Importantly, NaMPK4 plays a critical role in stomatal physiology: NaMPK4 negatively controls stomatal conductance and is required for ABA and H2O2 to promote stomatal closure, and it confers a very effective guard cell-based defense against invading pathogenic bacteria. We also demonstrate that NaMPK4 mediates defense against apoplastic *Pst* DC3000 in a SA-independent manner. These data highlight the important functions of NaMPK4 in mediating various ecologically important traits in both guard cells and mesophyll cells, and our analyses underscore the unexpected evolutionary complexity of MAPKs in different plant lineages.

**MATERIALS AND METHODS**

**Plant Cultivation**

*Nicotiana attenuata* (Solanaceae) seeds were from a line maintained in our laboratory that was originally collected in Utah and had inbred for 30 generations in the glasshouse. Unless otherwise noted, *N. attenuata* was grown in 1-L pots in soil and grown in the glasshouse at 26°C to 28°C under 16 h of light supplemented by Philips Sun-T Agro 400 Na lights (Philips Lighting). Homozygous Arabidopsis (*Arabidopsis thaliana*) **mpk11** (SALK_049352C) seeds were obtained from the European Arabidopsis Stock Center (http://arabidopsis.info/) and the homozygosity was confirmed by PCR (Supplemental Table S1). Arabidopsis Col-0 and **mpk11** were directly germinated in soil and were cultivated under long-day conditions (16-h photoperiod, 65% relative humidity, 21°C). For the field experiment in the Great Basin desert of southwest Utah, seeds were imported under Animal and Plant Health Inspection Service notification number 07-341-101n, and the field experiments were conducted under notification number 06-242-03r.

**Plant-Stable Transformation and Transient Transformation for Localization**

To create NaMPK4-silenced plants, a 344-bp fragment of the NaMPK4 gene was inserted into the pRSCE5 transformation vector in an inverted-repeat orientation (for primer sequences, see Supplemental Table S1) to form **pRSCE5-MPK4**. *Agrobacterium tumefaciens* harboring this vector was used for transforming *N. attenuata* (Kru¨gel 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 II* gene as a probe. Two homozygous T2 lines with single T-DNA insertions were identified and used in subsequent experiments.

For NaMPK4 localization, the NaMPK4 coding sequence was first fused with EGFP in pEGFP (Clontech), and then the NaMPK4-EGFP sequence was further amplified and cloned into pCAMBIA1301 vector to form pCAM-NaMPK4-EGFP. **EGFP** was also cloned into pCAMBIA1301 as a positive control (pCAM-EGFP; Supplemental Table S1). *Agrobacterium* (strain GV3101) was transformed with these vectors. Transformed *Agrobacterium* was grown overnight, and the medium was removed by centrifuging. Bacteria (optical density at 600 nm [OD600] = 0.5) were resuspended in an inoculation solution containing 10 mM MgCl2, 5 mM MES (pH 5.5), and 200 μM acetosyringone. *N. attenuata* (25 d old) was inoculated with *Agrobacterium* using 1-mL syringes, and plants were kept in the dark and at high humidity for 24 h. Two days after inoculation, inoculated areas were excised and protoplasts were prepared (Yoo et al., 2007). Images were obtained on a fluorescence microscope (Axiovert 200; Carl Zeiss).

**RNA Extraction and qPCR**

Total RNA was extracted from ground leaf samples using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. For qPCR analysis, five replicate biological samples were used. A total of 0.5 μg of total RNA sample was reverse transcribed with oligo(dT)18 and SuperScript II reverse transcriptase (Invitrogen). qPCR was performed on an ABI PRISM 7700 Plant Physiol. Vol. 158, 2012 773
sequence detection system (Applied Biosystems) using qPCR Core kits (Eurogentec). An \textit{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 S1.

**Stomatal Assays in Epidermal Peels**

For all assays of stomatal apertures, fully expanded young leaves from 4- to 5-week-old plants were used. Epidermal strips were peeled from the abaxial side of the leaves and were immediately placed in opening solution (50 ms KC1 and 10 ms MES-KOH, pH 6.13) with the adaxial side upward. Stomatal opening was induced by white light illumination (300 \(\mu mol m^{-2} s^{-1}\)) for 3 h and gentle shaking (40 rpm). The epidermal strips were examined with a microscope (Leica LMD6000) equipped with a CCD camera and a work station to determine the apertures of the stomatal pores at different times after the application of 2 \(\mu l\) ABA, 100 \(\mu l\) H2O2, 1 ms Ca\({}^{2+}\), or 100 \(\mu l\) SNP (Sigma).

**Measurement of Photosynthetic Rates, Transpiration Rates, Stomatal Conductance, and Chlorophyll Contents, and Quantification of Rates of Water Loss in Detached Leaves**

Photosynthetic rates, transpiration values, and stomatal conductance were measured using the LI-COR 6400 Portable Photosynthesis System (LI-COR Biosciences). A light-responsive curve of photosynthesis was generated from at least five replicate plants, each at ambient CO2 concentration (400 \(\mu l l^{-1}\)) and six different light intensities: 0, 200, 500, 1,000, 1,500, and 2,000 \(\mu l l^{-1}\). Photosynthesis rates were also measured at six different CO2 concentrations: 0, 200, 400, 600, 800, and 1,000 \(\mu l l^{-1}\), at a light irradiance of 1,200 \(\mu l l^{-2} s^{-1}\), using at least five replicate plants for each CO2 concentration. Chlorophyll contents were determined using a portable chlorophyll meter (Minolta SPAD-502; Konica Minolta). Leaves at the –1 position when plants were 30 d 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 \(\mu l l^{-1}\) \(ms^{-1}\). Immediately after lights were switched off, the transpiration rates were measured with the 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 the abaxial side facing up, and their masses were measured after various times. For ABA treatment, detached leaves were first incubated in a 20 \(\mu l\) ABA solution for 8 h prior to the air-drying treatment; leaves incubated in water (0.02% ethanol; the solvent for the 20 \(\mu l\) ABA) served as controls. Water losses were expressed as the percentage of initial fresh masses after excision.

**Analysis of SA and ABA Concentrations**

One milliliter of ethyl acetate spiked with 40 ng of D_2-ABA and 200 ng of D_3-SA, the internal standards for ABA and SA, was added to each briefly crushed frozen leaf sample (approximately 100 mg). Samples were then ground on a FastPrep homogenizer (Thermo Electron). After being centrifuged at 13,000g for 10 min at 4°C, 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 (15 min, 13,000g; 4°C) to remove particles. The supernatants were analyzed on an HPLC-tandem mass spectrometry device (1200L LC-MS system [Varian]; Wu et al., 2007).

**Pathogen Assays**

\textit{Pseudomonas syringae pv tomato} DC3000 was grown at 28°C in Luria-Bertani liquid medium with antibiotics until OD_{600} was approximately 0.6. After 10 min of centrifugation at 3,000g, the supernatant was discarded and cells were resuspended to OD = 0.8 (approximately \(4 \times 10^7\) cfu ml\(^{-1}\)) in a 10 ml MgCl2 solution without any detergents. To quantify stomata closure in response to \(Pst\) DC3000, leaves of intact plants were dipped into the bacterial solution for 5 s and transpiration rates were measured using the LI-COR 6400 Portable Photosynthesis System. 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) and then rinsed with sterile water, 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 Luria-Bertani agar plates containing antibiotics. Plates were incubated at 28°C until colonies in appropriate sizes appeared, and colony numbers were counted and values were presented as log(cfu cm\(^{-2}\)).

To examine the growth of intercellularly located \(Pst\) DC3000, leaves were inoculated with a \(Pst\) DC3000 suspension of OD = 0.001 using 1-mL syringes. Mock inoculation was done by infiltrating a 10 ms MgCl2 solution. Pathogen growth was quantified using the above-mentioned method without sterilizing the surfaces.

**Statistical Analysis**

Data were analyzed by ANOVA or unpaired \(t\) test using StatView, version 5.0 (SAS Institute).

**Phylogeny Analysis**

Protein sequences were retrieved from GenBank and were aligned using the ClustalW algorithm embedded in the MegAlign software (DNASTAR Lasergene 8). The unrooted neighbor-joining protein tree was constructed by MEGA4 software (1,000 replications; Tamura et al., 2007).

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

**Supplemental Data**

The following materials are available in the online version of this article.

- Supplemental Figure S1. Alignment of the protein sequences of NaMPK4, AtMPK4, AtMPK11, and AtMPK12.
- Supplemental Figure S2. NaMPK4 transcript accumulation in irNaMPK4 plants.
- Supplemental Figure S3. Silencing NaMPK4 reduces plant above-ground biomass.
- Supplemental Figure S4. irNaMPK4 exhibits smaller rosette sizes in the natural habitat of \textit{N. attenuata}.
- Supplemental Figure S5. SA levels in wild-type and irNaMPK4 plants at different developmental stages.
- Supplemental Figure S6. Photographs of the leaves excised from wild-type and irNaMPK4 plants (line 119; 56 d old).
- Supplemental Figure S7. Wild-type and irNaMPK4 plants have similar activity levels of ribulose-1,5-bisphosphate carboxylase oxygenase.
- Supplemental Figure S8. Exogenously applying ABA to detached irNaMPK4 leaves only partially recovers its stomatal closure response to dehydration.
- Supplemental Figure S9. Exogenously applying SNP to Arabidopsis leaf epidermal strips partially closes stomata.
- Supplemental Figure S10. Transpiration rates of paired wild-type and irNaMPK4 plants after being deprived of water.
- Supplemental Figure S11. Silencing NaMPK4 impairs plant defense against the surface-deposited bacterial pathogen \(Pst\) DC3000.
- Supplemental Figure S12. Surface-deposited bacterial pathogen \(Pst\) DC3000 enters irNaMPK4 but does not detectably infect wild-type \textit{N. attenuata}.
- Supplemental Figure S13. Exogenously applying SA does not alter the resistance levels of wild-type or irNaMPK4 plants.
- Supplemental Figure S14. NahG effectively minimizes SA accumulation in \(Pst\) DC3000-infected plants.
- Supplemental Figure S15. NahG plants do not have abnormal stomatal conductance.
Supplemental Figure S16. mpk11 mutant has the same transpiration rates as those in wild-type (Col-0) Arabidopsis.

Supplemental Table S1. Primer sequences.

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