Isochorismate synthase is required to synthesize salicylic acid for plant defence

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Salicylic acid (SA) mediates plant defences against pathogens, accumulating in both infected and distal leaves in response to pathogen attack[1–5]. Pathogenesis-related gene expression and the synthesis of defensive compounds associated with both local and systemic acquired resistance (LAR and SAR) in plants require SA. In *Arabidopsis*, exogenous application of SA suffices to establish SAR, resulting in enhanced resistance to a variety of pathogens. However, despite its importance in plant defence against pathogens, SA biosynthesis is not well defined. Previous work has suggested that plants synthesize SA from phenylalanine[6–10]; however, SA could still be produced when this pathway was inhibited[6,8], and the specific activity of radiolabelled SA in feeding experiments was often lower than expected[7,8]. Some bacteria such as *Pseudomonas aeruginosa* synthesize SA using isochorismate synthase (ICS) and pyruvate lyase[11]. Here we show, by cloning and characterizing an *Arabidopsis* defence-related gene (*SID2*) defined by mutation, that SA is synthesized from chorismate by means of ICS, and that SA made by this pathway is required for LAR and SAR responses.

In *Arabidopsis*, two putative ICS genes, *ICS1* (GenBank accession numbers AC011765 and AC008263) and *ICS2* (AC011809), are located at the bottom and top of chromosome I, respectively. To test the hypothesis that plants use ICS to synthesize SA, we first examined whether pathogen treatment of *Arabidopsis* induces either of these *ICS* genes. Expression of *ICS1* occurred in leaves infected with the fungal biotroph *Erysiphe orontii* or the bacterial necrotroph *Pseudomonas syringae* pv. *maculicola*. In addition, when *Arabidopsis* plants were inoculated with an avirulent strain of the *Pseudomonas* pathovar *maculicola*, resulting in SA accumulation and SAR in systemic leaves, *ICS1* was systemically induced (data not shown). The timing of *ICS1* expression is similar to that of SA accumulation for these pathogen treatments and is correlated with expression of the pathogenesis-related gene *PR1*, a molecular marker of SAR[12,13] (see Fig. 1). We detected no *ICS2* transcript in either infected or uninfected *Arabidopsis* leaves (Fig. 1).

If the product of the pathogen-inducible *ICS1* gene is involved in defence-related SA biosynthesis, an *ics1* mutant should exhibit reduced SA accumulation in response to pathogens, reduced pathogenesis-related gene expression, and enhanced susceptibility to pathogens. Two allelic *Arabidopsis* mutants, *sid2-1* (ref. 14) and *sid2-2/eds16-1* (ref. 15), exhibit these phenotypes and have been mapped to the bottom of chromosome I, near the *ICS1* locus. To ascertain whether the *sid2* mutants contain mutations in *ICS1*, we first examined the fast-neutron-generated *sid2-2* mutant. Fast neutron mutagenesis typically causes deletions resulting in loss of transcription of the affected gene. We did not detect *ICS1* transcript in *Erysiphe*-infected leaves of *sid2-2* (Fig. 2a). Moreover, amplification of *sid2-2* genomic DNA using a series of *ICS1*-specific primers resulted in aberrant polymerase chain reaction (PCR) products. Specifically, altered products using primers in exon IX of *ICS1* suggested that a region of at least 50 nucleotides was affected (data not shown). We confirmed the presence of a significant deletion/rearrangement in exon IX by DNA blot analysis (Fig. 2b, c). To determine whether *sid2-1* (a mutant generated by treatment with ethylmethane sulphonate, EMS) contains a mutation in *ICS1*, we sequenced *ICS1* genomic DNA from *sid2-1* and wild-type plants. *sid2-1* contains a single base-pair (bp) mutation resulting in a stop codon in exon IX (Fig. 2c, d). Consequently, the mutations in both *sid2* alleles should disrupt the highly conserved chorismate-binding domain (see below).

Comparison of *Arabidopsis ICS1* with plant *ICS* messenger RNA sequences (AJ006065 and AF078080) revealed that *Arabidopsis* ICS1 probably contains an additional amino-terminal extension not annotated for *Arabidopsis* ICS1. We therefore sequenced *Arabidopsis ICS1* complementary DNA, isolated using rapid amplification of cDNA ends (RACE)-PCR, and confirmed that an extra N-terminal extension of 66 amino acids is present in *ICS1* (Fig. 2c). This extension, absent from bacterial ICS sequences, is characteristic of chloroplast transit sequences and contains a putative cleavage site. The carboxy-terminal region of *Arabidopsis* ICS1 contains the highly conserved chorismate-binding domain (Fig. 2d). Within this domain, ICS1 contains residues essential for activity of another chorismate-binding enzyme, anthranilate synthase, except that ICS1 has an Ala rather than a Thr residue at position 472, consistent with other ICS sequences[16]. Overall, the *Arabidopsis* ICS1 sequence is 57% identical to ICS from *Catharanthus roseus* (Madagascar periwinkle), and greater than 20% identical to bacterial isochorismate synthases (Fig. 2e). Biochemical activity of these *ICS* gene products has been confirmed[16,11].

In *Arabidopsis*, addition of SA induces SAR and the removal of SA by the bacterial transgene *nahG* abolishes SAR[1,2]. In *sid2* mutants, total SA accumulation is about 5–10% of wild-type levels in response to either the virulent biotroph *Erysiphe* or avirulent strains of *Pseudomonas syringae*[14,15], and is similar in magnitude to that of uninfected plants (Fig. 3a). We also assessed levels of pathogenesis-related protein 1 (PR1) because *PR1* is a SAR molecular marker downstream of SA accumulation—*PR1* is induced at very low levels in *sid2* mutants, approximately 1–10% of wild-type levels[14,15] (Fig. 3a; see also Supplementary Information Fig. 5). Consistent with these observations, *sid2* mutants do not exhibit SAR in systemic leaves when infected with an avirulent pathogen[14], or produce SAR-like responses in leaves infected with the fungal biotroph *Erysiphe*[15]. These results suggest that *Arabidopsis* SAR defence responses require SA synthesized using ICS1.

To investigate the role of ICS1 in SAR, we examined *ICS1*

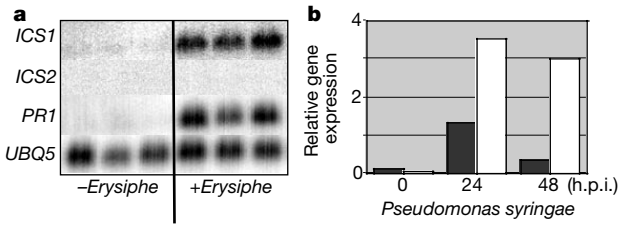

Figure 1 *ICS1* is induced in response to pathogens. RNA blot analysis of total RNA from wild-type (Columbia) *Arabidopsis* leaves probed with *ICS1*, *ICS2*, *PR1* or the loading control *UBQ5*. **a**, RNA extracted from *Erysiphe*-infected or control leaves. Triplicate samples are shown. **b**, RNA extracted from leaves inoculated with a suspension (optical density at 600 nm of 0.002) of *Pseudomonas* pathovar *maculicola* strain ES4326 at 0-, 24- and 48-h post inoculation (h.p.i.). *ICS1* (black) or *PR1* (white) normalized to UBQ5. *ICS1/UBQ5* values were multiplied by 100. *ICS1* probe has less than 0.1 times the activity of the *PR1* probe. No *ICS2* signal was detected. Average of duplicate samples is shown. Experiments in **a** and **b** were repeated with similar results.

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expression in *Arabidopsis* mutants exhibiting altered SAR responses. Genetic analyses have identified NPR1/NIM1 (refs 17–20) as a requisite regulator of SAR that acts downstream of SA. NPR1/NIM1 interacts with transcription factors to alter the expression of genes (such as *PR1*) in response to pathogen attack[21]. Figure 3b indicates that ICS1 acts upstream of NPR1/NIM1, as *ICS1* mRNA is still expressed in the *npr1* mutant, consistent with SA accumulation through ICS1 acting upstream of NPR1. Our data support a role for NPR1 as a negative feedback regulator of *ICS1* expression and SA accumulation. This role for NPR1 was first proposed when elevated SA levels were observed in infected leaves of *npr1/nim1* plants[18]. We found that *ICS1* expression was also elevated in infected leaves of *npr1* plants (Fig. 3b and Supplementary Information Fig. 5). We wanted also to determine whether SA regulates the expression of *ICS1*.*nahG* transgenic plants convert SA to catechol; they do not accumulate SA, they express little *PR1* in response to pathogen, and they do not exhibit SAR[1,2]. Expression of *ICS1* was not notably altered in *nahG* transgenic plants (Fig. 3b and Supplementary Information Fig. 5). The simplest interpretation of this result is that SA alone is not required for the induction or repression of *ICS1* expression and hence there is no direct autoregulation. Finally, to support our conclusion that SA synthesized through ICS1 mediates SAR, we examined *ICS1* (and *PR1*) expression in the *Arabidopsis cpr* mutants (*cpr1*, *cpr5* and *cpr6*; refs 22, 23 and 24, respectively), which exhibit constitutively elevated SA levels, *PR1* expression and SAR. We found that *ICS1* was constitutively expressed in the *cpr* mutants (Fig. 3b and Supplementary Information Fig. 5). Figure 3c shows the placement of ICS1 in the induction of SAR in *Arabidopsis* on the basis of these findings.

Our results indicate that SA synthesized through ICS1 acts upstream of NPR1/NIM1 in the induction of *PR1* and SAR. Therefore, we analysed the *ICS1* promoter for known *cis*-acting regulatory elements associated with defence responses in plants (Fig. 3d and Supplementary Information Table 1), as this might elucidate regulatory elements and their cognate transcription factors that act upstream of SA accumulation. The *ICS1* promoter was enriched in W-box elements, as are promoters of genes, such as *PR1* (ref. 13), that are downstream of SA. W boxes are recognized by WRKY plant-specific transcription factors that regulate pathogen and stress responses[25]. One potential explanation consistent with an enrichment of W-box elements in both the promoters of *ICS1* and downstream genes (for example, *PR1*) is that different WRKY factors regulate these responses. Reports of a variety of early- and late-acting WRKY factors activated by SA, elicitors and/or pathogens[25] support this view. Alternatively, shared WRKY factor(s) may suppress both *ICS1* and *PR1* expression. A W-box element in a region that negatively regulates *PR1* expression in response to a SA analogue[26] (linker scanning region 4, LS4) supports this explanation. Unlike the *PR1* promoter, the *ICS1* promoter does not contain the *cis*-acting regulatory elements in LS7 (bZip motif) or LS10 (NF-κB motif) required for the induction of *PR1* by SA[26]. This is consistent with our observation that removal of SA does not significantly affect expression of *ICS1* (Fig. 3b and Supplementary Information Fig. 5), and supports the hypothesis that a systemic signal other than SA is required for *ICS1* induction, SA accumulation and SAR[1].

The *ICS1* promoter also contains an Myb-binding site (MBSII) at a position (approximately −500 bp) similar to that of the other

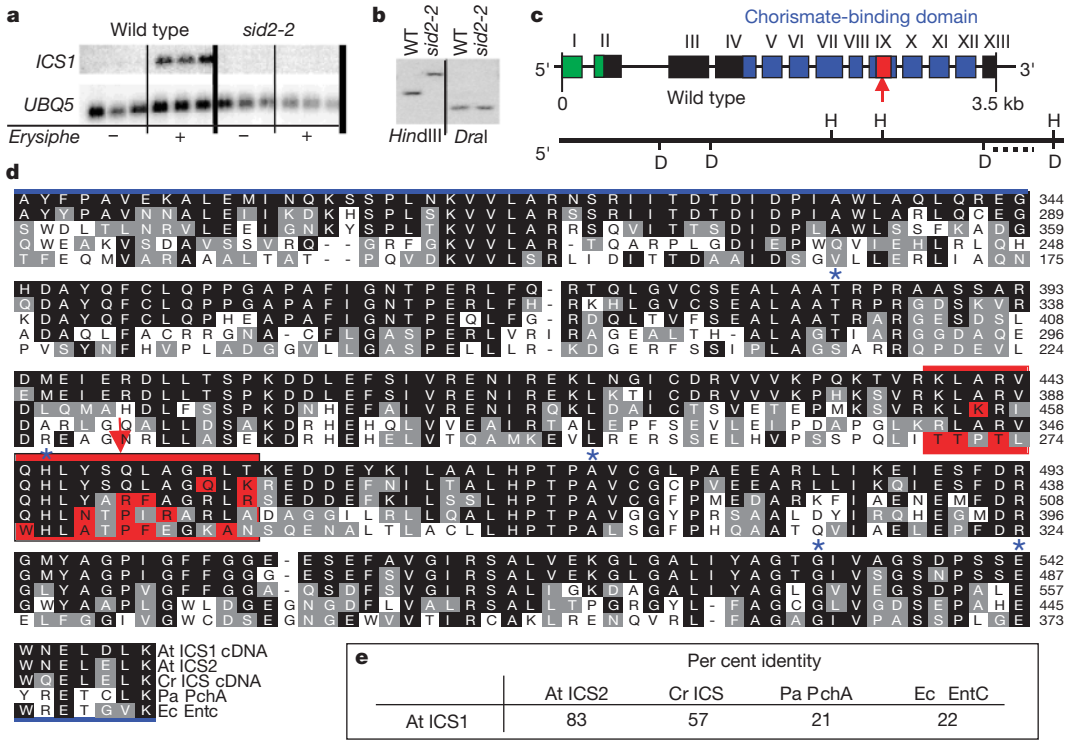

Figure 2 *SID2* encodes *ICS1*. **a**, Northern blot analysis of total RNA from *Erysiphe*-infected and uninfected leaves of *sid2-2* and wild-type *Arabidopsis* probed with *ICS1* or *UBQ5*. Triplicate samples are shown. **b**, Southern blot analysis of genomic DNA extracted from *sid2-2* or wild-type *Arabidopsis*, digested with *Hin*dIII or *Dra*I. **c**, Schematic representation of *ICS1*. Putative plastid transit sequence (green), chorismate-binding domain (blue), and location of mutations in *sid2-2* (red box) and *sid2-1* (red arrow) are shown. Cleavage sites *Hin*dIII (H) and *Dra*I (D) are indicated. The probe used in **b** is indicated by a dashed line. **d**, Clustal alignment of chorismate-binding domain (ProDom number P000779) of *Arabidopsis* ICS1 with selected isochorismate synthases. Identical (black), conserved (shaded) and residues deemed critical for ICS activity (blue asterisk)[16] are shown. T, residue 384; H, residue 445; A, residue 472; G, residue 532; E, residue 542. Location of mutations in *sid2-2* (red box) and *sid2-1* (red arrow) are shown. **e**, Overall amino-acid sequence comparison of *Arabidopsis* ICS1 and selected isochorismate synthases. At ICS1, *Arabidopsis thaliana* cDNA (AY056055); At ICS2, *A. thaliana* (AAF27094); Cr ICS, *C. roseus* cDNA (CAA06837); Pa PchA, *P. aeruginosa* (CAA57969); Ec EntC, *Escherichia coli* (AAA16100).

inducible chorismate-using enzymes, chorismate mutase, *CM1*, (Fig. 3d and Supplementary Information Table 1), and anthranilate synthase, *ASA1* (data not shown). Myb factors have a role in SA-mediated plant defences (for example, Myb1 (ref. 27)) and in the regulation of secondary metabolism including modulation of pathogen-inducible *ASA1* expression[28]. As the chorismate-using enzymes are critical control points of pathways resulting in many defensive compounds, it is likely that *ICS1* expression is regulated by a Myb factor(s).

We have described a SA biosynthetic pathway in *Arabidopsis* that uses isochorismate synthase to produce SA from chorismate, as do some bacteria (Fig. 4). The *ICS1* gene contains a putative plastid transit sequence and cleavage site consistent with its use of plastid-synthesized chorismate as a substrate. Plastid-localized synthesis of SA mediated by ICS is consistent with observations that many nuclear-encoded, plastid-localized metabolic pathways derive evolutionarily from prokaryotic endosymbionts. A prokaryotic origin of ICS1 is supported by the presence of an *ICS* gene in the chloroplast genome of the red algae *Cyanidium caldarium* (GenBank accession number NC_001840). It seems probable that other plant species also use the ICS pathway to synthesize SA, as plastid-localized periwinkle ICS has been identified (AJ006065)[16]. Moreover, expressed sequence tags for isochorismate synthase have been annotated for soybean (AW596452 and AW759689) and identified by homology and conservation of critical residues (see Fig. 2d) in other plant species, including wild tomato (AW398687).

An unresolved issue is whether SA is synthesized through the phenylalanine ammonium lyase (PAL) pathway, shown in Fig. 4, as well as through ICS. Our evidence suggests that SA synthesized through ICS1 has an important role in plant defence against pathogens and specifically, that it is required for *PR1* gene expression and LAR and SAR defence responses. However, SA also potentiates plant cell death in response to particular pathogens or fungal elicitors[1]. The importance of PAL in this type of cell death has been reported previously[5,6]. Furthermore, *sid2* mutants infected with necrotizing pathogens still exhibit cell death[14,15]. Therefore, the SA that potentiates plant cell death is probably synthesized through PAL. Notably, previous work on SA biosynthesis in plants focused on infected leaves undergoing cell death[6–10], and thus may have been biased towards elucidation of the PAL pathway. A related issue is the source of the low levels of SA present in uninfected leaves. Because these low levels are still present in *sid2* mutants, it seems that ICS1 is not responsible for this SA production. Perhaps ICS2 is involved in synthesis of this low level, constitutive SA. This would be analogous to the roles of the two anthranilate synthase genes in *Arabidopsis*—*ASA1* is highly induced in response to

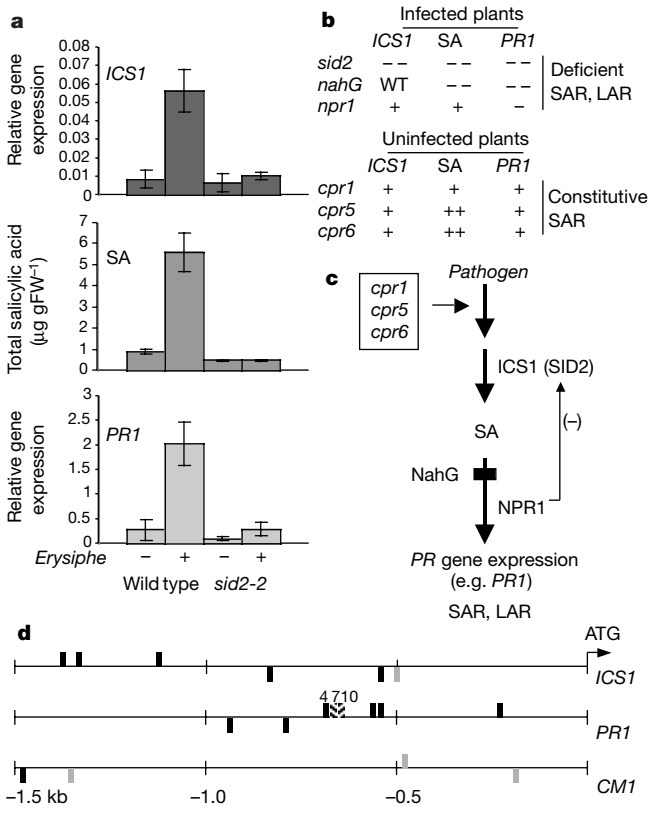

Figure 3 ICS1 mediates SA-dependent LAR and SAR defence responses. **a**, *ICS1*, total SA levels and *PR1* gene expression in wild-type and *sid2-2* plants infected or not infected with *Erysiphe*. The average of triplicate samples is shown. Expression levels were normalized to the loading control *UBQ5*. gFW, gram fresh weight. **b**, Relative total SA levels and *ICS1* and *PR1* gene expression levels in mutant or transgenic *Arabidopsis* compared to wild-type plants. Relative values are indicated as <0.1× (– –), <0.5× (–), wild type (WT), >2× (+) and >10× (+ +) compared with wild type. *ICS1* and *PR1* expression were assessed by northern blot analysis of total RNA infected with *Erysiphe*. Ranges represent findings from two–four separate experiments. (See Supplementary Information Fig. 5 for northern blots.) Total (free and sugar-conjugated) SA levels are compiled from Fig. 2a and published work[14,15,18,22–24]. **c**, Proposed placement of ICS1 in SAR induction on the basis of results tabulated in **b**. The SA-dependent NPR1-independent component of LAR/SAR is not explicitly shown. *PR1* mRNA levels are lower in leaves of *Erysiphe*-infected *sid2* than in *npr1* mutants. Therefore, ICS1 probably participates in SA-mediated NPR1-dependent and NPR1-independent pathways. **d**, Schematic of *cis*-acting DNA regulatory elements 1.5-kb upstream of translation start of *ICS1*, compared with *PR1* and *CM1* (chorismate mutase). Black box, W-box core (TTGAC) or extended core (TTGAC(C/T))[25]; grey box, Myb-binding site (MBS) II consensus (G(G/T)T(A/T)G(G/T)T)[27]. The hatched box indicates SA analogue-inducible elements in linker scanning regions LS10 (NF-kB; GGACTTTTC) or LS7 (bZIP; ACGTCA) of the *PR1* promoter[26]. The LS4 region (containing a W box) negatively regulates SA analogue-inducible expression of *PR1*. Boxes above the line represent elements found on the forward (becomes the coding) strand. Further details are provided in Supplementary Information Table 1.

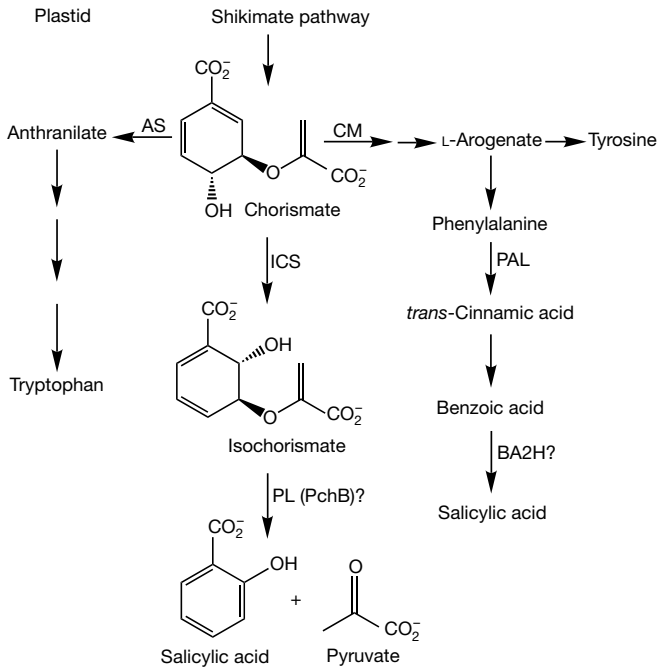

Figure 4 Proposed salicylic acid biosynthetic pathways in plants. Proposed pathway for SA biosynthesis through isochorismate synthase (ICS). In bacteria, this pathway also requires pyruvate lyase (PL; PchB in *P. aeruginosa*)[11]. The previously described pathway of SA biosynthesis from PAL and benzoic acid-2 hydroxylase (BA2H) is also shown. AS, anthranilate synthase; CM, chorismate mutase.

pathogens and wounding, whereas *ASA2* is constitutively expressed at low levels[29]. Alternatively, there is evidence that the PAL pathway may be operational under these conditions[7,8]. There are at least three *PAL* genes in *Arabidopsis*, one of which could function to produce these low levels of SA.

A mechanistic understanding of the functions of SA in plant defence requires knowledge not only of where, when, and how much SA is made, but how it is synthesized. Our work now allows for constructs and genetic mosaics to be created which will allow for the careful examination of the complex role of SA in plant defence, and for the identification of signals and regulators of SAR that act upstream of SA. □

Methods

RNA analysis

Arabidopsis mutants and transgenics are of the Columbia ecotype. The *nahG* transgenic *Arabidopsis* line and the *npr1-1*, *cpr1-1*, *cpr5-1* and *cpr6-1* mutant alleles have been described[17,22–24,30]. At 4–4.5 weeks of age, plants were infected with *P. syringae* pv. *maculicola* strain ES4326 or *E. orontii* isolate MGH, *Erysiphe*-infected leaves were collected at 7 days post inoculation, and RNA extractions and blot analysis (including synthesis of *PR1* and *UBQ5* probes) were performed as described[15]. We used a Molecular Dynamics PhosphorImager to quantify the levels of hybridization. *ICS1* and *PR1* hybridization levels were normalized to those obtained for the loading control *UBQ5*. *ICS1*- and *ICS2*-specific primers corresponded to the 3′-untranslated region. Forward and reverse primers used to make the *ICS* probe templates and probes were: *ICS1*, forward 5′-GGGGATAAGGGGTTC TCACAATA-3′ and reverse 5′-CTGCCCTAGTTACAACCCGAAAAG-3′; *ICS2*, forward 5′-TGTGTTTGGTCATTGGTGT-3′ and reverse 5′-TCATAGAGTCATAGTCGCTTCA-3′. One, five and ten picograms of *ICS1* and *ICS2* probe templates were spotted onto nylon membranes before crosslinking as controls for successful hybridization and to assess cross-hybridization (negligible).

Salicylic acid

We extracted and quantified total salicylic acid (free and sugar-conjugated) by HPLC as described[15]. Recovery was determined for each sample by spiking with *o*-anisic acid before leaf extraction.

sid2-2 and *sid2-1* mutations

Genomic DNA was extracted from the fast-neutron-generated mutant *sid2-2* and from wild-type *Arabidopsis*, and digested with *Hin*dIII or *Dra*I. DNA blot analysis was performed with the probe shown in Fig. 2c using standard procedures (CPMB). The unaltered *Dra*I cleavage pattern indicates that this deletion/rearrangement does not extend into the next gene. Additional restriction enzymes and probes gave results consistent with the alteration in exon IX (see Fig. 2c, d).

The *ICS1* sequence from the *sid2-1* mutant generated with EMS, and wild-type (Columbia) genomic DNA was amplified by PCR (using Pfu Taq), subcloned and sequenced using standard procedures (CPMB). Multiple overlapping sequences of *sid2-1* contained a mutation (C→T in the coding strand) in exon IX. The position of the EMS-generated mutation in *sid2-1* results in a stop codon (TAA) at residue 449 instead of glutamine. The wild-type (Columbia) sequence matched the GenBank genomic sequence. This experiment was repeated with the same result.

Isolation of cDNA, annotation and sequence analysis

ICS1 cDNA sequence was isolated using RACE-PCR (Clontech SMART RACE cDNA Amplification Kit). Predicted chloroplast localization and transit sequence cleavage site were determined by analysis with ChloroP (http://www.cbs.dtu.dk/services/ChloroP). We assessed comparisons of overall sequence identity using DNAStar MegAlign Clustal analysis program. Conserved residues are within three distance units (PAM250 matrix). The N-terminal extensions (putative plastid transit sequences) of plant isochorismate synthases were not included for this analysis.

Received 14 May; accepted 27 September 2001.

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Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We thank S. Volko for RNA from leaves infected with *Pseudomonas* pathovar *maculicola* strain ES4326, C. Nawrath for the *sid2-1* seeds, and D. Lee, J. Stone, J. Dangl and E. Meyerowitz for comments on drafts of the manuscript. This work was supported by a National Institutes of Health grant to F.M.A. and a National Research Initiative Competitive Grants Program/USDA postdoctoral fellowship to M.C.W.

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Acknowledgements

We thank R. Malkin, J. Gray and S. Merchant for discussions. This work was supported by the National Institutes of Health and the US Department of Energy (S.L.).

Competing interests statement

The authors declare that they have no competing financial interests.

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corrigendum

Isochorismate synthase is required to synthesize salicylic acid for plant defence

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Nature **414**, 562–565 (2001).

In this Letter, the GenBank accession number for the sequence of ICS1 cDNA was given incorrectly as AY056500. It should be AY056055. □