

Rapid Phosphorylation of a Syntaxin during the Avr9/Cf-9-Race-Specific Signaling Pathway¹

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The tomato (*Lycopersicon esculentum*) resistance (*R*) gene *Cf-9* is required for resistance to races of the fungal pathogen *Cladosporium fulvum* expressing the elicitor *Avr9* and also confers responsiveness to *Avr9* in *Cf-9*-containing transgenic tobacco (*Nicotiana tabacum*; *Cf9* tobacco). Although protein phosphorylation is required for many early *Avr9/Cf-9*-signaling events, so far the only phosphorylation targets known in this race-specific signaling pathway are three kinases: the two mitogen-activated protein kinases, wound-induced protein kinase and salicylic acid-induced protein kinase, and the calcium-dependent protein kinase *NtCDPK2*. Here, we provide evidence that a tobacco syntaxin is rapidly and transiently phosphorylated after *Avr9* elicitation. The syntaxin was detected with an antibody against *NtSyp121*, a plasma membrane-localized syntaxin implicated in abscisic acid responses and secretion. Consistent with the gene-for-gene hypothesis, syntaxin phosphorylation required the presence of both *Avr9* and *Cf-9*. This phosphorylation event occurred either upstream of the pathway leading to reactive oxygen species production or in a parallel pathway. Interestingly, rapid syntaxin phosphorylation was triggered by the race-specific elicitor *Avr9* but not by *flg22^{P.aer}*, a general elicitor capable of inducing other defense-related signaling events in *Cf9* tobacco such as reactive oxygen species production, mitogen-activated protein kinase activation, and *PR5* transcript up-regulation. Furthermore, *NtSyp121* transcript levels were increased at 24 h after elicitation with *Avr9* but not with *flg22^{P.aer}*. Because most other previously described *Avr9*- and *flg22^{P.aer}*-elicited responses are similar, syntaxin phosphorylation and *NtSyp121* transcript up-regulation may serve as novel early biochemical and late molecular markers, respectively, to elucidate further differences in the signaling responses between these two elicitors.

Plants resist pathogen attack by perceiving the invading organism and activating appropriate cellular responses that ultimately arrest growth of the pathogen. In the gene-for-gene interaction, a disease resistance (*R*) gene confers resistance to pathogens expressing the corresponding avirulence (*Avr*) gene. The *R* protein presumably functions as part of a receptor complex that recognizes the *Avr* elicitor and subsequently initiates defense responses (Hammond-Kosack and Jones, 1997; Martin et al., 2003). If either the *R* or *Avr* gene is absent or nonfunctional, disease will occur.

The tomato (*Lycopersicon esculentum*) *R* gene *Cf-9* is required for resistance against races of the leaf fungus *Cladosporium fulvum* expressing *Avr9*, which encodes a small Cys-rich peptide secreted into the plant apoplast during infection (Hammond-Kosack and Jones, 1997; Joosten and de Wit, 1999). Although *C. fulvum* is able to infect only tomato, *Cf-9* confers *Avr9* responsiveness in other solanaceous species (Hammond-

Kosack et al., 1998). The *Cf-9* gene encodes a highly glycosylated type I membrane protein with a domain structure characteristic of receptor-like proteins (Van der Hoorn et al., 2005). Its extracellular Leu-rich repeat (LRR) domain plays a major role in *Avr9* specificity (Van der Hoorn et al., 2001; Wulff et al., 2001). In contrast to receptor-like kinases, the short cytoplasmic domain of *Cf-9* lacks any apparent signaling domain, suggesting that *Cf-9* interacts with other signaling components to initiate defense responses (Rivas and Thomas, 2002; Rivas et al., 2004).

In the past, the *Cf-9/Avr9* system has served as an excellent model system to dissect early signaling events associated with *R*-gene-mediated defense responses, of which many occur at the plasma membrane (PM; Hammond-Kosack and Jones, 1997; Rivas and Thomas, 2002). In *Cf-9*-expressing solanaceous plants, elicitation with the race-specific elicitor *Avr9* triggers rapid changes in ion flux (Piedras et al., 1998; Blatt et al., 1999), the production of reactive oxygen species (ROS; Piedras et al., 1998), the activation of the mitogen-activated protein kinases (MAPKs) such as wound-inducible protein kinase (WIPK) and salicylic acid-inducible protein kinase (SIPK; Romeis et al., 1999), and the activation of calcium-dependent protein kinases (Romeis et al., 2000; Romeis et al., 2001). Addition of *Avr9* to tobacco (*Nicotiana tabacum*) suspension culture cells also induces rapid changes in expression levels of a large, diverse set of mRNAs, most of which are independent of ROS production (Durrant et al., 2000). Based on inhibitor studies, many early defense responses appear to depend on phosphorylation

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events (Piedras et al., 1998; Blatt et al., 1999; Romeis et al., 1999, 2000, 2001). Phosphorylated targets in the Avr9/Cf-9 response, however, remain mainly elusive; so far, MAPKs and the membrane-bound calcium-dependent protein kinase NtCDPK2 are the only proteins shown to be phosphorylated in response to Avr9 (Romeis et al., 1999, 2001).

Another class of elicitors referred to as pathogen-associated molecular patterns or general elicitors triggers early cellular responses very similar to those activated in the *R*-gene-mediated pathogen recognition. In contrast to race-specific elicitors, however, general elicitors are not specific for a particular pathogen but characteristic for whole classes of microorganisms (Nürnberg and Brunner, 2002; Nürnberg et al., 2004). Although the exact role of most general elicitors in disease resistance remains unclear, Zipfel et al. (2004) demonstrated that pretreatment of *Arabidopsis thaliana* leaves with flg22^{P.aer} increases resistance to pathogenic bacteria by restricting bacterial growth. This general elicitor represents a 22-amino acid peptide derived from *Pseudomonas aeruginosa* flagellin, the main building block of its flagella. In contrast to flg22^{P.aer}, a corresponding flagellin peptide derived from *Agrobacterium tumefaciens* (flg22^{A.tum}) is unable to restrict bacterial growth and thus considered an inactive flg22 peptide (Zipfel et al., 2004; see also Felix et al., 1999; Bauer et al., 2001).

Analogous to the Avr9/Cf-9 system, flg22^{P.aer} is perceived in *Arabidopsis* via FLS2, a type I membrane protein containing extracellular LRRs (Gomez-Gomez and Boller, 2000). FLS2 is classified as a receptor-like kinase due to its intracellular kinase domain, whose activity is required for flg22^{P.aer} responses (Gomez-Gomez and Boller, 2000; Gomez-Gomez et al., 2001). Although, thus far, Avr9 and flg22^{P.aer} responses have not been compared directly in the same plant species, many defense-related signaling events induced by flg22^{P.aer} are similar to those reported for Avr9. In *Arabidopsis*, elicitation with flg22^{P.aer} leads to an increase in *PR* gene transcript levels (Gomez-Gomez et al., 1999; Asai et al., 2002), and a recent comparison between *FLARE* (flagellin rapidly elicited) and *ACRE* (Avr9/Cf-9 rapidly elicited) genes points toward an overlap in early gene activation by flg22^{P.aer} in *Arabidopsis* and Avr9 in tobacco (Navarro et al., 2004). Furthermore, kinase inhibitors also block flagellin-induced cellular responses (Felix et al., 1991), and elicitation with flg22^{P.aer} leads to activation of MAPK signaling cascades (Nühse et al., 2000; Asai et al., 2002) and phosphorylation of mostly unknown targets (Peck et al., 2001; Nühse et al., 2003; see "Results" and "Discussion").

In this article, we identify a novel phosphorylation target in the early Avr9/Cf-9 signaling pathway, namely a syntaxin detected with an antibody against NtSyp121. NtSyp121 is a PM-localized syntaxin implicated in abscisic acid (ABA) responses (Leyman et al., 1999) and secretion (Geelen et al., 2002). As part of hetero-oligomeric SNARE complexes, syntaxins are

known to play a central role in the fusion of incoming transport vesicles with a target membrane throughout the endomembrane system (Lin and Scheller, 2000; Sanderfoot et al., 2000; Fasshauer, 2003). We show that a syntaxin was rapidly phosphorylated after elicitation with the race-specific elicitor Avr9, but not with the general elicitor flg22^{P.aer}. Thus, rapid syntaxin phosphorylation appears to represent a novel difference in the Avr9- and flg22^{P.aer}-dependent early signaling pathways.

RESULTS

Specific Appearance of an Additional Syntaxin Band in Response to Intercellular Fluid + Avr9

During our studies on the effects of Avr9 elicitation on the resistance protein Cf-9 in Cf9 tobacco leaves (A. Heese and J.D.G. Jones, unpublished data), we also probed leaf extracts with antibodies made against various cellular markers including an antibody against NtSyp121, a PM-localized syntaxin (Leyman et al., 2000). After infiltration of intercellular fluid (IF) either containing the Avr9 peptide (IF + Avr9) or without (IF - Avr9) into Cf9 tobacco leaves, leaf discs were harvested over a time period of 24 h after elicitation. Total protein extracts were prepared and analyzed by immunoblot analysis using the α NtSyp121 antibody (Fig. 1A). Equal loading was confirmed by Ponceau S staining of the protein blot (Fig. 1C). In immunoblots probed with α NtSyp121, the NtSyp121 protein was detected as the prominent band migrating at approximately 30 kD as judged by comparison to the pre-stained protein markers (data not shown). After elicitation with IF + Avr9, we consistently observed a slight decrease in NtSyp121 protein accumulation (or in that of a closely related syntaxin) within the first 30-min postelicitation (Fig. 1A; see "Discussion"). This decrease was followed by a significant increase in syntaxin accumulation over the next hours with the highest accumulation at 24 h. Changes in syntaxin protein accumulation at later time points were more pronounced after IF + Avr9 than after IF - Avr9 infiltration (see also transcript accumulation; Fig. 4C). No obvious changes in steady-state levels of BiP, a soluble endoplasmic reticulum protein (Fontes et al., 1991), were observed independent of whether leaves were infiltrated with IF + Avr9 or IF - Avr9 (Fig. 1B). At 24 h after Avr9 elicitation, we also detected an α NtSyp121 cross-reacting band that migrated slightly faster than NtSyp121 at approximately 28 kD (Fig. 1A, asterisk; see "Discussion").

Importantly, in extracts elicited with IF + Avr9, we observed the rapid appearance of an additional α NtSyp121 cross-reacting band that migrated at approximately 32 kD, thus slightly slower than NtSyp121 (Fig. 1A, arrowhead). This 32-kD band was only weakly induced after IF-Avr9 infiltration. The Avr9-dependent appearance of the 32-kD band was transient in that it was detected within 10 min (see also Fig. 4D),

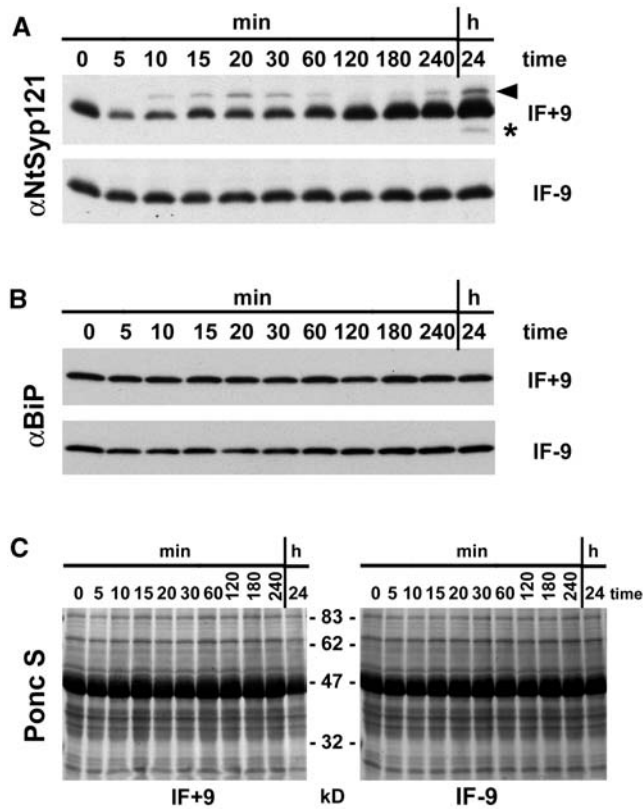


Figure 1. Avr9-dependent appearance of an additional α NtSyp121 band. A and B, After infiltration of Cf9 tobacco leaves with IF that lacked (IF-9) or contained Avr9 (IF+9), leaf discs were excised at indicated times in minutes (min) except for the last time point, which was taken at 24 h. Total protein extracts were subjected to immunoblot analysis using antibodies against α NtSyp121 (A) and α BiP (B). The position of the additional slower (32 kD) and faster (28 kD) migrating NtSyp121 cross-reacting bands are indicated by an arrowhead and an asterisk, respectively, in A. C, Equal protein loading was confirmed by Ponceau S (Ponc S) staining of membranes. Molecular mass standards are indicated in kiloDaltons (kD).

peaked at approximately 20 min, and disappeared after 2 h. It was visible again after 4 h and showed strongest accumulation 24 h after Avr9 elicitation. Based on its detection with the α NtSyp121 antibody (Fig. 1A) as well as its similar molecular mass to other syntaxins, including NtSyp121 (Leyman et al., 2000), we propose that this Avr9-inducible 32-kD band is a syntaxin that shares epitopes with NtSyp121. Consistent with it being a syntaxin, the protein of interest was also detected with another, unrelated syntaxin antibody (α AtSyp122; data not shown) that was made against the entire cytosolic part of the Arabidopsis syntaxin AtSyp122 and has been previously shown to detect both bacterial expressed AtSyp121 and AtSyp122 (Nühse et al., 2003).

Taking these results together, we observed a rapid Avr9-dependent accumulation of a 32-kD syntaxin that was detected with an α NtSyp121 antibody. In addition, over a period of hours, steady-state protein levels of NtSyp121 and/or a closely related syntaxin

accumulated to higher levels in response to IF + Avr9 than to IF - Avr9 infiltration.

Gene-for-Gene Dependency of the Appearance of the 32-kD Syntaxin

To determine whether in a gene-for-gene-dependent manner the rapid appearance of the 32-kD syntaxin band required the Avr9/Cf-9 combination, synthetic Avr9 peptide was infiltrated into tobacco leaves that stably expressed Cf-9 (*cf9:Cf9*; Cf9 tobacco) or that did not express Cf-9 (-). Tissue samples were taken at 0 and 15 min after infiltration, then processed and analyzed by immunoblot analysis using α NtSyp121 antibodies. Equal protein loading was confirmed by membrane staining with Ponceau S (data not shown). Elicitation with Avr9 led to a significant increase in the accumulation of the 32-kD α NtSyp121 band in leaves expressing Cf-9, irrespective of whether the Avr9 peptide was re-suspended in water (Fig. 2, *cf9:Cf9*/Avr9) or MES buffer at physiological pH 5.7 (see Fig. 4D, Avr9). No significant increase in the band of interest was observed when Cf9 tobacco leaves were infiltrated with water (Fig. 2, *cf9:Cf9*/H₂O), IF - Avr9 (Fig. 1A, IF-9), or MES buffer (Fig. 4D, mock). Furthermore, when infiltrated into leaves lacking Cf-9, Avr9 peptide (Fig. 2, -/Avr9) did not induce any significant increase in the accumulation of the additional syntaxin. In control experiments, no significant increase in the 32-kD syntaxin was detected after infiltration of tobacco leaves lacking Cf-9 with IF + Avr9, IF - Avr9, or water (data not shown). We conclude that, in accordance with the gene-for-gene hypothesis, the significant increase in protein accumulation of the 32-kD α NtSyp121 band required the presence of both the elicitor Avr9 and the resistance gene Cf-9.

Avr9-Dependent Syntaxin Phosphorylation

Because the transient nature of a small increase in apparent molecular mass would be consistent with phosphorylation of NtSyp121 or a closely related syntaxin, we tested whether the appearance of the 32-kD band was due to phosphorylation of an α NtSyp121 cross-reacting syntaxin. To this end, we challenged tobacco leaves expressing Cf-9 with IF + Avr9 for 15 min, collected leaf discs, and treated total proteins

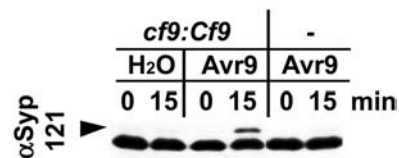


Figure 2. Gene-for-gene dependency of the appearance of the 32-kD α NtSyp121 band. Tobacco leaves that expressed Cf-9 (*cf9:Cf9*) or lacked Cf-9 (-) were infiltrated with Avr9 or water (H₂O). Leaf discs were excised at indicated times in minutes (min). Total protein extracts were subjected to immunoblot analysis using the α NtSyp121 (α Syp121) antibody. The position of the phosphorylated syntaxin is indicated by the arrowhead.

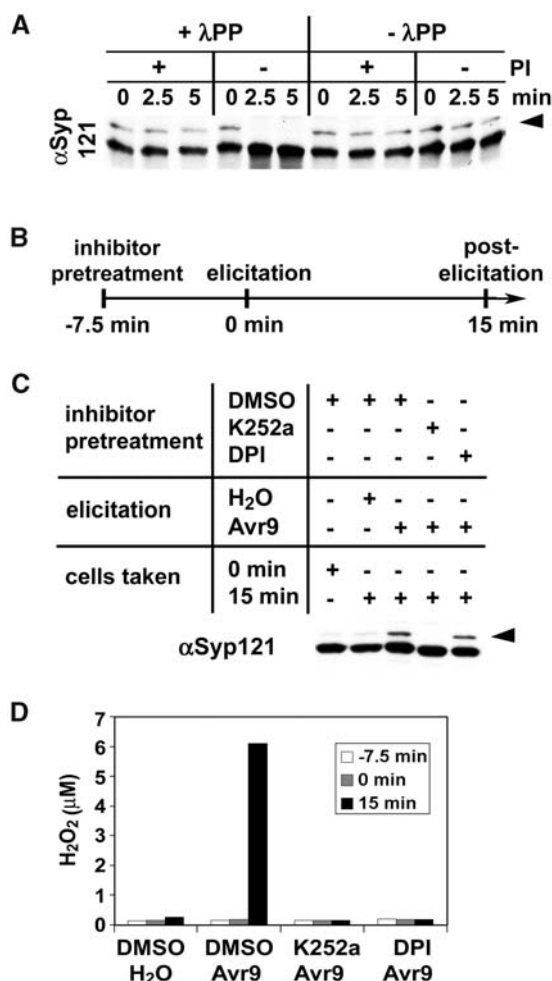


Figure 3. The appearance of the 32-kD syntaxin was due to Avr9-dependent phosphorylation. **A**, In vitro dephosphorylation studies. Cf9 tobacco leaves were elicited with IF + Avr9 for 15 min. Total protein extracts were then incubated with (+ λ PP) or without (- λ PP) lambda phosphatase in the presence (+) or absence (-) of PIs at 30°C. At indicated times in minutes (min), an equal amount of protein extract was withdrawn and subjected to immunoblot analysis using the α NtSyp121 (α Syp121) antibody. The position of the phosphorylated syntaxin is indicated by the arrowhead. **B**, Timeline for in vivo inhibitor studies and production of ROS shown in Figure 3, C and D, respectively. Cf9 tobacco-cultured cells (8808) were pretreated with DMSO, 1 μ M K252a, or 0.8 μ M DPI for 7.5 min (-7.5 min; inhibitor pretreatment). After addition of 20 nM Avr9 (Avr9) or water (0 min; elicitation), cells were withdrawn after 15 min for subsequent analysis (15 min; postelicitation). **C**, In vivo inhibitor studies in Cf9 tobacco-cultured cells. After pretreatment with DMSO, 1 μ M K252a, or 0.8 μ M DPI for 7.5 min (inhibitor pretreatment), Cf9 tobacco cells were harvested prior to (0 min) or 15 min after elicitation (15 min) and subjected to immunoblot analysis using the α NtSyp121 (α Syp121) antibody. The position of the phosphorylated syntaxin is indicated by the arrowhead. **D**, Oxidative burst in Cf9 tobacco-cultured cells. Aliquots of the Cf9 tobacco cells were taken at -7.5, 0, and 15 min (see timeline in B) and measured for ROS synthesis (expressed as H₂O₂ production) by ferricyanide-catalyzed oxidation of luminol. Although the absolute value of the H₂O₂ production varied from experiment to experiment, the trends were always similar. As an example, ROS measurements obtained from the same Cf-9 tobacco cell batch used for the in vivo inhibitor immunoblot analysis (shown in C) are depicted.

with lambda phosphatase (λ PP; Fig. 3A). Equal amounts of protein extract were withdrawn 0, 2.5, and 5 min after λ PP-treatment and subjected to immunoblot analysis using the α NtSyp121 antibody. Equal protein loading was confirmed by membrane staining with Ponceau S (data not shown). In the absence of phosphatase inhibitors (PIs), the slower migrating cross-reacting band disappeared within 2.5 min after addition of the phosphatase (+ λ PP/-PI). In control experiments, the band of interest did not disappear when protein samples were (1) incubated with both PIs and phosphatase (+ λ PP/+PI), (2) incubated with PI but without λ PP (- λ PP/+PI), or (3) without both λ PP and PIs (- λ PP/-PI). These results indicate that the disappearance of the band was due to the dephosphorylation activity of λ PP but not to non-specific protein degradation. Phosphatase treatment in the absence of PIs did not lead to the appearance of an additional α NtSyp121 cross-reacting band. We observed, however, a slight increase in the signal intensity of the 30-kD α NtSyp121 cross-reacting band, suggesting that NtSyp121 or a syntaxin with an apparent molecular mass of 30 kD may serve as the Avr9-dependent phosphorylation target. It is also possible, however, that, in its dephosphorylated form, the 32-kD syntaxin may be unstable or may not be recognized by the α NtSyp121 antibody. Furthermore, we cannot exclude that additional yet uncharacterized phosphorylated forms of NtSyp121 and/or of other closely related syntaxins were present in nonelicited and/or elicited leaves, which may be detected only by the α NtSyp121 antibody in a dephosphorylated state.

Next, we investigated whether pretreatment with a kinase inhibitor could prevent the appearance of the phosphorylated syntaxin in vivo using tobacco suspension culture cells, a system highly suitable for inhibitor studies (Piedras et al., 1998; Romeis et al., 1999, 2000; Durrant et al., 2000). To this end, we established a cell suspension culture from Cf9 tobacco leaves (8808; used in this study), which expressed the single Cf-9 gene under the control of its own promoter. As observed by ROS production (Fig. 3D, dimethyl sulfoxide [DMSO]/Avr9; data not shown), the responsiveness of these cells to Avr9 was comparable to the previously described cell culture 34.1B, which expressed not only the Cf-9 gene but also other closely related Cf-9 paralogs (Piedras et al., 1998; Romeis et al., 1999, 2000). For kinase inhibitor studies, cells were pretreated for 7.5 min (-7.5 min; see timeline, Fig. 3B) with either 1 μ M K252a, a general Ser/Thr kinase inhibitor resuspended in DMSO, or with DMSO alone. After 7.5 min pretreatment, the cells were elicited with Avr9 peptide (0 min, see also timeline, Fig. 3B), and at 15 min postelicitation, cells were withdrawn for subsequent immunoblot analysis using the α NtSyp121 antibody (Fig. 3C). Aliquots of the same cell batch used for immunoblot analysis were also analyzed for ROS production (Fig. 3D). Similar to the in planta results (Figs. 1A and 2), elicitation with Avr9 induced syntaxin phosphorylation in Cf9 suspension-cultured cells (Fig. 3C, DMSO/Avr9/15 min). Importantly, pretreatment

with K252a completely inhibited this response (Fig. 3C, K252a/Avr9/15 min), indicating that kinase activity was required for the appearance of the band of interest. Consistent with a previous report (Piedras et al., 1998), pretreatment with K252a also completely blocked ROS production (Fig. 3D, K252a/Avr9). In control experiments, no significant increase in syntaxin phosphorylation (Fig. 3C, DMSO/H₂O/15 min) or in ROS production (Fig. 3, DMSO/H₂O) was observed when cells were treated with water. We further attempted to place the syntaxin phosphorylation within the Avr9-dependent cellular-signaling cascade. In contrast to K252a, pretreatment with 0.8 μ M diphenyleneiodium (DPI), known to interfere with NADPH oxidase function (Piedras et al., 1998; Romeis et al., 2000), did not inhibit the phosphorylation shift (Fig. 3C, DPI/Avr9/15 min). This compound was functional, however, because DPI pretreatment completely blocked ROS production in suspension-cultured cells (Fig. 3D, DPI/Avr9; Piedras et al., 1998; Romeis et al., 2000). We conclude that syntaxin phosphorylation is not ROS dependent, indicating that this phosphorylation event occurs either upstream of or in parallel to the pathway leading to ROS production. Studies using calcium-dependent kinase inhibitors were inconclusive because the concentrations previously used were toxic in our experiments (see "Discussion").

Based on the dephosphorylation and the *in vivo* inhibitor studies, we conclude that the appearance of the additional α NtSyp121 cross-reacting band was due to an Avr9/Cf-9-dependent phosphorylation event of NtSyp121 or a closely related syntaxin.

Induction of Defense-Related Signaling Events by the Race-Specific Elicitor flg22^{P.aer} in Cf9 Tobacco Leaves

Recently, AtSyp122, an Arabidopsis PM syntaxin closely related to NtSyp121, was shown to be phosphorylated within 3 min of treatment with the general elicitor flg22^{P.aer}, the flagellin peptide derived from *P. aeruginosa* (Nühse et al., 2003). Both Avr9 and flg22^{P.aer} are perceived through type I membrane proteins containing extracellular LRRs, respectively Cf-9 and FLS2, and elicit similar early signaling events (see introduction). Thus, we were interested in determining whether syntaxin phosphorylation also occurred in response to flg22^{P.aer}. But although flg22^{P.aer} is a potent elicitor in a variety of different plant species including tobacco suspension culture cells (Felix et al., 1999; A. Heese, M. Smoker, and J.D.G. Jones, unpublished data), we needed to confirm that flg22^{P.aer} was able to elicit defense-related responses in tobacco leaves. For these *in planta* studies, we used flg22^{P.aer} at a concentration (1 μ M) previously used to elicit signaling events in Arabidopsis leaves and seedlings (Gomez-Gomez et al., 1999, 2001; Bauer et al., 2001).

First, we examined whether flg22^{P.aer} induced early defense-related responses such as oxidative burst and MAPK activation in Cf9 tobacco leaves. Consistent with results obtained in Cf9 suspension-cultured cells

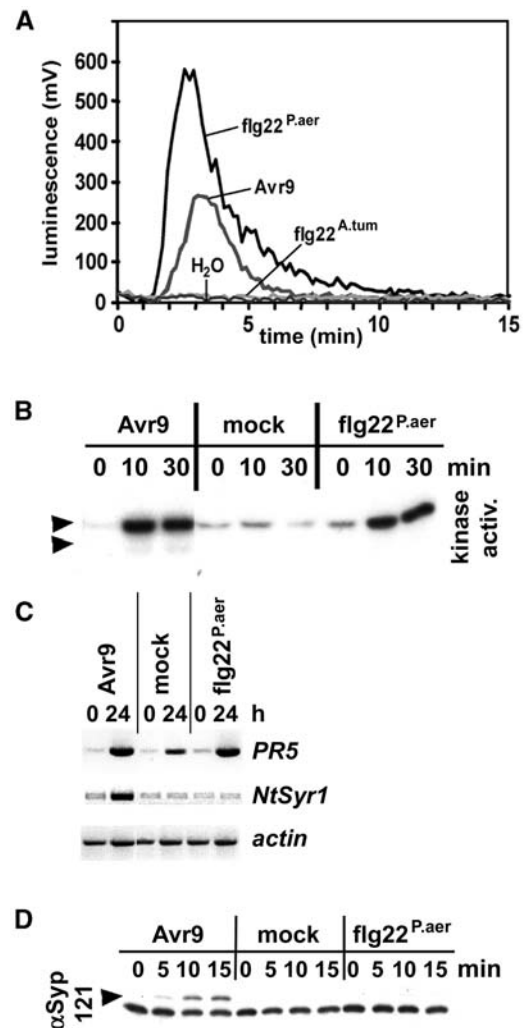


Figure 4. Syntaxin phosphorylation occurred in response to the race-specific elicitor Avr9 but not to the general elicitor flg22^{P.aer}. **A**, Oxidative burst in Cf9 tobacco leaf pieces. Oxidative burst was assayed by measuring the H₂O₂-dependent luminescence of luminol after addition of 40 nM Avr9, 1 μ M flg22^{P.aer}, 1 μ M inactive flg22^{A.tum}, or water over time in minutes (min). Although the absolute value of the H₂O₂ production varied from experiment to experiment, the trends were always similar, and one example of these responses is shown. **B**, Activation of MAPK. Leaves were infiltrated with 40 nM Avr9, 1 μ M flg22^{P.aer}, or 5 mM MES buffer, pH 5.7 (mock). Cf9 tobacco leaves discs were excised at indicated times in minutes (min). Kinase activity (kinase activ.) was analyzed using an in-gel kinase assay with myelin-basic protein as a substrate. The positions of SIPK and WIPK activities are indicated by arrowheads. **C**, Induction of gene transcript levels. Cf9 tobacco leaves were infiltrated with 40 nM Avr9 peptide (Avr9), 1 μ M flg22^{P.aer}, or water (mock). Leaf discs were excised at indicated times in hours (h). Total RNA was extracted and subjected to RT-PCR using primers for *PR5* and *NtSyp121*. *Actin* primers were used to confirm equal loading. **D**, Syntaxin phosphorylation. Cf9 tobacco leaves were infiltrated with 40 nM Avr9, 1 μ M flg22^{P.aer}, or 5 mM MES buffer, pH 5.7 (mock). Leaf discs were excised at indicated times in minutes (min). Total protein extracts were subjected to immunoblot analysis using the α NtSyp121 (α Syp121) antibody. The position of the phosphorylated syntaxin is indicated by the arrowhead.

(Fig. 3D), treatment of Cf9 tobacco leaf pieces with 40 nM Avr9 led to the rapid release of ROS as evident by an increase in the H₂O₂-dependent luminescence of luminol within 2 min (Fig. 4A, Avr9). Treatment with water as a control did not lead to any significant increase in ROS levels. Importantly, leaf pieces treated with 1 μ M flg22^{P.aer} responded with a significant release of ROS (flg22^{P.aer}), and this response was consistently slightly more rapid and stronger than the Avr9-induced oxidative burst. To confirm the specificity of the flg22^{P.aer} response in Cf9 tobacco leaves, we measured ROS levels after treatment with 1 μ M of the inactive flg22 peptide derived from *A. tumefaciens* (flg22^{A.tum}; Bauer et al., 2001; Zipfel et al., 2004). As shown in Figure 4A, addition of flg22^{A.tum} did not induce any significant increase in ROS levels, which were similar to those measured after water treatment. In additional control experiments, only flg22^{P.aer} but not Avr9, water, or flg22^{A.tum} led to any significant increase in ROS levels in tobacco leaves lacking Cf-9 (data not shown).

To determine whether MAPK activity increased after flg22^{P.aer} or Avr9 treatment, Cf9 tobacco leaf discs were collected at 0, 10, and 30 min after elicitor infiltration. Protein extracts were analyzed for an increase in MAPK activity by in-gel kinase assays using γ -³²P-ATP and myelin basic protein as a substrate. As previously shown in Cf9 tobacco leaves (Romeis et al., 1999), an increase in the MAPK activities of WIPK and SIPK is evident by an increase in the phosphorylation of myelin basic protein at approximately 46 kD and approximately 48 kD, respectively. Equal protein loading was confirmed by staining of the gel with Coomassie Blue (data not shown). Consistent with Romeis et al. (1999), elicitation with Avr9 led to a strong increase in SIPK activity (approximately 48 kD) within 10 min after elicitation, which prevailed for at least 30 min. Induction of WIPK activity (approximately 46 kD) was much weaker, but consistently observed after longer exposures (data not shown). When compared to the buffer control (mock), flg22^{P.aer} clearly elicited MAPK activity above the flooding/wounding response. The timing of MAPK activation by flg22^{P.aer} was similar to that by Avr9, although activation was consistently slightly weaker with flg22^{P.aer} than with Avr9. Diverse elicitors have been previously shown to lead to different intensities of MAPK activation (Cardinale et al., 2000).

We also investigated whether late defense responses were induced by flg22^{P.aer} in Cf9 tobacco leaves. Transcript level changes of *PR5*, a late defense response gene previously shown to be induced 24 h after 1 μ M flg22^{P.aer} elicitation in Arabidopsis (Gomez-Gomez et al., 1999), were determined by reverse transcription (RT)-PCR. Total RNA was isolated at 0 and 24 h after 40 nM Avr9, 1 μ M flg22^{P.aer} or mock treatment. Actin transcript levels were used as a loading control (Fig. 4C). When compared to mock-treated samples, addition of Avr9 or flg22^{P.aer} led to a strong increase in *PR5* transcript accumulation after 24 h (Fig.

4C). When examining the same RNA samples for effects on *NtSyp121* transcript levels, we observed that *NtSyp121* transcript levels were elevated 24 h after Avr9, but not after flg22^{P.aer} or mock treatment (Fig. 4C).

Taking these results together, flg22^{P.aer}-induced production of ROS, activation of MAPKs, and increase in *PR5* transcript levels clearly demonstrated that flg22^{P.aer} elicited early and late defense-related signaling events in Cf9 tobacco leaves. Because flg22^{A.tum} treatment failed to induce ROS production, recognition of flg22^{P.aer} in tobacco leaves was specific. Accumulation of *NtSyp121* transcript at 24 h appeared to be Avr9 but not flg22^{P.aer} dependent.

Syntaxin Phosphorylation Is Triggered by the Race-Specific Elicitor Avr9 But Not by the General Elicitor flg22^{P.aer}

To determine whether syntaxin phosphorylation occurred not only in response to the race-specific elicitor Avr9 but also to the general elicitor flg22^{P.aer}, Cf9 tobacco leaves were infiltrated with 40 nM Avr9, 1 μ M flg22^{P.aer}, or MES buffer alone (pH 5.7; mock). Leaf discs were collected 0, 5, 10, or 15 min after elicitation. Total proteins isolated from each time point were subjected to immunoblot analysis using the α NtSyp121 antibody (Fig. 4D). Equal protein loading was confirmed by membrane staining with Ponceau S (data not shown). In contrast to the Avr9-dependent syntaxin phosphorylation (Fig. 4D, Avr9), at concentrations shown to induce ROS production, MAPK activation, and *PR5* transcript accumulation in Cf9 tobacco (Fig. 4A, B and C), flg22^{P.aer} treatment did not lead to a significant accumulation of the phosphorylated syntaxin (Fig. 4D, flg22^{P.aer}). Its levels were comparable to those observed after mock treatment (Fig. 4D, flg22^{P.aer} and mock) indicating that syntaxin phosphorylation was not induced by the addition of any biologically active peptide. Furthermore, these data suggest that NtSyp121 or a closely related syntaxin was phosphorylated in response to the race-specific elicitor Avr9 but not to the general elicitor flg22^{P.aer}.

DISCUSSION

Examination of Avr9-induced changes in ion fluxes, gene expression, and protein activity or abundance has revealed signaling components of the Avr9/Cf-9-dependent pathway (Romeis et al., 1999, 2000; Rowland et al., 2005; J.D.G. Jones, unpublished data). Using an antibody against one of the few well-characterized PM proteins of tobacco, the syntaxin NtSyp121, we detected an additional, slower migrating α NtSyp121 cross-reacting band within 5 to 10 min of Avr9 elicitation (Figs. 1A and 4D). Based on its detection by two unrelated syntaxin antibodies, α NtSyp121 (Figs. 1–4) and α AtSyp122 (data not shown), and its apparent molecular mass of approximately 32 kD which is similar to other known syntaxins including NtSyp121

(Leyman et al., 2000), this additional protein band is likely to be a syntaxin. In vitro dephosphorylation and in vivo kinase inhibition assays indicated that the rapid appearance of the additional syntaxin was due to and required phosphorylation. As observed for many *Avr9/Cf-9*-dependent responses (for review, see Rivas and Thomas, 2002), this syntaxin phosphorylation was transient in that the accumulation of the phosphorylated syntaxin peaked around 20 min but returned to nearly basal levels within 2 h. The transient nature of this phosphorylation event suggests that in addition to kinase(s), phosphatase(s) may also regulate this response or alternatively that the phosphorylated syntaxin form may be preferentially degraded.

Because its apparent molecular mass is only a few kD larger than NtSyp121, one interpretation is that the additional syntaxin band could be a phosphorylated form of NtSyp121. So far, however, NtSyp121 is the only known syntaxin in tobacco. Although the α NtSyp121 antibody employed in this study was previously used to show PM localization of NtSyp121 by electron microscopy (Leyman et al., 2000), it is unclear whether this antibody is specific for NtSyp121 or also detects other closely related, yet unknown syntaxins. At this point, we can only speculate why the additional 32-kD syntaxin band was also detected at later time points (Fig. 1A, 4 and 24 h). One possibility is that rapid and later syntaxin modifications may not only be temporally but also spatially separated in that they occurred at different times in different cells, in particular because leaf discs were used for time-course analyses that contained a mixture of cell types. Alternatively, the appearance may represent a secondary phosphorylation event involved in later responses. We cannot exclude, however, that its appearance was caused by mere technical reasons.

In *Arabidopsis*, the PM-localized syntaxin AtSyp122 is rapidly phosphorylated in response to the general elicitor flg22^{P.aer} (Nühse et al., 2003). The closest relative of this syntaxin is AtSyp121, the proposed ortholog of NtSyp121 (see below). Based on sequence comparisons, the in vivo phosphorylation site in the N terminus of AtSyp122 (M¹NDLLSGSFK; phosphorylation site is underlined; Nühse et al., 2004) was conserved in NtSyp121 (M¹NDLFSGSFS). Phosphorylation of AtSyp122 is calcium dependent (Nühse et al., 2003), and calcium-dependent kinases are required for the *Avr9/Cf-9* defense response pathway (Romeis et al., 2000, 2001). Our attempts to determine whether the *Avr9*-dependent syntaxin phosphorylation was inhibited by calcium-dependent kinase inhibitors (such as trifluoperazine dimaleate (TFP) and W-7) were however inconclusive. When cells were pretreated with inhibitors at concentrations previously used (Romeis et al., 2000), we observed that TFP or W-7 blocked syntaxin phosphorylation; however, most cells died during the course of these experiments (within 20 min; A. Heese, M. Smoker, and J.D.G. Jones, unpublished data). Because lower concentrations of TFP and W-7 permitted cell survival but did not block

physiological responses, we were unable to draw any meaningful conclusions from these experiments. In contrast, pretreatment with K252a and DPI had no effect on cell viability as over 90% of the treated cells survived.

Interestingly, we observed that rapid phosphorylation of the tobacco syntaxin was triggered in response to the race-specific elicitor *Avr9* but not to the general elicitor flg22^{P.aer} (Fig. 4D). Despite these data, flg22^{P.aer} is recognized by Cf9 tobacco leaf cells because, similar to *Avr9*, this elicitor peptide induced defense-related signaling events such as ROS production, MAPK activation, and *PR5* transcript accumulation (Fig. 4, A–C), and these latter results are consistent with previous reports noting the resemblance between defense-signaling responses initiated through flg22^{P.aer}/FLS2 and race-specific *Avr/R-gene* combinations, including *Avr9/Cf-9* (Nühse et al., 2000; Navarro et al., 2004; Zipfel et al., 2004). We also confirmed that the flg22^{P.aer} response was specific in Cf9 tobacco leaf cells because an inactive flg22 peptide (flg22^{A.tum}; Bauer et al., 2001; Zipfel et al., 2004) did not induce ROS levels (Fig. 4A). Thus, we propose that the syntaxin phosphorylation identified in this study may serve as an early biochemical marker to elucidate further differences in the signaling pathways of these two elicitors. At this point, we cannot exclude the possibility that syntaxin phosphorylation may be important for the formation of the hypersensitive response (HR). In contrast to *Avr9*, we did not observe any HR after infiltration of 1 μ M flg22 into Cf9 tobacco leaves (data not shown), consistent with previous reports (Gomez-Gomez and Boller, 2002).

In contrast to syntaxin phosphorylation being an early biochemical marker, *NtSyp121* transcript up-regulation may serve as a late molecular marker in differentiating the signaling responses to *Avr9* and flg22^{P.aer} because *NtSyp121* transcript levels were elevated at 24 h after *Avr9* but not flg22^{P.aer} treatment. As suggested by Wick et al. (2003), levels of SNARE components may be up-regulated after pathogen attack to compensate for the increased secretion activity to export pathogenesis-related proteins and antimicrobial compounds required for the cellular protection against pathogens at a later stage of infection. It should be noted that *NtSyp121* transcript levels have been previously reported to be induced by mechanical wounding within 1 h (Leyman et al., 2000). Consistent with this study, we also observed an increase in *NtSyp121* transcript levels to comparable levels irrespective of whether Cf9 tobacco leaves were treated with *Avr9*, flg22^{P.aer}, or buffer for 1 h (data not shown). Thus, the added early wound stress may have masked any possible *Avr9*-dependent *NtSyp121* transcript changes, making it difficult to determine whether *Avr9* may affect NtSyp121 transcript levels at early time points.

Consistent with the *Avr9*-dependent up-regulation of *NtSyp121* transcript levels at 24 h, an increase in protein levels of NtSyp121 or of a closely related 30-kD

syntaxin was evident after infiltration with IF + Avr9 at later time points with its highest accumulation at 24 h (Fig. 1A), possibly representing an Avr9-dependent up-regulation of the 30-kD syntaxin(s). In addition, Avr9 may induce protein accumulation of other yet unknown syntaxin(s) because another α NtSyp121 cross-reacting band of approximately 28 kD that migrated slightly faster than NtSyp121 was detected 24 h after infiltration with IF + Avr9 but not with IF – Avr9 (Fig. 1A, asterisk). Alternatively, this faster migrating α NtSyp121 band may represent a degradation product of NtSyp121 because the timing of its appearance correlated well with the visible onset of Avr9-induced HR at 24 h (Hammond-Kosack et al., 1998; data not shown).

We also consistently observed a slight decrease in the immunoblot signal for NtSyp121 and/or of a closely related 30-kD syntaxin during the initial 30 min after infiltration with IF + Avr9 (Fig. 1A) and Avr9 peptide (Fig. 4D), possibly suggesting an Avr9-dependent degradation of the 30-kD syntaxin band(s) during earlier time points. It should be noted, however, that no specific Avr9-dependent decrease in the α NtSyp121 cross-reacting band of 30 kD was apparent when protein synthesis was blocked by pretreatment of leaves with cycloheximide (data not shown). Furthermore, in nearly all experiments, the timing of this decrease appeared to correlate with the appearance of the phosphorylated syntaxin consistent with the possibility that Avr9 elicitation may lead to a posttranslational modification, possibly phosphorylation, of NtSyp121 and/or a closely related syntaxin that impairs cross reactivity with the antibody.

At this point, we can also only speculate about a possible role(s) of the rapid Avr9-dependent syntaxin phosphorylation. Classically, syntaxins participate in the fusion of incoming transport vesicles with a target membrane as part of the heterotrimeric SNARE complex (Lin and Scheller, 2000; Sanderfoot et al., 2000; Fasshauer, 2003). Consistent with this, tobacco syntaxins whose functions are inhibited by overexpression of the cytosolic NtSyp121 fragment appear to function in general secretion (Geelen et al., 2002). Interestingly, in mammalian and yeast cells, phosphorylation of SNARE components seems to modulate SNARE complex formation during membrane trafficking and fusion. Its exact function, however, remains controversial because, depending on the cell system, phosphorylation of SNARE components has been reported to either promote (Cabaniols et al., 1999; Polgar et al., 2003) or inhibit SNARE complex interaction (Gurunathan et al., 2002; Marash and Gerst, 2003).

Functional evidence for the involvement of syntaxins in plant disease resistance comes from the recent discovery that mutations in the PM-localized syntaxin *AtSYP121/PEN1* lead to an increase in penetration frequency of the fungus *Blumeria graminis* f. sp. *hordei* into epidermal cells of the nonhost *Arabidopsis* (Collins et al., 2003). Furthermore, *HvSYP121/ROR2*, the functional barley (*Hordeum vulgare*) homolog of *AtSYP121/*

PEN1, is required for elevated basal penetration resistance against *B. graminis* f. sp. *hordei* in barley *mlo* mutants (Collins et al., 2003). Based on sequence alignment, NtSyp121 may be the tobacco ortholog of *AtSYP121/PEN1* (Leyman et al., 1999; Sanderfoot et al., 2000), yet so far NtSyp121 is the only characterized syntaxin in tobacco. It remains to be shown whether NtSyp121 is the functional homolog of *AtSyp121/Pen1*, in particular because mutations in *AtSYP121/PEN1* do not lead to any obvious defects in stomatal closing ability, root development, or general growth (Collins et al., 2003), functions previously attributed to NtSyp121 (Leyman et al., 1999; Geelen et al., 2002). Consistent with different PM syntaxins having different roles in defense, *AtSyp121/Pen1* appears to function in polarized secretion, possibly of antimicrobial compounds and PR proteins, and in papilla formation (Assaad et al., 2004). In contrast to *AtSYP121/PEN1*, *AtSYP122* mutant plants show a small but significant reduction in penetration resistance against *B. graminis* f. sp. *Hordei*, and *AtSyp122* protein has been proposed to function in diffuse secretion (Assaad et al., 2004).

In addition to mediating vesicle fusion, syntaxins localized to the PM have also been reported to modulate ion channel and transporter activity in animal cells (for review, see Catterall, 2000; Atlas, 2001; Peters et al., 2001). In plants, syntaxin(s) that is cleavable with *Clostridium botulinum* type C toxin appears to regulate ABA-mediated potassium and chloride channels in tobacco guard cells (Leyman et al., 1999). Furthermore, NtSyp121 was originally identified in a screen for ABA-signaling components involved in the control of ion channels when expressed in *Xenopus* oocyte cells (Leyman et al., 1999). Interestingly, Avr9 challenge also affects activities of potassium and chloride channels in Cf9 tobacco guard cells, and this response is blocked by kinase inhibitors (Blatt et al., 1999). Because the timing of Avr9-dependent channel activation (Blatt et al., 1999) and syntaxin phosphorylation (this study) coincide, it is possible that the Avr9-dependent syntaxin phosphorylation may participate directly or indirectly in channel activation. It is important to note that despite the similarities in response to the two stimuli, Avr9- and ABA-evoked phosphorylation events mediate channel activation through different pathways (Blatt et al., 1999).

CONCLUSION

In summary, we identified syntaxin(s) as a novel component in the Avr9/Cf9-race-specific signaling pathway. Rapid syntaxin phosphorylation and late *NtSyp121* transcript accumulation was triggered by Avr9 but not flg22^{P.aer}, identifying novel differences between these elicitor peptides. In the future, syntaxin phosphorylation and *NtSyp121* transcript accumulation may serve as early biochemical and late molecular biomarkers, respectively, to gain a better understanding in differences between the race-specific elicitor

signaling pathway through Avr9/Cf-9 and that of the general elicitor signaling pathway through flg22^{P.aer}/FLS2.

MATERIALS AND METHODS

Plant Culture Conditions

Cf9 tobacco (*Nicotiana tabacum*) plants of the genotypes *Petit Havana* (–; nontransformed) or SLJ8808 (*Petit Havana* transformed with *cf9:Cf9*; Hammond-Kosack et al., 1998) were grown in the greenhouse as described (Romeis et al., 1999). The fourth to sixth leaves of 6- to 10-week-old plants were used for all experiments. To enable proper comparison, a set of treatments were performed within the same leaf. The tobacco cell suspension culture line 8808 expressing the single gene *Cf-9* was generated from leaf pieces (SLJ8808) and was grown and maintained as described by Piedras et al. (1998).

Time-Course Experiments for Immunoblot Analyses

Leaves were infiltrated with IF isolated from transgenic tobacco leaves expressing Avr9 peptide apoplastically (IF + Avr9) or from control tobacco leaves lacking Avr9 (IF – Avr9) (Piedras et al., 1998). Experiments were repeated more than six times for earlier time points and at least two times for later time points. For analyses using synthesized peptide, 40 nM Avr9 (Piedras et al., 1998) or 1 μ M flg22^{P.aer} (Sigma, Poole, UK; Gomez-Gomez et al., 1999, 2001; Bauer et al., 2001) were diluted in 5 mM MES, pH 5.7, or water prior to infiltration. Experiments were performed two and three times for dilution in MES buffer and water, respectively.

For protein-blot experiments, 1-cm-diameter leaf discs were collected at indicated times, immediately frozen, and ground in liquid nitrogen. Each sample was thawed in 100 μ L of a 1:1 mixture of 2 \times sample buffer and buffer H (100 mM Tris-HCl, pH 7.5, 250 mM Suc, 15 mM EDTA, 5% glycerol, 0.5% polyvinylpyrrolidone K25, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/mL antipain, 2 μ g/mL leupeptin, 2 μ g/mL aprotinin, 50 mM sodium pyrophosphate, 25 mM sodium fluoride [NaF], 1 mM sodium molybdate) and stored at –20°C until immunoblot analysis. Protein samples were heated for 5 to 10 min at 65°C and centrifuged at 16,000g for 10 min. Total protein extracts (10 μ L, equivalent to 40 μ g protein) were separated on 13% SDS-PAGE and transferred to Protran BA85 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The apparent molecular masses of proteins were compared to the Prestained Protein Marker (broad range; Bio-Rad Laboratories, Hercules, CA). After staining with Ponceau S (Sigma) to confirm equal protein loading, membranes were cut into horizontal strips, which were subjected to immunoblot analysis using the appropriate primary antibodies (α Sp3/NtSyp121, 1:4,000; Leyman et al., 2000; BiP, 1:20,000; Fontes et al., 1991). After incubation with the secondary antibody (anti-rabbit IgG-peroxidase, 1:15,000; Sigma), signals were visualized using an enhanced chemiluminescence system (ECL kit, Amersham, Little Chalford, UK).

Dephosphorylation Studies

After elicitation of Cf9 tobacco leaves with IF + Avr9 for 15 min, 4- \times 1 cm-diameter leaf discs (SLJ8808) were collected, frozen, and ground in liquid nitrogen. Samples were resuspended in 400 μ L buffer H containing 3 mM dithiothreitol but lacking EDTA or any other phosphatase or kinase inhibitors. Crude protein extracts (80 μ L) were incubated with 400 U of nonspecific λ protein phosphatase (New England Biolabs, Beverly, MA) in the presence or absence of PIs (10 mM sodium vanadate, 50 mM NaF, 50 mM EDTA) at 30°C according to the manufacturer's specifications (120 μ L final volume). Control samples were treated exactly the same except that λ protein phosphatase was omitted. At 0, 2.5, or 5 min, 34- μ L samples were withdrawn and added to 16 μ L 4 \times sample buffer containing additional PI (see above) to stop the reaction. Samples were frozen and stored at –20°C. Twenty microliters of samples were subjected to immunoblot analysis. Dephosphorylation experiments were performed three times.

Inhibitor and Oxidative Burst Studies

Log phase suspension culture cells (3–5 d old) were prepared for inhibitor and elicitor treatments as described (Romeis et al., 1999). Prior to elicitation

with 20 nM Avr9 peptide or water, cells were pretreated with 0.8 μ M DPI (Sigma), 1 μ M K252a (*Nocardioopsis* sp.; Calbiochem, Darmstadt, Germany) or DMSO (mock) for 7.5 min. For protein analysis, 4.5 mL of cells were withdrawn at indicated time points, collected by filtration, added to 0.4 mL ice-cold homogenization buffer containing 3 mM dithiothreitol, 50 mM sodium pyrophosphate, 25 mM NaF, 1 mM sodium molybdate, mixed, and immediately frozen in liquid nitrogen. Cells were lysed by rapid five to six freeze-thaw cycles using a sonication water bath and freezing in liquid nitrogen. After addition of 6 \times sample buffer, samples were stored at –20°C until immunoblot analysis. Experiments were performed at least three times for each treatment.

In parallel to immunoblot analysis, 0.2-mL aliquots of the same cell cultures were tested at indicated time points for ROS production using the ferricyanide-catalyzed oxidation of luminol (Piedras et al., 1998). Data were repeated in at least three different experiments with similar results, and although the absolute value of the H₂O₂ production varied from experiment to experiment, the trends were always the same. An example of these responses is shown in Figure 3D.

Measurements of ROS in tobacco leaf tissue were assayed by measuring the H₂O₂-dependent luminescence of luminol as described for *Arabidopsis thaliana* leaf tissue (Gomez-Gomez et al., 1999). Briefly, 1-cm-diameter tobacco leaf discs were cut into small pieces and floated overnight in water. Immediately prior to elicitation with synthesized peptides (40 nM Avr9, 1 μ M flg22^{P.aer}, 1 μ M inactive flg22^{A.tum} [Sigma]) or water, the incubating reaction was exchanged with 300 μ L water containing 50 μ M luminol and 10 μ g/mL peroxidase. Luminescence was measured every 10 s for 15 min in a Photek HRPCS-3 PSU camera (Photek, East Sussex, UK). Experiments were repeated at least 10 or six times for Cf9 tobacco or those lacking Cf9, respectively. Although the absolute value of the H₂O₂ production varied from experiment to experiment, the trends were always the same. An example of the responses is shown in Figure 4A.

In-Gel MAPK Assay

After elicitor treatment with 40 nM Avr9, 1 μ M flg22^{P.aer}, or 5 mM MES buffer alone (pH 5.7; mock), leaf discs of 2 cm diameter were immediately frozen in liquid nitrogen. MAPK activity was determined by in-gel kinase assays with myelin basic protein (Sigma) as substrate as described previously (Romeis et al., 1999). Comparable results were obtained when peptides were resuspended in water instead of MES buffer, and experiments were performed at least twice for each treatment.

RNA Isolation and RT-PCR

After elicitor treatment with 40 nM Avr9, 1 μ M flg22^{P.aer}, or water (mock), 1-cm leaf discs were immediately frozen in liquid nitrogen and stored at –80°C. Total RNA from two 1-cm-diameter leaf discs was isolated using the Tri Reagent method according to the manufacturer's recommendations (Sigma). First-strand cDNA was synthesized from 2 μ g of total RNA using Expand Reverse Transcriptase (Roche, Lewes, UK). RT-PCR was performed as described previously (Romeis et al., 2001) for 27 cycles with an annealing temperature of 50°C. The following primers were used for amplification of the gene transcripts: NTSYP121-s (5'-AAATCTATTCCTCAATCCATCTTC-3') and NTSYP121-a (5'-GAGAGGACAAGTGATGGATACAGTT-3') for *NtSyp121*; PR5-F (5'-ATGAACTTCTCAAAAAGCTTCCCC-3') and PR5R (5'-AGGGCAGAAGACAACCCTGTAATT-3') for *PR5*; and actin1 (5'-ATGGCAGACGGTGAGGATATTCA-3') and actin2 (5'-GCCTTGCATCCACATCTGTG-3') for *Actin*. Comparable results were obtained when peptides were resuspended in 5 mM MES buffer, pH 5.7, instead of water, and experiments were performed at least twice for each treatment.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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