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)/AVRPHPB SUSCEPTIBLE3 (PBS3)/GH3-LIKE DEFENSE GENE1 (GDD1), 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...
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; Martinez 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 (Todisco 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). 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 (PmaDG3; 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 (10^5 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 hypersensitivity to Botrytis (Fig. 2, A and B). win3-1 partially
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 ± 0.1 µg g\(^{-1}\) fresh weight) and acd6-1 (4.4 ± 1.7 µg 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 PmaDG3. 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

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**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 µM flg22 or water for 24 h before infection with *Pma*DG3 (OD\(_{600}\) = 0.0001). Bacterial growth was assessed 3 d after infection. Data represent average bacterial numbers in six samples ± 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.

**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) ± 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.
Roles of WIN3 in Regulating Defense and Flowering Time

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-
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 CONSTANS (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...
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 both 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-
These experiments were repeated two times with similar results. Amplified from the genomic DNA template under the RT-PCR conditions, fragments for FLC are 2,150 bp for \( \text{FLC} \), 5,592 bp for FLC, and only the results from 30-cycle PCR are shown. RT-PCR of 25 and 30 cycles showed similar results of gene expression, and only the results from 30-cycle PCR are shown.

**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. 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 FT and FLIC 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.

**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 \(( P < 0.05 )\).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Flowering Time</th>
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<tbody>
<tr>
<td>Col</td>
<td>87.6 ± 1.2 c</td>
</tr>
<tr>
<td>win3-1</td>
<td>86.7 ± 1.7 c</td>
</tr>
<tr>
<td>acd6-1</td>
<td>90.4 ± 2.5 c,d</td>
</tr>
<tr>
<td>acd6-1win3-1</td>
<td>79.0 ± 2.0 b</td>
</tr>
<tr>
<td>acd6-1npr1-1</td>
<td>93.3 ± 0.9 d</td>
</tr>
<tr>
<td>acd6-1win3-1npr1-1</td>
<td>50.1 ± 3.0 a</td>
</tr>
<tr>
<td>npr1-1</td>
<td>74.3 ± 1.7 b</td>
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**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 -independent 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 -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
Roles of WIN3 in Regulating Defense and Flowering Time

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 _FT_ genes. 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.

__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.__
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 FT separate pathways to affect the expression of NPR1 are repressors of the floral transition through SA level can lead to accelerated flowering, WIN3 and repression, affecting the floral transition. While an increased SA signaling networks cross talk with plant development, likely involves both WIN3 and PAD4. In addition, the negative role of NPR1 on SA accumulation but also can negatively regulate SA only positively transduce SA signaling and affect SA amplification loops with SA. On the other hand, NPR1 function in modifying benzoate substrates. Genetic analysis showed that WIN3 acts additively with an-other 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 μmol m⁻² s⁻¹, 60% humidity, and 22°C. Unless otherwise indicated, plant materials used in this paper are in the Col-0 ecotype. 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). acd6-1/acd6-1, acd6-1/acd6-1pad4-1, acd6-1/acd6-1sad2-1, and acd6-1/acd6-1/npr1-1 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 seed 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'-GACCGGCACTCCTCCGGATATACAAGAAC-3') and AT5g46330_1326r (5'-CCGGAATCCTCCGAGTCACT-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 bacteria inoculation with a 1×10⁶ cfu mL⁻¹ 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 μM 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 × 10⁵ 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 μM 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 Leica DM 2500 stereomicroscope.
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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 vortexed at 37°C (Sigma G-0395) was added to digest glucosyl-conjugated SA (total SA) for 1.5 h on a fume hood overnight. The residual fraction was resuspended in 0.5 mL of 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 the fume hood. The pellet was dissolved by adding 500 μL 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 (ethylicacetetacyclopentane-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-μm 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 × 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/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 A5f9g13320.

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 phs3 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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