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Pseudomonas syringae Virulence Factor Syringolin A Counteracts Stomatal Immunity by Proteasome Inhibition

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The peptide derivative syringolin A, a product of a mixed nonribosomal peptide and polyketide synthetase, is secreted by certain strains of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*. Syringolin A was shown to be a virulence factor for *P. syringae* pv. *syringae* B728a because disease symptoms on its host *Phaseolus vulgaris* (bean) were greatly reduced upon inoculation with syringolin A-negative mutants. Syringolin A's mode of action was recently shown to be irreversible proteasome inhibition. Here, we report that syringolin A-producing bacteria are able to open stomata and, thus, counteract stomatal innate immunity in bean and *Arabidopsis*. Syringolin A-negative mutants, which induce stomatal closure, can be complemented by exogenous addition of not only syringolin A but also MG132, a well-characterized and structurally unrelated proteasome inhibitor. This demonstrates that proteasome activity is crucial for guard cell function. In *Arabidopsis*, stomatal immunity was salicylic acid (SA)-dependent and required NPR1, a key regulator of the SA-dependent defense pathway whose proteasome-dependent turnover has been reported to be essential for its function. Thus, elimination of NPR1 turnover through proteasome inhibition by syringolin A is an attractive hypothesis to explain the observed inhibition of stomatal immunity by syringolin A.

As a defense against pathogens, plants have evolved an innate immune system. As currently understood, it has two branches (Chisholm et al. 2006; Jones and Dangl 2006; Boller and Felix 2009; Cui et al. 2009). One branch concerns the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors located predominantly on the surface of host cells. Recognition triggers defense responses that collectively restrict pathogen growth, leading to basal, non–race specific resistance dubbed PAMP-triggered immunity (PTI) (Jones and Dangl 2006). However, pathogens have evolved effectors able to suppress PTI. In bacterial pathogens, which are particularly well studied, many effector molecules are proteins injected into the host cytoplasm by the type III secretion system (T3SS) where they interfere with PTI signal transduction, thus leading to effector-triggered susceptibility (He et al. 2007; Guo et al. 2009; Mansfield 2009; Métraux et al. 2009). As a second line of defense, plants have evolved effector recognition systems that trigger a strong but race-specific defense response (effector-triggered immunity) (ETI) that often involves programmed cell death (Jones and Dangl 2006).

To cause disease, plant pathogens have to invade host tissue. It has recently been reported that, apart from wounds, stomata are entry ports for bacterial pathogens that are relevant for disease progression, and that stomatal closure upon pathogen attack is part of plant innate immunity (Melotto et al. 2006, 2008; Liu et al. 2009). The importance of the stomatal innate immune response is also evident from the fact that bacterial effectors have been identified that counteract PAMP-induced stomatal closure. For example, the polyketide toxin coronatine, a virulence factor secreted by certain strains of *Pseudomonas syringae*, was shown to actively open stomata (Melotto et al. 2006). A similar activity was also reported of a factor of unknown identity secreted by *Xanthomonas campestris* (Gudesblat et al. 2009).

We have previously characterized syringolin A, a peptide derivative which is produced by a mixed nonribosomal peptide synthetase and polyketide synthetase and secreted by some strains of *P. syringae* pv. *syringae* (Wäspi et al. 1998, 1999; Amrein et al. 2004; Imker et al. 2009; Ramel et al. 2009). Recently, we reported syringolin A produced by *P. syringae* pv. *syringae* strain B728a to be a virulence factor because disease symptoms on its host *Phaseolus vulgaris* (bean) were greatly reduced upon inoculation with syringolin A-negative mutant compared with wild-type bacteria (Groll et al. 2008). The cellular target of syringolin A was identified to be the proteasome. It was demonstrated that syringolin A irreversibly inhibited all three catalytic activities of the eukaryotic proteasome and forms, together with the glidobactins, a new structural class of proteasome inhibitors that act by a novel mechanism (Schellenberg et al. 2007; Groll et al. 2008).

Because the proteasome plays a central role in numerous regulatory pathways such as hormone signaling and responses to environmental stimuli and pathogens (Vierstra 2009), inhibition of the proteasome is expected to elicit pleiotropic responses and it is not evident from which of these the pathogen would benefit. Here, we report that syringolin A-producing bacteria are able to open stomata and, thus, counteract stomatal innate immunity in bean and *Arabidopsis*. It likely does so by its capacity as a proteasome inhibitor because the same effect was also observed by treatment with MG132, a well-characterized and structurally unrelated proteasome inhibitor. This suggests that proteasome function is crucial for guard cell function. Stomatal immunity in *Arabidopsis* was dependent on NPR1, an important regulator of salicylic acid (SA)-dependent defense pathways. Because NPR1 function requires its turnover by the proteasome (Spoel et al. 2009), suppression of NPR1 turnover by proteasome inhibition is an attractive hypothesis explaining the observed action of syringolin A on stomata.

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RESULTS

Syringolin A counteracts pathogen-induced stomatal closure in bean by proteasome inhibition.

First, we tested whether the increased number of disease symptoms resulting from infections of bean with the syringolin A-producing *Pseudomonas syringae* pv. *syringae* strain B728a compared with infection with syringolin A-negative mutant strains might be directly linked to in planta growth differences between the two genotypes. Thus, leaves of 3-week-old bean plants (*Phaseolus vulgaris* cv. Bush Blue Lake) were spray inoculated with the wild-type strain B728a and the syringolin A-negative mutant derivative B728a KO_sylC (Groll et al. 2008). Endophytic bacterial growth was determined over 5 days. Whereas both the wild type and the mutant were able to grow endophytically, the mutant reached lower (within a factor of 10) population densities compared with the wild type (Fig. 1). In some experiments, as in the one shown in Figure 1, the mutant strain transiently seemed to catch up with the wild type but seemed to decline faster. However, in other experiments, this was not obvious (Supplementary Fig. S1). Similar patterns were observed in independent repetitions of the experiment and in a second cultivar (Winnetou) (data not shown). The most prominent difference was always observed 6 h post inoculation (hpi), the earliest time point measured. This suggested that wild-type bacteria reach the endophytic leaf space more efficiently than syringolin A-negative mutant bacteria.

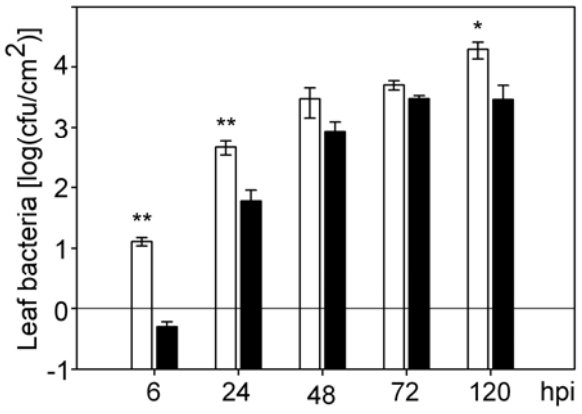

Fig. 1. Endophytic growth of wild-type and syringolin A-negative *Pseudomonas syringae* pv. *syringae* B728a strains on bean. Leaves of *Phaseolus vulgaris* (cv. Bush Blue Lake) were spray inoculated with suspensions (10^7 CFU/ml) of the wild-type *Pseudomonas syringae* pv. *syringae* B728a strain (open bars) or the syringolin A-negative mutant *P. syringae* pv. *syringae* B728a KO_sylC (black bars). Means ± standard error of five plants per treatment are given ($n = 5$). Asterisks indicate significant differences between the strains (Student's *t* test; ** and * indicate $P < 0.01$ and 0.05, respectively). The experiment was independently repeated once using cv. Bush Blue Lake and three times using cv. Winnetou with similar results.

Because bean leaves were spray inoculated without damaging tissue, plant stomata likely represented the only entry point into the inner leaf tissue. Therefore, we investigated whether syringolin A would affect stomatal aperture. Abaxial epidermal peels were prepared from primary leaves of 2-week-old bean plants (cvs. Bush Blue Lake and Winnetou) kept in the light prior to the experiment (stomata are open) and incubated in the light in control solution or bacterial suspensions of the syringolin A-producing wild-type strain *Pseudomonas syringae* pv. *syringae* B728a and the syringolin A-negative knock-out mutant of this strain (B728a KO_sylC). At 1 h after incubation, epidermal peels were mounted on glass slides in high humidity

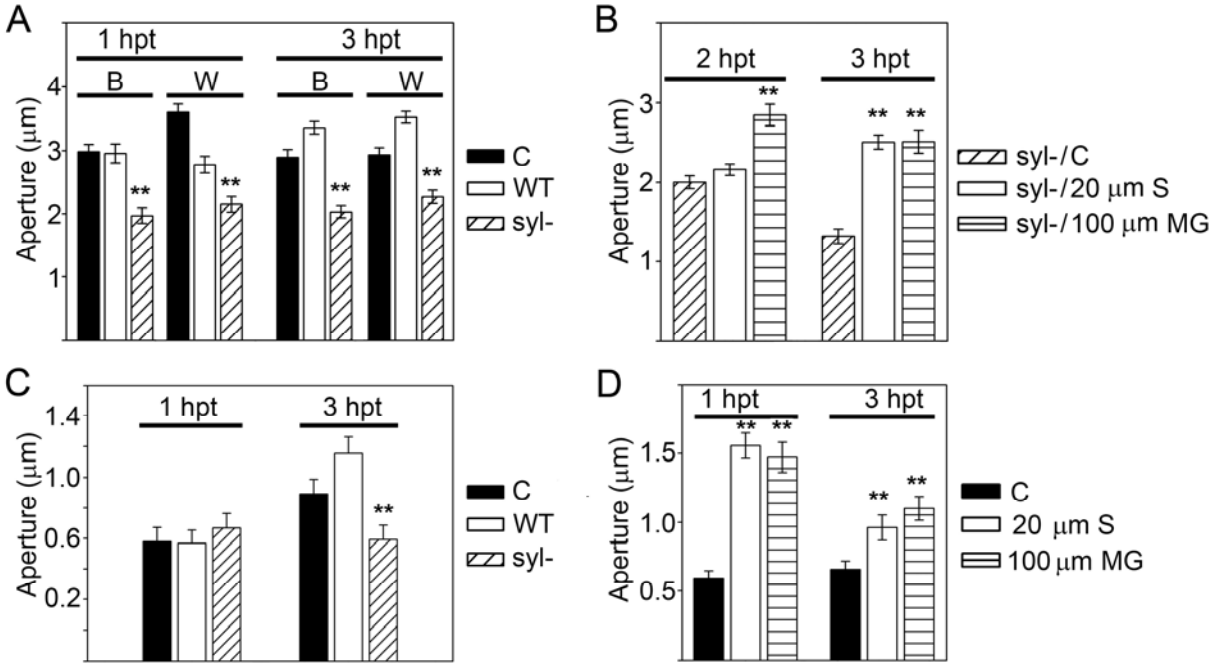

Fig. 2. Pathogen-induced stomatal closure is counteracted by syringolin A in bean. **A,** Apertures of stomata on epidermal peels of bean cvs. Bush Blue Lake (B) and Winnetou (W) 1 and 3 h after treatment with control buffer, wild-type, or syringolin A-negative *Pseudomonas syringae* pv. *syringae* B728a strains. **B,** Complementation of syringolin A-negative *P. syringae* pv. *syringae* B728a by exogenous addition of syringolin A or MG132. Epidermal peels of bean cv. Winnetou were preincubated with bacteria for 1 h before addition of the chemicals. Stomatal apertures were measured after another 1 and 2 h. **C,** Stomatal apertures of epidermal peels from bean leaves (cv. Winnetou) kept in the dark were incubated in the dark with control buffer, wild-type (WT), and syringolin A-negative *P. syringae* pv. *syringae* B728a bacteria. **D,** Stomatal apertures of epidermal peels from bean leaves (cv. Winnetou) kept in the dark were incubated in the dark with control buffer, syringolin A, and MG132. **A** through **D,** Means ± standard errors are given ($n \geq 70$ for each data point). WT, wild type; syl–, syringolin A-negative mutant; hpt, h post treatment. Experiments were independently performed twice (C) or three times (A, B, and D). C, control buffer; S, syringolin A; MG, MG132. Asterisks denote significant differences (Student's *t* test; ** indicates $P < 0.01$) between WT and syl– strains (A and C) or between treatment with syringolin A or MG132 and the control treatment (B and D).

and apertures of all stomata present on photomicrographs were measured. After 3 h of incubation under the same conditions, the stomatal aperture was measured again on photomicrographs taken from the same slides. The results (Fig. 2A) show that, after 3 h of incubation with the wild-type strain, epidermal peels exhibited open stomata with an aperture comparable with the control without bacteria, whereas the syringolin A-negative mutant led to significantly reduced stomatal apertures. Thus, syringolin A-producing bacteria were able to keep open or reopen stomata.

We next tested whether exogenous addition of isolated syringolin A could revert stomatal closure induced by syringolin A-negative bacteria. Epidermal peels of light-exposed bean plants were incubated with mutant bacteria for 1 h before syringolin A was added to a final concentration of 20 µM. As is evident from Figure 2B, 2 h after syringolin A addition, stomata openings were significantly wider than on the control-treated peels. The same result was obtained when syringolin A was substituted by MG132, a well-established, structurally unrelated, reversible proteasome inhibitor (Fig. 2B). These results suggest that syringolin A in its capacity as a proteasome inhibitor was responsible for the opening or reopening of stomata observed with wild-type bacteria.

To clarify whether syringolin A-synthesizing bacteria were capable to actively open stomata, bean plants were preincubated in the dark, where stomata are normally closed. Epidermal peels prepared from these plants were then incubated in the dark with suspensions of wild-type and syringolin A-negative mutant bacteria or in control solution. At 1 h after incubation, all epidermal peels showed closed stomata whereas, after 3 h, stomata on peels incubated with wild-type bacteria were significantly more open when compared with the ones incubated with suspensions of syringolin A-negative bacteria or with control solution (Fig. 2C). In a further experiment, epidermis peels of bean plants kept in the dark were incubated in the dark in the presence of 20 µM syringolin A or 100 µM MG132. Both proteasome inhibitors caused closed stomata to open (Fig. 2D), suggesting that proteasome activity is crucial for guard cell function in bean.

Of the many plant hormones that affect guard cell function, stomatal closure by abscissic acid (ABA) plays a particularly crucial role (Acharya and Assmann 2009). Therefore, we wanted to test whether syringolin A and MG132 counteract ABA-induced stomatal closure in bean. Epidermal peals of bean plants (cv. Winnetou) kept in the light were incubated in the light in the presence of 30 µM ABA and increasing concentrations of syringolin A or MG132. Both proteasome inhibitors partially counteracted ABA-induced stomatal closure in a concentration-dependent manner (Fig. 3).

Proteasome function is also crucial for the stomatal immune response in *Arabidopsis*.

Melotto and co-workers (Melotto et al. 2006, 2008) reported stomatal closure to be part of PTI in *Arabidopsis* toward bacterial pathogens such as *P. syringae* pv. *tomato* DC3000 or PAMPs, which was counteracted by the toxin coronatine produced by *P. syringae* pv. *tomato* DC3000. Therefore, we explored whether the proteasome played a role in the stomatal immune response in *Arabidopsis* using the coronatine-negative mutant *P. syringae* pv. *tomato* DC3118 (Ma et al. 1991) in experiments with *Arabidopsis* (accession Col-0) epidermal peels. The mutant but not the wild type led to stomatal closure (Fig. 4) as reported in the literature (Melotto et al. 2006). This stomatal immune response was counteracted by exogenous addition of solutions containing 20 µM syringolin A or 100 µM MG132, suggesting that proteasome function was necessary for PTI-associated stomatal immunity in *Arabidopsis*. Treatment of *Arabidopsis* (Col-0) epidermal peels with wild-type and syringolin A-negative mutant *P. syringae* pv. *syringae* B728a strains, for which *Arabidopsis* is not a host, led to open and closed stomata, respectively, as was observed in bean (data not shown). Results similar to those in bean were also obtained with dark-incubated *Arabidopsis* epidermal peels in experiments analogous to the ones shown in Figure 2C and D (data not shown).

Using mutants and pharmacological agents, Melotto and coworkers (2006, 2008) showed that, in *Arabidopsis*, pathogen- or PAMP-induced stomatal closure was dependent on the SA-regulated defense pathway and on ABA. The proteasome was reported to play a role in the regulation of NPR1, a key regula-

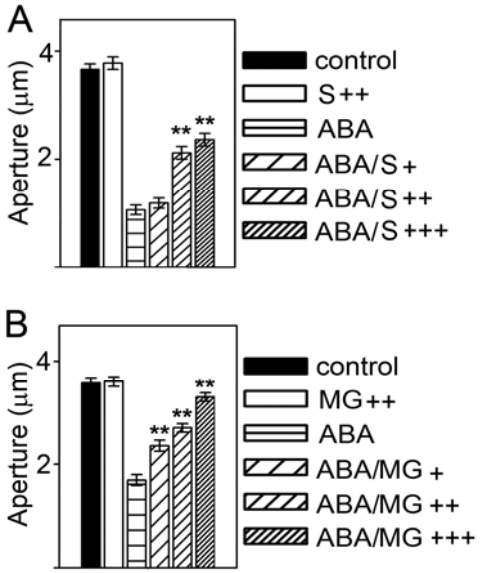

Fig. 3. Interference of proteasome inhibitors with abscisic acid (ABA)-induced stomatal closure. Stomatal apertures (means ± standard errors; $n \geq 70$ for each data point) on epidermal peels of bean leaves (cv. Winnetou) 3 h after treatment with **A,** ABA and syringolin A or **B,** ABA and MG132. Control, control solution; ABA, 30 µM ABA; S+, S++, and S+++ = 10, 20, and 40 µM syringolin A, respectively; MG+, MG++, and MG+++ = 50, 100, and 200 µM MG132, respectively. Asterisks indicate significant differences (Student's *t* test; ** indicates $P < 0.01$) between treatment with syringolin A or MG132 and treatment with ABA alone. Stomatal apertures after ABA treatment are also significantly smaller ($P < 0.01$) than after control treatment or treatment with syringolin A or MG132 alone.

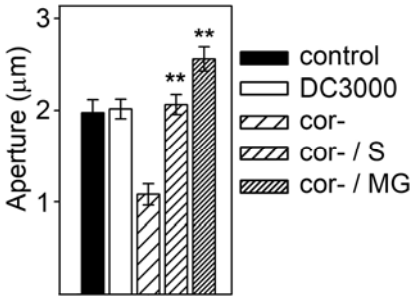

Fig. 4. Syringolin A and MG132 reverse pathogen-associated molecular pattern- or pathogen-induced stomatal closure in *Arabidopsis*. Epidermal peels of *Arabidopsis* (Col-0) were incubated with the *Pseudomonas syringae* pv. *tomato* DC3000 wild-type strain (DC3000) or coronatine-negative strain *P. syringae* pv. *tomato* DC3118 (cor–) with and without 20 µM syringolin A (S) or 100 µM MG132 (MG). Means ± standard errors of stomatal apertures ($n \geq 70$ for each data point) 3 h after treatment are given. The experiment was independently repeated once with similar results. Asterisks indicate significant differences (Student's *t* test; ** indicates $P < 0.01$) between treatment with the cor– strain and treatments with the cor– strain and syringolin A or MG132, respectively.

tor in the SA-dependent defense pathway (Spoel et al. 2009). The importance of proteasome activity for NPR1 function was corroborated by the observation that i) syringolin A greatly reduced SA-induced *PATHOGENESIS-RELATED PROTEIN 1* (*PR-1*) transcript accumulation in spray application experiments with *Arabidopsis* and ii) infiltration of *Arabidopsis* with wild-type *P. syringae* pv. *syringae* B728a resulted in significantly reduced *PR-1* transcript accumulation compared with infiltration with syringolin A-negative mutant bacteria (Supplementary Fig. S2). In addition to the SA pathway, in *Arabidopsis*, the proteasome was reported to be involved in the ABA signal transduction pathway, where it was proposed to control ABA sensitivity by regulating nuclear localization of the ABA-insensitive 1 (ABI1) protein (Moes et al. 2008). Proteasome inhibition by MG132 led to a marked increase of ABI1-mediated inhibition of ABA signaling (Moes et al. 2008). Thus, we explored whether these proteins were involved in the stomatal immune response to *P. syringae* pv. *syringae* B728a in *Arabidopsis*.

First, we tested whether the SA defense pathway was also necessary for the stomatal immune response triggered by the nonhost pathogen *P. syringae* pv. *syringae* B728a, as reported for *P. syringae* pv. *tomato* DC3000 (Melotto et al. 2006). Indeed, stomata did not close on epidermal peels from SA-deficient *nahG* transgenic *Arabidopsis* plants (Delaney et al. 1994) upon incubation with syringolin A-negative B728a KO_sylC mutant bacteria (Fig. 5A), confirming the importance of the SA defense pathway. We then tested the stomatal immune response in *npr1* mutant plants (Cao et al. 1994). As is evident from Figure 5B, *npr1* plants were unable to close stomata upon treatment with syringolin A-negative bacteria, indicating that NPR1 was necessary for the stomatal immune response. Thus, we consider inhibition by syringolin A of proteasome-mediated turnover of NPR1, which was shown to be essential for NPR1 function (Spoel et al. 2009), to provide an attractive hypothesis explaining the observed syringolin A-mediated inhibition of *P. syringae* pv. *syringae* B728a-induced stomatal closure.

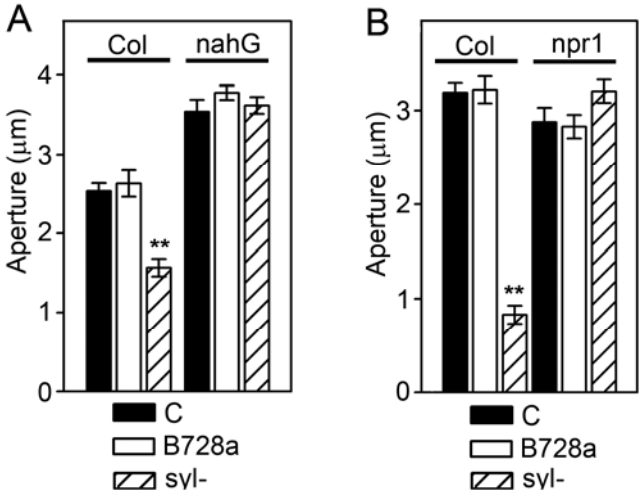

Fig. 5. Stomatal response of *Arabidopsis* mutants affected in the salicylic acid signaling pathway. Epidermal peels from Col-0 wild-type (Col) and **A,** *nahG* or **B,** *npr1* plants were treated with control solution (C), *Pseudomonas syringae* pv. *syringae* B728a, and syringolin A-negative *P. syringae* pv. *syringae* B728a KO_sylC (syl–) bacteria. Means ± standard errors of stomatal apertures ($n \geq 70$ for each data point) 3 h after treatment are given. Experiments were independently performed twice (A) and three times (B) with similar results. Asterisks denote significant differences (Student's *t* test; ** indicates $P < 0.01$) between treatment with wild-type and syringolin A-negative mutant bacteria in Col-0 plants.

Next, we explored whether pathogen-induced stomatal closure in *Arabidopsis* required the ABA signaling pathway in which ABI1 is involved. ABI1 belongs to the group A protein phosphatase 2C (PP2C) family of proteins which negatively regulate ABA signaling. They recently have been shown to be sequestered in a complex with the PYR/PYL/RCAR family of ABA receptor proteins in the presence of ABA, leading to their inactivation and the consequent derepression of ABA responses (Fujii et al. 2009; Melcher et al. 2009; Miyazono et al. 2009; Nishimura et al. 2009; Santiago et al. 2009a and b). Because functional redundancy within the PP2C family usually does not result in a mutant phenotype of individual loss-of-function PP2C mutants, we made use of the dominant ABA-insensitive *abi1-1* allele (Koornneef et al. 1984). Epidermal peels from both wild-type and *abi1-1 Arabidopsis* plants reacted to syringolin A-negative mutant *P. syringae* pv. *syringae* B728a by closing their stomata (Fig. 6A), indicating that the PP2C/PYL/PYR/RCAR-mediated ABA signaling pathway was not essen-

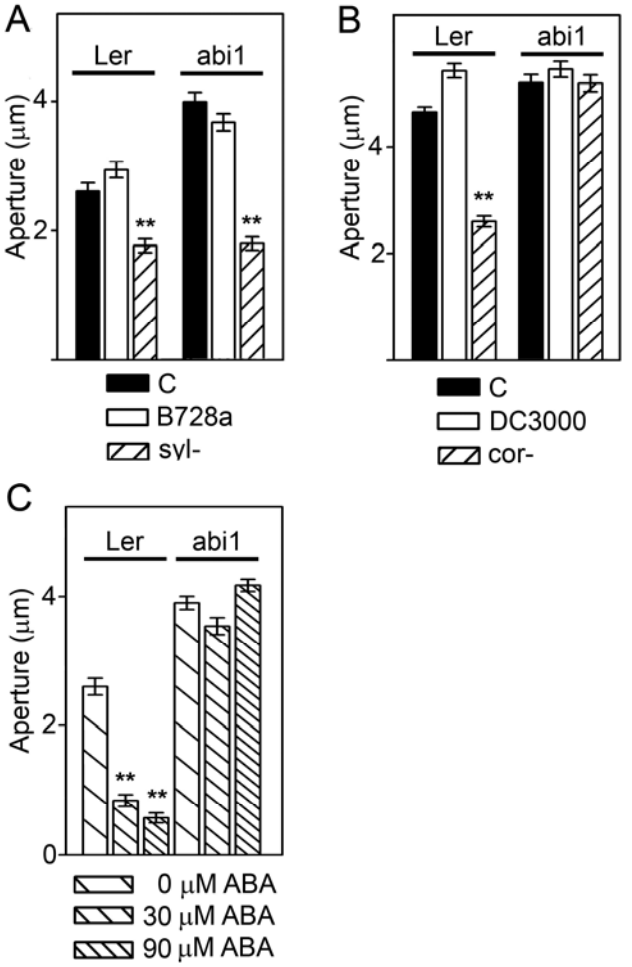

Fig. 6. Stomatal immune response in the *abi1-1 Arabidopsis* abscisic acid (ABA)-signaling mutant. Epidermal peels from L*er* wild-type and *abi1-1* mutant *Arabidopsis* plants were treated with **A,** control solution (C), *Pseudomonas syringae* pv. *syringae* B728a (B728a), and syringolin A-negative *P. syringae* pv. *syringae* B728a KO_sylC (syl–) bacteria; **B,** *P. syringae* pv. *tomato* DC3000 (DC3000) and coronatine-negative *P. syringae* pv. *tomato* DC3118 (cor–) bacteria; or **C,** the indicated concentrations of ABA. Means ± standard errors of stomatal apertures ($n \geq 70$ for each data point) 3 h after treatment are given. Experiments were independently performed twice (A and C) and three times (B) with similar results. Asterisks denote significant differences (Student's *t* test; ** indicates $P < 0.01$) between treatment with wild-type and syringolin A-negative (A) and coronatine-negative (B) bacteria, respectively, or between control treatment and ABA treatment (C).

tial for the stomatal response to this nonhost bacterium. This was in contrast to the stomatal response elicited by coronatine-negative *P. syringae* pv. *tomato* DC3118, which required this pathway because stomata on epidermal peels of *abi1-1* plants were unable to close upon treatment with *P. syringae* pv. *tomato* DC3118 bacteria (Fig. 6B). The control experiment (Fig. 6C) showed that, as expected, wild-type but not *abi1-1* plants responded with stomatal closure to exogenous addition of ABA.

DISCUSSION

Spray inoculation of bean plants with wild-type and syringolin A-negative mutant *P. syringae* pv. *syringae* B728a bacteria revealed that both strains were able to multiply endophytically, although the wild type reached moderately higher population densities than the mutant. Apparently, once inside the leaf, both bacterial genotypes are able to multiply at similar rates, although persistence may be somewhat lowered in the mutant. The facilitated entry that syringolin A-producing bacteria experience may well be relevant for disease etiology, at least under certain environmental conditions. Our results are compatible with earlier observations reported in the literature. Willis and associates (1990) infiltrated the B728a strain and the *lemA* (later renamed *gacS*) mutant NPS3136 into bean leaves where both strains were able to multiply to similar extents, although the mutant was deficient in lesion formation. The *lemA* mutant has a nonfunctional *gacA/gacS* global regulatory two-component system which also controls syringolin A biosynthesis (Reimmann et al. 1995; Wäspi et al. 1998). Spray inoculation of bean plants with these strains in field experiments revealed that the *lemA* gene contributed to the field fitness as the mutant strain reached lower population densities associated with bean leaves (Hirano et al. 1997). Although, in these experiments, epiphytic and endophytic growth were not distinguished, and although the *gacA/gacS* regulon certainly affects other properties of the bacterium in addition to syringolin A production, facilitated entry into leaves may have contributed to the reported observations.

The fact that the proteasome inhibitors syringolin A and MG132 are able to suppress stomatal immunity implied that proteasome function is essential for the stomatal immune reaction upon pathogen perception. The proteasomal degradation pathway has been implicated in at least some aspects of the signaling pathways identified by Melotto and associates (2006) to be important for pathogen- or PAMP-induced stomatal closure. The proteasome plays a pivotal role in the SA-mediated transcriptional defense response. The key regulator NPR1, a transcriptional co-activator, must be turned over in its phosphorylated form by the proteasome to activate target genes involved in systemic acquired resistance (Spoel et al. 2009). We have shown that the stomatal immune response to *P. syringae* pv. *syringae* B728a is impaired in the *npr1* mutant, indicating that NPR1 function is necessary for this response. Thus, inhibition of proteasomal NPR1 turnover by syringolin A provides a likely explanation for the observed syringolin A-mediated inhibition of stomatal closure.

With regard to ABA signaling, PP2Cs such as ABI1 and homologues must be located in the nucleus to bind and inhibit their target kinases (e.g., OST1) (Vlad et al. 2009). Nuclear location was at least in part regulated by the proteasome because MG132 led to preferential localization of wild-type ABI1 mutant abi1-1 proteins in the nucleus and to enhanced inhibition of ABA signaling (Moes et al. 2008). Thus, inhibition of *P. syringae* pv. *tomato* DC3118-triggered PTI-associated stomatal closure in *Arabidopsis* by exogenous syringolin A and MG132 (Fig. 4) could also be explained by interference with the PYR/PYL/RCAR-PP2C-mediated ABA signaling pathway which is required for the response to this pathogen (Fig. 6B). This may also apply to the *P. syringae* pv. *syringae*–bean pathosystem provided that the wiring of the PTI-associated stomatal immune response in this pathosystem is similar to the one in the *P. syringae* pv. *tomato*–*Arabidopsis* system, which is currently not known. In contrast, the stomatal immune response triggered in *Arabidopsis* by the nonhost pathogen *P. syringae* pv. *syringae* B728a was not dependent on the PPC2-mediated ABA signal transduction pathway (Fig. 6A).

The stomata are entry points into plants for many pathogens. Thus, it is not surprising that the regulation of stomatal aperture upon pathogen perception is part of the plant immune response (Melotto et al. 2006, 2008; Liu et al. 2009). On the other hand, it is increasingly becoming clear that pathogens have evolved diverse means to counteract stomatal immunity. The most widely known agent is the wilting toxin fusicoccin produced by *Fusicoccum amygdali*, a pathogen of almond and peach trees (Ballio et al. 1964; Marre 1979). It leads to stomatal opening by activating the plasma membrane H^+-ATPase in complex with a 14-3-3 protein (Baunsgaard et al. 1998; Olivari et al. 1998). Other agents that overcome stomatal immunity in *Arabidopsis* include a recently described small diffusible factor of unknown identity from *X. campestris* (Gudesblat et al. 2009) and the already mentioned toxin coronatine which is secreted by certain *P. syringae* strains such as *P. syringae* pv. *tomato* DC3000 (Melotto et al. 2006).

Coronatine is a mimic of isoleucine-conjugated jasmonic acid (JA) and activates the JA signaling pathway in a *CORONATINE INSENSITIVE-1* (*COI1*) dependent manner. COI1 is an F-box protein and part of the SCF^{COI1} E3 ubiquitin ligase that targets repressors of JA responses to proteasomal destruction (Chini et al. 2007; Thines et al. 2007). Because syringolin A is a proteasome inhibitor and coronatine needs host proteasome function for its action, syringolin A and coronatine seem to be incompatible if produced by the same pathogen at the same time. Indeed, none of the limited number of strains whose genome sequence is known or that we checked by polymerase chain reaction-based methods contains both syringolin A synthetase genes and genes for coronatine biosynthesis. Syringolin A production would seem also to be incompatible with the concurrent expression of effectors such as HopAB2 (formerly named AvrPtoB), which contains E3 ligase activity and targets host defense proteins to proteasomal destruction (Rosebrock et al. 2007). Although the *P. syringae* pv. *syringae* B728a genome, in contrast with that of *P. syringae* pv. *tomato* DC3000, does not contain a *hopAB2* gene (Sarkar et al. 2006), it is currently not known whether strains exist capable of expressing syringolin A and effectors requiring host proteasome activity for their action. If such strains exist, interference may be avoided by exclusive expression of one or the other, depending on time and conditions.

MATERIALS AND METHODS

Plant material, bacterial strains, and growth conditions.

Phaseolus vulgaris (bean) plants (cvs. Bush Blue Lake 274 and Winnetou) were cultivated with a regime of 16 h of light and 8 h of darkness at a mean air temperature of 21°C and a humidity of 50 to 60%. *Arabidopsis* seed were surface sterilized with a solution containing 1% (vol/vol) sodium hypochlorite and 0.03% (vol/vol) Triton X-100 for 15 min, washed three times with sterile water, and stratified for 3 to 4 days at 4°C in the dark. Seed were aligned on half-strength Murashige and Skoog (MS) plates containing 2% (wt/vol) sucrose and 0.6% phytagel (Sigma-Aldrich, Buchs, Switzerland). Plates were placed in vertical orientation in an incubator under long-day conditions (16 h of light and 8 h of darkness) at 21°C for

approximately 10 days. Seedlings were then transferred into soil and kept under the same conditions.

Construction of the syringolin A-negative *Pseudomonas syringae* pv. *syringae* mutant B728a KO_sylC by plasmid insertion into the *sylC* gene of the syringolin A synthetase gene cluster has been described (Amrein et al. 2004; Groll et al. 2008). All strains were grown at 28°C on Luria-Bertani (LB) plates (bactotryptone [Difco, Chemie Brunschwig AG, Basel, Switzerland] at 10 g/liter, bactoyeast extract [Difco] at 5 g/liter, NaCl at 10 g/liter, and agar [Difco] at 15 g/liter) supplemented with rifampicin (Rif) at 50 μg/ml for the wild type and Rif at 50 μg/ml and tetracycline at 10 μg/ml for mutant bacteria.

Inoculation of bean plants and determination of endophytic bacterial growth.

For spray inoculation of bean plants, bacterial inocula were essentially prepared as described (Hirano et al. 1997). In short, bacterial overnight cultures grown in LB without antibiotics were diluted to an optical density at 600 nm of 1.0 (approximately 5×10^8 CFU/ml). Then, 100 μl of this dilution were plated onto LB plates (9 cm in diameter) and incubated for 2 days at 28°C. Bacteria were scraped off the plates with a spatula, resuspended in distilled water, and diluted to 10^7 CFU/ml. The bacterial suspensions were sprayed onto both sides of primary leaves of 2-week-old bean plants which had been incubated in a climate chamber at 23°C with a light intensity of 17 μE for 2 h prior to inoculation. The spray-inoculated plants were covered with a clear, light-permeable plastic lid for 24 h to maintain high humidity.

For each treatment and time point, endophytic bacterial growth was determined from one leaf of five different plants. Per leaf, three samples of six discs with a diameter of 1 cm were excised. Each sample was surface-sterilized in 15% (vol/vol) H_2O_2 for 5 min, washed in sterile water, and homogenized in 1 ml 10 mM $MgSO_4 \cdot 7H_2O$ with a plastic pestle in an Eppendorf vial. The three extracts were pooled and diluted stepwise by a factor of 10 in 10 mM $MgSO_4 \cdot 7H_2O$. Six 20-μl droplets of each dilution were placed onto antibiotic-free LB plates. The plates were incubated at 28°C for 20 h. Bacterial colonies were counted, averaged over the replicate platings of the same dilution, and normalized to CFU per square centimeter of leaf area.

Preparation and treatment of epidermal peels and determination of stomatal apertures.

To determine stomatal apertures, bean and *Arabidopsis* plants were incubated in a climate chamber under light (130 μE) or in the dark at 23°C for at least 3 h prior to use. Bacterial overnight cultures grown in LB medium were washed and diluted in distilled water to 5×10^8 CFU/ml. Silwet L-77 was added to a final concentration of 0.025%. Distilled water containing 0.025% Silwet L-77 was used as the control solution and also used to prepare solutions of chemicals. Bacterial suspensions and solutions of chemicals were dropped onto a glass slide bordered with Scotch tape, and epidermal peels prepared from the abaxial leaf side were directly mounted on the liquid layer and covered with the corresponding suspensions or solutions and a cover slide. To prevent desiccation, glass slides were put into a tray lined with wet household paper and incubated in a climate chamber at 23°C.

In mutant complementation experiments, epidermal peels were incubated in small petri dishes containing bacterial suspensions of 5×10^8 CFU/ml for 1 h to trigger the basal resistance reaction. The peels were then transferred onto a glass slide carrying a liquid layer with the desired chemical solution, covered with the respective solution and a cover slide, and incubated as described above. The preparations were incubated under the same light conditions as the whole plants were prior to their use. At 1 and 3 h after treatment (hpt), or 2 and 3 hpt in the case of mutant complementation experiments, digital pictures of epidermal peels were taken with a Leica DFC420 camera (Leica Microsystems, Wetzlar, Germany) mounted on a Laborlux S microscope (Leitz, Wetzlar, Germany) equipped with a ×20 objective . Stomatal apertures were measured on photographs using ImageJ version 1.4.1 software (Abramoff et al. 2004). Apertures of all stomata in focus on a digital image were measured. Every data point represented the average from 70 to 200 stomata from a total of 10 nonoverlapping images taken.

Syringolin A was prepared as described (Wäspi et al. 2001; Amrein et al. 2004) and MG132 was purchased from Biomol (ANAWA Trading SA, Wangen Zürich, Switzerland). All other chemicals were obtained from Sigma-Aldrich.

RNA gel blot analysis of chemically treated and infiltrated *Arabidopsis* plants.

Three-week-old *Arabidopsis* (Col-0) plants were sprayed with 0.05% Tween 20 solutions containing 2.5 mM SA supplemented with or without 50 μM syringolin A until droplets started to role off the leaves. Alternatively, leaves were infiltrated with suspensions of wild-type *P. syringae* pv. *syringae* B728a or syringolin A-negative *P. syringae* pv. *syringae* B728a KO_sylC bacteria containing 10^6 CFU/ml of distilled water. Total RNA was extracted 18 h after treatment and subjected to gel blot analysis with a ^{32}P-labeled *PR-1* cDNA hybridization probe (Uknes et al. 1992) using standard procedures (Ausubel et al. 1987).

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