

# Signals for local and systemic responses of plants to pathogen attack

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## Abstract

**Activation of plant defences following recognition of pathogen attack involves complex reiterative signal networks with extensive signal amplification and cross-talk. The results of two approaches that have been taken to analyse signalling in plant–microbe interactions are discussed here. Activation tagging with T-DNA harbouring multiple 35S enhancer elements was employed as a gain-of-function approach to dissect signalling related to bacterial pathogen resistance in *Arabidopsis thaliana*. From a screen of ~5000 activation tagged lines, one line was identified as harbouring a T-DNA leading to over-expression of an apoplastic aspartic protease (CDR-1), that resulted in resistance to normally virulent *Pseudomonas syringae*. The second approach was to screen for loss-of-function mutants in T-DNA tagged populations. From a screen of 11 000 lines, one line, defective in induced resistance-1 (*dir-1*) lost resistance to normally avirulent *P. syringae*. Models for action of the products of the *CDR-1* and *DIR-1* genes suggest involvement of peptide and lipid signals in systemic disease resistance responses in *A. thaliana*.**

**Key words:** Aspartic protease, bacterial resistance, lipid transfer protein, salicylic acid, signal transduction.

## Introduction

The past 5–10 years have seen a major increase in understanding of the molecular mechanisms underlying the outcome of plant–pathogen interactions. In large part, this has resulted from genetic approaches, both in model systems such as *Arabidopsis thaliana*, and in crop plants including tomato and rice (Baker *et al.*, 1997; Dangl and Jones, 2001; Martin *et al.*, 1993; Ronald, 1997). Interactions between plants and most biotrophic fungal or bacterial pathogens are governed by complementary interactions between products of pathogen avirulence (*avr*) gene loci and alleles of the corresponding plant disease resistance (*R*) loci (Dangl and Jones, 2001). Many *R* genes have now been cloned, some of which have significant identity to genes involved in signalling during development in *Drosophila* and human (Dangl and Jones, 2001; Whitham *et al.*, 1994). Although downstream signal transduction between the *R* gene recognition event and the ultimate expression of defence responses has also been amenable to genetic dissection (Shah *et al.*, 1999; Zhou *et al.*, 1995, 1997, 1998), the frequency at which independent mutations in downstream signalling pathways are recovered is less than the frequency with which *R* genes are recovered.

Low frequency recovery of signalling mutants has been a problem for dissecting the phenomenon of systemic acquired resistance (SAR), whereby inoculation of one leaf of a plant, leading to local hypersensitive resistance, is followed by the generation of a systemic signal that

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transduces the resistance response to other parts of the plant, such that attempted secondary infections are rapidly and effectively blocked (Ryals *et al.*, 1996). SAR shares many features with local resistance, including the involvement of salicylic acid (SA) as a necessary component for full activation of downstream defence response genes and the oxidative burst (Lawton *et al.*, 1995; Mur *et al.*, 1996; Shah and Klessig, 1999; Shirasu *et al.*, 1997). However, in spite of many attempts, the nature of the mobile signal(s) involved in the establishment of SAR has yet to be unequivocally determined, and even the biochemical origin of the SA involved in plant disease resistance is still unclear (Shadle *et al.*, 2003; Wildermuth *et al.*, 2001).

Oxylipins and peptides are common signal transducers in mammalian cells, and biochemical approaches have provided evidence for the involvement of peptide and lipid-mediated signalling in the responses of Solanaceous plants to wounding and insect herbivory (Ryan and Pearce, 1998). A mobile 18 amino acid peptide, systemin, is released from a 200 amino acid precursor, pro-systemin, following physical damage to tomato or tobacco leaves (Pearce *et al.*, 1991). This process results in receptor binding (Scheer and Ryan, 1999) that leads to activation of a lipid-mediated signal transduction cascade involving the octadecanoid pathway, analogous to signalling in mammalian cells (Bergey *et al.*, 1996), with phytodienoic and jasmonic acids as key intermediates. SA-mediated signalling in plant defence to pathogens and JA-mediated wound signalling act antagonistically (Doares *et al.*, 1995; Felton *et al.*, 1999), and, in spite of the characterization of several peptide and proteinaceous elicitors of defence responses from plant pathogens (Baker *et al.*, 1993; Nürnberger *et al.*, 1994; Perez *et al.*, 1997) there is, to date, no direct evidence for the involvement of endogenous peptide signalling in the activation of either local or systemic microbial pathogen defence responses in plants. A serine protease has been implicated to act on a negative regulator of the SA-potentiated oxidative burst in soybean cell cultures (Guo *et al.*, 1998), but neither the protease nor its target has been characterized. The legume *ENOD40* gene, which is rapidly induced in roots in response to bacterial chito-oligosaccharide nodulation factors, contains several short open reading frames and is believed to encode two peptides that bind to, and may regulate, sucrose synthase (Rohrig *et al.*, 2002). Orthologues of *ENOD40* are found in non-nodulating plants such as tobacco ((van de Sande *et al.*, 1996), suggesting a broader role for peptide signalling in plants (Ryan, 1996) that is becoming increasingly realized as peptide regulators of plant growth and development, and their corresponding receptors, are being characterized for the first time (Bisseling, 1999; Matsubayashi *et al.*, 2001).

Most genetic screens for plant defence signalling components have used gene knock-out approaches, which may fail to uncover genes for which functional redundancy exists. T-DNA activation tagging provides a

means of generating dominant, gain-of-function mutations (Weigel *et al.*, 2000). Ectopic expression of defence response signalling molecules can result in constitutively expressed resistance phenotypes (Verberne *et al.*, 2000), and this type of screen might therefore be expected to uncover genes that quantitatively impact resistance even if, as is now believed, there is considerable redundancy and cross-talk within and between signalling pathways (Dangl and Jones, 2001). The ease of genetic transformation of many plant species also facilitates metabolic engineering strategies to address the involvement of specific biochemical pathways in plant defence. Recent studies from the authors' laboratories that have employed gain and loss of function genetic approaches and metabolic engineering to dissect signalling mechanisms during plant-pathogen interactions are reviewed here. New information is also presented on the mode of action and localization of CDR1, an extracellular aspartic protease involved in both local and systemic activation of defence response genes in *Arabidopsis*.

## Materials and methods

### Plant materials

Growth and characterization of the *Arabidopsis thaliana* *cdr1-D* and *dir1-1* mutants has been described previously (Maldonado *et al.*, 2002; Y Xia *et al.*, unpublished data). For experiments on the expression of CDR1 and its site-directed mutants, *A. thaliana* ecotype Columbia (Col-0) plants were grown at 23 °C under short-day conditions (9 h 150 µE light and 15 h dark) for 5 weeks, followed by growth under long-day conditions (16 h light and 8 h dark) until mature.

### Nucleic acid analysis

Total RNA was isolated from aerial parts of 4–5-week-old plants that had not started to bolt using Tri-reagent according to the manufacturer's instructions (MRC Inc., Cincinnati, Ohio). RT-PCR analysis was carried out using total RNA isolated from leaves of 4–5-week-old plants. First-strand cDNA was synthesized from 4 µg total RNA using Superscript II reverse transcriptase (GIBCO) and oligo dT primer. PCR was carried out for 25 cycles whereby cDNAs were exponentially amplified by ExTaq DNA polymerase (Takara) using gene-specific primers for CDR1, PR1, ICS1, and actin as follows: 5'-TACGAACGATAGCCATGGCCTCTC-3' and 5'-GGATCCTACATCTTTGCACAATCTGTTGGC-3' for CDR1; 5'-ATGAATGAAATGTCGTTCTTTGGTTATAG-3' and 5'-CCAATCACTAATATGGACGTTGACCGATG-3' for PR1; 5'-GGAACGGAAACGGAAACGGAAACAGTG-3' and 5'-GGCCTGCCTAGTTACAACCCGAAAAG-3' for ICS1; and 5'-GATATGAAAAGATCTGGCATCAC-3' and 5'-TCATACTCGGCCTTGAGATCCAC-3' for actin.

### Subcellular localization of CDR1 in *A. thaliana*

To generate a fusion protein of CDR1 with the green fluorescent protein (sGFP), the full-length cDNA encoding CDR1 was used as a template for PCR. The signal peptide (27 amino acids) from the N-terminus of CDR1 was amplified by PCR using pfu DNA polymerase (Stratagene) with the *NcoI* upstream primer 5'-CGATGCCATGGCCTCTCTATTCTCTTCAGTTCTCT-3' and the *NcoI* downstream primer 5'-CGATGCCATGGGCTTAGCGTTTGCATTTGAGAGAAA-3'. The PCR product was purified,

ligated into pGEMTeasy vector (Promega), sequenced, excised, and inserted into the *NcoI* sites of p35-sGFP (Niwa *et al.*, 1999) under control of the cauliflower mosaic virus 35S promoter. After digestion with *Bam*HI and *Eco*RI, the resulting chimeric DNA was inserted into the corresponding sites of pBI121 (Clontech). Similarly, the CDR1 open reading frame was amplified by PCR using pfu DNA polymerase (Stratagene) with the *NcoI* upstream primer 5'-CGATGCCATGGCCTCTCTATTCTCTTCAGTTCTCT-3' and the *NcoI* downstream primer 5'-CGATGCCATGGACATCT-TTGCACAATCTGTTGGCTTAAA-3'. The PCR product was ligated into pGEMTeasy vector (Promega), sequenced, excised, and inserted into the *NcoI* sites of p35-sGFP. After digestion with *Bam*HI and *Eco*RI, the resulting GFP fusion constructs were inserted into the corresponding sites of pBI121, mobilized into *Agrobacterium tumefaciens* strain GV3101, and re-introduced into wild-type Col-0 by *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998).

#### Generation of CDR1 site-directed mutants

For construction of CDR1 mutants, the full-length cDNA encoding CDR1 was used as a template for PCR. To generate D108N, in which the aspartic acid residue at position 108 was replaced by asparagine, the mutation was carried out using the *NcoI* upstream primer 5'-CGATGCCATGGCCTCTCTATTCTCTTCAGTTCTCT-3', the *Bam*HI downstream primer 5'-CGCGGATCCTACATCTTGCACAATCTGTTGGCTT-3', the internal forward primer 5'-TGGCCATCGCCAACACCGGAAGTGATCTCC-3', and the internal reverse primer 5'-CACTTCCGGTGTGGCGATGGCCATGATC-3'. The double underlined nucleotides indicate the positions of mutagenesis. The first PCR amplification was performed using the two sets of primers (one set, *NcoI* upstream primer and internal reverse primer, another set, internal forward primer and *Bam*HI downstream primer) in separate reaction tubes. A second PCR amplification was then performed to generate the D108N fragment using a mixture of the two PCR-amplified fragments, *NcoI* upstream primer and *Bam*HI downstream primer. The PCR product was purified, ligated into pGEMTeasy vector (Promega), sequenced and re-cloned between the *NcoI* and *Bam*HI sites of pRTL2 (Restrepo *et al.*, 1990) under control of the CaMV35S promoter with duplicated enhancer. The mutated CDR1 expression cassette was excised with *Hind*III and cloned into the binary vector pGA482 (An, 1986).

Other site-directed mutations in CDR1 were made in the same way using the following combinations of mutated internal primers: internal forward primer 5'-TTGTCTTACGGGAATAACTCAT-ACACAAAGGG-3' and internal reverse primer 5'-TGTATGAGTTATTCCTCGTAAGACAATGAG-3' for D174N; internal forward primer 5'-CATCATCATCAATTCAGGCACAACCTTA-ACG-3', and internal reverse primer 5'-AGTTGTGCCTGAATTGATGATGATGTTTCCC-3' for D319N. All constructs were mobilized into *A. tumefaciens* strain GV3101 and re-introduced into wild-type Col-0 by *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998).

#### Expression and assay of CDR1 fusion protein in *E. coli*

To generate CDR1-GST fusion protein, the insert of pCDR1C1 was PCR amplified using the primer pair 5'-CGCGGATCCATGGCCTCTCTATTCTCTTCAGTTCTC-3' and 5'-CGCGGATCCTACATCTTGCACAATCTGTTGGCTT-3', and the PCR product subcloned into the *Bam*HI site of the pGEX-4T-2 expression vector (Pharmacia) to generate pGEX-CDR1. pGEX-CDR1 was introduced into *E. coli* strain BL21 (DE3) (Novagen). Cells harbouring the expression construct were grown to an OD<sub>600</sub> of 0.6, and expression induced by addition of isopropyl 1-thio- $\beta$ -D-galactopyranoside

(IPTG) to a final concentration of 1 mM, with further incubation at 16 °C for 9 h.

Purification of the CDR1 fusion protein on a Glutathione Sepharose 4B column was performed according to the manufacturer's instructions. Aspartic protease activity of CDR1-GST was assayed using FITC-BSA as substrate (Murakami *et al.*, 2000). The reaction mixture contained, in a total volume of 60  $\mu$ l, 2 mg ml<sup>-1</sup> fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) (Sigma), purified CDR1 fusion protein (1.5  $\mu$ g), 400 mM Na citrate buffer (pH 3.2). The mixture was incubated at 37 °C for 2 h and the reaction stopped by the addition of 120  $\mu$ l of 5% trichloroacetic acid (TCA). TCA-insoluble protein was removed by centrifugation at 12 000 g for 5 min and levels of peptide-FITC in the supernatant quantified by fluorescence spectrophotometry (excitation 488 nm, emission 530 nm).

#### Transcript analysis

Transcript levels were measured by RT-PCR analysis using total leaf RNA from 4–5-week-old plants. First-stand cDNA was synthesized from 4  $\mu$ g total RNA using Superscript II reverse transcriptase (GIBCO) and oligo dT primer. PCR was carried out for 25 cycles using ExTaq DNA polymerase (Takara) with gene-specific primers for CDR1, PR1 and ICS1.

#### Extraction and determination of SA

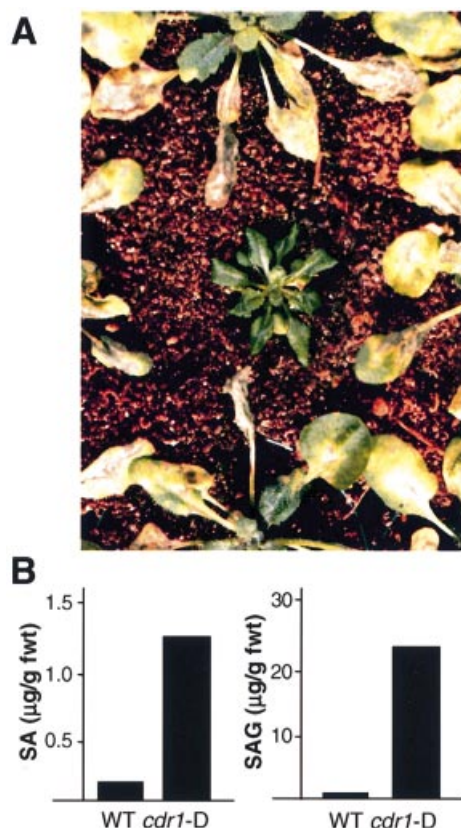
SA (free and glycosylated) was extracted according to published procedures (Meuwly and Metraux, 1993). Separation and determination was performed on an HP1100 (Agilent) HPLC, Waters Spherisorb ODS2 5  $\mu$  C<sub>18</sub> reverse phase column (250  $\times$  4.6 mm), flow rate 0.8 ml min<sup>-1</sup>, with a diode array detector (Agilent) and fluorescence detector (Jasco Model FP920) in tandem. SA levels were calculated based on fluorescence (excitation wavelength 305 nm, emission at 407 nm) compared with a standard curve using authentic SA.

## Results and discussion

#### T-DNA activation tagging uncovers a gene involved in peptide-mediated local and systemic disease response signalling in *A. thaliana*

About 5000 activation tagged T<sub>1</sub> *Arabidopsis thaliana* ecotype Columbia (Col-0) lines were screened for resistance to infection by virulent strains of *Pseudomonas syringae* pathovar *tomato* (*Pst*). Individuals that exhibited no disease symptoms, or less severe symptoms than wild-type Col-0 plants, were classified as putative mutants. One individual, *cdri-D* (for *constitutive disease resistance-Dominant*) was identified as resistant to *Pst*. Neighbouring plants were severely infected following spraying with virulent *Pst*, whereas the *cdri-D* plant was almost asymptomatic (Fig. 1A; Y Xia *et al.*, unpublished data). The *cdri-D* plant was dwarf and the leaves were slightly curled. However, the plants were otherwise developmentally normal and were fertile (Y Xia *et al.*, unpublished data).

The *cdri-D* mutation is dominant over its wild-type allele, and molecular analysis indicated that it is caused by T-DNA insertion and that the dwarf stature is likely to be a side-effect of constitutive expression of disease resistance mechanisms mediated by the mutation (Y Xia *et al.*,



**Fig. 1.** Visible phenotype and SA levels of *cdr1-D* compared with wild-type *Arabidopsis*. (A) Appearance of plants following infection with virulent *P. syringae*. The *cdr1-D* mutant is in the centre. (B) SA levels in non-infected wild-type and *cdr1-D* *A. thaliana* plants. SA, free salicylic acid; SAG, salicylic acid glucoside.

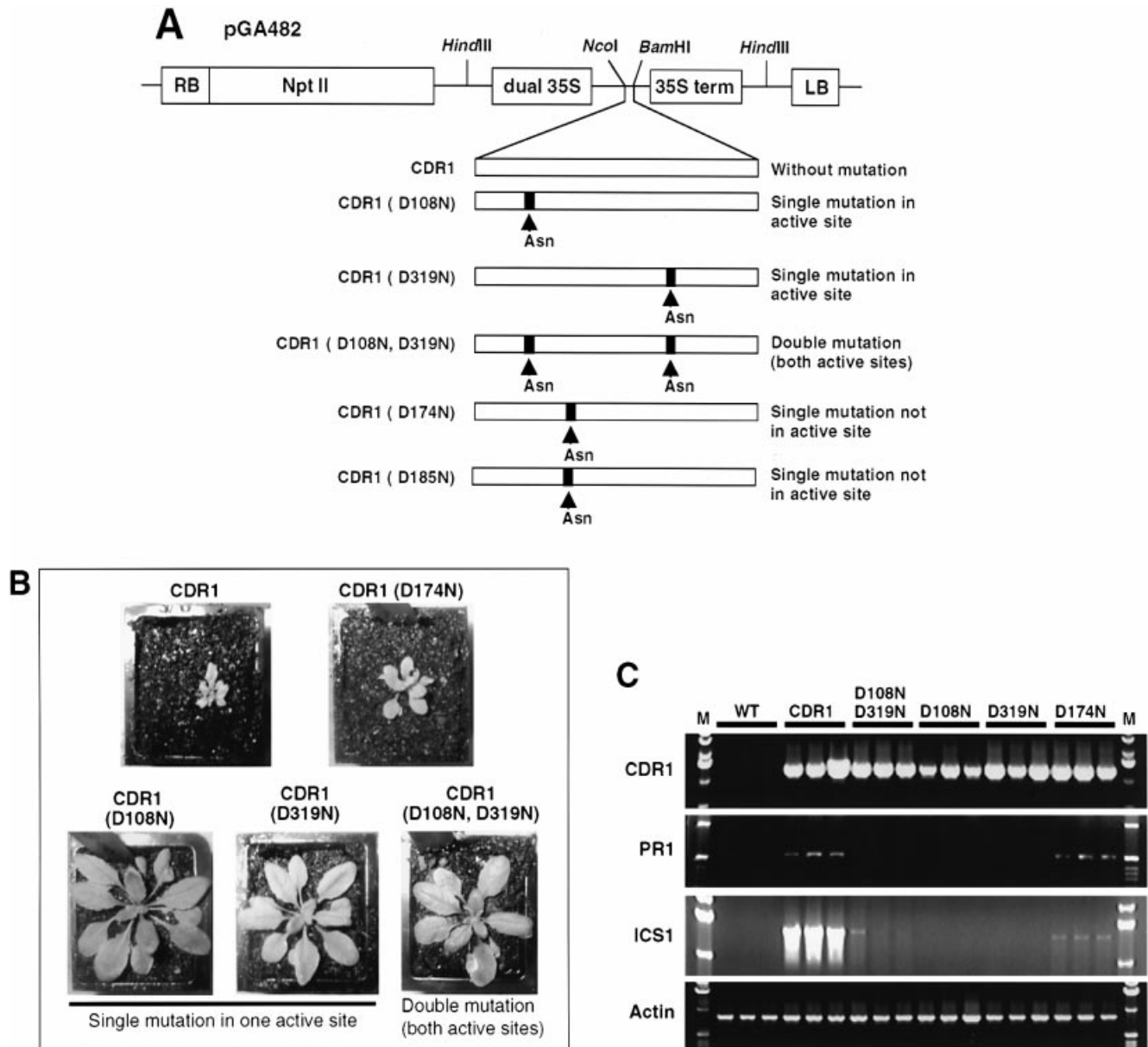
unpublished data). Figure 2C shows comparative transcript analysis of unchallenged wild-type and *cdr1-D* lines. Notable among the genes constitutively up-regulated in the *cdr1-D* mutant are *PR1*, a marker characteristic of salicylate-mediated defences (Verberne *et al.*, 2000), and a gene encoding isochorismate synthase (*ICS1*), an enzyme recently shown to be involved in a new pathway for SA biosynthesis in *Arabidopsis* (Wildermuth *et al.*, 2001). Perhaps surprisingly, transcripts encoding L-phenylalanine ammonia-lyase, the first enzyme in the phenylpropanoid pathway previously believed to be the origin of SA (Verberne *et al.*, 1999) are down-regulated in *cdr1-D* (Y Xia *et al.*, unpublished data). This is discussed further below. Consistent with these transcript changes, levels of SA are significantly elevated in *cdr1-D* compared with wild-type plants (Fig. 1B).

The mutant *cdr1* gene was cloned by plasmid rescue, and the wild-type *CDR1-D* gene isolated from an *Arabidopsis* genomic library (Y Xia *et al.*, unpublished data). Sequence information indicated that CDR1 appeared to be an aspartic protease enzyme, with an N-terminal signal peptide indicative of extracellular localization

(Y Xia *et al.*, unpublished data). Expression of CDR1 as a GST fusion protein in *E. coli* confirmed weak protease activity of the recombinant protein against the artificial substrate FITC-labelled bovine serum albumin (Fig. 3A). A higher specific activity would doubtless be expected if the enzyme could be tested with its yet to be discovered endogenous substrate. CDR1-GST had a very broad pH optimum when compared with the cysteine protease cathepsin (Fig. 3B), and was inhibited by high concentrations of NADPH (Fig. 3A), a feature common to some plant cysteine proteases (Murakami *et al.*, 2000). However, CDR1-GST, unlike cathepsin, was relatively insensitive to the protease inhibitor pepstatin A (Fig. 3A). This is surprising, as pepstatin appears to inhibit the biological function of CDR1 *in vivo* (Y Xia *et al.*, unpublished data). Perhaps other pepstatin-sensitive proteases are involved downstream of CDR1 activity.

To determine whether its protease activity is necessary for *in vivo* biological activity of CDR1, site-directed mutants were introduced into the CDR1 open reading frame so as to replace critical aspartate residues with asparagine residues. Wild-type *Arabidopsis* plants were transformed with constructs encoding D174N, D108N, D319N, and D108N:D319 (Fig. 2A). Figure 2B shows that expression of CDR1 harbouring a mutation of an aspartate residue outside of the active site area (D174N) results in plants that show the typical dwarf phenotype of *cdr1-D*, whereas plants transformed with CDR1 constructs in which either or both of the active site aspartate residues had been changed exhibited a wild-type growth phenotype. Furthermore, mutation of the active site aspartate residues also resulted in loss of constitutive expression of *PR1* and *ICS1* (Fig. 2C). Thus, the biochemical and biological phenotypes of CDR1 over-expression are a direct result of CDR1's protease activity.

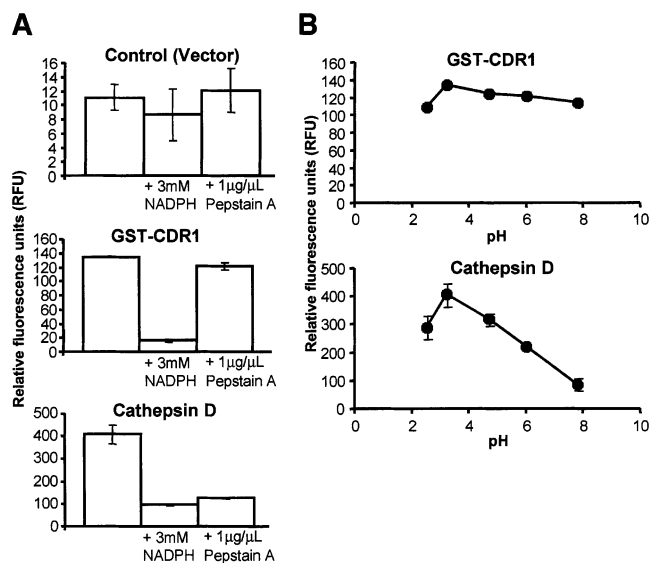
To test whether CDR1 is indeed an apoplastic enzyme, gene constructs encoding C-terminal fusions of green fluorescent protein (GFP) to the CDR1 27 amino acid signal peptide, or the intact CDR1 open reading frame, were made and stably transformed into *Arabidopsis* under the control of the constitutive 35S promoter (Fig. 4A). Confocal microscopy indicated a classical endoplasmic reticulum and perinuclear localization pattern for GFP fluorescence from CDR1-GFP and signal peptide-GFP within *Arabidopsis* leaf epidermal cells (Fig. 4C), very distinct from the localization of unfused GFP to cytoplasmic strands and nucleus. Analysis of GFP fluorescence in roots indicated that non-fused GFP accumulated throughout the cells of the root and root tip, whereas CDR1-GFP and signal peptide GFP were primarily localized to root cap and vascular tissue, with weaker staining in the cortex and epidermis (Fig. 4D). Most of the fluorescence appeared to be in the cell periphery and/or intercellular spaces (Fig. 4D) of cortical and epidermal cells. In trichomes (Fig. 4A), non-fused GFP again localized to



**Fig. 2.** Morphological phenotypes and defence gene expression of *A. thaliana* constitutively expressing CDR1 or site-directed mutants targeting critical and non-critical aspartate residues. (A) Constructs for transformation of *A. thaliana* with CDR1 and site-directed mutants. (B) Plant phenotypes. (C) Determination of defence gene transcripts by RT-PCR.

nucleus and cytoplasmic strands, whereas signal peptide GFP was clearly localized to the cell surface. CDR1-GFP showed a localization pattern that appeared to reflect a combination of ER and extracellular localization. These experiments confirm the function of the CDR1 signal peptide in targeting protein secretion to the extracellular space, but also suggest that the intact CDR1-GFP protein is not effectively trafficked out of the ER. This may be because of the size of the protein; if it is due to incorrect folding, the GFP is, nevertheless, still functional.

The involvement of an extracellular aspartic protease in defence gene activation is suggestive of the operation of an apoplastic peptide signalling system for microbial defence in *Arabidopsis*, as outlined in Fig. 5. This model is supported by the observation that intercellular fluids from leaves of CDR1 over-expressing plants cause both local and systemic induction of defence, including *PR* gene expression, when inoculated into a single leaf of an *Arabidopsis* plant (Y Xia *et al.*, unpublished data). Furthermore, the 'elicitor' activity in the intercellular



**Fig. 3.** Protease activity of CDR1 expressed in *E. coli*. (A) Activity against FITC-BSA of extracts from empty vector transformed *E. coli* cultures, purified CDR1-GST and cathepsin. Effects of 3 mM NADPH and 1 µg µL<sup>-1</sup> pepstatin A are shown. (B) pH profiles for the activity of CDR1-GST and cathepsin.

fluids can pass through a membrane filter that does not allow passage of the intact CDR1 protein. The nature of this elicitor is currently under investigation.

#### *DIR-1* and lipid signalling in systemic resistance in *A. thaliana*

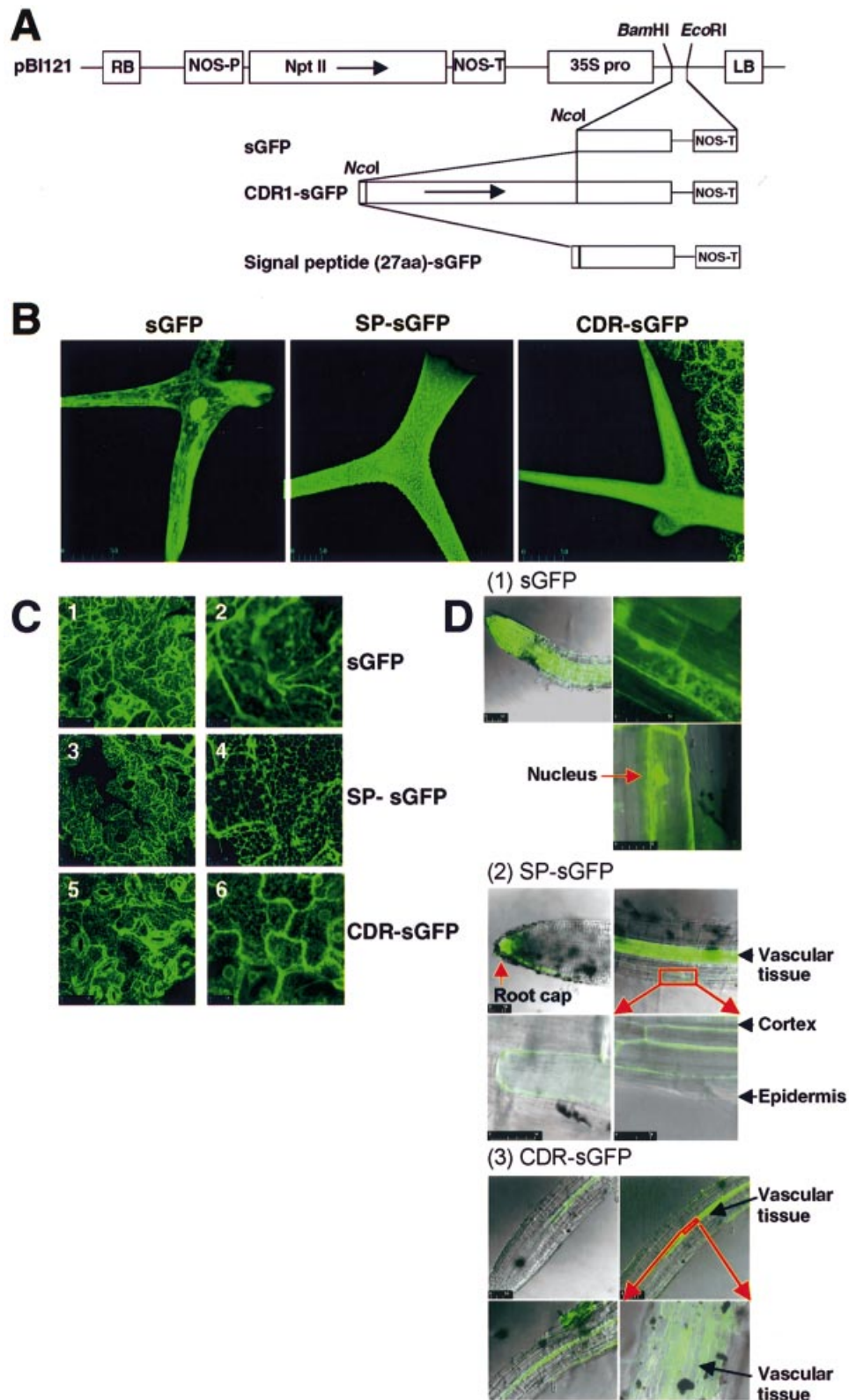
Although the phenomenon of SAR has been known for many years, few attempts have been made to employ genetic screens to identify genes specifically involved in long-distance signalling, the critical feature of SAR. This is presumably because screening for mutants in the entire SAR pathway requires both primary and secondary inoculations on spatially distinct leaves. This is labour intensive, and the absolute extent of expression of SAR is strongly affected by developmental stage and environmental conditions (Kus *et al.*, 2002), making scoring quite difficult. In spite of these limitations, it was possible to screen a collection of T-DNA tagged *A. thaliana* lines for mutants that failed to develop SAR after inoculation with avirulent *Pst*. After inoculating one leaf with *PstavrRpt2*, three other leaves on the same plant were inoculated with virulent *Pst* 2 d later and disease symptoms monitored 3 d later. Of 11 000 M1 T-DNA lines screened, one, *defective in induced resistance 1-1* (*dir1-1*), was finally shown to be compromised in SAR (Maldonado *et al.*, 2002). Either the *dir1-1* mutation was dominant to *DIR1* or the wild-type allele was haplo-insufficient.

Challenging *dir1-1/dir1-1* plants with virulent *Pst* 3 d after local inoculation with avirulent *PstavrRpt2* resulted in disease symptoms similar to those observed in non-induced wild-type plants, confirming the lack of SAR in

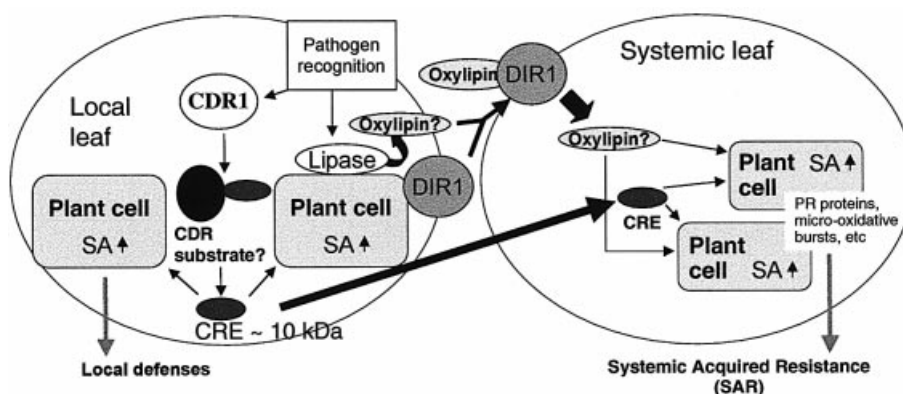
the homozygote lines (Maldonado *et al.*, 2002). Local accumulation of *PR-1*, *PR-5* and glutathione-*S*-transferase (*GST*) transcripts in leaves inoculated with *Pst-avrRpm1* was similar in wild-type and *dir1-1* plants, but was greatly reduced in the distant uninoculated leaves of *dir1-1* plants compared with the wild type. *Pst*-induced SAR to the oomycete pathogen *Peronospora parasitica* was also abolished in the *dir1-1* mutant. However, previously uninoculated *dir1-1* plants restricted the growth of *PstavrRpt2* to a similar extent as wild-type plants and there was no difference between the growth of virulent *Pst* in previously un-inoculated *dir1-1* compared with equivalent wild-type plants (Maldonado *et al.*, 2002). Basal resistance to *Pst* is, therefore, not compromised in *dir1-1*, unlike other defence response mutants, including *npr1*, *pad4*, or *eds1* (Delaney *et al.*, 1995; Falk *et al.*, 1999; Zhou *et al.*, 1998). Therefore, *DIR1* represents the first example of a defence gene that functions specifically for signalling during SAR.

Based on the molecular and disease phenotypes, *DIR1* might function in the production of the mobile SAR signal, its transmission from the inoculated leaf, its perception in distant leaves or downstream transduction of the signal. Petiole exudates from wild-type *Arabidopsis* leaves that had been inoculated with avirulent *Pst* elicited *PR-1* expression in leaves of healthy, untreated wild-type plants, whereas exudates collected from *PstavrRpt2*-induced *dir1-1* leaves elicited little *PR-1* gene expression (Maldonado *et al.*, 2002). However, petiole exudates from wild-type plants could induce PR expression when injected into leaves of *dir1-1*. Together, these data indicated that *DIR1* functions in the production of an essential mobile signal or its transmission from the induced, emitting leaf, rather than in the perception or transduction of the signal in distant, recipient leaves.

*DIR1* encodes a 102 amino acid protein with 53% amino acid similarity to the *Phaseolus vulgaris* non-specific lipid transfer-like protein (nsLTP). The protein contains a hydrophobic N-terminal signal sequence and the eight cysteine residues conserved in all LTPs (Kader, 1997), consistent with *DIR1* being an apoplastic LTP. LTPs bind to and transfer phospholipids between membranes *in vitro*. A number of plant LTPs are up-regulated in response to infection and exhibit anti-microbial activity (Kader, 1997). However, some animal LTPs act as lipid sensors or are involved in phospholipase-C-linked signal transduction via their lipid binding activity (Wirtz, 1997), so a role for a plant LTP in signalling is not without precedence. Although *DIR1* might, in principle, itself be the mobile signal, this is unlikely because over-expression of *DIR1* is not sufficient to induce SAR. Pathogen exposure is still required implying that either *DIR1* activity or localization is regulated in some way or *DIR1* functions in co-operation with the mobile signal.



**Fig. 4.** Cellular and subcellular localization of CDR1-eGFP fusions in transgenic *Arabidopsis*. (A) Constructs for transformation of *Arabidopsis* with sGFP, CDR1-sGFP and CDR1 signal peptide-sGFP. (B) GFP expression in trichomes. (C) GFP expression in leaf epidermal cells. (D) GFP expression in roots.

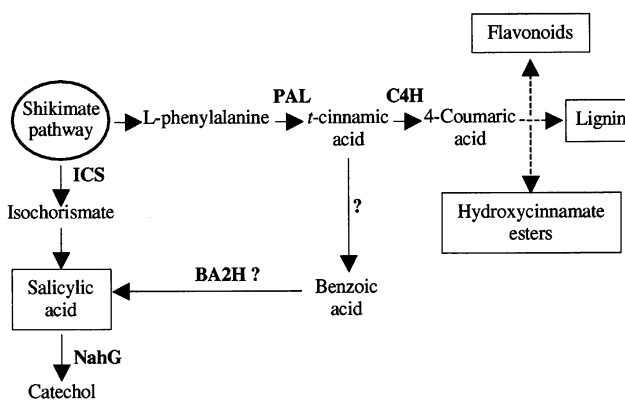


**Fig. 5.** Model for local and systemic signalling of defence responses involving CDR1 and DIR1. In this model activation of CDR1 activity releases a small mobile signal (CRE), presumably a polypeptide, which then serves to activate defence responses in secondary tissues to result in systemic acquired resistance (SAR). In parallel, oxylipin signals released from cell membranes in locally infected leaf cells are transported, through binding to the DIR1 lipid transfer protein, to systemic leaves where they also act to induce SAR responses.

Lipid molecules such as oxylipins (jasmonic acid), phosphatidic acid, and N-acyl ethanolamines (NAEs) are synthesized or released from membranes upon pathogen or insect attack and may act as second messengers for plant defence response signal transduction pathways (Chapman, 2000; Munnik, 2001; Wasternack and Parthier, 1997). Oxylipins, generated by the peroxidation of cellular lipids with reactive oxygen intermediates produced from the pathogen-induced oxidative burst (Lamb and Dixon, 1997), may interact with DIR1 to initiate long-distance SAR signalling (Fig. 5). It is interesting that two other genes known to be essential for pathogen resistance in *Arabidopsis*, *PAD4* and *EDS1* (Falk *et al.*, 1999; Jirage *et al.*, 1999) encode putative lipases, which may initiate release of a lipid-derived mobile SAR signal.

#### The biosynthesis of salicylic acid during plant defence

Many studies have shown the importance of salicylic acid for both local and systemic induced resistance in plant responses to pathogens (Lawton *et al.*, 1995; Mur *et al.*, 1996; Shah and Klessig, 1999; Shirasu *et al.*, 1997), and indeed, signalling through the CDR1 pathway described above requires SA and is thus impaired if SA is removed by transgenic expression of the NahG salicylate hydroxylase enzyme (Y Xia *et al.*, unpublished data), which converts SA to catechol (Friedrich *et al.*, 1995). In spite of the central role of SA in plant defence responses, the actual biochemical pathways involved in SA biosynthesis are still not fully resolved. Until recently, it was believed that SA was synthesized by a branch of the phenylpropanoid pathway, through which cinnamic acid, formed from phenylalanine by the action of L-phenylalanine ammonia-lyase (PAL), underwent chain shortening to form benzoic acid, which was then hydroxylated by a specific benzoate 2-hydroxylase to yield SA (Lee *et al.*, 1995; Verberne *et al.*, 1999) (Fig. 6). This pathway received genetic support from the observation that plants down-regulated in



**Fig. 6.** Pathways for the biosynthetic origin of the salicylic acid involved in plant defence signalling. Enzymes are: PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; BA2H, benzoate 2-hydroxylase; ICS, isochorismate synthase.

PAL expression had reduced levels of SA and compromised disease resistance (Maher *et al.*, 1994; Pallas *et al.*, 1996), and biochemical support from the partial purification of a benzoate 2-hydroxylase enzyme, believed to be an unusual, high molecular weight, soluble cytochrome P<sub>450</sub> (León *et al.*, 1995). However, the gene encoding this latter enzyme has not been identified, and, although many different mechanisms have been proposed for the side-chain shortening reaction involved in the formation of benzoic acids and benzaldehydes, the *in vivo* origin of these compounds is still unclear (Podstolski *et al.*, 2002).

Constitutive expression of normally inducible defences in the *cdl1-D* mutant involves increased SA levels, but PAL transcripts appear to be lower than in wild-type plants. The significance of this observation is unclear in the absence of a specific measurement of transcripts originating from each of the four *Arabidopsis* PAL genes, but, when viewed along with the strong induction of transcripts

encoding isochorismate synthase, the key enzyme in an alternative pathway to SA by-passing the PAL reaction and recently shown to be important for disease resistance in *Arabidopsis* (Wildermuth *et al.*, 2001), it would seem likely that PAL expression is not important for the biosynthesis of the pool of SA that is necessary for the resistance response of *Arabidopsis* to *Pst*.

Free SA levels are similar in wild-type *Arabidopsis* and the *dir1-1* mutant in the locally inoculated leaf, in distant uninoculated leaves and following challenge inoculation in distant leaves (Maldonado *et al.*, 2002). *DIR1* is therefore not necessary for SA accumulation either locally or systemically. Although *dir1-1* still accumulates SA, *PR-1* expression and SAR are compromised. SA alone is not a particularly potent inducer of defence mechanisms, but acts in an agonist-dependent manner to amplify the gene-for-gene HR and oxidative burst (Shirasu *et al.*, 1997) and perhaps also potentiates the activation of inducible defences in SAR-induced plants upon subsequent pathogen challenge. Although SA accumulates to wild-type levels in *dir1-1* leaves (Maldonado *et al.*, 2002), the agonist is not present in the induced leaf and, therefore, SAR is not observed. It is also possible that the SA accumulation observed in distant leaves and upon pathogen challenge in *dir1-1* is not associated with SAR, but rather with basal defence to *Pseudomonas* (Cameron *et al.*, 1999; Nawrath and Metraux, 1999; Zhou *et al.*, 1998). This model is consistent with the concept that different functional pools of SA may exist in plants in relation to different aspects of basal and induced defence, and may have different biosynthetic origins (Wildermuth *et al.*, 2001).

By contrast with the *Arabidopsis/Pst* system, PAL and downstream enzymes such as caffeic acid *O*-methyltransferase (COMT) are strongly induced following infection of tobacco plants with viral pathogens or exposure to a fungal elicitor (Jaekel *et al.*, 1992; Pellegrini *et al.*, 1993, 1994), and SA acts as a signal molecule for local and systemic resistance of tobacco and other plants to viral, fungal and bacterial pathogens (Dempsey *et al.*, 1999; Klessig and Malamy, 1994; Shah and Klessig, 1999; Silverman *et al.*, 1993). Reduction of phenylpropanoid biosynthesis in tobacco by the down-regulation of PAL compromises local resistance to fungal infection (Maher *et al.*, 1994), and both local and systemic resistance to viral infection (Pallas *et al.*, 1996). However, it is not clear which phenylpropanoid derivatives confer resistance in wild-type tobacco. Based upon the similarity in disease phenotypes between PAL-reduced tobacco (Pallas *et al.*, 1996) and plants expressing the bacterial *NahG* salicylate hydroxylase gene (Gaffney *et al.*, 1993) (Fig. 6), it has been assumed that reduction in SA levels is an important component of the PAL<sup>-</sup> phenotype. In view of the more recent findings of a non-phenylpropanoid origin for SA this hypothesis requires re-evaluation.

Transgenic tobacco plants over-expressing a bean PAL transgene (PAL<sup>+</sup>) (Howles *et al.*, 1996) were generated as a tool with which to examine the relationships between PAL activity, SA production, and the role of phenylpropanoid compounds in disease resistance. Such plants produce high levels of chlorogenic acid (CGA) and exhibit markedly reduced susceptibility to infection with the fungal pathogen *Cercospora nicotianae*, although their resistance to tobacco mosaic virus (TMV) is unchanged (Shadle *et al.*, 2003). Levels of SA were similar in PAL<sup>+</sup> and control plants whether uninfected or infected with TMV. In crosses of PAL<sup>+</sup> with *NahG*, progeny harbouring both transgenes lost resistance to TMV, indicating that SA is critical for resistance to TMV and that increased production of CGA or other phenylpropanoid compounds can not substitute for SA. By contrast, PAL<sup>+</sup>/*NahG* plants had strongly reduced susceptibility to *C. nicotianae* compared with the *NahG* parent line. These results further question the role of PAL in SA biosynthesis in *Arabidopsis*, and highlight the importance of phenylpropanoid compounds such as CGA or coumarins in plant disease resistance (Shadle *et al.*, 2003).

## Conclusion

The studies reviewed in this article indicate that both peptide and lipid signalling may play critical roles in the activation of plant defences in response to microbial attack. They also highlight the power of genetic approaches for dissecting plant signal transduction pathways. These must now, however, be combined with biochemical approaches in order to determine the nature of the systemically transported lipid signal carried by the DIR1 protein, the nature of the endogenous substrate for CDR1 and the resulting peptide signal, and the relative roles of the isochorismate and phenylpropanoid pathways in the biosynthesis of defence-related SA pools. Recent developments in proteomic and metabolomic technologies will surely advance these efforts.

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