The Synthetic Elicitor 3,5-Dichloroanthranilic Acid Induces *NPR1*-Dependent and *NPR1*-Independent Mechanisms of Disease Resistance in Arabidopsis[1][W][OA]

Colleen Knoth[2], Melinda S. Salus, Thomas Girke, and Thomas Eulgem*

ChemGen Integrative Graduate Education and Research Traineeship Program, Center for Plant Cell Biology, Institute for Integrative Genome Biology, Department of Botany and Plant Sciences, University of California at Riverside, Riverside, California 92521

Immune responses of Arabidopsis (*Arabidopsis thaliana*) are at least partially mediated by coordinated transcriptional up-regulation of plant defense genes, such as the *Late/sustained Up-regulation in Response to Hyaloperonospora parasitica* (*LURP*) cluster. We found a defined region in the promoter of the *LURP* member *CaBP22* to be important for this response. Using a *CaBP22* promoter-reporter fusion, we have established a robust and specific high-throughput screening system for synthetic defense elicitors that can be used to trigger defined subsets of plant immune responses. Screening a collection of 42,000 diversity-oriented molecules, we identified 114 candidate *LURP* inducers. One representative, 3,5-dichloroanthranilic acid (DCA), efficiently induced defense reactions to the phytopathogens *H. parasitica* and *Pseudomonas syringae*. In contrast to known salicylic acid analogs, such as 2,6-dichloroisonicotinic acid (INA), which exhibit a long-lasting defense-inducing activity and are fully dependent on the transcriptional cofactor NPR1 (for Nonexpresser of Pathogenesis-Related genes1), DCA acts transiently and is only partially dependent on NPR1. Microarray analyses revealed a cluster of 142 DCA- and INA-responsive genes that show a pattern of differential expression coinciding with the kinetics of DCA-mediated disease resistance. These *ACID* genes (for *Associated with Chemically Induced Defense*) constitute a core gene set associated with chemically induced disease resistance, many of which appear to encode components of the natural immune system of Arabidopsis.

Plants utilize an abundance of mechanisms to defend themselves against invading pathogens (Chisholm et al., 2006). These defenses can be induced or constitutive (Somssich and Halbrock, 1998; Dangl and Jones, 2001). Induced defenses are often triggered by the recognition of conserved pathogen-associated molecular patterns (PAMPs), resulting in PAMP-triggered immunity (PTI; Gomez-Gomez and Boller, 2002). To counteract PTI, pathogens evolved effector molecules that can attenuate PTI, making the plant susceptible and the pathogen virulent (compatible interaction; Chisholm et al., 2006). An additional form of induced defense is triggered in response to recognition of pathogen effectors by Leu-rich repeat-containing plant resistance (R) proteins, making the pathogen avirulent and the plant resistant (incompatible interaction). This type of plant immunity is called effector-triggered immunity (ETI) or R-mediated resistance (Jones and Dangl, 2006).

PAMP and effector recognition rapidly induce several well-characterized biochemical changes in the plant. These early defense features involve the production of signaling molecules, including reactive oxygen intermediates (ROIs; oxidative burst), nitric oxide, and salicylic acid (SA; Malamy and Klessig, 1992; Jabs et al., 1997; Dangl, 1998; Delledonne et al., 2002). Specifically associated with ETI is the hypersensitive response (HR), a programmed plant cell death localized to infection sites that in many cases effectively restricts pathogen spread and growth (Goodman and Novacky, 1994; Dangl et al., 1996). SA has been shown to be a key player in ETI and PTI (Malamy and Klessig, 1992; Klessig et al., 2000; Tsuda et al., 2008). Accumulation of SA is preceded by the oxidative burst, leading to downstream defense responses and potentiation of further ROI production (Shirasu et al., 1997). In many cases, plants deficient in their ability to accumulate or produce SA are unable to mount successful defense responses (Gaffney et al., 1993; Delaney et al., 1994). SA signaling is partially dependent on NPR1 (for Nonexpresser of Pathogenesis-Related genes1), a transcriptional cofactor that is required for the activation of multiple defense genes (Dong, 2004).

[1] This work was supported by the National Science Foundation-Integrative Organismal Biology (grant no. 0449439 to T.E.), by the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service (grant no. 2008–35301–19264 to T.E.), and by a predoctoral fellowship to C.K. from the National Science Foundation-funded ChemGen Integrative Graduate Education and Research Traineeship program (fellowship no. DGE 0504249).

[2] Present address: Kelley Scientific Resources, La Jolla, CA 92121.

* Corresponding author; e-mail thomas.eulgem@ucr.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Thomas Eulgem (thomas.eulgem@ucr.edu).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.108.133678

Plant defense responses can be induced by both biotic and abiotic stimuli, such as chemical elicitors. A chemical can be considered a defense activator if it triggers resistance to pathogens while inducing the same or similar molecular markers as biotic defense stimuli. In addition, such a compound should not be directly toxic to the pathogen (Kessmann et al., 1994). Exogenous application of chemicals such as SA, 2,6-dichloroisonicotinic acid (INA), and acibenzolar-*S*-methyl benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) has been shown to activate the plant's natural immune responses (Métraux et al., 1991; Ward et al., 1991; Uknes et al., 1992; Schob et al., 1997). Public concern over the dangers of pesticide use has spawned considerable interest in alternative methods for pest control. Many chemical pesticides currently in use rely on direct antibiotic activity, which often leads to undesirable toxic environmental side effects (Kessmann et al., 1994). Compounds that elicit a plant's innate immune response offer an attractive alternative to the application of toxic pesticides for disease-control regimes (Ward et al., 1991; Uknes et al., 1992).

INA and BTH were discovered in screens for chemical inducers of long-lasting broad-spectrum disease resistance in cucumber (*Cucumis sativus*; Métraux et al., 1991; Görlach et al., 1996). They have the qualities of efficient defense activators as they induce defense responses in a wide variety of plant species and do not exhibit any direct antimicrobial activity. Both compounds are considered functional analogs of SA because they induce the expression of known SA-responsive genes. Notably, INA and BTH are active in *nahG* plants, which are unable to accumulate SA, showing that they act independently of SA perception and biosynthesis (Kessmann et al., 1993; Friedrich et al., 1996) and suggesting that they interfere with biological targets operating downstream from these steps. While INA has never been used commercially, BTH, under the names Actigard and Bion, is commercially available from Syngenta. Besides their potential use for pest control, such synthetic defense elicitors can serve as versatile tools for chemical genetic analyses of plant defense mechanisms.

Previously, microarray analyses identified the *LURP* cluster, a set of Arabidopsis (*Arabidopsis thaliana*) genes that exhibit coordinated late/sustained up-regulation in response to the pathogenic oomycete *Hyaloperonospora parasitica* (*Hp*; Eulgem et al., 2004). A defining feature of these genes is a strong accumulation of their transcripts between 12 and 48 h after infection, while transcript levels of a second *Hp*-responsive gene cluster we identified predominantly accumulated within the first 12 h after infection (Evrard et al., 2009). The *Hp* inducibility of *LURP* expression was found to be independent from NPR1 (Eulgem et al., 2004). Genetic studies have functionally implicated several genes from this set in plant defense responses (Zhou et al., 1998; Knoth et al., 2007; Knoth and Eulgem, 2008). We found one member of this cluster, which encodes the WRKY70 transcription factor, to participate in the regulation of other *LURP*s (Knoth et al., 2007). WRKY70 appears to act downstream from ROI and SA signaling.

Another member of this cluster, *CaBP22*, closely matches the average *Hp*-induced *LURP* expression profile (Eulgem et al., 2004). *CaBP22* (At2g41090; also known as *CML10*) encodes a putative calmodulin-like calcium-binding protein (McCormack et al., 2005). Biological roles of this protein have not been described. Here, we report on the analysis of the *CaBP22* promoter and its use in screens for new synthetic defense elicitors. We have identified two *Hp*-responsive regions in the *CaBP22* promoter that contain $LURP^{A}$, a protein-binding motif. Using an *Hp*-responsive *CaBP22* promoter fragment fused to *GUS* (*uidA*), we screened chemical libraries for compounds that induce the expression of this reporter gene. We identified 114 candidate elicitors and designed a set of assays to characterize their modes of action. One of these synthetic elicitors, 3,5-dichloroanthranilic acid (DCA), was shown to rapidly and transiently induce resistance to two phytopathogens by simultaneously activating two distinct branches of the plant defense signaling network. Although structurally related, we found DCA and the SA analog INA to be functionally distinct with regard to their dependency on NPR1 and the kinetics of their activities. Microarray analyses revealed a cluster of genes showing transcriptional changes strictly associated with disease resistance mediated by DCA and INA.

RESULTS

Characterization of the *Hp*-Responsive *CaBP22* Promoter by 5′ Deletion Analysis

To locate *Hp*-responsive regions in the *CaBP22* promoter, we performed a 5′ deletion analysis using transgenic Arabidopsis lines containing promoter fragments ranging from 65 to 1,075 bp upstream of the *CaBP22* transcriptional start site translationally fused to the *Escherichia coli GUS* reporter gene (lines $CaBP22^{-1075}$, $CaBP22^{-590}$, $CaBP22^{-333}$, and $CaBP22^{-65}$; Fig. 1A). Figure 1 (B and C) shows *GUS* expression responses driven by each promoter deletion visualized by histochemical staining. In lines containing $CaBP22^{-1075}$ to $CaBP22^{-333}$, *GUS* expression is clearly visible 7 d postinfection (dpi), with avirulent *Hp*Emoy2 and virulent *Hp*Noco2, and is greatly reduced (or absent) in response to mock treatment (Fig. 1B; data not shown). There was no observable induction by *Hp*Emoy2 or mock treatment in the $CaBP22^{-65}$ lines (Fig. 1, B and C).

GUS expression in the $CaBP22^{-1075}$ to $CaBP22^{-333}$ lines was significantly inducible by *Hp*Emoy2 in quantitative 4-methyl-umbelliferyl-β-D-glucuronide fluorescence assays, while no induction was observed for the $CaBP22^{-65}$ lines (Fig. 1, D and E). Notably $CaBP22^{-333}$

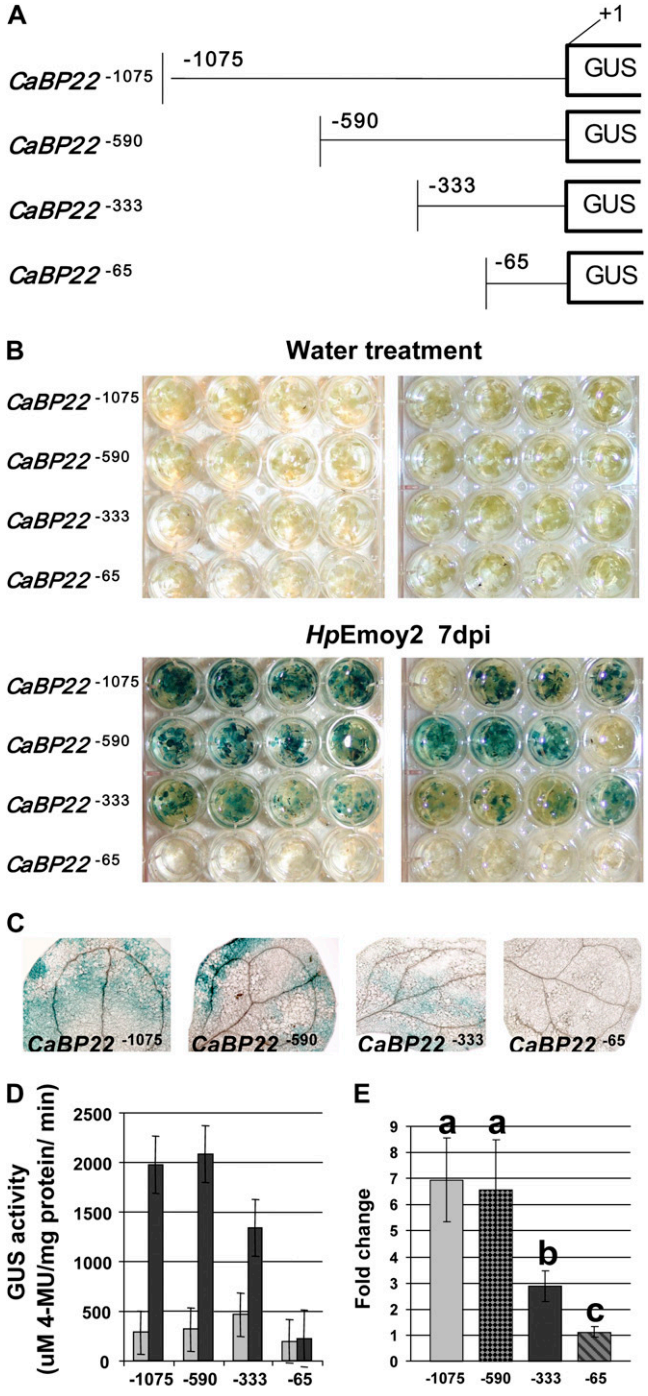


Figure 1. Analysis of *CaBP22-promoter*::*GUS* 5′ deletion constructs in Arabidopsis seedlings. A, Diagram of *CaBP22-promoter*::*GUS* 5′ deletion series. Numbers indicate 5′ promoter end points relative to the *CaBP22* transcriptional start site. B, Histochemical GUS staining of 3-week-old Arabidopsis seedlings transformed with each *CaBP22-promoter*::*GUS* 5′ deletion construct after spray inoculation with avirulent *Hp*Emoy2 (7 dpi with 5×10^4 spores mL^{-1}) or water (7 dpi). Each well contains five to 10 seedlings from independent transformation events. Shown are representative examples from eight independent transformation events per construct. C, Close-up views of GUS-stained *CaBP22-promoter*::*GUS* constructs at 7 dpi with *Hp*Emoy2. Ten independent lines per construct were analyzed. Shown are examples representing the typical behavior for each construct for at least five independent experiments. D, Fluorometric analysis of 10-d-old T2 transgenic *CaBP22-promoter*::*GUS* Arabidopsis seedlings. Light gray bars represent mock treatment (4 dpi), and dark gray bars represent *Hp*Emoy2 treatment (4 dpi with 5×10^4 spores mL^{-1}). Measurements were taken for eight independent lines for each construct. Shown are combined averages from all eight measurements from three independent experiments. 4-MU, 4-Methyl-umbelliferyl-β-D-glucuronide. E, Graphic representation of the fold-induction for each deletion construct. Mann-Whitney *U* test with a cutoff of $P < 0.05$ was used to test for significant differences, marked a, b, or c. The error bars in D and E represent SE.

lines exhibited an average induction of 3-fold, which is about half the induction produced in $CaBP22^{-1075}$ and $CaBP22^{-590}$ lines (6- to 7-fold). These data show that the promoter region contained in the $CaBP22^{-333}$ construct is sufficient to mediate *Hp*-induced *GUS* expression. Furthermore, there appear to be two *Hp*-responsive regions located in the *CaBP22* promoter operating in an additive fashion. One of these *Hp*-response regions must be located between positions −590 and −333 and one between positions −333 and −65.

Nuclear Proteins Interact with a Novel Motif in the *CaBP22* Promoter

Our reporter assays defined a minimal *Hp*-responsive region of 268 bp within the *CaBP22* promoter (between positions −333 and −65). While this region does not contain any known defense-associated cis-elements, it has a 25-bp stretch consisting of two inversely repeated sequences (5′-ATTGTTTTCTTCT-GTAGAAGACCAT-3′) that is strictly conserved in the second *Hp*-responsive region between positions −590 and −333. We termed this conserved region $LURP^A$ and the inversely repeated half sites consisting of the core motif AGAAGA $LURP^A$-CM (underlined in the 25-bp sequence shown above). This hexamer is statistically moderately enriched among promoters of the *LURP* cluster (as defined as cluster II by Eulgem et al. [2004]; $P = 3.3 \times 10^{-3}$).

To determine if $LURP^A$ can interact with nuclear proteins, we performed electrophoretic mobility shift assays (EMSAs) with nuclear protein extracts from Arabidopsis seedlings that were left untreated or harvested 48 h after defense induction, a time point that coincides with high *LURP* transcript levels (Eulgem et al., 2004). A probe containing the full 25-bp $LURP^A$ sequence ($LURP^A$-WT) led to a distinct constitutive shift, the intensity of which was clearly enhanced after infection with *Hp*Emoy2 or treatment with 1 mM SA (Fig. 2, A–C). This interaction was successfully competed by 100-fold excess of unlabeled $LURP^A$-WT probe. Additional EMSAs were conducted with several mutated probes to further delineate $LURP^A$'s key protein-interacting regions. $LURP^A$-M2 contains block mutations in both $LURP^A$ half sites ($LURP^A$-HS); while $LURP^A$-M1 and $LURP^A$-M3 are mutated in only the

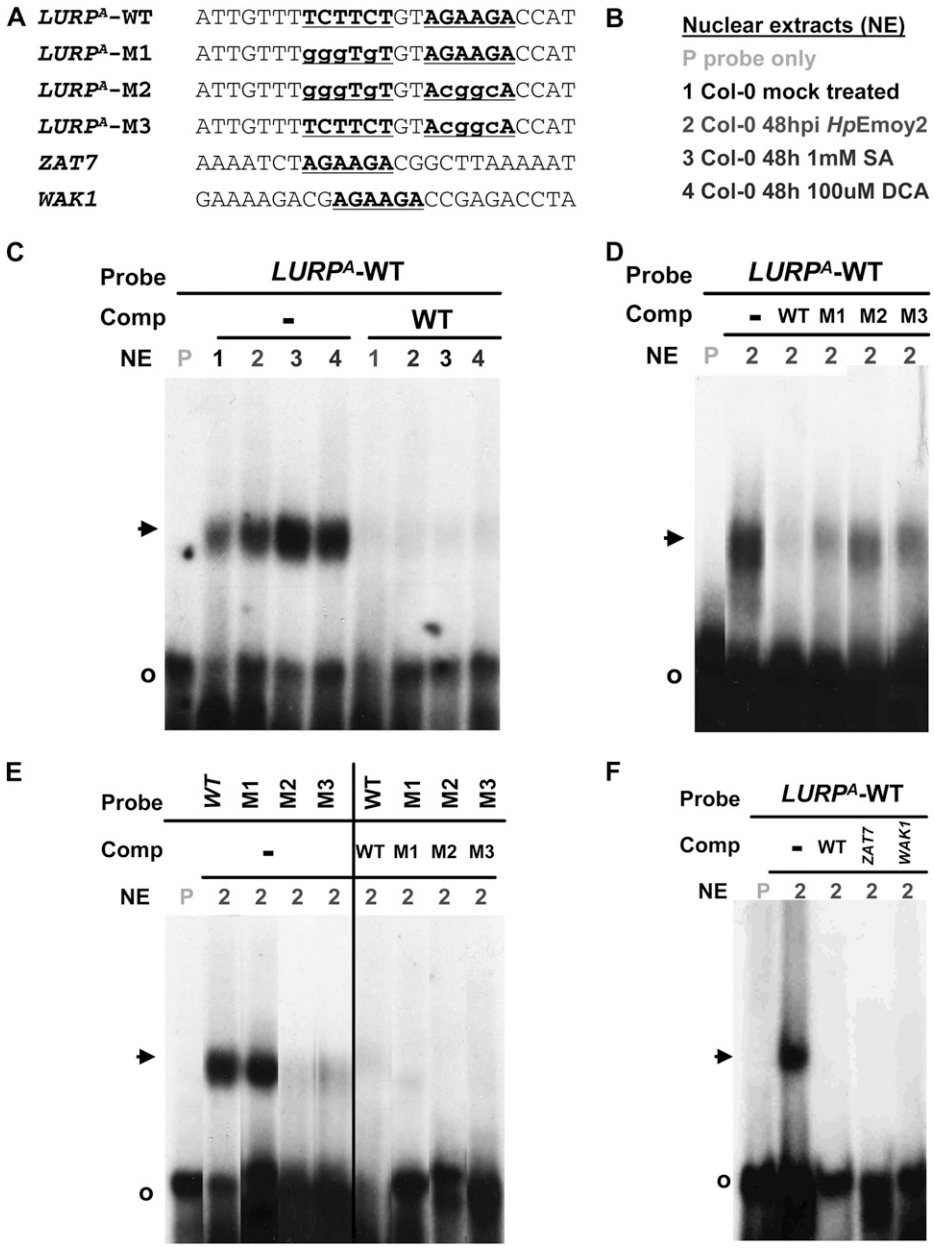


Figure 2. Nuclear factors interact in EMSAs with a conserved motif in the *CaBP22* promoter. A, Sequences of labeled probes and unlabeled competitors representing an inverted repeat region of the conserved motif found in *Hp*-responsive regions of the *CaBP22* promoter (*LURP*^{A}). The conserved core units of the *LURP*^{A} inverted repeats are underlined. Mutations are indicated by lowercase lettering. B, Nuclear extracts (NE) used in EMSAs. C to F, EMSAs reveal sequence-specific shifts with nuclear extracts. The arrows indicate specific shifts, and circles indicate free probe. EMSAs were performed with 15 μg of nuclear protein per lane. A 100-fold excess of the indicated unlabeled probe was used as competitor (Comp). Shown are results typical for at least three experiments. WT, Wild type.

first (TCTTCT; *LURP*^{A}-5′HS) or second (AGAAGA; *LURP*^{A}-3′HS) *LURP*^{A} half site, respectively (Fig. 2A). The *Hp*-induced *LURP*^{A} shift was successfully competed by 100-fold excess of unlabeled *LURP*^{A}-WT and *LURP*^{A}-M1 probes, while unlabeled *LURP*^{A}-M2 and *LURP*^{A}-M3 probes competed the *LURP*^{A}-mediated shift less efficiently (Fig. 2D). EMSAs performed with labeled *LURP*^{A}-M1 produced a shift similar to that of the unmutated *LURP*^{A}-WT probe, while no shift was detected using the labeled *LURP*^{A}-M2 probe (Fig. 2E). Labeled *LURP*^{A}-M3 produced a shift of similar size but severely reduced intensity compared with the wild-type probe.

In summary, these data show that nuclear DNA-binding factors interact with *LURP*^{A} in a sequence-specific manner that is mainly facilitated by the downstream half site *LURP*^{A}-3′HS. We observed a significant decrease in reporter activity between *CaBP22*$^{-590}$ (which contains two copies of *LURP*^{A}) and *CaBP22*$^{-333}$ lines (which contains a single copy of *LURP*^{A}) and complete loss of activity in *CaBP22*$^{-65}$ lines (which lacks *LURP*^{A}). This suggests that a single copy of this inverted repeat is sufficient for *Hp*-responsive *CaBP22* promoter activity, but the additional copy of it may enhance this promoter's responsiveness to defense stimuli. As *CaBP22* represents the average expression pattern of the *LURP* cluster and *LURP*^{A}-CM is statistically moderately enriched in promoters of this gene set, AGAAGA-containing protein-binding sites or related motifs may contribute to the defense-associated

coregulation of *LURP* members. Consistent with a possible role of AGAAGA-containing promoter sites in controlling *LURP* expression, derivatives of this motif from two other *LURP* promoters, *ZAT7* and *WAK1*, successfully competed with $LURP^{A}$ for nuclear DNA-binding factors (Fig. 2F).

A Chemical Screen Reveals a Small Molecule Elicitor of *LURP* Expression

As shown above, the $CaBP22^{-333}$ promoter mediates *GUS* expression in response to avirulent *Hp*Emoy2. To determine if this promoter is defense specific, we tested several known biotic and abiotic stimuli (Supplemental Table S1) in an Arabidopsis line homozygous for a single insertion site of the $CaBP22^{-333}$ construct. This line clearly expressed *GUS* in response to treatment with the bacterial pathogen *Pseudomonas syringae*, SA, wounding, and virulent *Hp*Noco2. However, reporter expression was not induced by any of the other stimuli tested (jasmonic acid, ethephon, kinetin, abscisic acid, $CaCl_2$, $MgCl_2$, NaCl, indole-3-acetic acid, GA_3, 2,4-dichlorophenoxyacetic acid, or submersion). Due to the apparent specificity of the response pattern of $CaBP22^{-333}$ for defense-inducing stimuli, we considered this line an excellent choice for a high-throughput screen for synthetic elicitors. One-week-old seedlings of the homozygous $CaBP22^{-333}$ line grown in liquid growth medium on 96-well plates were incubated for 24 h with library compounds at final concentrations of 4 to 20 μM followed by histochemical staining to visualize *GUS* expression (Fig. 3A). Screening a total of 42,000 diverse organic compounds (see "Materials and Methods") identified 114 candidates that reproducibly induced *GUS* expression in the $CaBP22^{-333}$ line. Many of these elicitors are structurally related to SA, while several others do not have any obvious similarity to known defense elicitors.

We found one of these 114 compounds, DCA (Fig. 3B), which has not been reported as a plant defense inducer, to be particularly active in detailed follow-up analyses. Histochemical staining of the $CaBP22^{-333}$ line submerged in varying DCA concentrations (saturation

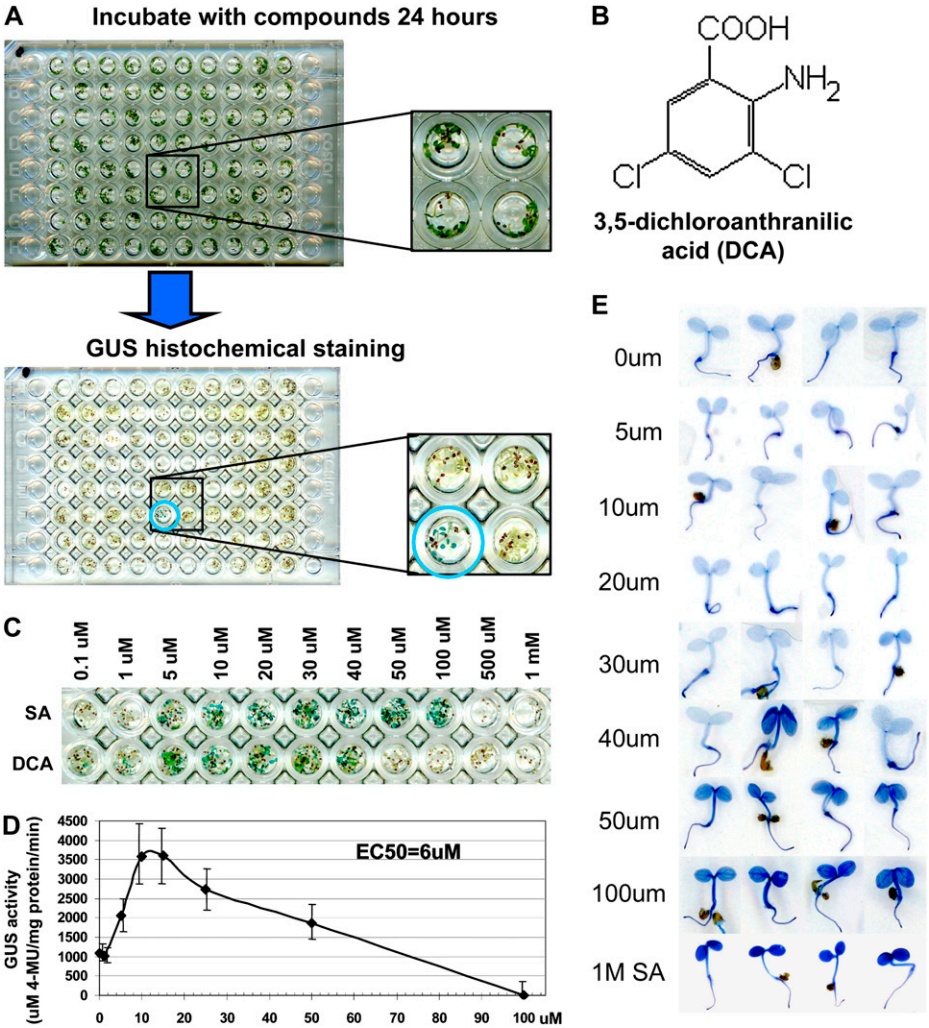


Figure 3. Chemical screen reveals a new elicitor of defense-specific reporter expression. A, Scheme illustrating the screening procedure. Top, Example of a 96-well screening plate containing 7-d-old liquid-grown $CaBP22^{-333}$ seedlings after a 24-h incubation with library compounds at concentrations ranging from 4 to 20 μM. Bottom, Screening plate after GUS histochemical staining. The blue circle indicates the "hit well." B, Structure of DCA. C, GUS histochemical staining of $CaBP22^{-333}$ seedlings comparing reporter responses to SA and DCA after a 24-h incubation treatment at the indicated concentrations. D, Dose-response curve plotted from GUS fluorometric assays measuring DCA-induced GUS activities at the indicated concentrations with proteins extracted from 7-d-old liquid-grown $CaBP22^{-333}$ seedlings after a 24-h incubation with DCA-treated medium. Mean and SE values calculated from three independent experiments are shown. 4-MU, 4-Methyl-umbelliferyl-β-D-glucuronide. E, Trypan Blue staining of Col-0 wild-type seedlings incubated for 24 h in DCA-containing medium at the indicated concentrations. Dark blue color indicates cell death (toxicity). All staining experiments were performed at least three times with similar results.

treatment) revealed that this elicitor is active at concentrations as low as 0.1 μM, making it significantly more potent than SA, which did not trigger GUS expression at concentrations under 5 μM (Fig. 3C). Quantitative dose-response curves after saturation treatments with DCA revealed a maximal effective concentration of approximately 15 μM. Application of higher concentrations resulted in a sharp decline in GUS activity (Fig. 3D). Its median effective concentration (EC50) was determined to be 6 μM. To examine DCA-induced phytotoxicity, we used trypan blue staining of seedlings after saturation treatment. Dark blue staining, indicating cell death, was prevalent in approximately 50% of the seedlings treated for 24 h with 40 μM DCA and in nearly 100% of the seedlings treated with DCA concentrations of 50 μM or higher (Fig. 3E). However, no cell death was observed at concentrations showing effective reporter activation (5–20 μM), suggesting that cell death was not responsible for *GUS* reporter activation but may be the cause of the observed decline of GUS activity at higher concentrations. Based on these assays, DCA is clearly a potent inducer of *GUS* expression in the *CaBP22*$^{-333}$ lines. Consistent with this effect, DCA triggered increased binding of nuclear Arabidopsis proteins to *LURP*^{A} (Fig. 2C).

DCA Induces Rapid and Transient Resistance to *Hp*

We further examined if the induction of *CaBP22*$^{-333}$:: *GUS* expression by DCA translates to defense activation in soil-grown plants. Figure 4A shows that application of DCA via foliar spray induced reporter activity in 2-week-old soil-grown *CaBP22*$^{-333}$ seedlings at concentrations ranging from 10 to 500 μM. We also pretreated 2-week-old soil-grown ecotype Columbia (Col-0) seedlings by foliar spray with varying concentrations of DCA 24 h prior to challenge with virulent *Hp*Noco2. The extent of *Hp* spore formation was assayed 7 d after pathogen challenge. Plants

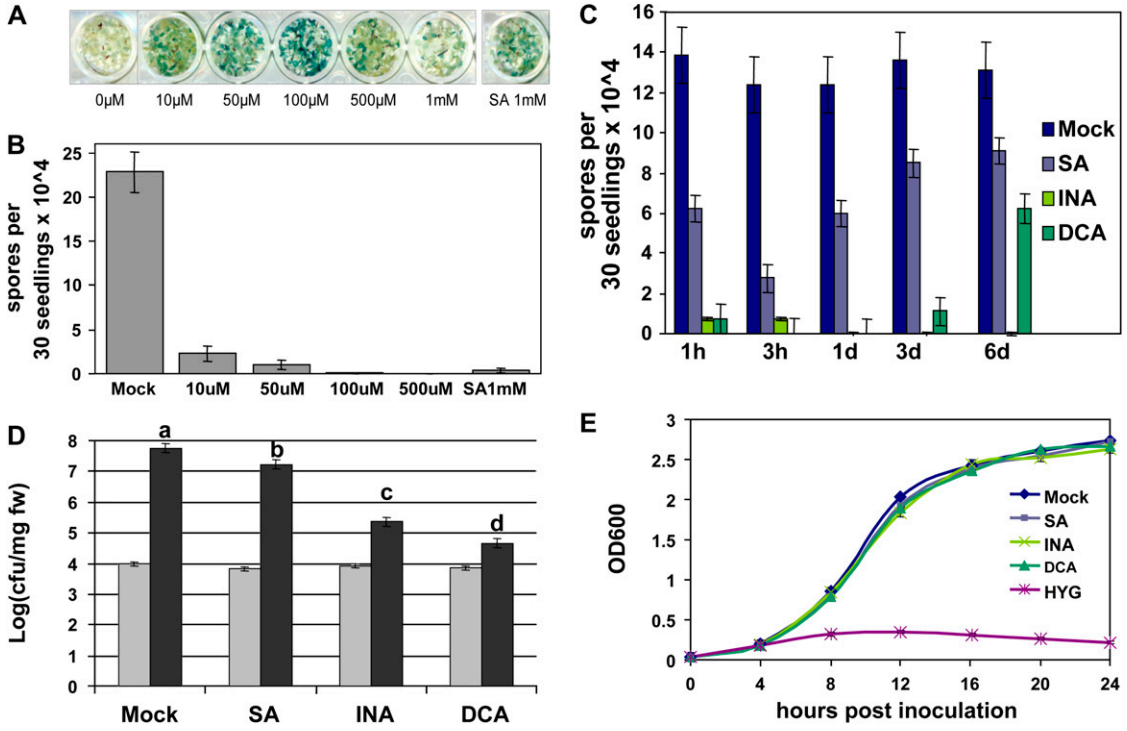


Figure 4. DCA induces disease resistance. A to D, All experiments were conducted with 14-d-old soil-grown *CaBP22*$^{-333}$ or Col-0 seedlings. Mean and SE values were calculated from three independent trials. A, GUS histochemical staining of *CaBP22*$^{-333}$ seedlings 24 h after chemical spray treatment with the indicated concentrations of DCA and SA. B, Col-0 seedlings were sprayed with DCA and SA at the indicated concentrations 24 h prior to spray infection with virulent *Hp*Noco2 (3×10^4 spores mL^{-1}). Spores were counted at 7 dpi. C, Kinetic analysis of chemically induced disease resistance; Col-0 seedlings were sprayed with 100 μM of each chemical at the indicated times prior to *Hp*Noco2 (3×10^4 spores mL^{-1}) spray infection. Spores were counted at 7 dpi. D, Quantification of *Pst* growth by colony-forming units (cfu). Col-0 seedlings were pretreated with 100 μM of the indicated chemicals 24 h prior to dip inoculation with virulent *Pst*. Bacteria were extracted at day 0 (gray bars) or day 3 (black bars). Significant differences were tested using the Mann-Whitney *U* statistical test ($P < 0.05$). fw, Fresh weight. E, *Pst* grown in liquid culture with 100 μM of the indicated chemicals or 100 μg mL^{-1} hygromycin (HYG). OD600, which represents the optical density of bacteria at 600 nm, was measured at the indicated times after inoculation. SE values were all less than 0.05 and so are not visible on the graph.

pretreated with DCA at concentrations as low as 10 μM displayed a significant reduction of *Hp* spore numbers compared with mock-pretreated plants (Fig. 4B). Maximal effects with regard to *CaBP22*$^{-333}$::*GUS* induction and suppression of *Hp*Noco2 spore formation were observed after spray application of 100 μM DCA (Fig. 4, A and B). Spray application of DCA to soil-grown plants at this concentration did not cause any detectable amount of cell death within 8 d (Supplemental Fig. S1). Therefore, for all further experiments, DCA was applied by foliar spray to 2-week-old soil-grown seedlings at a concentration of 100 μM.

To examine the kinetics of DCA-induced *Hp*Noco2 resistance compared with other defense elicitors (SA and INA), plants were pretreated with 100 μM of each compound at specific times ranging from 1 h to 6 d prior to pathogen challenge (Fig. 4C). As noted in the reporter assays, DCA is unmistakably more potent than SA, which was unable to induce full resistance under these conditions at any of the tested time points. Chemical pretreatment with DCA or INA 24 h prior to *Hp* infection induced full resistance, while mock- or 100 μM SA-pretreated plants showed prolific development of *Hp* sporangiophores, which contain mature asexual spores. Interestingly, DCA and INA both induced strong resistance as early as 1 h after treatment. However, despite the structural relatedness of INA and DCA (Fig. 5A), INA-induced resistance was long lasting, whereas DCA-induced resistance began to decline between 3 and 6 d after chemical treatment (Fig. 4C). The surprisingly early defense induction triggered already 1 h after DCA treatment coincided with a similarly fast induction of *WRKY70* and *CaBP22* expression (Supplemental Fig. S2). These data clearly show that DCA is a potent elicitor of *Hp* resistance and that its activity is both rapid and reversible.

DCA Reduces Growth of the Bacterial Pathogen *Pseudomonas syringae* in Planta Only

We also tested the ability of DCA to induce resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*). Plants pretreated with 100 μM DCA and INA 24 h prior to dip inoculation with *Pst* showed no visible disease symptoms at 5 dpi, while 100 μM SA- and mock-treated plants appeared highly diseased, exhibiting extensive chlorosis and necrosis (data not shown). Quantification of in planta bacterial growth revealed that plants pretreated with DCA showed the greatest reduction in bacterial growth, followed by INA and SA pretreatment, respectively (Fig. 4D). To determine if DCA possesses direct antibacterial properties, we monitored the growth of *Pst* in liquid medium containing 100 μM DCA, SA, INA, or the antibiotic hygromycin. None of the tested defense inducers reduced bacterial growth at their bioactive concentrations, while hygromycin completely eliminated growth of *Pst* (Fig. 4E). These data show that DCA, like INA or SA, induces resistance to *Pst* without exhibiting direct antibiotic activity.

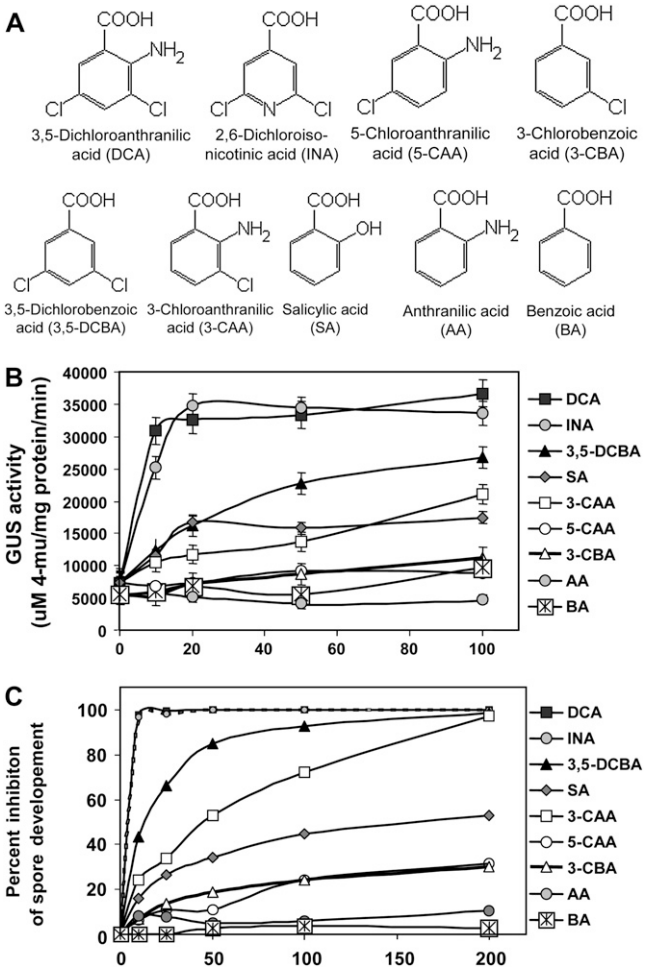


Figure 5. Structure-activity analysis. A, Chemical structures of DCA analogs analyzed. B, Fluorometric analysis of GUS activities induced by DCA analogs using proteins extracted from 2-week-old soil-grown *CaBP22*$^{-333}$ seedlings 48 h after spray treatment with compounds at the indicated concentrations (μM). The mean and SE values were calculated from three independent replicates. 4-mu, 4-Methyl-umbelliferyl-β-D-glucuronide. C, *Hp*Noco2 growth inhibition assay. Two-week-old soil-grown Col-0 seedlings were spray infected with *Hp*Noco2 at 48 h after treatment with varying concentrations (μM) of each DCA analog and then assayed at 7 dpi for disease symptoms (spores). A value of 100% inhibition = 0 spores. The assay was repeated three times with similar results.

Structure-Activity Analysis

In order to determine the features of DCA that are important for its defense-inducing activity, we analyzed structural analogs of this molecule. Initially, 18 DCA analogs were tested for their ability to induce reporter activity in *CaBP22*$^{-333}$ seedlings using the liquid growth assay established for high-throughput screening (Supplemental Table S2). From these, we selected six compounds that represent a range of activities while maintaining obvious structural similarities to DCA plus INA and SA for detailed analyses (Fig. 5A). Dose-response curves were generated illus-

trating the activities of each compound in two different types of assays: (1) GUS activity triggered in *CaBP22*$^{-333}$ seedlings using fluorometric 4-methylumbelliferyl-β-D-glucuronide assays (Fig. 5B); and (2) inhibition of *Hp* spore development during a normally compatible plant-*Hp* interaction (Fig. 5C). Each analog displayed similar behavior in both assays, and three major activity trends were revealed: strong, moderate, and weak. DCA, like INA, showed strong *GUS* induction (greater than 7-fold) and nearly 100% inhibition of spore development at low micromolar concentrations. At the opposite end of this spectrum, benzoic acid, anthranilic acid, 3-chlorobenzoic acid, and 5-chloroanthranilic acid were very weak inducers of GUS activity (less than 2-fold) and were unable to mediate full *Hp* defense at any of the tested concentrations. Both 3,5-dichlorbenzoic acid and 3-chloroanthranilic acid mediated a moderate inhibition of spore development (50%–75% at 100 μM) and triggered a medium level of GUS activity similar to that induced by SA (approximately 4-fold). Generally, dichlorinated molecules showed the strongest defense-inducing activity. Anthranilic acid and benzoic acid analogs with single chlorines exhibited strongly reduced or abolished bioactivity in both assays, while their completely dechlorinated derivatives were inactive. Removal of the amino group from DCA significantly reduced its biological activity. Additionally, DCA, 3,5-dicholorbenzoic acid, and 3-chloranthranilic acid were all more potent than SA. In summary the structure-activity analysis showed that no substitutions for DCA were well tolerated, as activity was lowered in all tested DCA analogs. Furthermore, chlorination, particularly in the 3 position, is required for biological activity, and anthranilic acid derivatives were consistently more active than their comparable benzoic acid analogs.

DCA Acts Downstream or Independently of SA Perception and Is Partially Dependent on WRKY70 and NPR1

To determine at what hierarchical level DCA interferes with defense signaling, we used reverse transcription (RT)-PCR to examine transcript levels of two *LURP*s (*CaBP22* and *WRKY70*) after treatment with 1 mM SA and 100 μM DCA in Col-0 and several well-characterized defense signaling mutant backgrounds (Fig. 6A). As anticipated, SA and DCA treatments transcriptionally induced the endogenous *LURP* genes *CaBP22* and *WRKY70* in Col-0 seedlings. DCA- and SA-induced *LURP* expression remained unaltered in the *eds1*, *ndr1*, and *pad4* mutants known to be blocked upstream from SA (Aarts et al., 1998; Jirage et al., 1999) and in the *npr1* mutant, which is compromised in some signaling processes downstream of SA perception (Dong, 2004). The *LURP*-inducing activity of DCA, unlike that of SA, was also not blocked in *nahG* plants. Only in the *wrky70* mutant was the DCA and SA inducibility of *LURP* transcript accumulation blocked. This outcome was not surprising, as WRKY70 was shown previously to affect the expression of *CaBP22* and *LURP1* (Knoth et al., 2007). These data show that DCA targets a *WRKY70*-dependent branch of the defense signaling network.

To confirm the effects of some of the tested mutations on DCA activity, we analyzed DCA-mediated resistance to *Hp*Noco2 (Fig. 6B). Col-0 and mutant plants were pretreated with DCA prior to spray infection with *Hp*Noco2. DCA induced strong *Hp* resistance in Col-0 and *nahG* plants, nearly fully suppressing the formation of *Hp*Noco2 spores. DCA-induced resistance was significantly compromised in the *wrky70* mutant but was not fully abolished. In contrast to the results of the RT-PCR analysis, DCA-mediated resistance was weakly reduced in *npr1* plants. The *npr1-3* and *wrky70-3* mutants used appear to be null alleles (Cao et al., 1997; Knoth et al., 2007). Thus, in the absence of NPR1 or WRKY70, DCA is still able to activate defense reactions to a certain extent. This may indicate that NPR1- and WRKY70-dependent mechanisms are partially redundant. Alternatively or additionally, DCA may also induce defense mechanisms that are completely independent from NPR1 and WRKY70.

The fact that the impact of *npr1* on DCA-mediated *Hp*Noco2 resistance is only weak was surprising, as the defense-inducing activity of the structurally related INA is known to be fully dependent on NPR1 (Cao et al., 1994; Delaney et al., 1995). In fact, a side-by-side comparison confirmed that *Hp*Noco2 resistance mediated by INA is fully blocked in *npr1* plants, while resistance mediated by DCA is somewhat reduced in this mutant (Fig. 6C). In the *npr1* background, DCA suppressed *Hp* spore formation to 43% of the level observed in untreated plants, which is approximately 4-fold less than the DCA-mediated suppression of spore levels to 10% in Col-0.

Taken together, these data show that DCA triggers both NPR1-dependent and NPR1-independent defense responses (Fig. 6D). Interaction of DCA with defense signaling pathways is likely to occur either downstream or independently of SA perception/accumulation and is partially dependent on WRKY70. The partial independence from NPR1 and the transient nature of its defense-inducing activity functionally discriminates DCA from INA.

Microarray Analyses Reveal Transcriptional Changes Associated with Chemically Induced Disease Resistance

We reasoned that DCA-triggered transcriptome changes that follow the temporal pattern of DCA-mediated resistance are very likely to be of key importance for a successful pathogen defense. To this end, we hybridized to Affymetrix ATH1 GeneChips RNA from Col-0 seedlings that were treated for 48 h or 6 d with mock solution, DCA, or INA. We examined responses at these two time points, because both DCA and INA efficiently suppressed *Hp* spore formation

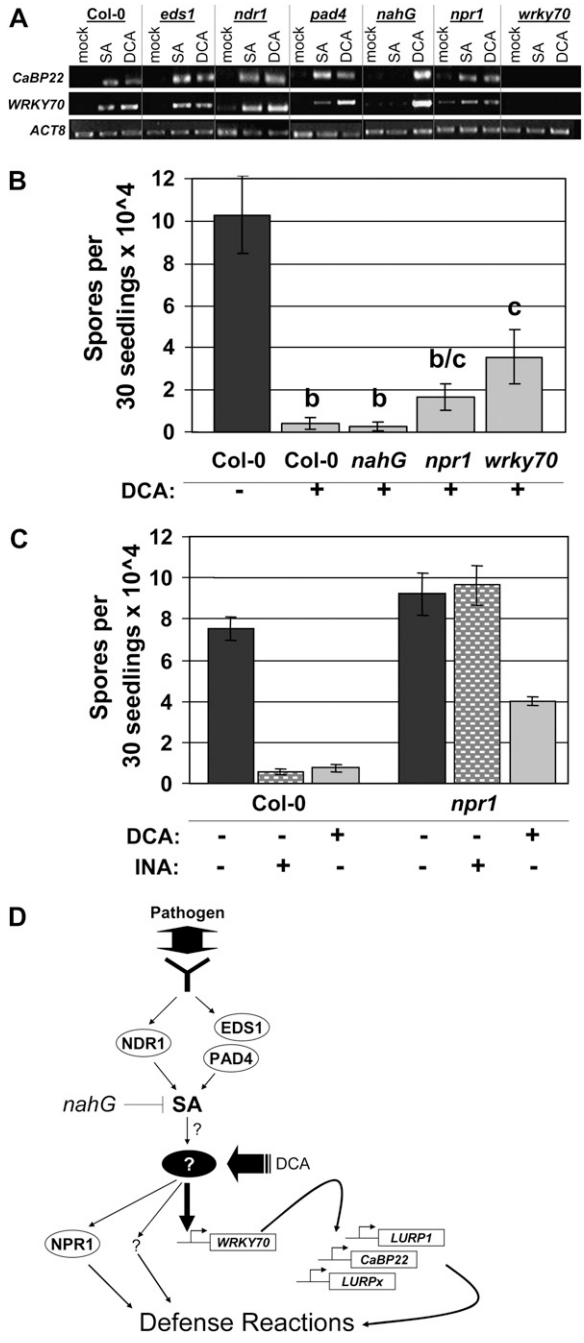
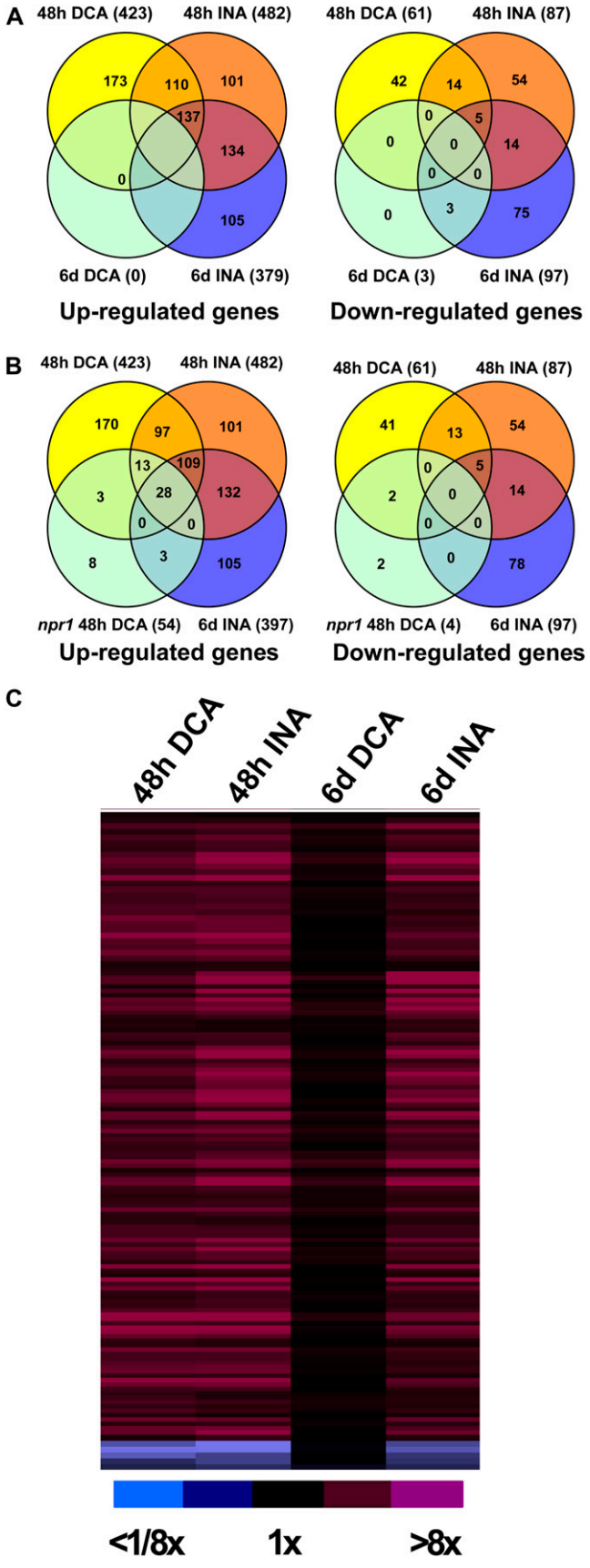


Figure 6. Analysis of DCA activity in known defense mutants. A, RT-PCR analysis of *CaBP22* and *WRKY70* expression in Col-0 (wild type) and mutant or *nahG* backgrounds 24 h after spraying 2-week-old seedlings with water (mock), 1 mM SA, or 100 μM DCA. *Actin8* (*ACT8*) is shown as a loading control. At least three biological replicates showed consistent results. B and C, Two-week-old seedlings were sprayed with virulent *Hp*Noco2 at 24 h after pretreatment with water, 100 μM DCA, or 100 μM INA as indicated. Spores were counted 7 d after spray infection. Mean and SE values were calculated from three independent experiments. Mann-Whitney *U* test ($P < 0.05$) was used to determine significant differences among the different plant lines. Dark gray bars indicate mock pretreatment, light gray bars indicate 100 μM DCA, and checked bars indicate 100 μM INA treatment. D, Model illustrating how DCA may interfere with defense signaling. DCA targets regulators operating downstream or independently of SA, triggering both NPR1-dependent and NPR1-independent defense responses. The latter branch targets a WRKY70-dependent node of the defense signaling network. Additional DCA-triggered pathways may involve NPR1 and WRKY70 paralogs or defense regulators unrelated to them.

between 24 and 72 h after treatment but substantially differed in their efficiency at 6 d after treatment. To further examine the role of NPR1 in DCA-mediated disease resistance, we also analyzed responses at 48 h after DCA or mock treatment in the *npr1* mutant. Differentially expressed genes (DEGs) in response to the chemical treatment were identified statistically among three biological replicates using as a cutoff a false discovery rate of less than 0.05. The up- and down-regulated DEGs identified by this analysis are summarized in Figure 7A (see also Supplemental Tables S3–S8). In Col-0 wild-type plants at 48 h after DCA treatment (48 h DCA), 423 genes were up-regulated and 61 genes were down-regulated. Notably, at 6 d after DCA treatment (6 d DCA), which does not correlate with disease resistance, only three genes displayed any significant change, confirming that the effects of DCA are reversible. A larger set of genes exhibited differential responses to INA at both tested time points. In Col-0 at 48 h after INA (48 h INA) application, a total of 482 genes were up-regulated and 87 genes were down-regulated, while 6 d after treatment with INA (6 d INA), 379 genes were classified as up-regulated and 97 genes as down-regulated. The transcriptional changes in response to the 48 h DCA and 48 h INA treatments overlap. Fifty-eight percent of the 48 h INA-inducible genes were also up-regulated by DCA at 48 h in Col-0, and 31% of the 48 h INA-suppressed genes were also down-regulated by DCA in Col-0 at this time point.

Most importantly, our Venn analyses shown in Figure 7A revealed a cluster of 142 DEGs whose patterns of transcriptional changes and disease resistance triggered by DCA or INA match. Hence, they represent transcriptional changes strictly associated with DCA- and INA-induced defense and are likely to be functionally important for these immune responses. These genes showed significantly altered transcript levels 48 h after DCA or INA treatments as well as 6 d after INA treatment but not 6 d after DCA treatment (Fig. 7C). This *ACID* (for *Associated with Chemically Induced Defense*) cluster contains 137 genes that were coordinately up-regulated and five genes that were coordinately down-regulated by all three defense-inducing treatments (48 h after DCA or INA treatments and 6 d after INA treatment). Consistent with the partial suppression of DCA-mediated defense responses by NPR1, 20% of the 137 up-regulated *ACID* genes exhibit *NPR1*-independent expression, while the remaining genes are expressed in an *NPR1*-dependent manner. All of the five coordinately down-regulated genes are *NPR1* dependent (Fig. 7B).



In order to infer possible molecular processes contributing to DCA/INA-mediated disease resistance, we used the FuncAssociate program to identify enriched Gene Ontology (GO) terms (Berriz et al., 2003). This program identified several statistically overrepresented GO terms in the set of 137 up-regulated *ACID* members (Supplemental Table S9). GO terms representing kinase activity, transferase activities, and calmodulin binding were ranked the highest in this set. Both calmodulin-binding proteins, which sense Ca^{2+} fluxes, and protein kinases and are known to be important for plant defense signaling (Zhou et al., 1995; Zhang and Klessig, 2001; Kim et al., 2002). The overrepresentation of these terms within the *ACID* cluster supports the conclusion that these genes are important for disease resistance, as suggested by their strict correlation with successful pathogen defense.

DISCUSSION

Plants have an intricate immune system that responds to pathogen infections via a complex regulatory network. Synthetic bioactive molecules that interfere or interact with defined signaling mechanisms can serve as powerful tools for the dissection of regulatory networks and complement the use of mutants, natural messenger molecules, or ligands (Kawasumi and Nghiem, 2007). In order to expand the existing repertoire of elicitors that can be used for analyses of the plant defense network, we initiated screens of several diversity-oriented chemical libraries for new inducers of pathogen-responsive reporter genes.

We performed elicitor screens targeting regulatory mechanisms controlling the expression of *LURP* genes. As *CaBP22* represents the average response pattern of the *LURP* cluster, we first dissected its promoter and

Figure 7. Gene sets differentially regulated after elicitor treatments. A, Nonproportional Venn diagrams depicting the overlap of gene sets up-regulated (left) or down-regulated (right) 48 h after DCA or INA treatment or 6 d after DCA or INA treatment. Not shown in the diagrams are the overlaps between 48 h DCA and 6 d INA (three up-regulated; zero down-regulated) and between 48 h INA and 6 d DCA (zero up-regulated; zero down-regulated). B, Venn diagrams showing the overlap of gene sets up-regulated (left) or down-regulated (right) 48 h after DCA or INA or 6 d after INA treatment in Col-0 as well as 48 h after DCA in the *npr1* mutant background. The number next to the treatment indicates total genes in this set. The significance cutoff (false discovery rate) is $P < 0.05$. Not shown in the diagrams are the overlaps between 48 h DCA and 6 d INA (three up-regulated; zero down-regulated) and between 48 h INA and *npr1* 48 h DCA (zero up-regulated; zero down-regulated). C, Hierarchical clustergram of the *ACID* set of 142 DEGs coordinately up-regulated or down-regulated 48 h after DCA or INA treatment or 6 d after INA treatment but not 6 d after DCA treatment ($P \leq 0.05$). Displayed are ratios of transcript levels triggered by the individual chemical treatments compared with the respective mock treatments. Magenta represents up-regulated relative to control, and blue represents down-regulated relative to control; the brightest color indicates a greater than 8-fold differential expression.

identified a *Hp*-responsive region containing *LURP*^{A}. This motif appears to be related to the defense-associated TL1 element (CTGA**AGAAGA**A; Wang et al., 2005), which contains the *LURP*^{A} core motif (*LURP*^{A}-CM). Thus, TL1/*LURP*^{A}-type promoter elements may play a wider role in defense gene regulation.

GUS reporter activity mediated by the *CaBP22*$^{-333}$ region was only observed after a variety of defense stimuli activating the SA signaling cascade or wounding, but not after other treatments. Hence, the *CaBP22*$^{-333}$ line seemed to be a specific and reliable reporter system for our elicitor screens. We observed a low hit rate of 0.3%, and as anticipated, many of the 114 *LURP* inducers we identified are structurally related to SA. However, we also identified several structurally novel compounds in this screen. One candidate, DCA, was particularly active in detailed follow-up analyses. The EC50 value for GUS expression in *CaBP22*$^{-333}$ seedlings after 24 h of DCA saturation treatment was found to be 6 μM, which is 10-fold lower than the published value for *PR1* induction by SA (65 μM; Pillonel, 2001). Low active concentrations are often correlated with high target specificity and a decrease in unwanted side effects (Burdine and Kodadek, 2004). DCA did not exhibit any herbicidal activity at the bioactive concentrations; however, it proved to be phytotoxic at higher concentrations. To our knowledge, there are no reports of defense induction by exogenous application of DCA, and despite some structural similarities, it is chemically distinct from known defense inducers. In addition, DCA represents a substructure repeatedly observed among the other 114 elicitors we identified. It is also readily commercially available. Combined, these results made DCA an interesting candidate for further analysis of its role in defense gene activation.

DCA induced resistance to two phylogenetically distinct pathogens at concentrations much lower than SA without exhibiting direct antibiotic activity. DCA did not induce HR-type cell death or an oxidative burst (data not shown) at biologically active concentrations, suggesting that it acts downstream or independently of these defense responses. The *wrky70* mutant was the only tested Arabidopsis line deficient in defense regulation, with suppressed DCA inducibility of *CaBP22* expression. Consistent with this, the *wrky70* mutant exhibited significantly reduced DCA-mediated resistance. In contrast to the results of the *Hp*Noco2 defense assays, *LURP* expression responses were not affected in the *npr1* mutant. To clarify the role of *NPR1* in DCA-induced resistance, we performed microarray analyses in the *npr1* mutant background 48 h after DCA treatment. Of the 137 DCA-inducible *ACID* genes, 20% exhibited NPR1-independent transcriptional up-regulation. As anticipated, the *NPR1*-independent *ACID* subset contains several *LURP*s, including *WRKY70*, *LURP1*, *NPR4*, and *WAK1*. Both the *NPR1*-dependent and *NPR1*-independent subsets of the *ACID* cluster contain a multitude of additional genes implicated in defense responses. Based on these observations, we propose that DCA operates downstream or independently of SA, activating a *WRKY70*/*LURP*-dependent branch of the defense signaling network as well as, weakly, a separate *NPR1*-dependent branch (Fig. 6D). We cannot exclude that additional NPR1-independent and *WRKY70*-independent signaling routes are activated by DCA. Such alternative pathways may involve paralogs of *NPR1* and/or *WRKY70*.

The defining features of DCA-type elicitors are the presence of the 3- and 5-position chlorines and an amino group at position 2. All of the tested analogs conform to Lipinski's rule of five (Lipinski et al., 1997) and have low polar surface area values, which suggests that they should all be readily absorbed by cells. However, compared with DCA, all of tested analogs showed a reduced defense-inducing activity, suggesting that each tested region of this compound is important for its activity. Although functionally distinct, DCA, SA, and INA have several common structural features. SA and DCA share the backbone structure of a benzoic acid substituted at the 2 position. However, this core structure (anthranilic acid in the case of DCA) is inactive with regard to defense induction. Comparative analyses of structure-activity relationships of DCA and INA turned out to be more complex, because their efficiencies were nearly identical regarding both *CaBP22*$^{-333}$::*GUS* induction and defense activation. DCA and INA share a common structure of a dichlorinated six-member ring with a carboxy group. Their closest common structure, 3,5-dichlorobenzoic acid, also induces *CaBP22*$^{-333}$::*GUS* expression and *Hp* resistance, albeit at a significantly reduced level. Exchange of a carbon atom by a nitrogen atom at position 4 of the ring converts this molecule to INA, while addition of an amino group to position 2 of the ring results in DCA. Hence, both INA and DCA can formally be considered as two representatives of a continuum of related defense-inducing molecules. However, some key differences between DCA and INA were observed in our defense assays. As discussed above, *npr1* only mildly affected DCA-induced resistance. This functionally discriminates DCA from SA, INA, and BTH, as their defense activation is fully blocked in *npr1* (Lawton et al., 1996; Lipinski et al., 1997; Knoth et al., 2007). Taken together, these data suggest that despite some structural similarities and a qualitatively related response, DCA seems to shift the balance between NPR1-dependent and NPR1-independent responses toward the NPR1-independent ones.

Besides the *ACID* set, our microarray analyses defined a second interesting cluster, which comprises genes that are specifically up-regulated by 48 h DCA and but not by INA treatment (173 genes). GO analysis of this set show that it is highly enriched for genes annotated for involvement in defense responses. Strongly overrepresented in this set are genes involved in phosphorylation, phosphate metabolism, phosphotransferases, and phosphokinase activity. Whereas in the set of genes specifically up-regulated by 48 h INA

(235 genes), GO analysis does not show any enrichment for phosphorylation-related mechanisms (Supplemental Table S10). This supports the conclusion that DCA and INA may be acting on different (but possibly related) targets, leading, on the one hand, to the activation of defense responses specific for each of these elicitors and, on the other hand, to a set of common defense responses.

In addition, DCA and INA differ substantially in their kinetic behavior. Consistent with previous reports (Uknes et al., 1992), we found INA to induce long-term disease resistance, whereas DCA-induced resistance is transient. This feature of DCA should allow rapid and reversible induction of immune responses at any developmental stage with limited side effects. Permanent defense activation often results in fitness costs, due to the toxicity of defensive products and resource allocation away from growth or reproduction. For example, possibly due to its long-term activity, INA was insufficiently tolerated by some crop plants to warrant practical use as a plant protection compound (Ryals et al., 1996). Furthermore, the mutants *cpr1-1* (for *constitutive expresser of PR1-1*) and *ssi1* (for *suppressor of SA-insensitivity1*) exhibit constitutive defense responses causing severe dwarf phenotypes (Shah et al., 1999; Jirage et al., 2001). Thus, chemicals that transiently activate plant immunity may be beneficial in combating virulent pathogens that threaten crops only during a limited period of time. A transiently active compound like DCA may allow fine-tuned control of defense induction coordinated with the plant's needs, thereby decreasing any unwanted side effects caused by long-term defense activation. The distinct kinetic characteristics of DCA and INA may be due to differences of their lifetimes in planta. Alternatively, the two compounds may differ regarding their modes of target interaction. Future studies will have to address their fate in planta, the identification of their biological targets, and details of their interference with these targets.

We defined the *ACID* cluster as a set of genes strictly associated with defense activation by two separate defense-inducing chemicals, DCA and INA. In addition, 118 of the 142 *ACID* members also respond to a third synthetic elicitor, BTH (Wang et al., 2006). The *ACID* cluster contains many known defense-related genes and is highly enriched for genes associated with calmodulin binding and kinase activity. Among the up-regulated *ACID* genes are six genes encoding WRKY transcription factors, which have been associated with plant immune responses (Eulgem and Somssich, 2007). Also, there are 19 genes encoding Leu-rich repeat-containing proteins represented in this cluster. Conspicuously absent from the overrepresented GO attributes for the *ACID* set are terms annotated for processes associated with upstream/early defense responses, like the HR, cell death, peroxidases, response to ROIs, and SA biosynthesis genes. The lack of enhancement of these terms is consistent with our observation that DCA targets only a subset of the defense signaling network downstream of these early responses. This can be further illustrated by comparing GO terms from transcriptome changes triggered by other defense stimuli. For example, GO analysis of genes up-regulated by flg22 treatment (Zipfel et al., 2004) contains all of the GO terms found in the *ACID* cluster. However, the flg22 set also includes terms for HR response, transcriptional regulation, and defense response that were not overrepresented in the *ACID* cluster. This implies that DCA activates a distinct subset of pathogen-inducible defense responses.

Several *LURP*s (originally defined as cluster II by Eulgem et al. [2004]) were found to be up-regulated by 48 h DCA or INA but were not included in the *ACID* cluster. *CaBP22* is found in this set, as it is not differentially expressed after the 6 d INA treatment. Consistent with this, *cabp22* T-DNA mutants did not display detectable defense-associated phenotypes (data not shown). This shows that while *CaBP22*, as a WRKY70 target gene, is an excellent marker for defense activation, it is functionally not essential for such processes. Another *LURP* member that falls into the same category as *CaBP22*, *ECS1*, is up-regulated in several plant-pathogen interactions (Aufsatz et al., 1998; Eulgem et al., 2004). However, *ECS1*, like *CaBP22*, is not vital for effective defense activation (Aufsatz et al., 1998). These observations highlight the usefulness of the *ACID* cluster for the identification of new components essential for full plant immune responses.

We have developed a specific and reliable high-throughput screening system for synthetic elicitors and identified DCA, a plant defense activator that triggers a defined aspect of the plant defense network. However, its target(s) remain(s) to be determined. Screens for proteins directly targeted by DCA or operating downstream from DCA perception may reveal new components of the plant immune response. The fact that DCA strongly triggers NPR1-independent defense makes such screens very useful and may overcome limitations of previous strategies that often identified *npr1* alleles (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). We are currently analyzing some other candidates identified in our chemical screen and continue to screen chemical libraries for inducers of $CaBP22^{-333}$::*GUS* as well as other pathogen-responsive reporter genes that are not inducible by DCA. We expect to provide a collection of compounds that interact with distinct hierarchical levels of the plant defense signaling network. These synthetic elicitors will be invaluable tools for the fine dissection of defense mechanisms and may lead to the development of novel pesticides tailored to enhance a crop's inherent defense capabilities.

MATERIALS AND METHODS

Plant Growth Conditions and Pathogen Infections

Arabidopsis (*Arabidopsis thaliana*) plants were grown on soil under fluorescent lights (14 h of light/10 h of dark, 21°C, 100 μE m^{-2} s^{-1}) unless

otherwise noted. The mutants *eds1-1* (Parker et al., 1996), *ndr1-1* (Century et al., 1997), *wrky70-3* (Knoth et al., 2007), *npr1-3* (Cao et al., 1997), and transgenic *nahG* (Delaney et al., 1994) have been described. *Hyaloperonospora parasitica* was grown and propagated as described previously (McDowell et al., 2000). Plants were spray infected with *Hp* spore suspensions at 5×10^4 spores mL^{-1} for *Hp*Emoy2 and 3×10^4 spores mL^{-1} for *Hp*Noco2 with Preval sprayers (http://www.prevalspraygun.com). Plants were scored at 7 d after infection for severity of infection by Trypan Blue staining (McDowell et al., 2000), visual sporangiophore counts, and counting spores/seedlings using a hemicytometer. Arabidopsis plants were dip inoculated with *Pseudomonas syringae* pv *tomato* DC3000 with an inoculum concentration (optical density at 600 nm) of 0.05. For these experiments, infections and scoring were performed as described previously (Tornero and Dangl, 2001). Plants were visually scored for disease symptoms 4 to 7 d (as indicated) after inoculation.

RT-PCR

RNA was isolated from seedlings using TRIZOL (Invitrogen). cDNAs were prepared as described previously (Knoth and Eulgem, 2008). In each case, 1 μL of cDNA was used for all PCRs (20 μL total volume). A 425-bp fragment of *Actin8* was amplified as a loading control using primers RT-*ACT8*-F (5′-ATGAAGATTAAGGTCGTGGCAC-3′) and RT-*ACT8*-R (5′-GTTTTTATCCGAGTTTGAAGAGGC-3′) with an annealing temperature of 60°C for 21 cycles. A 301-bp fragment of *CaBP22* was amplified using the primer pair RT-*CaBP22*-FP (5′-CGGAACCATCAATTTCACTGAGT-3′) and RT-*CaBP22*-RP (5′-CAAAGTGCCACCAGTTGTGTCAT-3′) with an annealing temperature of 62°C for 24 cycles. The 477-bp fragment of *WRKY70* was amplified using the primer pair RT-*WRKY70*-FP (5′-AACGACGGCAAGTTTGAAGATTC-3′) and RT-*WRKY70*-RP (5′-TTCTGGCCACACCAATGACAAGT-3′) with an annealing temperature of 63°C for 24 cycles. The 338-bp fragment of *PR1* was amplified using the primer pair RT-*PR1-FP* (5′-GCCCACAAGATTATCTAAGGG-3′) and RT-PR1-RP (5′-ACCTCCTGCATATGATGCTCCT-3′) with an annealing temperature of 53°C for 27 cycles. All PCRs used the following thermal program, deviating as indicated for annealing temperatures and cycles: 94°C for 1 min; X cycles of 95°C for 30 s, annealing temperature of X°C for 1 min; and 72°C for 40 s. PCR products were electrophoresed on 1.6% agarose gels containing 0.5 μg mL^{-1} ethidium bromide. Negative controls omitting reverse transcriptase in the cDNA production process and PCR without cDNA yielded no products.

Generation of Transgenic Arabidopsis Plants

CaBP22-promoter::*GUS* translational fusion constructs were cloned from PCR products as described previously (Knoth and Eulgem, 2008). PCR products were generated using Col-0 genomic DNA as a template. All *CaBP22-promoter*::*GUS* constructs were designed with a *Hin*dIII restriction site at their 5′ end and a *Bam*HI restriction site at their 3′ end to allow directional restriction enzyme/ligation-mediated cloning into the pBI101.1 vector (Clontech). The sequences of the primers were as follows (numbers indicate the size of the fragment [bp] from the transcriptional start site): *CaBP22*$^{-1075}$-FP, 5′-AATTAAGCTTCTTGAGTCAGGAACATGAAGTGG-3′; *CaBP22*$^{-590}$-FP, 5′-AATTAAGCTTATTGGTCCAGTTACTCATCC-3′; *CaBP22*$^{-333}$-FP, 5′-AATTAAGCTTGTAGCGATTGGTCCACTACCC-3′; *CaBP22*$^{-65}$-FP, 5′-AATTAAGCTTGCAAAAGCTGACATGGCAGTG-3′; and *CaBP22*-RP, 5′-AATTGGATCCCATTGTTTTTTATTTTCTGTGATGACTGAGAGAAG-3′. Plasmids were transformed by electroporation into *Agrobacterium tumefaciens* strain AGLO2 (Sambrook et al., 1989). Col-0 plants were transformed by *Agrobacterium*-mediated transformation using the floral dip method (Clough and Bent, 1998). Plants were selected for the presence of the transgene on half-strength Murashige and Skoog (MS)/0.8% agar medium containing 25 mg L^{-1} kanamycin.

Analysis of GUS Activity

GUS histochemical staining was performed using whole seedlings stained in a 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) solution containing 1 mg mL^{-1} X-gluc, 50 mM Na_2PO_4, pH 7.2, 0.5 mM $K_3Fe(CN)_6$, and 0.5 mM $K_4Fe(CN)_6$ at 37°C and cleared with 70% ethanol. Soil-grown seedlings were incubated at 37°C for 5 h. One-week-old seedlings grown in liquid medium were incubated at 37°C for 18 h. Fluorometric analyses of GUS activities were performed on 10-d-old soil-grown seedlings as described previously (Knoth and Eulgem, 2008). Fold changes were calculated for each replicate experiment and then averaged for final fold change.

EMSAs with Nuclear Proteins

EMSAs were performed with synthetic oligonucleotides (Invitrogen) and nuclear proteins extracted from whole Arabidopsis seedlings. The sequences of the oligonucleotide probes representing *CaBP22*- and other *LURP*-promoter stretches used were as follows (mutated sequences are indicated by lowercase lettering): *LURP*^{A}-WT, 5′-ATTGTTTTCTTCTGTAGAAGACCAT-3′; *LURP*^{A}-M1, 5′-ATTGTTTgggTgTGTAGAAGACCAT-3′; *LURP*^{A}-M2, 5′-ATTGTTTgggTgTGTAcggcACCAT-3′; *LURP*^{A}-M3, 5′-ATTGTTTTCTTCTGTAcggcACCAT-3′; *ZAT7*, 5′-AAAATCTAGAAGACGGCTTAAAAAT-3′; and *WAK1*, 5′-GAAAAGACGAGAAGACCGAGACCTA-3′. Nuclear proteins were extracted as described previously (Desveaux et al., 2004). Bradford protein assays were used to determine protein concentrations (Bio-Rad Laboratories). EMSAs were performed as described previously (Desveaux et al., 2000; Knoth and Eulgem, 2008). Gels were then vacuum dried and autoradiographed on HyBlot Cl Autoradiography film (Denville Scientific). Statistical enrichments of motifs in promoters were calculated using the Arabidopsis Functional Genomics Consortium's MotifFinder tool (http://Arabidopsis.org/tools/bulk/motiffinder/index.jsp).

Chemical Screen for Elicitors of *CaBP22*$^{-333}$-*Promoter*::*GUS* Arabidopsis Plants

Homozygous T4 *CaBP22*$^{-333}$-*promoter*::*GUS* Arabidopsis seedlings were grown in 200 μL of liquid half-strength MS medium on 96-well plates (Costar) for 7 d on an orbital shaker under long-day conditions (16 h of light, 8 h of dark, 22°C, 100 μE m^{-2} s^{-1}). After 7 d, the liquid half-strength MS medium volume was returned to 200 μL, and 0.2 μL of each compound was administered by a robotic pin tool (Biomek FX Laboratory Automation Workstation) to each well for a final concentration of 4 to 20 μM in 0.001% dimethyl sulfoxide (DMSO). Plates were returned to the orbital shaker for 24 h and then stained (histochemically) for *GUS* expression. A total of 42,000 compounds were screened in duplicate. The libraries used were as follows: Microsource Spectrum, containing 2,000 known bioactive compounds; Sigma TimTec Myria Screen, containing 10,000 diversity-oriented compounds; Chembridge Nova Core, containing 10,000 diversity-oriented compounds of novel building blocks and scaffolds; and Chembridge Diverset, containing 20,000 diversity-oriented compounds. Chemicals that induced *GUS* expression (leading to a blue precipitate after staining) in both repetitions were scored visually for intensity of blue color (high, medium, or low). The EC50 of a compound was calculated as the concentration that induces *GUS* expression halfway between the baseline (bottom) and maximum (top).

Chemical Treatments

Stock solutions were prepared in DMSO. Stocks were added directly to the growth medium for treatment of liquid-grown plants. Stock solutions were diluted in water and sprayed on soil-grown plants at the indicated times and concentrations with Preval sprayers until imminent runoff. Final DMSO concentrations never exceeded 0.002%. Mock treatment was application of 0.002% DMSO in water. Chemicals were supplied from Sigma. To test for chemically induced disease resistance, the plants were sprayed with chemicals at the indicated concentrations and times prior to pathogen challenge. Disease symptoms were analyzed as described above.

Microarray Preparation and Data Analysis

Total RNA was isolated from seedlings using TRIZOL as outlined above (Invitrogen). RNA was processed and hybridized to the Affymetrix Arabidopsis ATH1 genome array GeneChip following the manufacturer's instructions (Affymetrix) by the University of California at Riverside Core Instrument Facility. Three independent biological replicates were performed for each treatment. Microarray analysis was performed in the statistical programming environment R using Bioconductor packages. Raw expression values were normalized using the robust multichip averaging algorithm. Analysis of DEGs was performed with the LIMMA package (Smyth, 2004). The Benjamini and Hochberg method was selected to adjust *P* values for multiple testing and to determine false discovery rates (Benjamini et al., 2001).

As a confidence threshold, an adjusted *P* value of ≤0.05 was chosen (compared with mock treatments with water). To visualize the DEG sets, hierarchical clustering was performed using the Cluster and TreeView programs (Eisen et al., 1998). Overrepresented GO terms were identified with the FuncAssociate program (Berriz et al., 2003).

The microarray data have been deposited in MIAME-compliant format in the GEO database under the accession number GSE13833.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Trypan blue staining of 2-week-old soil-grown Col-0 wild-type seedlings 8 d after spray application of mock solution (water), 100 μM DCA, 500 μM DCA, or 2 mM SA; dark blue color indicates cell death (toxicity).

Supplemental Figure S2. DCA induces rapid accumulation of *WRKY70* and *CaBP22* transcript levels.

Supplemental Table S1. Comparison of the ability of different stimuli to induce *GUS* reporter expression in the *CaBP22*$^{-333}$ line.

Supplemental Table S2. Comparison of *GUS* reporter induction for several DCA analogs.

Supplemental Table S3. Genes differentially expressed in Col-0 48 h after DCA.

Supplemental Table S4. Genes differentially expressed in Col-0 6 d after DCA.

Supplemental Table S5. Genes differentially expressed in Col-0 48 h after INA.

Supplemental Table S6. Genes differentially expressed in Col-0 6 d after INA.

Supplemental Table S7. Genes differentially expressed in *npr1* 48 h after DCA.

Supplemental Table S8. Members of the *ACID* cluster.

Supplemental Table S9. Enriched GO terms in the *ACID* cluster.

Supplemental Table S10. Comparison of enriched GO terms after treatment with two distinct defense activators.

ACKNOWLEDGMENTS

We thank Drs. Julia Bailey-Serres, Natasha Raikhel, Cynthia Larive, Sean Cutler, and Michael Pirrung (all University of California at Riverside) for helpful discussions and advice.

Received December 3, 2008; accepted March 17, 2009; published March 20, 2009.

LITERATURE CITED

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene mediated signalling pathways in Arabidopsis. Proc Natl Acad Sci USA **95:** 10306–10311

Aufsatz W, Amry D, Grimm C (1998) The ECS1 gene of Arabidopsis encodes a plant cell wall-associated protein and is potentially linked to a locus influencing resistance to Xanthomonas campestris. Plant Mol Biol **38:** 965–976

Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I (2001) Controlling the false discovery rate in behavior genetics research. Behav Brain Res **125:** 279–284

Berriz GF, King OD, Bryant B, Sander C, Roth FP (2003) Characterizing gene sets with FuncAssociate. Bioinformatics **19:** 2502–2504

Burdine L, Kodadek T (2004) Target identification in chemical genetics: the (often) missing link. Chem Biol **11:** 593–597

Cao H, Bowling SA, Gordon AS, Dong X (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6:** 1583–1592

Cao H, Glazebrook J, Clark JD, Volko S, Dong X (1997) The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88:** 57–64

Century KS, Shapiro AD, Repetti PP, Dahlbeck D, Holub E, Staskawicz BJ (1997) NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science **278:** 1963–1965

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell **124:** 803–814

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Dangl JL (1998) Plants just say NO to pathogens. Nature **394:** 525–527

Dangl JL, Dietrich RA, Richberg MH (1996) Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell **8:** 1793–1807

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826–833

Delaney T, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessman H, Ward E, et al (1994) A central role of salicylic acid in plant disease resistance. Science **266:** 1247–1250

Delaney TP, Friedrich L, Ryals JA (1995) Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc Natl Acad Sci USA **92:** 6602–6606

Delledonne M, Murgia IDE, Sbicego PF, Biondani A, Polverari A, Lamb C (2002) Reactive oxygen intermediates modulate nitric oxide signaling in the plant hypersensitive disease-resistance response. Plant Physiol Biochem **40:** 605–610

Desveaux D, Despres C, Joyeux A, Subramaniam R, Brisson N (2000) PBF-2 is a novel single-stranded DNA binding factor implicated in PR-10a gene activation in potato. Plant Cell **12:** 1477–1489

Desveaux D, Subramaniam R, Despres C, Mess JN, Levesque C, Fobert PR, Dangl JL, Brisson N (2004) A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in Arabidopsis. Dev Cell **6:** 229–240

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

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA **95:** 14863–14868

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol **10:** 366–371

Eulgem T, Weigman VJ, Chang HS, McDowell JM, Holub EB, Glazebrook J, Zhu T, Dangl JL (2004) Gene expression signatures from three genetically separable resistance gene signaling pathways for downy mildew resistance. Plant Physiol **135:** 1129–1144

Evrard A, Ndatimana T, Eulgem T (2009) FORCA, a promoter element that responds to crosstalk between defense and light signaling. BMC Plant Biol **9:** 2

Friedrich L, Lawton K, Reuss W, Masner P, Specker N, Gut Rella M, Meier B, Dincher S, Staub T, Uknes S, et al (1996) A benzothiadiazole induces systemic acquired resistance in tobacco. Plant J **10:** 61–70

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

Gomez-Gomez L, Boller T (2002) Flagellin perception: a paradigm for innate immunity. Trends Plant Sci **7:** 251–256

Goodman RN, Novacky AJ (1994) The Hypersensitive Response in Plants to Pathogens. APS Press, St. Paul

Görlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessman H, et al (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in barley. Plant Cell **8:** 629–643

Jabs T, Colling C, Tschöpe M, Hahlbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and reactive oxygen species from the oxidative burst signal defense gene activation and phytoalexin synthesis in parsley. Proc Natl Acad Sci USA **94:** 4800–4805

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, 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 JD, Dong X, Glazebrook J (2001) Constitutive salicylic acid-dependent signaling in *cpr1* and *cpr6* mutants requires PAD4. Plant J **26:** 395–407

Jones JD, Dangl JL (2006) The plant immune system. Nature **444:** 323–329

Kawasumi M, Nghiem P (2007) Chemical genetics: elucidating biological systems with small-molecule compounds. J Invest Dermatol **127:** 1577–1584

Kessmann H, Staub T, Hofmann C, Ahl Goy P, Ward E, Uknes S, Ryals J (1993) Induced disease resistance by isonicotinic acid derivatives. Jpn J Pestic Sci **10:** 29–37

Kessmann H, Staub T, Hofmann C, Maetzke T, Herzog J, Ward E, Uknes S, Ryals J (1994) Induction of systemic acquired resistance in plants by chemicals. Annu Rev Phytopathol **32:** 439–459

Kim MC, Lee SH, Kim JK, Chun HJ, Choi MS, Chung WS, Moon BC, Kang CH, Park CY, Yoo JH, et al (2002) Mlo, a modulator of plant defense and cell death, is a novel calmodulin-binding protein: isolation and characterization of a rice Mlo homologue. J Biol Chem **277:** 19304–19314

Klessig DF, Durner J, Noad R, Navarre DA, Wendehenne D, Kumar D, Zhou JM, Shah J, Zhang S, Kachroo P, et al (2000) Nitric oxide and salicylic acid signaling in plant defense. Proc Natl Acad Sci USA **97:** 8849–8855

Knoth C, Eulgem T (2008) The oomycete response gene LURP1 is required for defense against Hyaloperonospora parasitica in Arabidopsis thaliana. Plant J **55:** 53–64

Knoth C, Ringler J, Dangl JL, Eulgem T (2007) Arabidopsis WRKY70 is required for full RPP4-mediated disease resistance and basal defense against Hyaloperonospora parasitica. Mol Plant Microbe Interact **20:** 120–128

Lawton K, Friedrich L, Hunt M, Weymann K, Delaney T, Kessman H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J **10:** 71–82

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev **23:** 3–25

Malamy J, Klessig DF (1992) Salicylic acid and plant disease resistance. Plant J **2:** 643–654

McCormack E, Tsai YC, Braam J (2005) Handling calcium signaling: Arabidopsis CaMs and CMLs. Trends Plant Sci **10:** 383–389

McDowell JM, Cuzick A, Can C, Beynon J, Dangl JL, Holub EB (2000) Downy mildew (*Peronospora parasitica*) resistance genes in Arabidopsis vary in functional requirements for *NDR1*, *EDS1*, *NPR1*, and salicylic acid accumulation. Plant J **22:** 523–530

Métraux JP, Ahl Goy P, Staub T, Speich J, Steinemann A, Ryals J, Ward E (1991) Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. *In* H Hennecke, DPS Verma, eds, Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 1. Kluwer, Dordrecht, The Netherlands, pp 432–439

Parker JE, Holub EB, Frost LN, Falk A, Gunn ND, Daniels MJ (1996) Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. Plant Cell **8:** 2033–2046

Pillonel C (2001) Identification of a 2,6-dichloroisonicotinic-acid-sensitive protein kinase from tobacco by affinity chromatography on benzothiadiazole-Sepharose and NIM-metal chelate adsorbent. Pest Manag Sci **57:** 676–682

Ryals JL, Neuenschwander UH, Willits MC, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell **8:** 1809–1819

Sambrook J, Fritsch S, Maniatis T (1989) Molecular Cloning: A Laboratory Manual., Vol 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schob H, Kunz C, Meins F Jr (1997) Silencing of transgenes introduced into leaves by agroinfiltration: a simple, rapid method for investigating sequence requirements for gene silencing. Mol Gen Genet **256:** 581–585

Shah J, Kachroo P, Klessig DF (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

Shah J, Tsui F, Klessig DF (1997) Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana* identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol Plant Microbe Interact **10:** 69–78

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb CJ (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. Plant Cell **9:** 261–270

Smyth G (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol **3:** 3–7

Somssich IE, Halbrock K (1998) Pathogen defense in plants: a paradigm of biological complexity. Trends Plant Sci **3:** 86–90

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

Tsuda K, Sato M, Glazebrook J, Cohen JD, Katagiri F (2008) Interplay between MAMP-triggered and SA-mediated defense responses. Plant J **53:** 763–775

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in *Arabidopsis*. Plant Cell **4:** 645–656

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog **2:** 1042–1050

Wang D, Weaver ND, Kesarwani M, Dong X (2005) Induction of protein secretory pathway is required for systemic acquired resistance. Science **308:** 1036–1040

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux JP, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094

Zhang S, Klessig DF (2001) MAPK cascades in plant defense signaling. Trends Plant Sci **6:** 520–527

Zhou J, Loh Y, Bressan RA, Martin GB (1995) The tomato gene Pti encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. Cell **83:** 925–935

Zhou N, Tootle TL, Klessig DF, Glazebrook J (1998) *PAD4* functions upstream of salicylic acid to control defense responses in *Arabidopsis*. Plant Cell **10:** 1021–1030

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428:** 764–767