The Innate Immune Signaling System as a Regulator of Disease Resistance and Induced Systemic Resistance Activity Against *Verticillium dahliae*

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In the last decades, the plant innate immune responses against pathogens have been extensively studied, while biocontrol interactions between soilborne fungal pathogens and their hosts have received much less attention. Treatment of *Arabidopsis thaliana* with the nonpathogenic bacterium *Paenibacillus alvei* K165 was shown previously to protect against *Verticillium dahliae* by triggering induced systemic resistance (ISR). In the present study, we evaluated the involvement of the innate immune response in the K165-mediated protection of *Arabidopsis* against *V. dahliae*. Tests with *Arabidopsis* mutants impaired in several regulators of the early steps of the innate immune responses, including *fls2*, *efr-1*, *bak1-4*, *mpk3*, *mpk6*, *wrky22*, and *wrky29* showed that FLS2 and WRKY22 have a central role in the K165-triggered ISR, while EFR1, MPK3, and MPK6 are possible susceptibility factors for *V. dahliae* and *bak1* shows a tolerance phenomenon. The resistance induced by strain K165 is dependent on both salicylate and jasmonate-dependent defense pathways, as evidenced by an increased transient accumulation of *PR1* and *PDF1.2* transcripts in the aerial parts of infected plants treated with strain K165.

Verticillium dahliae Kleb. is a widely distributed soilborne pathogen causing vascular wilt on more than 160 plant species. It causes estimated billions of dollars in crop losses annually and worldwide (Pegg and Brady 2002). Colonization of the host plant by *V. dahliae* leads to leaf flaccidity, chlorosis and necrosis, stunting, vascular discoloration in stems, as well as flower and fruit reduction (Pegg 1981). The pathogen is difficult to control due to the long viability of the resting structures, the broad host range, and the inability of fungicides to affect the fungus once it enters the xylem (Fradin and Thomma 2006). The lack of efficacious pesticides and host resistance for the control of *V. dahliae* has stimulated efforts to develop alternative disease management strategies such as biocontrol agents (BCA).

Interestingly, Tjamos and associates (2004) reported the isolation of a plant growth–promoting rhizobacterium (PGPR),

identified as *Paenibacillus alvei* K165, with biocontrol activity against *V. dahliae* in greenhouse and field experiments. The suppression of *Verticillium* disease by *P. alvei* K165 was attributed to induced systemic resistance (ISR) via the salicylic acid (SA)-dependent defense pathway (Tjamos et al. 2005). It is well-established that root colonization by PGPR can result in ISR via the SA or ethylene and jasmonate (ET/JA)-dependent pathway that is accompanied by the accumulation of pathogenesis-related proteins (Iavicoli et al. 2003; Pieterse et al. 1998; Tjamos et al. 2005; Ton et al. 2002). However, little is known about the perception of the BCA by the plant and the cascade of the early signaling events that eventually lead to ISR.

The presence of pathogenic microorganisms is sensed by plants through perception of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRR) located mostly on the cell surface, leading to the PAMP-triggered immunity (Jones and Dangl 2006; Zipfel 2008). In *Arabidopsis thaliana*, the leucine-rich repeat receptor kinases (LRR-RK) flagellin-sensitive 2 (FLS2) and elongation factor Tu receptor (EFR) act as PRR for the bacterial PAMPs flagellin and elongation factor Tu (EF-Tu) (Gómez-Gómez and Boller 2000; Zipfel et al. 2006). Upon activation with their respective ligands, FLS2 and EFR form heteromeric complexes with the coreceptor BAK1 (Roux et al. 2011). This binding leads to the activation of a mitogen-activated protein kinase cascade, followed by the induction of defense genes, production of reactive oxygen species, callose deposition, and synthesis of SA (Asai et al. 2002; Schwessinger and Zipfel 2008).

Most of the data about bacterial perception by plants have been generated by studying the interaction of *Pseudomonas syringae* pv. *tomato* DC3000 with *A. thaliana* on leaves. The early events of pathogen or microbe perception have been less studied in roots (Millet et al. 2010). It will, therefore, be informative to investigate how PGPRs are perceived by roots and to determine the early signaling steps that lead to soilborne pathogen resistance.

In the present study, we investigated the biocontrol interaction of the BCA *P. alvei* K165 against *V. dahliae* in *A. thaliana*. In particular, we examined the importance of the PAMP receptors FLS2 and EFR and the signaling cascades associated with these receptors, along with the expression of marker genes of the SA and ET/JA-dependent signaling pathways.

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RESULTS

**FLS2 and WRKY22 are essential factors
for the K165-triggered ISR, while EFR1, BAK1, MPK3,
and MPK6 are possible susceptibility factors for *V. dahliae*.**

The signaling pathway of K165-mediated induced resistance against *V. dahliae* was investigated using the *A. thaliana* mutants *fls2*, *efr-1*, *bak1-4*, *mpk3*, *mpk6*, *wrky29*, and *wrky22*, as inspired by the model, proposed by Asai and associates (2002), of an innate immune signaling cascade activated by bacterial LRR receptors in *Arabidopsis*.

Verticillium symptoms, mainly in the form of wilting, were rated in the different genotypes from 7 to 25 days postinoculation (dpi) (Figs. 1 and 2; Supplementary Fig. S1). Stunting (determined by length of the leaves) was also observed in the most susceptible genotypes (Col-0, *wrky29*, *fls2*, and *wrky22*); in those genotypes, the disease incidence was 100% at 25 dpi (data not shown).

The pathogenicity experiments revealed that the application of K165 conferred protection to Col-0 and *wrky29*, decreasing disease severity and, consequently, the overall amount of disease, referred to as area under the disease progress curve
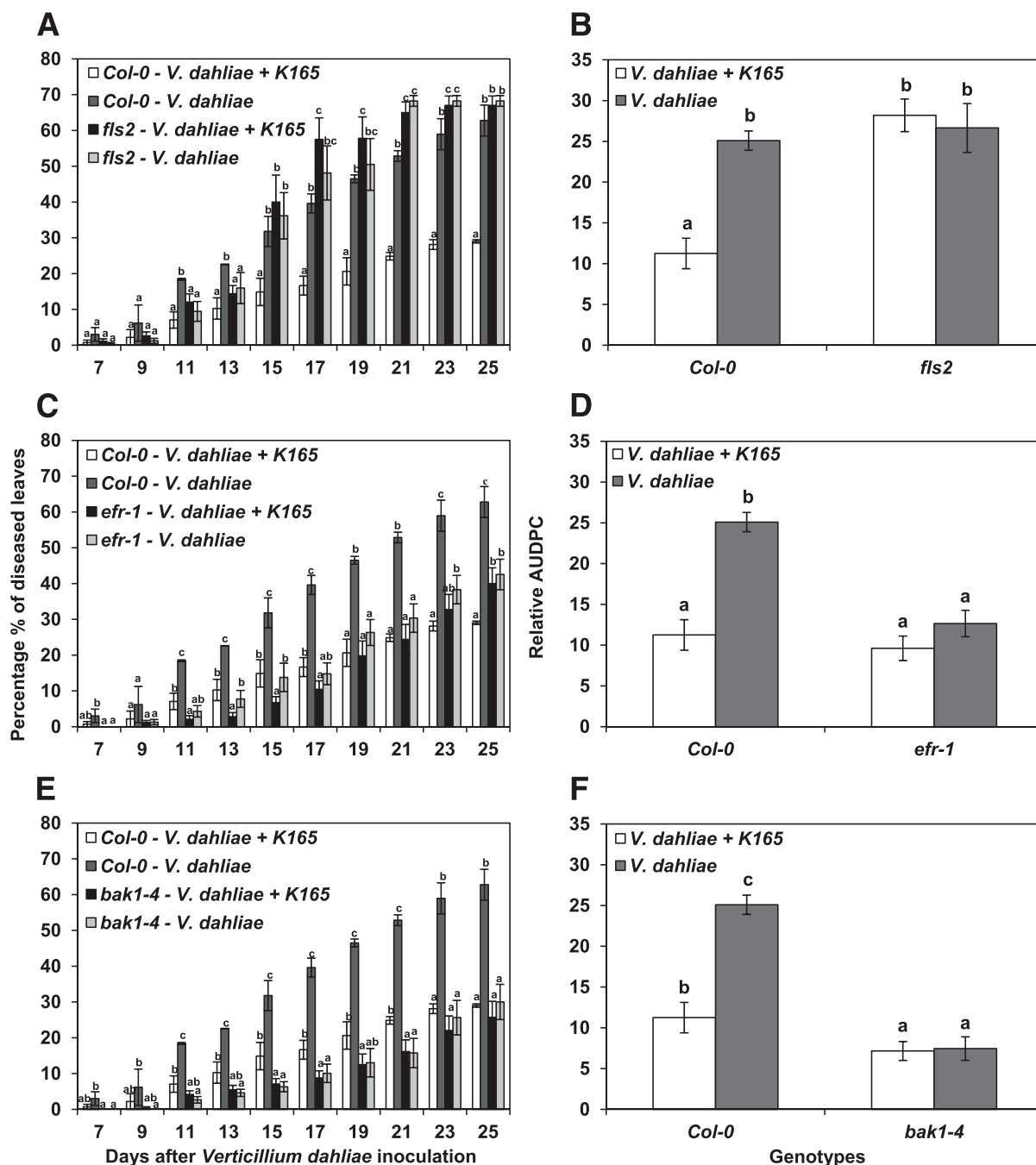

Fig. 1. Protection against *Verticillium dahliae* induced by *Paenibacillus alvei* K165 in the receptor and receptor-associated *Arabidopsis* mutants *fls2*, *efr-1*, and *bak1-4*, respectively, expressed as percentage of diseased leaves. **A, C,** and **E,** At each disease rating day, columns with different letters are significantly different according to Tukey's multiple range test at *P* < 0.05. **B, D,** and **F,** Results expressed as the relative area under the disease progress curve, i.e., the disease level as a percentage of the maximum possible area for the whole period of the experiment. The columns represent the means of 60 plants (two replications with 30 plants per replication and treatment) and the vertical bars indicate standard errors. Columns with different letters are significantly different according to Tukey's multiple range test at *P* < 0.05.

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(AUDPC) (Fig. 2G and H). Thus, protection appears to be independent of WRKY29.

On the other hand, the application of K165 did not reduce *Verticillium* symptom development in *fls2* and *wrky22* compared with the corresponding controls (Figs. 1A and B and 2E and F). Therefore, functional FLS2 and WRKY22 are required for the development of K165-mediated protection.

The application of K165 did not decrease the percentage of disease severity in *efr-1*, *bak1-4*, *mpk3*, and *mpk6* mutants (Figs. 1C, D, E, and F and 2A, B, C, and D). However, EFR, BAK1, MPK3, and MPK6 are unlikely to participate in the K165-triggered plant defense mechanism, since significantly lower AUDPC values were obtained for *Verticillium* wilt symptoms in the control (*V. dahliae*–treated plants) *efr-1*,
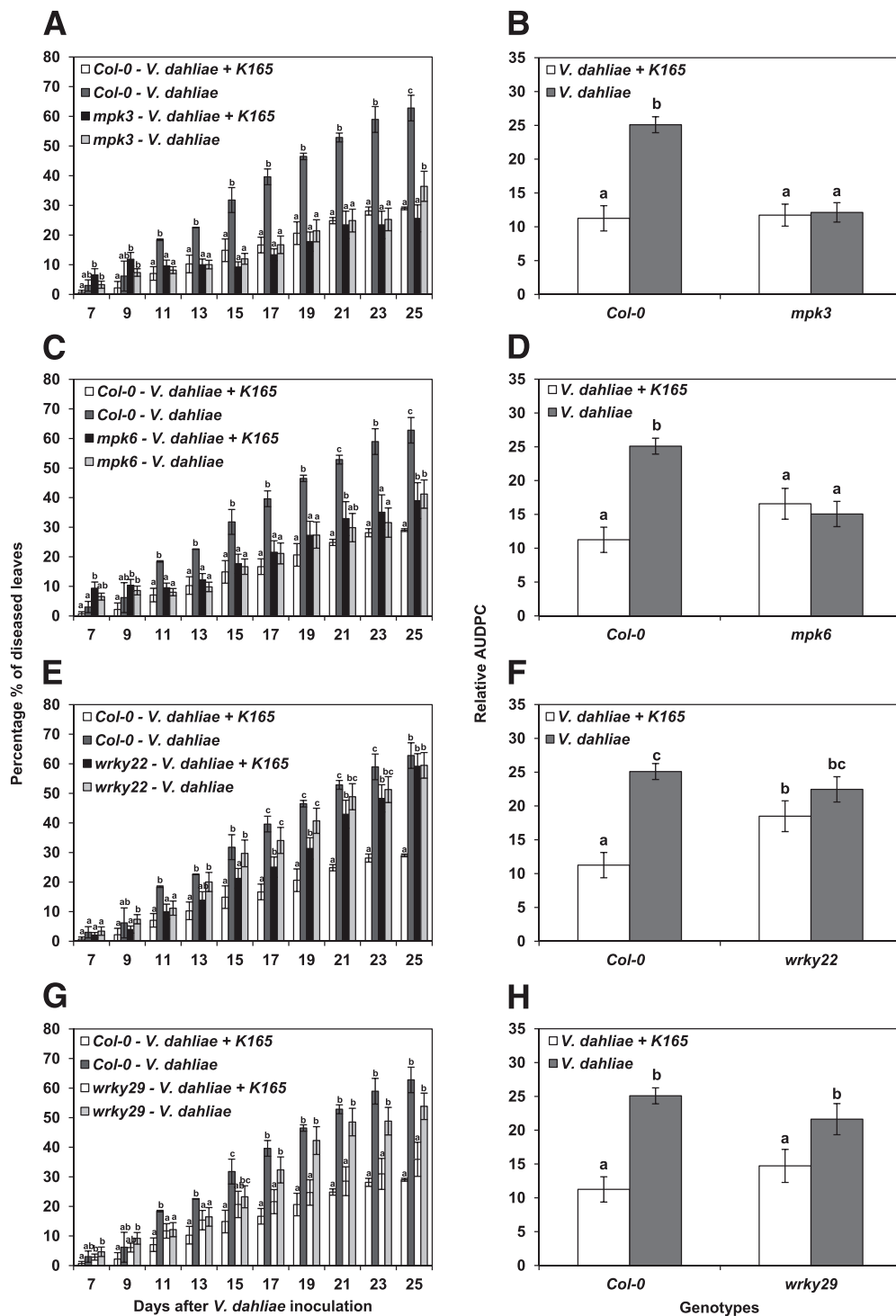

Fig. 2. Protection against *Verticillium dahliae* induced by *Paenibacillus alvei* K165 in the pattern-recognition receptor–driven defense signaling pathway *Arabidopsis* mutants *mpk3*, *mpk6*, *wrky22*, and *wrky29*, expressed as percentage of diseased leaves. **A, C, E,** and **G,** At each disease rating day, columns with different letters are significantly different according to Tukey's multiple range test at *P* < 0.05. **B, D, F,** and **H,** Results expressed as the relative area under the disease progress curve, i.e., the disease level as a percentage of the maximum possible area for the whole period of the experiment. The columns represent the means of 60 plants (two replications with 30 plants per replication and treatment) and the vertical bars indicate standard errors. Columns with different letters are significantly different according to Tukey's multiple range test at *P* < 0.05.

bak1-4, *mpk3*, and *mpk6* mutants compared with the Col-0 controls (Figs. 1C, D, E, and F and 2A, B, C, and D). This partial resistance indicates a hitherto unsuspected role of EFR, BAK1, MPK3, and MPK6 in the interaction of *V. dahliae* with the host plant.

efr1, mpk3, and mpk6 are partially resistant to Verticillium disease, whereas bak1 is tolerant.

Are the recorded wilt symptoms really reflecting *V. dahliae* growth and colonization in vascular tissues? To approach this question, the level of fungal colonization was assessed in each
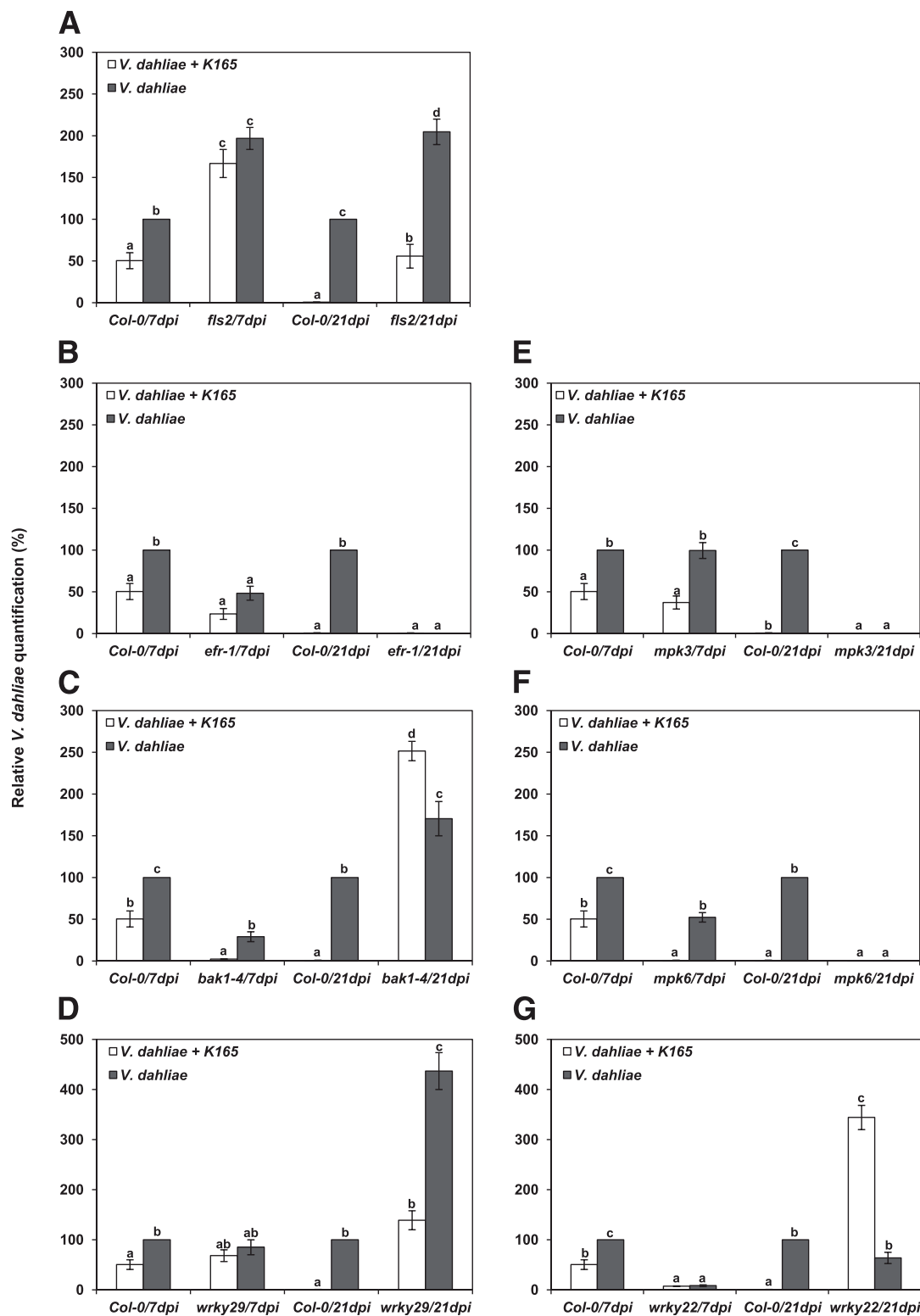
Fig. 3. Relative quantification of the *Verticillium dahliae* DNA levels in the *Arabidopsis* mutants *fls2*, *efr-1*, *bak1-4*, *mpk3*, *mpk6*, *wrky22*, *wrky29*, and wild-type Col-0 plants. Fungal DNA levels were estimated by quantitative polymerase chain reaction using total DNA isolated from the aerial parts of plants at 7 and 21 days postinoculation (dpi). The columns represent the means of two biological repeats (with 10 plants per treatment and repeat) and three technical repeats per biological repeat (total of six reactions per treatment). The vertical bars indicate the standard errors. Col-0 is set to 100%. Columns with different letters are significantly different according to Tukey's multiple range test at $P < 0.05$.

genotype and treatment by real-time quantitative polymerase chain reaction (qPCR) at early (7 dpi) and late stages (25 dpi) of the disease.

Col-0 plants treated with K165 displayed a marked reduction of relative DNA levels of *V. dahliae* compared with untreated controls at both time points (Fig. 3). Therefore, the protective activity of K165 against *V. dahliae* is based on the activation of plant resistance rather than tolerance mechanisms, since tolerance does not limit infection but, instead, reduces or offsets its negative consequences on fitness (Horns and Hood 2012). The *fls2* mutants exhibited high relative levels of *V. dahliae* DNA in agreement with the observed disease severity recordings at both time points, in contrast to *efr-1*, in which lower levels of the pathogen were observed relative to Col-0 (Fig. 3A and B). A decoupling between *Verticillium* wilt symptoms and relative quantification of pathogen DNA was observed in *bak1-4*, in which the DNA levels increased substantially with time in contrast to the AUDPC values (Figs. 1E and F and 3C). The situation in *bak1-4* suggests a tolerance phenomenon.

On the other hand, the relative quantification of *V. dahliae* in *mpk3* and *mpk6* indicates partial resistance; since, in *mpk3* and *mpk6*, the relative levels of *Verticillium* DNA were significantly lower than in Col-0 at 21 dpi (Fig. 3E and F). In *mpk6*, the detected levels of pathogen were significantly lower than in Col-0 at both sampling time points, while in *mpk3* the reduction compared with Col-0 was only visible at 21 dpi.

In *wrky22*, qPCR analysis revealed an increase in the relative amount of the pathogen with time, especially in the case of the K165-treated plants, reflecting the susceptibility of *wrky22* to the pathogen and the loss of the K165-mediated plant protection (Figs. 2E and F and 3G). Likewise, in the case of the *V. dahliae*–treated *wrky29*, the results of the qPCR showed a considerable increase of the relative levels of *V. dahliae* DNA with time (Fig. 3D). In agreement with the pathogenicity experiments, the K165-treated *wrky29* exhibited lower levels of pathogen DNA than the control plants (*V. dahliae*–treated *wrky29*), confirming that WRKY29 is not required in the K165-triggered ISR (Fig. 3D).

The rhizosphere population of K165 in the different genotypes does not influence the disease outcome.

To investigate whether the disease outcome in the K165-treated *fls2*, *efr-1*, *bak1-4*, *mpk3*, *mpk6*, and *wrky22* mutants was caused by insufficient colonization of the rhizosphere by strain K165, we determined the titer of the rifampicin-resistant K165 bacteria at 10 and 20 days after their application.

At 10 dpi, the highest K165 populations were recorded in *wrky22*, *fls2*, and Col-0 (Fig. 4). The finding that *wrky22* and *fls2* were colonized as much as the Col-0 shows that the rhizosphere population of K165 does not influence the disease outcome since, despite its high rhizosphere population, K165 was unable to protect *wrky22* and *fls2* against *V. dahliae*. This idea is also supported by the K165 titers recorded at 20 dpi, when the K165 population in *wrky22* ranged between those of *wrky29* and Col-0, both of which were genotypes protected by K165.

K165-triggered ISR occurs in aerial tissues, possibly through both SA- and ET/JA-dependent defense pathways.

The expression of *PR1*, *PR2*, and *PDF1.2*, marker genes for the SA- and ET/JA-associated defense mechanisms, was analyzed by qPCR in K165-treated and *V. dahliae*-infected Col-0 plants, both in root and in aerial tissues sampled at 3, 7, and 14 dpi.

The most significant changes in transcript levels of *PR1*, *PR2*, and *PDF1.2* were observed in the aerial tissues of the K165/*V. dahliae*–treated plants (Fig. 5B, D, and F). Interestingly, the expression of the monitored genes was suppressed in the aerial tissues of the *V. dahliae*-treated plants at the early

infection stage of 3 dpi (Fig. 5 B, D, and F). On the other hand, the pathogen-inoculated K165-treated plants exhibited the highest *PR1* and *PDF1.2* transcript levels between treatments at all sampling points (Fig. 5B, D, and F). The single application of K165 also resulted in the upregulation of *PR1*, *PR2*, and *PDF1.2* at all sampling points (Fig. 5B, D, and F).

In roots, the only significant differences in gene expression between treatments were observed for *PR1* and *PR2* at 14 dpi (Fig. 5A, C, and E). Therefore, a more significant role is suggested for the aerial than the root tissues for the deployment of the K165-triggered ISR upon *V. dahliae* invasion.

DISCUSSION

In the last decades a number of studies have been devoted to plant-pathogen recognition events and the downstream signaling pathways leading to disease resistance or susceptibility. However, early recognition of BCA that trigger ISR and the subsequent signaling cascade are poorly described. Similarly, the importance of receptor-like kinases such as FLS2 and EFR and of the downstream signaling components in response to root-invading pathogens is unknown. In the present study, mutants blocked at various steps in the defense-signaling cascade revealed that EFR, MPK3, and MPK6 seem to be susceptibility factors for *Verticillium* disease, while K165-mediated ISR against *V. dahliae* depends on FLS2 and WRKY22.

The partial resistance of *efr-1* and, therefore, the interplay of the EFR receptor with the pathogenicity mechanism of *V. dahliae* is an intriguing finding, since EFR binds specifically to the ubiquitous bacterial protein EF-Tu (Gómez-Gómez and Boller 2000; Zipfel et al. 2006). Interestingly, *V. dahliae* possesses a gene (VDAG_01458.1) homologous to EF-Tu that might interfere with EFR and could possibly promote disease, although this would have to be studied further. It is also known that ET modulates EFR-triggered immunity (Tintor et al. 2013). In parallel, the ET receptor ETR1 is a susceptibility factor for *V. dahliae* and *V. longisporum* (Johansson et al. 2006; Pantelides et al. 2010). Thus, it is tempting to speculate from those observations that *Verticillium* takes advantage of components of EFR-regulated immunity to infect the plant.

The *fls2* plants were as susceptible to *Verticillium* disease as the wild types and K165-mediated ISR against *V. dahliae* was compromised in *fls2* (Fig. 1A and B). It is evident that FLS2 has a central role in the recognition of K165 and the triggering of the downstream signaling cascade, as in the interaction of *Arabidopsis* with *Pseudomonas syringae* pv. *tomato* (Asai et al.
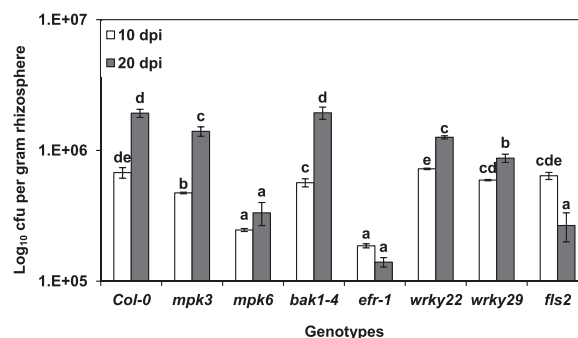

Fig. 4. Titer of *Paenibacillus alvei* K165 in the rhizosphere of *Arabidopsis thaliana* Col-0 plants and *mpk3*, *mpk6*, *bak1-4*, *efr-1*, *wrky22*, *wrky29*, and *fls2* mutants, at 10 and 20 days postinoculation (dpi). The vertical bars indicate standard errors. At each sampling day, columns with different letters are significantly different according to Tukey's multiple range test at $P < 0.05$.

2002). The presence of an amino acid sequence (EKLSSGYR-INRAADDAAGLAI) with 67% homology to flg22 (QRLSTG-SRINSAKDDAAGLQIA) translated from the WP021258858 locus of the *P. alvei* species (taxid: 44250) suggests a direct interaction of FLS2 with the flagellin subunit of K165. Is this interaction the trigger for the K165-mediated ISR? In previous studies, it has been shown that treatment with flg22 leads to the accumulation of SA and that FLS2 is dynamically regulated by SA (Mishina and Zeier 2007; Tateda et al. 2014; Tsuda et al. 2008). FLS2 might, indeed, be the crucial receptor in the K165-mediated protection of the plant since K165-triggered ISR is SA-dependent (Tjamos et al. 2005).

Like other PRR, the FLS2 and EFR receptors require BAK1 for function. BAK1 does not have a direct role in elicitor perception but seems to form heteromeric complexes with FLS2 and EFR after microbe-associated molecular pattern (MAMP) detection (Chinchilla et al. 2007; Heese et al. 2007; Schulze et al. 2010). Accumulating experimental evidence points to BAK1 as a central regulator of plant immunity and a target of several effectors of pathogen virulence (Fradin et al. 2011; Shan et al. 2008). In a previous study, Fradin and associates (2011) reported that *bak1-4* shows enhanced susceptibility to *V. dahliae*, as documented by a higher pathogen level in the aerial tissues of *bak1-4* than Col-0 at 21 dpi; similar results were also obtained in the present study (Fig. 3C). However, symptom development as well as the relative AUDPC values were much lower in *bak1-4* than in Col-0 (Fig. 1E and F). These results suggest the existence of a tolerance mechanism in *bak1-4*. In a pioneering study on molecular quantification of *Verticillium* disease in a tolerant tomato cultivar, it has been
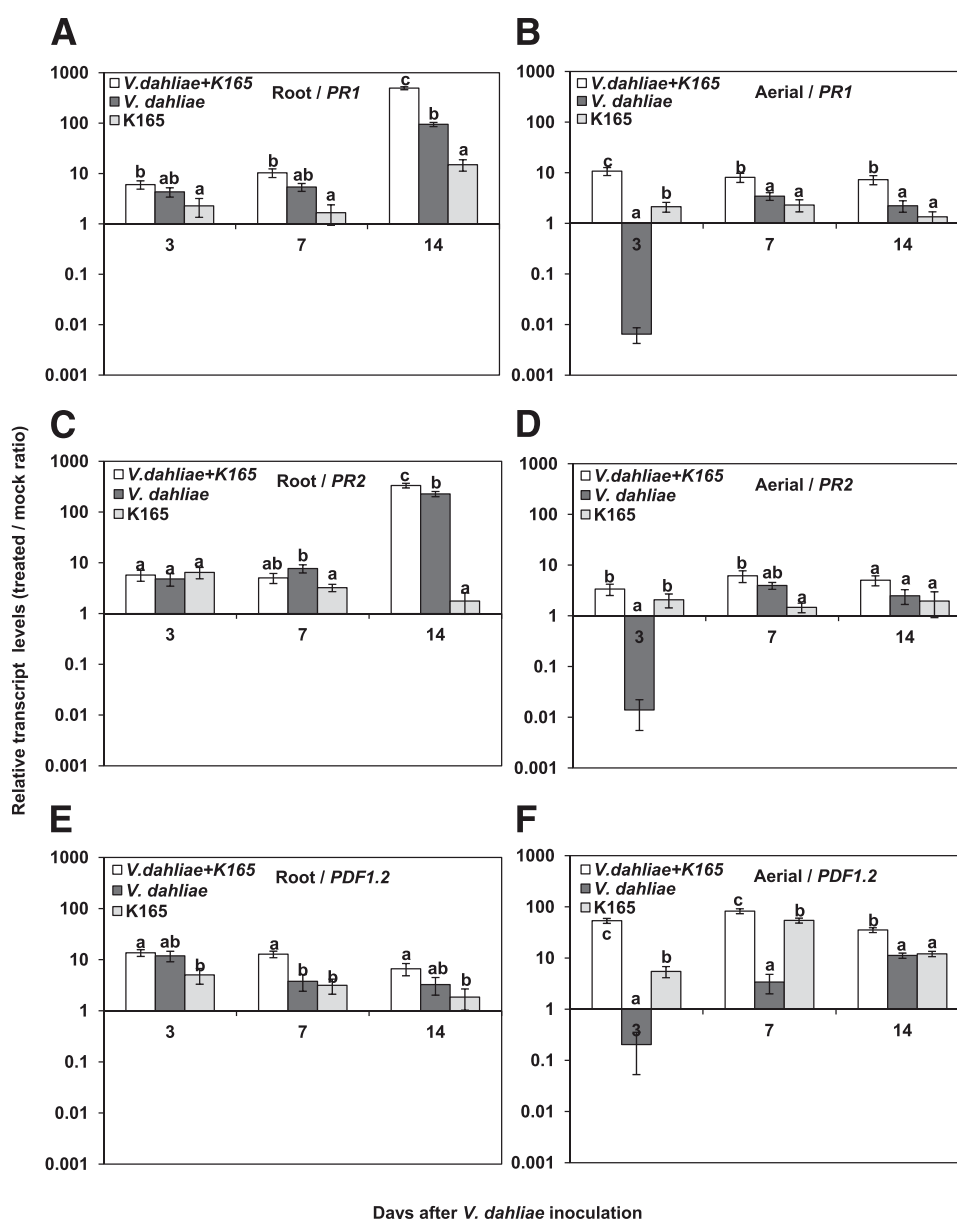

Fig. 5. A and **B,** Relative transcript levels of *PR1,* **C** and **D,** *PR2,* and **E** and **F,** *PDF1.2* in the root (A, C, E) and aerial tissues (B, D, F) of *Arabidopsis* Col-0 plants in response to infection with *Verticillium dahliae* or treatment with *Paenibacillus alvei* K165. Total RNA was isolated from plants at 3, 7, and 14 days postinoculation, was converted to cDNA, and was used as template in quantitative polymerase chain reaction assays. Transcript levels of the examined genes were normalized to the expression of At4g26410 measured in the same samples and were expressed relative to the normalized transcript levels in mock-treated plants. The columns represent the means of three biological repeats (with 10 plants per treatment and repeat) and three technical repeats per biological repeat (total of nine reactions per treatment). The vertical bars indicate the standard errors. Columns with different letters are significantly different according to Tukey's multiple range test at $P < 0.05$.

concluded that, in a tolerant host, an active coating response traps the pathogen as effectively as in a resistant plant, but the amounts of the pathogen remain higher than in resistant plants, suggesting that fungal sporulation and lysis are either delayed in the tolerant interactions or do not occur (Chen et al. 2004). The pathogen is controlled rather than eliminated in the resistant plants, according to different studies (Chen et al. 2004; Heinz et al. 1998; Veronese et al. 2003). A plausible explanation about the tolerance of *bak1-4* to *V. dahliae* could lie on the positive role of brassinosteroids (BR) in ET biosynthesis (Arteca 1995; Joo et al. 2006). BAK1 serves as an enhancer of BR signaling affecting the feedback regulation of BR biosynthesis (Wang et al. 2008; Yun et al. 2009) and, consequently, the effect of BR on ET biosynthesis. ET has been long-associated with *Verticillium* wilt symptoms, as the main contributing factor for the symptoms of epinasty and wilting (Cronshaw and Pegg 1976; Pegg 1976). Along these lines, Robison and associates (2001) examined the effects of reduced ET synthesis on *Verticillium* wilt of tomato by transforming tomato with 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC, the immediate biosynthetic precursor of ET in plants. It was observed that reduced ET synthesis results in increased disease tolerance.

ET might also explain the *Verticillium* resistance of *mpk3* and *mpk6*. In *Arabidopsis*, it has been demonstrated that ACS2 and ACS6 are substrates of MPK3 and MPK6 (Han et al. 2010; Liu and Zhang 2004). Phosphorylation of ACS2/ACS6 by MPK3 and MPK6 stabilizes the ACC synthase (ACS) protein in vivo, resulting in increases in cellular ACS activity and in ET production. Interestingly, MPK3 and MPK6 not only function in the phosphorylation-induced stabilization of ACS2/ACS6 proteins but also signal the *ACS2* and *ACS6* gene activation after *Botrytis cinerea* infection (Li et al. 2012). As proposed earlier, ET synthesis and perception might have a significant role in *Verticillium* pathogenesis and plant resistance or tolerance (Robb et al. 2007).

In contrast, *wrky22* and *wrky29* were highly infected by *Verticillium* spp. compared with the resistance observed in *mpk3* and *mpk6*. Notably the *V. dahliae*–treated *wrky29* and the K165-treated *wrky22* harbored the highest pathogen levels among all the tested genotypes. In the case of K165-treated *wrky22*, the high endophytic amounts of the pathogen reflect the participation of WRKY22 in the K165-mediated ISR. WRKY22 regulates innate immunity genes, defense genes, and PRR-related genes (Hsu et al. 2013) and its activation is induced by flg22 and *N*-acyl-homoserine lactones (AHL) (Schikora et al. 2011). AHL are involved in bacterial quorum sensing. Several reports have also provided indirect evidences that AHL play a role in plant immunity (Pang et al. 2009; Schuhegger et al. 2006); however, the molecular basis of the effect of AHL on the plant immune system remains unknown. The findings of the present study suggest a crucial role for WRKY22 in K165-triggered ISR and further studies are needed to elucidate whether WRKY22 is activated solely upon perception from K165-derived flagellin or by other compounds such as AHL.

The observed decoupling between disease severity and the estimated relative quantity of the pathogen observed in some treatments (*V. dahliae*–treated *fls2* at 21 dpi and *wrky29* at 21 dpi; K165 + *V. dahliae*–treated *wrky29* at 21 dpi and *wrky22* at 21 dpi) can be attributed to the cyclical periods of fungal elimination that characterize the lifestyle of *V. dahliae* in the vascular system of plants such as tomato and oilseed rape (Chen et al. 2004; Eynck et al. 2007; Heinz et al. 1998). Also, the levels of DNA of the pathogen reflect numbers of propagules more than actual biomass; spores are small, relative to mycelial cells, but have the

same DNA content (Chen et al. 2004). This disparity between pathogen proliferation and symptom development has also been observed in bacterial (Bent et al. 1992; Lund et al. 1998; O'Donnell et al. 2001) and viral (Cecchini et al. 2002) plant-pathogen interactions, and it has been partly explained by the possibility that symptoms can result from pathogen-induced signals that cause changes in normal plant growth and development (Cecchini et al. 2002; Dietrich et al. 1994; Lund et al. 1998; O'Donnell et al. 2001; Pilloff et al. 2002). It is evident that, in vascular wilts in which phytohormones, such as ET, are involved in the development of disease symptoms, both symptoms and pathogen quantification need to be measured to draw reliable conclusions about resistance, tolerance, or susceptibility.

It has been long-stated that, for *Verticillium*-caused wilt diseases, the resistance or susceptibility of the host plant is generally considered to be determined mainly by the cellular interactions between the plant and the fungus occurring in the stem (Robb et al. 2007). The present study also showed most striking differences in the expression of *PR1*, *PR2*, and *PDF1.2* between treatments in the aerial tissues after K165 and *V. dahliae* treatment or *V. dahliae* treatment of Col-0 plants. The strong activation of defense-associated genes observed after pathogen inoculation of K165-treated plants suggests the existence of a priming mechanism. Interestingly, the application of K165 induced the expression of markers for SA (*PR1* and *PR2*) and ET/JA (*PDF1.2*) signaling pathways. The upregulation of *PR1* in the aerial parts of the K165- and *V. dahliae*–treated plants can be crucial for restricting the pathogen at the early infection stages, when *V. dahliae* follows a biotrophic life style (Thaler et al. 2004). The importance of *PR1* during the biotrophic stages of plant infection by *V. dahliae* has also been proposed by Zhang and associates (2013). In addition, a previous study has already highlighted the significance of the SA-dependent defense pathway in the K165-mediated ISR against *V. dahliae* (Tjamos et al. 2005). The triggering of the *PDF1.2* expression by K165 is consistent with the data showing that PGPR-primed plants often display JA-dependent defense responses upon pathogen invasion (Pieterse et al. 1998; Pozo et al. 2008; van Hulten et al. 2006). However, since JA-related mutants did not show changes in disease severity caused by *V. dahliae* or *V. longisporum*, JA-signaling might be of less importance in the presently studied system (Johansson et al. 2006; Pantelides et al. 2010; Veronese et al. 2003). JA/ET-mediated defense responses are considered as more effective against necrotrophic than biotrophic pathogens (Glazebrook 2005) and, although most reports indicate a mutually antagonistic interaction between SA- and JA-dependent signaling, synergistic interactions have been described as well (Mur et al. 2006). Therefore, we cannot completely exclude a role for the JA pathway in the K165-mediated plant protection, especially at the later necrotrophic stages of the disease.

Furthermore, the qPCR analysis revealed that *V. dahliae* suppressed the plant immune system, since the expression of *PR1*, *PR2*, and *PDF1.2* was downregulated in the aerial tissues of the *V. dahliae*–treated plants at the early infection stages (3 dpi). Similarly, a substantial suppression of *PAL* mRNA levels occurred after inoculation of a tomato line susceptible to *Verticillium alboatrum*, while the suppression was absent or substantially reduced in a resistant line (Lee et al. 1992). The suppression of the immune system and, mainly, the SA-dependent defenses could be a strategy of *V. dahliae* to promote disease, since it would allow the pathogen to overcome the plant defense responses during its initial biotrophic stage. The use of EFR1, MPK3, and MPK6 as susceptibility factors from *V. dahliae* highlights the interplay of the pathogen with the plant defense signaling pathway.

In summary, this study shows the involvement of FLS2 and WRKY22 in a BCA-mediated ISR in *A. thaliana* (Fig. 6). The results also suggest that EFR, MPK3, and MPK6 are

susceptibility factors for *V. dahliae* (Fig. 7) and help promote disease development.

MATERIALS AND METHODS

Fungal culture.

V. dahliae isolated from *Raphanus sativus* L. (provided by E. Ligoxygakis, National Agricultural Research Institute, Crete, Greece), with known pathogenicity against *A. thaliana* plants (Tjamos et al. 2005), was used in the experiments. The fungal strain was cryopreserved by freezing a conidial suspension in 25% aqueous glycerol at –80°C (Maniatis et al. 1982). Before being used, the fungus was transferred to potato dextrose agar (Merck, Darmstadt, Germany) at 24°C for 5 days. For the bioassays, a suspension of 10^7 conidia of distilled sterile water per milliliter was prepared from a culture grown for 5 days at 24°C in sucrose sodium nitrate liquid medium (Sinha and Wood 1968).

Bacterial culture.

A K165 rifampicin-resistant mutant (Tjamos et al. 2004) was used throughout the experiments. The K165 strain was cryopreserved by freezing a 1×10^8 CFU/ml suspension in 25% aqueous glycerol at –80°C (Maniatis et al. 1982). Before being used, K165 was transferred to nutrient broth agar plus glycerol (NAG) at 28°C for 2 days. For the bioassays, bacterial cells were prepared in nutrient broth plus glycerol in an orbital incubator at 180 rpm and 28°C for 2 days. Suspensions were centrifuged at $5,600 \times g$ at 20°C for 10 min and were resuspended in sterile distilled water before treatment of the plants.

Plant material and growth conditions.

A. thaliana ecotype Columbia (Col-0) was used as the wildtype control. The mutant lines used in this study are *fls2* (Zipfel et al. 2004), *bak1-4* (Chinchilla et al. 2007), *mpk3* (Beckers et al. 2009), *mpk6* (Beckers et al. 2009), *wrky22* (GK-311E07.01), *wrky29* (GK-793H12.03), and *efr-1* (Zipfel et al. 2006). All seeds were stored at 4°C. *A. thaliana* seeds were sown in $9 \times 9 \times 10$-cm pots containing pasteurized soil mix of humus and perlite (3:1) and were maintained at 25°C with a 12-h photoperiod at 60 to 70% relative humidity in a controlled-environment growth chamber. After 10 days, the plants were singled to plastic pots containing approximately 80 cm^3 of pasteurized soil mix of humus and perlite (3:1).

K165–*V. dahliae* bioassays.

Eighteen-day-old plants were inoculated with K165 by root drenching, using 10 ml of 1×10^8 CFU/ml. After 5 days, the plants were challenge-inoculated with *V. dahliae* by root drenching with 10 ml of a suspension of 1×10^7 conidia per milliliter of sterile distilled water (Tjamos et al. 2005). Control plants were mock-inoculated with 10 ml of sterile distilled water. Disease severity at each observation was calculated from the number of leaves that showed wilting as a percentage of the total number of leaves of each plant and was periodically recorded for 25 days after inoculation. Disease ratings were plotted over time to generate disease progression curves. AUDPC was calculated by the trapezoidal integration method (Campbell and Madden 1990). Disease was expressed as a percentage of the maximum possible area for the whole period of the experiment, which is referred to as the relative AUDPC. The experiment was repeated two times with 30 plants per treatment and plant genotype (a total of 60 plants).
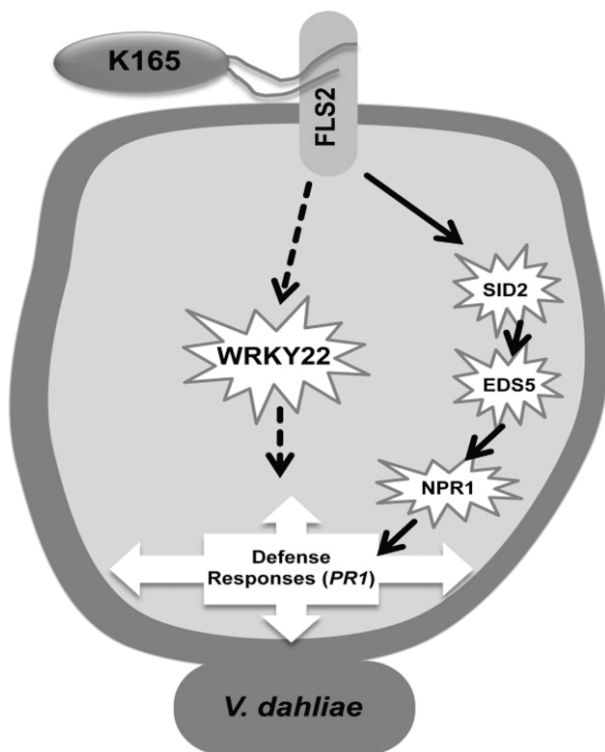
Fig. 6. Proposed model for the *Paenibacillus alvei* K165 induced systemic resistance signaling pathway leading to plant protection against *Verticillium dahliae*. K165 is perceived by the FLS2 receptor, leading to increased *PR1* transcripts in the aerial plant parts. The loss of the K165 protective activity in *wrky22* suggests the participation of the WRKY22 transcription factor in the K165-triggered signaling cascade. In a previous study, the suppression of *V. dahliae* by *P. alvei* K165 was attributed to the induction of systemic resistance via the salicylic acid (SA)-dependent defense pathway (Tjamos et al. 2005). It is known that flg22 treatment results to SA accumulation, so the FLS2 binding with K165 flagellin subunit may trigger the SA-dependent plant defense mechanism. Dashed lines represent the results of this study, whereas solid lines represent the results of Tjamos and associates (2005) and the presumed connection between FLS2 and the SA-dependent defense.
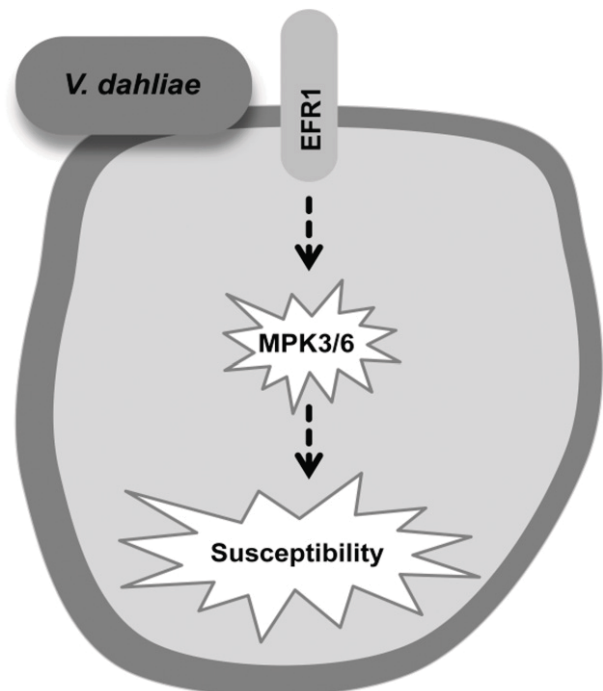
Fig. 7. Proposed model for a signaling pathway mediating *Verticillium dahliae* susceptibility. The *efr1*, *mpk3*, and *mpk6* mutants showed resistance to *V. dahliae* colonization and symptom development. Therefore, it is suggested that *V. dahliae* hijacks EFR1, MPK3, and MPK6 to promote disease.

Rhizosphere population of K165.

Colonization of the rhizosphere of wild-type and mutant plants by rifampicin-resistant K165 bacteria was determined at 10 and 20 days after the application of the bacterial strain. To estimate rhizosphere populations, 2 g of rhizosphere soil (soil particles in close contact with roots within a distance of approximately 1 to 5 mm) was collected and shaken for 45 min in 50 mM phosphate buffer, pH 7.0, containing 0.02% Tween 20, and the suspension was plated onto NAG medium supplemented with cycloheximide (100 µg/ml) and rifampicin (100 µg/ml). After 48 h of incubation at 28°C, the number of bacterial CFU per gram of rhizosphere soil was determined.

The experiment was repeated three times with 15 replicates per experiment.

DNA extraction and qPCR for fungal quantification.

The aerial parts of 10 plants from each treatment were harvested for real-time qPCR analysis at 7 and 21 dpi. In brief, the aerial parts were cut at soil level, were pooled, were rinsed with sterile distilled water, and were ground to a fine powder, using an autoclaved mortar and pestle in the presence of liquid nitrogen.

Total DNA was isolated according to Dellaporta and associates (1983) and was quantified by spectrophotometry. qPCR assays for the quantification of *V. dahliae* were conducted as described previously by Fradin and associates (2011), using the primer pair ITS1-F 5′-AAAGTTTTAATGGTTCGCTAAGA-3′ (Gardes and Bruns 1993) and ST-VE1-R 5′-CTTGGTCATTTA GAGGAAGTAA-3′ (Lievens et al. 2006). qPCR was performed in a Stratagene Mx3005P thermocycler and, for the amplification reactions, QuantiFast SYBR Green PCR (Qiagen, Valencia, CA, U.S.A.) master mix was used. The results were analyzed with MxPro qPCR software.

For sample calibration, the *Arabidopsis* gene At4g26410, previously described as a stable reference gene (Czechowski et al. 2005), was targeted, using the primer pair F 5′-GAGCTGA AGTGGCTTCCATGAC-3′ and R 5′-GGTCCGACATACCCA TGATCC-3′. PCR cycling started with an initial step of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. PCR efficiency for each amplicon was calculated by employing the linear regression method on log (fluorescence) per cycle number data, using Lin-RegPCR software (Ramakers et al. 2003). The qPCR assays were performed in triplicates. The absence of nonspecific products and primer dimers was confirmed by the analysis of melting curves. The experiment was repeated two times with 10 plants per treatment and plant genotype (a total of 20 plants).

Determination of transcript levels using reverse transcription-PCR assay.

Aerial and root tissues, shortly rinsed with water to remove soil particles, were collected for RNA analysis. Samples were collected from 10 Col-0 plants per treatment (K165-, K165 + *V. dahliae*-, and *V. dahliae*- and mock-inoculated plants) at 3, 7, and 14 dpi and were immediately frozen in liquid nitrogen and were stored at −80°C. For each sample, total RNA was extracted from 100 mg of tissue ground with liquid nitrogen, using TRIzol reagent (Invitrogen, Paisley, U.K.) according to the manufacturer's instructions. The RNA samples were treated with DNase I (Invitrogen) to eliminate traces of contaminating genomic DNA. The RNA concentration was measured on a Nanodrop ND-1000 spectrophotometer (Saveen Werner, Malmö, Sweden). First-strand cDNA was synthesized, using SuperScript II (Invitrogen) following the manufacturer's procedure. For the amplification of *PR1* (At2g19990), *PDF1.2* (At5g44420), and *PR2* (At3g57260), the primer sets designed by Trusov and associates (2009) and Pantelides and associates (2010) were used. The qPCR assays were performed in triplicate. Normalization

of gene expression, PCR efficiency, absence of nonspecific products and primer dimers, and data analysis were performed as described previously in the qPCR fungal quantification procedure. Under the experimental infection conditions, the gene expression of At4g26410, used for normalization of gene expression, was stable between treatments, with cycle threshold values equal to approximately 19.5. The experiment was repeated three times with 10 plants per treatment and plant genotype (a total of 30 plants).

Statistics.

Data on relative AUDPC, K165 rhizosphere population, *V. dahliae* DNA quantification, and gene expression were transformed with the $\sqrt{x}$ +1 transformation before analysis of variance was applied. When a significant ($P < 0.05$) F test was obtained for treatments, data were subjected to means separation by Tukey's multiple range test.

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