Regulation of RNA-Dependent RNA Polymerase 1 and Isochorismate Synthase Gene Expression in Arabidopsis

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Abstract

Background: RNA-dependent RNA polymerases (RDRs) function in anti-viral silencing in Arabidopsis thaliana and other plants. Salicylic acid (SA), an important defensive signal, increases RDR1 gene expression, suggesting that RDR1 contributes to SA-induced virus resistance. In Nicotiana attenuata RDR1 also regulates plant-insect interactions and is induced by another important signal, jasmonic acid (JA). Despite its importance in defense RDR1 regulation has not been investigated in detail.

Methodology/Principal Findings: In Arabidopsis, SA-induced RDR1 expression was dependent on 'NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1', indicating regulation involves the same mechanism controlling many other SA-defense-related genes, including pathogenesis-related 1 (PR1). Isochorismate synthase 1 (ICS1) is required for SA biosynthesis. In defensive signal transduction RDR1 lies downstream of ICS1. However, supplying exogenous SA to ics1-mutant plants did not induce RDR1 or PR1 expression to the same extent as seen in wild type plants. Analysing ICS1 gene expression using transgenic plants expressing ICS1 promoter:reporter gene (β-glucuronidase) constructs and by measuring steady-state ICS1 transcript levels showed that SA positively regulates ICS1. In contrast, ICS2, which is expressed at lower levels than ICS1, is unaffected by SA. The wound-response hormone JA affects expression of Arabidopsis RDR1 but jasmonate-induced expression is independent of CORONATINE-INSENSITIVE 1, which conditions expression of many other JA-responsive genes. Transiently increased RDR1 expression following tobacco mosaic virus inoculation was due to wounding and was not a direct effect of infection. RDR1 gene expression was induced by ethylene and by abscisic acid (an important regulator of drought resistance). However, rdr1-mutant plants showed normal responses to drought.

Conclusions/Significance: RDR1 is regulated by a much broader range of phytohormones than previously thought, indicating that it plays roles beyond those already suggested in virus resistance and plant-insect interactions. SA positively regulates ICS1.

Introduction

RNA silencing refers to a set of gene regulation mechanisms occurring in most eukaryotes, whereby transcript stability or translatability is suppressed in a sequence-specific manner, guided by small 19–24 nt RNA molecules [1,2]. RNA silencing is an important component of anti-viral defense in plants [3,4]. Double-stranded structures within viral RNA can be cleaved by dicer-like (DCL) nucleases to generate double-stranded small interfering (si)RNAs. In Arabidopsis thaliana, there are four DCL enzymes, of which DCL4 and DCL2 are the most important in the generation of virus-derived siRNAs [5,6,7,8]. After further processing, single-stranded forms of virus-derived siRNA molecules associate with Argonaute (AGO) nucleases and direct AGO-catalysed slicing of complementary viral RNA molecules [9]. Of the ten AGOs encoded by the Arabidopsis genome, AGO1 is the primary ‘antiviral’ AGO, with secondary roles for AGO2, and in certain instances for AGO7 [10,11,12].

Another important feature of the anti-viral RNA silencing pathway in plants is referred to as amplification, whereby more virus-specific dsRNA substrates for DCLs are generated de novo by cellular RNA-dependent RNA polymerases (RDRs) [13]. The Arabidopsis thaliana genome encodes six RDRs, characterized by the DFDGD catalytic domain, of which RDRs 1, 2 and 6 are known to be involved in biogenesis of siRNAs [14]. In Arabidopsis and other plants, RDRs 1 and 6 contribute to antiviral RNA silencing, whilst RDR2 is involved in establishment of transcriptional gene silencing [8,13,15,16,17,18,19,20,21,22,23,24]. RDRs also contribute to silencing mediated turnover of transcripts encoded by endogenous plant genes and transgenes [1,25].
Xie and colleagues [15] reported that in tobacco (\textit{Nicotiana tabacum}) \textit{RDR1} gene expression is induced by the defensive phytohormone salicylic acid (SA). This was a notable finding because it provided for the first time a possible connection between RNA silencing and two well-studied resistance phenomena that are dependent upon SA-mediated signal transduction: (i) the hypersensitive response, a genetically defined and highly pathogen-specific defense; and (ii) systemic acquired resistance (SAR) a broad-spectrum resistance to pathogens that is often triggered by a hypersensitive response [26]. However, it was also reported by Xie et al. [15] that although knockdown of \textit{NtRDR1} expression in transgenic tobacco enhanced the susceptibility of these plants to infection by tobacco mosaic virus (TMV) and potato virus X, resistance to these viruses could still be induced by treatment of the plants with exogenous SA. Subsequently, it was shown that \textit{Arabidopsis} mutants compromised in \textit{AtRDR1} expression showed normal responses to bacterial infection and normal SA-induced expression of pathogenesis-related protein 1 (PR1: a marker for SA-induced resistance to bacteria, oomycetes and fungi), while no effect on SA-induced virus resistance in these \textit{Atrdr1} mutants was reported [16]. Constitutive expression of the \textit{Medicago truncatula} RDR1 in \textit{N. benthamiana} (a natural \textit{rdr1} mutant: [18]) did not enhance SA-induced resistance or rescue chemically-induced resistance in plants compromised in induced resistance by expression of a mutant form of alternative oxidase [27]. Curiously, expression of \textit{NtRDR1} in transgenic \textit{N. benthamiana} plants enhanced, rather than ameliorated, infection by plum pox virus [28]. Thus, although it is possible that RDR1 may contribute to SA-induced resistance to certain viruses, it is not an indispensable component of anti-viral resistance, and in some cases its expression may enhance susceptibility.

The regulation of \textit{RDR1} gene expression is not well understood. It was reported that TMV infection triggered increased \textit{NtRDR1} transcript accumulation in the tobacco cultivar Xanthi (mm genotype) [15] but this could not be due to increased levels of SA, since infection of this cultivar with TMV does not induce SA accumulation [29]. Interestingly, in \textit{N. attenuata}, \textit{RDR1} was induced by jasmonic acid (JA) [30], a phytohormone that is often assumed to be antagonistic to SA-mediated defensive signaling [31]. JA regulates induced resistance to herbivorous insects, and experiments with transgenic \textit{N. attenuata} plants deficient in \textit{RDR1} expression showed that NaRDR1 regulates inducible genes conferring resistance to insect herbivory [32]. Diminishing \textit{NtRDR1} expression in transgenic tobacco decreased the expression of several endogenous transcripts related to virus resistance including \textit{Alternative Oxidase 1a} and \textit{NtRDR6} [33]. Thus, in addition to its hypothesized role in enhancing antiviral RNA silencing [15,16], RDR1 may play indirect roles in plant defense via silencing-mediated regulation of cellular mRNAs encoding resistance factors. In this study we have investigated in more detail the regulation of \textit{AtRDR1} gene expression by SA and JA, and found that other phytohormones, including abscisic acid (ABA) and ethylene, trigger its expression.

\section*{Results}

\subsection*{SA-induced \textit{AtRDR1} Expression is NPR1-dependent}

Wild-type \textit{Arabidopsis} plants (ecotype Col-0) were treated with 1 mM SA, then samples were taken over a 24 h time course spanning 72 hours (h) and were analysed by reverse transcription coupled with quantitative PCR (RT-qPCR) for \textit{RDR1} and \textit{PR1} expression (Figure 1). In agreement with previous studies using northern blotting [16], \textit{AtRDR1} transcript accumulation increased following SA treatment. In this study SA treatment peaked (at approximately four-fold basal level) between 2 and 6 h post-treatment before decreasing to approximately two-fold at 24 h post-treatment and returning to near starting levels by 72 h post-treatment (Figure 1A). The increase in accumulation of \textit{AtPR1} transcripts confirmed that the treatment with SA had been effective (Figure 1). This is in contrast to the work of Yu and colleagues, who reported that \textit{AtRDR1} induction took longer to become detectable (4 to 8 h) with no diminution of \textit{AtRDR1} expression apparent at 24 h post-treatment, the point at which the analysis was terminated [16]. The current work shows that, in contrast to SA-induced \textit{AtPR1} gene expression, the effect of SA on \textit{AtRDR1} gene expression is transient (Figure 1).

The transcriptional activator ‘Non-Expressor of PR proteins 1’ (NPR1) is required for \textit{PR} gene induction and SAR against a wide range of microbial pathogens [34], although it is not required for SA-induced resistance to viruses [35,36]. To determine if \textit{RDR1} gene expression is NPR1-dependent, we used two independent mutant lines: \textit{npr1-1} (Col-0 background: [37]) and \textit{npr1-5}, which was originally named \textit{salicylic acid insensitive 1} (\textit{sai1}) (Nössen background: [38]), and examined \textit{AtRDR1} transcript accumulation in plants at 6 h post-treatment with SA. As a control, the induction of \textit{AtPR1} by SA, which is dependent upon NPR1, was also examined in wild-type and \textit{npr1} mutant plants. SA-induced \textit{AtRDR1} expression in both \textit{npr1}
mutant lines was markedly lower than that in wild-type plants (Figure 2), indicating that it is NPR1-dependent. It was noted that in all experiments AtRDR1 expression was consistently higher at 6 h post-treatment in plants of the Nössen ecotype than in plants of the Col-0 ecotype (Figure 2).

ICS1 expression is auto-regulated by SA and is required for maximal SA-induced expression of RDR1

Plants of the sid2 line are impaired in their ability to synthesize SA due to a lesion in the gene encoding the isochorismate synthase isozyme, ICS1, upon which Arabidopsis is dependent for the bulk of its stress-induced SA biosynthesis, and which is a key factor in the induction of SAR in this species [39,40,41]. It was observed during initial experiments on the role of NPR1 in AtRDR1 induction that SA treatment of sid2 mutant plants caused induction of less AtRDR1 and PR1 expression than was seen in wild-type plants (data not shown and Figure 3A). This result was unexpected since although these plants are compromised in their ability to produce SA, it was anticipated that addition of exogenous SA would rescue expression of the two SA-inducible transcripts. As expected, in plants of the transgenic NahG line, which expresses a bacterial salicylate hydroxylase [42], SA-induced accumulation of both transcripts was greatly diminished (Figure 3A).

We investigated the effect of exogenous SA application on expression of the AtICS1 gene, as well as the other Arabidopsis ICS ortholog, ICS2. Transgenic plants harboring ICS1:β-glucuronidase (GUS) and ICS2:GUS promoter:reporter gene fusion constructs were treated with SA. ICS1:GUS-transgenic plants consistently exhibited increased GUS activity 24 and 48 h after SA treatment (histochemical analysis of 24 h samples are shown in Figure 3B). In contrast, GUS activity was already detectable in untreated ICS2:GUS-transgenic plants, and showed no induction at either time-point.

The responsiveness of ICS gene expression was investigated further by examining transcript accumulation for ICS1 and ICS2 using RTqPCR (Figure 3C). ICS2 transcript accumulation increased transiently after SA treatment but decreased again by 6 h post-treatment. ICS1 transcript accumulation increased by 2–2.5 fold within 6 h of SA treatment but this elevated level was sustained over 24 hours, followed by a gradual decline (Figure 3C). These results were consistent over three biological replicates. Therefore, ICS1 gene expression appears to be positively auto-regulated by SA. ICS1 is the isozyme responsible for the bulk of SA biosynthesis [40,43,44] and unlike the gene for ICS2, the ICS1 gene is stimulated in a sustained fashion by SA (Figure 3C). This positive auto-regulation of ICS1 expression by SA appears to explain why in sid2 mutant plants the increase in PR1 and RDR1

Figure 2. SA induces AtRDR1 expression in an NPR1-dependent manner. (A) AtRDR1 and AtPR1 expression in ecotype Col-0 wild type and npr1-1 control and SA treated plants 6 h after treatment. (B) AtRDR1 and AtPR1 expression in Nössen (NO) wild-type and npr1-5 control and SA treated plants 6 h after treatment. Error bars represent standard error of the mean. doi:10.1371/journal.pone.0066530.g002
gene expression triggered by exogenous SA was weaker than in wild-type plants (Figure 3A).

Mock Inoculation Induced RDR1 Expression

TMV infection was reported to increase accumulation of RDR1 transcripts in the inoculated leaves of Arabidopsis [16]. However, we found that the kinetics of RDR1 transcript accumulation were similar in mock-inoculated and TMV-inoculated leaves (Figure 4A). In both cases, increased RDR1 expression was transient, peaking and declining during the first 24 h following treatments and in the TMV-inoculated leaves there was no obvious relationship between RDR1 expression and the kinetics of viral RNA accumulation (Figure 4A). This suggests that the process of inoculation, involving abrasion of the adaxial surfaces of the leaves with Carborundum, rather than virus infection per se, was responsible for increased AtRDR1 expression.

To explore the possibility that RDR1 expression was triggered by abrasion, further experiments were carried out to follow the expression of both RDR1 and a well-characterized wound-induced gene, terpene synthase 10 (TPS10), over a period of 72 h following mock inoculation (Figure 4B). Although the response of RDR1 to mock-inoculation in terms of fold-increase in expression was at least two orders of magnitude less than the response of TPS10, the timing of expression following mock-inoculation was similar for both transcripts, supporting the idea that wounding had triggered increased RDR1 gene expression in TMV-inoculated plants (Figure 4A, B).

JA Induces RDR1 Expression in a COI1-independent Manner

JA-mediated signaling co-ordinates a large proportion of wound-induced gene expression [45]. Indeed, expression of the wound-inducible TPS10 transcript is stimulated by treatment with methyl-JA [46]. In N. attenuata, NaRDR1 expression was shown to be induced by JA [30], suggesting that the abrasion-induced expression of AtRDR1 is regulated by JA-dependent signaling. To examine this further, wild type Col-0 plants were treated with 250 μM methyl-JA and RNAs were extracted at various times over a 72 h time-course for analysis of gene expression by Q-RT-PCT (Figure 5A). Methyl-JA treatment induced transient increases in expression of both AtRDR1 and TPS10, used here as a positive control for JA-induced gene expression (Figure 5A). AtRDR1 expression peaked at 6 h post-treatment, and by 24 h had decreased to pre-treatment levels (Figure 5A). The results show

Figure 3. AtICS1 expression is positively regulated by SA and is required for optimal expression of AtRDR1 and PR1 in response to exogenous SA treatment. (A) RTqPCR analysis of transcript accumulation for AtRDR1 and AtPR1 expression in Arabidopsis ecotype Col-0 wild type (WT), sid2-mutant (compromised in expression of ICS1) and NahG-transgenic plants 6 h post SA treatment. (B) Transgenic Arabidopsis plants harboring the promotor: reporter constructs ICS1:GUS and ICS2:GUS stained for GUS activity 24 h after control (water) or SA treatment. SA treated Col-0 WT and 35S:GUS plants, 24 h after treatment are included as controls. (C) RTqPCR analysis of AtICS1, AtICS2, and AtPR1 transcript accumulation in wild-type plants treated with SA, over a 48 h time course. Error bars represent standard error of the mean. doi:10.1371/journal.pone.0066530.g003
that regulation of \textit{RDR1} gene expression by JA is conserved between \textit{N. attenuata} and \textit{Arabidopsis}.

To investigate further the relationship of JA-mediated signal transduction to \textit{AtRDR1} expression, plants of the mutant line \textit{coronatine insensitive 1–16} (\textit{coi1-16}), which is compromised in perception of the active form of JA, JA-Ile, were treated with methyl-JA and samples were harvested at 6 h post-treatment for RNA extraction and analysis of expression of \textit{AtRDR1} and \textit{TPS10} by RTqPCR (Figure 5B). It has been shown previously that the induction by methyl-JA of increased \textit{TPS10} expression is COI1-dependent [45] and our data were consistent with this. Thus, we found that induction of \textit{TPS10} transcription accumulation by methyl-JA was inhibited in \textit{coi1-16} mutant plants (Figure 5B). In contrast, \textit{AtRDR1} expression following methyl-JA treatment was similar in wild-type and \textit{coi1-16} mutant plants (Figure 5B), demonstrating that JA-induced \textit{AtRDR1} expression is COI1-independent.

Ethylene and ABA Induce \textit{RDR1} Expression

There is significant cross-talk between JA- and SA-regulated signaling and signaling mediated by ethylene and ABA [43]. Therefore, we investigated if these other stress-related phytohormones affected \textit{RDR1} expression. \textit{Arabidopsis} plants were treated with solutions of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or ABA. ACC treatment induced the expression of \textit{AtRDR1} in parallel with induction of the ethylene-responsive \textit{AtPR4} gene peaking at 6 h post treatment (Figure 6A). ABA treatment induced \textit{AtRDR1} expression and expression of the ABA-responsive gene \textit{RD29A}, also with a peak in expression at 6 h post-treatment (Figure 6B).

ABA is an important regulator of drought responses [48]. As \textit{AtRDR1} is induced by ABA treatment, this suggested the possibility that \textit{RDR1} might play a role in resistance to drought stress. However, when plants were subjected to drought by 9 days of water deprivation, there was no significant difference between the percentage water content of wild-type or \textit{rdr1} mutant plants (Figure 6C). The same result was seen in three independent experiments.

\textbf{Discussion}

Although often viewed as being SA-inducible, \textit{RDR1} expression displays some intriguing differences, as well as similarities too, to the behaviour of other SA-regulated plant genes. As is the case
with the well-studied PR genes, SA-induced AtRDR1 expression was shown to be NPR1-dependent, which was confirmed using two independent npr1 mutant lines from different Arabidopsis ecotypes. However, unlike the transcript for the PR1 protein, the increase in AtRDR1 transcript accumulation was transient and its peak expression was markedly lower than that for AtPR1. This suggests that AtRDR1 transcript accumulation is under tighter transcriptional and post-transcriptional control than AtPR1.

Interestingly, SA-induced accumulation of both AtPR1 and AtRDR1 transcripts was diminished in plants of the SA biosynthetic mutant line sid2 (which is compromised in ICS1). This led to our finding that the ICS1 gene but not the ICS2 gene is under a form of positive feedback from SA, the ultimate end product of ICS1 activity. The results also confirmed the primacy of ICS1 over ICS2 in facilitating SA biosynthesis in Arabidopsis [44].

The induction by SA of resistance to viruses is not dependent upon NPR1 [35,36]. The dependence on NPR1 of SA-induced AtRDR1 expression provides additional evidence, along with previous studies with transgenic plants [15,27], that the major contribution of RDR1 to virus resistance lies in its role in basal defense, and that it is not essential for SA-induced resistance to viruses. The role of RDR1 in stress tolerance and defense via the silencing of endogenous genes is something that has been suggested previously by Pandey and Baldwin (2007) as an explanation for the susceptibility observed in rdr1 mutant N. attenuata lines to herbivory [30]. Furthermore, tobacco lines deficient in RDR1 have been shown to have altered expression of other defense related genes, suggested by the authors that RDR1 plays a role in regulating other endogenous defense-related genes by suppressing the expression of regulatory molecules [33].

The JA-mediated and SA-mediated defensive signaling pathways are to a great extent antagonistic, and few transcripts are positively regulated by both [49]. Thus, the responsiveness of RDR1 to both of these phytohormones seen in N. attenuata [30] and in Arabidopsis (this study) sets this ‘SA-responsive’ gene apart from typical SA-responsive genes like PR1. RDR1 is also not a typical JA-responsive gene, since its induction by methyl-JA was not dependent upon COI1, a F-box protein responsible for degradation of JASMONATE ZIM-domain proteins that negatively regulate most JA-responsive genes [50,51,52]. Although most...
JA-responsive genes are dependent upon COI1 for induction, several, including genes involved in plant defense, have been discovered to be COI1-independent [53]. Perhaps the independence from COI1-mediated jasmonate perception allows RDR1 regulation to be outside the typical SA-JA antagonism, and may allow the gene to be similarly responsive to such a wide range of distinct stress signals as JA, SA, ethylene and ABA.

In *N. attenuata*, simulating herbivory by wounding leaves and applying oral secretions from leaf-chewing larvae caused NaRDR1 expression to increase, due to the JA-responsiveness of the gene [30]. We found that gentle wounding, specifically the abrasion used during mechanical inoculation with virus, is sufficient to induce *AtRDR1* transcript accumulation and it was wounding, rather than an effect of the virus, that caused *AtRDR1* induction in directly-inoculated leaves. The finding is reminiscent of findings of induction by abrasion of host RDR enzyme activity in plant tissues in early studies of plant viral RNA synthesis [54]. In previous work it was suggested that induction of *RDR1* expression in inoculated and systemically infected tissues of virus-infected Arabidopsis was due to effects of the virus [15,16]. However, our results indicate that these findings should be re-assessed and that *RDR1* induction in inoculated tissue was most likely due to wounding, while induction in systemically infected leaves is probably attributable to localized induction of RNA silencing, such as which occurs during ‘green-island’ formation [55], rather than as a direct effect of the virus or its gene products.

The responsiveness of *AtRDR1* expression to a wide range of stress related hormones might imply a role in co-ordination of resistance to both biotic and abiotic insult. The responsiveness to ABA suggested that one of the stresses that RDR1 may help protect against is drought. However, *rdr1*-mutant plants were neither more nor less resistant to water loss than wild-type plants. In one way this was a surprising result because in a number of studies it has been shown that small RNA pathways affect drought responses. For example, where RNA silencing pathways have been compromised through mutation of AGO1 or DCLs 1–4, the mutant plants showed increased resistance to water loss [56,57,58]. Hence, although RDR1 is a component of the silencing pathway it does not appear to play a critical role in drought resistance, unlike the DCLs and AGO1. Thus, the biological implications of the ABA-responsiveness of the *RDR1* gene remain to be discovered.

**Materials and Methods**

**Arabidopsis Mutants and Growth Conditions**

*Arabidopsis thaliana* wild type Col-0, Col-0gl, and Nössen (NO) ecotypes were used in this study, either alone or alongside mutant...
lines with the corresponding ecotype. Arabidopsis mutants used included the previously characterised NahG, sid2, npr1-1, npr1-5, coi1-16 and rbo1, ICS1:GUS, ICS2:GUS, and 35S:GUS lines. All plants were grown in short-day condition growth chambers (Conviron Ltd., Winnipeg, Manitoba, Canada): 8 h of light at 200 micromol (photons) m⁻² s⁻¹ and 22°C, with 60% humidity.

Hormone Treatments

Hormone treatments were conducted on plants four weeks of age. The plant hormone treatments were SA (1 mM), methyl-JA (250 μM), ABA (50 μM) and ACC (the precursor of ethylene; 1 mM) dissolved in water. Hormone concentrations were selected on the basis of previous optimization for SA and methyl-JA [46], ACC [59], and ABA [38,60]. Control treatment used water only. Plants were sprayed until surface run-off and samples (aerial tissues of six plants) were taken and frozen in liquid nitrogen at time points of 0.5, 1, 2, 4, 6, 24, 48 and, in some experiments, 72 h post treatment. All experiments were carried out at least three times.

Wounding Treatment

Four-week-old, wild-type Col-0 plants were wounded by squeezing leaves twice with a pair of tweezers. Two leaves per plant were wounded. Aerial plant tissue (from six plants per time point) was harvested and immediately frozen in liquid nitrogen at time points of 0.5, 1, 2, 4, 6, 24, 48 and, in some experiments, 72 h post treatment. All experiments were carried out at least three times.

TMV Inoculation

TMV strain U1 (20 μg ml⁻¹ purified virions in sterile water) was mechanically inoculated onto Carborundum-dusted leaves. Mock-inoculation used sterile water only. Arabidopsis plants were inoculated at the 4- to 6-true-leaf stage. Successful inoculation was confirmed by RT-PCR on extracted plant RNA, using primers for the TMV coat protein gene (forward primer 5'-CAGCGTGGGCCG-3'; reverse primer 5'-GAGGTCCAGACCAA-3').

Reverse Transcription-coupled Quantitative Polymerase Chain Reaction

Total RNA for RTqPCR analysis was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was then purified by a phenol-chloroform extraction and subsequently treated with TURBO-DNase (Ambion, Austin, TX, USA) according to the manufacturer's instructions. First strand cDNA synthesis was carried out on 0.5 μg total RNA using GoScript (Promega, Madison, WI, USA) with random hexamer primers according to the manufacturer's instructions. The cDNA produced was diluted 1 to 5 and RTqPCR performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, St Louis, MO, USA) in 15 μl reactions according to the manufacturer's instructions. Reactions were conducted in triplicate. Primers sequences are given in Table 1. The gene glyceraldehyde-3-phosphate dehydrogenase was used as the reference gene as its expression was identified as being stable under the experimental conditions. The instrument used was a BioRad C1000 thermal cycler connected to a CFX96 Real-Time PCR Detection System and a PC running on CFX manager software (BioRad). The data was analysed using LinRegPCR [61] to give Ct and amplification efficiency values. Relative gene expression was calculated using efficiency adjusted ΔΔCt methodology, incorporating the reference transcript to control for variation in loading. Gene expression was expressed relative to mock-treated wild-type plants.

Generation of ICS:GUS Transgenic Lines

Generation of ICS1:GUS transgenic lines was previously described by Lewsey et al. [46]. PCR amplification of the ICS2 promoter region for cloning was performed using oligonucleotides designed to incorporate 5' XbaI (forward primer, target sequence underlined) and 3' XmaI (reverse primer, target sequence underlined) restriction sites (forward primer 5'-ATATCTCAGATTAATGGTTAGAGAACG-3' and reverse primer 5'-TATCCGGGAGAGAGACTGCAAG-3'). The 1.5-kb product was cloned into pGEM-T Easy (Promega), sequenced to check for mutations and sub-cloned into pGreen-GUS [62] using XbaI and Xmal. The resulting plasmid was introduced into Agrobacterium tumefaciens GV3101::pMP90::pSOUP by electroporation and used to transform A. thaliana Col-0 by floral dipping [63].

Detection of GUS Activity

Plants of four-week-old ICS1:GUS and ICS2:GUS lines were sprayed with 1 mM SA until surface run-off. As a control plants of the same age were treated with water. Aerial plant tissue was harvested at time points of 24 and 48 h post treatment for both control and SA-treated plants. Immediately after harvesting, plant tissue was stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) by submerging the rosettes in 5 ml of X-Gluc solution and infiltrating them under a vacuum for 15 min. Samples were incubated at 37°C overnight. The indigo stain develops and indicates regions where the GUS reporter gene has
Drought Experiments

Water content analysis was performed according to the methods of Xu et al. [61]. At least 20 four-week-old plants were drenched in water for 30 min to achieve 100% soil saturation. The plants were divided into equal numbers, half receiving water as a control, whilst the other half did not receive any more water. The position of individual plants was randomized and plants were observed daily. After 9 days without water, the aerial tissues of well-watered whilst the other half did not receive any more water. The position of Xu

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