Running title:
DCA, a new synthetic plant defense elicitor

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The synthetic elicitor 3,5-Dichloroanthranillic acid (DCA) induces NPR1-dependent and NPR1-independent mechanisms of disease resistance in Arabidopsis thaliana

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ABSTRACT

Immune responses of *Arabidopsis thaliana* are at least partially mediated by coordinated transcriptional upregulation of plant defense genes, such as the *Late/sustained Uregulation 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 sub-sets 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 co-factor NPR1, 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* (associated with chemically induced defense) genes 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.
INTRODUCTION

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, which 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 leucine-rich repeat (LRR)-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 (NO) 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 (non-expresser of pathogenesis-related genes 1), a transcriptional co-factor that is required for the activation of multiple defense genes (Dong, 2004).

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 induces 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 plants’ innate immune responses offer an attractive alternative to 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 (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 they act independently of SA perception and biosynthesis (Kessmann et al., 1993); (Friedrich et al., 1996) and suggesting 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 thaliana (Arabidopsis) genes that exhibit coordinated late/sustained upregulation 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 hours post infection (hpi), 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 LURPs (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 LURPA1, a protein binding motif. Using an Hp-responsive CaBP22 promoter fragment fused to
GUS (β-glucuronidase, uidA) we screened chemical libraries for compounds that induce 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 activity. 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 1075-base pairs (bp) upstream of the CaBP22 transcriptional start site translationally fused to the Echerichia coli β-glucuronidase (uidA, GUS) reporter gene (lines: CaBP22 -1075, CaBP22 -590, CaBP22 -333, CaBP22 -65; Figure 1A). Figures 1B&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 seven days post infection (7 dpi) with avirulent HpEmoy2 and virulent HpNoco2, while greatly reduced (or absent) in response to mock treatment (Figure 1B and data not shown). There was no observable induction by HpEmoy2 or mock treatment in the CaBP22 -65 lines (Figures 1B & C).

GUS expression in the CaBP22 -1075 to CaBP22 -333 lines was significantly inducible by HpEmoy2 in quantitative 4-methyl-umbelliferyl-β-D-glucuronide (MUG) fluorescence assays, while no induction was observed for the CaBP22 -65 lines (Figures 1D & E). Notably CaBP22 -333 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'-ATTGTTTTCTTCTGTAGAAGACC-3') that is strictly conserved in the second Hp-responsive region between positions -590 and -333. We termed this conserved region LURP\textsuperscript{A} and the inversely repeated half sites consisting of the core motif AGAAGA LURP\textsuperscript{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\textsuperscript{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 timepoint which coincides with high LURP transcript levels (Eulgem et al., 2004). A probe containing the full 25-bp LURP\textsuperscript{A} sequence (LURP\textsuperscript{A}-WT) led to a distinct constitutive shift the intensity of which was clearly enhanced after infection with HpEmoy2 or treatment with 1mM SA (Figures 2A-C). This interaction was successfully competed by 100-fold excess of unlabeled LURP\textsuperscript{A}-WT probe. Additional EMSAs were conducted with several mutated probes to further delineate LURP\textsuperscript{A}'s key protein interacting regions. LURP\textsuperscript{A}-M2 contains block mutations in both LURP\textsuperscript{A} half sites (LURP\textsuperscript{A}-HS); while LURP\textsuperscript{A}-M1 and LURP\textsuperscript{A}-M3 are mutated in only the first (TCTTCT, LURP\textsuperscript{A}-5'HS) or second (AGAAGA, LURP\textsuperscript{A}-3'HS) LURP\textsuperscript{A} half site, respectively (Figure 2A). The Hp-induced LURP\textsuperscript{A} shift was successfully competed by 100-fold excess of unlabeled LURP\textsuperscript{A}-WT and LURP\textsuperscript{A}-M1 probes, while unlabeled LURP\textsuperscript{A}-M2 and LURP\textsuperscript{A}-M3 probes competed the LURP\textsuperscript{A}-mediated shift clearly less efficiently (Figure 2D). EMSAs performed with labeled LURP\textsuperscript{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 (Figure 2E). Labeled $LURP^A$-M3 produced a shift of similar size, but severely reduced intensity compared to the wild-type probe.

In summary, these data show that nuclear DNA-binding factors interact with $LURP^A$ in a sequence-specific manner which is mainly facilitated by the downstream halfsite $LURP^A$-3’HS. We observed a significant decrease in reporter activity between $CaBP22$ -590 (which contains 2 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 co-regulation 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 (Figure 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 1) 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* (*Pst*), SA, wounding, and virulent $Hp$Noco2. However, reporter expression was not induced by any of the other
stimuli tested (Jasmonic acid, Ethephon, Kinetin, ABA, CaCl$_2$, MgCl$_2$, NaCl, IAA, GA$_3$, 2,4D, 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 in 96-well plates were incubated for 24 hours with library compounds at final concentrations of 4-20uM followed by histochemical staining to visualize GUS expression (Figure 3A). Screening a total of 42,000 diverse organic compounds (see Materials & 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, 3,5-dichloroanthranilic acid (DCA; Figure 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 treatment) revealed that this elicitor is active at concentrations as low as 0.1uM, making it significantly more potent than SA, which did not trigger GUS expression at concentrations under 5 uM (Figure 3C). Quantitative dose-response curves after saturation treatments with DCA revealed a maximal effective concentration of approximately 15uM. Application of higher concentrations resulted in a sharp decline in GUS activity (Figure 3D). It’s EC50 (median effective concentration) was determined to be 6uM. To examine DCA-induced phytotoxicity we used trypan blue staining of seedlings after saturation treatment. Dark blue staining, indicating cell death, was prevalent in ~50% of the seedlings treated for 24 hours with 40uM DCA and in nearly 100% of the seedlings treated with DCA concentrations of 50uM or higher (Figure 3E). However, no cell death was observed at concentrations showing effective reporter activation (5 – 20uM),
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 (Figure 2C).

**DCA induces rapid and transient resistance to Hyaloperonospora parasitica**

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 – 500uM. We also pretreated 2 week old soil grown Col-0 seedlings by foliar spray with varying concentrations of DCA 24 hours prior to challenge with virulent HpNoco2. The extent of Hp spore formation was assayed seven days after pathogen challenge. Plants pretreated with DCA at concentrations as low as 10uM displayed a significant reduction of Hp spore numbers compared to mock pretreated plants (Figure 4B). Maximal effects with regard to CaBP22-333::GUS-induction and suppression of HpNoco2 spore formation were observed after spray-application of 100 uM DCA (Figures 4A & B). Spray-application of DCA to soil grown plants at this concentration did not cause any detectable amounts of cell death within 8 days (Supplemental Figure 1). Therefore for all further experiments DCA was applied by foliar spray to 2 week old soil grown seedlings at a concentration of 100 uM.

To examine the kinetics of DCA-induced HpNoco2 resistance compared to other defense elicitors (SA and INA), plants were pretreated with 100uM of each compound at specific times ranging from one hour to six days prior to pathogen challenge (Figure 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 pre-treatment with DCA or INA 24 hours prior to Hp infection induced full resistance while mock or 100uM 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 one hour after treatment. However, despite the structural relatedness of INA and DCA (see Figure 5A) INA-induced resistance was long-lasting, whereas DCA-induced resistance began to decline between three and six days after chemical treatment (Figure 4C). The surprisingly early defense induction triggered already 1h after DCA treatment coincided with a similarly fast induction of WRKY70 and CaBP22 expression (Supplemental Figure 2). These data clearly show that DCA is a potent elicitor of Hp-resistance and 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 pre-treated with 100uM DCA and INA 24 hours prior to dip inoculation with Pst showed no visible disease symptoms 5 dpi, while 100uM SA and mock-treated plants appeared highly diseased exhibiting extensive chlorosis and necrosis (not shown). Quantification of in-planta bacterial growth revealed that plants pre-treated with DCA showed the greatest reduction in bacterial growth, followed by INA and SA, respectively (Figure 4D). To determine if DCA possesses direct antibacterial properties, we monitored the growth of Pst in liquid medium containing 100mM of either 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 (Figure 4E). These data show that DCA like INA or SA induces resistance to Pst without exhibiting direct antibiotic activity.
**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 2). From these we selected 6 compounds that represent a range of activities while maintaining obvious structural similarities to DCA plus INA and SA for detailed analyses (Figure 5A). Dose-response curves were generated illustrating the activities of each compound in two different types of assays: (1) GUS activity triggered in CaBP22-333 seedlings using fluorometric MUG assays (Figure 5B); (2) inhibition of Hp spore development during a normally compatible plant/Hp interaction (Figure 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 (>7-fold) and nearly 100% inhibition of spore development at low uM 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 (< 2-fold) and were unable to mediate full Hp-defense at any of the tested concentrations. Both 3,5-dichlorobenzoic acid, and 3-chloroanthranilic acid mediated a moderate inhibition of spore development (50 – 75% at 100uM) and triggered a medium level of GUS activity similar to that induced by SA (~4-fold). Generally di-chlorinated 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 de-chlorinated derivatives were inactive. Removal of the amine group from DCA significantly reduced its biological activity. Additionally, DCA, 3,5-dicholorbenzoic acid, and 3-chloranthranilic acid were all more potent than SA, respectively. In summary the structure-activity analysis showed that no substitutions to 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 salicylic acid perception and is partially dependent on WRKY70 and NPR1**

To determine at what hierarchical level DCA interferes with defense signaling, we used reverse transcription-PCR (RT-PCR) to examine transcript levels of two LURPs (CaBP22 and WRKY70) after treatment with 1mM SA and 100uM DCA in Col-0 and several well characterized defense signaling mutant backgrounds (Figure 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) or 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 the DCA and SA-inducibility of LURP transcript accumulation was blocked. This outcome was not surprising as WRKY70 was shown previously to affect 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 HpNoco2 (Figure 6B). Col-0 and mutant plants were pretreated with DCA prior to spray-infection with HpNoco2. DCA induced strong Hp-resistance in Col-0 and nahG plants nearly fully suppressing the formation of HpNoco2 spores. DCA-induced resistance was significantly compromised in the wrky70 mutant, but not fully abolished. In contrast to the
results of the RT-PCR analysis, DCA-mediated resistance was weakly reduced in \textit{npr1} plants. The \textit{npr1-3} and \textit{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 \textit{npr1} on DCA-mediated \textit{HpNoco2} 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 \textit{HpNoco2} resistance mediated by INA is fully blocked in \textit{npr1} plants, while resistance mediated by DCA is somewhat reduced in this mutant (Figure 6C). In the \textit{npr1} background DCA suppressed \textit{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 (Figure 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 on 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 48h or 6d with mock solution, DCA or INA. We examined responses at these two timepoints, because both DCA and INA efficiently suppressed *Hp* spore formation between 24h and 72h post treatment, but substantially differed in their efficiency 6d post treatment. To further examine the role of NPR1 in DCA-mediated disease resistance we also analyzed responses at 48h post 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 cutoff a false discovery rate (FDR) < 0.05. The up- and down-regulated DEGs identified by this analysis are summarized in Figure 7A (see also Supplemental Tables 3-8). In Col-0 wild type plants 48h after the DCA treatment (48h DCA) 423 genes were upregulated and 61 genes were downregulated. Notably 6d after DCA treatment (6d DCA), which does not correlate with disease resistance, only 3 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 timepoints. In Col-0 48h after INA (48h INA) application a total of 482 genes were upregulated and 87 were downregulated, while six days after treatment with INA (6d INA), 379 genes were classified as upregulated and 97 genes as downregulated. The transcriptional changes in response to the 48h DCA and 48h INA treatments overlap. 58% of the 48h INA inducible genes were also upregulated by DCA at 48h in Col-0 and 31% of the 48h INA-suppressed genes were also downregulated 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 48h after DCA or INA treatments as well as 6d after INA treatment, but not 6d after DCA treatment (Figure 7C). This ACID (associated with chemically induced defense) cluster contains 137 genes that were coordinately upregulated and 5 genes that were coordinately downregulated by all three defense-inducing treatments (48hours after DCA or INA treatment as well as 6 days after INA treatment). Consistent with the partial suppression of DCA-mediated defense responses by NPR1, 20% of the 137 upregulated ACID genes exhibit NPR1-independent expression, while the remaining ones are expressed in an NPR1-dependent manner. All of the 5 coordinately downregulated genes are NPR1-dependent (Figure 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 over-represented GO terms in the set of 137 upregulated ACID members (Supplemental Table 9). 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 expression of \textit{LURP} genes. As \textit{CaBP22} represents the average response pattern of the \textit{LURP} cluster, we first dissected it's promoter and identified a \textit{Hp}-responsive region containing \textit{LURP}\textsuperscript{A}. This motif appears to be related to the defense associated TL1 element (CTGAAGAAGAA) (Wang et al., 2005) which contains the \textit{LURP}\textsuperscript{A}-core motif (\textit{LURP}\textsuperscript{A}-CM). Thus TL1/\textit{LURP}\textsuperscript{A}-type promoter elements may play a wider role in defense gene regulation.

\textit{GUS} reporter activity mediated by the \textit{CaBP22} \textsuperscript{-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 \textit{CaBP22} \textsuperscript{-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 \textit{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 EC\textsubscript{50} value for GUS expression in \textit{CaBP22} \textsuperscript{-333} seedlings after 24h DCA saturation treatment was found to be 6\textmu M which is ten-fold lower then the published value for \textit{PR1} induction by SA (65\textmu M) (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. 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 amongst 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 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 HpNoco2 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 48h 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 LURPs 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 (Figure 6D). We can not 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 amine 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 suggest that they should all be readily absorbed by cells. However, compared to 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. The 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 structure 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 compounds 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 towards the NPR1-independent ones.

Besides the ACID set, our microarray analyses defined a second interesting cluster, which comprises genes that are specifically upregulated by 48h DCA and but not by INA treatments (173 genes). GO analysis of this set show 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 upregulated by 48h INA (235) GO analysis does not show any enrichment for phosphorylation related mechanisms (Supplemental Table10). 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 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 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 plant protection compound (Ryals et al., 1996). Furthermore, the mutants cpr1-1 (constitutive expresser of PR1-1) and ssi-1 (suppressor of SA-insensitivity-1) 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 interferences 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 respond also 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 LRR containing proteins represented in this cluster. Conspicuously absent from the over-represented GO attributes for the ACID set were terms annotated for processes associated with upstream/early defense responses, like the hypersensitive response, cell death, peroxidases, response to reactive oxygen intermediates 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 upregulated by flg22 treatment (Zipfel et al., 2004) contain 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
everpresent in the ACID cluster. This implies that DCA activates a distinct
subset of pathogen inducible defense responses.

Several LURPs (originally defined as cluster II by (Eulgem et al., 2004) were
found to be upregulated by 48h 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
6d 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 upregulated 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 crops' inherent defense capabilities.
Materials and Methods

Plant growth conditions and pathogen infections

Arabidopsis thaliana plants were grown on soil under fluorescent lights (14 hours light, 10 hours dark, 21°C, 100 Einstein/m²/s) 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) plants have been described. Hyaloperonospora parasitica was grown and propagated as previously described (McDowell et al., 2000). Plants were spray-infected with Hp spore suspensions at 5x10⁴ spores/ml for HpEmoy2 and 3x10⁴ spores/ml for HpNoco2 with preval sprayers (Preval, http://www.prevalspraygun.com). Plants were scored 7 days post 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 (Pst DC3000) with an inoculum concentration of OD₆₀₀ =0.05. For these experiments infections and scoring were performed as previously described (Tornero and Dangl, 2001) Plants were visually scored for disease symptoms four to seven days (as indicated) after inoculation.

Reverse-transcription polymerase chain reaction (RT-PCR)

RNA was isolated from seedlings using TRIZOL (Invitrogen). cDNAs were prepared as previously described (Knoth and Eulgem, 2008). In each case 1ul of cDNA was used for all PCR reactions (20ul total volumes). A 425 bp fragment of Actin8 (Act8) was amplified as a loading control using primers RT-ACT8-F: 5’-ATGAAGATTAAGGTCGTGGCAC-3’ and RT-ACT8-R: 5’-GTTTTTATCCGAGTTTGAAGGCG-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’-CAGGAACCATCAATTTCACTGAGT and RT-CaBP22-RP 5’-CAGAATGCGACCAGTTGTGTCAT with an annealing temperature of 62°C for
24 cycles. The 477 bp fragment of \textit{WRKY70} was amplified using the primer pair RT-WKY70-FP 5’- AACGACGCAAGTTTGAAGATTC and RT-WKY70-RP 5’-TTCTGGCCACACCAATGACAAGT with an annealing temperature of 63°C for 24 cycles. The 338 bp fragment of \textit{PR1} was amplified using the primer pair RT-PR1-FP 5’-GCCCAACAAGATTATCTAAGGG and RT-PR1-RP 5’-ACCTCCTGCATATGATGCTCCT with an annealing temperature of 53°C for 27 cycles. All PCR reactions used the following thermal program deviating as indicated for annealing temperatures (AT) and cycles: 94°C for 1 min; X cycles of 95°C for 30 sec, AT of X°C for 1 min, and 72°C for 40 sec. PCR products were electrophoresed on 1.6% agarose gels containing 0.5ug/ml ethidium bromide. Negative controls omitting reverse transcriptase in the cDNA production process and PCR reactions without cDNA yielded no products.

\textbf{Generation of transgenic Arabidopsis plants}

\textit{CaBP22-promoter::GUS} translational fusion constructs were cloned from PCR products as previously described (Knoth and Eulgem, 2008). PCR products were generated using Col-0 genomic DNA as a template. All \textit{CaBP22-promoter::GUS} constructs were designed with a HindIII restriction site at their 5’ end and BamHI restriction site at their 3’ end to allow directional restriction enzyme/ligation-mediated cloning into the pBI 101.1 vector (Clontech; Mountain View, CA). The sequences of the primers were (numbers indicate the size of fragment (base pairs) from the transcriptional start site):

\begin{itemize}
  \item \textit{CaBP22}, 1075-FP, 5’-AATTAAGCCTTTGAGTCAGGAACATGAAGTGG
  \item \textit{CaBP22}, 590-FP, 5’-AATTAAGCCTTTGAGTCAGGAACATGAAGTGG
  \item \textit{CaBP22}, 333-FP, 5’-AATTAAGCCTTTGAGTCAGGAACATGAAGTGG
  \item \textit{CaBP22}, 65-FP, 5’-AATTAAGCCTTTGAGTCAGGAACATGAAGTGG
  \item \textit{CaBP22}-RP, 5’-AATTAAGCCTTTGAGTCAGGAACATGAAGTGG
\end{itemize}

Plasmids were transformed by electroporation into \textit{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 presence of the transgene on 0.5MS/0.8% agar media containing 25mg/L Kanamycin.

Analysis of GUS Activity
GUS histochemical staining was performed using whole seedlings stained in a 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc) solution containing 1mg/ml X-gluc, 50mM Na$_2$PO$_4$ pH 7.2, 0.5mM K$_3$Fe(CN)$_6$, 0.5mM K$_4$Fe(CN)$_6$ at 37°C and cleared with 70% EtOH. Soil grown seedlings were incubated at 37°C for 5 hours. One week old seedlings grown in liquid media were incubated at 37°C for 18 hours. Fluorometric analyses of GUS activities were performed on ten-day-old soil grown seedlings as previously described (Knoth and Eulgem, 2008). Fold changes were calculated for each replicate experiment then averaged for final fold change.

Electrophoretic mobility shift assays with nuclear proteins
Electrophoretic mobility shift assays (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 (mutated sequences indicated by lower case lettering):

- **LURP$^A$-WT** 5’-ATTGTTTTCTTCTGTAgaAGACCAT
- **LURP$^A$-M1** 5’-ATTGTTTgggTgTGAaGACCAT
- **LURP$^A$-M2** 5’-ATTGTTTgggTgTGTAcggcACCAT
- **LURP$^A$-M3** 5’-ATTGTTTTCTTCTGTAcggcACCAT
- **ZAT7** 5’-AAAATCTAGAAGACCGGCTTTAAAAAT
- **WAK1** 5’-GAAAAGACGAgaAGAACCgAGACCTA.

Nuclear proteins were extracted as previously described (Desveaux et al., 2004). Bradford protein assays were used to determine protein concentrations (Bio-Rad
laboratories, Inc). EMSAs were preformed as previously described (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 AFGC’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 200ul liquid 0.5 MS media in 96-well plates (Costar) for 7 days on an orbital shaker under long day conditions (16 hours light, 8 hours dark, 22°C, 100 Einstein/m$^2$/s). After 7 days, the liquid 0.5 MS media volume was returned to 200ul and 0.2ul of each compound was administered by robotic pin tool (Biomek FX Laboratory Automation Workstation) to each well for a final concentration of 4-20uM in 0.001% DMSO. Plates were returned to the orbital shaker for 24 hours and then stained (histochemical) for GUS expression. 42,000 compounds were screened in duplicate. The libraries used were: Microsource Spectrum containing 2000 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, low). The EC50 (effective concentration leading to a half-maximal response) of a compound is 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 eminent runoff. Final DMSO concentrations never exceeded 0.002%. Mock treatments were 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 Affymetrix Arabidopsis ATH1 genome array GeneChip following manufacturer’s instructions (Affymetrix) by the University of California, 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 (R Development Core Team, 2008, Gentleman et al, 2005). Raw expression values were normalized using the robust multichip averaging (RMA) algorithm. Analysis of differentially expressed genes (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 FDRs (Benjamini et al., 2001). As confidence threshold an adjusted p-value of ≤ 0.05 was chosen (compared to mock treatments with water). To visualize the DEG sets, hierarchical clustering was performed using the Cluster and TreeView programs (Eisen et al., 1998). Over-represented GO terms were identified with the FuncAssociate program from (Berriz et al., 2003). The Microarray data have...
been deposited in MIAME compliant format to the GEO database under the accession number GSE13833.
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FIGURE LEGENDS

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 HpEmoy2 (7 dpi with 5 x 10^4 spores/ml) or water (7 dpi). Each well contains 5-10 seedlings from independent transformation events. Shown are representative examples from 8 independent transformation events per construct.

(C) Close-up views of GUS stained CaBP22-promoter::GUS constructs 7 dpi with HpEmoy2. Ten independent lines per construct were analyzed. Shown are examples representing the typical behavior for each construct for at least 5 independent experiments.

(D) Fluorometric analysis of 10-day-old T2 transgenic CaBP22-promoter::GUS Arabidopsis seedlings. Light grey bars represent mock treatment (4 dpi). Dark grey bars represent HpEmoy2 treatment (4 dpi with 5 x 10^4 spores/ml). Measurements were taken for 8 independent lines for each construct. Shown are combined averages from all 8 measurements from 3 independent experiments.

(E) Graphic representation of the fold induction for each deletion construct. Mann-Whitney U with a cut off of p<0.05 was used to test for significant differences. The error bars in panels (d) and (e) represent the standard error of the mean.
Figure 2. Nuclear factors interact in EMSAs with a conserved motif in the CaBP22 promoter.

(A) Sequences of labeled probes and unlabelled competitors representing an inverted repeat region of conserved motif found in Hp-responsive regions of CaBP22 promoter (LURPA). The conserved core units of the LURPA inverted repeats are underlined (LURPA-HS). Mutations are indicated by lowercase lettering.

(B) Nuclear extracts (NE) used in Electrophoretic mobility shift assays (EMSAs). DCA signifies treatment with the defense inducer 3,5-dichloroanthranilic acid.

(C-F) EMSAs reveal sequence specific shifts with nuclear extracts. The arrow indicates specific shifts; open circles indicate free probe. EMSAs were performed with 15ug of nuclear protein per lane. One-hundred fold excess of indicated unlabeled probe was used as unlabelled competitor (comp). Shown are results typical for at least 3 experiments.
Figure 3. Chemical genomics screen reveals a new elicitor of defense specific reporter expression.

(A) Scheme illustrating screening procedure. Example of 96-well screening plate containing 7 day-old liquid grown CaBP22<sup>333</sup> seedlings after a 24 hour incubation with library compounds at concentrations ranging from 4-20μM (top). Screening plate after GUS histochemical staining (bottom); blue circle indicates “hit well”.

(B) Structure of 3,5-dichloroanthranilic acid (DCA).

(C) GUS histochemical staining of CaBP22<sup>333</sup> seedlings comparing reporter response to Salicylic acid (SA) and DCA after 24 hour incubation treatment at indicated concentrations.

(D) Dose response curve plotted from GUS fluorometric assays measuring DCA induced GUS activities at indicated concentrations (μM) with proteins extracted from 7 day-old liquid grown CaBP22<sup>333</sup> seedlings after 24 hour incubation with DCA treated media. Mean and standard errors calculated from 3 independent experiments.

(E) Trypan blue staining of Col-0 wild type seedlings incubated for 24 hours in DCA containing media the indicated concentrations. Dark blue color indicates cell death (toxicity). All staining experiments were performed at least 3 times with similar results.
Figure 4. DCA induces disease resistance.

(A-D) All experiments were conducted with 14 day old soil grown CaBP22-333 or Col-0 seedlings. Means and standard errors were calculated from 3 independent trials.

(A) GUS histochemical staining of CaBP22-333 seedlings 24 hours after chemical spray treatment with indicated concentrations of DCA or SA.

(B) Col-0 seedlings were sprayed with DCA and SA at the indicated concentration 24 hours prior to spray-infection with virulent HpNoco2 (3x10^4 spores/ml). Spores were counted 7dpi.

(C) Kinetic analysis of chemically induced disease resistance; Col-0 seedlings were sprayed with 100uM of each chemical at the indicated times prior to HpNoco2 (3x10^4 spores/ml) spray-infection. Spores were counted 7dpi.

(D) Quantification of Pst DC3000 growth by colony forming units (cfu). Col-0 seedlings were pre-treated with 100uM of indicated chemical 24h prior to dip-inoculation with virulent Pst DC3000. Bacteria were extracted at day 0 (grey bars) or day 3 (black bars). Significant differences were tested using the Mann-Whitney U statistical test (p<0.05).

(E) Pst DC3000 grown in liquid culture with 100uM of the indicated chemicals or 100ug/ml hygromycin (HYG). The OD600 which represents the density of bacteria was measured at indicated times (hours) after inoculation. Standard errors were all less than 0.05 so are not visible on the graph.
**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 hours after spray treatment with compounds at the indicated concentrations (uM). The means and standard errors were calculated from 3 independent replicates.

(C) \(HpNoc\)o2 growth inhibition assay. Two week old soil grown Col-0 seedlings were spray infected with \(HpNoc\)o2 48 hours after treatment with varying concentrations (uM) of each DCA analog and then assayed 7dpi for disease symptoms (spores). 100% inhibition = 0 spores. The assay was repeated three times with similar results.
Figure 6. Analysis of DCA activity in known defense mutants.

(A) RT-PCR analysis of \textit{CaBP22} and \textit{WRKY70} expression in Col-0 (wild type) and mutant or \textit{nahG} backgrounds 24 hours after spraying 2 week old seedlings with water (mock), 1mM SA, or 100uM DCA. \textit{ACT8} is shown as loading control. At least three biological replicates showed consistent results.

(B-C) Two week old seedlings were sprayed with virulent HpNoco2 24 hours after pre-treatment with water, 100um DCA or 100um INA as indicated. Spores were counted 7 days after spray infection. Means and standard errors 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 grey bars indicate mock pretreatment, light grey indicates 100uM DCA and checkered bars indicate 100uM 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 \textit{WRKY70}-dependent branch of the defense signaling network. Additional DCA-triggered pathways may involve NPR1 and \textit{WRKY70} paralogs or defense regulators unrelated to them.
Figure 7. Gene sets differentially regulated after elicitor treatments.

(A) Non-proportional venn diagrams depicting the overlap of gene sets up-regulated (left) or down-regulated (right) 48h after DCA or INA treatment or 6d after DCA or INA treatment. Not shown in the diagrams are the overlaps between 48h DCA and 6d INA (3 upregulated; 0 downregulated) and between 48h INA and 6d DCA (0 upregulated; 0 downregulated).

(B) Venn diagrams showing the overlap of gene sets up-regulated (left) or down-regulated (right) 48h after DCA or INA or 6d after INA treatments in Col-0 as well as 48h DCA in npr1 mutant background. The number next to the treatment indicates the total genes in this set. Significance cutoff (false discovery rate) is P-value < 0.05. Not shown in the diagrams are the overlaps between 48h DCA and 6d INA (3 upregulated; 0 downregulated) and between 48h INA and npr1 48h DCA (0 upregulated; 0 downregulated).

(C) Hierarchical clustergram of 142 DEGs coordinately up- or down-regulated 48h after DCA or INA treatments or 6d after INA treatment, but not 6d after DCA treatment (p-value ≤ 0.05). Displayed are ratios of transcript levels triggered by the individual chemical treatments compared to the respective mock treatments; magenta: up-regulated relative to control; blue: down-regulated relative to control; the brightest color indicates a >8-fold differential expression.
Legends of supplemental figures:

**Supplemental Figure 1:** Trypan blue staining of 2 week-old soil-grown Col-0 wild type seedlings 8 days after spray application of mock solution (water) 100μM DCA, 500μM DCA or 2mM SA. Dark blue color indicates cell death (toxicity). Shown are leaf sections representing typical examples from two independent experiments. Cell death was not observed after treatment with 100 μM or 500μM DCA, while ~ 50% of the seedlings exposed to 2mM SA showed cell death in at least some parts of their tissue.

**Supplemental Figure 2:** DCA induces rapid accumulation of *WRKY70* and *CaBP22* transcript levels. RT-PCR analysis of *CaBP22* and *WRKY70* expression in Col-0 (wild type) at various timepoints after spraying 2 week old seedlings with 100μM DCA. *ACT8* is shown as loading control.
A

Water treatment

CaBP22 -1075
CaBP22 -590
CaBP22 -333
CaBP22 -65

B

Water treatment

HpEmoy2 7dpi

CaBP22 -1075
CaBP22 -590
CaBP22 -333
CaBP22 -65

C

D

GUS activity (μM 4-MU/mg protein/min)

E

Fold change

0 1 2 3 4 5 6 7 8 9

CaBP22 -1075
CaBP22 -590
CaBP22 -333
CaBP22 -65

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A Incubate with compounds 24 hours

GUS histochemical staining

B 3,5-dichloroanthranilic acid (DCA)

C SA

DCA

D EC50=6 uM

GUS activity

0.1 uM 1 uM 5 uM 10 uM 20 uM 30 uM 40 uM 50 uM 100 uM 500 uM 1 mM

GUS activity (uM 4-MU/mg protein/min)

0 10 20 30 40 50 60 70 80 90 100

0um 5um 10um 20um 30um 40um 50um 100um 1M SA
A

0µM 10µM 50µM 100µM 500µM 1mM 10µM SA 1mM

B

OD600

C

Mock SA INA DCA

D

Log(cfu/mg fw)

Mock SA INA DCA

E

OD600

hours post inoculation

0 4 8 12 16 24

Mock SA INA DCA HYG
3,5-Dichloroanthranilic acid (DCA)
2,6-Dichloroisonicotinic acid (INA)
5-Chloroanthranilic acid (5-CAA)
3-Chlorobenzoic acid (3-CBA)

3,5-Dichlorobenzoic acid (3,5-DCBA)
3-Chloroanthranilic acid (3-CAA)
Salicylic acid (SA)
Anthranilic acid (AA)
Benzoic acid (BA)

B

GUS activity (μM 4-mu/mg protein/min)

C

Percent inhibition of spore development

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