Published in The Plant Journal 4, issue 1, 155-166, 2005,
which should be used for any reference to this work

The Arabidopsis *csb3* mutant reveals a regulatory link between salicylic acid-mediated disease resistance and the methyl-erythritol 4-phosphate pathway

M[a] José Gil[1], Alberto Coego[1], Brigitte Mauch-Mani[2], Lucia Jordá[1,†] and Pablo Vera[1,*]
[1]*Instituto de Biología Molecular y Celular de Plantas (IBMCP), UPV-CSIC, Camino de Vera, s/n, 46022 Valencia, Spain, and*
[2]*University of Neuchâtel, Faculty of Sciences, Institute of Botany, Rue Emil-Argand 11, CP 2, CH-2007 Neuchâtel, Switzerland*

[*]For correspondence (fax +34 96 3877859; e-mail vera@ibmcp.upv.es).
[†]Present address: Departamento de Biotecnología, Universidad Politécnica de Madrid, Avda Complutense s/n, 28040 Madrid, Spain.

Summary

We report on *constitutive subtilisin3* (*csb3*), an Arabidopsis mutant showing strikingly enhanced resistance to biotrophic pathogens. Epistasis analyses with *pad4, sid2, eds5, NahG, npr1, dth9* and *cpr1* mutants revealed that the enhanced resistance of *csb3* plants requires intact salicylic acid (SA) synthesis and perception. *CSB3* encodes a 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase, the enzyme controlling the penultimate step of the biosynthesis of isopentenyl diphosphate via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in the chloroplast. *CSB3* is expressed constitutively in healthy plants, and shows repression in response to bacterial infection. We also show the pharmacological complementation of the enhanced-resistance phenotype of *csb3* plants with fosmidomycin, an inhibitor of the MEP pathway, and propose that *CSB3* represents a point of metabolic convergence modulating the magnitude of SA-mediated disease resistance to biotrophic pathogens.

Keywords: plant defense, Pst DC3000, biotrophic pathogens, defense response.

Introduction

Plants react to microbial attack with an array of inducible defenses that are integrated by a complex signaling apparatus. Significant progress in our understanding of the molecular mechanisms by which plants defend themselves against microbial attack has been achieved through the cloning and characterization of plant disease-resistance factors that recognize the presence of pathogen avirulence factors to trigger the so-called hypersensitive response (HR; Dangl and Jones, 2001). In most cases, the onset of the HR results in activation of systemic acquired resistance (SAR), a defense response that provides long-lasting protection throughout the plant against a broad spectrum of pathogens (Durrant and Dong, 2004). Salicylic acid (SA) is an essential signal molecule that plays a central role in the induction of SAR (Delaney *et al.*, 1994). Numerous plant mutants compromised in SA accumulation or perception show enhanced susceptibility to biotrophic pathogens, whereas mutants that overproduce SA display enhanced resistance. In particular, several mutants of *Arabidopsis thaliana* have been isolated that are unable to activate SA-dependent defense responses (Durrant and Dong, 2004). The *eds16*/*sid2* and *eds5*/*sid1* (Nawrath and Metraux, 1999; Rogers and Ausubel, 1997; Wildermuth *et al.*, 2001) mutants do not accumulate SA after inoculation with virulent and avirulent pathogens. *SID2* encodes a putative chloroplast-localized isochorismate synthase (Wildermuth *et al.*, 2001), while *EDS5* encodes a chloroplast-localized and membrane-associated protein that shows homology to multi-drug and toxin extrusion (MATE) transporters (Nawrath *et al.*, 2002). These findings demonstrated the existence of an SA-synthesis pathway localized in the chloroplast, and revealed the importance of this organelle in mediating/controlling critical aspects of the plant defense response (Kachroo *et al.*, 2003; Shah, 2003). Furthermore, the *eds1* and *pad4* mutants block the biosynthesis of SA triggered by infection with virulent pathogens (Feys *et al.*, 2001; Jirage *et al.*, 1999), and also that observed to occur in different disease-resistance mutants (Clarke *et al.*, 2001; Jirage *et al.*, 2001). *EDS1* and *PAD4* are required for the resistance conferred by a subset of R genes (*TIR-NB-LRR*). Both proteins have homology to lipases, and both operate

upstream of *SID2* and *EDS5* in the regulation of SA synthesis (Feys *et al.*, 2001; Glazebrook *et al.*, 1997; Jirage *et al.*, 1999; Nawrath *et al.*, 2002).

The ankyrin-repeat protein NPR1 (non-expressor of *PR-1*) was initially identified in Arabidopsis through a genetic screen for SAR-compromised mutants (Cao *et al.*, 1997). NPR1 is an essential regulator of SAR (Dong, 2004) and of the SAR-independent defense pathway for induced systemic resistance (Pieterse and Van Loon, 2004). On SA treatment and cognate redox imbalance, NPR1 is translocated to the nucleus where it acts as a modulator of *PR* gene expression through selective interaction with members of the TGA subclass of the basic leucine zipper family of transcription factors (Dong, 2004; Mou *et al.*, 2003).

In addition to *npr1*, other SAR-compromised mutants have been identified. These include *dir1* (Maldonado *et al.*, 2002), which carries a mutation in a gene encoding a protein with sequence similarity to lipid-transfer proteins, and points towards a mobile lipid-based signal mediating SAR. Also, the *dth9* mutant (Mayda *et al.*, 2000) differs from *npr1* in that *PR* expression is unaltered. The fact that SA treatment does not restore resistance to disease-susceptible *dth9* plants places DTH9 downstream of SA in an NPR1 parallel pathway. In addition, several genetic screens have been performed to search for suppressors of an *npr1*, allowing the identification of novel components of the SAR-signaling pathway such as *SNI1*, *SNC1*, *SON1*, or *SSI2* and SSI4, among others (Kachroo *et al.*, 2001; Kim and Delaney, 2002; Li *et al.*, 1999, 2001; Shah *et al.*, 1999; Shirano *et al.*, 2002; Zhang *et al.*, 2003). Interestingly, the *snc1*, *sni1* and *ssi2* mutants show constitutive *PR* expression and increased resistance to biotrophs. Conversely, the *son1* mutant delivers SAR without *PR* gene induction, and defines a new phenomenon that has been termed SAR-independent resistance or SIR (Hammond-Kosack and Parker, 2003).

We describe here the isolation and characterization of the *constitutive subtilisin3* (*csb3*) mutant from Arabidopsis with enhanced resistance to biotrophic pathogens. *CSB3* encodes a 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase, an enzyme that controls one of the terminal steps of the biosynthesis of isopentenyl diphosphate (IPP) via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. We present evidence indicating that *CSB3* controls critical aspects of the SA-mediated disease-resistance pathway in Arabidopsis plants.

Results

Isolation of the csb3 *mutant*

The *P69C* gene encodes a subtilisin-like protease originally identified in tomato plants (Jorda and Vera, 2000). Transgenic Arabidopsis plants containing a 2.5-kb region of the *P69C* 5′ promoter fused to the β-glucuronidase (GUS) reporter gene reveal a rapid transcriptional activation during the course of compatible and incompatible interactions with *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) (Jorda and Vera, 2000). Induced expression of this gene is observed in the inoculated leaves, at a distance from the zone where the HR is executed (on inoculation with Pst DC3000 carrying *avrRpm1*: Pst DC3000 *avrRpm1*), as well as in distal non-inoculated leaves of the same plant (see Supplementary Material). This expression pattern is different from that observed for other SA-regulated genes (Bowling *et al.*, 1994).

To identify signals and pathways controlling activation of the *P69C* promoter, we addressed the search for mutants in Arabidopsis. We therefore mutagenized one of our homozygous transgenic lines containing a single insertion of *P69C*–GUS with ethyl methanesulfonate (EMS) and screened approximately 12 800 M_2 seeds for individuals with constitutively elevated GUS activity. We named these mutants *csb* (*constitutive subtilisin*). One high GUS-expressing mutant, named *csb3*, was selected and is described here in detail.

csb3 plants are distinguishable from wild-type plants by their characteristic dwarf phenotype and curled leaves (Figure 1a). Histochemical staining for GUS activity is shown in Figure 1(b,c), and revealed that *csb3* seedlings showed permanent GUS activity that is retained throughout development.

As local application of SA induces expression of *P69C*–GUS in wild-type Arabidopsis plants (Jorda and Vera, 2000), we hypothesized that SA accumulation might be increased in *csb3* plants. We first examined the expression of several SA-inducible genes (*PR-1*, *PR-2* and *GST6*) in *csb3* plants. Northern blots shown in Figure 1(d) revealed that the selected genes were upregulated in intact *csb3* plants but not in wild-type plants, thus pointing towards a constitutive activation of the SA-dependent signaling pathway. This could be explained by an impaired accumulation of SA in *csb3* plants. Thus levels of free SA and conjugated salicylate glucoside (SAG) were examined in leaf tissues from *csb3* and wild-type plants (Figure 1e). In healthy *csb3* plants, an eightfold increase in SAG content was observed when compared with wild-type plants. No significant differences were found in free-SA concentrations. However, in infected leaf tissue, at 3 days post-inoculation (d.p.i.) with Pst DC3000, the amounts of free SA increased 11-fold in *csb3* plants, whereas it increased only threefold in wild-type plants. Similar proportional increases in the amount of SAG were observed between *csb3* and wild-type plants on inoculation (Figure 1e). This finding suggests that the *csb3* mutation not only confers an enhancement of SA accumulation under resting conditions, but also renders plants more prone to increasing the synthesis and accumulation of this molecule on infection.

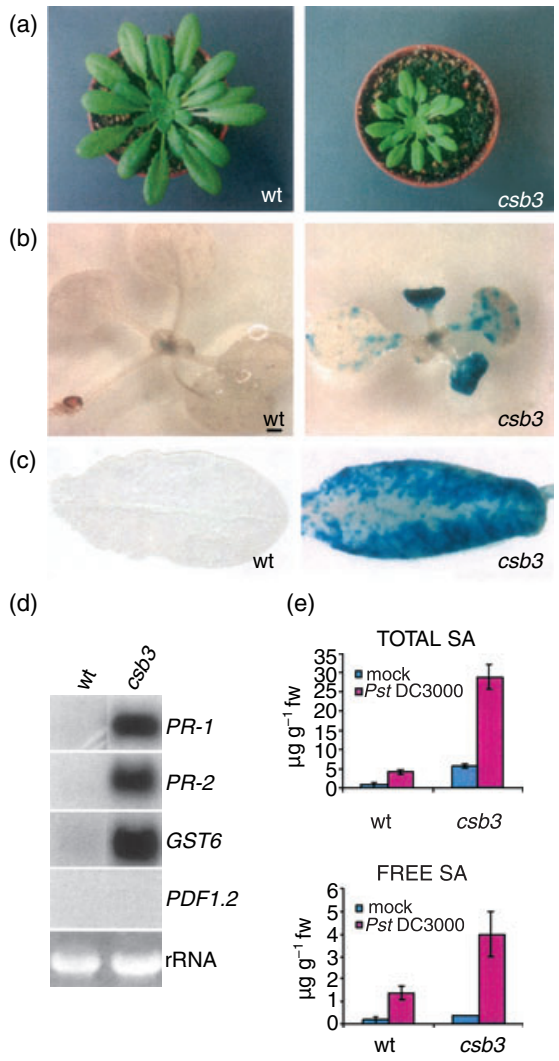

Figure 1. Characterization of *csb3* plants and comparison with wild-type plants.
(a) A comparison of the external appearance of *csb3* plants (right) and wild-type (wt) plants (left). Plants were photographed at 3.5 weeks old.
(b) Histochemical staining of GUS activity driven by the *P69C* promoter in a 14-day-old wild-type transgenic seedling (left) and *csb3* seedlings (right).
(c) Fully expanded rosette leaf from a wild-type transgenic plant (left) and *csb3* plants (right) stained for GUS activity.
(d) Expression of *PR-1*, *PR-2*, *GST6* and *PDF1.2* marker genes in wild-type and *csb3* plants.
(e) Levels of free and total salycilic acid (SA) in wild-type (wt) and *csb3* plants. Plants were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) or 10 mM $MgSO_4$ (mock) and the amount of free SA and SA glucoside (SAG) was determined 24 h after inoculation. Each bar represents the mean of three replicate samples with standard deviation. Free SA and SAG were assayed in the same samples.

To analyze the pattern of inheritance of the *csb3* mutation, *csb3*/*csb3* plants were back-crossed to wild-type *CSB3*/*CSB3* plants containing the *P69C*–GUS transgene, and the resulting progeny were tested for GUS staining. In the F_2 plants, expression was present in 67 of 240 plants (3 : 1 non-expressers : constitutive expressers; $\chi^2 = 0.4$, $0.1 > P > 0.5$), supporting the conclusion that *csb3* is a single recessive nuclear mutation.

The csb3 *mutant has enhanced resistance to biotrophic but not to necrotrophic pathogens*

We tested the response of *csb3* and wild-type plants to different pathogens that generate disease in Arabidopsis. The response of plants to the obligate biotrophic oomycete *Hyaloperonospora parasitica* is shown in Figure 2(a,b). We inoculated 25 *csb3* plants as well as 25 wild-type plants with the *H. parasitica* isolate NOCO (105 conidiospores ml^{-1}), which is virulent on Arabidopsis Col-0. Growth of the pathogen was assayed on infected leaves (Figure 2a,b) and it revealed that, whereas wild-type plants became heavily colonized by the oomycete and allowed it to complete its life cycle (forming sexual oospores and asexual conidia on conidiophores), *csb3* plants drastically inhibited the growth of this pathogen. This indicates that resistance to this biotrophic pathogen was dramatically enhanced or susceptibility was blocked as a consequence of the mutation in the *CSB3* locus.

We also challenged wild-type and *csb3* plants with the virulent bacterial pathogen Pst DC3000, another biotrophic pathogen of Arabidopsis. After inoculation, the growth rate of the pathogen was monitored in infected leaves (Figure 2c) and it was revealed that this growth was 30- to 60-fold lower in *csb3* than in wild-type plants. Thus *csb3* plants also show enhanced resistance to this bacterial pathogen.

The susceptibility of *csb3* plants to pathogens was analyzed further using *Plectosphaerella cucumerina* and *Botrytis cinerea*, which are necrotrophic fungal pathogens in Arabidopsis. Infection of plants with these pathogens leads to a strong degradation of the leaf tissue, manifested as extended lesions that increase in diameter as the infection progresses along the inoculated leaf. No differences in extension of the lesion were observed between *csb3* and wild-type plants for either of these two fungal pathogens. In both cases necrosis was accompanied by extensive proliferation of the fungal mycelia (Figure 2d–f). Therefore the *csb3* mutation apparently did not affect the susceptibility of the plant to necrotrophic pathogens.

Thus, based on the phenotype of the recessive *csb3* mutant, it could be hypothesized that *CSB3* could be negatively regulating certain aspects of the defense response directed towards biotrophic pathogens.

Response of csb3 *double mutants to Pst DC3000*

To provide further insights into *csb3*-activated signaling to biotrophs, we generated a series of double mutants between *csb3* and different mutants affected in the SA-related pathway. The resistance response to Pst DC3000 for all these mutants was investigated and is summarized in Figure 3.

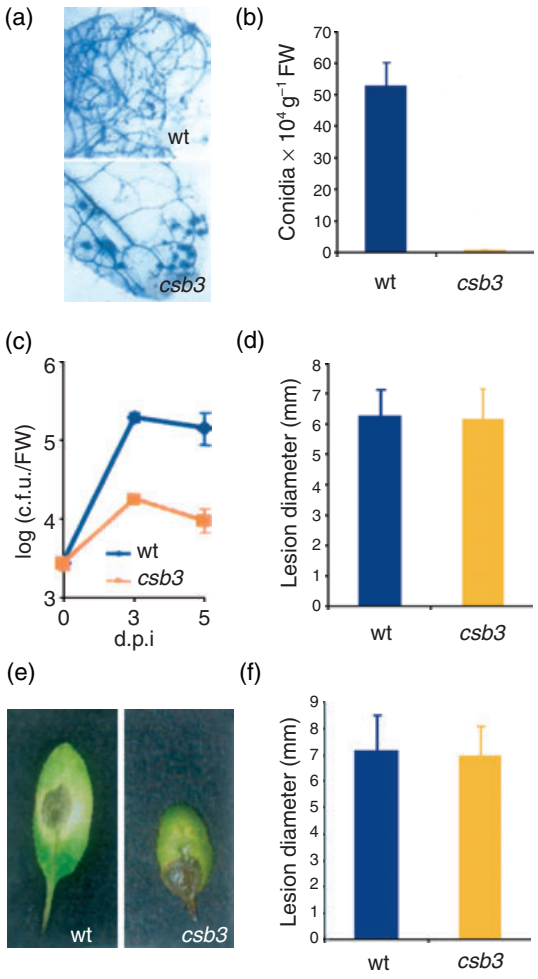

Figure 2. Enhanced resistance of *csb3* plants to biotrophic but not to necrotrophic pathogens.
(a) Resistance response of wild-type and *csb3* mutant Arabidopsis plants to virulent *Hyaloperonospora parasitica*. Inoculated leaves were stained with lactophenol-trypan blue to reveal growth of hyphae. Growth of this pathogen was strikingly inhibited in *csb3* plants.
(b) To quantify resistance to *H. parasitica*, production of conidia was counted 7 days after inoculation. Plants carrying the *csb3* mutation were highly resistant to this pathogen.
(c) Growth of *Pseudomonas syringae* pv. *tomato* DC3000 in wild-type and *csb3* plants. Colony-forming units (CFU) per fresh weight were determined at 0, 3 and 5 days after infection for wild-type (blue lines) and *csb3* (orange lines). Data points represent average ± SD of logarithms. Plants carrying the *csb3* mutation were highly resistant to this pathogen.
(d) Resistance response of wild-type and *csb3* plants to *Plectosphaerella cucumerina*. Disease symptoms were evaluated 4 days after inoculation by determining average lesion diameters. Data points represent average lesion size ± SE of measurements.
(e) Leaves from wild-type and *csb3* plants 4 days after inoculation with a 6-μl droplet of *Botrytis cinerea* spores (2.5×10^4 conidia ml^{-1}).
(f) Lesion size as generated by *B. cinerea* was measured 4 days after inoculation. Data points represent average lesion size ± SE of measurements from a minimum of 30 lesions. wt, Wild type.

The *pad4* mutation compromises the SA accumulation triggered by infection with virulent *P. syringae* (Feys *et al.*, 2001; Jirage *et al.*, 1999). As shown in Figure 3(a,b), both the enhanced resistance to Pst DC3000, and the constitutive expression of the SA-inducible *PR-1* gene conferred by the *csb3* mutation, are suppressed in the *csb3 pad4* double-mutant plants. Thus *PAD4* is fully required for downstream signaling in *csb3* plants.

The *sid2* and *eds5* mutants are impaired in the synthesis and accumulation of SA, and show increased susceptibility to pathogens (Nawrath and Metraux, 1999; Wildermuth *et al.*, 2001). In *csb3 sid2* plants, as well as in *csb3 eds5* plants, the resistance to Pst DC3000 conferred by *csb3* in isolation is notably reduced (Figure 3a). Also, the constitutive expression of the *PR-1* marker gene observed in *csb3* plants was blocked in *csb3 sid2* and, to a lesser extent, also in *csb3 eds5* plants (Figure 3b). Hence, the enhanced resistance of *csb3* plants also requires functional copies of *SID2* and *EDS5*.

To further extend our understanding of the role of SA for the enhanced resistance of *csb3* plants, the *NahG* transgene was introgressed into *csb3* plants. *NahG* encodes a salicylate hydroxylase that blocks SA accumulation in the cytosol by degrading SA to catechol (Delaney *et al.*, 1994). In the *csb3 NahG* plants, the increased resistance conferred by the *csb3* mutation is again suppressed and the *NahG* hypersusceptibility to Pst DC3000 is imposed (Figure 3a). Likewise, the constitutive expression of the *PR-1* gene is abolished when the *NahG* transgene is present (Figure 3b).

npr1 shows increased susceptibility to biotrophic pathogens and does not respond to SA treatment (Cao *et al.*, 1997; Dong, 2004). Interestingly, *csb3 npr1* plants remained as susceptible as *npr1* plants to Pst DC3000 (Figure 3a), and expression of the *PR-1* gene was also abrogated (Figure 3b). This indicates that the *csb3* SA-mediated resistance requires a functional NPR1 protein. Another SAR-compromised mutant is *dth9*. However, unlike *npr1* plants, *dth9* plants still retain the ability to express *PR* genes in response to SA or pathogen attack (Mayda *et al.*, 2000). Again, the *csb3 dth9* double-mutant plants showed the enhanced susceptibility to Pst DC3000 attributable to the *dth9* mutation (Figure 3a) but, in contrast to *npr1*, expression of the *PR-1* marker gene attributable to the *csb3* mutation is kept intact (Figure 3b), indicating that the *csb3* resistance is also DTH9-dependent.

The constitutive expression of GUS activity, driven by the *P69C* promoter as observed in *csb3* plants, is also abrogated in all the double mutants generated except *csb3 dth9*, as seen in Figure 3(c) for *csb3 NahG* plants.

Another interesting mutant analyzed by epistasis was *cpr1*. The *cpr1* plants are more resistant to Pst DC3000, have increased levels of SA, show constitutive expression of the *PR-1* gene, and show an abnormal growth habit (Bowling *et al.*, 1994). Therefore *cpr1* shares with *csb3* several aspects of disease resistance and growth stature. Interestingly, when the *cpr1* mutation was introgressed into the *csb3* background, the resultant *csb3 cpr1* double-mutant plants showed additive effects that resulted in an extremely dwarf

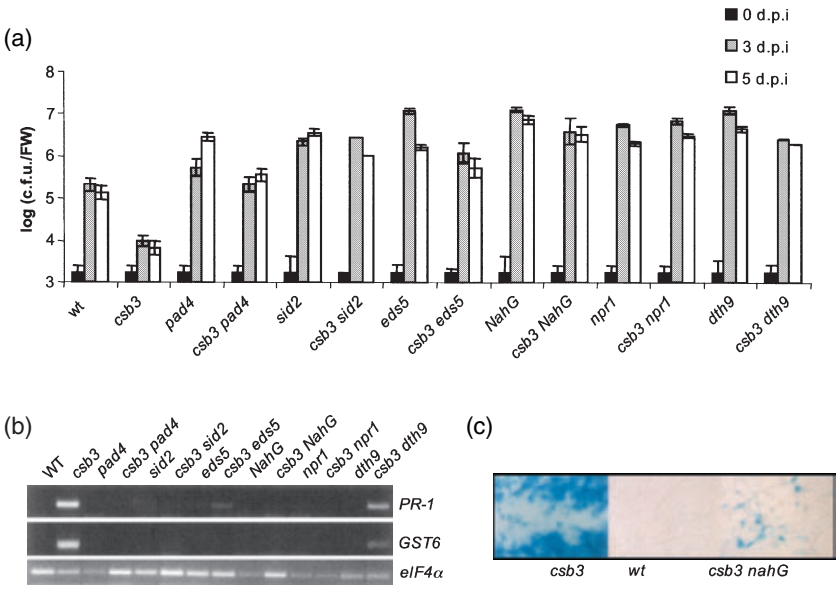

Figure 3. Effect of SA-related mutations on the disease resistance response of *csb3* plants.
(a) Growth of *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) on the *csb3 pad4*; *csb3 sid2*; *csb3 eds5*; *csb3 NahG*; *csb3 dth9*; and *csb3 npr1* double mutants compared with growth on single mutants and wild-type plants. Bacterial growth was recorded in leaf samples collected immediately after infection (day 0, black); 3 d.p.i. (grey); and 5 d.p.i (white). Eight samples were taken for each genotype at each time point. The experiment was repeated twice with similar results.
(b) RT–PCR analysis of *PR-1* and *GST6* gene expression in salicylic acid-related mutants as well as in *csb3* double mutants and wild-type plants. The bottom gel shows RT–PCR for the housekeeping gene *eIF4a* used here as a loading control.
(c) Fully expanded rosette leaf from a wild-type parental plant (center), *csb3* plants (left) and *csb3 NahG* plants (right) stained for GUS activity driven by the *P69C* gene promoter.

phenotype (see Supplementary Material). The homozygous double-mutant plants never reached the reproductive phase and thus had to be propagated in heterozygosis. Therefore the *csb3* and *cpr1* mutations may contribute to their respective enhanced-resistance phenotype through independent pathways downstream of SA accumulation. However, at this stage we cannot disregard the possibility that the double *csb3 cpr1* mutant produces even more SA than each of the single mutants, thus leading to an even stronger dwarf phenotype.

All these observations reinforce the consideration that the synthesis and perception of SA are pivotal for *csb3*-associated resistance.

Cloning of CSB3

The *CSB3* gene was cloned by a map-based cloning approach (see Experimental procedures and Supplementary Material), and was found to match the annotated At5g60600 ORF localized on chromosome 5. The gene contains 20 exons, and the *csb3* mutation (hereafter *csb3-1*) represented a G-to-A transition on the coding strand of exon 19 that led to a Gly-to-Asp amino acid change at position 696 (Figure 4a). *CSB3* is a single-copy gene that encodes the chloroplast-localized 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP) synthase (HDS) that catalyses the formation of HMBPP from 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) by an as-yet-unknown mechanism (Hecht *et al.*, 2001; Kollas *et al.*, 2002). The HDS enzyme controls the penultimate steps of the biosynthesis of IPP and dimethylallyl diphosphate (DMAPP) via the MEP pathway, which occurs in the chloroplasts of higher plants (Figure 5). The plastid-localized MEP pathway, together with the cytosolic mevalonate-dependent pathway, are in charge of the synthesis of IPP and its isomer DMAPP, which are the precursors for the biosynthesis of all types of isoprenoids in plants (Rohmer, 1999). The HDS enzyme is conserved in microorganisms, where it is known as GCPE (Kollas *et al.*, 2002), notwithstanding the difference from the mature plant HDS enzyme which contains an internal large protein domain absent in the bacterial counterpart (Figure 4b). This extra domain might confer distinct regulatory or catalytic functions on the plant enzyme (Querol *et al.*, 2002). Plant and microbial enzymes show a highly conserved amino acid region close to the C-terminus where a set of canonical Cys residues are located, which are involved in the assembly of a [4Fe–4S] cluster (amino acids 640–684, Figure 4b). The identification of different mutations in *Escherichia coli* located within or next to the 4Fe–4S motif point to this region being critical for the enzymatic function (Sauret-Güeto *et al.*, 2003). As the *csb3* mutation identified is close to this motif (Figure 4b), we hypothesize that the mutation introduces a structural alteration that may destabilize the folding and, in turn, the correct function of the Fe–cluster.

We identified an Arabidopsis T-DNA insertion (SALK 017595) in the *CSB3* gene (Figure 4a). This mutation disrupts *CSB3* at the level of the 16th exon. Interestingly, plants heterozygous for this T-DNA insertion segregated albino seedlings that were lethal (not shown). Moreover, in a search for mutants affected in chloroplast development, Gutiérrez-Nava *et al.* (2004) recently identified the *clb4-1* (chroplast biogenesis) mutant carrying an EMS-induced mutation in the same locus where a transition change results in the appearance of a premature stop codon in exon 9 (Figure 4a). The *clb4-1* mutation also gave rise to albino seedlings that were lethal (Gutiérrez-Nava *et al.*, 2004). To

keep the nomenclature uniform, we rename our original *csb3* mutant as *clb4-3*, and the allele identified as a T-DNA insertion as *clb4-2*. It is interesting to note here that other T-DNA mutants disrupting ORFs of other genes of the MEP pathway (Budziszewski *et al.*, 2001) cause the same seedling-lethal albino phenotype. This suggests that the MEP pathway is essential for the plant, and that the *clb4-3* allele identified in the present work is a partial loss of function, not a null mutant.

To confirm that At5g60600 corresponds to the *CSB3* gene, *Agrobacterium tumefaciens*-mediated transformation was used to introduce into *clb4-3*/*csb3* plants a cDNA corresponding to At5g60600 under control of the CaMV 35S promoter (35S–*CSB3* construct). We generated several transgenic lines with the 35S–*CSB3* construct, and studied some in detail. All the transgenic lines generated had lost the characteristic morphological phenotype attributable to the *clb4-3*/*csb3* mutation and recovered a wild-type growth habit (Figure 4c). In addition, the transformants lost the constitutive expression of the *P69C*–GUS gene (Figure 4c), as well as the expression of SA-related genes (Figure 4e) characteristic of the non-transformed *clb4-3*/*csb3* plants. Furthermore, expression of At5g606000 under the control of the 35S promoter in transgenic *csb3-1* plants abolishes the enhanced resistance to Pst DC3000. In these transgenic plants, resistance to Pst DC3000 returns to levels similar to those attained in wild-type plants (Figure 4d). Full complementation of all the different aspects of the *clb4-3*/*csb3* phenotype confirms that At5g60600 is equivalent to *CLB4*/*CSB3*.

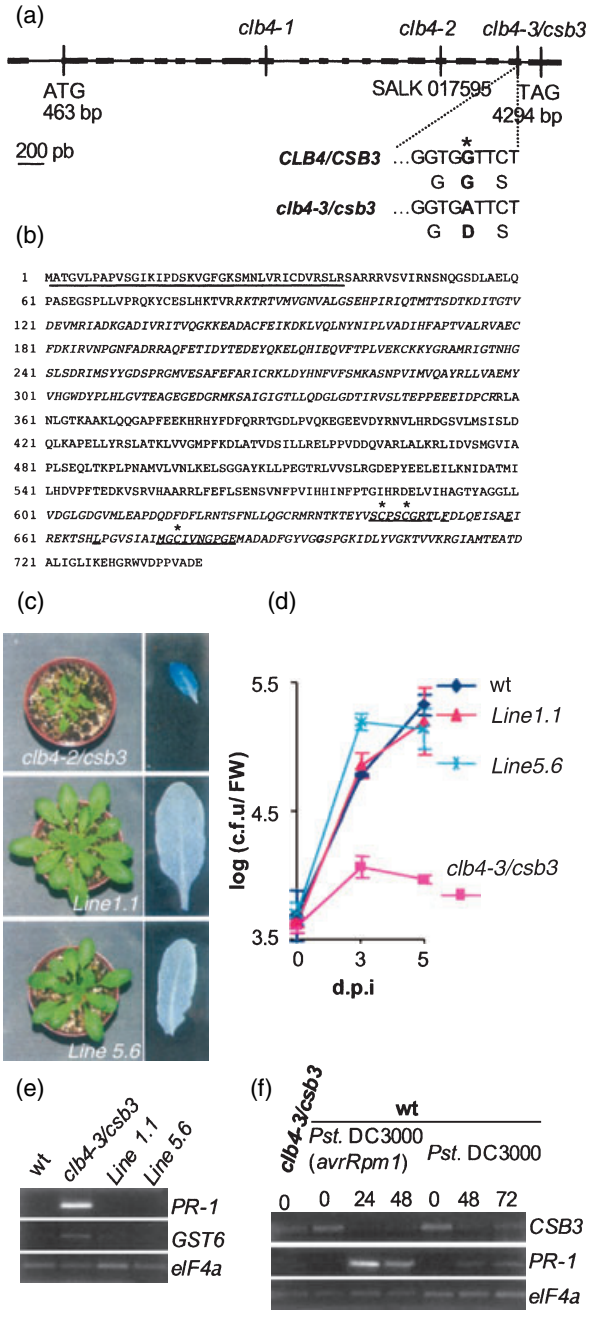

Figure 4. Structure of *CSB3*, complementation of *csb3* plants and downregulation of *csb3* gene expression following infection with *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000).

(a) Exon/intron structure of *CLB4*/*CSB3*. Coding regions are indicated by thick lines; positions of mutant alleles are shown on top of each exon. Insert shows the nucleotide exchange found in the original ethyl methanesulfonate (EMS)-induced *csb3* mutant and its influence on the protein sequence. The mutant allele is indicated below the wild-type sequence. Upper-case letters mark nucleotide sequences in the middle of exon 19. The G to A transition due to the mutagen is indicated in bold. The deduced amino acid sequences are indicated as single-letter code below each nucleotide triplet; letters in bold type mark amino acid changes (G to D) in the protein sequences.

(b) Deduced amino acid sequence of the *CLB4*/*CSB3*-encoded plastid-associated hydroxymethylbutenyl 4-diphosphate synthase (HDS). The predicted signal sequence for plastid import (CHLOROP program) is shown underlined in the N-terminus. Regions homologous between plant HDS homologs and the GcpE homolog from microorganisms are shown in italics. At the C-terminus, cysteine residues highly conserved in all the HDS and GcpE enzymes are shown with an asterisk. Conserved residues that comprise the signature where the [4Fe–4S] cluster allocates are underlined. The glycine residue converted to an aspartic residue in the *csb3-1* mutant is shown in bold at position 695.

(c) Morphological appearance of untransformed *clb4-3*/*csb3* plants compared with two transgenic lines transformed with a 35S–*CSB3* gene construct. Right, histochemical staining of GUS activity driven by the *P69C* promoter in fully expanded rosette leaves.

(d) Growth of Pst DC3000 in two independent transgenic *clb4-3*/*csb3* lines (lines 1.1 and 5.6) stably transformed with a 35S–*CSB3* gene construct, and comparison with wild-type (wt) and *csb3-1* mutant. Bacterial growth was recorded at 0, 3 and 5 d.p.i. The experiment was repeated three times with similar results.

(e) RT–PCR analysis of *PR-1* and *GST6* gene expression in *clb4-3*/*csb3*, in wild-type plants and in the transgenic lines (1.1 and 5.6) expressing the 35S–*CSB3* gene construct. The bottom gel shows RT–PCR for the housekeeping gene *eIF4a* used here as a loading control.

(f) RT–PCR analysis of *CLB4*/*CSB3* and *PR-1* gene expression in wild-type plants following infection with Pst DC3000, carrying or not carrying the avirulence *avrRpm1* gene. Numbers indicate hours after inoculation. The expression level of these two genes was also analyzed in healthy *clb4-3*/*csb3* plants and is shown on the left for comparison. The bottom gel shows a RT–PCR for the housekeeping gene *eIF4a* used here as a loading control. The experiment was repeated twice with similar results. Pictures of agarose gels were subjected to image analysis to measure the intensity of bands in the different replicates of the RT–PCR experiments (see Supplementary Material).

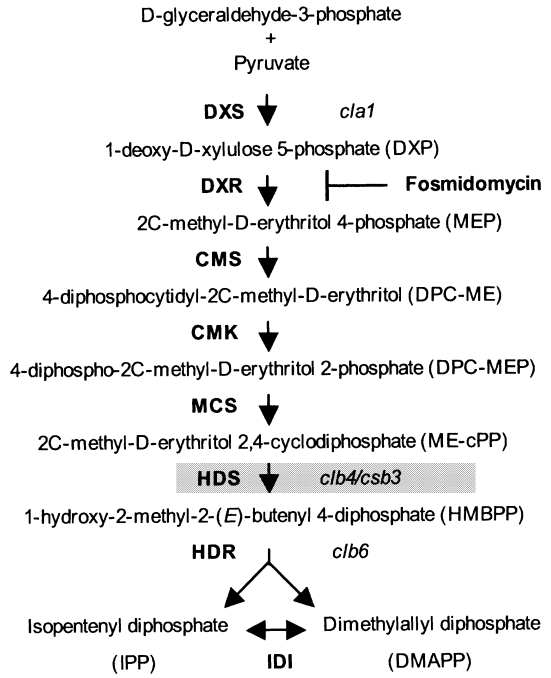

Figure 5. Diagram of the methyl-D-erythritol 4-phosphate (MEP) metabolic pathway.
Abbreviations for enzymes catalyzing each step in the pathway are shown on the left side of the figure in bold: DXS, 1-deoxy-D-xylulose 5-phospate synthase; DXR, 2C-methyl-D-erythritol 4-phosphate synthase; CMS, 4-diphosphocytidyl-2C-methyl-D-erythritol 4-phosphate synthase; CMK, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; MCS, 2C-methyl-D-erytrithol 2,4-diphosphate synthase; HDS, 2C-methyl-D-erytrithol 2,4-cyclodiphosphate reductase; HDR, 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase; IDI, isopentenyl diphosphate isomerase. The names of some of the mutants found are indicated on the right. The position of the *clb4/csb3* mutation is shown in grey. The position where fosmidomycin is known to inhibit the pathway is also indicated.

CLB4/CSB3 *expression is partially repressed by Pst DC3000 infection*

Expression of *CLB4*/*CSB3* in response to *P. syringae* infection was analyzed in leaves from wild-type plants at different time intervals after inoculation, and in a context that generates either a compatible interaction (on inoculation with Pst DC3000) or an incompatible one (on inoculating with Pst DC3000, *avrRpm1*). The presence of mRNAs was studied by RT–PCR. These analyses revealed that infection with Pst DC3000 carrying the avirulence gene *avrRpm1* gene, and thus inciting a HR response in inoculated tissue, was followed by a marked decrease in the level of accumulation of the *CLB4*/*CSB3* mRNAs (Figure 4f). This reduction was already observed 24 h post-inoculation, and was further maintained even after 48 h post-inoculation. Concomitantly, and inversely correlated with this downregulation of *CLB4*/*CSB3*, the SA-inducible marker gene *PR-1* was upregulated. Similar results, albeit of a less intensive magnitude, were recorded in a compatible interaction such as that following inoculation with Pst DC3000 lacking *avrRpm1* (Figure 4f). Moreover, expression of *CLB4*/*CSB3* in *clb4-3*/*csb3* plants also revealed a reduction in the steady-state levels of its mRNA compared with wild-type plants (Figure 4f). Thus the downregulation of *CLB4*/*CSB3* on bacterial infection, or in the mutant background, inversely correlates with the induced expression of *PR-1*.

Fosmidomycin inhibits resistance of wild-type plants and blocks the enhanced resistance of clb4-3/csb3 *plants to Pst DC3000*

An increase in the accumulation of either the substrate (MEcPP) of the HDS enzyme encoded by *CLB4*, or that of any of the metabolic intermediates preceding MEcPP synthesis in the MEP pathway (Figure 5), might account for the enhanced resistance of *clb4-3*/*csb3* plants. Alternatively, a decrease in synthesis of the product (HMBPP) of the same enzyme might compromise the synthesis and accumulation of the final products (IPP and DMAPP; Figure 5), which, in turn, might compromise the subsequent biosynthesis of the different isoprenoids to be derived from the MEP pathway. This latter possibility may also account for the enhanced resistance of *clb4-3*/*csb3* plants. To study these two possibilities, the impact of an inhibitor of the MEP pathway on the enhanced disease resistance of our mutant to Pst DC3000 was studied. For this approach we used fosmidomycin [3-(*N*-formyl-*N*-hydroxyamino)-propylphosphonic acid], a competitive inhibitor of 2C-methyl-D-erythritol 4-phosphate synthase (DXR), the enzyme controlling the second commitment step of the MEP pathway (Figure 5) which reduces the levels of the final products of this pathway (IPP and DMAPP) (Laule *et al.*, 2003; Schwender *et al.*, 1999). To disengage this response from that derived from a chloroplast developmental arrest, the fosmidomycin treatments were performed at much lower doses, and for more restricted periods, than those used experimentally by others (Laule *et al.*, 2003). Wild-type and *clb4-3*/*csb3* seedlings were sprayed once with either a 25 or a 50-μM fosmidomycin solution at 48 and 24 h prior to their inoculation with Pst DC3000, and bacterial growth in the inoculated plants was recorded at 0, 3 and 5 d.p.i. Treated plants were also assayed for an effect on the normal expression of the *P69C*–GUS gene. As shown in Figure 6(a), a 25-mM fosmidomycin treatment on mutant plants was sufficient to reduce notably the enhanced resistance to Pst DC3000. This inhibition was even more apparent after a treatment with 50-mM fosmidomycin, which completely abolished the enhanced resistance attributed to the *clb4-3*/*csb3* mutation (Figure 6a). At this latter concentration of inhibitor, the observed resistance response of the mutant was comparable with that attained in untreated wild-type plants. The inhibitory effect of fosmidomycin on the enhanced resistance of *clb4-3*/*csb3* plants

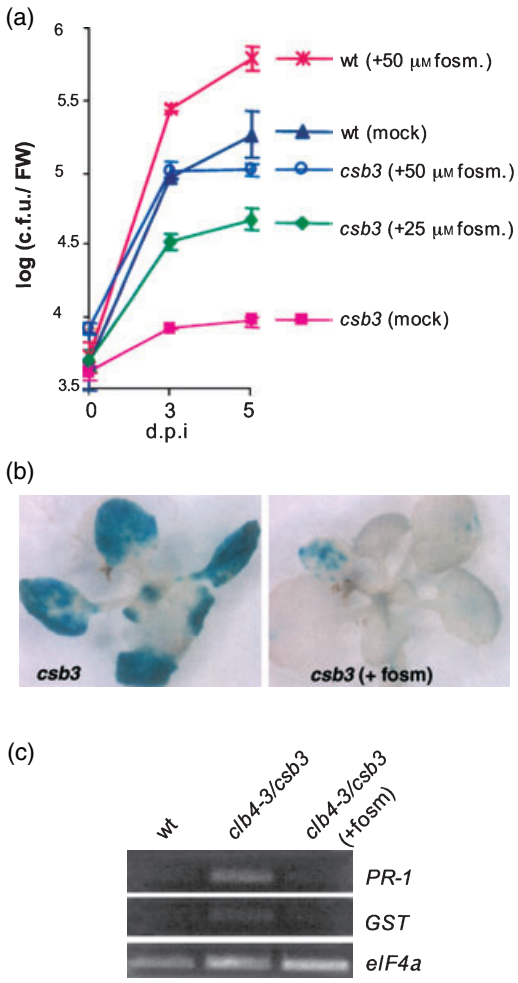

Figure 6. Pharmacological complementation of *clb4-3*/*csb3*-enhanced resistance with fosmidomycin.
(a) Arabidopsis wild-type (wt) and *clb4-3*/*csb3* seedlings were sprayed once at 48 and 24 h prior to challenge with *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) with a buffer solution supplemented with 25 or 50 μM fosmidomycin (+ fosm). Controls were treated similarly with buffer solution alone. Plants were challenged with Pst DC3000 and growth of the pathogen was recorded at 0, 3 and 5 d.p.i.
(b) GUS staining before and after treatment with fosmidomycin.
(c) Effect of fosmidomycin treatment [25 μM, treated as indicated in (a)] on the constitutive expression of *P69C*–GUS in *csb3-1* seedlings (right) compared with buffer-treated *clb4-3*/*csb3* seedlings (left).
(d) Effect of fosmidomycin treatment [25 μM, treated as indicated in (a)] on the constitutive expression of salicylic acid-regulated marker genes in *clb4-3*/*csb3* Arabidopsis seedlings compared with buffer-treated seedlings.

was also accompanied by downregulation of the constitutive expression of the *P69C*–GUS transgene (Figure 6b) and that of the marker *PR-1* and *GST6* genes (Figure 6c).

Fosmidomycin was also able to inhibit the normal disease resistance to Pst DC3000 observed in wild-type plants. A single treatment of wild-type plants with a 50-μM solution of fosmidomycin is sufficient to block resistance to Pst DC3000 and allows a five- to eightfold higher titer of bacterial growth than that observed in mock-treated wild-type plants (Figure 6a). This increase in disease susceptibility is close in magnitude to that observed in some Arabidopsis mutants compromised in the SA-mediated disease resistance. Moreover, analysis of gene expression in wild-type plants following fosmidomycin treatment revealed that this chemical also represses induction of defense-related genes normally activated by the endogenous SA (data not shown). All these observations further reinforce the consideration that keeping the MEP pathway intact is pivotal for mounting an effective resistance response to pathogens. Furthermore, from the analysis of the effect of fosmidomycin on *clb4-3*/*csb3* plants, it can be concluded that the enhanced resistance of this mutant appears to be related not to a reduction of IPP and its isomer DMAPP, but rather to the result of an excess accumulation of the direct substrate of the encoded HDS enzyme (MEcPP) or that of any of its immediate precursors.

Discussion

We identified a novel Arabidopsis mutant, initially designated *csb3* and later renamed *clb4-3*, that shows high levels of resistance to the biotrophic pathogens *P. syringae* and *H. parasitica*, while normal susceptibility to the necrotrophic pathogens *P. cucumerina* and *B. cinerea* is retained. Phenotypic and genetic analyses indicate that *clb4-3*/*csb3* is a recessive partial loss-of-function mutation resulting in the activation of the plant's defenses. Consistent with the observed enhancement of resistance towards biotrophs, *clb4-3*/*csb3* plants show enhanced accumulation of SA under resting conditions, and a further ability to increase biosynthesis over wild-type levels on challenge with Pst DC3000. This observation offers an explanation as to why *clb4-3*/*csb3* plants also show constitutive activation of several defense-related marker genes (e.g. *PR1*, *PR2* and *GST6*) that are ultimately controlled by the SA-mediated signaling pathway (Durrant and Dong, 2004). Particularly instructive in understanding the role of *CLB4* in this defense pathway was the observation that SA is absolutely required for *clb4-3*/*csb3*-conferred resistance, as inferred from the suppression of this phenotype in the various double-mutant backgrounds that we generated. Steady-state *PR-1* gene transcript levels, expression of *P69C*–GUS, and enhanced resistance to Pst DC3000 were strikingly abolished in the *csb3 pad4*; *csb3 sid2*; *csb3 eds5*; and *csb3 NahG* double mutants compared with those seen in *csb3* homozygous plants. All these observations indicate that keeping SA synthesis and accumulation intact is pivotal for the enhancement of disease resistance in *clb4-3*/*csb3* plants. Thus our studies indicate that *clb4-3*/*csb3*-mediated control of the SA-dependent defense response appears to operate at some stage in the very early events of the plant–pathogen interaction and upstream of *PAD4*, *SID2* and *EDS5*. Furthermore, enhanced resistance

of *clb4-3*/*csb3* also requires components that function as transducers of the SA-derived defense signal (such as NPR1), as demonstrated by the *csb3 nrp1* double-mutant plants, which behave like *npr1* with respect to lack of *PR* gene expression and hypersusceptibility to Pst DC3000 infection. Moreover, reversion of the resistance phenotype from resistant to hypersusceptible, without a suppression of the steady-state *PR-1* gene transcript level in the *csb3 dth9* double mutant, reveals a requirement for an intact DTH9-dependent pathway downstream of SA accumulation for the *clb4-3*/*csb3*-associated resistance. Thus, intact NPR1 and DTH9 pathways for regulating disease resistance are also required in *clb4-3*/*csb3* plants.

Using map-based cloning, we have identified the *CLB4*/*CSB3* gene and demonstrated by reverse genetics that it fully complements the *clb4-3*/*csb3* phenotype. Moreover, analysis of gene expression in wild-type plants revealed that *CLB4*/*CSB3* is downregulated following infection with Pst DC3000. This downregulation correlates inversely with establishment of resistance and induction of SA-regulated *PR-1* (Figure 5f). All these observation accord with the hypothesis that *CLB4*/*CSB3* may function as negative regulator of the SA-dependent pathway, leading to resistance towards biotrophic pathogens.

Interestingly, the *clb4-3*/*csb3* mutant is not predicted to be a null mutant, as other null alleles in the *CLB4* gene (*clb4-1* and *clb4-3*) are lethal. The *CLB4*/*CSB3* gene encodes the HMBPP synthase (HDS) that catalyses the formation of HMBPP from MEcPP (Kollas *et al.*, 2002). This enzymatic activity controls the penultimate step in the biosynthesis of IPP via the MEP metabolic pathway that occurs in the chloroplast (Figure 5) (Rohmer, 1999).

Because the partial loss of function associated with the *clb4-3*/*csb3* mutation results in the activation of the SA pathway that leads to the observed enhanced resistance, we deduced that the dismantling of a downstream compound (due to the reduced enzymatic activity of the *clb4-3*/*csb3* enzyme) or, alternatively, the overaccumulation of an upstream compound (such as MEcPP or any of its metabolic precursors in the pathway), must have a function in the SA-controlled defense response. We tested this conjecture by examining the effect of an inhibitor of the MEP pathway (fosmidomycin) on the enhanced resistance of *clb4-3*/*csb3* plants, as well as on the normal resistance attained in wild-type plants. Fosmidomycin blocks the activity of the DXR, the enzyme controlling the second commitment step of the MEP pathway (Figure 6), and thus inhibits the pathway upstream of HDS (Laule *et al.*, 2003; Schwender *et al.*, 1999). We observe that fosmidomycin fully blocks the *clb4-3*/*csb3*-associated enhanced-resistance phenotype as well as the expression of the associated defense-related marker genes (Figure 6). This inhibitory effect of fosmidomycin favors the interpretation that activation of the SA pathway in the *clb4-3*/*csb3* mutant is not caused by a reduction in the level of a critical metabolite downstream of HDS that may compromise subsequent enzymatic steps leading to isoprenoid biosynthesis. Rather, the activation of the SA pathway is the result of an excess accumulation of the direct substrate of HDS (MEcPP) or any of its immediate precursors. Moreover, we demonstrate that in wild-type plants inhibition of the MEP pathway with fosmydomycin also provokes a corresponding inhibition of disease resistance to Pst DC3000, as it gives rise to plants in which the rate of bacterial growth is much higher (Figure 6a). These findings give strong support to the consideration that the MEP pathway, and in particular the step controlled by the HDS enzyme, is a point of metabolic control of SA-dependent disease resistance in wild-type plants. However, at this stage we cannot disregard the possibility that high levels of MEcPP, or of any other metabolic intermediate of the MEP pathway, could also cause stress in the plant and indirectly result in increased pathogen resistance. This would be a non-specific response, as against a scenario where levels of MEcPP or its precursors regulate SA levels. Further experiments directed towards understanding the direct effects of MEcPP accumulation and SA signaling will help to clarify whether the role of the MEP pathway in disease resistance is direct or indirect.

In summary, our results point to the participation of a chloroplastic signal/factor, as derived from one of the early intermediary steps of the MEP pathway, in control of the pathogen-activated SA pathway leading to disease resistance to biotrophic pathogens. This supports the importance of the chloroplast in mediating critical aspects of the plant defense response. The exact nature of the signal molecule(s) that exert such a positive effect on the SA pathway; the nature of the target(s); and how this interplay is controlled are our challenges for the future.

Experimental procedures

Plants, growth conditions and treatments

Arabidopsis thaliana plants were grown in soil or on plates containing Murashige and Skoog (MS) medium as described previously (Mayda *et al.*, 2000). The *csb3* mutant was isolated in a screen for constitutive expressers of the *P69C*–GUS reporter gene in transgenic Columbia (Col-0) plants mutagenized with ethyl methanesulfonate (EMS), as described previously for other mutants (Mayda *et al.*, 2000). The *csb3* mutant line used in these experiments had been back-crossed four times to the wild-type parental line. Plants were grown in a growth chamber (19–23°C, 100 μE m^{-2} sec^{-1} fluorescent illumination) on a 10-h light/14-h dark cycle.

Pathogen infection

Pseudomonas syringae pv. *tomato* DC3000 was grown and prepared for inoculation as described previously (Mayda *et al.*, 2000). Plants were inoculated by the dipping method according to Tornero and Dangl (2001). Data are reported as means and standard deviations of the log (CFU g^{-1} FW) of six to eight replicates.

Hyaloperonospora parasitica (10^5 conidiospores ml^{-1}), *Plectosphaerella cucumerina* (5×10^6 spores ml^{-1}) and *Botrytis cinerea* (2.5×10^4 conidia ml^{-1}) resistance assays were carried out as described previously (Coego *et al.*, 2005).

Genetic analysis

Crosses were performed by emasculating unopened buds and using the pistils as recipients for pollen. Back-crosses with the parental transgenic line were performed using *P69C*–GUS plants as the pollen donor. The reciprocal crosses were also performed. F_1 and F_2 plants were grown on MS plates and tested for GUS activity. Segregation of phenotype in the F_2 generation was analyzed for goodness of fit using the chi-square test.

Map-based cloning

To define the chromosomal map position of *CSB3*, the *csb3* mutant, which is in a Col-0 background, was crossed to wild-type Landsberg *erecta* (L*er*) plants. Homozygous *csb3* mutant plants were identified among the F_2 progeny by their characteristic morphological phenotype. The F_2 mapping population selected was examined for segregation of PCR-based molecular markers. For the initial mapping, 38 F_2 plants were tested with single sequence-length polymorphism (SSLP) markers from each of the five chromosomes (Bell and Ecker, 1994). The CIW10 marker on chromosome 5 showed cosegregation with the *csb3* phenotype. Further analysis of this region showed the mutation to be localized between the nga129 and MBK-5 markers, which are 15 cM apart. To narrow down the region in which *CSB3* was located, the Cereon Genomics large insertion/deletion database was used to design SSLP markers. After screening 748 F_2 plants, *CSB3* was narrowed down to two BAC clones (MUF9 and MUP24) comprising a 66-kb region that contained 21 ORFs (see Supplementary Material). The entire region of each of these genes was amplified from *csb3* plants and the sequences of the PCR products were determined.

Generation of double mutants

The mutant alleles used throughout this study were *npr1-1* (Cao *et al.*, 1997); *pad4-1* (Zhou *et al.*, 1998); *sid2-1* (Wildermuth *et al.*, 2001); *eds5* (Nawrath *et al.*, 2002); and *dth9* (Mayda *et al.*, 2000). The *csb3 npr1*; *csb3 pad4*; *csb3 sid2*; *csb3 eds5*; *csb3 dth9*; and *csb3 NahG* double mutants were generated using *csb3* as recipient for pollen. The homozygosity of the loci was confirmed using a molecular marker for each of the alleles in segregating populations. For the double mutant containing *dth9*, hypersusceptibility to Pst DC3000 was the criterion used to assess homozygocity. All the double mutants were confirmed in the F_3 generation.

cDNA cloning and reverse complementation

The genomic sequence was used as the basis for cloning the *CSB3* cDNA. Poly(A+) RNA was isolated from wild-type plants and reverse-transcribed using oligo(dT) primers as described (Mayda *et al.*, 1999). This cDNA was amplified by PCR using gene-specific primers designed to include the region upstream of the start codon and part of the 3′ region that follows the stop codon of the *CSB3* gene. The sequences of the *CSB3* forward and reverse primers used were: (5′-CTTCCTCTGGATCCTTCTCTTCTC-3′) and (5′-GGACACTAGTTCAAAATGATGATG-3′) respectively. The cDNA was cloned into the binary vector pBI121 (Clontech, Palo Alto, CA, USA) under control of the constitutive CaMV 35S promoter. From there the construct was transferred to pCAMBIA1300 yielding pCAMBIA35S*CSB3* which, in turn, was transferred to *Agrobacterium* and used to transform *csb3-1* plants by the floral-dip method (Bechtold *et al.*, 1993).

Determination of salicylic acid

Free and conjugated SA content in leaves was determined in methanolic extracts by HPLC analysis, as described previously (Mayda *et al.*, 1999).

Expression analysis

To analyze the level of gene expression by reverse transcriptase-mediated PCR, total RNA samples were prepared from leaf tissues using the Totally RNA kit from Ambion (Austin, TX, USA). Reverse transcription was performed using the RT-for-PCR kit from Clontech. The oligonucleotide primer sets (50 pmol each) used to amplify *CSB3* were: *CSB3PCR1* (5′-GGAGGCCTTCTTGTGGATGG-3′)/*CSB3PCR2* (5′-GCTGACCCAACGACCATGTTC-3′). The primers used to amplify *GST6* were *GST6PCR1* (5′-ATGGCAGGAATCAAAGTTTCGGTC3′)/*GST6PCR2* (5′-GAGATTCACTTAAAGAACCTTCTG-3′). The primers used to amplify *PR1* were *PR1PCR1* (5′-ATGAATTTTACTGGCTATTC-3′)/*PR1PCR2* (5′-AACCCACATGTTCACGGCGGA-3′). PCR conditions for each of the genes indicated are available on request.

Acknowledgements

We thank C. Castresana, P. Tornero and B. Wulff for helpful discussion and for comments on the manuscript. We also thank A. Agorio for collaborating in the RT–PCR experiments and S. Saurí for excellent technical assistance. We acknowledge the support of the Spanish Ministry of Science and Technology (Grant BMC2003-00267 to P.V.) for financial support. We thank the SALK collection of T-DNA insertions for providing seeds for some of the mutants.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Histochemical localization of GUS activity driven by the *P69C* gene promoter in transgenic Arabidopsis plants.

Figure S2. Phenotype of *csb3-1*, *cpr1-1* and *csb3-1 cpr1-1* plants compared with wild-type (wt) Arabidopsis plants.

Figure S3. Positional cloning of *CSB3*.

Figure S4. Relative abundance of *CSB3* and *PR1* mRNA following bacterial inoculation.

References

Bechtold, N., Ellis, J. and Pelletier, G. (1993) In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *CR Acad. Sci. Paris Life Sci.* **316**, 1194–1199.

Bell, C.J. and Ecker, J.R. (1994) Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. *Genomics*, **19**, 137–144.

Bowling, S.A., Guo, A., Cao, H., Gordon, S., Klessig, D.F. and Dong, X. (1994) A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. *Plant Cell*, **6**, 1845–1857.

Budziszewski, G.J., Lewis, S.P., Glover, L.W. *et al.* (2001) Arabidopsis genes essential for seedling viability: Isolation of insertional mutants and molecular cloning. *Genetics*, **159**, 1765–1778.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X. (1997) The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell*, **88**, 57–63.

Clarke, J.D., Aarts, N., Feys, B.J., Dong, X. and Parker, J.E. (2001) Constitutive disease resistance requires EDS1 in the Arabidopsis mutants *cpr1* and *cpr6* and is partially EDS1-dependent in *cpr5*. *Plant J.* **26**, 409–420.

Coego, A., Ramirez, V., Gil, M.J., Flors, V., Mauch-Mani, B. and Vera, P. (2005) An *Arabidopsis* homeodomain transcription factor, OCP3, mediates resistance to infection by necrotrophic pathogens. *Plant Cell*, **17**, 2123–2137.

Dangl, J.L. and Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. *Nature*, **411**, 826–833.

Delaney, T.P., Uknes, S., Vernooij, B. *et al.* (1994) A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1247–1250.

Dong, X. (2004) NPR1, all things considered. *Curr. Opin. Plant Biol.* **7**, 547–552.

Durrant, W.E. and Dong, X. (2004) Systemic acquired resistance. *Annu. Rev. Phytopathol.* **42**, 185–209.

Feys, B.J., Moisan, L.J., Newman, M.A. and Parker, J.E. (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. *EMBO J.* **20**, 5400–5411.

Glazebrook, J., Rogers, E.E. and Ausubel, F.M. (1997) Use of Arabidopsis for genetic dissection of plant defense responses. *Annu. Rev. Genet.* **31**, 547–569.

Gutiérrez-Nava, M.L., Gillmor, C.S., Jiménez, L.F., Guevara-García, A. and León, P. (2004) *Chloroplast biogenesis* genes act cell and noncell autonomously in early chloroplast development. *Plant Physiol.* **135**, 471–482.

Hammond-Kosack, K.E. and Parker, J.E. (2003) Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding. *Curr. Opin. Biotechnol.* **14**, 177–193.

Hecht, S., Eisenreich, W., Adam, P., Amslinger, S., Kis, K., Bacher, A., Arigoni, D. and Rohdich, F. (2001) Studies on the nonmevalonate pathway to terpens: the role of the GcpE (IspG) protein. *Proc. Natl Acad. Sci. USA*, **98**, 14837–14842.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M. and Glazebrook, J. (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl Acad. Sci. USA*, **96**, 13583–13588.

Jirage, D., Zhou, N., Cooper, B., Clarke, J.D., Dong, X. and Glazebrook, J. (2001) Constitutive salicylic acid-dependent signaling in *cpr1* and *cpr6* mutants requires PAD4. *Plant J.* **26**, 395–407.

Jorda, L. and Vera, P. (2000) Local and systemic induction of two defense-related subtilisin-like protease promoters in transgenic Arabidopsis plants. Luciferin induction of *PR* gene expression. *Plant Physiol.* **124**, 1049–1058.

Kachroo, P., Shanklin, J., Shah, J., Whittle, E.J. and Klessig, D.F. (2001) A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc. Natl Acad. Sci. USA*, **98**, 9448–9453.

Kachroo, A., Lapchyk, L., Fukushige, H., Hildebrand, D., Klessig, D.F. and Kachroo, P. (2003) Plastidial fatty acid signaling modulates salicylic acid- and jasmonic acid-mediated defense pathways in the Arabidopsis *ssi2* mutant. *Plant Cell*, **15**, 2952–2965.

Kim, H.S. and Delaney, T.P. (2002) Arabidopsis SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. *Plant Cell*, **14**, 1469–1482.

Kollas, A.-K., Duin, E.C., Eberl, M. *et al.* (2002) Functional characterization of GcpE, an essential enzyme of the non-mevalonate pathway of isoprenoid biosynthesis. *FEBS Lett.* **532**, 432–436.

Laule, O., Fürholz, A., Chang, H., Zhu, T., Wang, X., Heifetz, P.B., Gruissem, W. and Lange, M. (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **100**, 6866–6871.

Li, X., Zhang, Y., Clarke, J.D., Li, Y. and Dong, X. (1999) Identification and cloning of a negative regulator of systemic acquired resistance, SNI1, through a screen for suppressors of *npr1-1*. *Cell*, **98**, 329–339.

Li, X., Clarke, J.D., Zhang, Y. and Dong, X. (2001) Activation of an EDS1-mediated *R*-gene pathway in the *snc1* mutant leads to constitutive, NPR1-independent pathogen resistance. *Mol. Plant–Microbe Interact*, **14**, 1131–1139.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J. and Cameron, R.K. (2002) A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature*, **419**, 399–403.

Mayda, E., Tornero, P., Conejero, V. and Vera, P. (1999) A tomato homeobox gene (HD-Zip) is involved in limiting the spread of programmed cell death. *Plant J.* **20**, 591–600.

Mayda, E., Mauch-Mani, B. and Vera, P. (2000) Arabidopsis *dth9* mutation identifies a gene involved in regulating disease susceptibility without affecting salicylic acid-dependent responses. *Plant Cell*, **12**, 2119–2128.

Mou, Z., Fan, W. and Dong, X. (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell*, **113**, 935–944.

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

Nawrath, C., Heck, S., Parinthawong, N. and Métraux, J-P. (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. *Plant Cell*, **14**, 275–286.

Pieterse, C.M. and Van Loon, L.C. (2004) NPR1: the spider in the web of induced resistance signaling pathways. *Curr. Opin. Plant Biol.* **7**, 456–464.

Querol, J., Campos, N., Imperial, S., Boronat, A. and Rodríguez-Concepción, M. (2002) Functional analysis of the *Arabidopsis thaliana* GCPE protein involved in plastid isoprenoid biosynthesis. *FEBS Lett.* **514**, 343–346.

Rogers, E.E. and Ausubel, F.M. (1997) Arabidopsis enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. *Plant Cell*, **9**, 305–316.

Rohmer, M. (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat. Prod. Rep.* **16**, 565–574.

Sauret-Güeto, S., Ramos-Valdivia, A., Ibanez, E., Boronat, A. and Rodriguez-Concepcion, M. (2003) Identification of lethal mutations in *Escherichia coli* genes encoding enzymes of the methylerythritolphosphate pathway. *Biochem. Biophys. Res. Commun.* **307**, 408–415.

Schwender, J., Müller, C., Zeidler, J. and Lichtenthaler, H.K. (1999) Cloning and heterologous expression of a cDNA encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase of *Arabidopsis thaliana*. *FEBS Lett.* **455**, 140–144.

Shah, J. (2003) The salicylic acid loop in plant defense. *Curr. Opin. Plant Biol.* **6**, 365–371.

Shah, J., Kachroo, P. and Klessig, D.F. (1999) The Arabidopsis *ssi1* mutation restores pathogenesis-related gene expression in *npr1* plants and renders defensin gene expression salicylic acid dependent. *Plant Cell*, **11**, 191–206.

Shirano, Y., Kachroo, P., Shah, J. and Klessig, D.F. (2002) A gain-of-function mutation in an Arabidopsis toll interleukin1 receptor–nucleotide binding site–leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. *Plant Cell*, **14**, 3149–3162.

Tornero, P. and Dangl, J.L. (2001) A high-throughput method for quantifying growth of phytopathogenic bacteria in *Arabidopsis thaliana*. *Plant J.* **28**, 475–481.

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*, **414**, 562–565.

Zhang, Y., Goritschnig, S., Dong, X. and Li, X. (2003) A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of *npr1-1*, *constitutive 1*. *Plant Cell*, **15**, 2636–2646.

Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F. and Glaze brook, J. (1998) PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell*, **10**, 1021–1030.