

Transcriptomics and Functional Genomics of ROS-Induced Cell Death Regulation by *RADICAL-INDUCED CELL DEATH1*

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Abstract

Plant responses to changes in environmental conditions are mediated by a network of signaling events leading to downstream responses, including changes in gene expression and activation of cell death programs. *Arabidopsis thaliana* RADICAL-INDUCED CELL DEATH1 (RCD1) has been proposed to regulate plant stress responses by protein-protein interactions with transcription factors. Furthermore, the *rcd1* mutant has defective control of cell death in response to apoplastic reactive oxygen species (ROS). Combining transcriptomic and functional genomics approaches we first used microarray analysis in a time series to study changes in gene expression after apoplastic ROS treatment in *rcd1*. To identify a core set of cell death regulated genes, RCD1-regulated genes were clustered together with other array experiments from plants undergoing cell death or treated with various pathogens, plant hormones or other chemicals. Subsequently, selected *rcd1* double mutants were constructed to further define the genetic requirements for the execution of apoplastic ROS induced cell death. Through the genetic analysis we identified WRKY70 and SGT1b as cell death regulators functioning downstream of RCD1 and show that quantitative rather than qualitative differences in gene expression related to cell death appeared to better explain the outcome. Allocation of plant energy to defenses diverts resources from growth. Recently, a plant response termed stress-induced morphogenic response (SIMR) was proposed to regulate the balance between defense and growth. Using a *rcd1* double mutant collection we show that SIMR is mostly independent of the classical plant defense signaling pathways and that the redox balance is involved in development of SIMR.

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Introduction

Plants live in a world of change - fluctuating light, temperature, water availability and pathogen attack are among the conditions that require an appropriate response from the plant. Stress responses are energetically costly [1–3], hence environmental and growth signals must be integrated and balanced. Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, which can be generated in different subcellular compartments and fulfill signaling roles during both abiotic/biotic stress and development are among these key signals in plants [4–6]. While regulation of ROS production is to some extent understood, the mechanisms of ROS perception and downstream signaling are mostly unknown [7].

Activation of programmed cell death (PCD) is one of the aspects of plant defense responses where ROS play a crucial role [8–10]. The understanding of plant cell death execution lags behind that of mammals; however, some key differences have been identified. Apoptosis does not exist in plants; based on morphological criteria plant PCD has been categorized as vacuolar cell death and necrosis, plus several other types that are not easily categorized [11]. Most studies on PCD in the model plant *Arabidopsis thaliana* have been in the context of immune responses where a localized rapid cell death program termed the hypersensitive response (HR) is a feature of resistance [8,12]. The air pollutant ozone (O₃), when applied in short and high pulses, mimics and activates the plant's own production of an apoplastic ROS burst, similar to that seen in

Author Summary

Reactive oxygen species (ROS) are utilized in plants as signaling molecules to regulate development, stress responses and cell death. One extreme form of defense uses programmed cell death (PCD) in a scorched earth strategy to deliberately kill off cells invaded by a pathogen. Compared to animals, the regulation of plant PCD remains largely uncharacterized, particularly with regard to how ROS regulate changes in gene expression leading to PCD. Using comparative transcriptome analysis of mutants deficient in PCD regulation and publicly available cell death microarray data, we show that quantitative rather than qualitative differences in cell death gene expression appear to better explain the cell death response. In a genetic analysis with double mutants we also found the transcription factor WRKY70 and a component of ubiquitin mediated protein degradation, SGT1b, to be involved in regulation of ROS induced PCD.

immune responses [13]. Hence, O₃ can be used as a tool to study the role of ROS during defense and PCD, where the PCD elicited by O₃ shares many similarities with HR [10]. However, many of the regulatory steps governing plant PCD in different contexts remain to be elucidated.

Massive changes in gene expression are observed during plant defense responses, after application of ROS treatments, and during cell death [14–19]. The requirement of changes in gene expression for cell death execution is illustrated by the reduction of O₃ activated cell death by application of α -amanitin, a transcriptional inhibitor [10]. Furthermore, several lesion mimic mutants, which display spontaneous HR-like cell death in absence of pathogens [20], or mutants with altered pathogen tolerance have been used in various experiments including suppressor mutant screens and protein interaction studies to identify more regulators of cell death. Some of these regulators are involved in epigenetics, transcription or mRNA processing and include the transcription factors MYB30 [21], bZIP10 [22], SR1 [23], WRKY70 [24], mRNA processing proteins MOS2 and MOS4 [25], and chromatin remodeling factors to fine tune the transcriptional status of chromatin (*LAZ2*, encoding the histone methyltransferase SDG8; [26]). Although numerous abiotic/biotic stress microarray studies have been performed, relatively few experiments available in the public domain directly address the transcriptomics of cell death [27]. Further, the variety of cell death forms, and triggers used to initiate them complicate experimental design and hinder identification of cell death gene expression signatures that would not also simultaneously contain genes regulated during plant defense. Indeed cell death and defense are intimately linked, perhaps even inseparable. Clearly there is need for further analysis of gene expression during cell death in diverse experimental systems to identify potential core regulators of cell death execution.

The O₃ sensitive Arabidopsis mutant *radical-induced cell death1* (*rdl1*) is one of the mutants with defects in PCD control; it develops cell death lesions in response to a normally sub-lethal dose of apoplastic superoxide, but not H₂O₂ [28,29]. RCD1 belongs to a plant specific SRO (SIMILAR TO RCD-ONE) gene family with five other genes (SRO1-SRO5) [30–32], some of which are also involved in stress signaling [33]. RCD1 and its closest homologue SRO1 possess a nuclear localization signal and a WWE domain, which is predicted (but not experimentally shown) to be involved in protein-protein interactions [34]. RCD1 and SRO1 display unequal genetic redundancy: whereas the *rdl1* mutant has pleiotropic phenotypes in development and stress responses, the

rdl1 mutant has only subtle phenotypes. However, the *rdl1 sro1* double mutant has severe developmental phenotypes and requires rescue on sugar containing media to generate viable plants [31,35,36]. RCD1 has been shown to interact with 21 transcription factors via a novel C-terminal RST (RCD1-SRO-TAF4) domain, and the known target genes of these interaction partners (such as DREB2A, DEHYDRATION-RESPONSIVE ELEMENT BINDING2A) have altered expression in *rdl1* [31]. These results suggest that RCD1 may regulate PCD at the level of transcription.

The balancing act of regulating growth while maintaining an appropriate level of defenses includes a response termed stress-induced morphogenic response (SIMR) [14,36–39]. SIMR includes inhibition of shoot elongation and stimulation of auxiliary branching [37,38]. SIMR is thought to be an adaptive response to stress and is regulated by a complex interplay between ROS, auxin, ethylene and antioxidants [14,37,40]. The morphological phenotypes of *rdl1* indicate that it could be classified as a mutant with constitutively heightened SIMR response [14,36,38]. A screen for modulators of defense responses identified *rdl1* as an enhancer of the growth inhibition caused by constitutive activation of defense responses in the mutant *sncl* (*suppressor of npr1, constitutive1*), giving further support for a role of RCD1 in balancing between growth and defense [41]. A deeper understanding of SIMR could offer new breeding target(s) for plants with increased tolerance to abiotic/biotic stresses without accompanying growth defects.

Apoplastic ROS, in the form of O₃, alter the expression of thousands of genes assigned to biotic and abiotic stress responses [14,42]; however there are only a few studies comparing both O₃ sensitive and tolerant genotypes at gene expression level which is required to dissect signaling pathways involved in PCD regulation [43]. To gain deeper mechanistic understanding of this process we used the *rdl1* mutant to perform: (1) microarray analysis of an O₃ time course in comparison to Col-0 to explore the role of RCD1 in defense and PCD signaling; (2) analysis of genes differentially regulated in *rdl1* gene expression data using public array experiments to find genes regulated during the PCD process; (3) detailed quantitative real time PCR (qPCR) analysis to find marker genes for PCD; (4) a screen of *rdl1* double mutants to find regulators of apoplastic ROS induced PCD and SIMR. We identify through the genetic analysis WRKY70 and SGT1b as regulators of cell death in *rdl1* and show that quantitative rather than qualitative differences in cell death gene expression appear to better explain outcomes in cell death.

Results and Discussion

Gene expression in Col-0 and O₃ sensitive *rdl1-1*

The O₃-induced transcriptional response in *rdl1* was studied in order to define cell death signaling events and to address the potential role of RCD1 as a transcriptional co-regulator. Samples of *rdl1* and Col-0 were collected 0, 1, 2, 4, 8 and 24 hours after the onset of O₃ exposure (6 h 350 nL L⁻¹). All hybridizations were performed with two-color oligonucleotide microarrays against a common reference RNA, facilitating multidirectional comparisons between the genotypes and treatments. Data were analyzed with linear mixed models and genes having two-fold or higher change of expression (log₂ ratio ± 1 , $q < 0.05$) in at least one time point were regarded as differentially regulated in each comparison (Figure 1A). In *rdl1*, 4102 O₃-responsive transcripts were identified over the experimental time course (Figure 1A), slightly more than in the O₃-tolerant Col-0 control (3635 transcripts, [14]). In total expression of 475 genes differed between the genotypes; 274 in

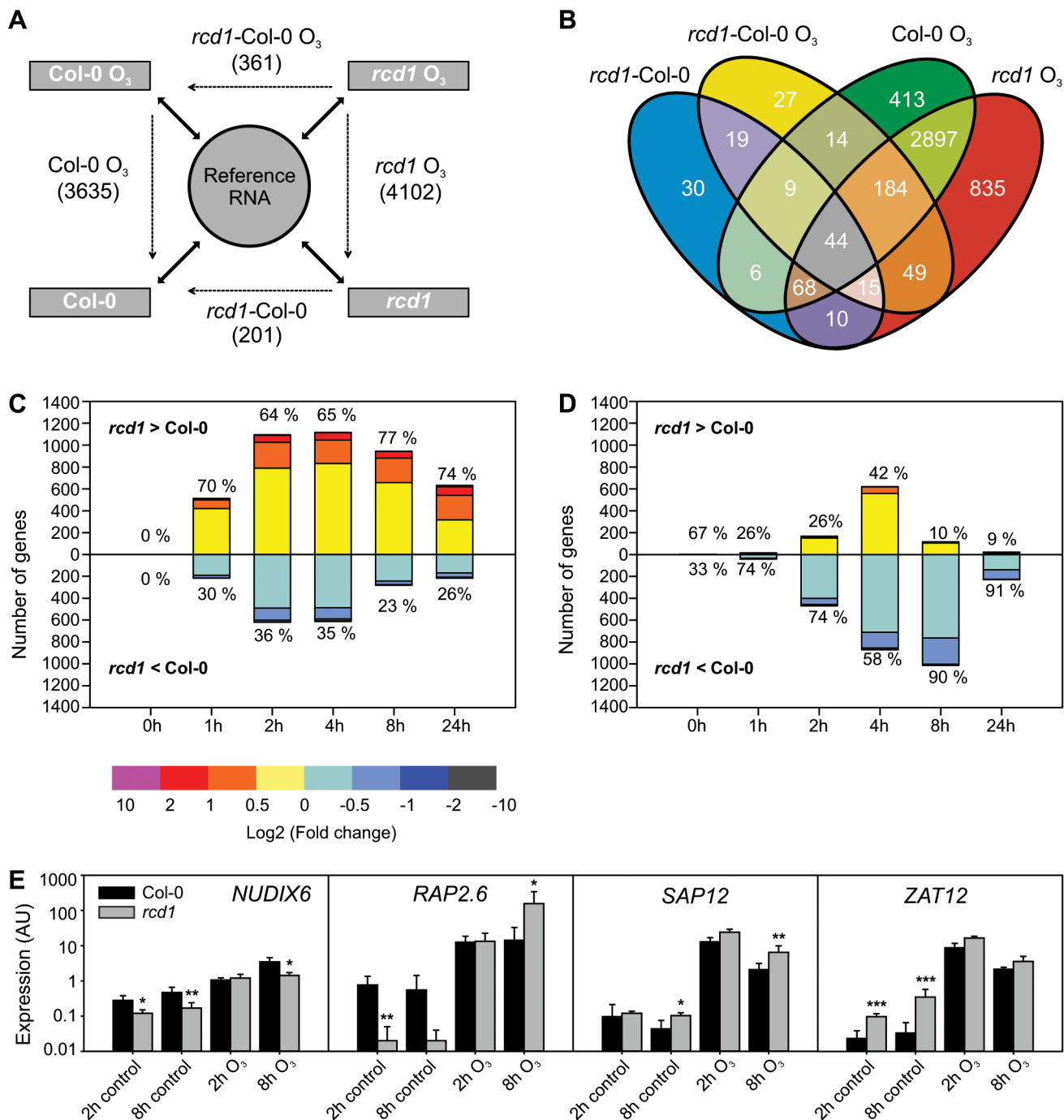


Figure 1. Gene expression of Col-0 and *rcd1* mutant in clean air and O₃-treated plants. A) Experimental design with each sample hybridized against a reference RNA (solid arrows) enables multidirectional comparisons between genotypes and treatments with a linear mixed model (dotted arrows). The experiment described was repeated at each time point (0, 1, 2, 4, 8 and 24 h) and lists of differentially expressed transcripts (\log_2 ratio ± 1 , $q < 0.05$) were made from each comparison. Number of differentially expressed unique genes at all time points is shown. The 0 h comparison of O₃-treated *rcd1* and O₃-treated Col-0 was analyzed together with *rcd1* and Col-0 grown in clean air to comprise the list of genes differentially regulated in *rcd1* in normal growth conditions (*rcd1*-Col-0). Simultaneously, this 0 h comparison was omitted from the *rcd1*-Col-0 O₃ data set. B) Venn diagram showing the overlap of gene lists. Transcripts at least two-fold differentially regulated between *rcd1* and Col-0 in response to apoplastic ROS are divided into several subcategories discussed in the results. C, D) Transcript levels of 4544 genes responsive to O₃-treatment (\log_2 ratio ± 1 , $q < 0.05$) in one or both of the genotypes were compared. Genes were divided into O₃-induced (C) and O₃-repressed (D) according to the O₃-response specifically at each time point (0, 1, 2, 4, 8 and 24 h). For each gene the difference between O₃-treated *rcd1* and Col-0 was calculated (\log_2 ratio) and the number of genes in each range of differential expression (depicted in color) is shown on the y-axis. The percentage of genes with higher (*rcd1*>Col-0) and lower (*rcd1*<Col-0) expression in *rcd1* compared to Col-0 was calculated. E) Expression of selected marker genes was studied in Col-0 and *rcd1* plants with qPCR. Bars represent means of three biological repeats, error bars show standard deviation. Statistically significant difference between genotypes is depicted with asterisk (P<0.05;*; P<0.01;** and P<0.001***). doi:10.1371/journal.pgen.1004112.g001

response to O₃ and 114 genes in clean air and 87 under both conditions (Figure 1B). Comparison of the O₃ responses of Col-0 and *rcd1* revealed remarkable similarity; most O₃-responsive transcripts (2897) overlapped between the genotypes (Figure 1B). Transcript levels of altogether 4544 genes responsive to O₃-treatment in one or both of the genotypes (Figure 1B) were directly compared to study the quantitative difference in the ROS response between the genotypes. First, lists of O₃-induced and -repressed genes were created for each time point and genotype separately. At all time points, there were more O₃-regulated genes in *rcd1* than in Col-0, and both genotypes possessed uniquely regulated genes, but no transcripts were oppositely regulated by apoplastic ROS in Col-0 and *rcd1* backgrounds, i.e., increased in one genotype and decreased in the other at the same time point. Therefore, the genotype-specific lists of regulated genes were combined and the differences in absolute levels between O₃-treated *rcd1* and O₃-treated Col-0 were calculated. The magnitude of O₃-induced changes was greater in *rcd1* than in Col-0 at all O₃ time points for both induced (64–77% of the O₃-induced genes) and repressed (58–91% of the O₃-repressed genes) transcripts (Figure 1C and 1D, respectively). Therefore, O₃-regulated genes had a more pronounced expression change in *rcd1*, suggesting that the O₃ response is quantitatively heightened in *rcd1*. However, most of the differences between the genotypes were subtle, less than two-fold (Figure 1C and 1D). Four genes were quantified in more detail with qPCR, and the results from this analysis were in agreement with the array data (Figure 1E).

Expression of RCD1 and interacting transcription factors

At the whole tissue level, *RCD1* transcript levels are slightly responsive to many stresses including O₃ (Figure S1, [30,32]) and show strong induction only in response to high light [44]. To test for local cellular responses in ROS-induced lesions, activity of the *RCD1* promoter was monitored by β-glucuronidase (GUS) staining in *RCD1*-promoter::*uidA* fusion lines exposed to high O₃-concentrations that induced lesions, or treated with the herbicide methyl viologen (MV; paraquat) which induces chloroplastic ROS production. The *RCD1* promoter was active specifically in the cells inside O₃-induced cell death lesions and also in the cells directly treated with MV (Figure S2). As a comparison, lines expressing *uidA* under control of the promoter from *SRO1*, the paralog most similar to *RCD1*, were also monitored. Although *SRO1* and *RCD1* share similar developmental expression [31], unlike *RCD1*, *SRO1* expression was not increased in O₃-induced lesions or in response to MV treatment (Figure S1). Another member of this gene family, *SRO5*, regulates salt stress responses [33] and is induced by O₃ [32]. The *sro1* and *sro5* mutants did not display increased O₃ sensitivity and the *rcd1 sro5* double mutant had unaltered responses compared to *rcd1* (Figure S2). The lack of stress regulation of the *SRO1* gene or O₃ stress phenotypes in the *sro1* and *sro5* mutants suggests that RCD1 regulates ROS responses and cell death independent of *SRO1* and *SRO5*.

RCD1 interacts with several transcription factors (TFs) and may regulate transcription via protein-protein interactions [31,45]. Many RCD1 interacting TFs have no established biological functions. Co-expression analysis has been used to suggest the function of previously uncharacterized proteins [46]. Expression profiles of 15 RCD1 interacting TF genes and *RCD1* itself were studied in data sets comprising hormone-, abiotic stress-, biotic stress- and O₃-treatments (Figure S3; see below for a full discussion of data sets used). *DREB2A*, *ANAC013* and *ANAC046* were the only TFs with major expression changes in response to diverse stresses. *ANAC013* is localized to both cytosol and nucleus [47] and is a possible membrane anchored protein that after proteolytic

cleavage would move to the nucleus [48]. None of the TFs had altered expression in the *rcd1* mutant under O₃, indicating that RCD1 does not transcriptionally regulate genes encoding its interaction partners. Overall, the *RCD1* expression profile was not similar to that of its interaction partners, thus other types of data, for example *in vivo* protein stability or double mutants will be required to position RCD1 in stress signaling pathways.

Clustering of RCD1 regulated genes

To gain further information on processes downstream of RCD1, the expression profiles of 423 RCD1 regulated genes were clustered together with publicly available data from experiments performed on the Affymetrix ATH1 chip (Figure 2). These experiments were selected to distinguish between genes regulated by abiotic and biotic stresses, stress hormones, ROS and cell death (see Materials and Methods for the complete set of experiments). Several constitutive defense mutants (*siz1*, *smi1* [suppressor of *npr1-1*, inducible1], *lht1* [lysine histidine transporter1], *cs26* [cysteine synthase26]) clustered together with the salicylic acid (SA) analog benzothiadiazole (BTH) treatment (Figure 2). The strongest change in gene expression was in cell death associated treatments including *Pseudomonas syringae* pv. *maculicola* (Psm) ES4326 infection [49], the *acd11* (accelerated cell death11) lesion mimic mutant [26], and ROS challenged *rcd1* at 8 and 24 h (Figure 2). These late O₃ time points exhibited the largest differences between O₃-treated *rcd1* and Col-0, whereas early O₃ time points of both genotypes clustered together with H₂O₂ and flagellin 22 (flg22) treatments (Figure 2). The *rcd1* mutant in clean air had a unique gene expression profile, as discussed in detail below.

RCD1-regulated genes clustered into six different major groups, two of which further divided into subclusters. These were further analyzed for enrichment of gene ontology (GO) classes and promoter elements (Figure 2, see Table S1 for genes belonging to each cluster and statistical results). Clusters Ia and Ib contained genes with reduced transcript accumulation in most experiments analyzed. Cluster Ib was enriched for genes encoding 17 chloroplast located proteins and 5 apoplast proteins (Table S1). Photosynthesis and chloroplast related genes have reduced expression during biotic stress as a defense strategy, likely for energy conservation [50]. However, photosynthesis as a biological process was not enriched in cluster Ib (Table S1).

The heterogeneous cluster II included a few genes with increased expression in *rcd1* clean air samples, increased expression in light grown COP9 signalosome mutants (*csn3*, *csn4*, *csn5*; [51]) and a late time point after treatment with the proteasome inhibitor Syringolin [52] (Figure 2). The COP9 signalosome regulates the activity and stability of cullin-RING-type E3 ubiquitin ligases (CRL), and Arabidopsis *csn* mutants arrest growth at the seedling stage, possibly through a DNA damage pathway [51]. Through most other clusters (I, IIIb–VI), expression profiles in *csn* mutants were very similar to *acd11*, suggesting that they may undergo cell death during seedling growth arrest. However, in cluster II several genes had increased expression in *csn* mutants and in clean air *rcd1*, but were not regulated by other stresses. E3 ligases are involved in targeting specific proteins for degradation by the proteasome, similarly one proposed function of RCD1 is to regulate stability of transcription factors [31]. The specific targets of COP9 and CRLs in Arabidopsis are mostly unknown, but more detailed characterization of genes regulated by both COP9 and RCD1 might reveal new insights into the role of protein degradation in stress responses.

Genes in cluster IIIa had a trend towards increased expression in nearly all treatments studied and included ethylene biosynthesis

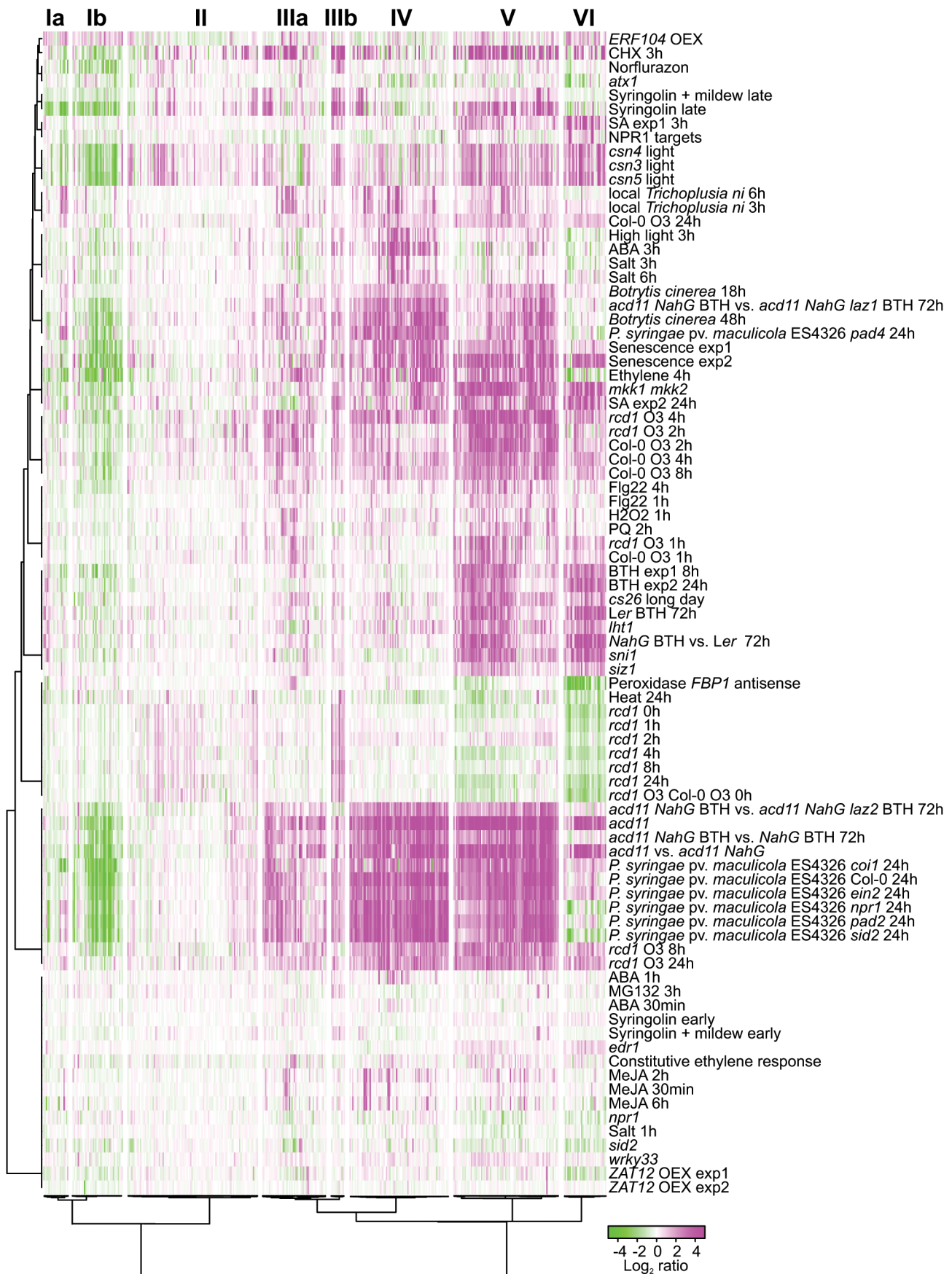


Figure 2. Cluster analysis of genes differentially regulated in *rcd1* compared to Col-0. Bootstrapped Bayesian hierarchical clustering of 423 genes with at least two-fold changed expression (\log_2 ratio ± 1 , $q < 0.05$) in clean air or O_3 -treated *rcd1* is shown. Data sets used were *rcd1* mutant grown in control conditions, O_3 -treated Col-0, O_3 -treated *rcd1* and several other available experiments related to stress signaling and cell death (see “Materials and Methods” for the complete set of experiments). Six main clusters (I to VI) with subclusters (marked with a or b) were identified. GO and promoter element enrichment results are provided in Table S1. Magenta and green indicate increased and decreased expression as \log_2 ratio compared with untreated or wild type plants, respectively.
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and signaling genes (*ACS6* [1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (*ACC*) SYNTHASE 6], *ACO2* [ACC OXIDASE 2], *ERF13* [ETHYLENE RESPONSE FACTOR13], *ERF104*, and the ROS signaling kinase *OXI1* (OXIDATIVE SIGNAL-INDUCIBLE1) [53]. Genes in cluster IIIb were characterized by very high expression in clean air *rcd1*. This cluster of eleven genes contained *AOX1a* (ALTERNATIVE OXIDASE 1A), *UPOX1* (UPREGULATED BY OXIDATIVE STRESS1) and a putative RCD1 interacting transcription factor *ANAC013* [31]. The high expression of *AOX1a* and cluster IIIb genes indicated that *rcd1* was under constitutive stress as previously proposed [30,31]. The major RCD1 interactor DREB2A is a regulator of both drought and heat stress [54]. Of the stress treatments included in this study, *rcd1* expression profile in clean air shared the highest similarity to heat stress (Figure 2). Many DREB2A targets are also heat-responsive [54], of which the *NFXL1* (NF-X-LIKE1) transcription factor, involved in heat acclimation [55], was found in cluster IIIb. Therefore, the *rcd1* mutant may have a heat tolerance phenotype in line with its higher accumulation of heat shock proteins [45]. Transcript levels in cluster IIIb were decreased by MV (Figure 2), which might be connected to the MV tolerance of *rcd1* [29,30]. Further studies with cluster IIIb genes may also reveal pathways contributing to the MV tolerance of the *rcd1* mutant. Intriguingly, the cell death regulator *LAZ2* involved in chromatin remodeling [26] reversed the expression of cluster IIIb genes hinting that these genes may be involved in cell death and defense responses.

Cluster IV genes generally exhibited increased transcript accumulation under most stress treatments including salt stress, high light and abscisic acid (ABA) (3 h), which suggested that expression of these genes is governed by a “general” stress regulatory circuit. Consistent with a role for ABA, *NCED3* (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3), an early stress-induced ABA biosynthesis gene [56,57] was found in this cluster, and the GO category “response to water deprivation” was significantly enriched together with *cis*-elements related to ABA responses and abiotic stress (Table S1). *ALD1* (AGD2-LIKE DEFENSE RESPONSE PROTEIN 1), a regulator of biotic stress responses and cell death [58], was found in this cluster together with transcription factors *RAP2.6* (RELATED TO AP2 6), *WRKY28* and *ANAC019*.

Genes in cluster V had reduced expression in clean air *rcd1* and were strongly induced by O_3 , biotic stress, ethylene, SA, BTH, senescence and in constitutive defense mutants. This cluster included multiple TFs, including *WRKY18*, *WRKY25*, *WRKY48*, *WRKY75* and *bZIP60*. Promoters of cluster V genes were enriched in CONSERVED MOTIF2 (CM2), a binding site for CAMTA TFs [59] (Table S1). Furthermore, this cluster contained several regulators of biotic stress responses including *PAD4* (PHYTOALEXIN DEFICIENT4), *SAG101* (SENESCENCE-ASSOCIATED GENE101), *FMO1* (FLAVIN-DEPENDENT MONOOXYGENASE1) and *NUDX6* (NUCLEOSIDE DIPHOSPHATES LINKED TO SOME MOIETY X 6). EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) and its interacting partners *PAD4* and *SAG101*, are regulators of biotic stress, SA responses and ROS signaling [60]. Cluster VI had genes with strongly reduced expression in clean air *rcd1* and increased expression by

biotic stress, SA, BTH and in constitutive defense and cell death mutants. Expression of these genes was reduced by ethylene and cluster VI was the only cluster where ethylene gave the opposite result to SA/BTH. Reduced expression of these genes in the SA biosynthesis mutant *sid2* (*salicylic acid induction deficient2*) and the SA receptor/transcriptional co-factor *npr1* (*nonexpressor of pathogenesis-related genes1*) strongly suggested that SA and SA signaling were required for expression of these genes. Cluster VI included several direct targets of NPR1, such as *WRKY38*, *WRKY54* and *WRKY70* [61] and the SA marker genes *PR-2* (PATHOGENESIS-RELATED PROTEIN 2) and *PR-5* (Figure 2). GO analysis revealed significant enrichment of “response to salicylic acid stimulus” and “response to bacterium” in cluster VI (Table S1). The SA-responsive cell death regulator *ACD6* (ACCELERATED CELL DEATH6) [62] also belonged to cluster VI. The *rcd1* mutant in clean air did not display any similarity to the constitutive defense mutants *sni1*, *siz1*, *cs26*, *mkk1 mkk2* [*mitogen activated protein kinase kinase1/2*] or *lht1* [63–67], which all had high expression of SA and BTH responsive genes (clusters V and VI). In contrast, *rcd1* had reduced expression of these genes suggesting that RCD1 is a previously unrecognized positive regulator of SA signaling. In cluster VI, *rcd1* in clean air was also very similar to plants with silenced apoplastic peroxidases [68]. Since the *rcd1* mutant is specifically sensitive to apoplastic ROS, this expression profile similarity indicates a role for apoplastic signaling events resulting in lowered expression of SA responsive genes. Consistent with this interpretation, signaling activated by *flg22* (which is perceived by the FLAGELLIN SENSITIVE2 (FLS2) receptor in the apoplast) leads to reduced expression of SA responsive genes [69]. Importantly, this apoplastic signaling does not involve cluster IIIb genes regulated by heat stress, hence partially dissecting these signaling pathways.

Overall the clustering of experiments indicated that there was no specific cell death profile. Under O_3 , the early *rcd1* time points clustered closely with Col-0. Indeed, many of the genes with decreased expression in *rcd1* in clean air, for instance *WRKY70*, *PR-2* and *PR-5*, exhibited normal O_3 -induction in *rcd1* (Figure 2). At late time points, post O_3 treatment, expression levels nearly returned to basal clean air levels in Col-0. In contrast, *rcd1* at 8 and 24 hours maintained a highly altered expression profile similar to biotic stress treatments and the *acd11* mutant undergoing cell death. Especially genes in cluster IV were strongly induced in these experiments (Figure 2). Cluster IV was enriched for GO classes related to water stress and the ABA response element (ABRE), but not defense (Table S1), and therefore this suggests that cell death and ABA responses coincide at late time points. Spontaneous cell death mutants with associated high expression of various defense genes can arise from various disturbances in cellular homeostasis or signaling [20], which makes it difficult to deconvolute the response to cell death versus activation of defense gene expression. Both basal and effector triggered immunity are qualitatively similar, i.e., the same set of defense genes are induced, but in the latter there is a quantitative difference in that the genes are induced higher and faster [70]. We observed a similar phenomenon in the O_3 response of *rcd1* where apoplastic ROS induced a gene expression response qualitatively similar to Col-0, but faster

and to a higher level. Overall, clustering of genes differentially expressed in *red1* suggests that the ROS-triggered lesion formation might depend on a fine-tuned threshold and timing, rather than of an on-off regulation of gene expression.

Depending on the particular stress, the hormones ABA, SA, jasmonic acid (JA) and ethylene can show synergistic or antagonistic interactions [71,72]. SA and ethylene promote ROS-induced cell death, when JA antagonizes cell death [73]. The clustering clearly separated the function of the defense hormones ABA, JA, SA and ethylene in regulating ROS-induced gene expression in *red1* (Figure 2). ABA and JA mostly had a minor role with only a few genes having strong induction (ABA only regulated genes in cluster IV). In contrast SA/BTH at 24 h and ethylene at 4 h had a very similar and strong effect on many genes in clusters I and IV–V. Timing is clearly important since SA at 3 h did not have strong effect on clusters I and IV. Unfortunately, the only publicly available ethylene Affymetrix experiment using fully grown plants was done only at the 4 h time point [74], and most public SA/BTH experiments only have late time points (8 or 24 h). Given this limitation, and the known kinetics for O₃ induced biosynthesis of SA (5 h; [10] and ethylene (1 h or earlier; [28,75]) it is likely that ethylene is the initial stress hormone, later being augmented with SA in regulation of O₃ induced gene expression. One exception to the synergistic role of ethylene and SA were the cluster VI genes which were regulated positively by SA and negatively by ethylene. A similar SA-ethylene antagonism in gene expression was detected for a subset of genes responding to P₃ME4326 treatment [76].

Cell death marker genes

To further characterize marker genes and potential cell death regulatory genes, a subset of RCD1 regulated genes with functions related to defense, stress or cell death signaling were studied with qPCR in mutants related to cell death and defense signaling (Figure 2). These genes were *NUDX6*, *SAP12* (*STRESS-ASSOCIATED PROTEIN 12*) and transcription factors *RAP2.6*, *WRKY38*, *WRKY62*, *WRKY70*, *WRKY75* and *ZAT12*. In addition, O₃-responsive genes *ACS6*, *ALD1*, *JAZ1* (*JASMONATE-ZIM-DOMAIN PROTEIN1*) and *FMO1* were included in the analysis. Some of these genes have also been shown to be regulators of cell death based on mutant analysis (*ald1*, *fmo1*, [58]) or to suppress constitutive defense signaling (*wrky70*, [24]). Before using these genes in O₃ experiments they were validated in an independent cell death experimental system with lesion mimic mutants *acd2* (*accelerated cell death2*), *acd5* (*accelerated cell death5*) and *lsd1* (*lesion simulating disease resistance1*). The three lesion mimic mutants were selected to have contrasting mechanisms for lesion formation. *ACD2* encodes a protein with multiple subcellular localizations (chloroplast, mitochondria, cytosol) which likely antagonizes cell death via binding to PCD-inducing metabolic products [77]. *ACD5* encodes a ceramide kinase involved in lipid metabolism and signaling [78,79], and *LSD1* has been proposed to act as a cellular hub negatively regulating PCD by interacting with other proteins, such as bZIP10 [22], AtMC1 (METACASPASE1; [80]) and GILP (GSH-INDUCED LITAF DOMAIN PROTEIN; [81]).

The lesion mimic mutants were first grown lesion free in permissive conditions for three weeks and then shifted to lesion-inducing long day (LD) conditions. Three days after the shift to LD, extensive lesion formation was observed in *acd5* and *lsd1* plants, and to a lesser extent in *acd2* plants. Samples were harvested separately from Col-0 and from lesioned leaves (+), lesion-free leaves from lesion-containing plants (–) and lesion free plants (0). This harvesting scheme allowed the separation of gene expression effects before lesions (the 0 samples), cell death (the +

samples) and systemic signaling from dying cells (the – samples). Overall, the expression of marker genes before visible lesions (0 samples) was similar to Col-0 and increased with the appearance of lesions, especially in *acd2*, and to some extent elevated in systemic (–) leaves (Figure 3). However, *NUDX6* expression decreased in lesioned leaves (Figure 3). *NUDX6* has pyrophosphohydrolase activity towards NADH and regulates redox balance and gene expression in SA signaling [82]. Its close homologue *NUDX7* has been extensively characterized in ROS related cell death and defense responses, where knock-out mutants display spontaneous cell death, altered redox balance and constitutive defense gene expression [83–85]. SA signaling is dependent on redox changes of NPR1 [86] and *NUDX6* is proposed to be involved in regulating this redox balance [82]. Since SA is a positive regulator of cell death [10,73], and knock-out of *NUDX6* leads to increased sensitivity to SA [82], the lower expression of *NUDX6* in lesion-leaves could be involved in fine-tuning the redox balance and hence downstream events in cell death execution.

Having established that the chosen set of marker genes were regulated during PCD (Figure 3), qPCR analysis of *red1* and several other single and corresponding double mutants with *red1* was performed at 2 and 8 h after the start of O₃ treatment. The mutants were chosen to disrupt signaling of the major stress hormones JA, ethylene and SA (*coi1-16* the receptor for JA, *etr1-1* a dominant negative allele of the ethylene receptor, *npr1* the SA receptor and transcriptional co-regulator). Furthermore, two other mutants were included: *mpk6*, a knockout for *MAP KINASE6*, a regulator of many stress responses including O₃ [87,88] and *wrky70*, a positive regulator of SA signaling and negative regulator of JA signaling [89]. Previously we have established that removing JA function enhances, and depletion of SA reduces cell death in *red1* [10,14].

The qPCR data was clustered with bootstrapped Bayesian hierarchical clustering to find similarities between genes and mutants (Figure 4A) and statistically analyzed with linear mixed models for differences to the respective Col-0 (Figure 4B and 4C). In control conditions many differences were observed in the mutants, in particular *ZAT12* expression appeared to be very sensitive to any perturbation since its expression increased in almost all mutants compared to Col-0 (Figure 4C). Increased expression of *WRKY38*, *WRKY62*, *ALD1*, *FMO1* and *NUDX6* in *wrky70* suggests that *WRKY70* is a negative regulator of these genes (Figure 4C). NPR1 was a positive regulator of *WRKY38*, *WRKY62* and *WRKY70*, consistent with previous analysis of NPR1 regulated genes [61]. JAZ1 is a transcriptional repressor of JA responses and is rapidly degraded after binding of the bioactive JA-Ile to the receptor COI1 [90]. The *JAZ* genes are also regulated at the transcriptional level by JA [91], and in *coi1-16* there was low *JAZ1* expression (Figure 4C); collectively this indicates that *JAZ1* makes a good marker for the output of JA signaling (Figure 4A and 4B).

Many of the genes (*SAP12*, *ZAT12*, *ACS6*, *ERF104*, *JAZ1*, *ALD1* and *WRKY75*) were induced by apoplastic ROS early at 2 h, after which their expression started to decline 8 h after the start of the O₃ treatment (Figure 4A). *ACS6* encodes a stress-inducible ethylene biosynthesis gene often used as a marker for ethylene signaling and consistent with this, *ACS6* expression was lower after O₃ in the *etr1* mutant (Figure 4B). The remaining marker genes (*FMO1*, *RAP2.6*, *NUDX6*, *WRKY38*, *WRKY62* and *WRKY70*) had the highest expression at 8 h (Figure 4A). The most dramatic change from WT expression pattern was seen for *WRKY38*, and to a lesser extent *WRKY62* in *npr1* and *red1 npr1*, where the O₃ induction was abolished; hence, NPR1 is an essential positive O₃

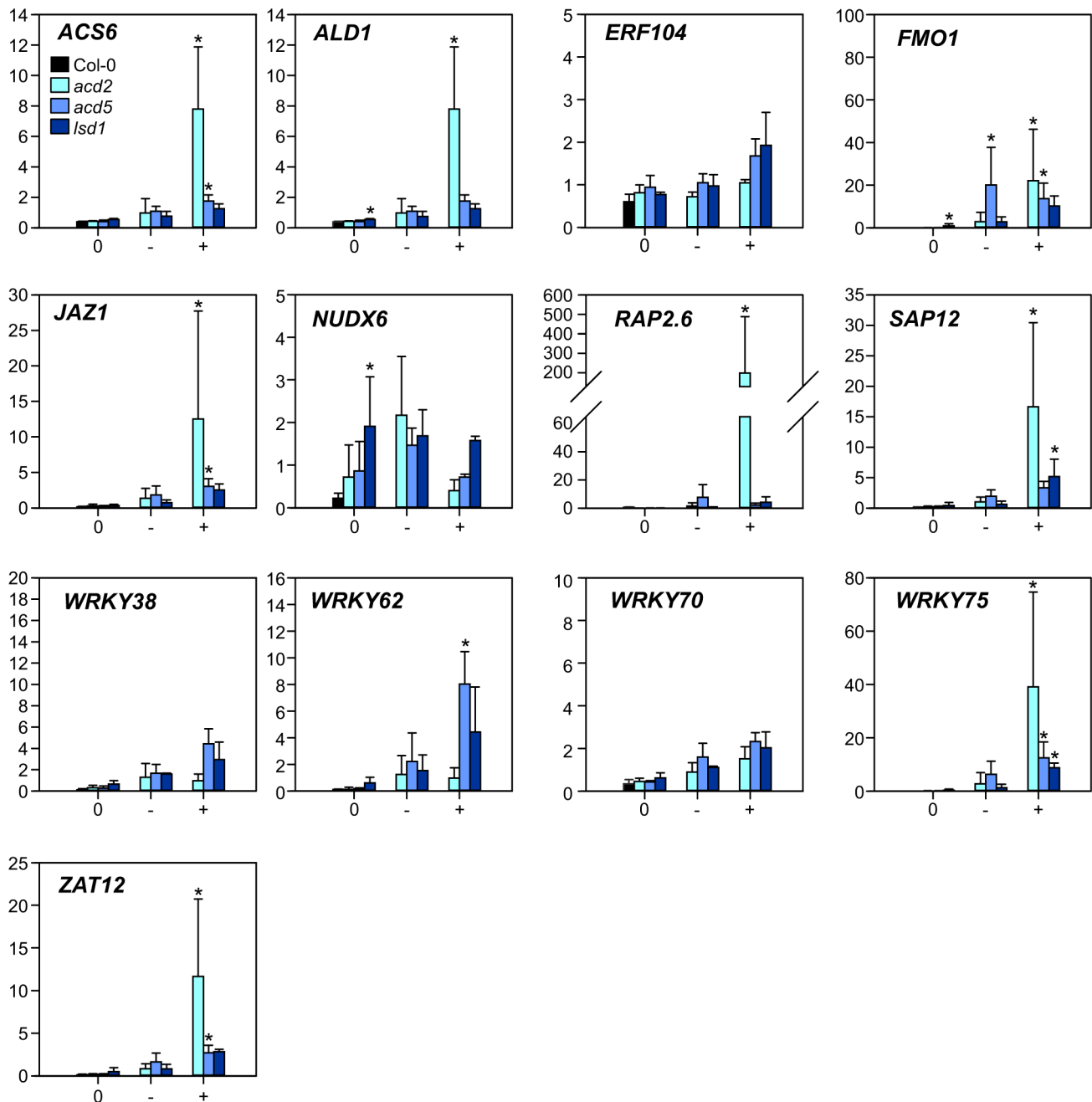


Figure 3. Expression of selected marker genes in lesion mimic mutants. Samples from three lesion mimic genotypes (*acd2*, *acd5* and *lsd1*) 3 d after the transfer to LD were classified as samples from plants with no lesions (labelled 0); leaves with no lesions from plants with lesions (labelled -); leaves with lesions (labelled +). Averages of qPCR results (arbitrary units) from three biological replicates are shown; error bars depict standard deviation. Asterisks depict statistical significance ($P < 0.05$) between to Col-0 (within 0 samples) or to 0 samples within the respective genotype (- and + samples).
doi:10.1371/journal.pgen.1004112.g003

regulator of these genes (Figure 4A and 4B). SA and *Pseudomonas syringae* induction of these WRKY TFs also requires NPR1 [92], thus in particular *WRKY38* can be used as a convenient reporter to follow signaling via NPR1. In contrast, *FMO1* expression was enhanced in *npr1*, *wrky70* and corresponding *rd1* double mutants (Figure 4B), thus NPR1 has both positive and negative signaling roles in apoplastic ROS signaling. The *coi1-16* mutant also revealed both positive and negative roles for JA signaling: *RAP2.6* expression was reduced and *NUDX6* enhanced at 2 h O_3 in

coi1-16 (Figure 4B). Apparently, JA regulation is most important at early signaling since at 8 h there were no longer any differences compared to WT (Figure 4B).

The marker genes chosen are related to cell death or defense signaling, and were induced during lesion formation (Figure 3). Could they also provide insights to the cell death process in *rd1*? The relative severity of cell death at 8 h is in the order *rd1 coi1-16* > *rd1*, *rd1 mpk6*, *coi1-16* > *rd1 wrky70*, *rd1 npr1* ([10,14]; Figure 5), but there was no indication that *rd1 coi1-16* would be

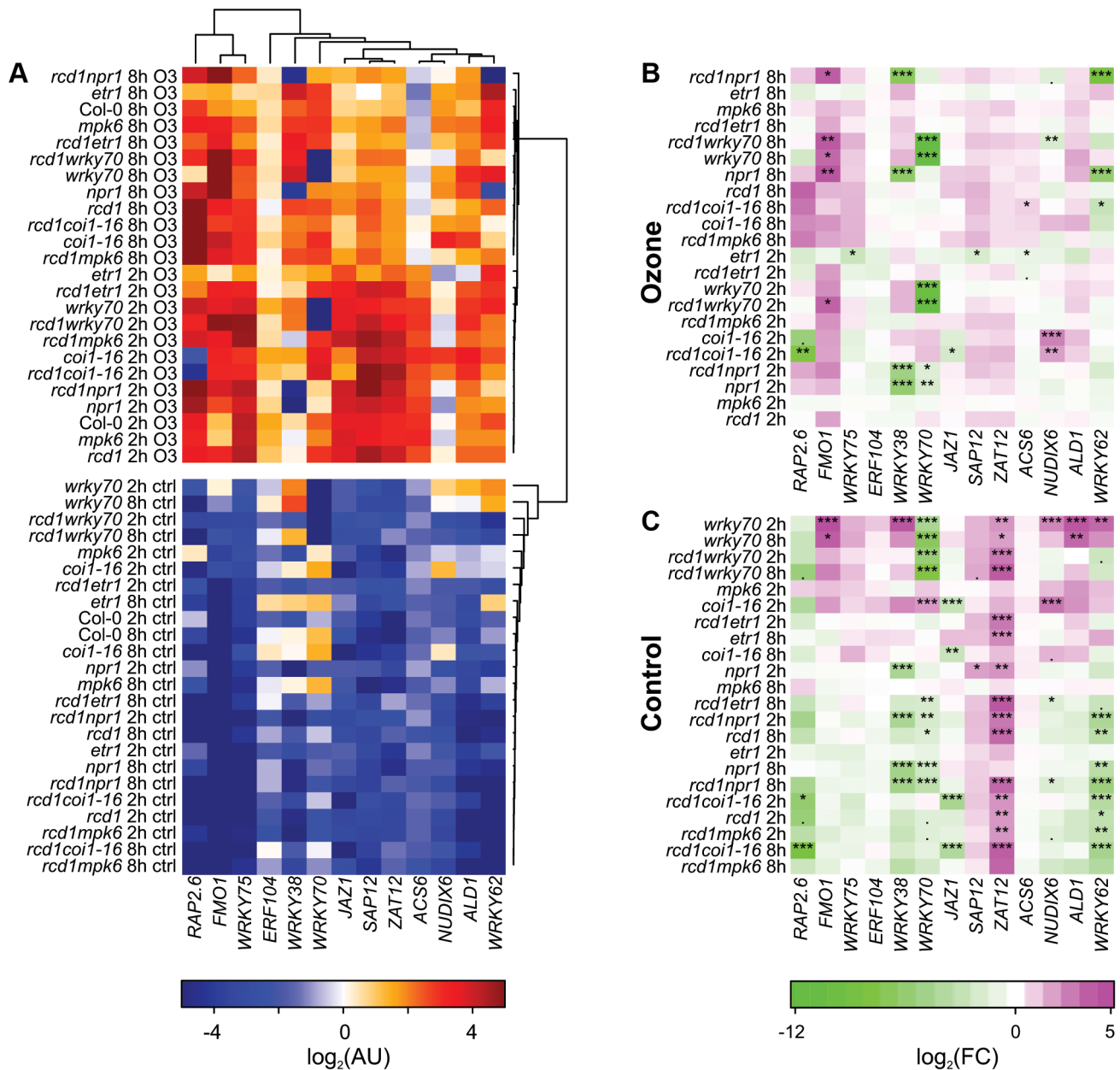


Figure 4. Cluster analysis of gene expression in clean air and O₃-treated plants. Three-week-old plants were treated with 6 h of O₃ (350 nL L⁻¹) and samples harvested at 2 and 8 h after the start of the O₃ exposure. Expression of selected marker genes in each genotype was studied with qPCR and bootstrapped Bayesian hierarchical clustering was applied to log₂-transformed expression values in arbitrary units (A). Gene expression in mutant genotypes was compared to the respective Col-0 sample at each time point (2 h, 8 h) and log₂-transformed fold changes were calculated for O₃ treatment (B) and control plants (C). Asterisks mark statistical significance according to the linear model (P<0.10:“.”; P<0.05:“*”; P<0.01:“**”; P<0.001:“***”).

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strikingly different from *rcd1* at this time point, except for somewhat enhanced *ACS6* expression. Instead, it appeared that signaling prior to visible lesion formation (i.e., the 2 h time point) could determine the extent of later cell death. In particular *NUDX6* expression was enhanced in the O₃ sensitive *rcd1 coi1-16* and *coi1-16*. Although both the cluster analysis (Figure 2) and qPCR results (Figure 3 and 4) revealed some interesting correlations between cell death and gene expression, it appears that gene expression data alone is insufficient to identify “unique” cell death regulators, i.e., genes that would be regulated only during cell death and not by other abiotic or biotic stresses.

Identification of genes specifically regulated during cell death could, e.g., be identified with a much higher temporal and spatial resolution, i.e., cells undergoing cell death (as well as neighboring cells) would have to be microdissected out from leaves preferably in a time course [93,94]. Analysis of whole leaves/rosettes from dying plants is likely to contain a mix of cell death process and neighboring cells with activated defense responses. It is also a distinct possibility that unique cell death regulators might be rare and instead, regulators (such as various transcription factors) are recruited at different times to fulfill signaling roles both during stress and cell death.

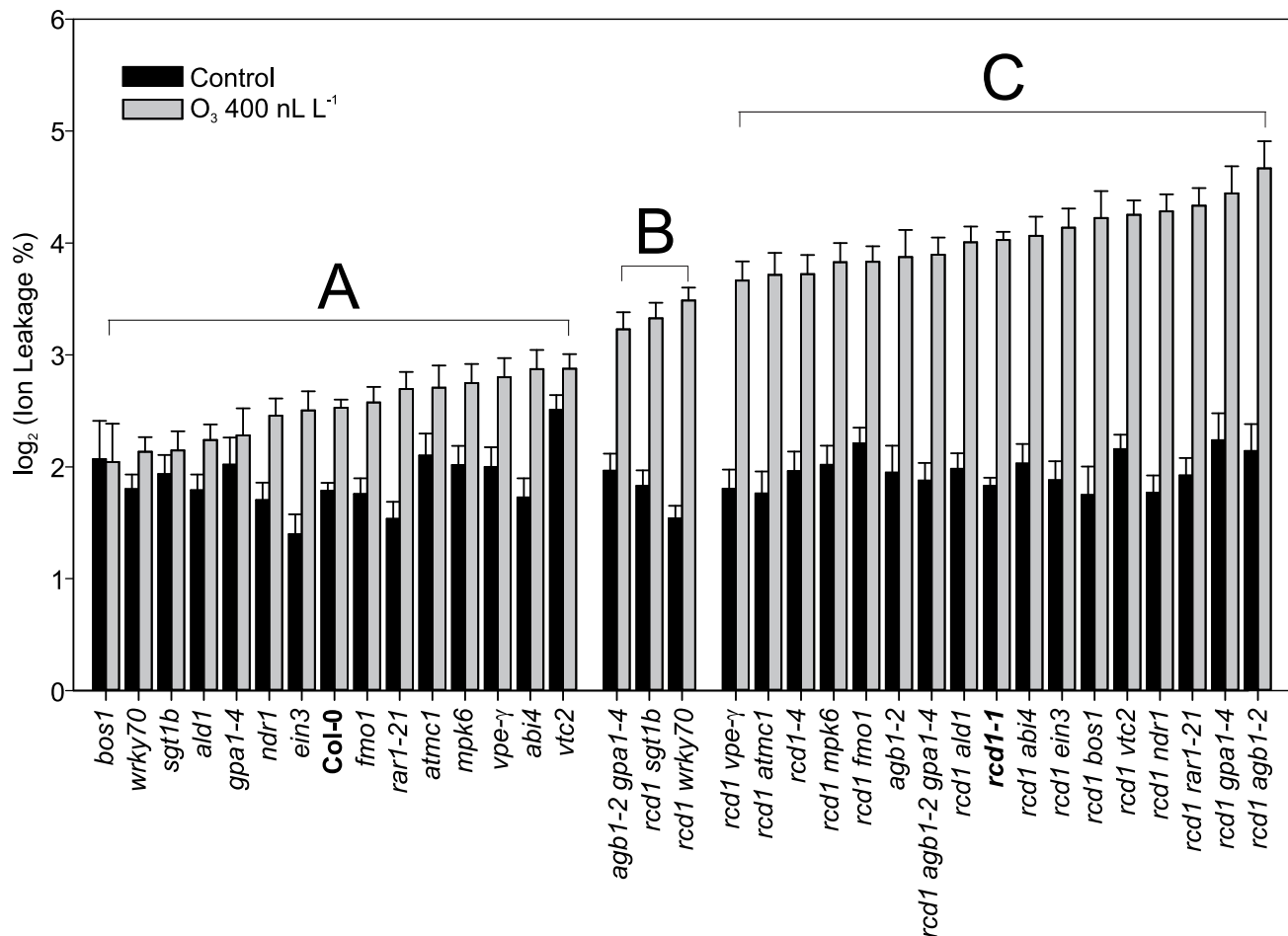


Figure 5. Quantification of O₃-induced cell death in Col-0, *rcd1* and various double mutants. Plants were exposed to 6 h of O₃ (400 nL L⁻¹) and after 2 h recovery in the clean air, they were harvested for ion leakage measurements. Samples of untreated plants grown in clean air were simultaneously collected. Samples are ranked according to their ion leakage in O₃. Ion leakage percentages of O₃ samples were compared to O₃-treated Col-0 and *rcd1-1* with linear models. Ion leakages of samples belonging to groups A and C did not differ from Col-0 and *rcd1*, respectively. Group B and C samples showed elevated O₃-damage compared to Col-0 ($P < 0.01$), whereas samples in groups A and B had decreased O₃-damage in comparison to *rcd1* ($P < 0.01$). In clean air samples, only *vtc2* differed from Col-0 ($P < 0.001$). Bars represent means of two to seven biological repeats with standard error.

doi:10.1371/journal.pgen.1004112.g005

rcd1 cell death execution is distinctly different from pathogen induced cell death processes

Cell death in *rcd1* is reduced by application of a transcriptional inhibitor [10]. This suggests that some O₃ responsive genes in *rcd1* are candidate regulators of cell death. Also, many cell death regulators have been identified through the study of pathogen induced cell death. Mutant analysis was used to directly test the role of these cell death regulatory genes in O₃-induced cell death. Based on *rcd1* and O₃ gene expression data, as well as known regulators of pathogen defenses and suppression of lesion mimic phenotypes, several *rcd1* double mutants were constructed and evaluated for O₃ induced PCD (Figure 5). Mutants included *ald1* and *fmo1*, suppressors of a SYNTAXIN lesion mimic mutant *syb121 syb122* [58]; *mpk6*, a regulator of stress responses [95]; *abi4*, a TF regulator of stress signals originating from chloroplasts and mitochondria [96]; *ein3*, a TF in the ethylene signaling pathway; *wrky70* a TF with positive regulation of SA signaling and negative regulator of JA signaling [89]; *rar1* (required for *MLA12* resistance) and *sgt1b* (suppressor of the G2 allele of *SKP1b*) regulators of various aspects of pathogen defenses, SA signaling and PCD [97]; *ndr1* (*nonrace*

specific disease resistance1) a regulator of ROS mediated cell death in *lsd1*, and avirulent *Pseudomonas* infection [98,99]; *mc1* (*metacaspase1*) a positive regulator of PCD [80]; *vtc2* (*vitamin C defective2*) a mutant with low concentration of the important antioxidant ascorbic acid [100]; *vpe-gamma* (*vacuolar processing enzyme*) [101], *gpa1* and *agb1*, the alpha and beta subunits of heterotrimeric G-protein signaling complex [102].

Despite the well-documented role for many of these genes in regulating cell death during pathogen infection and in lesion mimic mutants, only *sgt1b* and *wrky70* were able to partially suppress O₃ induced cell death in *rcd1* (Figure 5). WRKY70 acts as an integrator between SA and JA signaling [89]. In addition, *wrky70* was isolated from a suppressor screen for mutants that restore normal growth to a dwarfed constitutive defense mutant *sn2-1D* (*suppressor of npr1-1, constitutive 2*) [24]. Hence the decreased cell death in *rcd1 wrky70* could be the result of reduced expression of a WRKY70-dependent positive regulator of cell death. SGT1b is an accessory factor to SCF (Skp1/Cullin1/F-box) E3 ligases, which are master regulators of ubiquitin targeted protein degradation [103]. Since the SCF E3 ligases have multiple targets

in plants, most of which are unknown, we speculate that in *rcd1 sgt1b* there is negative regulator of cell death that is stabilized when SGT1b function is removed. The lack of influence on O₃-induced cell death by other regulators previously shown to alter pathogen-induced cell death or to suppress lesion mimic phenotypes, including *ald1* and *fmo1*, indicate that despite many similarities between pathogen and O₃-induced cell death [10], the execution of the O₃ cell death program requires different components. Alternatively, RCD1 could function as one of the final downstream steps in cell death execution, hence previously identified cell death regulators might be mostly up-stream of RCD1 and epistatic in double mutant analysis. A suppressor mutant screen of *rcd1* has the potential to identify new regulators of PCD execution.

Cluster IIIb (Figure 2) contained genes with very high expression in clean air *rcd1* and included two mitochondria localized proteins AOX1a and UPOX1. AOX1a acts to bypass the last step of mitochondrial electron transport and is proposed to reduce ROS production in times of stress and to act as a regulator of PCD [104]. To determine whether constitutively higher expression of these two genes is committing *rcd1* for PCD, respective *rcd1* double mutants were constructed. Furthermore, plants overexpressing AOX1a (*AOX1a* OE) or a constitutively active AOX1a (*AOX1a* OE-CA) were included in the experiments [105]. In contrast to the situation in tobacco where overexpression of AOX1 leads to O₃ sensitivity [106], there was no increased cell death in any of the *AOX1a* OE or *AOX1a* OE-CA lines, nor in the single *aox1a*, and no changes in the O₃ damage in *rcd1-1 aox1a* plants in comparison to *rcd1* (Figure 6). Increased AOX activity has been proposed to lead to stress tolerance by reducing

mitochondrial ROS production and/or maintenance of mitochondrial function during stress [104,107]. However, the lack of O₃ phenotypes in various transgenic lines with altered AOX1a levels suggest that the role of mitochondrial ROS production during cell death is more complicated than anticipated.

O₃-induced cell death in Col-0 and *rcd1* was also independent of the mitochondrial protein UPOX1 [108] (Figure 6), which is universally induced by oxidative stress [15]. Recently, cytosolic localization of UPOX1 was also demonstrated [47]. It should be noted that the T-DNA insertion in *UPOX1* is located at the end of the coding sequence and would only remove the last five amino acids of the protein, and although there was an altered *UPOX1* transcript size in *upox1* and *rcd1 upox1* (data not shown), these plants may still have a functional protein. Apparently, neither *AOX1a* nor *UPOX1* modulate apoplastic ROS cell death of *rcd1*.

Regulation of *AOX1a* expression has been extensively studied to reveal components of stress and/or mitochondrial retrograde signaling [109,110]. A mutant screen for regulators of *AOX1a* expression identified *rao1* (*regulator of alternative oxidase1*), encoding CYCLIN-DEPENDENT KINASE E1 (CDKE1), as a regulator of stress and growth responses [111]. Since both CDKE1 and RCD1 are regulators of *AOX1a* expression this prompted us to compare the expression profiles of both mutants. Of 423 genes misregulated in *rcd1* (Figure 2), 123 were also misregulated in *rao1* [111]. Subsequent clustering of these genes using raw data from [111] and the *rcd1*/O₃ data did not reveal any striking similarities between the two mutants (data not shown). However, the opposite *AOX1a* expression phenotypes of the two mutants, i.e., higher expression in *rcd1* and lower in *rao1*, suggest that *rcd1* may be a valuable tool to further dissect mitochondrial retrograde signaling.

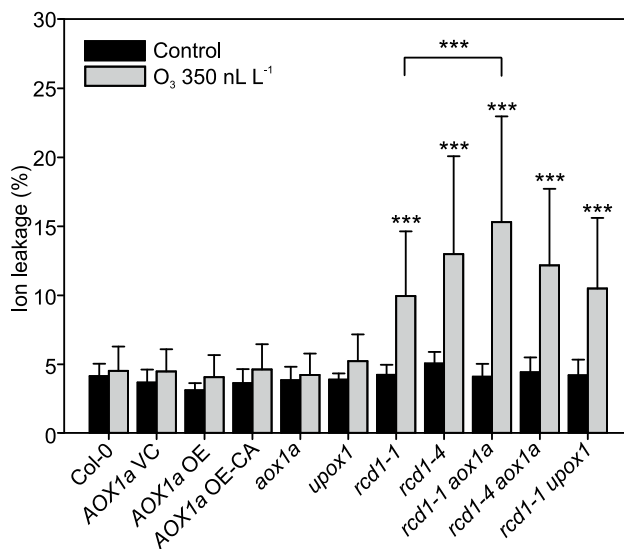


Figure 6. Response to apoplastic ROS in *rcd1* is not influenced by AOX1 or UPOX. Genotypes studied were Col-0, *AOX1a* OE (overexpression), *AOX1a* OE-CA (overexpression of constitutively active AOX1a), the corresponding vector control (VC) and the mutants *aox1a*, *upox1*, *rcd1-1*, *rcd1-4*, *rcd1-1 upox1*, *rcd1-1 aox1a* and *rcd1-4 aox1a*. Plants were exposed to 6 h of O₃ (350 nL L⁻¹) and after 2 hour recovery in the clean air they were harvested for ion leakage measurements. Samples of untreated plants grown in clean air were simultaneously collected. Clean air and O₃ samples were compared to wild type Col-0 with linear models (P<0.05:*; P<0.01:** and P<0.001:***). Double mutants (*rcd1-1 aox1a*, *rcd1-4 aox1a* and *rcd1-1 upox1*) were also compared to *rcd1-1* and *rcd1-4*. Bars represent means of four to six biological repeats with standard deviation. doi:10.1371/journal.pgen.1004112.g006

rcd1 growth defects are not suppressed by defense signaling or cell death regulators

The *rcd1* mutant displays an altered growth phenotype indicative of constitutive stress-induced morphogenic response (SIMR), a growth response including inhibition of shoot elongation and stimulation of auxiliary branching [37,38]. SIMR is thought to be an adaptive response to stress and is regulated by a complex interplay between ROS, auxin, ethylene and antioxidants [14,37]. The effect of ROS may at least partially be mediated by direct oxidation of indole-3-acetic acid to form inactive 2-oxindole-3-acetic acid [112]. Since *rcd1* displays constitutive SIMR, the *rcd1* double mutant collection allows the dissection of signaling pathways contributing to SIMR. Most double mutants exhibited no alterations in *rcd1* growth habitus, thus excluding a role for ethylene, SA, JA as well as several other defense regulators in the regulation of SIMR (Table 1). RCD1 has also been shown to regulate growth suppression down-stream from defense activation, a response dependent on ROS production and proper redox balance [41]. The few *rcd1* double mutants with altered growth phenotypes include *rcd1 axr1*, which displayed an additive growth inhibition [14] thus highlighting a role for auxin in regulation of SIMR. Furthermore, both ascorbic acid biosynthesis mutants, *vtc1* and *vtc2*, enhanced growth suppression of *rcd1* (Figure 7). In conclusion, the SIMR response was governed by a set of regulators distinct from classical defense signaling and the *rcd1* mutant represents a useful tool in dissecting SIMR.

Conclusions

Numerous regulators of cell death have been identified through work on plant PCD and lesion mimic mutants. O₃-elicited cell death in *rcd1* requires a partially distinct set of regulators, indicating further complexity in plant cell death regulation. At the gene expression level quantitative higher expression of stress

Table 1. Enhanced SIMR in *rcd1* double mutants.

Double mutant	Biological process altered	Reference to double mutant	Reference to single mutant
<i>rcd1 axr1</i>	Subunit of RUB1 activating enzyme that regulates protein degradation activity of Skp1-Cullin-F-box complexes.	[14]	[120]
<i>rcd1 noa1</i>	Decreased nitric oxide production+required for chloroplast ribosome assembly	[121]	[122]
<i>rcd1 noa2</i>	Unknown	[121]	Not available
<i>rcd1 sro1</i>	Closest homolog to <i>rcd1</i>	[31,35],	[31,35],
<i>rcd1 vtc1</i>	Decreased ascorbic acid concentration+altered stress responses	Figure 7	[123]
<i>rcd1 vtc2</i>	Decreased ascorbic acid concentration+altered stress responses	Figure 7	[123]
<i>rcd1 snc1</i>	Immune response	[41]	[41]
<i>rcd1 rbohF</i>	ROS production	[41]	[124]
Other <i>rcd1</i> double mutants in hormone signaling i.e., <i>rcd1 ein2</i> , <i>rcd1 jar1</i> , <i>rcd1 coi1-16</i> , <i>rcd1 npr1</i> have the same growth phenotype as <i>rcd1</i> .		[10,14,28]	

Enhanced SIMR, defined as increased growth defects and/or dwarfism, is observed in the listed *rcd1* double mutants. The biological process(es) altered in the mutant crossed with *rcd1* is briefly summarized. Of the double mutants used for cell death experiments (Figure 5 and 6) only *rcd1 vtc2* displayed enhanced SIMR. In addition *rcd1 agb1-2*, *rcd1 gpa1-4* and *rcd1 agb1-2 gpa1-4* displayed leaf characteristics of both parents: round leaves like the G-protein mutants and wavy, bushy leaves like *rcd1*. doi:10.1371/journal.pgen.1004112.t001

related genes was more important than qualitative difference in individual genes. The identification of *wrky70* and *sgt1b* as partial suppressors of *rcd1* cell death indicate that there are more cell death regulators to be identified by studying WRKY70 target genes and SGT1B-SCF E3-ligase target proteins. The convergence of stress, growth response and mitochondrial retrograde signaling in RCD1, its nuclear localization and interaction with various TFs, indicate a role for RCD1 in transcriptional regulation or possibly chromatin regulation or other epigenetic changes.

Materials and Methods

Plant material and O₃ treatment

The growth conditions used and collection of plant material for microarray experiments are described in [14]. In brief, *Arabidopsis thaliana* ecotype Col-0 and *rcd1-1* were grown in controlled environment chambers (Weiss Bio1300; Weiss Gallenkamp,

<http://www.weiss-gallenkamp.com/>) with 12-h/12-h (day/night) cycle, temperature 22/19°C, relative humidity 70/90%. O₃ experiments (6 hours of 350 nL L⁻¹) were performed with three-week-old plants, which were collected at 0, 1, 2, 4, 8 and 24 h after the start of the O₃ treatment. The experiment was repeated three times, in addition to which a fourth identical repeat was used as the common reference RNA. Lesion mimic mutants *acd2-2* and *lsd1-3* [aka *chs4-1* [113]] and T-DNA knockouts were obtained from NASC (<http://arabidopsis.info/>) and *acd5* was a gift from Dr Jean Greenberg. Lesion mimics were grown in growth rooms with the same conditions as above for 22 days (no lesions were visible at this point), and then moved to long day greenhouse to induce lesions (18/6 h day/night). Samples were harvested 72 hours later. Col-0 and lesion mimic genotypes with no visible lesions were marked with 0, individuals with lesion leaves were marked with + and leaves without lesions from the same plant as lesion leaves were marked with -. In this experimental design leaves undergoing cell death (+ leaves) are separated from systemic leaves (- leaves) which might receive a signal from dying leaves; and leaves/plants which have not yet started the cell death program (0 leaves).

Plants were harvested for cell death quantification with ion leakage after 6 h of 350 (or 400 nL L⁻¹) of O₃ and 2 h in clean air into 15 ml MilliQ-water. Ion leakage caused by O₃ was measured with a conductivity meter 2 h after harvesting. For total ion content measurements, samples were frozen and thawed to release the content of cells. The *rcd1* double mutants were constructed with *rcd1-1* or *rcd1-4* as pollen acceptor and various other defense related mutants as pollen donors, see Table S2 for full details. Mutants were obtained from NASC (<http://arabidopsis.info/>) or were gifts from Dr Günter Brader (*wrky70*), Dr Hans Thordal-Christensen (*ald1* and *fmo1*), Dr Patricia Conklin (*vtc1* and *vtc2*), Dr Heribert Hirt (*mpk6*), Dr Tesfay Mengiste (*bos1*), Dr. Jeff Dangl (*ndr1*, *mc1*, *rar1-21*, and *edm1*), Dr Alan Jones (*gpa1*, *agb1*) and Dr. Ikuko Hara-Nishimura (*vpe-gamma*). Double mutants were initially screened for the visible phenotype of *rcd1* (curly leaves and compact rosette), subsequently the mutations were verified with PCR based markers (Table S2). Other *rcd1* double mutants have been described previously [10,14]. GUS staining and RCD1 and SRO1 promoter *uidA*-lines were described previously [31].

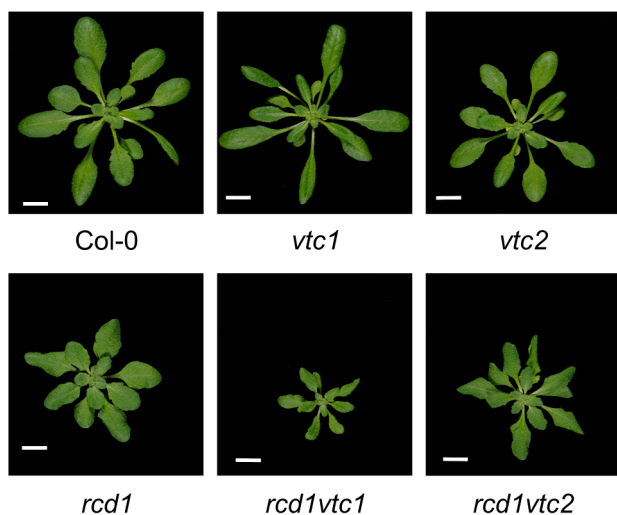


Figure 7. Constitutive SIMR in the *rcd1* mutant is enhanced by ascorbate deficiency. Plants were grown for four weeks and photos taken. Scale bar = 1 cm. doi:10.1371/journal.pgen.1004112.g007

Arabidopsis overexpressing AOX1a or a constitutively active AOX1a and vector controls are described in [105] and [114]. These lines are available from NASC with the stock codes N6589–N6598. Initially all lines were screened for O₃ sensitivity, subsequently lines N6589, N6591 and N6595 were characterized in more detail.

Microarray hybridizations and data analysis

RNA extraction, microarray hybridizations, data preprocessing and analysis with scripts in R are reported in [14]. Genes with at least 2-fold change in expression with statistical significance $q < 0.05$ were considered as differentially expressed in each comparison made between treatments, genotypes and time points. Overlap between multiple gene lists was studied with Venn diagrams [115]. Gene expression data is deposited into ArrayExpress, accession number: E-MTAB-662.

Analysis of *rcd1* gene expression in comparison with publicly available gene expression data

The raw .cel files were downloaded from public databases, normalized with Robust Multi-array Average (RMA) normalization, and manually annotated to control and treatment conditions. For each experiment the log₂-base fold changes of treatment versus control were computed. The preprocessed data was clustered using bootstrapped Bayesian hierarchical clustering as described in [116]. Publicly available experiments using the Affymetrix ATH1-121501 platform were obtained from several data sources: NASC Arrays <http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl> (ABA - NASCARRAYS-176; CHX - NASCARRAYS-189; MG132 - NASCARRAYS-190; SA experiment 1 - NASCARRAYS-192; BTH experiment 1 - NASCARRAYS-392; ZAT12 OEX experiment 1 - NASCARRAYS-353; Senescence experiment 1 - NASCARRAYS-52; Senescence experiment 2 - NASCARRAYS-150). ArrayExpress <http://www.ebi.ac.uk/microarrayas/ae/> (MeJA - EATMX-13; PQ - E-ATMX-28; Syringolin A - E-MEXP-739). Gene Expression Omnibus <http://www.ncbi.nlm.nih.gov/geo/> (H₂O₂ and ZAT12 OEX experiment 2 - GSE5530; Salt - GSE5623; Heat - GSE19603; High light - GSE7743; Flg22 - GSE5615; *sid2* - GSE9955; *lht1* - GSE19109; *edr1* - GSE26679; *Pseudomonas syringae* ES4326 - GSE18978; *smi1* - GSE6827; *csn3*, *csn4* and *csn5* - GSE9728; *cs26* long day - GSE19241; Norflurazon - GSE12887; SA experiment 2 - GSE14961; *siz1* - GSE6583; BTH experiment 2 and *mkk1mkk2* - GSE10646; Ethylene and amiR-ETP1/2 (constitutive ethylene response) - GSE14247; *npr1* - GSE13833; ERF104 OEX - GSE11807; *Botrytis cinerea* infection - GSE5684). The Integrated Microarray Database System <http://ausubellab.mgh.harvard.edu/imds> (Experiment names: NPR1 direct targets full genome, FBP1-antisense transgenic and Local and systemic responses to *Trichoplusia ni* feeding). Raw data for *wky33*, *acd11*, *laz1* and *laz2* [26,117] were obtained from Dr John Mundy. Raw data for *atx1* [118] was obtained from Dr Zoya Avramova.

Gene ontology and promoter element enrichment analysis

Gene ontology enrichment analysis was carried out with GO information downloaded from TAIR (<ftp://ftp.arabidopsis.org/>) on 30th November 2010. Enrichment was computed with scripts in R implementing Fisher exact test for the set of genes in each cluster in comparison to the clustering gene list (423 genes). Promoter analysis of the genes was carried out with 500 base pair upstream promoter sequences from TAIR 10, available from <ftp://ftp.arabidopsis.org/>. Matching of 196 known binding

motifs was carried out with scripts in R for both plus and minus DNA strands of the promoter areas as described by [14]. Cluster-specific enrichment of motifs was determined with Fisher exact test.

qPCR gene expression analysis

Gene expression analysis of selected marker genes was performed with qRT-PCR (Table S2 has primer sequences and primer amplification efficiencies). RNA was isolated and treated with DNaseI as in [31]. Reverse transcription was performed with 5 µg of RNA with RevertAid Premium RT and Ribolock RNase inhibitor (Thermo Scientific Fermentas) and the reaction diluted to the final volume of 100 µl. PCR was performed in triplicate using iQ SYBR GREEN supermix (Bio-Rad). The cycle conditions with Bio-Rad CFX384 were: 1 cycle initiating with 95°C 10 min, 39 cycles with 95°C 15 s, 60°C 30 s, 72°C 30 s and ending with melting curve analysis. Normalization of the data was performed in qBase 2.0 (Biogazelle), with three reference genes *TIP41*, *At5g08290* and *PP2AA3* selected from [119] and validated with geNorm to have stable expression in the samples used in this study. Primer amplification efficiencies were determined in qBase from a cDNA dilution series. Statistical significances in qPCR data were evaluated with scripts in R. In statistical analysis, a 2-base logarithm was first taken from the data to improve the model fit. Then a linear mixed model was fitted in using R package nlme, having fixed effects for Genotype, Treatment and Time and their interactions, plus a random effect for the biological repeat. The model contrasts were then computed with multcomp package, and the subsequent p-values were adjusted for multiple comparisons by Benjamini-Hochberg correction.

Analysis of ion leakage data

Statistical analysis of ion leakage data was carried out with scripts in R. A linear mixed model with fixed effects for Genotype, Treatment and their interaction was fitted to the data, plus a random effect for biological repeat. The model contrasts were estimated with multcomp package, and the estimated p-values were subjected to single-step p-value correction.

Supporting Information

Figure S1 *RCD1* and *SRO1* promoter activity during ROS induced cell death. The expression of β-glucuronidase fused with the promoter of *RCD1* and *SRO1* genes was visualized with GUS staining after treatment with 6 h 350 nL L⁻¹ O₃ or 4 h 0.5 mM MV. * indicates position of lesions caused by O₃ or MV. (EPS)

Figure S2 The *sro1* and *sro5* mutants are not O₃ sensitive. Plants were exposed to 6 h of O₃ (350 nL L⁻¹) and after 2 h recovery in the clean air, harvested for ion leakage measurements. Samples of untreated plants grown in clean air were simultaneously collected. Samples with different letters were significantly different from each other (P<0.001). Bars represent means of three to nine biological repeats with standard error. (EPS)

Figure S3 Expression of *RCD1* interacting transcription factors and *RCD1* in different stress and cell death inducing experiments. Bootstrapped Bayesian hierarchical clustering of genes is shown in stress or hormone treated plants compared with normal growth conditions, or in mutant versus wild type. Magenta and green indicate increased and decreased expression as log₂ ratio compared with untreated or wild type plants, respectively. (EPS)

Table S1 GO and *cis*-element enrichments and annotations of *rcd1* misexpressed genes. (XLSX)

Table S2 Primers used in this study. (XLSX)

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