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Multiple Roles of WIN3 in Regulating Disease Resistance, Cell Death, and Flowering Time in Arabidopsis^{1[C][W][OA]}

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The salicylic acid (SA) regulatory gene *HOPW1-1-INTERACTING3* (*WIN3*) was previously shown to confer resistance to the biotrophic pathogen *Pseudomonas syringae*. Here, we report that *WIN3* controls broad-spectrum disease resistance to the necrotrophic pathogen *Botrytis cinerea* and contributes to basal defense induced by flg22, a 22-amino acid peptide derived from the conserved region of bacterial flagellin proteins. Genetic analysis indicates that *WIN3* acts additively with several known SA regulators, including *PHYTOALEXIN DEFICIENT4*, *NONEXPRESSOR OF PR GENES1* (*NPR1*), and *SA INDUCTION-DEFICIENT2*, in regulating SA accumulation, cell death, and/or disease resistance in the Arabidopsis (*Arabidopsis thaliana*) mutant *acd6-1*. Interestingly, expression of *WIN3* is also dependent on these SA regulators and can be activated by cell death, suggesting that *WIN3*-mediated signaling is interconnected with those derived from other SA regulators and cell death. Surprisingly, we found that *WIN3* and *NPR1* synergistically affect flowering time via influencing the expression of flowering regulatory genes *FLOWERING LOCUS C* and *FLOWERING LOCUS T*. Taken together, our data reveal that *WIN3* represents a novel node in the SA signaling networks to regulate plant defense and flowering time. They also highlight that plant innate immunity and development are closely connected processes, precise regulation of which should be important for the fitness of plants.

Successful defense against pathogens is crucial for plant growth and development. Plants have evolved sophisticated defense mechanisms against pathogen attacks. In addition to preformed physical and chemical barriers, plants have basal defense, triggered by perception of pathogen-associated molecular patterns, which are conserved molecules derived from many pathogens. More specific and robust defense responses can be induced when plant resistance proteins recognize their cognate pathogen effector proteins (Zipfel and Felix, 2005; Chisholm et al., 2006; Jones and Dangl, 2006). Such recognitions can subsequently lead to systemic acquired resistance, a form of long-lasting resistance to broad-spectrum pathogens at the

whole plant level (Ryals et al., 1996; Durrant and Dong, 2004).

During different periods of plant defense, the small molecule salicylic acid (SA) plays a central role in activating defense signaling (Hammond-Kosack and Jones, 1996; Ryals et al., 1996; Tsuda et al., 2008). Genes regulating SA signaling networks can be grouped into three types, which affect three intricately interconnected subcircuits of the networks (Lu, 2009). The type I SA genes are directly involved in SA biosynthesis, including *SA INDUCTION-DEFICIENT2/ENHANCED DISEASE SUSCEPTIBILITY16* (*SID2/EDS16*), which encodes isochorismate synthase for the synthesis of the majority of SA (Wildermuth et al., 2001) and genes in *SID2*-independent pathways for the minor production of SA (Chen et al., 2009; Lu et al., 2009). The type II SA genes are not directly involved in SA synthesis but influence SA levels through mechanisms that are not yet well understood. Examples of such SA regulators include *ACCELERATED CELL DEATH6* (*ACD6*), *AGD2-LIKE DEFENSE1*, *EDS1*, *PHYTOALEXIN DEFICIENT4* (*PAD4*), *SID1/EDS5*, *HOPW1-1-INTERACTING3* (*WIN3*)/*AVRPPHB SUSCEPTIBLE3* (*PBS3*)/*GH3-LIKE DEFENSE GENE1* (*GDG1*), and the *MODIFIER OF SNC1* genes (Falk et al., 1999; Jirage et al., 1999; Nawrath et al., 2002; Lu et al., 2003; Song et al., 2004; Palma et al., 2005, 2007; Zhang et al., 2005; Zhang and Li, 2005; Goritschnig et al., 2007; Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007). The type III SA genes act downstream of SA signaling, among which *NONEXPRESSOR OF PR GENES1* (*NPR1*) is the best-characterized SA signal transducer. The *NPR1* protein translocates from the

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cytoplasm to the nucleus in response to the change of cellular redox and influences defense gene expression by interacting with transcription factors (Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997; Fan and Dong, 2002; Mou et al., 2003; Dong, 2004). Besides being the key defense signaling molecule, accumulating evidence indicates that SA regulates plant development, such as flowering time (Cleland and Tanaka, 1979; Cleland and Ben-Tal, 1982; Martínez et al., 2004; Endo et al., 2009; Wada et al., 2009). Consistent with this role of SA, light and phytochrome signaling, which are among the main factors contributing to the determination of flowering time, were shown to be part of the defense signaling networks (Genoud et al., 2002; Griebel and Zeier, 2008). Hence, studies from different fields of plant research clearly indicate an intimate relationship between plant development and innate immunity. However, our understanding of how these two processes are linked mechanistically is still in its infancy.

While little is known about the mechanisms by which many SA regulators act, information on how they interact to form distinct pathways within the networks is even scarcer. Biochemical and microarray approaches have proven useful to resolve some of the complexity of the defense networks (Feys et al., 2001; Bartsch et al., 2006; Wang et al., 2008). Genetic analysis based on the unique *Arabidopsis* (*Arabidopsis thaliana*) mutant, *acd6-1*, has also provided novel insights on the interactions among some SA regulators (Song et al., 2004; Lu et al., 2009). ACD6, a type II SA regulator with an ankyrin-repeat motif and a transmembrane domain, was shown to be a major determinant of fitness in *Arabidopsis* (Todesco et al., 2010). The *acd6-1* mutant displays constitutive defense, severe cell death, and extreme dwarfism (Rate et al., 1999; Vanacker et al., 2001; Lu et al., 2003). Interestingly, the small size of *acd6-1* is sensitive to the change of defense levels in the plant (Lu et al., 2009). Such a feature of *acd6-1* makes it a useful tool to gauge the change of defense levels due to genetic interactions among some defense regulators.

The type II SA regulator WIN3 is one of the 19 members of the firefly luciferase family (Staswick et al., 2005). WIN3, also named PBS3, GDG1, and GH3.12, was previously shown to act upstream of SA signaling and to regulate resistance to the biotrophic pathogen *Pseudomonas syringae* (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007). The WIN3 protein conjugates amino acids to 4-substituted benzoates (Okrent et al., 2009), which can be used as precursors for SA biosynthesis (Chen et al., 2009). In this report, we show that WIN3 regulates broad-spectrum disease resistance to both biotrophic and necrotrophic pathogens. Our genetic analysis indicates that WIN3 acts independently of PAD4 and NPR1 in regulating SA accumulation, disease resistance, and cell death in *acd6-1*. WIN3 also contributes to both SID2-dependent and -independent SA biosynthesis. Surprisingly, we found that WIN3 and NPR1 act synergistically in regulating flowering time, highlight-

ing the interconnectedness of plant innate immunity and development.

RESULTS

WIN3 Contributes to Flg22-Induced Defense

WIN3 was previously shown to regulate basal defense against virulent *P. syringae* strains in *Arabidopsis* (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007). To further investigate this role of WIN3, we pretreated *win3-1* and wild-type control (Columbia [Col-0]) plants with a well-characterized elicitor of basal defense, flg22, a 22-amino acid peptide derived from the conserved region of bacterial flagellin proteins (Felix et al., 1999). Twenty-four hours after flg22 or water pretreatment, we infected the plants by infiltration with the virulent strain *P. syringae* pv *maculicola* ES4326 DG3 (*Pma*DG3; optical density at 600 nm [OD₆₀₀] = 0.0001). Compared with Col-0, *win3-1* showed more bacterial growth and severe disease symptoms in both flg22 and water pretreatments (Fig. 1; Lee et al., 2007). Flg22 pretreatment induced disease resistance in both Col-0 and *win3-1*. Compared with Col-0, which had 62-fold reduction of bacterial growth in flg22- versus water-pretreated samples, *win3-1* only had 14-fold reduction of bacterial growth in flg22- versus water-pretreated samples (Fig. 1A). These data suggest that *win3-1* is less responsive to flg22 pretreatment. Consistent with these observations, we found that the *win3-1* seedlings were also less responsive to the inhibition of flg22 on root growth (Supplemental Fig. S1). Together, our data indicate a partial contribution of WIN3 in regulating flg22-induced defense.

WIN3 Regulates Disease Resistance against *Botrytis cinerea*

WIN3 is known to regulate resistance to the biotrophic pathogen *P. syringae* (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007). To further investigate the breadth of disease resistance conferred by WIN3, we infected *win3-1* and Col-0 plants by spraying with *Botrytis cinerea*, a necrotrophic fungal pathogen. Compared with Col-0, *win3-1* exhibited more severe necrotic lesions on the leaves 2 d after the spray with *Botrytis* spores (10⁵ spores mL⁻¹; Fig. 2A). Disease rating indicated that *Botrytis* susceptibility conferred by *win3-1* was comparable to two known *Botrytis*-susceptible mutants, *jasmonic acid response1-1* (*jar1-1*) and *ethylene insensitive2-1* (Ferrari et al., 2003; Genger et al., 2008; Fig. 2B). It is worth noting that both WIN3 and JAR1 are members of the firefly luciferase family (Staswick et al., 2005). *Botrytis* susceptibility conferred by *win3-1* was further confirmed with a second WIN3 allele, *pbs3-1* (Supplemental Fig. S2).

In contrast to *win3-1*, *acd6-1*, a gain-of-function mutant with constitutive defense to *P. syringae* strains (Rate et al., 1999; Lu et al., 2003), displayed hyper-resistance to *Botrytis* (Fig. 2, A and B). *win3-1* partially

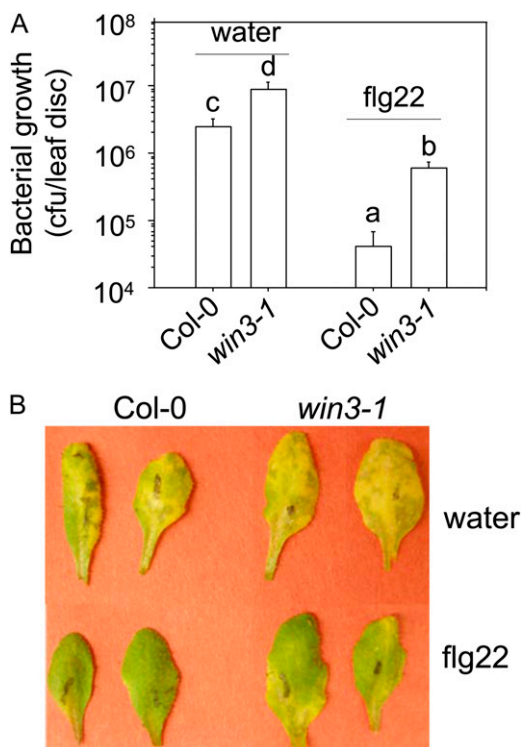


Figure 1. WIN3 contributes to flg22-induced resistance to *Pseudomonas* infection. A, Bacterial growth assay. Twenty-five-day-old plants grown in 12-h-light/12-h-dark conditions were pretreated with 1 μ M flg22 or water for 24 h before infection with *Pma*DG3 (OD₆₀₀ = 0.0001). Bacterial growth was assessed 3 d after infection. Data represent average bacterial numbers in six samples \pm SE. Statistical analysis was performed with Student's *t* test (StatView 5.0.1). Letters indicate significant differences among the samples (*P* < 0.05). B, Disease symptoms. Infected leaves from plants in A were photographed 4 d after infection. These experiments were repeated twice with similar results.

suppressed *Botrytis* resistance in *acd6-1*. Camalexin is a phytoalexin that was previously shown to be important to *Botrytis* resistance (Ferrari et al., 2003; Stefanato et al., 2009). However, we did not observe any significant difference in the levels of camalexin in *acd6-1win3-1* ($7.1 \pm 0.1 \mu\text{g g}^{-1}$ fresh weight) and *acd6-1* ($4.4 \pm 1.7 \mu\text{g g}^{-1}$ fresh weight). Further analyzing camalexin levels in a 48-h time course after *Botrytis* infection revealed no difference in the amount of camalexin and the kinetics of camalexin accumulation in Col-0 and *win3-1* (Fig. 2C). These results suggest that WIN3-mediated resistance to *Botrytis* is camalexin independent.

WIN3 and Several SA Regulators Act Together to Affect *acd6-1*-Conferred Phenotypes

We previously used *acd6-1* as a genetic tool to understand the functional relationships between several SA regulators (Song et al., 2004; Lu et al., 2009). To elucidate the genetic interaction between WIN3 and other SA regulators, we crossed *win3-1* to several SA

mutants, including *pad4-1*, *npr1-1*, and *sid2-1*, in the *acd6-1* background and assessed plant size and defense phenotypes. Like these other SA mutants, we found that *win3-1* suppressed small size, high SA accumulation, and constitutive defense in *acd6-1* (Figs. 2–4). In addition, the triple mutants, *acd6-1win3-1pad4-1*, *acd6-1win3-1npr1-1*, and *acd6-1win3-1sid2-1*, were significantly larger than their respective double mutants (Fig. 3, A and B). Consistent with the change of plant size, free and glucosyl-conjugated SA (total SA) levels were drastically reduced in *acd6-1win3-1pad4-1* and *acd6-1win3-1npr1-1* compared with the respective double mutants (Fig. 3C). We also observed a small but significant decrease of total SA level in *acd6-1win3-1sid2-1* compared with *acd6-1win3-1* and *acd6-1sid2-1* (Fig. 3C, inset in top panel).

To see if changes in plant size and/or SA levels are correlated with the change in disease resistance, we infected these plants with *Pma*DG3. The triple mutants showed a similar susceptibility to *win3-1* but were significantly more susceptible than their respective double mutants, suggesting that *win3-1* and these SA

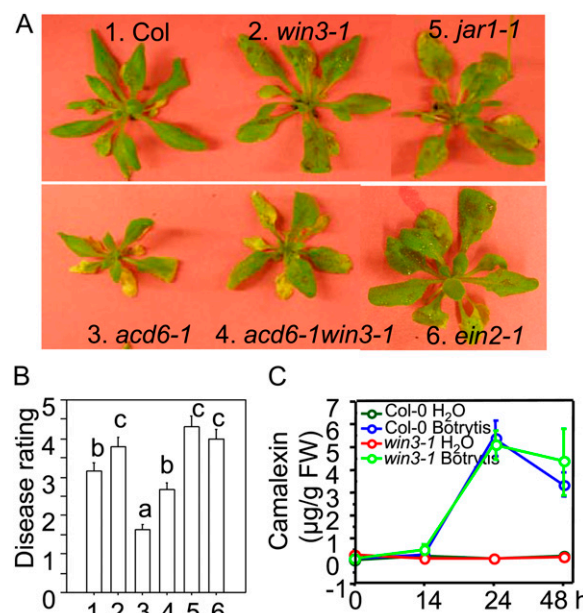


Figure 2. WIN3 regulates resistance to *B. cinerea*. A, Disease symptoms. Twenty-five-day-old plants grown in 12-h-light/12-h-dark conditions were sprayed with *Botrytis* spore suspension (2×10^5 spores mL⁻¹) and photographed for disease symptoms 2 d after inoculation. B, Disease rating. Disease symptoms were rated 2 d after inoculation according to the previously described scale (0 = no disease to 6 = extensive disease; Genger et al., 2008). Data represent means of the ratings (*n* > 12 plants per genotype) \pm SE. Statistical analysis was performed with Student's *t* test (StatView 5.0.1). Letters indicate significant differences among the samples (*P* < 0.05). The key for the genotypes is the same as shown in A. C, Camalexin accumulation. Camalexin was extracted from the infected plants at the indicated time points and quantified by HPLC. The experiments in A and B were repeated four times and that in C was repeated two times, and similar results were obtained. FW, Fresh weight.

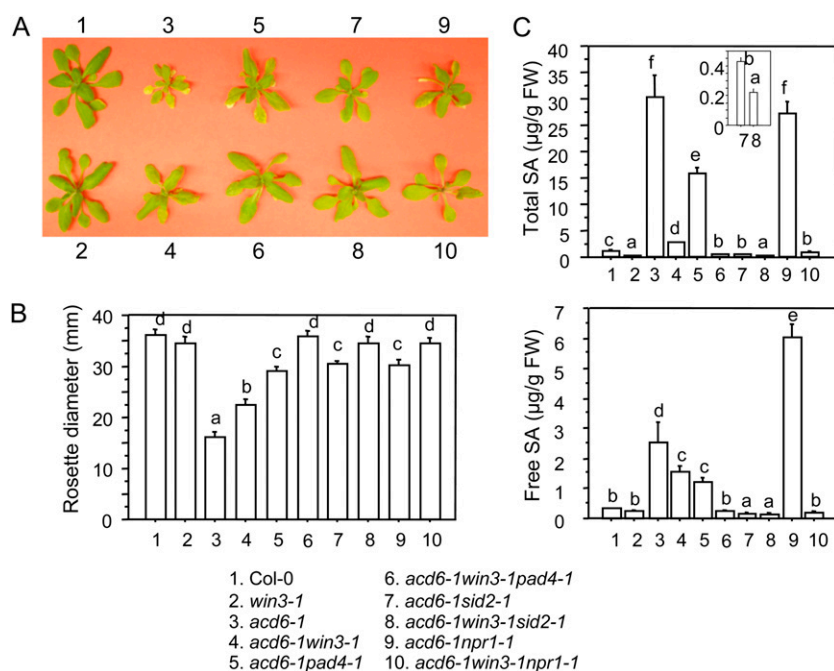


Figure 3. *win3-1* suppresses dwarfism and SA accumulation in *acd6-1* independently of *pad4-1*, *sid2-1*, or *npr1-1*. A, Twenty-five-day-old plants grown in 16-h-light/8-h-dark conditions. B, Plant size comparison. Plants from A were measured for their rosette diameters ($n > 12$). C, SA quantitation. Total and free SA were extracted from plants shown in A and analyzed by HPLC. The inset in the top panel shows SA values of *acd6-1sid2-1* and *acd6-1win3-1sid2-1* to highlight the difference between the two samples. Statistical analysis was performed with Student's *t* test (StatView 5.0.1). Letters indicate significant differences among the samples ($P < 0.05$). The key for the genotypes of all panels is shown at the bottom of the figure. These experiments were repeated twice with similar results. FW, Fresh weight. [See online article for color version of this figure.]

mutants contribute additively to the suppression of *acd6-1*-conferred disease resistance.

We further examined the expression of defense marker genes in the absence of *P. syringae* infection. We used *PR1* as a marker for SA signaling and *PDF1;2* as a marker for ethylene and jasmonic acid signaling (Glazebrook et al., 1997; Penninckx et al., 1998; Reymond and Farmer, 1998). We found that expression of *PR1* was completely suppressed but that of *PDF1;2* was highly induced in the triple mutants compared with the double and single mutants (Fig. 4B). Such an expression pattern of *PR1* and *PDF1;2* is consistent with the antagonistic effect of SA on ethylene and jasmonic acid in *acd6-1* (Lu et al., 2009), further supporting a role of WIN3 in regulating SA levels. Together, our results from Figures 3 and 4 suggest that WIN3 acts additively with PAD4, NPR1, and SID2 in regulating dwarfism, SA accumulation, and defense responses in *acd6-1*.

WIN3 Modulates Cell Death in *acd6-1*

The SA regulators PAD4, NPR1, and SID2 were shown before to modulate cell death in *acd6-1* (Lu et al., 2009). To see if WIN3 plays a role in regulating cell death, we examined *acd6-1win3-1* and related triple mutants for their cell death phenotype with trypan blue staining. We found that, like *pad4-1*, *npr1-1*, and *sid2-1*, *win3-1* also reduced the severity of cell death, in particular the large patches of cell death in *acd6-1*. In addition, the triple mutants *acd6-1win3-1pad4-1*, *acd6-1win3-1npr1-1*, and *acd6-1win3-1sid2-1* had much reduced cell death, compared with the corresponding double mutants (Fig. 5). These observations suggest that WIN3 acts additively with PAD4, NPR1, and SID2

in controlling cell death. However, residual cell death was still observed in these triple mutants even though their SA levels are less than those in the wild type, corroborating the notion that cell death conferred by *acd6-1* is only partially SA dependent (Lu et al., 2009).

SA Signaling and Cell Death Synergistically Promote the Expression of WIN3

Previous studies showed that expression of WIN3 was inducible with *P. syringae* infection or SA treatment and requires multiple SA components (Jagadeeswaran et al., 2007; Lee et al., 2007). Consistent with these results, we found that WIN3 transcripts were highly accumulated in *acd6-1* (Fig. 4B). The SA mutants *pad4-1*, *npr1-1*, and *sid2-1* partially suppressed the abundance of WIN3 transcripts in *acd6-1*. In addition, we found that the small amount of WIN3 transcripts detected in *acd6-1win3-1* was completely abolished in the triple mutants. These results further support that the full expression of WIN3 requires functional PAD4, NPR1, and SID2.

Interestingly, we noticed that the level of WIN3 transcripts in *acd6-1* was much higher than that in Col-0 induced by treatment with benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH), a SA agonist (Fig. 6). This result led us to speculate that activation of SA signaling alone is not enough to activate the high level of WIN3 expression and that perhaps severe cell death in *acd6-1* also contributes to WIN3 expression. To test this possibility, we used an inducible system, *acd6-1nahG*, to activate SA signaling and cell death. The transgene *nahG*, which encodes a bacterial SA hydroxylase, is known to completely suppress *acd6-1*-conferred phe-

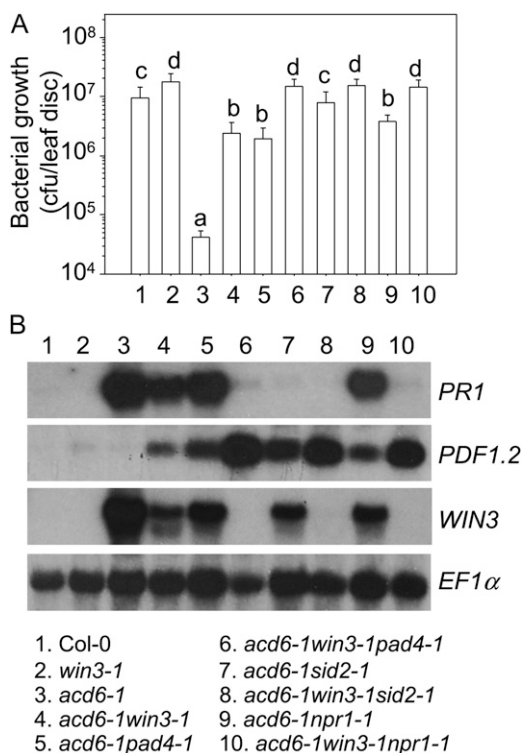


Figure 4. *win3-1* suppresses constitutive defense conferred by *acd6-1* independently of *pad4-1*, *sid2-1*, or *npr1-1*. A, Bacterial growth assay. Twenty-five-day-old plants grown in 12-h-light/12-h-dark conditions were inoculated with *PmaDG3* (OD₆₀₀ = 0.0001), and bacterial growth was assessed 3 d after infection. Data for bacterial growth represent averages of six samples \pm SE. Statistical analysis was performed with Student's *t* test (StatView 5.0.1). Letters indicate significant differences among the samples ($P < 0.05$). The number of bacteria inoculated at day 0 was similar in each genotype (data not shown). B, Expression of defense marker genes. Total RNA was extracted from uninfected 25-d-old plants for northern-blot analysis. Probes specific to *PR1*, *PDF1.2*, and *WIN3* were used to study the expression of these genes. *EF1α* was used as a loading control. The key for the genotypes is shown at the bottom of B. These experiments were repeated twice with similar results.

notypes (Rate et al., 1999; Vanacker et al., 2001). SA signaling and cell death can be reactivated in *acd6-1nahG* with BTH treatment in the presence of light (Vanacker et al., 2001; Lu et al., 2003). Under this condition, we found that the abundance of *WIN3* transcripts was even higher in *acd6-1nahG* than in *acd6-1*. Although they expressed a similar level of *PR1* (a marker for SA signaling) to that in BTH-treated *acd6-1nahG*, BTH-treated Col-0 and *nahG* showed no cell death and expressed only weak or undetectable *WIN3* (Fig. 6). These results clearly indicate that activation of both SA signaling and cell death is necessary for the high expression of the *WIN3* gene. Thus, our data show that while *WIN3* is required for SA-mediated defense and cell death formation, the expression of this gene is likely under a feedback control by these two processes.

WIN3 and NPR1 Additively Regulate Flowering Time in *acd6-1*

One of the noticeable phenotypes of the *win3-1* mutant was early flowering. To further investigate whether *WIN3* plays a role in regulating Arabidopsis flowering, we recorded plant flowering time. In long-day condition (16 h of light/8 h of dark), Col-0 flowered about 22 d after planting and produced an average of 11 leaves at the time of the first appearance of flower buds. The *win3-1* mutant not only flowered earlier (about 17.6 d after planting) but also produced fewer leaves (about 8.5 leaves) at bolting (Fig. 7, A and B; Supplemental Table S1). While *acd6-1* flowered slightly earlier than Col-0 in one of the two experiments that we conducted, *acd6-1win3-1* showed the same early flowering as *win3-1*. Since *acd6-1win3-1* only accumulated 10% of total SA levels seen in *acd6-1* (Fig. 3C), we concluded that *WIN3*-mediated early flowering is largely SA independent.

We also observed an early-flowering phenotype in *npr1-1*. Strikingly, the *acd6-1win3-1npr1-1* triple mutant flowered earliest among all the plants tested (Fig. 7, A and B). These results suggest that both *WIN3* and *NPR1* negatively regulate the transition to flowering, possibly in two independent pathways. Interestingly, *acd6-1npr1-1* flowered about the same time as Col-0, possibly due to the substantial expression of *WIN3* in *acd6-1npr1-1* that can antagonize early flowering conferred by *npr1-1* (Fig. 4B). Unlike *acd6-1npr1-1*, *npr1-1* expressed undetectable *WIN3* (Supplemental Fig. S3). In addition, our analysis of *pad4-1* and *sid2-1* mutants grown in long-day conditions did not reveal any significant difference in the flowering time in the single mutants and the double mutants in the *acd6-1* background compared with Col-0 and *acd6-1* (Supplemental Table S1). However, in the presence of *win3-1*, the triple mutants *acd6-1win3-1sid2-1* and *acd6-1win3-1pad4-1* showed accelerated flowering, further supporting a role of *win3-1* in regulating floral transition.

In short-day conditions (8 h of light/16 h of dark), the *win3-1* and *npr1-1* mutants had overall delayed flowering with a largely similar pattern as seen in long-day conditions, with *acd6-1win3-1npr1-1* flowering the earliest (Table I). These results suggest a normal response to the change of light period in these plants. The fact that the single mutants *win3-1* and *acd6-1* had wild-type-like flowering time but the double mutant *acd6-1win3-1* flowered earlier than the two parental mutants (Table I) implies that *win3-1* also acts additively with *acd6-1* in regulating flowering time in short-day conditions.

To gain further insight into how *WIN3* and *NPR1* regulate the floral transition, we examined the expression of three genes promoting flowering time, *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; Samach et al., 2000; Wigge et al., 2005; Yoo et al., 2005), and one flowering repressor, *FLOWERING LOCUS C* (*FLC*; Michaels and Amasino, 2001; Helliwell

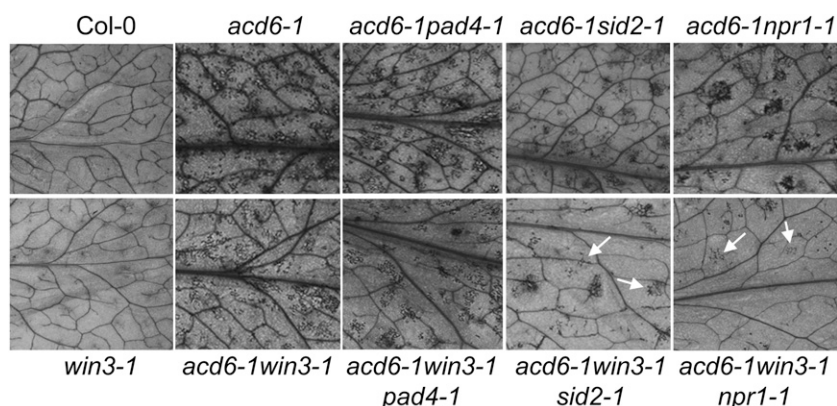


Figure 5. Suppression of cell death in *acd6-1* by *win3-1* is additive to the effect by *pad4-1*, *sid2-1*, or *npr1-1*. The fourth to sixth leaves of the indicated genotypes grown in 16-h-light/8-h-dark conditions were stained with trypan blue as described (Rate et al., 1999) and photographed with a dissecting microscope connected to an Axio-Cam MRC5 camera (Zeiss). Arrows indicate the minor cell death in *acd6-1win3-1sid2-1* and *acd6-1win3-1npr1-1*. No cell death was detected in *pad4-1*, *sid2-1*, and *npr1-1* (data not shown).

et al., 2006; Searle et al., 2006). In 16-d-old plants grown in long-day conditions, we found that *FLC* transcripts were lower in the *win3-1* background and in *npr1-1* compared with Col-0 and *acd6-1*. On the other hand, *FT* transcript level remained constant in these plants (Fig. 7C). In 25-d-old plants grown in the same light regime, we observed a similar suppression of *FLC* expression. By contrast, *FT* transcripts were induced to a higher level in the presence of *win3-1* and in the *npr1-1* mutant (Fig. 7D). Expression of *CO* and *SOC1*, however, remained unchanged in these mutants in both 16- and 25-d-old plants (Supplemental Fig. S4). These results suggest that the early flowering conferred by *win3-1* and *npr1-1* mutants is via the regulation of *FLC* and *FT* but is independent of *CO* and *SOC1*.

DISCUSSION

The type II SA regulator WIN3 was previously shown to regulate resistance to the biotrophic pathogen *P. syringae*. Data reported in this study indicate that WIN3 is also involved in controlling broad-spectrum disease resistance to the necrotrophic pathogen *Botrytis*. Genetic analysis indicated that WIN3 represents a new branch on SA signaling networks, acting together with PAD4, NPR1, and SID2 to regulate plant defense. Our data also revealed novel roles of WIN3 and NPR1 in influencing the transition from vegetative growth to reproductive growth, highlighting the interconnectedness of plant innate immunity and development.

WIN3 Confers Resistance to Biotrophic and Necrotrophic Pathogens

Evidence from this report and previous studies indicate that WIN3 confers resistance to biotrophic and necrotrophic pathogens. SA is the key signaling molecule critical for broad-spectrum disease resistance. The susceptibility of the *win3* mutants to bacterial pathogens can be rescued by SA treatment (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta

et al., 2007), suggesting that WIN3 acts upstream of SA signaling. We further show here that, like the *sid2* mutant impaired in the major SA biosynthesis, the *win3-1* mutant is partially compromised in response to flg22-induced resistance (Fig. 1). In addition, our data demonstrated that suppression of *acd6-1*-conferred constitutive defense to *P. syringae* by *win3-1* was well correlated with the change of SA levels in *acd6-1win3-1* (Figs. 3 and 4). Thus, these data indicate that WIN3-conferred resistance to *P. syringae* is largely SA dependent.

We show here that WIN3 confers resistance to the necrotrophic fungal pathogen *Botrytis*. Our data indicate that such resistance is independent of the phytoalexin camalexin. Interestingly, we observed a correlation of reduced SA accumulation and enhanced *Botrytis* susceptibility in *acd6-1win3-1* (Fig. 3C). Since *SID2* contributes to the majority of SA biosynthesis, the fact that the SA level in *acd6-1win3-1* is only 10% of that in *acd6-1* but higher than that in *acd6-1sid2-1* suggests that WIN3 only partially affects *SID2*-mediated SA biosynthesis. In addition, an even lower SA level was observed in the triple mutant *acd6-1win3-1sid2-1* compared with *acd6-1win3-1* and *acd6-1sid2-1*, imply-

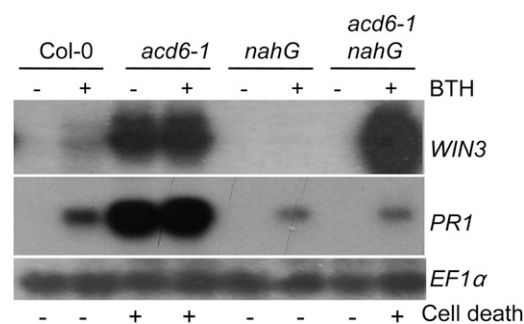


Figure 6. Activation of *WIN3* expression by SA signaling and cell death. Twenty-five-day old plants grown in a 16-h-light/8-h-dark cycle were treated with 100 μ M BTH or water for 24 h. Cell death was detected by trypan blue staining as described (Rate et al., 1999). Total RNA was extracted and analyzed in northern blotting. *EF1 α* was used as a loading control.

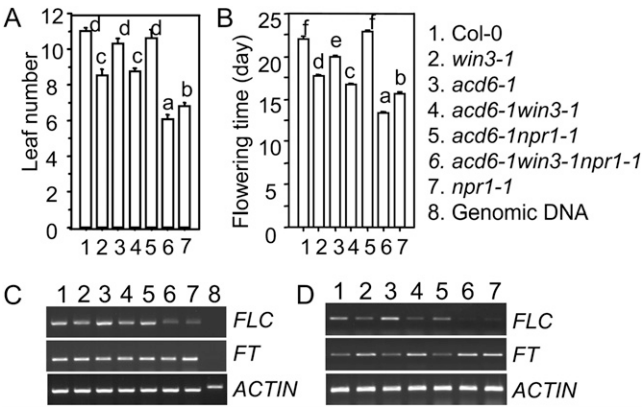


Figure 7. WIN3 and NPR1 act synergistically to regulate the transition to flowering in long-day conditions (16 h of light/8 h of dark). A, Leaf number quantitation. For each genotype ($n > 10$), the number of leaves at the appearance of the first flower bud was counted. B, Flowering time measurement. For plants shown in A, the number of days post planting required for the appearance of the first flower bud was recorded. For A and B, statistical analysis was performed with Student's t test (StatView 5.0.1). Letters indicate significant differences among the samples ($P < 0.05$). C, Gene expression in 16-d-old plants. D, Gene expression in 25-d-old plants. For C and D, plant tissue was harvested at 4 PM (10 h after light was on in the chamber). Total RNA was extracted from the indicated genotypes and reverse transcribed with the First-Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. RT-PCR of 25 and 30 cycles showed similar results of gene expression, and only the results from 30-cycle PCR are shown. *ACTIN* was used as a loading control. The RT-PCR product sizes are as follows: 591 bp for *FLC*, 529 bp for *FT*, and 513 bp for *ACTIN*. The sizes of the corresponding genomic fragments for the genes that can also be amplified with the RT-PCR primers are as follows: 5,592 bp for *FLC*, 2,150 bp for *FT*, and 588 bp for *ACTIN*. Note the absence of genomic fragments for *FLC* and *FT*, due to their large sizes that were not amplified from the genomic DNA template under the RT-PCR conditions. The key for the genotypes in all panels is placed to the right of B. These experiments were repeated two times with similar results.

ing that WIN3 also contributes to SID2-independent SA biosynthesis. Since SID2 is not required for resistance to *Botrytis* (Govrin and Levine, 2002; Ferrari et al., 2003), WIN3-mediated resistance to *Botrytis* is possibly through a SID2-independent SA pathway. Alternatively, since SID2-independent SA accumulation is minor (Fig. 3C), it is also possible that WIN3 regulates defense against *Botrytis* in a SA-independent manner.

WIN3 Represents a Novel Branch in the SA Signaling Networks

acd6-1 is a small mutant whose size is inversely correlated with its defense levels. We have used this unique feature of *acd6-1* to assess interactions among several known defense genes in a genetic analysis (Song et al., 2004; Lu et al., 2009). Using a similar approach, we investigated in this study the interaction of WIN3 with several SA regulators. Our data indicate that WIN3 contributes to SID2-dependent and -inde-

pendent SA biosynthesis (Fig. 3C). Although further reduction of SA levels in *acd6-1win3-1sid2-1* was small, suppression of *acd6-1*-conferred disease resistance, cell death and dwarfism was more obvious in the triple mutant than in *acd6-1sid2-1*. One explanation could be that the WIN3-mediated, SID2-independent SA biosynthetic pathway plays a significant role in regulating disease resistance, cell death, and plant size in *acd6-1win3-1sid2-1*. Alternatively, a WIN3-mediated SA-independent pathway is required for these phenotypes conferred by *acd6-1sid2-1*. This interpretation of WIN3 function is supported by the biochemical activity of WIN3. WIN3 was shown to conjugate specific amino acids to its preferred benzoates (Okrent et al., 2009). Some of these benzoate substrates of WIN3 might be used as precursors for and/or signaling molecules to prime SA biosynthesis in a SID2-dependent and/or SID2-independent manner (Yalpani et al., 1993; Ribnicky et al., 1998; Chong et al., 2001; Okrent et al., 2009). It is also possible that WIN3 substrates are engaged in physiological processes not affected by SA. Consistent with the latter notion, microarray analysis using a custom microarray to monitor the expression of 464 pathogen-responsive genes in several defense mutants, *win3*, *sid2*, *eds5*, and *npr1*, revealed that the number of genes affected by WIN3 was greater than that affected by the major SA regulators, SID2 and NPR1 (Sato et al., 2007; Wang et al., 2008).

Previous genetic analysis with *pad4-1* and *npr1-1* mutants in the *acd6-1* background led us to conclude that NPR1 plays multiple roles in regulating SA-mediated defense, acting as a positive SA signal transducer and a positive and a negative regulator of SA accumulation. The negative role of NPR1 likely requires PAD4 to form a negative feedback loop in regulating SA levels (Lu et al., 2009; Fig. 8). Our analysis of *acd6-1win3-1npr1-1* further corroborates multiple roles of NPR1 in regulating SA-mediated defense. Similar to *pad4-1*, *win3-1* greatly suppressed the high levels of SA in *acd6-1npr1-1*, suggesting that WIN3 is also part of a negative feedback loop of NPR1 in regulating SA accumulation. In addition, our data show that the suppression of *acd6-1*-conferred phenotypes by

Table 1. WIN3 and NPR1 act synergistically to regulate floral transition in short-day conditions (8 h of light/16 h of dark)

Flowering time is recorded as the days post planting at the appearance of the first flower bud for each genotype ($n > 10$). Statistical analysis was performed with Student's t test (StatView 5.0.1). Letters indicate significant differences among the samples ($P < 0.05$).

Genotype	Flowering Time
Col	87.6 ± 1.2 c
win3-1	86.7 ± 1.7 c
acd6-1	90.4 ± 2.5 c,d
acd6-1win3-1	79.0 ± 2.0 b
acd6-1npr1-1	93.3 ± 0.9 d
acd6-1win3-1npr1-1	50.1 ± 3.0 a
npr1-1	74.3 ± 1.7 b

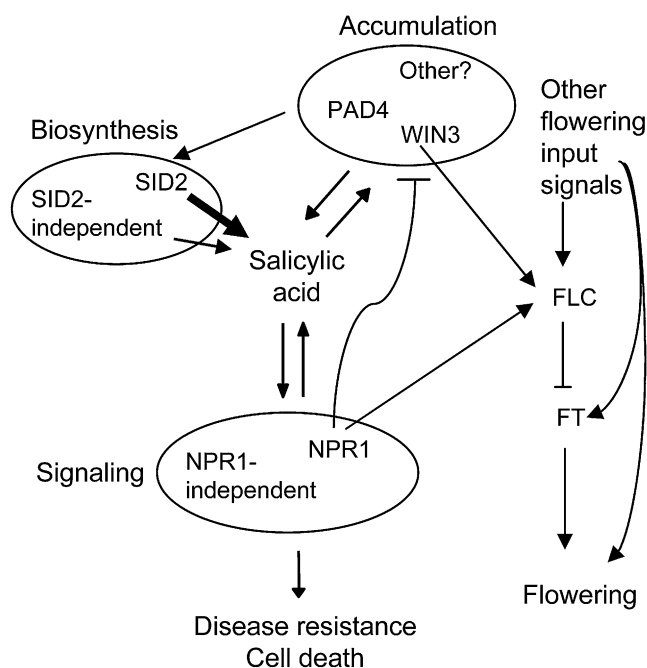


Figure 8. A model of action for WIN3. The SA signaling networks can be viewed in three interconnected regulatory subcircuits. WIN3 is a type II SA regulator that affects the accumulation of SA not by directly participating in SA biosynthesis but possibly through modifying precursors or signaling molecules for SA synthesis. WIN3 contributes to SID2-dependent and -independent pathways. WIN3 acts additively with another type II SA regulator, PAD4, to affect SA accumulation. Both WIN3 and PAD4 form positive signal amplification loops with SA. NPR1 can positively transduce SA signaling and affect SA accumulation. NPR1 can also negatively regulate SA levels, involving both WIN3 and PAD4. WIN3 and NPR1 act in separate pathways to regulate the floral transition by affecting the expression of *FLC* and *FT* genes.

pad4-1 and *win3-1* is additive, suggesting that WIN3 and PAD4 act in separate pathways (Fig. 8). Together, these observations implicate more than one negative feedback loop involving NPR1 and PAD4, WIN3, and/or possibly other SA regulators to keep SA signaling networks in check. Interestingly, expression of WIN3 is partially dependent on several SA regulators (Fig. 4B; Jagadeeswaran et al., 2007; Lee et al., 2007), possibly due to the effects of these SA regulators on SA accumulation and/or signaling. Together, these data suggest that WIN3 represents a novel node in the SA signaling networks. A model to summarize the role of WIN3 and other SA regulators in controlling SA signaling is shown in Figure 8.

WIN3 and NPR1 Act Additively in Regulating the Transition to Reproduction

Previous studies implicate a positive role of SA in influencing flowering time. For instance, exogenous SA application accelerates the floral transition in many plants (Cleland and Tanaka, 1979; Cleland and Ben-Tal, 1982; Martínez et al., 2004; Endo et al., 2009;

Wada et al., 2009). UV-C treatment or infection with *P. syringae*, which potentially activate SA accumulation and/or signaling, also shortens flowering time in *Arabidopsis* (Korves and Bergelson, 2003; Martínez et al., 2004). In addition, some mutants with reduced SA levels demonstrate delayed flowering, while other mutants with increased SA levels show accelerated flowering (Martínez et al., 2004; Jin et al., 2008; March-Díaz et al., 2008).

In contrast to the positive role of SA and some SA genes in regulating flowering time suggested by these previous studies, our data implicate a negative role of WIN3 and NPR1 in flowering time control. Although *acd6-1win3-1* and *acd6-1win3-1npr1-1* accumulated much reduced SA levels compared with *acd6-1*, these plants flowered much earlier than *acd6-1* and Col-0, suggesting that WIN3 and NPR1 control of flowering time is largely SA independent (Fig. 7). The fact that *acd6-1win3-1npr1-1* flowered the earliest among all the plants tested under both long-day and short-day conditions suggests that WIN3 and NPR1 act in two independent pathways to regulate flowering time. A previous study showed that the SA-deficient mutant *sid2-1* flowered late in short-day conditions but not in long-day conditions (Martínez et al., 2004). Consistent with these results, we did not observe delayed flowering in *sid2-1* in long-day conditions (Supplemental Table S1).

How does WIN3 control flowering time? As discussed earlier, biochemical analysis of the WIN3 protein and gene expression profiling analysis by microarray with *win3* and other mutants disrupting SA signaling suggest the possibility that WIN3 acts in a SA-independent pathway (Wang et al., 2008; Okrent et al., 2009). Our data indicate that WIN3-mediated flowering is largely SA independent, possibly through the suppression of the major flowering repressor FLC and the activation of the positive flowering regulator FT. Since WIN3 is an enzyme that does not have a nucleus localization motif, it is less likely that WIN3 exerts a direct transcriptional control of *FLC* and *FT* genes. However, it is possible that in the absence of WIN3, accumulation of WIN3-preferred benzoate substrate(s) or their upstream compounds, and/or the lack of a certain product(s) downstream of benzoate production and/or modification, signal the control of expression of these flowering regulatory genes.

Like WIN3, NPR1 also regulates the expression of *FLC* and *FT*. As a transcription coregulator, NPR1 might function by interacting with transcription factors, such as members in the TGA protein family, to directly affect the expression of these flowering regulatory genes (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). One such TGA protein is likely TGA4, which was shown to bind to the promoter of *FT* to control flowering time (Song et al., 2008). In addition, our data showed that higher expression of *FT* was only observed in 25-d-old but not in 16-d-old plants, suggesting that WIN3- and NPR1-regulated *FT* expression is also development related.

Multiple intrinsic and extrinsic factors are known to regulate plant flowering. Since *win3-1* and *npr1-1* mutants demonstrated a similar early flowering pattern in both long-day and short-day conditions, we speculate that WIN3 and NPR1 function independently of the photoperiod pathway. The biological clock is another main factor that determines flowering time (McClung, 2001). Increasing evidence shows that the biological clock also controls the innate immunity in Arabidopsis. For instance, the component of the central oscillator of the Arabidopsis clock, CIRCADIAN CLOCK ASSOCIATED1, was recently shown to play a direct role in plant defense (Wang et al., 2011b). In addition, the expression of some defense genes is also known to be under the control of the biological clock (Wang et al., 2001, 2011a; Sauerbrunn and Schlaich, 2004; Weyman et al., 2006). However, we did not detect any change in the expression of the circadian clock-regulated gene *CO* in both *win3* and *npr1* mutants (Fig. 7; Suárez-López et al., 2001) compared with Col-0. In addition, we recently showed that expression of *NPR1* was not affected by the biological clock under our growth conditions (Wang et al., 2011a). Therefore, evidence from our studies did not support a role of the biological clock in regulating WIN3- and NPR1-mediated flowering time control. Further investigation should elucidate if WIN3 and NPR1 cross talk to other flowering pathways, such as the autonomous, hormone, and vernalization pathways, and uncover how these other flowering pathways are linked to plant innate immunity.

To summarize the role of WIN3 and NPR1 in regulating SA-mediated defense and flowering time, we present the model in Figure 8. In this model, we view the SA signaling networks in three interconnected regulatory subcircuits. WIN3 is a type II SA regulator that represents a novel node in the SA signaling networks. WIN3 contributes to SID2-dependent and -independent SA biosynthesis, possibly through its function in modifying benzoate substrates. Genetic analysis showed that WIN3 acts additively with another type II SA regulator, PAD4, to affect SA accumulation. Both WIN3 and PAD4 form positive signal amplification loops with SA. On the other hand, NPR1 has multiple roles in regulating SA defense. It cannot only positively transduce SA signaling and affect SA accumulation but also can negatively regulate SA levels. The negative role of NPR1 on SA accumulation likely involves both WIN3 and PAD4. In addition, the SA signaling networks cross talk with plant development, affecting the floral transition. While an increased SA level can lead to accelerated flowering, WIN3 and NPR1 are repressors of the floral transition through separate pathways to affect the expression of *FLC* and *FT* genes.

Taken together, we show that the SA regulator WIN3 plays multiple roles in controlling broad-spectrum disease resistance, cell death, and flowering time. The fact that two major SA regulatory genes, WIN3 and NPR1, are involved in determining the floral

transition strongly supports the idea that plant innate immunity and development are intimately connected. Diseases, of course, can be devastating to crop plants, but disease resistance is also energetically costly. Under defense conditions, making an accelerated transition to the reproductive phase is crucial to the long-term fitness of plants. A better understanding of how plants adjust their growth and reproduction in response to pathogen attack should make it possible to positively manipulate overall crop yield.

MATERIALS AND METHODS

Plant Materials

All Arabidopsis (*Arabidopsis thaliana*) plants were grown in growth chambers with light intensity at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, 60% humidity, and 22°C. Unless otherwise indicated, plant materials used in this paper are in the Col-0 background. Pathogen infection, flg22 and BTH treatments, and sample collection for RNA, SA, and camalexin analyses and cell death staining were done 6 h after light was on in the growth chamber. For each experiment, appropriate controls were included. Transgenic plant *nahG* and mutants (*acd6-1*, *npr1-1*, and the double mutants in the *acd6-1* background) were described before (Rate et al., 1999; Lu et al., 2003, 2009; Song et al., 2004). *win3-1* was previously designated *win3-T* (Lee et al., 2007). *acd6-1win3-1*, *acd6-1win3-1pad4-1*, *acd6-1win3-1sid2-1*, and *acd6-1win3-1npr1-1* were made by genetic crosses and confirmed with cleaved amplified polymorphic sequence markers or other PCR markers corresponding to each single mutant (Lee et al., 2007; Lu et al., 2009). The *pbs3-1* and *pbs3-2* seeds were provided by Mary Wildermuth (Nobuta et al., 2007). The *fls2* mutant (SALK_062054) was obtained from the Arabidopsis Biological Resource Center and confirmed with PCR primers At5g46330_937f (5'-CAAGCACTCCGGATATACAAGAAC-3'), At5g46330_1323r (5'-CGGGATCTCGCCAGTCATT-3'), and LbB1 (5'-GCGTG-GACCGCTTGCTGCAACT-3').

Bacterial and Fungal Infection

Pseudomonas syringae pv *maculicola* ES4326 strain DG3 (Guttman and Greenberg, 2001) was used to infect 25-d-old plants grown in a chamber with a 12-h-light/12-h-dark cycle. The infection was conducted by infiltrating bacterial solution with a 1-mL needleless syringe to the abaxial side of the fifth to seventh leaves. Bacterial culturing, infection, and growth analysis were performed as described previously (Greenberg et al., 2000; Lu et al., 2003). For flg22 treatment, $1 \mu\text{M}$ flg22 (GenScript USA) was infiltrated into the leaves 24 h prior to *Pma* infection.

Botrytis cinerea was provided by Tesfaye Mengiste at Purdue University. *Botrytis* culture and infection were performed as described (Veronese et al., 2004). Briefly, *Botrytis* was cultured on a plate containing 4% potato dextrose agar at room temperature for 3 to 4 weeks. Spores were harvested and resuspended in a broth containing 1% Difco sabouraud maltose. The 25-d-old plants grown in a chamber with a 12-h-light/12-h-dark cycle were sprayed with 2×10^5 spores mL^{-1} and covered with a clear plastic dome to maintain high humidity. The disease rating was performed 2 d after the infection with a rating scale similar to one described previously (Genger et al., 2008). The rating scale was as follows: 0 = no detectable lesions; 1 = small rare lesions; 2 = lesions on up to 10% of leaves; 3 = lesions on up to 10% to 30% of leaves; 4 = lesions on up to 30% to 60% of leaves; 5 = lesions on up to 60% to 80% of leaves; and 6 = less than 10% of green leaves remained.

Cell Death Activation in *acd6-1nahG* and Cell Death Staining

To activate cell death, we sprayed 25-d-old *acd6-1nahG* and control plants, Col-0, *acd6-1*, and *nahG*, with $100 \mu\text{M}$ BTH or water. BTH was kindly provided by Robert Dietrich (Syngenta). The plants were grown in a chamber with a 16-h-light/8-h-dark cycle. Twenty-four hours after the treatments, leaves of all treated plants were collected for RNA analysis.

Trypan blue staining for cell death was performed as described (Rate et al., 1999). Stained leaves were examined with a Stemi SV 1.1 stereomicroscope

(Zeiss), and photographs were taken with an AxioCam MRc5 camera (Zeiss) connected to the microscope.

RNA Analysis

Total RNA was isolated from infected leaves or whole mutant plants using TRIzol reagent (Invitrogen). Northern blotting was performed as described previously (Lu et al., 2003). DNA templates for making radioactive probes were obtained by PCR with specific primers for each gene and labeled with [³²P]dCTP in a PCR with a gene-specific antisense primer. For reverse transcription (RT)-PCR, total RNA was reverse transcribed into cDNAs using the First-Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. Primers specific for each gene were used in PCR to amplify the corresponding gene. These primers were designed to specifically amplify RT-PCR products and avoid the contamination from the PCR products amplified from genomic DNA template. The RT-PCR product sizes for these genes should be 300 bp for *CO*, 591 bp for *FLC*, 529 bp for *FT*, 536 bp for *SOC1*, and 513 bp for *ACTIN*. The sizes of the corresponding genomic fragments for these genes that can be amplified with the RT-PCR primers are 5,592 bp for *FLC*, 2,150 bp for *FT*, 2,275 bp for *SOC1*, and 588 bp for *ACTIN*. Due to their large sizes, the genomic DNA fragments for *FLC*, *FT*, and *SOC1* should not be amplified from genomic DNA under the conditions used for RT-PCR. For *CO*, the primers anchor on the junctions of two exons; therefore, no PCR product should be amplified with the genomic DNA template. Primers used for making radioactive probes and for RT-PCR are listed in Supplemental Table S2.

SA and Camalexin Measurement

SA and camalexin were extracted as described previously (Lu et al., 2003; Song et al., 2004). Briefly, 200 mg of leaf tissue from plants grown in a chamber with a 16-h-light/8-h-dark cycle was ground to fine powder with liquid nitrogen and extracted once with 1.5 mL of 90% methanol followed by one extraction with 1.5 mL of 100% methanol. For each sample, 500 ng of *o*-anisic acid (Sigma 169978) was added as the internal control during the first extraction. Each data point had three replicates. The methanol fraction was equally split into two microcentrifuge tubes and dried in the fume hood overnight. The pellet was dissolved by adding 500 μ L of 100 mM sodium acetate (pH 5.5). To half of the duplicated samples, 40 units of β -glucosidase (Sigma G-0395) was added to digest glucosyl-conjugated SA (total SA) for 1.5 h at 37°C. All the samples were treated with an equal volume of 10% TCA and centrifuged at 10,000g for 10 min. The supernatant was extracted twice with 1 mL of extraction solvent (ethylacetate:cyclopentane:2-propanol 100:99:1, v/v). The top (organic) phase was collected in a microcentrifuge tube and dried in a fume hood overnight. The residual fraction was resuspended in 0.5 mL of 55% methanol by vortex and was passed through a 0.2- μ m nylon spin-prep membrane (Fisher 07-200-389) via centrifugation for 2 min (14,000g) before being subjected to HPLC analysis. A Dionex AS50 HPLC instrument with an Acclaim 120 C18 reverse column (4.6 \times 250mm) and an RF2000 fluorescence detector was used to separate and detect *o*-anisic acid, SA, and camalexin. The mobile phase included a gradient of methanol and 0.5% acetic acid. *o*-Anisic acid was detected at 4.9 min with 301-nm excitation/365-nm emission, SA was detected at 6.5 min with 301-nm excitation/412-nm emission, and camalexin was detected at 8.6 min with 318-nm excitation/385-nm emission. The standard curve was made from quantification of *o*-anisic acid, SA, and camalexin at concentrations of 50, 100, 250, 500, and 1,000 ng mL⁻¹ and was used to calculate the final concentration in each sample with Excel software (Microsoft).

Flowering Time Determination and Plant Size Quantitation

To determine flowering time, the number of rosette leaves at bolting and/or the number of days post planting at the appearance of the first flower bud were counted for plants grown in long-day (16 h of light/8 h of dark) and short-day (8 h of light/16 h of dark) chambers. For size determination, 25-d-old plants grown in a long-day chamber were measured for rosette diameter.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number At5g13320.

Supplemental Data

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

Supplemental Figure S1. The *win3-1* mutant is less responsive to flg22 treatment in root growth assay.

Supplemental Figure S2. The *pbs3* mutants are more susceptible to *Botrytis* infection.

Supplemental Figure S3. WIN3 expression in SA mutants.

Supplemental Figure S4. Expression of *CO* and *SOC1* is constant in *win3-1* and *npr1-1* mutants in long-day conditions (16 h of light/8 h of dark).

Supplemental Table S1. Flowering time in plants grown in long-day conditions (16 h of light/8 h of dark).

Supplemental Table S2. Primer sets used in northern blotting and RT-PCR.

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