Journal of Experimental Botany, Vol. 60, No. 4, pp. 1261–1271, 2009
doi:10.1093/jxb/ern363 Advance Access publication 2 February, 2009
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Salicylic acid deficiency in *NahG* transgenic lines and *sid2* mutants increases seed yield in the annual plant *Arabidopsis thaliana*

Maria Elizabeth Abreu and Sergi Munné-Bosch*

Departament de Biologia Vegetal, Universitat de Barcelona, Facultat de Biologia, Avinguda Diagonal 645, E-08028 Barcelona, Spain

Received 15 December 2008; Accepted 23 December 2008

Abstract

Salicylic acid-deficient *NahG* transgenic lines and *sid2* mutants were used to evaluate the role of this compound in the development of the short-lived, annual plant *Arabidopsis thaliana*, with a particular focus on the interplay between salicylic acid and other phytohormones. Low salicylic acid levels led to increased growth, as well as to smaller abscisic acid levels and reduced damage to PSII (as indicated by F_v/F_m ratios) during the reproductive stages in rosette leaves of *NahG* transgenic lines and *sid2* mutants, compared with wild-type plants. Furthermore, salicylic acid deficiency highly influenced seed yield and composition. Seed production increased by 4.4-fold and 3.5-fold in *NahG* transgenic lines and *sid2* mutants, respectively, compared to the wild type. Salicylic acid deficiency also improved seed composition in terms of antioxidant vitamin concentrations, seeds of salicylic acid-deficient plants showing higher levels of α- and γ-tocopherol (vitamin E) and β-carotene (pro-vitamin A) than seeds of wild-type plants. Seeds of salicylic acid-deficient plants also showed higher nitrogen concentrations than seeds of wild-type plants. It is concluded that (i) the *sid2* gene, which encodes for isochorismate synthase, plays a central role in salicylic acid biosynthesis during plant development in *A. thaliana*, (ii) salicylic acid plays a role in the regulation of growth, senescence, and seed production, (iii) there is a cross-talk between salicylic acid and other phytohormones during plant development, and (iv) the concentrations of antioxidant vitamins in seeds may be influenced by the endogenous levels of salicylic acid in plants.

Key words: Abscisic acid, antioxidant vitamins, phytohormones, salicylic acid, seed production, senescence.

Introduction

The first indication that salicylic acid (SA), a common plant phenolic, plays an important hormonal role in plants came from the study on thermogenesis in the inflorescences of *Arum* lilies (Raskin *et al.*, 1987). Subsequently, SA was shown to be a signal in systemic acquired resistance and to have a role in the resistance to viral, fungal, and bacterial pathogens (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Gaffney *et al.*, 1993; Delaney *et al.*, 1994). The development of acquired resistance often follows a localized tissue death at the site of pathogen penetration called the hypersensitive response, in which SA plays a central role (Delaney *et al.*, 1994). Aside from its effects in plant responses to biotic stress, SA is also believed to play a role in plant responses to abiotic stresses such as ozone and UV light (Yalpani *et al.*, 1994; Sharma *et al.*, 1996; Rao and Davis, 1999), heat stress (Dat *et al.*, 1998; Larkindale and Knight, 2002), chilling and drought (Senaratna *et al.*, 2000), and salt and osmotic stresses (Borsani *et al.*, 2001).

Furthermore, although far less studied, SA has also been demonstrated to be implicated in the regulation of plant development. It has been shown that this compound has a role in the regulation of gene expression during leaf senescence (Morris *et al.*, 2000) and in advancing flowering time in *A. thaliana* (Martínez *et al.*, 2004). Furthermore, it has been shown that SA plays a key role in inhibiting seed germination (Xie *et al.*, 2007). Since SA is thought to

* To whom correspondence should be addressed: E-mail: smunne@ub.edu
© 2009 The Author(s).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

accelerate flowering and leaf senescence, it is hypothesized that these effects might have an impact in seed production.

The recent discovery and use of transgenic plants and mutants in the study of the regulation of developmental processes by phytohormones has been of major significance in our understanding of the role of these compounds in plants. Taking advantage of these approaches, *NahG* transgenic lines and *sid2* mutants of *A. thaliana* were used to evaluate the role of SA in plant development. In higher plants, it is well established that SA derives from the shikimate-phenylpropanoid pathway (Métraux, 2002). Two routes from phenylalanine to SA have been described that differ at the step involving hydroxylation of the aromatic ring. Phenylalanine is first converted into cinnamic acid by phenylalanine ammonia lyase. Then, cinnamic acid can be hydroxylated to form *ortho*-coumaric acid followed by oxidation of the side chain or, alternatively, the side chain of cinnamic acid is initially oxidized to give benzoic acid, which is then hydroxylated in the *ortho* position to give rise to SA. Recently, an important breakthrough in the understanding of SA biosynthesis has been reported (Wildermuth *et al.*, 2001). These authors have mapped the SA-induction-deficient *sid2* mutation to a gene (*ICS1*) encoding isochorismate synthase. *ICS1* is induced locally and systemically upon pathogen infection. The level of SA after infection in *sid2* mutants is only 5–10% of the wild-type levels and resistance to fungal or bacterial pathogens is reduced, making it likely that higher plants produce significant amounts of SA from isochorismate, a biosynthetic pathway typical for bacteria. However, the relative contribution of this pathway to SA biosynthesis during plant development has not yet been tested. Finally, it has been shown that expression of a bacterial *NahG* gene encoding SA hydroxylase leads to large decreases in endogenous SA levels in *NahG* transgenic tobacco and *A. thaliana* plants (Friedrich *et al.*, 1995). These transgenic lines have been used in several studies despite the fact that resulting catechol accumulation could exert some effect on the roles attributed to SA deficiency. It is therefore very important to use these transgenic lines together with mutants deficient in SA biosynthesis unequivocally to unravel particular roles of this compound in plants.

The aim of the present study was to evaluate the role of SA in plant development, with an emphasis on the relationship between SA and other phytohormones. With this purpose, *sid2* mutants and *NahG* transgenic lines, which are deficient in SA, were compared with wild-type plants that accumulate large amounts of SA in the short-lived, annual *A. thaliana* plants.

Materials and methods

Plant material and conditions of study

Seedlings of *Arabidopsis thaliana* Columbia (Col) ecotype, wild-type (Col-0), and the SA-deficient mutant (*sid2*) and transgenic (*NahG*) lines, which were provided by Luis AJ Mur (Institute of Biological Sciences, University of Wales), were grown in pots containing a mixture of peat/perlite/vermiculite (1:1:1, by vol.) in a constant-environment chamber (8 h photoperiod, 90–110 μmol quanta m^{-2} s^{-1}, air temperature 21–23 °C). After 9 weeks of growth, plants were transferred to 16 h photoperiod under the same light and temperature regime to accelerate the developmental programme under long days. Measurements were performed on leaves collected in the middle of the photoperiod after 0, 6, and 11 d (pre-reproductive stages) and 21, 26, and 30 d (reproductive stages) of transfer to 16 h photoperiod. For analyses of chlorophylls, malondialdehyde, phytohormones, and antioxidant vitamins levels, samples were collected, immediately frozen in liquid nitrogen and stored at –80 °C until analysis. For each analysis, samples corresponded to a mixture of leaves of the whole rosette and samples of at least four different individuals situated in different trays were collected. Mature seeds were also collected at the end of the experiment, frozen in liquid nitrogen and stored at –80 °C until analysis.

Photosynthetic pigments, F_v/F_m ratio and lipid peroxidation

Levels of photosynthetic pigments (chlorophylls and carotenoids), the maximum efficiency of PSII photochemistry (F_v/F_m ratio), and the extent of lipid peroxidation in leaves, estimated as the accumulation of malondialdehyde, were measured as described by Munné-Bosch and Lalueza (2007) by using HPLC analyses, a fluorimeter Imaging-PAM (Walz, Effeltrich, Germany), and the thiobarbituric acid-reactive substances assay, respectively.

Leaf water contents and elemental analyses

Relative leaf water content (*RWC*) was determined as $100\times(FW–DW)/(TW–DW)$, where *FW* is the fresh matter, *TW* is the turgid matter after rehydrating the leaves for 24 h at 4 °C in darkness, and *DW* is the dry matter after oven-drying the leaves for 24 h at 80 °C. Total C and N concentrations in leaves and seeds were measured by the Dumas elemental analysis method by using a protein nitrogen analyser NA2100 (Thermo, Milan, Italy).

Phytohormone analyses

Levels of SA, jasmonic acid (JA), abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellin 4 (GA_4), zeatin, and zeatin riboside were simultaneously analysed by HPLCMS/MS with a modification of the methods described by Chiwocha *et al.* (2003) and Abreu and Munné-Bosch (2008). In short, leaf samples were ground in liquid nitrogen and extracted with methanol using sonication. After centrifugation, the pellet was extracted with isopropanol to allow full extraction of cytokinins. The two supernatants were separately collected, dried completely under a nitrogen stream and the extracts immediately reconstituted in methanol. Then the extracts were combined, filtered

through a 0.45 μm PTFE filter (Waters, Milford, MA, USA), and injected into the LC–MS/MS system.

The HPLC system consisted of a Perkin Elmer Series 200 (Norwalk, CT, USA) quaternary pump equipped with an autosampler and an UV detector. For the analysis of the extracts, a Supelco Discovery C18 (Bellefonte, PA, USA) column (150×2.1 mm, 5 μm) was used with a ternary solvent system comprising acetonitrile (A), deionized water (B), and 5% (v/v) glacial acetic acid in water (C). Separations were performed using a gradient of increasing acetonitrile content, a constant glacial acetic acid concentration of 7 mM (pH 3.4), and an initial flow rate of 0.250 ml min^{-1}. The gradient was increased linearly from 1.0% A, 98.2% B, 0.8% C to 45% A, 54.2% B, 0.8% C over 20 min and held for 2 min. The acetonitrile content was then increased linearly to 99.2% A, 0.0% B, and 0.8% C over 6 min. These conditions were held for an additional 2 min with an increased flow rate of 0.35 ml min^{-1}. After 1 min, the initial conditions were restored and allowed to equilibrate for 10 min before the next injection.

MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ont, Canada). All the analyses were performed using the Turbo Ion spray source in positive ion mode (for cytokinins) and negative ion mode (for SA, ABA, JA, IAA, and GA_4) in a single run by switching the mode at 19 min. MRM acquisition was done monitoring the following transitions: SA, 137/93; ABA, 263/153: JA, 209/59; IAA, 174/130; GA_4, 345/221; zeatin, 220/136; zeatin riboside, 352/220). The declustering potential and collision energy were optimized for each compound. Quantification by MS/MS using the MRM method was performed as described by Abreu and Munné-Bosch (2008). The MRM mode was required because many compounds could present the same nominal molecular mass, but the combination of the parent mass and unique fragment ions was used selectively to monitor each of the compounds analysed. Results were corrected taking into account the specific recovery rates for each compound (recovery rates were above 80% in all cases).

Antioxidant vitamins analyses

Levels of carotenoids and tocopherols were measured by HPLC as described by Munné-Bosch and Alegre (2000).

Statistical analyses

Differences between treatments were evaluated by an analysis of variance (ANOVA) or Student's *t* tests, and were considered significant at a probability level of $P \leqslant 0.05$.

Results

SA deficiency in NahG *transgenic lines and* sid2 *mutants*

Expression of SA hydroxylase in *NahG* transgenic lines and the mutation of *sid2*, the gene encoding isochorismate synthase, both led to drastic decreases in SA levels in leaves and seeds (Fig. 1). Leaves of *NahG* transgenic lines and *sid2* mutants showed around 25% SA levels compared with those of wild-type plants at the beginning of the experiment (under short days, day 0). While SA levels increased by 2.2-fold during the transition to flowering after 21 d exposure of plants to long days in wild-type plants, SA levels remained at low levels in the *sid2* mutants, thus indicating that isochorismate synthase plays a major role in SA biosynthesis during the transition to flowering in *A. thaliana*. Indeed, SA levels decreased progressively with plant development in both *NahG* transgenic lines and *sid2* mutants, reaching very low SA levels at the end of the experiment (1 ng and 25 ng [g dry wt]$^{-1}$, respectively, compared to *ca.* 820 ng [g dry wt]$^{-1}$ in wild-type plants at day 31). *NahG* transgenic lines and *sid2*

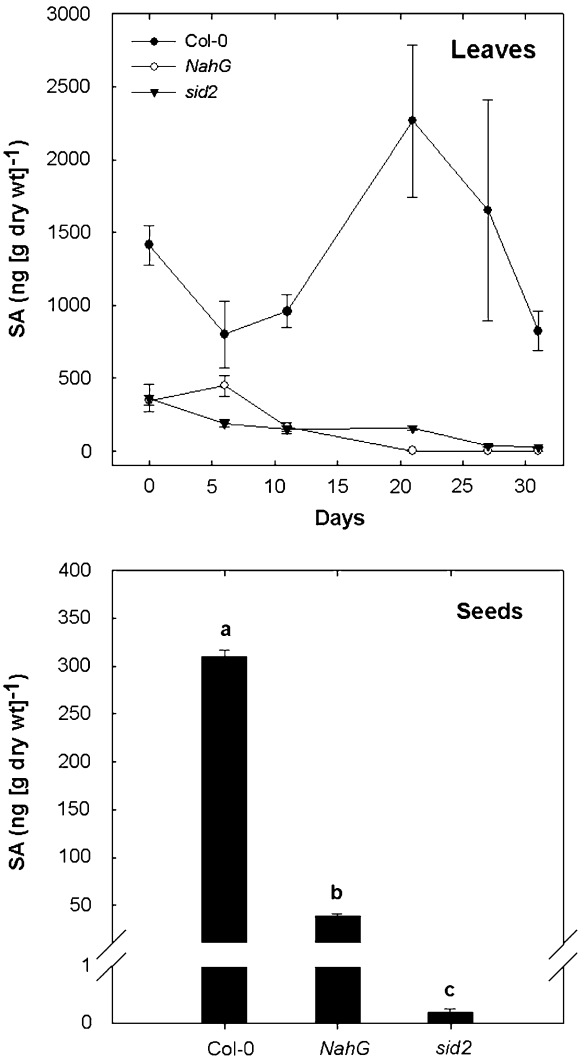

Fig. 1. Endogenous salicylic acid (SA) concentrations in leaves and seeds of wild type and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*. Plants were transferred from short days to long days and leaf samples analysed at pre-reproductive (days 0, 6, and 11) and reproductive stages (days 21, 27, and 31). Data represent the mean ±SE of four measurements. Letters indicate statistically significant differences between seeds of different plant groups at a probability level of $P \leqslant 0.05$.

mutants also displayed a sharp depletion of SA levels in seeds, which was particularly evident in the latter, thus indicating that the *sid2* gene plays a central role in SA biosynthesis not only in leaves, but also in seeds. These differences in SA accumulation led us to evaluate the role of this compound in plant development, with a special emphasis on leaf senescence and seed production and to examine how these two developmental processes are interconnected in *A. thaliana*.

SA deficiency increases growth and influences leaf senescence in A. thaliana

It is well known that when *A. thaliana* plants are transferred from short days to long days, the developmental programme is accelerated to take advantage of favourable conditions for reproduction. To study whether or not SA could play a role in this process, biochemical markers of senescence in leaves of SA-deficient and wild-type plants were compared. The F_v/F_m ratio revealed that SA deficiency in *NahG* transgenic lines and *sid2* mutants is associated with reduced damage to the photosynthetic apparatus compared with wild-type plants, despite the fact that leaf water and nutrient contents, as well as chlorophyll levels and C/N ratios, were similar in the three plant groups examined (with the exception of slightly, although not significantly higher chlorophyll levels and C/N ratios in *NahG* transgenic lines) (Fig. 2). It is noteworthy that leaf yellowing started just after the transfer to the long photoperiod in the three plant groups, while the F_v/F_m ratio decreased in wild-type plants only. Differences in the F_v/F_m

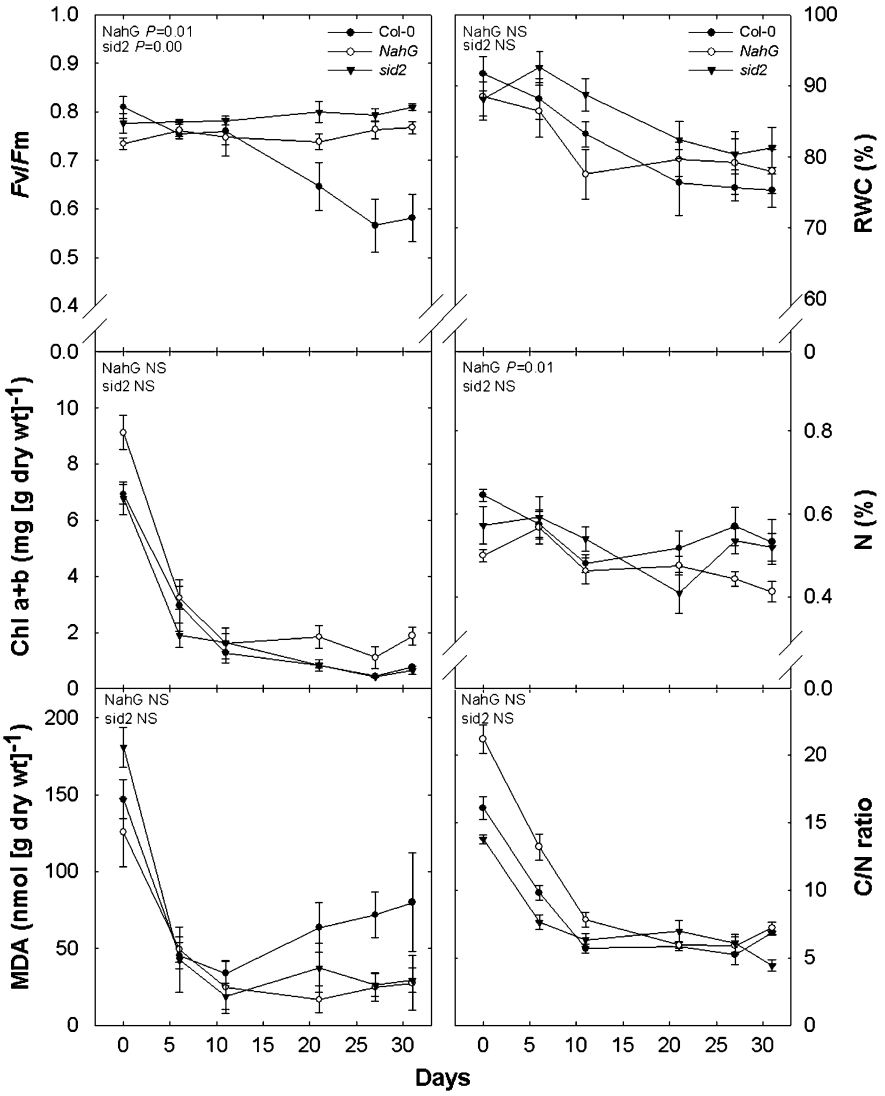

Fig. 2. Chlorophyll *a+b* levels, maximum efficiency of PSII photochemistry (F_v/F_m ratio, indicative of damage to PSII), malondialdehyde levels (MDA, an estimation of lipid peroxidation), relative leaf water content (*RWC*), nitrogen (N) concentration, and carbon/nitrogen (C/N) ratio in leaves of wild type and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*. Plants were transferred from short days to long days and leaf samples analysed at pre-reproductive (days 0, 6, and 11) and reproductive stages (days 21, 27, and 31). Data represent the mean ±SE of four measurements. Significance of plant group-generated changes (*NahG* and *sid2* versus the wild type) is depicted inside the panels (results of ANOVA). Differences were considered significant at a probability level of $P \leqslant 0.05$. NS, not significant.

ratio were apparent during the reproductive stages only, after SA increased by 2.2-fold in wild-type plants (Fig. 1). It is also worth mentioning that leaf rosette biomass at the early stages of reproduction (once flowering has occurred in the three plant groups, day 21) was of 2.04±0.10, 3.50±0.51, and 3.48±0.18 g fresh weight in the wild type, *sid2* mutants, and *NahG* transgenic lines, respectively, thus indicating increased leaf biomass production in SA-deficient plants (Fig. 3).

Hormonal balance in wild-type and SA-deficient plants

An analysis of the hormonal balance of leaves during plant development (Fig. 4) showed that SA accumulation during the transition to flowering in wild-type plants led to parallel (around 2-fold) increases in ABA and JA levels, while the levels of IAA, zeatin, and zeatin riboside were not significantly altered during this period. SA-deficient plants (both *NahG* transgenic lines and *sid2* mutants) showed smaller ABA levels in leaves during the reproductive stages than wild-type plants, thus indicating that those changes in the hormonal balance were mediated, either directly or indirectly, by SA. Furthermore, JA remained at low amounts and IAA levels increased during the reproductive stages in leaves of *NahG* transgenic lines, but not in *sid2* mutants. These mutants contained instead lower GA_4 and cytokinin levels in leaves throughout the experiment, particularly of zeatin, compared to the wild type. In this case, however, differences were already apparent at the beginning of the experiment.

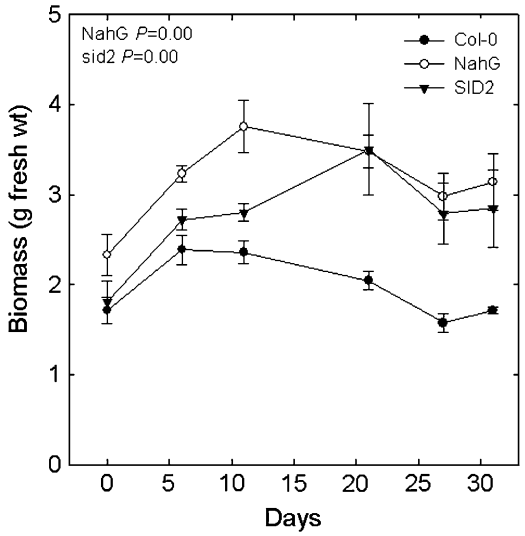

Fig. 3. Time-course evolution of plant growth in wild type and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*. Plants were transferred from short days to long days and leaf rosette biomass measured at pre-reproductive (day 0, 6, and 11) and reproductive stages (days 21, 27, and 31). Data represent the mean ±SE of five individuals. Significance of plant group-generated changes (*NahG* and *sid2* versus the wild type) is depicted inside the panels (results of ANOVA). Differences were considered significant at a probability level of $P \leqslant 0.05$.

SA deficiency influences seed yield and composition

Seed production was drastically increased in *NahG* transgenic lines and *sid2* mutants compared to wild-type plants (Table 1). Although the seed weight and seed number per fruit was similar in the three plant groups, total seed and fruit numbers per plant were drastically increased in SA-deficient compared to wild-type plants. The total number of seeds produced per plant was of 56 500 seeds for wild-type plants compared to 244 500 and 194 600 seeds for *NahG* transgenic lines and *sid2* mutants, respectively. This was caused by the decreased production of the number of reproductive side shoots, consequently reducing the number of fruits and seeds produced per plant (Table 1).

The analysis of hormonal levels in seeds did not reveal significant differences between wild-type plants and *NahG* transgenic lines, whose seeds at maturity contained SA levels of *ca.* 310 ng and 40 ng [g dry wt]$^{-1}$, respectively. Seeds of *sid2* mutants, which contained SA levels of *ca*. 0.2 ng [g dry wt]$^{-1}$ showed, however, significant alterations in the hormonal balance. The levels of IAA in seeds of the *sid2* mutants increased 5-fold compared with those of the wild type and *NahG* transgenic lines, while the levels of the cytokinins, zeatin, and zeatin riboside were reduced by *ca.* 50% in seeds of this mutant compared to wild type (Fig. 5).

The concentrations of antioxidant vitamins in seeds were also highly influenced by SA (Fig. 6). The levels of β-carotene (pro-vitamin A) were drastically increased in SA-deficient plants, the seeds of wild-type plants showing some traces of this antioxidant compared to high levels in SA-deficient plants (*ca.* 6 and 15 ng [g dry wt]$^{-1}$ in *NahG* and *sid2*, respectively). α- and γ-tocopherol levels also increased (5-fold and 2-fold, respectively) in *sid2* mutants, which showed the highest concentrations of these antioxidant vitamins in seeds, compared to wild-type plants. Elemental analyses of seeds also revealed important differences in nutrient composition. Total nitrogen concentrations were 13% and 50% higher in seeds of *NahG* transgenic lines and *sid2* mutants, respectively, than in those of wild-type plants. Furthermore, C/N ratios were 26% higher in seeds of *sid2* mutants compared to those of wild-type plants, while C/N ratios did not differ significantly between *NahG* transgenic lines and the wild type (Fig. 7). It is worth noting that, despite seeds of both *NahG* transgenic lines and *sid2* mutants being deficient in SA compared to the wild type, major differences in SA levels were observed between *NahG* transgenic lines and *sid2* mutants (40 versus 0.2 ng [g dry wt]$^{-1}$, Fig. 1).

Discussion

Diversity in life style leads to profound differences in growth and reproductive strategies, as well as in the progression of leaf and whole-plant senescence among different plant species (Munné-Bosch, 2008). The model plant, *A. thaliana* is a short-lived monocarpic plant, with a life history characterized by a single massive reproductive

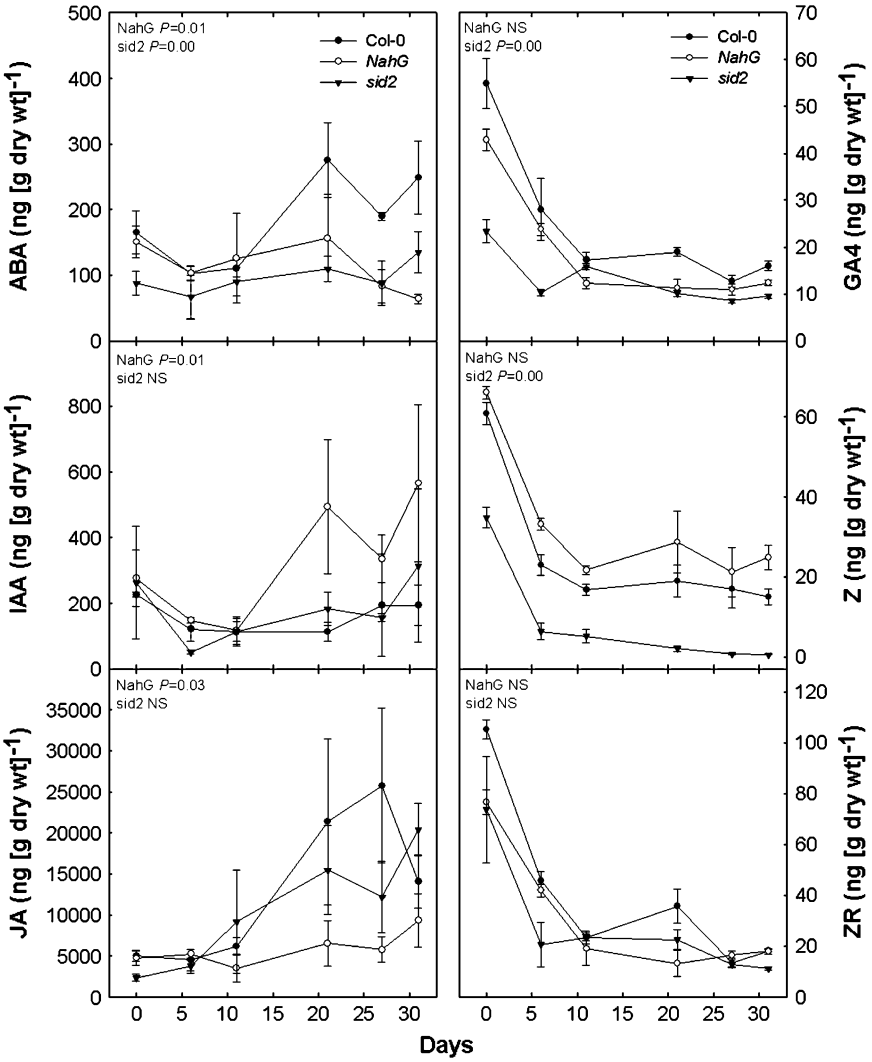

Fig. 4. Endogenous concentrations of abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA), gibberellin 4 (GA_4), zeatin (Z), and zeatin riboside (ZR) in leaves of wild type and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*. Plants were transferred from short days to long days and leaf samples analysed at pre-reproductive (days 0, 6, and 11) and reproductive stages (days 21, 27, and 31). Data represent the mean ±SE of four measurements. Significance of plant group-generated changes (*NahG* and *sid2* versus the wild type) is depicted inside the panels (results of ANOVA). Differences were considered significant at a probability level of $P \leqslant 0.05$. NS, not significant.

Table 1. Seed and fruit production in wild-type plants and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*

Data represent the mean ±SE of four measurements. Letters indicate statistical significant differences between different plant groups at a probability level of $P \leqslant 0.05$.

	Seed weight (mg/100 seeds)	Seed number per fruit	Fruit number	Total seed number (×1000)	Side shoot number
Col-0	2.21±0.08	37.8±2.9	167.0±15.6 a	56.5±8.1 a	7.8±0.5 a
*Nah*G	1.95±0.08	41.8±2.4	407.0±19.0 b	244.5±48.1 b	13.2±2.0 b
sid2	1.93±0.04	45.4±3.6	300.4±31.7 c	194.6±51.4 b	12.2±1.4 b

episode, which is followed by the death of the organism. In these plants, reproductive development often governs the senescence of leaves, such that nutrients are mobilized from senescing leaves to reproductive tissues before plant death occurs.

Source–sink relations (e.g. for nitrogen and carbohydrates) between the senescing leaves (source) and the reproductive organs (sink) influence the progression of leaf senescence to a great extent (Wingler *et al.*, 1998; Díaz *et al.*, 2005). In previous studies, it has been shown that SA has a role in the regulation of gene expression during leaf senescence (Morris *et al.*, 2000; Buchanan-Wollaston *et al.*, 2005) and that SA accelerates the progression of leaf senescence, particularly during its latest stages inducing cell death (García-Heredia *et al.*, 2008). It has been shown in the present study that SA deficiency reduces damage to PSII during plant senescence, thus extending leaf lifespan and increasing seed production. It appears therefore that SA accumulation in wild-type *A. thaliana* plants accelerates the developmental programme at the expense of reducing seed

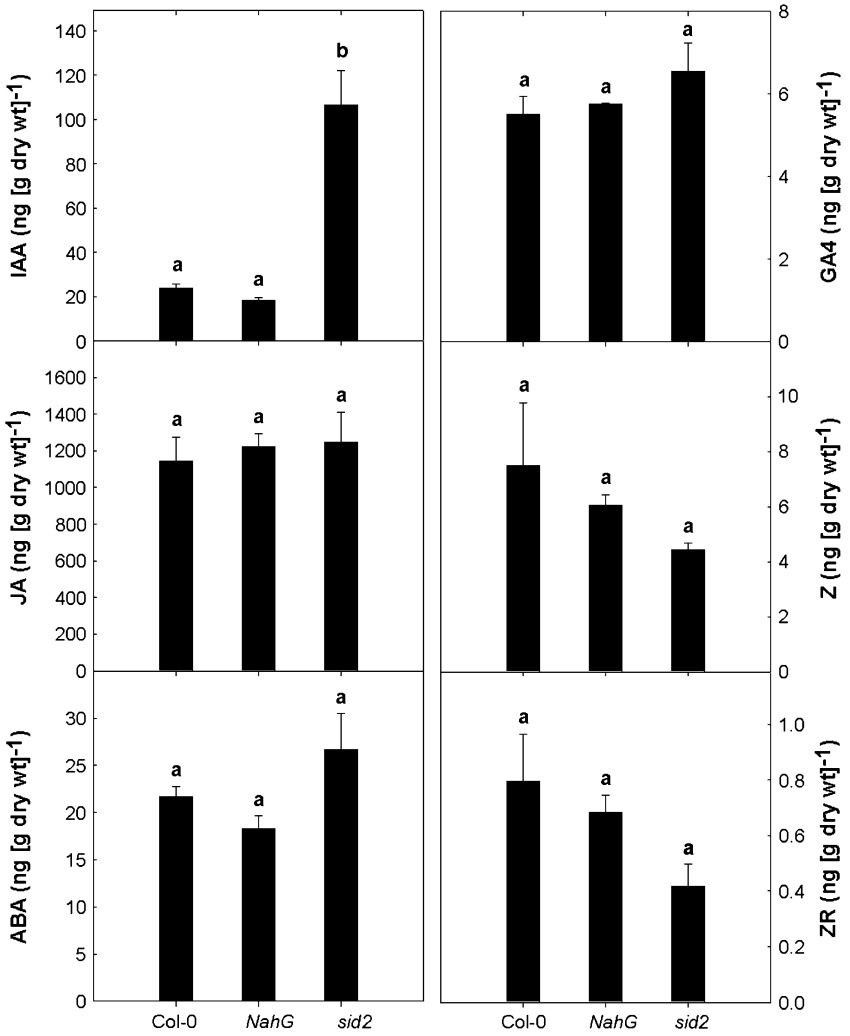

Fig. 5. Endogenous concentrations of indole-3-acetic acid (IAA), jasmonic acid (JA), abscisic acid (ABA), gibberellin 4 (GA_4), zeatin (Z), and zeatin riboside (ZR) in seeds of wild type and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*. Data represent the mean ±SE of four measurements. Letters indicate statistical significant differences between seeds of different plant groups at a probability level of $P \leqslant 0.05$.

production, so that rapid reproduction is achieved at the cost of reducing seed yield in this short-lived species. Furthermore, it is known that SA has additional adaptative advantages, increasing plant resistance to pathogens (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Gaffney *et al.*, 1993; Delaney *et al.*, 1994) and several environmental stresses (Yalpani *et al.*, 1994; Sharma *et al.*, 1996; Dat *et al.*, 1998; Rao and Davis, 1999; Senaratna *et al.*, 2000; Borsani *et al.*, 2001; Larkindale and Knight, 2002). It should therefore be noted that although it is shown in the present study that SA deficiency increases seed production and ameliorates seed composition in terms of antioxidant vitamins and nitrogen contents, a deficiency in SA can be deleterious to plant function, especially under adverse growth conditions. So it is the case that, to date, no mutants or transgenic plants with complete SA deficiency have been shown to be viable.

To date, the role of SA in seed production has been poorly understood. While exogenous application of this compound has been shown to increase seed production in some species (Joaquín *et al.*, 2007), another study has shown that this compound inhibits seed production in *A. thaliana* plants that constitutively over-produce SA in leaves (Mauch *et al.*, 2001). In the latter study, genetic manipulation of SA biosynthesis was achieved by fusing the two bacterial genes *pchA* and *pchB* from *Pseudomonas aeruginosa,* which encode isochorismate synthase and isochorismate pyruvate-lyase and the *pchB-A* fusion was expressed in *A. thaliana* under the control of the constitutive cauliflower mosaic virus (CaMV) *35S* promoter. In these plants, however, constitutive over-production of SA not only caused a reduction of seed yield, but also resulted in a dwarfed phenotype. In the present study, a direct relationship between SA and seed production is demonstrated for the first time by using SA-deficient *A. thaliana* plants.

The increase in seed production in SA-deficient plants may be explained by several reasons that underlie interconnected processes. First, damage to the photosynthetic

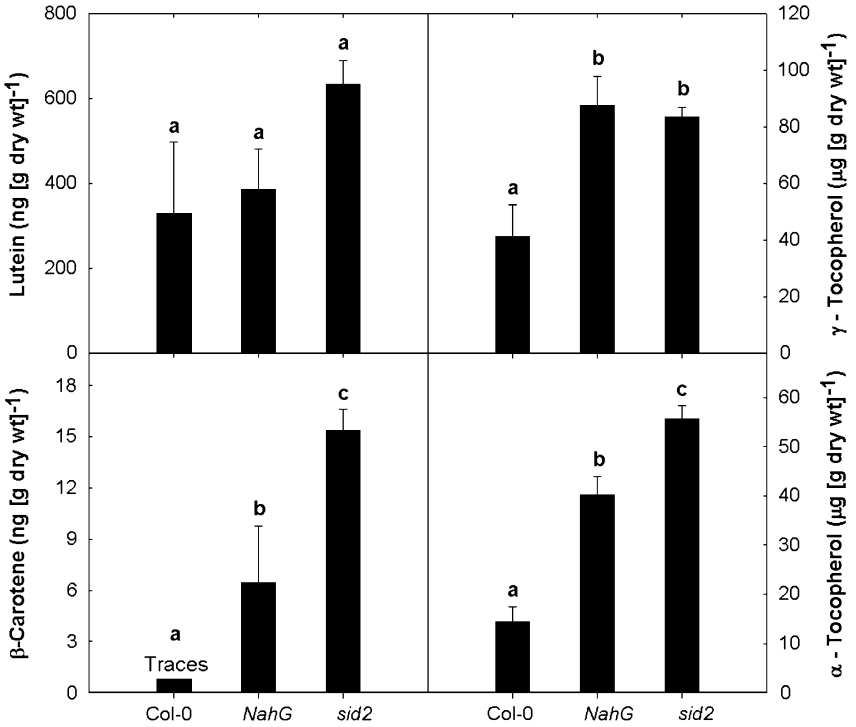

Fig. 6. Endogenous concentrations of the carotenoids, lutein, and β-carotene (pro-vitamin A) and α- and γ-tocopherols (vitamin E) in seeds of wild type and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*. Data represent the mean ±SE of four measurements. Letters indicate statistical significant differences between seeds of different plant groups at a probability level of $P \leqslant 0.05$.

apparatus was reduced in SA-deficient plants, while leaf yellowing progressed similarly in the three plant groups, thus suggesting that the terminal phase of leaf senescence is delayed in SA-deficient plants, which is in agreement with previous studies (García-Heredia *et al.*, 2008). Slowing down the progression of leaf senescence in SA-deficient plants increases the time needed, and therefore the possibilities to transport nutrients from source, senescing leaves to sink, reproductive organs. Second, increased biomass production during the vegetative phase also favours the translocation of nutrients from senescing leaves to reproductive organs. Third, it appears that the increase in seed yield was caused by an increased production of the number of reproductive side shoots, consequently increasing the number of fruits and seeds produced per plant. It is worth noting that senescence was associated with a similar rate of nutrient translocation from sources to sinks, as suggested by the time-course evolution of the C/N ratios in the leaf rosettes of the three plant groups. Furthermore, seeds of SA-deficient plants contained higher N concentrations than the wild type. It appears, therefore, that increased seed production was mainly supported, among other factors, by an extension of the leaf lifespan, increased vegetative growth, and increased formation of side reproductive shoots in SA-deficient plants.

Furthermore, we did intend to identify relationships between SA and other phytohormones in the regulation of plant development in *A. thaliana*. One of the most interesting observations was the parallel increase in the endogenous levels of SA and ABA in leaf rosettes during the transition to flowering in wild-type plants, thus suggesting that SA may influence, at least to some extent, the endogenous concentrations of ABA in *A. thaliana*. However, probably due to the analyses of a single compound, we failed to demonstrate an increase of GAs during the transition to flowering in *A. thaliana*, when it is known that this numerous group of phytohormones control flowering time in this species (Achard *et al.*, 2004). Indeed, GA_4 levels in leaf rosettes were constant during the transition to flowering. In any case, the endogenous levels of GA_4 were slightly higher in wild type than in SA-deficient plants, thus indicating that SA also influences to some extent GA levels in plants.

Another interesting observation made in the present study was a putative relationship between SA, JA, auxins, and cytokinins. JA remained at low amounts and IAA levels increased during the reproductive stage in leaves of *NahG* transgenic lines, thus suggesting a link between SA, JA, and auxins. Indeed, it has been shown that plants accumulating SA in leaves display morphological phenotypes that are reminiscent of auxin-deficient or auxin-insensitive mutants, indicating that SA might interfere with auxin responses (Wang *et al.*, 2007). However, this was not confirmed in *sid2* mutants, in which IAA levels in leaves did not increase. These mutants, however, contained lower cytokinin levels in leaves, particularly of zeatin. By contrast, the levels of IAA in seeds of the *sid2* mutants increased 5-fold compared with those of the wild type and *NahG* transgenic lines, while the levels of zeatin and zeatin riboside were reduced by *ca.* 50% in seeds of the *sid2* mutant compared with the wild type. Although the results are not

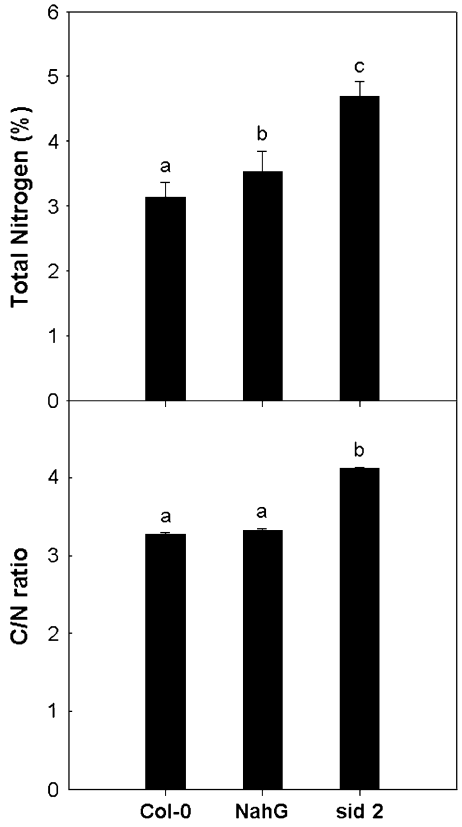

Fig. 7. Total N concentration and C/N ratio in seeds of wild type and SA-deficient *NahG* transgenic lines and *sid2* mutants of *A. thaliana*. Data represent the mean ±SE of four measurements. Letters indicate statistical significant differences between seeds of different plant groups at a probability level of $P \leqslant 0.05$.

conclusive and further research is needed, these results suggest that, aside from the SA-mediated control of ABA levels, there is also cross-talk between SA, JA, auxins, and cytokinins during plant development in *A. thaliana*, thus reflecting that all phytohormones interact in the regulation of plant development.

It has also been shown that the *sid2* gene, which encodes for isochorismate synthase, plays a central role in SA biosynthesis during plant development, not only in leaves but also in seeds of *A. thaliana*. Furthermore, it appears that this effect is more evident at advanced developmental stages, since rosette leaves of *sid2* mutants contained much less SA at the reproductive stages than prior to flowering. Only minimal levels of SA have been detected in *sid2* mutants after infection, UV or ozone exposure (Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001). Furthermore, it is shown here that seeds of *sid2* mutants contain very low SA levels compared to seeds of the wild type (0.2 versus 310 ng [g dry wt]$^{-1}$, respectively) Taken together, these results provide a strong support for isochorismate as a precursor of developmental, aside from stress-, induced SA accumulation in *A. thaliana*. The biosynthetic pathway for SA in *A. thaliana* is therefore related to that described in bacteria where SA is synthesized from chorismate via the rate-limiting enzyme isochorismate synthase and isochorismate pyruvate lyase (reviewed in Verberne *et al.*, 1999).

It has been shown that the levels of antioxidant vitamins (tocopherols and carotenoids) and nitrogen concentrations in seeds may be highly influenced by SA. The concentrations of vitamin E and pro-vitamin A increased sharply, while nitrogen concentrations increased up to 50% in seeds of SA-deficient plants compared with the wild type. Furthermore, C/N ratios increased in seeds of SA-deficient plants, although in *sid2* mutants only, in which SA deficiency is almost complete. It is interesting to note differences between seeds of *NahG* transgenic lines and *sid2* mutants; the latter containing much less salicylic acid, but 5-fold higher levels of indole-3-acetic acid, as well as higher nitrogen concentrations and C/N ratios. Although the interplay between SA and auxins in seeds and the molecular mechanisms implicated in the SA-dependent regulation of seed composition in *Arabidopsis* remains unknown, these results provide new insights into alternative ways of manipulating levels of antioxidant vitamins and nutrient concentrations in seeds. Manipulating the biosynthesis or degradation of SA to reduce the levels of this compound in plants may therefore represent a means not only to increase seed yield but also to improve seed composition, which applied to crops, may have a strong impact in human health.

Finally, it is worth noting that *sid2* mutants and *NahG* transgenic lines differed in some of the physiological parameters analysed. While differences in seeds (for instance, enhanced IAA accumulation and increased C/N ratios in *sid2* mutants compared to *NahG* transgenic lines) might be attributed to differences in SA accumulation (0.2 ng versus 40 ng [g dry wt]$^{-1}$, respectively), differences in physiological parameters in leaves (for instance, concentrations of some phytohormones) are more difficult to explain based on differences in SA levels, since endogenous concentrations of SA did not differ significantly between *sid2* mutants and *NahG* transgenic lines (Table 2). The possibility exists that the observed phenotype in *sid2* mutants is a result of a collateral mutation mediated by the mutagen treatment applied and not only due to the inactivation of SID2, as well as the fact that catechol accumulation in *NahG* transgenic lines could influence phytohormone accumulation in plants. Further studies with the inclusion of individuals with different SID2 alleles are therefore warranted to unravel the causes of differences observed between *sid2* mutants and *NahG* transgenic lines.

It is concluded that (i) the *sid2* gene, which encodes for isochorismate synthase, plays a central role in SA biosynthesis during plant development not only in leaves, but also in seeds of *A. thaliana*, (ii) SA plays a role in the regulation of growth, senescence, and seed production, (iii) there is a cross-talk between SA and other phytohormones during plant development, and (iv) the concentrations of antioxidant vitamins in seeds may be influenced by the endogenous levels of SA in plants. Further research aimed at better understanding the mechanisms involved in the regulation of these physiological and biochemical processes by SA

Table 2. Statistical differences in the physiological parameters measured in leaves between plant groups

Differences, indicated by an asterisk, were considered significant at a probability level of $P \leqslant 0.05$. NS denotes no significant difference.

	sid2 versus *NahG*	*sid2* versus wild type	*NahG* versus wild type
SA	NS	*	*
Chl *a+b*	NS	NS	NS
F_v/F_m	NS	*	*
MDA	NS	NS	NS
RWC	NS	NS	NS
Biomass	NS	*	*
N	*	NS	*
C/N	NS	NS	NS
ABA	NS	*	*
IAA	NS	NS	*
JA	*	NS	*
GA_4	*	*	NS
Z	*	*	NS
ZR	NS	NS	NS

and how this compound interacts with other phytohormones in their regulation is warranted.

Acknowledgements

We are grateful to Luis AJ Mur (Institute of Biological Sciences, University of Wales, UK) for providing us with the seeds of SA-deficient plants. We also thank the Serveis de Camps Experimentals and Serveis Científico-Tècnics (University of Barcelona, Spain) for technical assistance. This work was supported by the BFU2006-01127 grant from the Spanish Government.

References

Abreu ME, Munné-Bosch S. 2008. Salicylic acid may be involved in the regulation of drought-induced leaf senescence in perennials: a case study in field-grown *Salvia officinalis* L. plants. *Environmental and Experimental Botany* **64,** 105–112.

Achard P, Herr A, Baulcombe DC, Harberd NP. 2004. Modulation of floral development by a gibberellin-regulated microRNA. *Development* **131,** 3357–3365.

Borsani O, Valpuesta V, Botella MA. 2001. Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. *Plant Physiology* **6,** 1024–1030.

Buchanan-Wollaston V, Page T, Harrison E, *et al*. 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. *The Plant Journal* **42,** 567–585.

Chiwocha SDS, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross ARS, Kermode AR. 2003. A method for profiling classes of plant hormones and their metabolites using liquid chromatography–electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. *The Plant Journal* **35,** 405–417.

Dat JF, Foyer CH, Scott IM. 1998. Changes in salicylic acid and antioxidants during induction of thermotolerance in mustard seedlings. *Plant Physiology* **118,** 1455–1461.

Delaney TP, Ukness S, Vernooij B, *et al*. 1994. A central role of salicylic acid in plant disease resistance. *Science* **266,** 1247–1250.

Díaz C, Purdy S, Christ A, Morot-Gaudry J-F, Wingler A, Masclaux-Daubresse C. 2005. Characterization of markers to determine the extent and variability of leaf senescence in Arabidopsis. A metabolic profiling approach. *Plant Physiology* **138,** 898–908.

Friedrich L, Vernooij B, Gaffney T, Morse A, Ryals J. 1995. Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Molecular Biology* **5,** 959–968.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* **261,** 754–756.

García-Heredia JM, Hervás M, De la Rosa MA, Navarro JA. 2008. Acetylsalicylic acid induces programmed cell death in *Arabidopsis* cell cultures. *Planta* **228,** 89–97.

Joaquín TBM, Trejo C, Hernández-Garay A, Pérez PJ, García S, Quero CAR. 2007. Effects of ethephon, salicylic acid and cidef-4 on the yield and quality of guinea grass seed. *Tropical Grasslands* **41,** 55–60.

Larkindale J, Knight MR. 2002. Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiology* **128,** 682–695.

Malamy J, Carr JP, Klessig DF, Raskin I. 1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250,** 1002–1004.

Martínez C, Pons E, Prats G, León J. 2004. Salicylic acid regulates flowering time and links defence responses and reproductive development. *The Plant Journal* **37,** 209–217.

Mauch F, Mauch-Mani B, Gaille C, Kull B, Haas D, Reimmann C. 2001. Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. *The Plant Journal* **25,** 67–77.

Métraux JP. 2002. Recent breakthroughs in the study of salicylic acid biosynthesis. *Trends in Plant Science* **7,** 332–334.

Métraux JP, Signer H, Ryals J, Ward E, Wyssbenz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* **250,** 1004–1006.

Morris K, AH-Mackerness S, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V. 2000. Salicylic acid has a role in regulating gene expression during leaf senescence. *The Plant Journal* **23,** 677–685.

Munné-Bosch S. 2008. Do perennials really senesce? *Trends in Plant Science* **13,** 216–220.

Munné-Bosch S, Alegre L. 2000. Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. *Planta* **210,** 925–931.

Munné-Bosch S, Lalueza P. 2007. Age-related changes in oxidative stress markers and abscisic acid levels in a drought-tolerant shrub, *Cistus clusii* grown under Mediterranean field conditions. *Planta* **225,** 1039–1049.

Nawrath C, Métraux JP. 1999. Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *The Plant Cell* **11,** 1393–1404.

Raskin I, Ehmann A, Melander WR, Meusse BJD. 1987. Salicylic acid: a natural inducer of hear production in *Arum* lilies. *Science* **237,** 1601–1602.

Rao M, Davis KR. 1999. Ozone-induced lesion formation occurs via two distinct mechanisms in Arabidopsis: the role of salicylic acid. *The Plant Journal* **17,** 603–614.

Senaratna T, Touchell D, Bunn T, Dixon R. 2000. Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. *Plant Growth Regulation* **30,** 157–161.

Sharma YK, León J, Raskin I, Davis KR. 1996. Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defence-related transcripts and induced resistance. *Proceedings of the National Academy of Sciences, USA* **93,** 5099–5104.

Verberne MC, Budi Muljono RA, Verpoorte R. 1999. Salicylic acid biosynthesis. In: Libbenga K, Hall M, Hooykaas PJJ, eds. *Biochemistry and molecular biology of plant hormones*, Vol. 33. London: Elsevier, 295–312.

Wang D, Pajerowska-Mukhtar K, Hendrickson A, Dong X. 2007. Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. *Current Biology* **17,** 1784–1790.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414,** 562–565.

Wingler A, von Schaewen A, Leegood RC, Lea PJ, Quick WP. 1998. Regulation of leaf senescence by cytokinin, sugars, and light. *Plant Physiology* **116,** 329–335.

Xie Z, Zhang ZL, Hanzlik S, Cook E, Shen QXJ. 2007. Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic acid-inducible WRKY gene. *Plant Molecular Biology* **64,** 293–303.

Yalpani N, Enyedi AJ, León J, Raskin I. 1994. Ultraviolet light and ozone stimulate accumulation of salicylic acid and pathogenesis related proteins and virus resistance in tobacco. *Planta* **193,** 373–376.