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A Key Role for the *Arabidopsis* WIN3 Protein in Disease Resistance Triggered by *Pseudomonas syringae* That Secrete AvrRpt2

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Effector proteins injected by the pathogenic bacteria *Pseudomonas syringae* into plants can have profound effects on the pathogen–host interaction due to their efficient recognition by plants and the subsequent triggering of defenses. The AvrRpt2 effector triggers strong local and systemic defense (called systemic acquired resistance [SAR]) responses in *Arabidopsis thaliana* plants that harbor a functional *RPS2* gene that encodes an R protein in the coiled-coil, nucleotide-binding domain, leucine-rich repeat class. The newly identified *win3-T* mutant shows greatly reduced resistance to *P. syringae* carrying *avrRpt2*. In *win3-T* plants, RIN4 cleavage, an early AvrRpt2-induced event, is normal. However, salicylic acid accumulation is compromised, as is SAR induction and the local hypersensitive cell death response after infection by *P. syringae* carrying *avrRpt2*. *WIN3* encodes a member of the firefly luciferase protein superfamily. Expression of *WIN3* at an infection site partially requires PAD4, a protein known to play a quantitative role in RPS2-mediated signaling. *WIN3* expression in tissue distal to an infection site requires multiple salicylic acid regulatory genes. Finally, *win3-T* plants show modestly increased susceptibility to virulent *P. syringae* and modestly reduced SAR in response to *P. syringae* carrying *avrRpm1*. Thus, *WIN3* is a key element of the RPS2 defense response pathway and a basal and systemic defense component.

Additional keywords: avirulence, GH3, hypersensitive response, leaf spot disease.

The bacterial pathogen *Pseudomonas syringae* causes leaf spot disease on diverse plant hosts in a manner dependent on the delivery of effector proteins through the specialized type-three secretion apparatus. The host range of a particular *P. syringae* strain is a function of the repertoire of the effectors encoded by the strain (Greenberg and Vinatzer 2003). The model host *Arabidopsis thaliana* has the capacity to recognize some effectors (called avirulence [Avr] proteins) and trigger a resistance response that limits the growth of bacteria at the infection site. Recognition is a highly specific event, conditioned by the presence in the host of a cognate functional resistance (R)

protein (Chisholm et al. 2006). For the cognate *Arabidopsis* R proteins of well-characterized *P. syringae* Avr effectors, detection of a modified plant target leads to a signaling active state of the R protein (Coaker et al. 2005; Mackey et al. 2002, 2003; Shao et al. 2003). In some cases, a resistance response is accompanied by a hypersensitive response that causes programmed cell death and also leads to the systemic activation of defenses (Greenberg and Yao 2004), an event termed systemic acquired resistance (SAR). The defense molecule salicylic acid (SA) is essential for SAR and is sometimes required for the signaling activated by Avr–R interactions (Glazebrook 2001).

One of the best-studied cognate Avr–R pairs in the *P. syringae*–*Arabidopsis* model is that of the AvrRpt2–RPS2 interaction. When AvrRpt2 enters the plant cell, it is aided to fold into an active form by the *Arabidopsis* ROC1 cyclophilin (Coaker et al. 2005). Upon correct folding, AvrRpt2, a protease, acts on itself, an event necessary for the correct localization of AvrRpt2 to the plasma membrane (Jin et al. 2003). AvrRpt2 then cleaves the membrane-localized host target RIN4 (and probably other targets), an event that is highly correlated with the activation of RPS2 (Coaker et al. 2005). The RPS2-mediated signaling pathway requires the action of the NDR1, a signaling component also important for other R protein-mediated defense responses (Century et al. 1995, 1997). HSP90 and RAR1 are essential for RPS2, probably due to their chaperone functions that aid in protein folding and stability (Takahashi et al. 2003; Tornero et al. 2002). Additionally, RPS2-mediated disease resistance requires SA production (Nawrath and Métraux 1999) that is regulated by NDR1 (Shapiro and Zhang 2001), SA signal transduction mediated by the NPR1 protein (Rate and Greenberg 2001), and signaling by the interacting PAD4 and EDS1 proteins (Feys et al. 2001).

Here, we report that the recently identified *Arabidopsis* HopW1-interacting (WIN3) protein plays a key function in the local and systemic responses of the *Arabidopsis* Columbia (Col) accession to *P. syringae* that carries *avrRpt2*. In the *Arabidopsis* Ws accession, WIN3 is important for resistance activated in response to *P. syringae* carrying the Avr effector *hopW1*, whereas WIN3 overexpression confers resistance to virulent *P. syringae* (M. W. Lee, J. Jelenska, and J. T. Greenberg, *unpublished data*). We show here that WIN3 expression in infected tissue of the Col accession is partially controlled by PAD4. Its expression in systemic tissue requires numerous regulators of SA accumulation and responses. Finally, we find WIN3 to be important for partially restricting the growth of two virulent *P. syringae* strains. We suggest that WIN3 is an important downstream component in the defense responses activated in response to AvrRpt2 and also plays a role in basal defenses that limit the growth of virulent *P. syringae*.

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Accession number: WIN3, At5g13320, NP_196836.

RESULTS

WIN3 is rapidly induced in response to the *P. syringae* Avr effectors AvrRpt2 and AvrRpm1.

WIN3 mRNA levels in the Col accession of *Arabidopsis* previously were found to be elevated after infection with virulent *P. syringae* PtoDC3000 (Thilmony et al. 2006). Similarly, WIN3 transcript levels were induced by strain *Pma*DG3 (a fully virulent derivative of strain *Pma*ES4326) (Guttman and Greenberg 2001) by 12 h after infection (Fig. 1). However, avirulent derivatives of *Pma*ES4326 carrying the effectors *avrRpt2* (strain *Pma*DG6) or *avrRpm1* (strain *Pma*DG34) induced WIN3 transcript levels more rapidly than the congenic virulent strain *Pma*DG3 (Fig. 1). This suggests that there could be a role for WIN3 in one or more defense response pathways induced by *P. syringae* carrying different Avr effectors.

A *win3* loss of function mutant is severely compromised for AvrRpt2-induced resistance.

To further characterize the role of WIN3, we isolated a mutant with a T-DNA insertion in the third intron of the gene (At5g13320) in the Col background (Fig. 2A). Plants with the *win3-T* allele failed to accumulate WIN3 mRNA under infection conditions that usually result in WIN3 induction (Fig. 2B). Thus, *win3-T* is likely a null mutant.

Infection of the *win3-T* mutant plants with different avirulent derivatives of *Pma*ES4326 resulted in plants that were highly susceptible to *Pma*ES4326 carrying *avrRpt2*, but showed normal resistance to *Pma*ES4326 carrying *avrRpm1* or *avrB* (Fig. 3A and B). Additionally, avirulent derivatives of virulent strain PtoDC3000 carrying *hopA1* or *avrRps4* showed high-level resistance in *win3-T*, although there was a trend of reduced resistance elicited by AvrRps4 that was not statistically significant ($P = 0.14$) (Fig. 3C). Loss of resistance in response to AvrRpt2 also was accompanied by the loss of a macroscopic hypersensitive response. However, AvrB and AvrRpm1 elicited robust hypersensitive responses (Fig. 3D). Finally, *win3-T* plants had increased susceptibility to the virulent strains *Pma*DG3 and PtoDC3000 (Fig. 3B and E). The most dramatic phenotype, however, was the loss of resistance to *Pma*ES4326 carrying *avrRpt2*.

To confirm that the loss of resistance in response to AvrRpt2 was due to the T-DNA in *win3-T*, we transformed the mutant

with a dexamethasone (dex)-inducible version of WIN3. *win3-T* mutants carrying dex:WIN3 were treated with two different concentrations of dex and, at the same time, infected by *Pma*ES4326/vector (*Pma*DG3) or *Pma*ES4326/*avrRpt2* (*Pma*DG6). Complementation was observed at both concentrations of dex for both strains (Fig. 4A and B). In the absence of dex, most *win3-T* lines carrying dex:WIN3 were complemented for the growth of *Pma*ES4326 carrying vector, but not *Pma*ES4326/*avrRpt2* (Fig. 4A and B). This is likely due to some leakiness of the dex-inducible system. These data confirm that the *win3-T* mutation is responsible for the enhanced susceptibility to *Pma*ES4326 and *Pma*ES4326 carrying *avrRpt2*.

RIN4 cleavage in response to AvrRpt2 occurs normally in *win3-T* plants.

An early event in the AvrRpt2 response pathway is the cleavage of RIN4 by AvrRpt2, a process that is highly correlated with defense activation (Coaker et al. 2005; Mackey et al. 2002, 2003). *win3-T* plants showed normal proteolysis of RIN4 upon infection with *Pma*ES4326 carrying *avrRpt2* (Fig. 5). This suggests that a signaling event downstream of RIN4 cleavage is affected by WIN3.

WIN3 is important for SA accumulation and SAR in response to AvrRpt2.

To further characterize the defects in the *win3-T* mutant responses to AvrRpt2, we monitored several additional events known to occur after AvrRpt2 recognition. Because the biosyn-

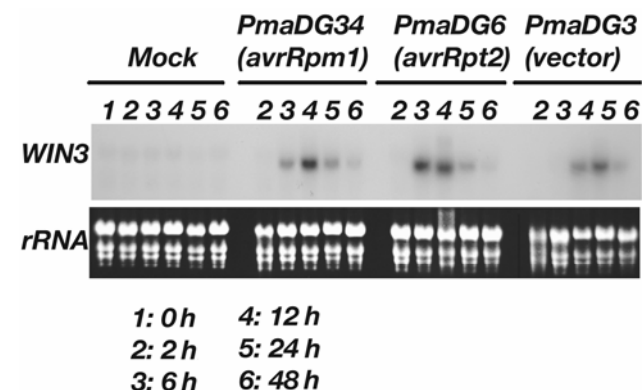


Fig. 1. Rapid induction of WIN3 transcript accumulation after infection with several derivatives of *Pma*ES4326. RNA blot analysis was performed on RNA samples from 20-day-old leaves grown in light-and-dark cycle of 16 and 8 h, respectively, isolated at the indicated times after mock inoculation with 10 mM MgSO_4 or infection with an optical density at 600 nm = 0.01 of *Pma*DG3 (vector control of *Pma*ES4326), *Pma*DG6 (*Pma*ES4326 carrying *avrRpt2*), or *Pma*DG34 (*Pma*ES4326 carrying *avrRpm1*). Ethidium-stained rRNA is shown as a loading control. This experiment was repeated twice with similar results.

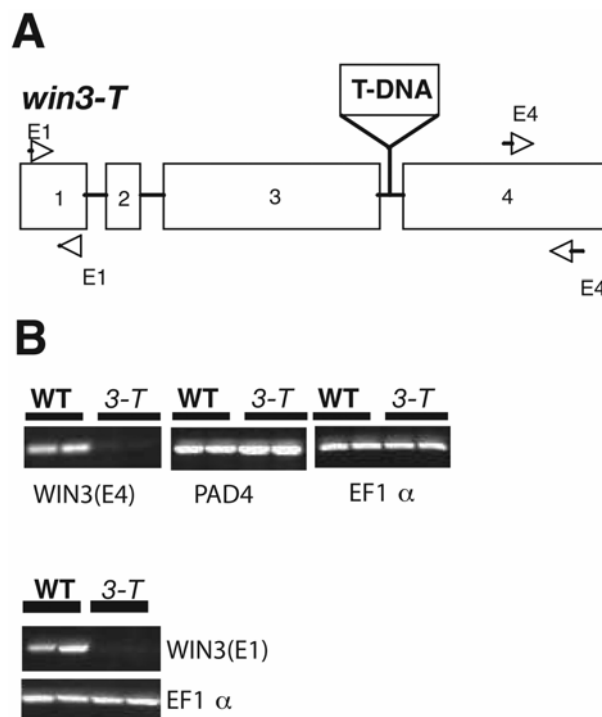


Fig. 2. Isolation of the *win3-T* mutant in the Col accession. **A**, Schematic gene structure of *WIN3* carrying a T-DNA insertion in the third intron. Arrows show the position of primers used to amplify exon 1 (E1) and exon 4 (E4), respectively. **B**, Lack of detectable *WIN3* transcript accumulation in the *win3-T* mutant 24 h after infection with an optical density at 600 nm = 0.01 of *Pma*DG3. WT is wild-type Col and 3-T is *win3-T*. Transcript accumulation of *PAD4*, inferred from data after 27 cycles of reverse-transcriptase polymerase chain reaction was not reduced in the *win3-T* samples. Transcript corresponding to exon 1 *WIN3*(E1) and exon 4 *WIN3*(E4) were detectable in WT but not *win3-T* plants. *EF1 α* serves as a loading control. This experiment was repeated twice with similar results. Plants were grown in a light-and-dark cycle of 16 and 8 h, respectively, at 22°C.

thesis of SA is important for an AvrRpt2-induced disease resistance (Nawrath and Métraux 1999), we measured SA accumulation in *win3-T* plants after infection. Both free and sugar-conjugated SA levels were reduced in *win3-T* plants that were infected with *PmaES4326* carrying the vector (*PmaDG3*) or *PmaES4326* carrying *avrRpt2* (*PmaDG6*) at 6 and 24 h after

infection (Fig. 6A). In contrast, although *PmaES4326* carrying *avrRpm1* (*PmaDG34*) elicited reduced levels of sugar-conjugated SA levels in *win3-T* plants, the free SA levels were not reduced in these plants (Fig. 6B). Interestingly, at 24 h after infection, the free SA level in *win3-T* reached the same level as that seen in wild-type plants (Fig. 6B). At 24 h, the free SA showed a trend of being higher in the *win3-T* plants, but this was not statistically significant ($P = 0.086$). Interestingly, treatment of *win3-T* leaves with the SA agonist benzo(1,2,3) thiadiazole-7-carbothioic acid (benzothiazole [BTH]) restored the ability of *PmaES4326* carrying *avrRpt2* to elicit a hypersensitive response in a dose-dependent manner (Table 1). Therefore, it is likely that the lack of timely accumulation of free SA was responsible for the reduced resistance response to *avrRpt2*.

The hypersensitive response can activate a long-distance signal that leads to enhanced resistance of the whole plant to subsequent infections, the SA-dependent process called SAR. Therefore, we tested SAR activation in *win3-T*. After infection with *PmaES4326* carrying *avrRpt2*, *win3-T* plants failed to become resistant to virulent *PmaDG3* in the systemic leaves or to express elevated transcript levels of the defense-related gene *PR1* (Fig. 7A and B). However *win3-T* plants retained the ability to respond in a systemic fashion to BTH, a chemical inducer of resistance and an SA agonist (Fig. 7A). Interestingly, BTH induced systemic accumulation of *WIN3* transcript, suggesting that *WIN3* may be in the SA response pathway (Fig. 7B). Finally, in *win3-T* plants infected with *PmaES4326* carrying *avrRpm1* (*PmaDG34*), SAR was still activated. However, in *win3-T*, the magnitude of SAR activated in response to *PmaDG34* was reduced several-fold compared with wild-type plants (Fig. 7C). This data suggest that *win3-T* has a minor role in general pathogen-induced SAR activation per se, but a major role in SAR activated as a consequence of infection with *PmaES4326* carrying *avrRpt2*.

Multiple defense components are important for proper WIN3 expression.

Both PAD4 and *WIN3* play roles in the induction of disease resistance in response to the Avr effectors AvrRpt2 and HopW1

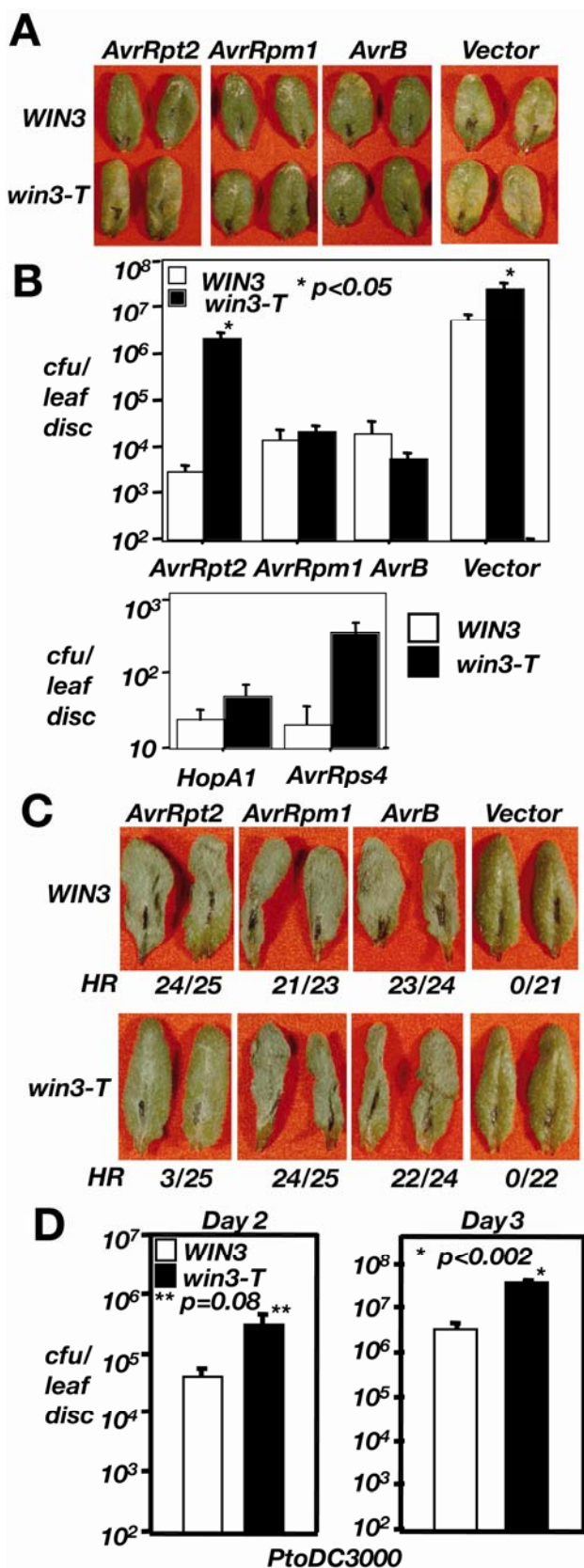


Fig. 3. WIN3 is required for basal and AvrRpt2-elicited disease resistance. Plants were grown in a light-and-dark cycle of 16 and 8 h, respectively, at 22°C. These experiments were repeated twice with similar results. **B**, **C**, and **E**, bars represent standard error; analysis of bacteria was done at different times and should not be directly compared. **A**, Disease symptom development of *PmaES4326* carrying vector, *avrRpt2*, *avrRpm1*, or *avrB* on Col and *win3-T* plants after infection of 3-week-old plants with an optical density at 600 nm (OD_{600}) = 0.0001 of the indicated strain. Leaves were photographed 4 days after infection. **B**, Growth of *PmaES4326* carrying vector (*PmaDG3*), *avrRpt2* (*PmaDG6*), *avrRpm1* (*PmaDG34*) and *avrB* (*PmaES4326* carrying plasmid pVSP61 containing the *avrB* gene) on Col and *win3-T* plants. Bacterial growth was measured 3 days after inoculation (as in **A**) using six samples. *win3-T* plants show highly increased susceptibility to *PmaES4326* carrying *avrRpt2* (*, $P < 0.05$, *t* test) and increased susceptibility to *PmaES4326* carrying the vector control (*, $P < 0.05$, *t* test). Error bars show standard error. **C**, Growth of *PtoDC3000* carrying *hopA1* or *avrRps4* on Col or *win3-T* plants. Bacterial growth was measured 3 days after inoculation with OD_{600} = 0.0001. Growth of bacteria on the two plant genotypes was not significantly different ($P = 0.27$ for *HopA1* and $P = 0.14$ for *AvrRps4*). **D**, Leaves of Col (*WIN3*) and *win3-T* plants were inoculated at a high dose (OD_{600} = 0.05) with *PmaES4326* carrying the vector control or expressing the indicated avirulence (Avr) effector. Plants were photographed 17 h after inoculation. Plants showing macroscopic collapse were scored as undergoing a hypersensitive response (HR). Beneath each pictured leaf is the number of leaves that showed an HR out of the total number of leaves infiltrated. **E**, Leaves of Col and *win3-T* plants were inoculated with *PtoDC3000* at OD_{600} = 0.0001. Bacterial growth at 2 and 3 days after infection was measured. *PtoDC3000* grew to higher levels in *win3-T* than the wild type (*, $P < 0.002$; **, $P = 0.08$; *t* test, $n = 6$). Bars represent standard error.

(Fig. 3) (Feys et al. 2001; M. W. Lee and J. T. Greenberg, *unpublished observation*). After infection with *PmaES4326* carrying *avrRpt2*, the transcript level of WIN3 was reduced in a *pad4* mutant in three out of four independent experiments (Fig. 8A). In contrast, PAD4 transcript levels were unaffected by the *win3-T* mutation (Fig. 2B). Many other defense mutants we tested did not show any clear reproducible alteration in WIN3 induction in directly infected tissue (data not shown). To independently confirm the PAD4-dependent expression of WIN3, we determined the WIN3 transcript level in the constitutive defense mutant *acd6-1* and *acd6-1pad4* plants we constructed previously (Lu et al. 2003). WIN3 transcript levels were ele-

vated in *acd6-1* and highly attenuated in *acd6-1pad4* in two separate experiments (data not shown). These data suggest that PAD4 plays a role in regulating WIN3 transcript levels.

Interestingly, WIN3 transcript levels were induced in systemic tissue after infection in a manner dependent on several signaling components known to be important for the regulation, biosynthesis, or response to SA. These include ALD1, NPR1, NDR1, PAD4, SID1, and SID2 (Fig. 8). In contrast, DTH9, which does not involve SA responses but is important for SAR (Mayda et al. 2000), was dispensable for induction of WIN3 in systemic tissue (Fig. 8).

DISCUSSION

WIN3 joins a small number of known proteins that play key roles in the resistance pathway that is triggered in response to the *P. syringae* effector AvrRpt2. A summary of how WIN3 fits into the *Arabidopsis* response pathway to AvrRpt2 is shown in Figure 9. We found WIN3 to act downstream of RIN4 cleavage but upstream of the hypersensitive response and early SA accumulation after infection. The requirement for WIN3 for the AvrRpt2-induced defense response extends to the induction of SAR, which is severely compromised. However, WIN3 appears to be largely dispensable for local plant responses to an SA agonist or a number of other Avr effectors. PAD4, known to contribute quantitatively to the induction of resistance during infection with *P. syringae* carrying *avrRpt2* (Feys et al. 2001), is partially required for local induction of WIN3. Finally, systemic accumulation of WIN3 transcripts after a local infection requires several genes important for SA accumulation or perception. This suggests that SA plays an important role (directly or indirectly) in regulating WIN3 during SAR, but not in the local infection zone.

WIN3 is 1 of 19 proteins that compose the *Arabidopsis* firefly luciferase superfamily (also called the GH3 family), of which some members have enzymatic activities that modify plant hormones (Staswick et al. 2002). Notably, the defense component JAR1 is a member of this family and has jasmonic acid-amino synthetase activity (Staswick and Tiriyaki 2004). No specific enzymatic activity has been reported for WIN3,

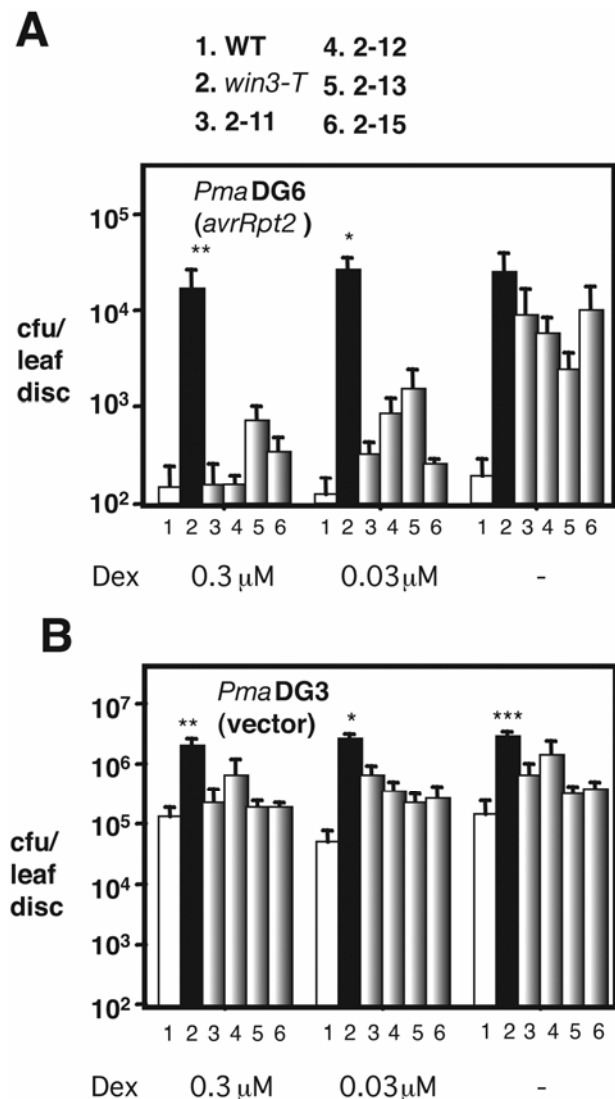


Fig. 4. Dexamethasone (dex)-induced WIN3 rescues the loss of response of *win3-T* to AvrRpt2. Plants were grown in a light-and-dark cycle of 16 and 8 h, respectively, at 22°C. **A**, Growth of *PmaES4326* carrying *avrRpt2* (*PmaDG6*) with an optical density at 600 nm (OD_{600}) = 0.0001 on the indicated plant lines (including four independent transgenic lines with dex-inducible WIN3 called 2-11, 2-12, 2-13, and 2-15) co-inoculated with 0.3 and 0.03 μ M dex or without dex treatment. Bacterial growth ($n = 6$) was measured 3 days after inoculation of all plants. Growth of bacteria in *win3-T* was higher than in the wild type and transgenic lines (*, $P < 0.03$ for 0.03 μ M dex and **, $P \leq 0.092$ for 0.3 μ M dex). **B**, Growth of *PmaES4326* carrying vector (*PmaDG3*) with $OD_{600} = 0.0001$ on indicated lines co-inoculated with 0.3 and 0.03 μ M dex or without dex treatment. Bacterial growth was measured in same manner as described in A. Growth of bacteria in *win3-T* was higher than in the wild type and transgenic lines (*, $P \leq 0.05$ for 0.03 μ M dex; **, $P < 0.002$ for 0.3 μ M dex; and ***, $P < 0.01$, except for line 2-12, for which $P = 0.24$). Bars represent standard error.

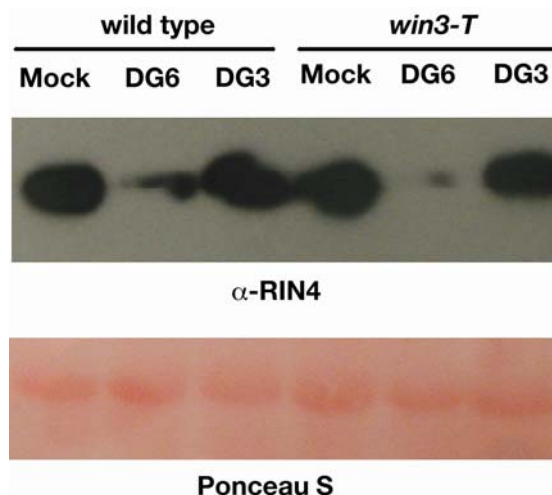


Fig. 5. AvrRpt2 cleaves RIN4 independent of the WIN3. Top: RIN4 immunoblots of total proteins derived from wild type and *win3-T* 20 h after mock treatment (10 mM $MgSO_4$) or inoculation at an optical density at 600 nm = 0.01 with *PmaES4326* carrying either AvrRpt2 (*PmaDG6*) or vector (*PmaDG3*). Bottom: total protein stained with Ponceau S to show equal loading. Each sample contained 10 μ g of total protein. Plants were grown in a light-and-dark cycle of 16 and 8 h, respectively, at 22°C. This experiment was repeated twice with similar results.

although it seems likely that WIN3 functions to modify a plant defense signal by an adenylating reaction similar to that proposed for other family members (Staswick et al. 2002). Possibly, WIN3 directly modifies SA or another signal that affects SA accumulation and the level of SA-derived molecules.

Is WIN3 required for additional defense response pathways? Interestingly, WIN3 also apparently is mutated in the *Arabi-*

dopsis pbs3 mutant (R. Innes, *personal communication*). Like *win3-T*, *pbs3* was largely compromised for resistance induced in response to AvrRpt2 (Warren et al. 1999). However, *win3-T* plants are not compromised for resistance to *P. syringae* carrying *avrB*, *avrRpm1*, or *avrRps4*, as was reported for *pbs3* (Warren et al. 1999). The fact that the original *pbs3* mutant also carried a second mutation in *sid2* could explain the discrepancy, although it is not known whether the *sid2* mutation results in loss of function for SID2 (Roger Innes, *personal communication*). Differences in the *P. syringae* strains used to deliver the *avrB* and *avrRpm1* effectors in the two studies also could account for the different results: herein, strain PmaES4326 was used for most infections whereas, in the *pbs3* study, strain PtoDC3000 was used.

WIN3 does appear to have multiple roles in the plant defense response network, depending on the genetic make up of both the pathogen and the host. There is a modest role for WIN3 in the induction of SAR in response to *P. syringae* carrying *avrRpm1*, although the local resistance response to this strain was robust. We identified WIN3 as a possible protein that interacts with *P. syringae* Avr effector HopW1. WIN3 is important for HopW1-induced resistance and sufficient to confer resistance to virulent *P. syringae* in the Ws accession (M. W. Lee and J. T. Greenberg, *unpublished observations*). However, the HopW1-induced defense pathway differs in some respects from the AvrRpt2-induced defense pathway. HopW1 does not induce a hypersensitive response, whereas AvrRpt2 induces a WIN3-dependent hypersensitive response. Additionally, HopW1-induced responses have a strong requirement for the PAD4 signaling component (M. W. Lee and J. T. Greenberg, *unpublished observations*), whereas AvrRpt2-induced defenses show only a minor requirement for PAD4 (Feys et al. 2001).

Interestingly, loss of WIN3 in the Col accession results in increased susceptibility to virulent *P. syringae*. However, reduced WIN3 levels (using RNAi) in the Ws accession do not result in such increased susceptibility to the virulent *P. syringae*

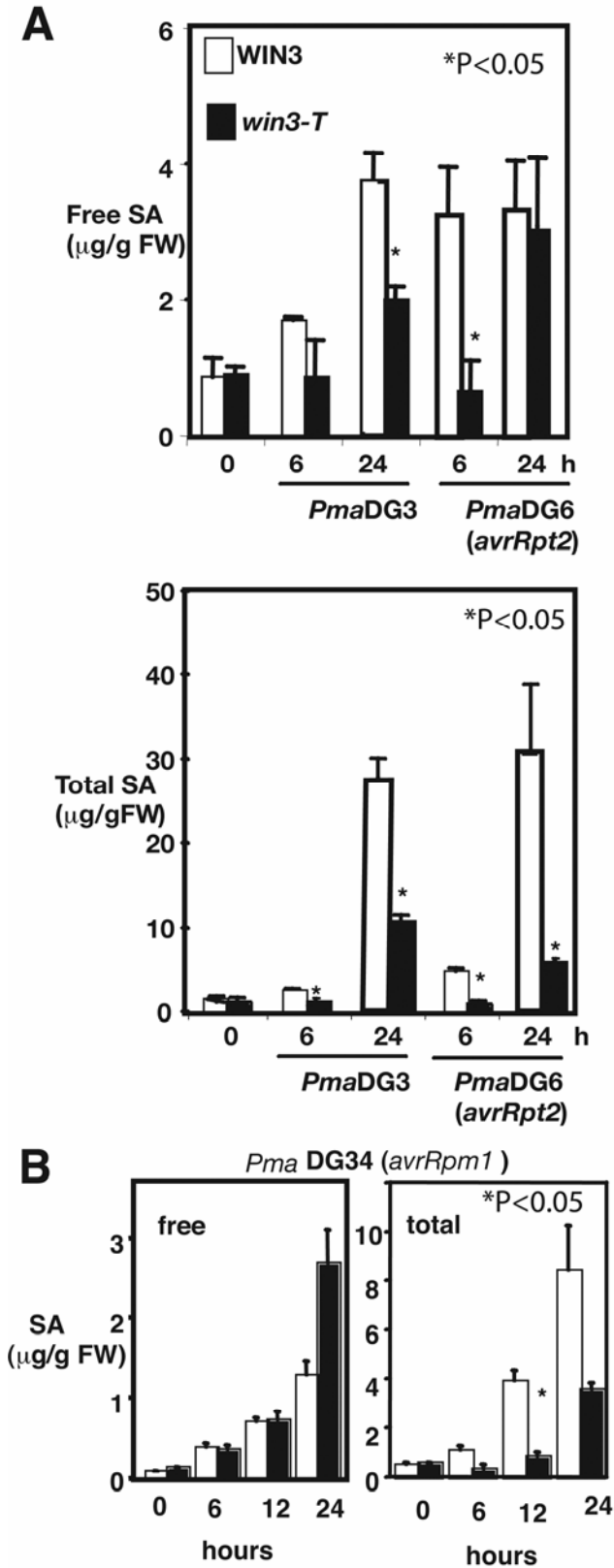


Table 1. Hypersensitive response of *win3-T* mutants is rescued by treatment with BTH^a

Treatment, strain	Number of leaves showing an HR	
	Wild type	<i>win3-T</i>
<i>PmaDG6</i> (<i>avrRpt2</i>)	10/10 (all strong)	0/10
<i>PmaDG3</i> (vector)	0/8	0/8
BTH (10 μM) + <i>PmaDG3</i>	0/12	0/10
BTH (10 μM) + <i>PmaDG6</i>	10/10 (all strong)	7/14 (4 strong, 3 weak)
BTH (100 μM) + <i>PmaDG6</i>	10/10 (all strong)	14/16 (10 strong, 4 weak)

^aWild-type Col and *win3-T* leaves were coinoculated with the indicated concentrations of benzo(1,2,3) thiadiazole-7-carbothioic acid (benzothiazole [BTH]) and *Pseudomonas syringae* at an optical density at 600 nm = 0.05. At 17 h after inoculation, leaves showing macroscopic collapse were scored as undergoing a hypersensitive response (HR). Numbers indicate leaves that showed an HR out of the total number tested. A replicated experiment gave similar results.

Fig. 6. *win3-T* plants are compromised for salicylic acid (SA) accumulation during infection. Free and sugar-conjugated SA levels were measured in 20-day-old leaves at the indicated times after inoculation with bacteria at an optical density at 600 nm = 0.01. Plants were grown in a light-and-dark cycle of 16 and 8 h, respectively. **A**, Inoculation with PmaES4326 carrying the vector (*PmaDG3*) or *avrRpt2* (*PmaDG6*) **B**, Inoculation with PmaES4326 carrying *avrRpm1* (*PmaDG34*). SA measurements for the *PmaDG34* infections were done at a different time than the other infections; therefore, the data should not be directly compared. Bars represent standard deviation (*n* = 2). This experiment was repeated three times with similar results. An asterisk (*) indicates that the SA level was reduced relative to the wild type (*P* < 0.05).

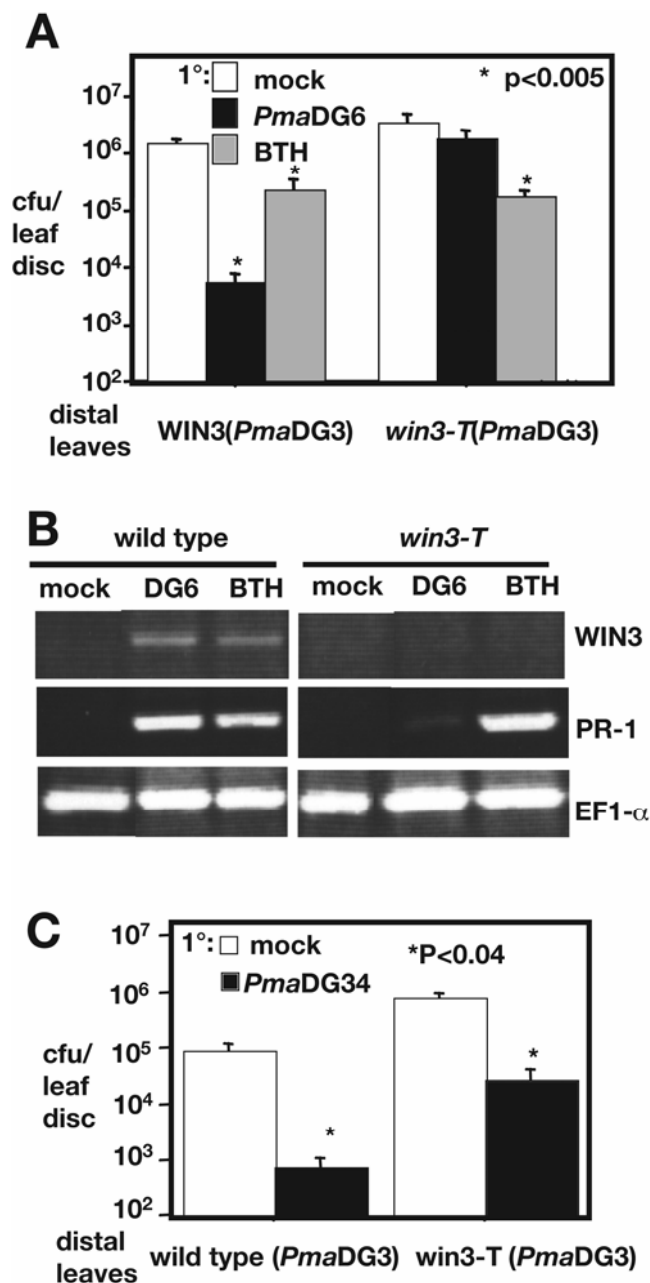


Fig. 7. Requirement for WIN3 in the induction of systemic resistance in response to *AvrRpt2*. Plants were grown in a 12-h light-and-dark cycle at 22°C. **A**, Leaves 3 and 4 of 24-day-old wild-type and *win3-T* plants (labeled 1°) were inoculated with 10 mM MgSO₄ (mock), optical density at 600 nm (OD₆₀₀) = 0.01 of strain *PmaES4326* carrying *avrRpt2* (*PmaDG6*) or benzo(1,2,3) thiadiazole-7-carbothioic acid (benzothiazole [BTH]) (100 μM) 3 days prior to infection of upper leaves (fifth and sixth, labeled “distal”) with *PmaES4326* carrying vector (DG3) at OD₆₀₀ = 0.0001. Bacterial growth was measured 3 days after infection. An asterisk (*) indicates that the growth was reduced relative to the mock treatment ($P < 0.005$, *t* test). Bars represent standard error. *win3-T* plants were compromised for systemic acquired resistance (SAR) induced by *PmaES4326* carrying *avrRpt2*. **B**, WIN3 and PR1 expression in uninfected leaves in the wild type and *win3-T* mutants. In all, 27 cycles of reverse-transcriptase polymerase chain reaction was performed in RNA extracted from uninfected upper leaves three days after primary inoculation of *PmaES4326* carrying *avrRpt2* (DG6) at OD₆₀₀ = 0.01 and BTH (100 μM). These experiments were repeated three times with similar results. **C**, SAR was assayed as in A, except that the primary infection was with *PmaES4326* carrying *avrRpm1* (*PmaDG34*). Wild-type and *win3-T* plants showed SAR after primary inoculation with *PmaES4326* carrying *avrRpm1*, albeit the magnitude of the response of *win3-T* plants was reduced. An asterisk (*) indicates that the growth was reduced relative to the mock treatment ($P < 0.04$, *t* test). This experiment was repeated twice with similar results.

PtoDC3000 (M. W. Lee and J. T. Greenberg, unpublished observations). These differences could be due to incomplete RNAi-mediated silencing of WIN3 in the Ws background. However, it is possible that basal resistance (composed of responses to pathogen-associated molecular patterns [PAMPs] and weak Avr effectors) differs in Col and Ws. This is plausible for two reasons. First, Ws lacks a function receptor for flagellin, a key *P. syringae* PAMP (Zipfel et al. 2004). Second, *PtoDC3000* may possess quantitative (weak) Avr effectors that are differentially recognized by Col but not Ws. WIN3 may be important for a weakly triggered defense response to virulent *P. syringae* in Col but not Ws. A similar scenario was found for the *P. syringae* *PsyB728a*, which has several quantitative Avr effectors that are differentially recognized by different plant species (Vinatzer et al. 2006).

MATERIALS AND METHODS

Plant material.

All *A. thaliana* plant material was in the Col background. *ald1-T*, *acd6-1*, *acd6-1pad4*, *dth9*, *pad4-1*, *ndr1-1*, *npr1-1*, *sid1-1*, and *sid2-1* were described previously (Cao et al. 1994; Glazebrook et al. 1996; Mayda et al. 2000; Nawrath and Métraux 1999; Rate et al. 1999; Song et al. 2004a,b). The

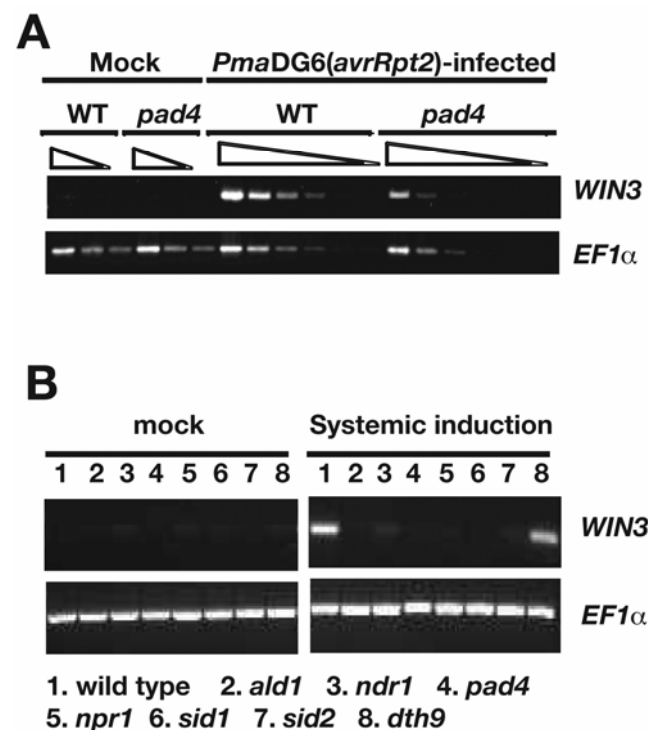


Fig. 8. A requirement for several defense components for WIN3 expression during *Pseudomonas syringae* infection. Plants were grown in a 12-h light-and-dark cycle at 22°C. Three-week-old plants of different genotypes were inoculated with *PmaES4326* carrying *avrRpt2* (*PmaDG6*) at optical density at 600 nm = 0.01 or mock inoculated with 10 mM MgSO₄. Local (infected) and systemic (uninfected leaves from infected plants) samples were taken 14 h and 2 days after infection, respectively. Twenty-five cycles of reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to monitor the induction of WIN3 from these samples. As a control, 21 cycles of RT-PCR was performed to monitor the mRNA levels of the control gene *EF1α*. **A**, Dilution series (twofold for each lane) of local samples showing that *pad4* mutant plants had reduced WIN3 mRNA levels after infection. Other mutants responded similarly to the wild type (not shown). This result was found in three of four independent trials (see text also). **B**, Many salicylic acid-related defense mutants showed reduced WIN3 mRNA levels in systemic samples. This experiment was repeated three times with similar results.

win3-T mutant harboring a T-DNA in WIN3 (SALK_018225) in Col was obtained from the Salk collection.

Identification of *win3-T* plants.

The *win3-T* plants were identified using polymerase chain reaction (PCR). The primers from the WIN3 gene (5' CCGTTGTCAGTGGTTCATGGGACAGT 3' and 5' ACTGAGGCGCGTTGTTGTAGAAACCAG 3') and a primer from the T-DNA insertion in *win3-T* (5' ATGGTTCACGTAGTGGGCCATCG 3') were used to identify plants carrying the *win3-T* mutation.

Complementation experiments.

We used recombinational cloning to generate a dex-inducible complementation clone of WIN3. A PCR product containing the cDNA (no introns) for the coding region of the WIN3 protein was used to generate an entry clone in vector DONR207 that was further used to generate an expression clone in pBAV154 (Vinatzer et al. 2006). Primers MWL3-F (5' GGGGACAAGTTTGTACAAAAAGCAGGCTTCGAAGGA GATAGAACC ATGAAGCCAATCTTCGATATCAACGAA 3') and MWL3-R (5' GGGGACCACTTTGTACAAGAAAGCTGG GTCAATACTGAAGAATTGGCTAC 3') were used to generate the entry clone. Underlined sequences indicate the recombinational (gateway) cloning site.

Transgenic plants were generated by vacuum infiltrating *Agrobacterium tumefaciens* GV3101 carrying WIN3 into flowering *Arabidopsis* (Clough and Bent 1998). Transgenic progeny were selected by spraying with BASTA solution (Crescent Biochemical Company) at 250 µg/ml. Four independent T₂ lines containing dex-inducible WIN3 were used for the complementation assays. Complementation lines were confirmed using PCR assays diagnostic for the *win3-T* mutation and the dex:WIN3 transgene. To verify the presence of the *win3-T* mutation, we amplified a 750-bp DNA fragment using primer WIN3(1100) (5' CCGTTGTCAGTGGTTCATGGGACAGT 3') in exon 3 and primer Lba1 (5' ATGGTTCACGTAGTGGG

CCATCG 3') in the T-DNA. To verify the presence of the dex:WIN3 transgene, we amplified a 306-bp DNA fragment using the WIN3(1100) primer and primer WIN3(1400) (5' AC-TGAGGCGCGTTGTTGTAGAAACCAG 3') in exon 4.

P. syringae strains and plasmids.

P. syringae pv. *tomato* DC3000 and derivatives of strain *P. syringae* pv. *maculicola* ES4326 were used for this study. Strains *PmaDG6* (*PmaES4326* harboring *avrRpt2* integrated at the *recA* locus), *PmaDG34* (*PmaES4326* harboring *avrRpm1* integrated at the *recA* locus), and *PmaDG3* (*PmaES4326* harboring a vector control integrated at the *recA* locus) were described previously (Guttman and Greenberg 2001). *PmaES4326* carrying *avrB* was constructed by transferring plasmid pVSP61 containing the *avrB* gene into *PmaES4326* by triparental mating. Strain *PtoDC3000* carrying *avrRps4* (plasmid pV316-1A) or *hopA1* (formerly called *hopPsyA*, on plasmid pLN92) from W. Gassmann (University of Missouri) were described previously (Gassmann 2005).

Pseudomonas infections and measurement of bacterial growth in leaves.

P. syringae strains grown overnight in King's B (KB) liquid medium (King et al. 1954) with appropriate antibiotics were diluted and regrown to an optical density at 600 nm (OD₆₀₀) = 0.7 to 1.0. Cultures (2 ml) were collected and resuspended in 10 mM MgSO₄ to dilution for plant inoculations. For in vivo growth assays, bacteria were diluted to OD₆₀₀ = 0.0001. Bacterial inoculations were performed with a blunt 1-ml syringe. After inoculation, plants were covered with a dome and disease symptoms were observed each day. Six independent samples were used for the *P. syringae* growth curves. Leaf punches (6 mm in diameter) were surface sterilized in 70% ethanol for 10 s, homogenized in 200 µl of 10 mM MgSO₄ with a power drill (Skil, Chicago), diluted in 10 mM MgSO₄, and plated on KB agar plates containing appropriate antibiotics. For the hy-

Summary of WIN3's Regulation and Role in AvrRpt2-Triggered Defense

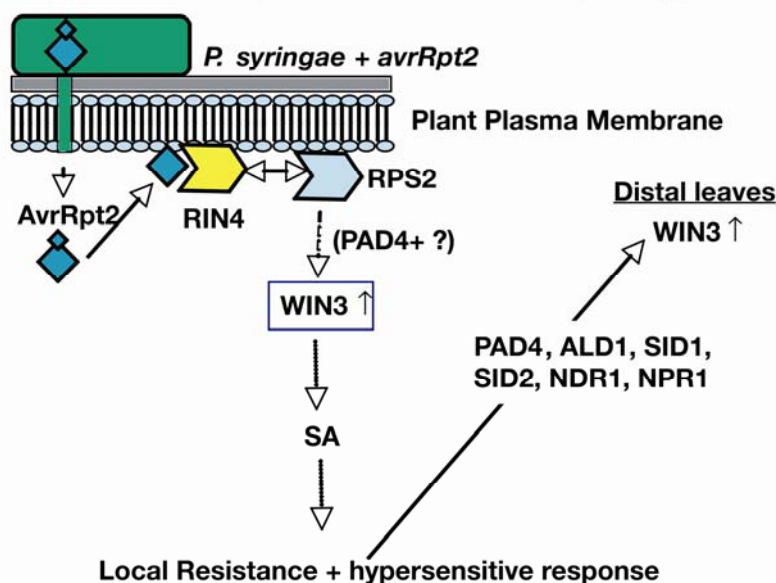


Fig. 9. Summary of the role and regulation of WIN3 in response to AvrRpt2. *Pseudomonas syringae* injects AvrRpt2 in a pro form, which is activated to fold and self-proteolyze inside plant cells. Processed AvrRpt2 localizes to the membrane, where it cleaves RIN4, an event that is highly correlated with activation of RPS2 signaling. WIN3 is upregulated in response to infection, a process that partially requires PAD4 in local tissue and multiple defense components for systemic expression. Our data cannot distinguish between multiple defense components (i) acting in local tissue to transduce a systemic signal and (ii) acting in the distal tissue in response to a systemic signal to regulate WIN3 expression in distal leaves. Whether upregulation of WIN3 is specifically necessary for the RPS2 response pathway is not yet known. WIN3 is important for the rapid accumulation of salicylic acid, a necessary signal molecule in the RPS2 pathway. Not all aspects of the RPS2 signaling pathway are shown; see text for a discussion of additional components.

persensitive response assay, bacteria culture was diluted to $OD_{600} = 0.05$ and inoculated into leaves. Inoculated, uncovered plants were scored for hypersensitive response (cell death) symptoms after 17 h.

Except for SAR experiments, 18- to 20-day-old plants grown in a light-and-dark cycle of 16 and 8 h, respectively, at 22°C were used. For the SAR experiments, wild-type (Col) and *win3-T* plants were grown in a 12-h light-and-dark cycle at 22°C. The third and fourth leaves of 24- to 26-day-old plants were inoculated with strain *PmaDG6* (carrying *avrRpt2*) or *PmaDG34* (carrying *avrRpm1*) at $OD_{600} = 0.01$. Three days later, leaves five and six were inoculated with virulent strain *PmaDG3* to assess the effectiveness of SAR. For BTH treatment, plants were injected with a 1-ml blunt syringe of BTH, a kind gift from Robert Dietrich, Syngenta) at 10 or 100 μ M.

Reverse-transcriptase PCR analysis.

Total RNA from leaves was extracted using the Trizol reagent method according to the manufacturer's procedure (Invitrogen). First-strand cDNA was synthesized from 500 ng of total RNA using superscriptII reverse transcriptase (Invitrogen). Reverse-transcriptase PCR was performed as described by Burton and associates (2000). To monitor *WIN3* transcript levels, we used primers for exon 1 *WIN3*(10) (5' ATCTTCGAT ATCAACGAACTTTT 3') and *WIN3*(350) (5' TTTCACATG CTTGGTTATAACTTGC 3') or exon 4 *WIN3*(CF) (5' CGAGT CATCAGGTCTCGATCTGA 3') and *WIN3*(CR) (5' TGACTT AATGCATCTAGGAGTCTTG 3'). To monitor *EF1 α* expression, we used primers *EF1 α* (F) (5' GCTGTCCTTATCATTGA CTCCACC 3') and *EF1 α* (C) (5' TCATACCAGTCTCAACAC GTCC 3'). To monitor *PAD4* expression, we used primers *PAD4-F* (5' GGACTAGTGGCGCGCCAAACATCAG AGGTAC 3') and *PAD4-R* (5' CGGGATCCATTAAATCACTCCTCAG GCACTT 3'). To monitor *PR1* expression, we used primers *PR1*(CF) (5' CACATAATCCCCACGAGGATC 3') and *PR1*(CR) (5' GTAGGTGCTCTTGTCTTCCC 3').

Northern blot analysis.

Leaves from 20-day-old plants grown at 22°C with 16 h of light and 8 h of dark were harvested for RNA extraction and RNA blot analysis according to Lu and associates (2003). RNA blots were hybridized with the [α - 32 P]dCTP-labeled 3' end fragment (1,350 to 1,650 bp) for *WIN3*.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting.

To get total protein extracts, *Arabidopsis* leaves were homogenized in liquid nitrogen, resuspended in one volume of extraction buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% TritonX-100; 0.1% sodium dodecyl sulfate [SDS]; 5 mM dithiothreitol; and 1 \times proteinase inhibitor) and centrifuged at 10,000 \times g for 10 min at 4°C. Approximately 10- μ g protein samples in sample buffer (0.0625 M Tris, pH 6.8; 10% glycerol; 2% SDS; 2.25 M urea; 100 mM dithiothreitol; and 0.04% bromophenol blue) were separated on 12% SDS-polyacrylamide gels using Tris-glycine electrophoresis buffer as described (Sambrook et al. 1989). Western blots were performed using standard methods and detected with ECL (Pierce, Rockford, IL, U.S.A.). To detect RIN4, we used a primary polyclonal RIN4 antibody (a kind gift from Jeff Dangl, University of North Carolina) at 1:5,000 dilution and the secondary ImmunoPure Antibody rabbit immunoglobulin G (H+L) (product number 31458; Pierce) at 1:15,000 dilution.

SA measurements.

Free and total SA was extracted and quantified as described previously (Seskar et al. 1998).

NOTE ADDED IN PROOF

While this work was under review, two papers also reported that *WIN3* (also called *PBS3* and *GDG3*) plays an important role in regulating SA production.

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signal.salk.edu/cgi-bin/tdnaexpress