Overexpression of CRK13, an Arabidopsis cysteine-rich receptor-like kinase, results in enhanced resistance to *Pseudomonas syringae*

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**Summary**

Protein kinases play important roles in relaying information from perception of a signal to the effector genes in all organisms. Cysteine-rich receptor-like kinases (CRKs) constitute a sub-family of plant receptor-like kinases (RLKs) with more than 40 members that contain the novel C-X₈-C-X₂-C motif (DUF26) in the extracellular domains. Here we report molecular characterization of one member of this gene family, CRK13. Expression of this gene is induced more quickly and strongly in response to the avirulent compared with the virulent strains of *Pseudomonas syringae*, and peaks within 4 h after pathogen infection. In response to dexamethasone (DEX) treatment, plants expressing the CRK13 gene from a DEX-inducible promoter exhibited all tested features of pathogen defense activation, including rapid tissue collapse, accumulation of high levels of several defense-related gene transcripts including *PR1*, *PR5* and *ICS1*, and accumulation of salicylic acid (SA). In addition, these plants suppressed growth of virulent pathogens by about 20-fold compared with the wild-type Col-0. CRK13-conferred pathogen resistance is salicylic acid-dependent. Gene expression analysis using custom cDNA microarrays revealed a remarkable overlap between the expression profiles of the plants overexpressing CRK13 and the plants treated with *Pst* DC3000 (*avrRpm1*). Our studies suggest that upregulation of CRK13 leads to hypersensitive response-associated cell death, and induces defense against pathogens by causing increased accumulation of salicylic acid.

**Keywords:** cysteine-rich receptor-like kinase, salicylic acid, pathogen defense, Arabidopsis, signaling, cell death.

**Introduction**

Plants are well equipped to sense invasion of pathogenic micro-organisms and have the ability to employ multiple strategies for self-defense. The hypersensitive response (HR), a type of programmed cell death (PCD) induced at the site of infection, is one of the most robust forms of active defenses employed by plants to prevent pathogen invasion. Recent studies have shown that, in most plant–pathogen interactions, activation of HR requires effector proteins produced by the pathogen and their indirect recognition by R gene products produced by the plant (reviewed in Chisholm *et al.*, 2006). Activation of R genes leads to induction of several defense responses by the plant, including changes in ion fluxes, production of reactive oxygen species (ROS), accumulation of salicylic acid (SA), reinforcement of the cell wall, synthesis of phytoalexins, and transcriptional activation of many defense and pathogenesis-related genes (Dempsey *et al.*, 1999; Hammond-Kosack and Jones, 1996). In many cases, HR is followed by activation of systemic acquired resistance (SAR), which provides resistance against a wide variety of pathogens. SA potentiates HR-associated cell death and plays a crucial role in establishment of SAR (Durrant and Dong, 2004; Ryals *et al.*, 1996).
The Arabidopsis genome has more than 600 receptor-like kinases (RLKs). Of these, 400 have an extracellular receptor domain, a transmembrane domain and an intracellular kinase domain (Shiu and Bleecker, 2001a). However, the function of very few RLKs is known. RLKs are involved in regulating a variety of plant physiological processes, including growth and development, symbiosis, hormone signaling, transpiration and pathogen defense (reviewed in Dievart and Clark, 2004; Johnson and Ingram, 2005; Shiu and Bleecker, 2001b). RLK genes implicated in pathogen defense include (i) FLS2, encoding an Arabidopsis flagellin-sensing LRR-RLK that plays an important role in plant innate immunity (Gomez-Gomez and Boller, 2000), (ii) rice resistance gene Xa21, which confers resistance to Xanthomonas oryzae pv. oryzae in a race-specific manner (Song et al., 1995), and (iii) barley Rpg1, which confers resistance to the stem rust fungus Puccinia graminis (Brueggeman et al., 2002).

A sub-family of 41 RLKs of Arabidopsis contains 1–4 copies of the ‘domain of unknown function 26’ (DUF26) consisting of a C-X8-C-X2-C motif that is located in the extracellular part of the protein in most members (Chen, 2001; Shiu and Bleecker, 2001b). Because of this domain, members of this sub-family have been classified as cysteine-rich receptor-like kinases (CRKs; Chen, 2001). The functions of only very few members of the CRK sub-family are known. Seven members of this sub-family are induced by SA and pathogens (Chen et al., 2003, 2004; Czernic et al., 1999). Among these, CRK5 and its three closest homologs have been implicated in regulating cell death (Chen et al., 2003, 2004).

In this study, we have characterized CRK13, a member of the CRK gene sub-family. The CRK13 gene is induced rapidly in response to bacterial pathogens. Our results demonstrate that overexpression of CRK13 leads to HR-like cell death, activation of all tested cellular and molecular markers of defense, accumulation of SA, and enhanced resistance to virulent and avirulent strains of bacterial pathogens.

Results

CRK13 is a member of the CRK sub-family of receptor-like kinases

We previously constructed several suppression subtractive hybridization (SSH) libraries enriched for Arabidopsis genes that are differentially expressed in response to defense-inducing chemicals and pathogens (Mahalingam et al., 2003). One of the cDNAs in these libraries represents a gene that is induced in response to virulent and avirulent strains of Pst DC3000. A BLAST search of GenBank revealed that this cDNA represents a receptor-like kinase gene (At4g23210) that contains cysteine-rich repeat (CRR) domains and corresponds to the CRK13 gene of the 41-member CRK sub-family of RLKs. The full-length cDNA of CRK13 was isolated by RACE and RT-PCR (see Experimental procedures) and was found to contain an open reading frame of 2019 bp. Search of the public sequence databases revealed two more cDNAs representing this gene. These cDNAs contain open reading frames of 1830 and 1572 bp (accession numbersAY142503 and BT001110), respectively. Together, these three cDNAs represent alternatively spliced transcripts of the CRK13 gene (Figure 1a,b). We named these alternatively spliced transcripts CRK13.1 (identified in our cDNA library, accession number DQ680080), CRK13.2 (accession numberAY142503) and CRK13.3 (accession number BT001110). To determine which of these alternative spliced transcripts are preferentially produced, if any, in response to pathogen infection, we used splicing variant-specific primers to determine the level of each transcript by

![Figure 1. Genomic organization and expression of the CRK13 gene.](image)

(a) CRK13 codes for at least three alternatively spliced forms: CRK13.1, CRK13.2 and CRK13.3. Exons are represented by boxes. Arrows represent primers used for RT-PCR in (c). Primer 1 spans the boundaries of exons 5 and 6 of CRK13.1 and CRK13.2, primer 2 spans the boundaries of exons 6 and 7 of CRK13.1 and exons 5 and 6 of CRK13.2, primer 3 corresponds to exon 5 of CRK13.3, and primer 4 corresponds to exon 6 of CRK13.2.

(b) Schematic representation of CRK13.1 protein. TM, transmembrane domain (amino acids 5–24 and 301–320); CRR, cysteine-rich repeat/DUF26 domain (amino acids 73–130 and 198–249); STYKc, dual-specificity serine/threonine and tyrosine kinase domain (amino acids 358–627). Protein domains were predicted using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de) and the NCBI domain searching databases (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

(c) Expression analysis of splicing variants of the CRK13 gene. The relative abundance of the three splicing variants in response to pathogen infection was determined using the transcript-specific primers shown in (a). Actin gene-specific primers were used as internal control. M, RNA isolated from 10 mM MgCl2-treated plants; R, RNA isolated from plants treated with Pst DC3000 (avrRpm1); S, RNA isolated from plants treated with Pst DC3000 (avrRpm1; 5 × 105 CFU ml−1) in 10 mM MgCl2. PCR was performed for 24 and 40 cycles. PCR amplification at 40 cycles demonstrates that all three splicing variants do exist and can be amplified using the primers shown in (a). This experiment was performed twice using different sets of RNA samples with similar results.
semi-quantitative RT-PCR. We found that the CRK13.1 transcript was predominantly produced in response to avirulent pathogen Pst DC3000 (avrRpm1; Figure 1c). Therefore, for studies involving use of CRK13 cDNA in this study, we used the cDNA corresponding to the CRK13.1 splicing variant.

To determine the structural similarity of CRK13 with other members of CRK sub-family, we performed phylogenetic analysis of predicted full-length proteins of all members by the CLUSTAL W method. Our analysis revealed that CRK13 falls in a sub-group of seven members, consisting of CRK22, CRK11, CRK33, CRK34, CRK12 and CRK14 (Figure S1). The sequence identity of CRK13 with other members in the sub-family ranges from 26% to 67% at amino acid level, and CRK22 is its closest homolog.

CRK13 is rapidly induced in response to bacterial pathogens

To determine the kinetics of induction of the CRK13 gene in response to bacterial pathogens, we monitored accumulation of CRK13 transcripts over a 24 h period in response to Pst DC3000 (virulent) and Pst DC3000 (avrRpm1; avirulent) strains of bacterial pathogen by Northern blot analysis. Expression of the CRK13 gene was induced within 1 h post-infiltration (hpi) in response to Pst DC3000 (avrRpm1) and within 2 hpi in response to Pst DC3000. In both cases, transcript accumulation peaked within 4 hpi (Figure 2a).

To compare the kinetics of accumulation of the CRK13 transcript relative to a known pathogen-inducible gene, we hybridized these blots with PR1 gene probe. As expected, PR1 gene transcript was readily detected by 8 hpi in response to Pst DC3000 (avrRpm1) and by 12 hpi in response to Pst DC3000 (Figure 2a). These results demonstrate that CRK13 is an early bacterial pathogen-inducible gene, and as expected for a typical defense-related gene, it is induced more quickly and to higher levels in response to an avirulent pathogen compared with a virulent pathogen. Similarly, the CRK13 gene was also induced in response to other bacterial pathogens (Figure 2b). Expression of the CRK13 gene was also analyzed in response to fungal pathogens (Alternaria brassicicola and Fusarium oxysporum), virus (turnip crinkle virus), insects (Spodoptera exigua and Myzus persicae), abiotic stresses (cold, heat, salinity, osmotic, freezing, wounding, drought), chemicals that induce oxidative stress (H₂O₂, paraquat, glucose/glucose oxidase, xanthine/xanthine oxidase), and the defense hormones SA and jasmonic acid (JA). None of these treatments induced expression of the CRK13 gene (Figure S2, and data not shown). These results suggest that the CRK13 gene is induced specifically in response to bacterial pathogens. Because CRK13 seems to be specifically induced in response to bacterial pathogens, we tested its expression in response to flg22 peptide, a well-characterized bacterial pathogen-associated molecular pattern molecule (PAMP; Gomez-Gomez and Boller, 2000).

Northern analysis revealed that flg22 induces expression of CRK13 within 4 hpi (Figure 2c). This result suggests a possible involvement of CRK13 in PAMP-triggered immune responses.

To determine whether expression of the CRK13 gene is regulated in a tissue- or developmental-specific manner, we analyzed expression of CRK13 in 1-week-old seedlings and in leaves, stems, roots and flowers of 6-week-old Col-0 plants and senescing tissue by Northern blot analysis. We were unable to detect expression of CRK13 in the seedlings, or any of the organs of the 6-week-old plants or senescing tissue (Figure S2d).

Overexpression of CRK13 leads to HR-like cell death

To investigate the role of the CRK13 gene in regulating resistance against pathogens, we identified two T-DNA insertion lines that had a T-DNA insertion in the CRK13 gene. One of these (crk13-1) was obtained from the Salk T-DNA insertional collection (Alonso et al., 2003), and the other
Overexpression of CRK13 induces pathogen defense

Overexpression of CRK13 leads to activation of pathogenesis-related genes

HR-associated programmed cell death is frequently accompanied by activation of many pathogenesis-related genes (Nimchuk et al., 2003). In order to test whether cell death associated with overexpression of the CRK13 gene mimics HR-associated cell death, we analyzed the expression of a variety of pathogenesis-related (PR) genes in DEX-26, DEX-33 and control DEX-V1 and DEX-V3 plants in response to 5.0 μM DEX treatment. High levels of the CRK13 transcript were detected within 4 h of DEX infiltration and remained high until at least 48 hpi (Figure 3c). Overexpression of CRK13 induced accumulation of transcripts of SA-responsive PR genes such as PR1 and PR5. We also analyzed these
lines for the expression of the *isochorismate synthase 1* (ICS1) gene, which is involved in biosynthesis of SA (Wildermuth *et al*., 2001). Similar to other PR genes, the ICS1 gene was also induced within 12 hpi. The abundance of the JA-regulated PDF1.2 transcript did not increase in these plants (data not shown). These results suggest that ectopic expression of CRK13 induces expression of defense-related genes that are regulated by the SA-mediated defense pathway.

**Overexpression of CRK13 leads to activation of defense-related cellular markers**

Activation of PR genes in CRK13 overexpressing plants prompted us to test whether defense-related cellular markers are also induced in these plants. Among these, we tested production of H$_2$O$_2$ and electrolyte leakage caused by membrane damage. Production of H$_2$O$_2$ was tested by *in situ* histochemical staining with 3,3’-diaminobenzidine (DAB) (Thordal-Christensen *et al*., 1997). A reddish-brown precipitate of oxidized DAB was observed in response to DEX treatment in the leaves of the transgenic DEX-26 and DEX-33 plants collected 24 hpi, but not in the treated control vector lines (Figure 3d). This result suggests that H$_2$O$_2$ accumulation in these plants parallels (and perhaps precedes) cell death, a response typically associated with many pathogen-induced HR responses. To test whether CRK13-conferred cell death is associated with membrane damage, we analyzed electrolyte leakage from DEX-26, DEX-33 and empty vector lines DEX-V1 and DEX-V3 in response to DEX treatment. DEX treatment lead to significantly higher ion leakage in DEX-26 and DEX-33 lines compared with the control DEX-V1 and DEX-V3 vector lines, suggesting that CRK13 overexpression leads to membrane damage (Figure S5a).

**Overexpression of CRK13 leads to accumulation of SA**

Induction of ICS1 in plants overexpressing CRK13 prompted us to test whether overexpression of the CRK13 gene also led to accumulation of SA. To this end, we determined the levels of SA and SA glucoside (SAG) in DEX-26, DEX-33, DEX-V1 and DEX-V3 plants in response to DEX treatment at 0, 12 and 24 hpi. The DEX-26 and DEX-33 plants, but not the DEX-V1 or DEX-V3 plants, accumulated high levels of SA and SAG in response to DEX treatment. Within 12 h after DEX treatment, levels of SA and SAG were approximately 100-fold and 20-fold higher, respectively, than at 0 h (Figure 4a,b). SAG levels continued to rise to 24 hpi, while SA levels declined modestly between 12 and 24 hpi, perhaps due to continued conversion of SA to SAG. The high levels of SA and SAG accumulation by 12 hpi suggest that accumulation of SA precedes cell death. These data suggest that SA may be important for CRK13-conferred cell death.

![Figure 4](image_url)

**Figure 4.** SA positively regulates CRK13-conferred cell death. (a, b) Free SA and SAG were extracted from leaves of transgenic plant overexpressing CRK13 (DEX-26, DEX-33) and empty vector lines (DEX-V1 and DEX-V3) at 0, 12 and 24 h after 5.0 μM DEX treatment. The values are presented as means ± SD of three replicates, each consisting of leaves from eight plants per genotype. (c) Rosette leaves of 4-week-old plants of T$_1$ transgenic lines of NahG/DEX::CRK13, sid2/DEX::CRK13 and Col/DEX::CRK13 were infiltrated with 5.0 μM DEX, and tissue samples were harvested 24 hpi for RNA isolation. Blots were probed with the CRK13 gene probe, stripped and reprobed with the *PR1* gene probe.

To test the role of SA in regulating CRK13-conferred cell death and induction of defense genes, we transformed DEX::CRK13 construct into plants that have reduced levels of SA. We used plants expressing the bacterial salicylic hydroxylase gene (*NahG*) and the *sid2* mutant for this purpose. The presence of the *NahG* gene in transgenic plants prevents accumulation of SA by catabolizing it to catechol (Friedrich *et al*., 1995), and the *sid2* mutant is deficient in SA biosynthesis (Wildermuth *et al*., 2001). In
Overexpression of CRK13 induces pathogen defense

Overexpression of CRK13 confers resistance to Pst DC3000

The results described above demonstrate that overexpression of CRK13 results in activation of several pathogen defense responses. To assess whether overexpression of CRK13 would also confer resistance to virulent strains of bacterial pathogens, we determined the growth of Pst DC3000 in response to DEX treatment in DEX-26, DEX-33 and control DEX-V1 and DEX-V3 plants. Because DEX treatment of DEX-26 and DEX-33 plants leads to rapid cell death, first we determined the minimum concentration of DEX that was sufficient to induce expression of PR genes without any visible cell death. For this, we syringe-infiltrated increasing concentrations of DEX solution (0.001–20.0 μM) into the DEX-26 and DEX-33 plants and monitored accumulation of PR1 gene transcripts at 24 hpi and cell death over a period of 5 days, which is the time required to determine in planta growth of virulent bacterial pathogens. As little as 0.05 μM DEX was sufficient to induce PR1 gene expression in DEX-26 and DEX-33 plants without causing visible cell death, although some yellowing of leaves was observed at 5 dpi (Figure 5a). No PR1 transcript was detected in response to 0.01 μM DEX. Based on these results, a concentration of 0.05 μM DEX was chosen for determining the growth of the virulent bacterial pathogen in these plants (Figure 5b).

Leaves of 4-week-old plants were infiltrated with 0.05 μM DEX, and 18 h later these leaves were re-infiltrated with Pst DC3000 at a titer of 1 × 10⁶ CFU ml⁻¹. At 4 dpi, an approximately 20-fold reduction in the growth of bacteria was observed in DEX-26 and DEX-33 lines compared with the Col-0, DEX-V1 and DEX-V3 vector control lines (Figure 5c, and data not shown). These results demonstrate that overexpression of CRK13 results in enhanced resistance against virulent Pst DC3000. We also analyzed growth of the avirulent pathogen Pst DC3000 (avrRpm1) in these plants. Similar to Pst DC3000, at 4 dpi, an approximately 10-fold reduction in the growth of bacteria was observed in DEX-26 and DEX-33 lines compared with the DEX-V1 and DEX-V3 vector control lines (data not shown). These results suggest that CRK13 also potentiates resistance against avirulent bacterial pathogen Pst DC3000 (avrRpm1).

To analyze the role of SA in regulating CRK13-conferred defense, we tested the growth of Pst DC3000 in response to DEX treatment in NahG/DEX::CRK13 and sid2/DEX::CRK13 plants as described above. The suppression of SA accumulation in these plants compromised the resistance conferred by DEX::CRK13 (Figure 5c). These results suggest that CRK13-conferred resistance is dependent on SA and are consistent with the suppression of defense gene expression in NahG/DEX::CRK13 and sid2/DEX::CRK13 plants (Figure 4c).

Transcriptome profiling reveals significant overlap between CRK13 and avirulent pathogen-modulated gene expression

The results described above suggest that the CRK13-conferred response mimics many of the responses induced by avirulent pathogens. To further identify the genes regulated by CRK13, we analyzed the transcriptome of plants overexpressing the CRK13 gene using a custom microarray representing 199 Arabidopsis genes. Most of these genes are differentially expressed in response to a variety of pathogen defense-related biotic stresses [predominantly Pst DC3000 (avrRpm1)] and abiotic stresses (Table S1). We analyzed changes in the transcriptome of DEX-26 and DEX-33 in response to 0.05 μM DEX treatment using these arrays. DEX-treated empty vector lines DEX-V1 and DEX-V3 were used as controls. Only those genes whose expression changed by more than two-fold in both lines (DEX-26 and DEX-33) compared with the vector controls were considered differentially expressed. Out of 199 genes, 101 (51%) were differentially expressed; of these, 93 were induced and eight were suppressed (Table S2). The results of the microarray analysis were evaluated by Northern blot analysis of a few randomly selected genes that were either induced or
suppressed in microarray experiments. The results of Northern blot analysis were in agreement with those of the microarray experiments (Figure S6).

For a different project, we performed transcriptome analysis of Col-0 in response to a variety of biotic stresses, including Pst DC3000 (avrRpm1), and abiotic stresses (Maqbool et al., unpublished). To compare changes in gene expression due to overexpression of CRK13 with avirulent pathogen-mediated responses, we compared the transcriptome of DEX-treated DEX::CRK13 plants with that of Col-0 plants treated with Pst DC3000 (avrRpm1). Tissue samples were collected 24 h after DEX treatment or pathogen infiltration. This analysis revealed a significant overlap between the transcriptome of CRK13- and pathogen-induced genes (Table S2). Among the genes induced by CRK13, 60% were also induced by Pst DC3000 (avrRpm1), and all eight genes that were suppressed by CRK13 were also suppressed by Pst DC3000 (avrRpm1; Table S2). Together, these results suggest that induction of CRK13 mimics the plant’s response to avirulent pathogens at the molecular and cellular level.

CRK13 expression in defense signaling mutants

To determine the epistatic relationship of CRK13 with other defense-related genes, we determined expression of the CRK13 gene in response to bacterial pathogens in variety of defense mutants and transgenic plants unable to accumulate SA. These include eds1 (Parker et al., 1996), ndr1 (Century et al., 1995), pad3 (Glazebrook and Ausubel, 1994), pad4 (Glazebrook et al., 1997), eds5 (Rogers and Ausubel, 1997), npr1 (Cao et al., 1997), NahG (Lawton et al., 1995) and etr1 (Pieterse et al., 1998). Leaves of 4-week-old plants of these mutants and control wild-type Col-0 plants were infiltrated with Pst DC3000 or Pst DC3000 (avrRpm1). Infiltrated leaf tissues were harvested at 4 hpi, and CRK13 gene expression was analyzed by Northern blot analysis. None of these mutations had any significant effect on accumulation of CRK13 transcripts in response to virulent or avirulent bacterial pathogens (Figure 6a, and data not shown). These results suggest that the CRK13 gene might function upstream of these defense signaling genes or that its expression is regulated through a novel signaling pathway(s) independent of these genes.

Many lesion-mimic mutants constitutively express several defense-related genes. We tested the accumulation of the CRK13 gene in several lesion-mimic mutants, namely lsd1 (Dietrich et al., 1997), acd2 (Mach et al., 2001), hr11 (Devadas et al., 2002) and dll1 (Pilolff et al., 2002). While these mutants accumulated high levels of PR1 transcripts, CRK13 transcripts were not detected in any of these mutants (Figure 6b).

Finally, to test whether expression of CRK13 required bacterial pathogens or whether the presence of avirulence factor was sufficient to induce its expression, we analyzed the accumulation of CRK13 transcript in transgenic plants...
expressing avirulence gene *avrRpt2* from a DEX-inducible promoter (DEX::avrRpt2). In response to DEX treatment, DEX::avrRpt2 plants in the *RPS2* genetic background, but not in the *rps2* mutant background, show induced HR-like cell death and activation of several *PR* genes (McNellis et al., 1998). CRK13 transcript accumulated at 4 hpi in response to DEX treatment in DEX::avrRpt2 plants in the *RPS2* genetic background but not in the *rps2-101C* mutant background (Figure 6c). These results suggest that *in planta* expression of the *avrRpt2* avirulence gene is sufficient to induce CRK13 transcript accumulation in the presence of a functional *RPS2* gene in the absence of any other pathogen-derived factors.

**Discussion**

CRKs have been identified in various plant species, and some members of this gene sub-family are induced in response to a variety of stimuli, including pathogens, symbionts and defense-inducing signals (Czernic et al., 1999; Du and Chen, 2000; Lange et al., 1999; Montesano et al., 2002; Ohtake et al., 2000). We have isolated and characterized the CRK13 gene of Arabidopsis. Several observations suggest that CRK13 is associated with activation of HR-like cell death and defense against virulent and avirulent bacterial pathogens. First, like a typical defense-related gene, CRK13 is induced more rapidly and to higher levels in response to avirulent bacterial pathogens compared with virulent pathogens (Figure 2a). Second, *in planta* expression of *avrRpt2* in wild-type Arabidopsis plants with a functional *RPS2* is sufficient to induce expression of the CRK13 gene (Figure 6c), suggesting that *avr-R* interaction is sufficient for induction of the CRK13 gene. Third, overexpression of the CRK13 gene leads to rapid HR-like cell death (Figure 3), accumulation of SA (Figure 4), induction of several defense-related genes (Figure 3, Table S2) and accumulation of cellular and biochemical markers of defense against pathogens (Figures 3 and S5a). Finally, overexpression of CRK13 suppresses the growth of virulent bacterial pathogens about 20-fold (Figure 5c) and that of avirulent pathogens about 10-fold. Furthermore, we tested expression of CRK13 in response to a variety of other biotic and abiotic stresses, none of which induced expression of CRK13. These results suggest that CRK13 might be a bacterial pathogen-responsive gene, probably involved in regulating an early step of defense signaling. Induction of CRK13 in response to bacterial elicitor flg22 supports this proposition (Figure 2c). However, it should be noted that we have analyzed transcript levels of CRK13 in these studies, and early transcript accumulation does not necessarily imply early accumulation or activation of the protein.

Loss of function of the CRK13 gene had no significant effect on the resistance against pathogens. There are at least two possible explanations for this. First, CRK13 is not involved in regulating resistance against pathogens. This seems unlikely because overexpression of CRK13 leads to activation of all tested defense responses, including resistance against virulent and avirulent bacterial pathogen. Second, CRK13 represents a member of a large gene sub-family represented by 41 members with sequence identity at the amino acid level ranging from 26% to 67%, and this genetic redundancy may explain the lack of altered phenotype in loss-of-function alleles. Genetic redundancy has been suggested to be one of the possible reasons for no phenotype in loss-of-function mutants of several plant genes, including other members of the CRK13 gene sub-family (Bouche and Bouchez, 2001; Chen et al., 2003).

SA is an important signal in defense against pathogens. Levels of SA dramatically increase in response to pathogen attack (Dempsey et al., 1999; Malamy et al., 1990; Metraux et al., 1990). Studies in *Nicotiana tabacum* and other higher plants have shown that SA is synthesized from cinnamate via benzoic acid (Ribnicky et al., 1998; Yalpani et al., 1993). More recent studies have shown that SA can also be synthesized from chorismate, and that the bulk of SA
produced in response to pathogen infection is produced from chorismate in Arabidopsis (Wildermuth et al., 2001). Our results show that overexpression of CRK13 leads to induction of ICS1 (involved in biosynthesis of SA from chorismate) within 12 h and of EDS5 (suggested to be involved in the transport of precursors of SA) (Nawrath et al., 2002) within 24 h. However, PAL1, PAL2 and PAL3 genes (involved in SA biosynthesis from phenylalanine) were not induced significantly (data not shown). This raises the possibility that overexpression of CRK13 might induce accumulation of SA and SAG probably by upregulating expression of ICS1 and possibly other genes involved in SA biosynthesis from chorismate.

How does ectopic expression of CRK13 lead to cell death and defense activation? Our results demonstrate that, despite overexpression of CRK13, cell death was delayed in NahG/DEX::CRK13 and sid2/DEX::CRK13 plants compared with Col-0/DEX::CRK13 plants. These findings suggest that CRK13-conferring cell death is regulated by both SA-dependent and -independent pathways. Studies on lesion-mimic mutants and pathogen-induced cell death have indicated the existence of SA-dependent and -independent pathways for regulating cell death (Lorrain et al., 2003; Nawrath and Metraux, 1999; Rate et al., 1999). These studies indicate that some pathogens, as well as some lesion-mimic mutants, activate pro-cell death signals distinct from SA. Our studies suggest that CRK13 promotes both of these signals. Reactive oxygen species may be one such signal involved in inducing cell death in plants overexpressing CRK13. Nonetheless, induction of cell death by CRK13 overexpression due to metabolic perturbation cannot be ruled out. Our results suggest that CRK13-conferring resistance against pathogens is mainly regulated by a SA-dependent pathway(s), because resistance was compromised in NahG/DEX::CRK13 and sid2/DEX::CRK13 plants.

CRK13 is likely to be an early component of the plant defense response, because of the following observations. First, expression of CRK13 in response to pathogen infection was not attenuated in any of the tested defense mutants, including eds1, ndr1, pad4, eds5, NahG, npr1 and etr1. In addition, expression of CRK13 was not induced in any of the tested lesion-mimic mutants, including lsd1, acd2, hrl1 and dll1. Some of these genes, such as NDR1 and EDS1, function early in defense signaling. These results suggest that either CRK13 functions upstream of these defense genes or it is part of a novel defense signaling pathway that converges into a signaling pathway upstream of ICS1, as overexpression of CRK13 induces expression of ICS1. Second, analysis of gene expression revealed a remarkable overlap between the expression profiles of the plants overexpressing CRK13 and the plants treated with avirulent bacterial pathogen Pst DC3000 (avrRpm1). For example, many of the known defense-related genes such as PR1, PR5, ICS1, chitinase genes, AIG1, several WRKY protein-encoding genes, protein kinase genes, RLKs, etc. were induced in plants overexpressing CRK13 and in response to Pst DC3000 (avrRpm1).

Similarly, all eight genes suppressed in plants overexpressing CRK13 were also suppressed in response to Pst DC3000 (avrRpm1). Because CRK13 gene expression is induced in plants expressing avrRpt2 in planta with functional RPS2, CRK13 appears to function downstream of resistance genes. In addition, induction of CRK13 in response to a variety of avirulence factors suggests that signals generated by these avr–R interactions might funnel into CRK13-mediated signaling to activate downstream pathogen defense pathways. Functional characterization of other CRK13 splicing variants and identification of CRK13-interacting proteins should help to elucidate mechanisms regulating defense and host cell death conferred by CRK13.

Experimental procedures

Plant growth, chemical treatment and pathogen infection of plants

Plants were grown and treated with SA or methyl jasmonate (MJ) as described previously (Devadas et al., 2002). Dexamethasone (DEX) treatment was performed by syringe infiltration of the indicated concentrations of DEX solution in 0.016% ethanol. Bacterial pathogen infiltration and growth estimation were performed as described previously (Devadas et al., 2002). Plants were syringe-infiltrated with 1 μM flg22 peptide (Sigma Genosys, www.sigmaaldrich.com) in 0.01% dimethyl sulfoxide (DMSO).

RNA analysis, RACE PCR and RT-PCR

Total RNA was isolated and Northern blot analysis was performed as described previously (Devadas et al., 2002). To obtain the full-length cDNA corresponding to CRK13, amplification of the 5′ and 3′ ends of the CRK13 gene was performed by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) using a GeneRacer kit (Invitrogen; http://www.invitrogen.com/) and four gene-specific primers according to the manufacturer’s instructions. GSP1 (5′-GTCCGTTGTCTCGAGAAGG-3′), GSP2 (5′-CACAGGACGGTGGAG-3′), GSP3 (TGCCCAACAGAAGTGCTGACT) and GSP4 (GGAGGACGGTGAGGATTGC) for 3′ RACE. This sequence information was used to design forward and reverse gene-specific primers to amplify the full-length cDNA by RT-PCR using 1 μg of total RNA and Powerscript reverse transcriptase according to the manufacturer’s protocol (BD Biosciences, www.bdbiosciences.com). Primers for RT-PCR to check splicing variants were: primer 1, 5′-CCGGAACTCCCGGTACATGC-3′; primer 2, 5′-AC-CTCCCACTATGTTGACA-3′; primer 3, 5′-CCGGAACTCCCGTAAACAT-3′; primer 4, 5′-CTCCCACTAGCAGCTGAAACAAA-3′.

Construction of transgenic plants

To construct transgenic plants overexpressing CRK13, the full-length CRK13 cDNA was amplified by RT-PCR. For cloning purposes, AscI and BamHI sites were introduced at the 5′ end of the forward primer and at the 3′ end of the reverse primer, respectively. The sequences of the forward and reverse primers were 5′-CAGGC-GCCGCTGTCTAGAAGAAGCAGACAGTG-3′ and 5′-CAGGATCCCGGATT-3′, respectively (restriction sites

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underlined). The PCR-amplified products were cloned in pCR2.1
TOPO TA cloning vector (Invitrogen) to construct TOPO-CRK13. The
sequence of the amplified product was confirmed by DNA sequenc-
ing. The insert was isolated by restriction digestion and cloned
downstream of the 35S promoter in Ascl–BamHl-digested binary
vector pSR3000 (derived from pCAMBIA 2300). Arabidopsis plants
(Col-0) were transformed by Agrobacterium-mediated transforma-
tion (Clough and Bent, 1998), and transgenic plants were selected on
MS medium containing 75 μg ml⁻¹ kanamycin.

To construct transgenic plants expressing DEX-inducible CRK13,
full-length cDNA was excised from TOPO-CRK13 as an Ascl–BamHl
fragment, ends were flushed by a fill-in reaction using Klenow
enzyme (New England Biolabs, www.neb.com), and the fragment
was cloned in Xhol-digested, Klenow filled-in binary vector
pTA7002. The orientation of the insert was confirmed by PCR using
an hptl-specific forward primer (5′-TTTAGCCAGAGCCTGACC-
TATTGC-3′) and a CRK13-specific reverse primer (5′-ACT-
CAAGTACGCTCGTGG-3′). Arabidopsis plants (Col-0), NahG
transgenic plants and sid2 mutant plants were transformed by
Agrobacterium-mediated transformation (Clough and Bent, 1998).
Transgenic plants were selected on MS medium containing
40 μg ml⁻¹ hygromycin.

Analyses of crk-1 and crk-2 T-DNA insertion mutants

T-DNA insertion lines crk13-1 (SAIL_085128) and crk13-2
(SAIL_G296G1) were obtained from the SALK T-DNA collection
(Alonso et al., 2003) and the SAIL T-DNA collection (Sessions et al.,
2002), respectively. Sequences flanking the T-DNA insertion sites
were amplified using the CRK13 gene-specific reverse primer (5′-
GCTTTTCCATAACCTCCAGAC-3′) and a CRK13-specific forward primer (5′-
TAGCATCTGAATTTC-3′) and (5′-TTTAGCCAGAGCCTGACC-
TATTGC-3′) for crk13-1 and crk13-2, respectively.

Amplified products were sequenced to determine the site of
insertion of T-DNA in each line. Homozygous mutants were
confirmed to be null alleles by Northern blot analyses.

Histochemical analysis and electrolyte leakage
measurements

Staining for the presence of H₂O₂ by the DAB uptake method was
performed as described previously (Thordal-Christensen et al.,
1997). Electrolyte leakage measurements were performed as
described previously (Devadas and Raina, 2002) with some modifica-
tions. For each time point, 10 leaf discs were collected from 10
Arabidopsis thaliana

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Supplementary material

The following supplementary material will appear for this article
online:

- Figure S1. Phylogenetic analysis of CRK proteins.
- Figure S2. Expression analysis of the CRK13 gene.
- Figure S3. Analysis of T-DNA mutants of CRK13 gene.
- Figure S4. Analysis of CRK13 overexpressing plants.
- Figure S5. Analysis of electrolyte leakage and CRK13-conferred cell
death.
- Figure S6. Validation of results of micro array analysis.

Table S1. List of genes on microarray.
Table S2. Genes differentially expressed in plants overexpressing
CRK13.

This material is available as part of the online article from http://

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