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A role for salicylic acid and NPR1 in regulating cell growth in Arabidopsis

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Summary

Salicylic acid (SA) plays a key role in activating defenses and cell death during plant–pathogen interactions. In response to some pathogens, SA also limits the extent of cell death, indicating that it acts positively or negatively depending on the host–pathogen interaction. In addition, we previously showed that SA affects cell growth in the Arabidopsis defense-related mutants *accelerated cell death 6–1* (*acd6–1*) and *aberrant growth and death 2* (*agd2*). Using *acd6–1*, *agd2* and two other defense-related mutants, *lesion simulating disease 6* (*lsd6*), *suppressor of SA-insensitivity* (*ssi1*), we show here in detail that SA regulates cell growth by specifically affecting cell enlargement, endoreduplication and/or cell division. We find that SA can act either positively or negatively to regulate cell growth depending on the context in which signaling occurs. Additionally, Nonexpressor of PR 1 (NPR1), a key SA signaling protein important for regulating defenses and cell death, also acts to promote cell division and/or suppress endoreduplication during leaf development. We propose that SA interacts with multiple receptors or signaling pathways to control cellular alterations during normal development, pathogen attack and/or stress situations. We suggest that SA and NPR1 play broader roles in cell fate control than has previously been understood.

Keywords: salicylic acid, cdc2B, cell death, endoreduplication, cell division.

Introduction

Since pioneering experiments first convincingly established an important role for the phenolic salicylic acid (SA) in defense regulation and disease resistance (Ryals *et al.*, 1996), it has become clear that SA also functions in cell fate control. For example, under some conditions, SA promotes cell death associated with disease (Greenberg *et al.*, 2000; O'Donnell *et al.*, 2001), while in other conditions, SA promotes or suppresses cell death associated with pathogen recognition during disease resistance responses (Brading *et al.*, 2000; Gaffney *et al.*, 1993; Rate *et al.*, 1999; Rate and Greenberg, 2001). A report showing that acetyl SA can promote colony formation in maize protoplasts also suggests a role for SA in the regulation of the cell cycle (Carswell *et al.*, 1989).

We previously reported that the pathogen-resistant dominant gain-of-function *accelerated cell death 6* (*acd6–1*) mutant of Arabidopsis shows SA-dependent spontaneous cell death and cell enlargement in whole leaves (Rate *et al.*, 1999). Under conditions when SA signaling in *acd6–1* occurs transiently and/or at a reduced level, the growth phenotype of *acd6–1* is amplified leading to the appearance of abnormal tumor-like growths (Rate *et al.*, 1999). Additionally, we recently found that another mutant, called *aberrant growth and death 2* (*agd2*), shows spontaneous tumor-like growths that are exacerbated by SA depletion (Rate and Greenberg, 2001). *agd2* also shows spontaneous cell death that requires Nonexpressor of PR1 (NPR1), an SA signaling component.

Our previous studies with *acd6–1* and *agd2* suggest a complex role for SA in cell fate control. It is unclear how SA regulates cell death and growth. For example, SA may influence growth by regulating cell division, enlargement

and/or activating DNA replication without concomitant nuclear division or cytokinesis (endoreduplication). In addition, the details about the cell-type specificity and timing of the cellular growth and death alterations in *acd6–1* and *agd2* in their regulation by SA is also unknown. It is possible that cell death, cell enlargement, endoreduplication and/or cell division could occur in distinct cell types or different populations of cells within leaves or these cellular alterations could occur in cells in close proximity and communication. Because the timing of cellular alterations regulated by SA could be different, one or more event may cause the induction of other event(s). To address these questions, we performed detailed morphological studies of *acd6–1* and *agd2*. Similar to *acd6–1* and *agd2*, *lesions simulating disease 6* (*lsd6*) and *suppressor of SA-insensitivity 1* (*ssi1*) mutants show SA-dependent cellular alterations, including cell death and dwarfism (Greenberg, 2000; Shah *et al.*, 1999; Weymann *et al.*, 1995). This raised the possibility that these defense-related mutants might also be affected in growth regulation in an SA-dependent manner. Therefore, we also examined the morphology of *lsd6* and *ssi* plants.

We show here that in *acd6–1*, *lsd6* and *ssi1* plants SA is important for activating cell enlargement and/or cell division. Furthermore, we establish that in *acd6–1* SA stimulates endoreduplication, while in the context of *agd2*, SA suppresses both cell enlargement and endoreduplication. Finally, we report a role for NPR1 in simulating cell division and/or suppressing endoreduplication during leaf development. This study therefore establishes novel functions for SA and NPR1 in growth control.

Results and discussion

We previously found that cell death and enlargement in *acd6–1* were SA dependent, but it was not clear whether these alterations were due to altered constitutive levels of SA. Quantitation of both free and total SA pools showed that *acd6–1* had constitutively high free and total SA (Figure 1), consistent with a model in which elevated SA contributes to the *acd6–1* phenotypes.

We next addressed the cell type specificity and the timing of cell death and cell growth induction by the SA pathway by examining thin sections of leaves of *acd6–1* and SA-depleted *acd6–1* in which the SA pathway was reactivated. In *acd6–1* leaves, but not in wild type, the palisade parenchyma cell layer showed collapsed, densely stained dead cells often near modestly enlarged cells (Figures 2a, 2b). To confirm that SA was necessary for these cellular alterations in *acd6–1*, we depleted SA using a *nahG* transgene, whose product metabolizes SA to the inactive catechol (Figure 1). Examination of thin sections showed that the alterations in cell viability and size were completely suppressed in *acd6–1-nahG* leaves (Figure 2),

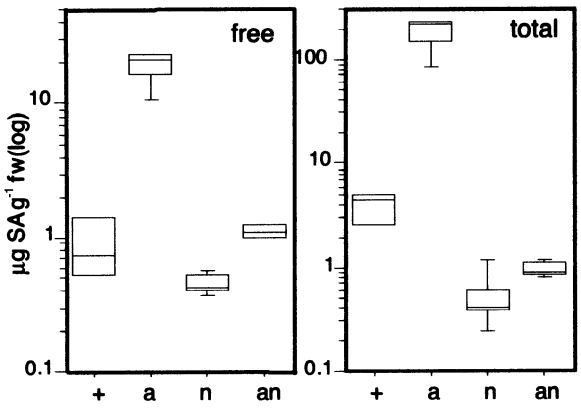

Figure 1. Salicylic acid content of *acd6–1* plants. Box plot shows the mean (internal bar), second and third quartiles indicating 50% of the dispersed data (open box) and data range (external bars) of the free and total SA levels of 4–6 samples from *acd6–1* (a), wild-type (+), *nahG* (*n*) and *acd6–1-nahG* (an) plants. Free and total SA levels in *acd6–1* were significantly different from all other genotypes ($P < 0.0006$, Fisher's Protected Least Significant Difference test, a posthoc multiple *t*-test). Values obtained from two separate experiments were similar.

consistent with our previous analysis of intact leaves (Rate *et al.*, 1999). To assess whether the formation of dead and enlarged cells occurs at or about the same time, we reactivated the SA pathway in *acd6–1-nahG* plants with a low level (10 µM) of the NahG-insensitive SA agonist benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) and followed the cellular morphology every 4 h over a 24-h period. Dead and enlarged cells adjacent to each other appeared simultaneously and were visible after 24 h (Figure 2h). Thus, cell death and cell enlargement are restricted to mesophyll cells in close proximity in *acd6–1* and both events occur at or about the same time.

Since low level activation of the SA pathway in SA-depleted *acd6–1-nahG* plants results in hyperactivation of the cell growth phenotype (Rate *et al.*, 1999), we investigated whether these cell growths were formed from newly divided as well as enlarged cells. We followed the morphology of *acd6–1-nahG* and control leaves after low level BTH treatment over an 8-day period. In *acd6–1-nahG* after the initial cell death and enlargement induced by BTH on days 1 and 2, we saw cell fragmentation (on day 3) and massive enlargement (on day 4). By day 8, two types of cellular alterations were equally represented: in one, regions between secondary veins were filled with many small cells and some enlarged cells (Figure 2h–k), and in another, regions between the secondary veins were filled with a few giant cells (data not shown). To determine whether an increase in cell numbers was induced by BTH, we counted cells in fully expanded water- and BTH-treated leaves. To allow direct comparisons of the cell numbers between samples with different sizes of cells, we used the developmental landmarks of a defined set of secondary

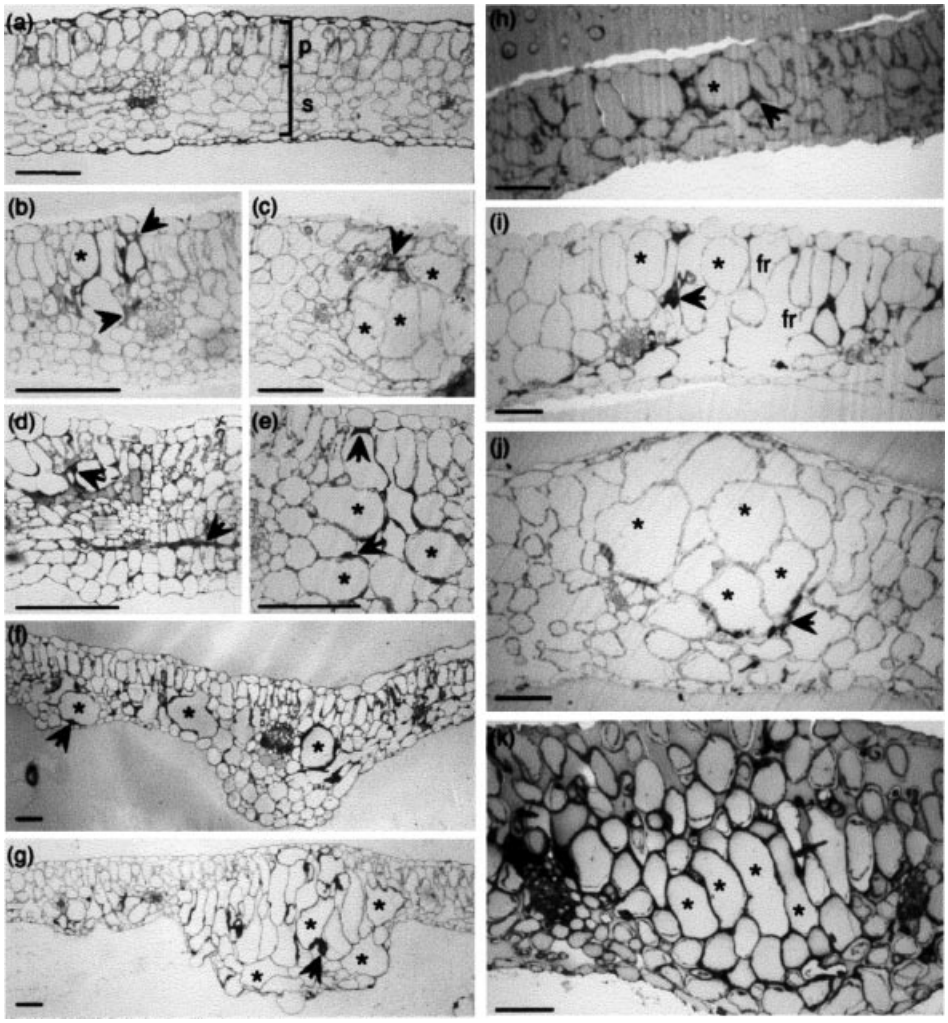

Figure 2. Morphological analysis of several Arabidopsis mutants.
Leaf tissue was fixed, embedded and cross-sectioned. Bar is 0.06 mm. Arrows indicate dead cells. Asterisks indicate enlarged cells. 'fr' marks cell fragments. Palisade parenchyma (p) and spongy mesophyll (s) cells are labeled. Samples are oriented adaxial side up.
(a) wild-type; (b) *acd6–1*; (c) *ssi1/SSI1*; (d) and (e) *lsd6/LSD6*; (f) *agd2*; (g) *agd2-nahG*; (h) to (k) *acd6–1-nahG* treated with 10 μM BTH after 1 (h), 3 (i), 4 (j) and 8 (k) days.
Not shown: Water-treated *nahG* and *acd6–1-nahG,* and BTH-treated wild type and *nahG* were indistinguishable from water-treated wild type (a); *acd6–1* with BTH was identical to *acd6–1* with water (b).

veins to demarcate the boundaries of the leaf areas used for the analysis. The vein pattern was similar in all leaves in which cell counts were made (data not shown). Where many small and enlarged cells were found in BTH-treated *acd6–1-nahG*, the average number of cells was two-fold higher in BTH-treated samples than the water-treated controls (Figure 3). BTH had no effect on the control samples (Figure 3). Only the palisade parenchyma and spongy mesophyll cells were obviously affected in the *acd6–1* abnormal growths (Figure 2k). Thus, the low level activation of the SA pathway stimulates both enlargement and division of mesophyll cells in *acd6–1* plants.

Cell enlargement has been correlated with the activation of an endoreduplication cycle (Melaragno *et al.*; 1993). The DNA content of the enlarged cells in the region formerly composed of palisade parenchyma and spongy mesophyll of BTH-treated *acd6–1-nahG* showed over 50% of nuclei with 16–64C or higher (Figure 4). The increased DNA content of nuclei likely resulted from the stimulation of an endoreduplication cycle, as there was no evidence of multinucleate cells as would be expected if cell fusion occurred. The majority of water- and BTH-treated palisade parenchyma and spongy mesophyll nuclei of wild type, *acd6–1*, *nahG* or water-treated *acd6–1-nahG* showed a DNA content of 2–8C (Figure 4), consistent with a previous report (Galbraith *et al.*, 1991). Thus, low level activation of the SA pathway stimulates an increase in nuclear DNA content in *acd6–1* plants.

An *npr1–1* mutation, which partially blocks SA signaling (Cao *et al.*, 1994), reduces and delays the amount of cell

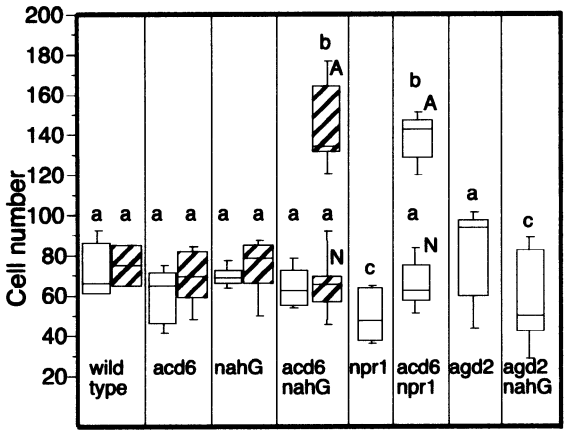

Figure 3. Cell content of leaves with altered SA signaling.
Average cell number from leaf cross sections of each indicated genotype was used for cell counting (n = 5–15). Open boxes, water treated; striped boxes, 10 μM BTH treated for 8 days. 'A', a region of abnormal growth; 'N', a morphologically normal region. Lower case letters represent significance groups as determined by Fisher's Protected Least Significant Difference measure. Each letter group differs from the other letter groups at a level of $P < 0.05$ or less.

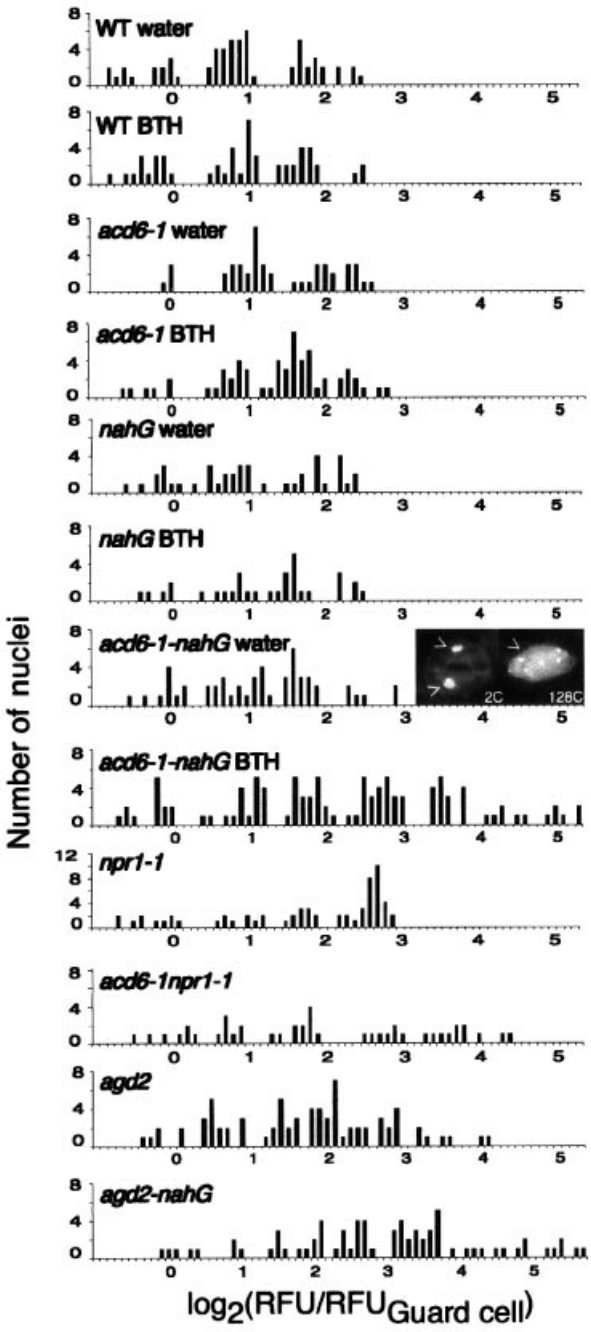

Figure 4. Nuclear DNA quantitation of mesophyll cells.
WT is wild type. RFU is relative fluorescence unit. *C*-values correspond to $\log_2$ (RFU/$RFU_{guard\ cells}$) as follows: up to 0 is 2C; 0–1 is 4C; 1–2 is 8C; 2–3 is 16C; 3–4 is 32C; 4–5 is 64C and 5–6 is 128C. Inset shows two 2C guard cell nuclei and a 128C nucleus; arrow indicates an individual nucleus. For treated samples, water or 10 μM BTH was used and tissue was assayed 8 days later.

death and exacerbates the cell growth phenotype of *acd6–1* (Rate *et al.*, 1999). This suggests that in *acd6–1npr1–1* there is an alteration in the balance of cell death and growth promoting signals (including SA) and/or their signal transduction (Rate *et al.*, 1999). To determine whether the growths of *acd6–1npr1–1* were similar to those seen in *acd6–1* leaves with low level SA signaling, we performed the same morphological analysis, cell counting and nuclear quantitation experiments as in *acd6–1*. *acd6–1npr1–1* leaves showed a mixture of enlarged and extra cells, and excessive cell numbers, similar to what was observed with day 8 BTH-treated *acd6–1-nahG* plants (Figure 3; data not shown). At least 40% of nuclei in *acd6–1npr1–1* showed 16–64C DNA content (Figure 4), whereas most nuclei in wild type and *acd6–1* were below 16C (Figure 4). Thus, low level stimulation of the SA pathway in SA-depleted *acd6–1* or a partial block of SA signal transduction in *acd6–1npr1–1* results in a similar activation of cell enlargement, cell division and endoreduplication.

To investigate whether NPR1 was important for growth regulation in otherwise wild-type plants, we quantitated cell numbers and DNA content of *npr1–1* leaf cells. Interestingly, *npr1–1* had fewer cells than wild type (Figure 3) and about half of the nuclei in *npr1–1* had 16C DNA content, whereas very few cells of wild type had 16C content (Figure 4). Thus, NPR1 promotes cell division and/or represses endoreduplication during leaf development. In contrast to the results with *npr1–1*, *nahG* leaves showed normal cell numbers and DNA content (Figures 3, 4). This difference in phenotypes may be due to the nearly normal basal level of free SA present in *nahG* plants (Figure 1), which allows for normal growth control, whereas a partial block in SA signaling in *npr1–1* results in a more dramatic effect on cell growth during development.

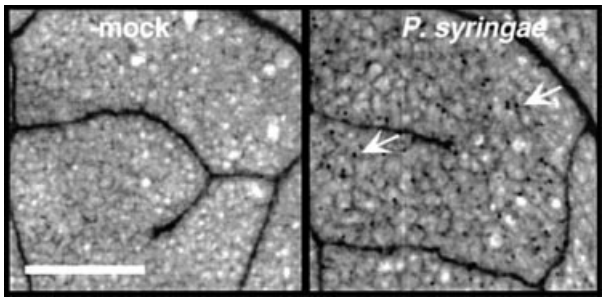

Figure 5. Cdc2B-β-glucuronidase expression in *P. syringae* infected cells. Wild-type Arabidopsis harboring *cdc2bAt-uidA* were hand-inoculated into fully expanded, non-senescent leaves of 3-wk-old plants with *P. syringae* pv. *maculicola* strain PsmES4326 at a dose of 2×10^3 cm^{-2}. Mock inoculation was done with 10 mM $MgSO_4$, the bacterial resuspension solution. One day after infection leaves were stained for 48 h (Butt *et al.*, 1998). Arrows indicate GUS-expressing nuclei. Bar is 0.5 mm.

To assess whether the activation of cell growth by the SA pathway was specific to *acd6–1*, we examined additional mutants that, similar to *acd6–1*, show SA-dependent cell death (Greenberg, 2000; Shah *et al.*, 1999; Weymann *et al.*, 1995). The pathogen-resistant dominant gain-of-function *lsd6* and haploinsufficient loss-of-function *ssi1* mutants of Arabidopsis both have this phenotype (Greenberg, 2000; Shah *et al.*, 1999; Weymann *et al.*, 1995). *ssi1/SSI1* heterozygous leaves showed regions of cell death and cell enlargement and/or cell fragmentation in the mesophyll cell layers similar to the day 3 and day 4 samples of BTH-treated *acd6–1-nahG* plants (Figure 2c; data not shown). In *lsd6/LSD6* heterozygous leaves, some regions showed cell death and a few enlarged cells (Figure 2e). Strikingly, however, some regions showed cell death mixed with abnormal numbers of very small cells, while some cells showed abnormal polarities or the oblong morphology of palisade parenchyma cells in the place of spongy mesophyll (Figure 2d). *ssi1/nahG* and *lsd6/nahG* transheterozygous plants showed normal cell morphology (data not shown), indicating an important role for SA in these cellular alterations. Thus, SA stimulates cell growth and cell death in the context of several different defense-related mutants of Arabidopsis.

While SA has a positive role in controlling cell growth in *acd6–1*, *ssi1* and *lsd6*, it can also repress cell growth and endoreduplication in the context of the recessive *agd2* mutant of Arabidopsis. The *agd2* mutant has modestly elevated SA levels, increased pathogen resistance and a low level of spontaneous cell death (Rate and Greenberg, 2001). *agd2* leaves frequently showed one or a few enlarged cells near vascular bundles, but did not have altered cell numbers (Figures 2f, 3). Interestingly, *agd2-nahG* plants showed a dramatic enhancement of the number of enlarged cells (Figure 2g) and had fewer cells than *agd2, nahG* or wild type (Figure 3). The enlarged cells in *agd2* and *agd2-nahG* leaves showed enlarged nuclei with at least 30% of the mesophyll nuclei showing a DNA content of 16–32C or higher in *agd2* and approximately 70% of the mesophyll nuclei showing a DNA content of 16–128C in *agd2-nahG* leaves. Leaves that emerged after BTH treatment of *agd2-nahG* plants showed the same morphology as *agd2* alone (data not shown), indicating that the effect of *nahG* was reversible. Thus, in the context of *agd2*, SA represses cell enlargement and endoreduplication.

Some plant pathogens cause the induction of cell enlargement and/or division and cell death (Duan *et al.*, 1999). While the SA pathway is clearly important for the activation or repression of cell death during different plant–pathogen interactions (Brading *et al.*, 2000; Gaffney *et al.*, 1993; Rate *et al.*, 1999), it is not yet known whether the SA pathway is exploited by pathogens to alter plant cell growth. Interestingly, *Pseudomonas syringae*, a pathogen that induces cell death and SA in Arabidopsis (Zhou *et al.*, 1998), also stimulates the expression of Cdc2b, a protein that marks dividing cells as well as those competent to divide (Segers *et al.*, 1996). Cdc2b induction was evidenced by the induction of β-glucuronidase activity in transgenic plants bearing a Cdc2b-β-glucuronidase reporter (Figure 5). Additionally, *P. syringae* stimulated localized cell enlargement at low doses (J.T.G., unpublished observations). Possibly pathogenesis (or other SA-inducing conditions) causes the cell cycle to become engaged, which can result in a number of cellular alterations such as cell death, enlargement and/or division depending on the cellular context. This phenomenon of cell cycle activation leading to alternative cell fates has been well documented in animals (Evan and Littlewood, 1998). For example, under- or over-expression of E2F-1 in mice causes tissue specific effects resulting in tissue atrophy and hyperplasia and/or tumor production in the same animal due to the interaction of E2F-1 with several proteins present in differing amounts in different cell types (Yamasaki, 1999).

Plants in which SA is elevated or moderately reduced, but not absent (Figure 1), do not have obviously altered morphogenesis (Gaffney *et al.*, 1993; Verberne *et al.*, 2000). However, plants genetically engineered to produce elevated levels of SA have not been examined in detail for alterations in cell number or DNA content. SA does activate cell enlargement, endoreduplication and/or cell division in both gain-of-function (*acd6–1* and *lsd6*) and hypomorphic (*ssi1*) mutant plants, and represses cell enlargement and endoreduplication in the recessive *agd2* mutant. Consistent with a role for SA in growth control, the SA signal transduction protein NPR1 promotes cell division and/or represses endoreduplication during leaf development. Interestingly, acetyl salicylate activates cell division in maize protoplasts (Carswell *et al.*, 1989).

In addition to its role in growth control, SA also regulates cell death. It promotes cell death during disease

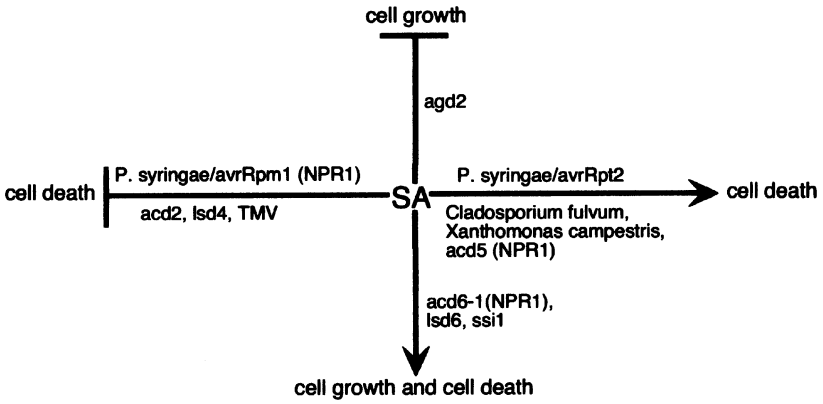

Figure 6. Summary of the effects of SA on cell fate.
SA can inhibit cell growth (cell enlargement, endoreduplication and/or cell division), shown with a bar, or promote cell death and cell growth, shown with an arrow. Also shown are the cell death repressive and promoting roles of SA in response to pathogens or in different Arabidopsis mutants. Where the effects of SA are known to be exerted through the action of NPR1, this is indicated in parentheses. Not shown is that during normal development, NPR1 is important for promoting cell division and/or inhibiting endoreduplication and the SA-inducing pathogen *P. syringae* can induce cell enlargement. Most examples were taken from published work (Brading *et al.* 2000; Gaffney *et al.*, 1993; Greenberg *et al.*, 2000; Hunt *et al.*, 1997; Rate *et al.*, 1999; Shah *et al.*, 1999; Weymann *et al.*, 1995; Mach *et al.*, 2001) or the data presented here.

in tomato infected with *Xanthomonas campestris* pv. *vesicatoria* (O'Donnell *et al.*, 2001). SA also promotes a type of cell death associated with disease resistance (the hypersensitive response, HR) in Arabidopsis infected with *P. syringae* carrying the *avrRpt2* gene (Rate *et al.*, 1999) and in tomato treated with the Avr9 elicitor (Brading *et al.*, 2000). However, SA also suppresses the HR in response to TMV in tobacco (Gaffney *et al.*, 1993) or *P. syringae* carrying the *avrRpm1* gene in Arabidopsis (Rate and Greenberg, 2001). NPR1 negatively regulates the HR in Arabidopsis infected with *P. syringae* carrying *avrRpm1* and positively regulates cell death in *agd2* (Rate and Greenberg, 2001), *acd6–1* and *acd5* mutants (Greenberg *et al.*, 2000; Rate *et al.*, 1999). At least in one case, the growth promoting and cell death-inducing effects of the SA pathway are dose dependent (Rate *et al.*, 1999). Together these findings, summarized in Figure 6, show that SA and NPR1 can alter cell death and/or growth in different ways depending on the cellular context and the level of signal transduction. We propose that SA may interact with various receptors and/or signaling pathways that are active under different conditions to cause opposing effects on cell death and/or growth.

The observation that SA and NPR1 function in cell death and cell growth control highlights the striking similarity between plants and animals, since in both cases defense signaling components play a role in development. In animals, NFκB is an important component of the innate immune system and is also critical for suppressing cell death and promoting cell cycle progression during development (Hatada *et al.*, 2000). Thus, the innate immune response and the plant defense response may have both evolved from developmental pathways.

Experimental procedures

Plant material and reagents

All plant material was in the Columbia background except *ssi1*, which was in the Nossen background (Shah *et al.*, 1999). For *ssi1*, all crosses were with plants in the Columbia background and controls were Nossen crosses to plants in the Columbia background. *acd6–1, acd6–1-nahG, acd6–1npr1, agd2, agd2-nahG, ssi1,* and *lsd6* seeds, plant treatments and growth conditions for Arabidopsis were described previously (Greenberg *et al.*, 2000; Greenberg, 2000; Rate *et al.*, 1999; Rate and Greenberg, 2001). Transgenic Arabidopsis carrying *cdc2bAt-uidA* was a gift from John Celenza (Boston University, Boston, MA, USA). The *cdc2bAt-uidA* transgene includes the 5 kb upstream promoter region and DNA encoding the first codon of Cdc2B translationally fused to an 800-bp genomic region of *cycB1*; *1* (formerly *cdc1A*) encoding two introns and the first 150 amino acids harboring the cyclin destruction box (to render the reporter protein proteolytically labile (Colon-Carmona *et al.*, 1999)) and nuclear localization signal of CycB1 followed by the *uidA* gene encoding the β-glucuronidase reporter protein. Benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) was a gift from Novartis (Research Triangle Park, NC, USA) as a 50% wettable powder. In the BTH treatment experiments, plants were sprayed with 10 μM BTH until all the leaves were wet.

Salicylic acid quantitation

Extracts were prepared from 3-wk-old plants and analyzed by reverse phase HPLC as previously described (Greenberg *et al.*, 2000; Greenberg, 2000; Rate *et al.*, 1999). SA yields, recovered from spiked samples, were 29%.

Morphological analysis

Leaf tissue from 3- to 4-wk-old plants was fixed as described (Donnelly *et al.*, 1999) and embedded in Histocryl Resin (London Resin Co., Hampshire, UK) or LR White (Polysciences, Inc.,

Warrington, PA, USA) as per the manufacturers' instructions. For morphological analyses, one micron sections stained with toluidine blue as described (Ruzin, 1999) were examined using an Axioskop microscope (Zeiss, Inc., Jena, Germany). For cell counting, all the cells between the second and third secondary veins (counting out from the midvein) from the middle region of the fifth and sixth leaves of the different genotypes were used.

DNA quantitation

For DNA quantitation, tissue was fixed, paraffin embedded, sectioned (15 μm), and stained with 5 μg ml^{-1} Hoechst 33258. Digital images of nuclei captured using a cryo-cooled CCD camera attached to an Axioplan microscope (Carl Zeiss, Inc., Jena, Germany) using a constant exposure time were analyzed using NIH image (Version 1.62). This method allowed the DNA content of each nucleus to be correlated with the overall size and spatial position of the cell. Fluorescence from stained mesophyll nuclei of the indicated genotypes was compared with the fluorescence values from nuclei of reference diploid guard cells from the same section, after subtraction of the background from each measurement.

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