Cytokinin Response Factor 6 Represses Cytokinin-Associated Genes during Oxidative Stress


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Cytokinin is a phytohormone that is well known for its roles in numerous plant growth and developmental processes, yet it has also been linked to abiotic stress response in a less defined manner. Arabidopsis (Arabidopsis thaliana) Cytokinin Response Factor 6 (CRF6) is a cytokinin-responsive AP2/ERF-family transcription factor that, through the cytokinin signaling pathway, plays a key role in the inhibition of dark-induced senescence. CRF6 expression is also induced by oxidative stress, and here we show a novel function for CRF6 in relation to oxidative stress and identify downstream transcriptional targets of CRF6 that are repressed in response to oxidative stress. Analysis of transcriptomic changes in wild-type and crf6 mutant plants treated with H2O2 identified CRF6-dependent differentially expressed transcripts, many of which were repressed rather than induced. Moreover, many repressed genes also show decreased expression in 35S:CRF6 overexpressing plants. Together, these findings suggest that CRF6 functions largely as a transcriptional repressor. Interestingly, among the H2O2 repressed CRF6-dependent transcripts was a set of five genes associated with cytokinin processes: (signaling) ARR6, ARR9, ARR11, (biosynthesis) LOG7, and (transport) ABCG14. We have examined mutants of these cytokinin-associated target genes to reveal novel connections to oxidative stress. Further examination of CRF6-DNA interactions indicated that CRF6 may regulate its targets both directly and indirectly. Together, this shows that CRF6 functions during oxidative stress as a negative regulator to control this cytokinin-associated module of CRF6-dependent genes and establishes a novel connection between cytokinin and oxidative stress response.

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play a role in systemic stress signaling, as defined elsewhere (Petrov and Van Breusegem, 2012; Wrzaczek et al., 2013; Suzuki et al., 2012).

Phytohormones also play important roles in stress response signaling (O’Brien and Benková, 2013). One such hormone, cytokinin, is generally considered to be an antagonist of stress tolerance (Argueso et al., 2009; Ha et al., 2012; Zwack and Rashotte, 2015; Nguyen et al., 2016). In Arabidopsis (Arabidopsis thaliana), reduced cytokinin levels as a result of decreased synthesis or increased degradation have been found to increase tolerance to drought stress (Nishiyama et al., 2011; Macková et al., 2013). Compromised cytokinin signaling in mutants of various components of the cytokinin signaling pathway enhance drought, salt, and freeze tolerance (Tran et al., 2007; Jeon et al., 2010; Mason et al., 2010). Each of these conditions is known to stimulate ROS production; however, oxidative stress has not been directly examined in these mutants.

CYTOKININ RESPONSE FACTORS (CRFs) are a subset of the plant specific AP2/ERF domain-containing transcription factor family that function both downstream, and as a side branch, of the primary cytokinin signaling pathway (Rashotte et al., 2006; Cutcliffe et al., 2011). Arabidopsis CRF6 is transcriptionally induced by cytokinin and plays a role in delaying leaf senescence (Zwack et al., 2013). CRF6 expression is also induced in response to a wide range of stress stimuli, including oxidative stress in the form of both treatment with and endogenous production of H$_2$O$_2$ (Zwack et al., 2013; Inzé et al., 2012). In addition, CRF6 is a direct target of retrograde signaling in response to organellar dysfunction (De Clercq et al., 2013; Ng et al., 2013). As such, this transcription factor has been proposed to integrate cytokinin and stress responses as part of a finely tuned response network (Zwack et al., 2013).

Here, we demonstrate that CRF6 functions in mediating the response to oxidative stress, in part through the repression of a set of genes involved in cytokinin metabolism, transport, and signaling. Thus, we propose that CRF6 acts to attenuate cytokinin signaling as part of an adaptive response to stress.

RESULTS
Increased Expression of CRF6 Alters Response to Oxidative Stress

Expression of CRF6 is induced in response to a wide range of stress-associated conditions; therefore, we examined whether increased expression of CRF6 could alter oxidative stress response. CRF6 overexpression lines (35S:CRF6, denoted as CRF6oe) were generated, and phenotypes were observed under a range of oxidative stress conditions. Individual leaves excised from transgenic (CRF6oe) and azygous plants (wild type) were floated for 24 h on a solution containing either 20 mM H$_2$O$_2$ as an exogenous treatment or 10 mM 3-amino-1,2,4-triazole (3-AT), which inhibits catalase activity, resulting in an accumulation of endogenously produced H$_2$O$_2$. The maximal PSII quantum efficiency (F$_{v}$/F$_{m}$) of individual leaves was examined to determine the effect of these oxidative stress treatments on photosynthetic parameters (Fig. 1; Supplementary Fig. S1; Baker, 2008). Interestingly, the average F$_{v}$/F$_{m}$ of wild-type leaves (0.82) was significantly greater than that of CRF6oe leaves (0.78) prior to treatment (Fig. 1A). However, the reduction in F$_{v}$/F$_{m}$ after oxidative stress treatments was significantly different for the two genotypes, where the average F$_{v}$/F$_{m}$ of CRF6oe leaves was greater (0.75 and 0.60, respectively for H$_2$O$_2$ and 3-AT) than leaves of wild type (0.72 and 0.53; two-way ANOVA P = 0.0002; Fig. 1A).

We further examined the effects of overexpression of CRF6 using a bioassay in which gas exchange was restricted (restricted gas [RG]) to increase photorespiratory-dependent H$_2$O$_2$ accumulation (see “Methods” for details). Similar F$_{v}$/F$_{m}$ levels were observed for leaves of intact dark-adapted wild-type (0.75) and CRF6oe (0.76) plants (as opposed to excised leaves as in Fig. 1A) under standard in vitro growth conditions. However, after 4 d of RG stress, the average F$_{v}$/F$_{m}$ was higher in CRF6oe (0.67) compared to wild type (0.61; Fig. 1, B and C; Supplementary Fig. S1A). We also measured the operating PSII efficiency (ΦII) during a time course following exposure to normal actinic light and found that ΦII was significantly greater for CRF6oe plants under standard and RG conditions (Supplemental Fig. S1). ΦII can be broken down into its F$_{v}$/F$_{m}$ and qP components (Genty et al., 1989), and a stronger increase in the efficiency of excitation energy transfer to the reaction centers that are open (F$_{v}$/F$_{m}$) was observed for CRF6oe under stress conditions (Fig. 1E). Accordingly, we also observed lower heat produced as a result of dissipation of excitation energy (nonphotochemical quenching [NPQ]) for CRF6oe plants in a stress-specific manner (Fig. 1F). Under these conditions, we did not observe significant differences in any nondark adapted parameters for crf6 loss-of-function mutant plants. Nonetheless, the altered response to both exogenous treatment with and endogenous production of H$_2$O$_2$ demonstrated for plants overexpressing CRF6, along with the previously reported transcriptional regulation of CRF6 under oxidative stress conditions, suggests that this transcription factor plays a key role in the response to ROS, in particular to H$_2$O$_2$. Furthermore, to determine if CRF6 could affect responses to oxidative stress in different aspects of plant growth, root growth was examined in altered CRF6 expression lines in the presence and absence of antimycin A (AA), which interferes with the respiratory electron transport chain leading to the accumulation of ROS (Fig. 1D). Wild-type plants show a significant (P < 0.001) decrease in root growth to only 35% of untreated controls after AA treatment. CRF6oe roots were significantly (P < 0.01) less affected than wild type, whereas the knockout mutant crf6-2 showed the opposite and was more (P < 0.05) affected (Fig. 1D).
We hypothesized that as a transcription factor, CRF6 may regulate oxidative stress response by regulating the expression of certain genes as part of a genome-wide transcriptional reprogramming. To test this, we examined global transcriptome changes in response to H$_2$O$_2$ (20 mM) treatment after 6 h in 10-d-old wild-type and crf6 loss-of-function mutant plants by microarray analysis (Supplementary Dataset S1). Levels of 956 transcripts (606 induced and 351 repressed; fold change >2, adj. $P < 0.05$) were altered by H$_2$O$_2$ in wild type. Analysis of functional annotations associated with these transcripts indicated that the Gene Ontogeny (GO) terms “response to oxidative stress” and “response to hydrogen peroxide” were enriched with the highest confidence scores (Benjamini Hochberg, $P = 3.2E-18$ and $9.5E-18$, respectively), indicating that the

**Figure 1.** Overexpression of CRF6 affects alterations in chlorophyll fluorescence parameters induced by oxidative stress. A, Maximum PSII $F_{v}/F_{m}$ of excised leaves before and 24 h after treatment with 20 mM H$_2$O$_2$ or 10 mM 3-AT. B, $F_{v}/F_{m}$ of leaves of intact plants exposed to either normal growth conditions (control) or photorespiratory-induced oxidative stress conditions (RG). C, False-color heat map image of plants from B showing differences across plants. After determination of $F_{v}/F_{m}$ followed by 40 s of darkness, plants were exposed to actinic light, and maximum and steady-state fluorescence were determined every 20 s for 5 min. $F^{'}/F^{'}_m$, $E$, NPQ, $F_{PSII}$, PSII operating efficiency, and $q_{PSII}$ and $q_{P}$ (Supplemental Fig. S1) were calculated. D, Primary root growth (from day 4 to 9) under oxidative stress (5 mM AA) relative to untreated growth control for the given genotypes. Data points represent the mean ± SEM of $n = 8$ leaves for treatments and $n = 5$ leaves for controls, and $n = 10$ for roots from three independent experiments.

CRF6 Partially Mediates Transcriptional Response to H$_2$O$_2$

We hypothesized that as a transcription factor, CRF6 may regulate oxidative stress response by regulating the expression of certain genes as part of a genome-wide transcriptional reprogramming. To test this, we examined global transcriptome changes in response to H$_2$O$_2$ (20 mM) treatment after 6 h in 10-d-old wild-type and crf6 loss-of-function mutant plants by microarray analysis (Supplementary Dataset S1). Levels of 956 transcripts (606 induced and 351 repressed; fold change >2, adj. $P < 0.05$) were altered by H$_2$O$_2$ in wild type. Analysis of functional annotations associated with these transcripts indicated that the Gene Ontogeny (GO) terms “response to oxidative stress” and “response to hydrogen peroxide” were enriched with the highest confidence scores (Benjamini Hochberg, $P = 3.2E-18$ and $9.5E-18$, respectively), indicating that the
treatment had the intended effects on overall expression patterns. When we examined the expression of these oxidative stress-regulated genes under identical conditions in the crf6 mutant background, we found that a subset (147 or 15%) was regulated in a CRF6-dependent manner (having a >50% decrease in magnitude of fold change compared to the wild-type background; Fig. 2A). While only 49 (8.1%) of the oxidative stress-induced genes were CRF6 dependent (Supplemental Table S1), 98 of the 351 (28%) genes repressed in wild type showed CRF6 dependence (Supplemental Table S2). This bias suggests that CRF6 plays a larger role in repressing the expression of genes in response to oxidative stress. Importantly, we found the expression of 132 transcripts to be altered in crf6 under control conditions, yet these included only 6 CRF6-dependent-induced and 2 CRF6-dependent-repressed genes (Supplemental Tables S1 and S2). The magnitude of fold change of some transcripts was increased in crf6 compared to wild type (17 [2.8%] greater induction and 3 [0.9%] greater repression; Supplemental Table S3); for such genes, loss of CRF6 may have a more indirect effect.

Based on known DNA-binding specificity of other CRF proteins, CRF6 is predicted to interact with both GCC (AGCCG GCC) and DRE ([A/G]CCGAC) motifs (Weirauch et al., 2014). In addition, CRF6 was recently shown to interact with a cytokinin responsive element in the promoter of PIN genes encoding auxin efflux carriers. This element was shown to contain the core G2, G5, and C7 bases shared by the GCC and DRE motifs (Šimášková et al., 2015). We examined the 2-kb upstream promoters of genes found to be differentially expressed in a CRF6-dependent manner and found that 27 of the 49 potential positive targets and 63 of the 98 potential negative targets contained at least one DRE or GCC element. Therefore, regulation of many of these genes by CRF6 could be mediated by direct transcription factor-DNA binding interactions.

Given the H2O2-responsive expression of CRF6 and the H2O2-response phenotypes of CRF6oe, we investigated whether the constitutively elevated levels of CRF6 in CRF6oe plants was sufficient to regulate the potential target genes we had identified. To this end, transcriptomes of untreated wild-type and CRF6oe plants were compared. Upon examination of the 49 CRF6-dependent-induced genes, we found that transcript levels of 16 of these were significantly higher in the CRF6oe background. Similarly, 41 of the 98 CRF6-dependent-repressed genes were expressed at significantly lower levels in CRF6oe (Fig. 2B; Supplemental Tables S1 and S2). Thus, for these genes, CRF6 is necessary for a normal transcriptional response to H2O2 stress, and elevated levels of CRF6, independent of H2O2.

Figure 2. CRF6 regulation of transciptional response to H2O2. A, Heat maps comparing fold change in expression of transcripts in wild-type (WT) and crf6 backgrounds after treatment with H2O2. The genes represented are those found to be differentially expressed in WT. B, Pie charts showing the number of transcripts in each comparison that are CRF6 independent (blue), CRF6 dependent (red) and CRF6 dependent as well as similarly changed in CRF6oe vs. WT comparison (green). C, GO terms enriched among CRF6-dependent repressed genes with P value < 0.05.
stimulus, are sufficient for a similar transcriptional response. Those genes found to be regulated in a CRF6-dependent manner, for which CRF6 overexpression was not sufficient, may require other context-specific factors for proper regulation.

CRF6 Represses Cytokinin Related Genes in Response to Oxidative Stress

To identify specific processes potentially regulated by CRF6 during oxidative stress, we examined GO terms associated with the potential CRF6 targets. When we performed an enrichment analysis of CRF6-dependent-repressed genes, the biological processes over-represented with the greatest degree of confidence were “response to cytokinin stimulus” (13.3-fold enriched, \( P = 5.1 \times 10^{-4} \)) and “cytokinin mediated signaling” (17.3-fold enriched, \( P = 1.5 \times 10^{-3} \); Fig. 2C). Given the previously identified connections between CRF6 and cytokinin-mediated signaling/response, we chose to further examine potential targets related to these processes.

Among the CRF6-dependent-repressed genes, we identified four that encode for proteins involved in the two-component cytokinin signaling pathway (AHP1, ARR6, ARR9, and ARR11). AHP1 (ARABIDOPSIS HIS PHOSPHOTRANSFER PROTEIN 1) is involved in relaying the cytokinin signal from the membrane-bound receptors into the nucleus (Hutchison et al., 2006). ARR11 (ARABIDOPSIS RESPONSE REGULATOR 11) is one of 11 type-B response regulators (RR-Bs), which are GARP domain-containing MYB-related transcription factors activated within the nucleus by phosphorylation by AHPs. ARR11 is one of a subset of RR-Bs responsible for the majority of the transcriptional response to cytokinin (Hill et al., 2013). ARR6 and ARR9 are type-A response regulators, which lack DNA-binding capability and are able to compete with RR-Bs for phosphorylation to fine-tune the output of the signaling pathway (To et al., 2007). In addition to these signaling components, we identified two additional genes associated with cytokinin: LOG7 (LONELY GUY 7) and ABCG14 (ATP-BINDING CASSETTE G14). LOG7 catalyzes a key step in the synthesis of the active form of cytokinin, and ABCG14 is a transporter essential for long-distance translocation of cytokinin (Tokunaga et al., 2012; Ko et al., 2014). Together, these six genes are necessary components of three major aspects of cytokinin function in plants: signaling, biosynthesis, and transport.

We examined the expression of these potential targets in the transcriptomes of untreated CRF6oe and wild-type plants to determine whether constitutive overexpression of CRF6 is sufficient to repress these target genes. Strikingly, all but one (AHP1) were also expressed at lower basal levels in CRF6oe plants than in wild type (Fig. 3A). To confirm the regulation of these genes by CRF6 (as well as the transcriptome analysis in general), we examined expression patterns using qRT-PCR. The five cytokinin-related targets examined were found to be repressed by \( \text{H}_2\text{O}_2 \) stress in wild-type plants; this repression was absent or attenuated in crf6 mutants (Supplemental Fig. S2). Reduced expression in CRF6oe plants was also supported by qRT-PCR (Fig. 3B).

To determine whether CRF6 might repress these genes through direct promoter-binding interactions, we carried out a targeted yeast one-hybrid screen. Promoter segments from within 2 kb upstream of ATG, containing any GCC-motifs and/or degenerate versions thereof, were used as baits for the full-length CRF6 coding sequence fused to the GAL4 activation domain as prey. The only high-confidence interaction detected was between CRF6 and the promoter of ARR6 (Fig. 3C; Supplementary Figs. S3 and S4). Interestingly, ARR6 was also the only promoter sequence tested that contained a true, nondegenerate GCC-motif (AGCGCGCC). A recent study using a root protoplast-based luciferase activation assay demonstrated that CRF6 regulates the expression of auxin transporters (PINs) in Arabidopsis roots. This regulation was shown to occur through interactions of CRF6 with upstream enhancer elements containing the motif AG[A/C]AGACG which has the conserved G2, G5, and C7 bases (underlined) that are essential for interaction with AP2/ERF family transcription factors (Simášková et al., 2015). As similar motifs are present in the promoters of each of the cytokinin-related targets, we employed the same assay here to determine whether CRF6 could activate these promoters within the context of a plant cell. In this assay, CRF6 was again found to activate with the ARR6 promoter similar to the Y1H direct binding (Fig. 3D). We also found CRF6 interactions with the promoters of ARR9 and LOG7 not found in Y1H, suggesting that additional plant-specific factors are required for these interactions. Different from Y1H, the protoplast-luciferase-activation assay has been shown to be capable of differentiating between inductive and repressive regulation. Although our prior results indicate that CRF6 acts to repress these genes, expression was shown as induced in this system. This unexpected result may again reflect the context-specific nature of CRF6 repression during oxidative stress. For example, stress-induced posttranslational modifications or additional transcription factors not expressed in root protoplasts may be required for proper regulation. Despite this ambiguity, these data indicate that CRF6 regulates the expression of ARR6 (and possibly others) through direct interaction with its promoter.

To gain further insight into and support for the role of the cytokinin-associated genes downstream of CRF6 in oxidative stress response, we examined whether mutants of these target genes have altered oxidative stress response phenotypes. We obtained T-DNA insertional mutants of the five cytokinin-associated genes with reduced basal expression in CRF6oe plants (arr6, arr9, arr11, abcg14, and log7; To et al., 2007; Ko et al., 2014; Alonso et al., 2003). Both whole seedlings and leaves from these mutants, along with the lines used for transcriptome analyses (CRF6oe19.3, crf6-2, and Col-0) and an additional, previously described line overexpressing
CRF6 (CRF6ox3 from Zwack et al., 2013), were treated with H2O2 (20 mM) or AA (5 mM) for 48 h. As previously observed, CRF6-overexpressing plants demonstrated significantly less reduction in Fv/Fm after H2O2 treatment of both seedlings and detached leaves compared to mock-treated controls. We observed a similar stress-related phenotype for all five target gene mutants (arr6, arr9, arr11, log7, and abcg14; Fig. 4, A and B). To further examine these lines after oxidative stress along with Fv/Fm measurements, Rfd the fluorescence decline ratio was measured in leaves (Lichtenthaler and Miehé, 1997; Zhang et al., 2016). Results of Rfd levels after oxidative stress H2O2 (20 mM) and AA (5 mM) treatments (Fig. 4, C and D) parallel the Fv/Fm findings after H2O2 (Fig. 4, A and B). CRF6oe lines had significantly less reduction in Rfd than wild type, while crf6-2 had a significantly greater reduction and target gene mutants were generally affected in a manner similar to the CRF6oe lines. In addition to these changes in photosynthetic efficiency, CRF6oe and cytokinin mutant leaves also displayed less visible discoloration than wild type after oxidative stress exposure (Fig. 4E). Finally, chlorophyll levels were determined in leaves of these same lines in the presence and absence of H2O2 (20 mM). The reduction in chlorophyll levels, shown as a percent of untreated levels, again paralleled both Fv/Fm and Rfd measurements (Fig. 4F; Supplementary Fig. S5). Overall, these stress-related phenotypic similarities to CRF6oe lines support these five genes as targets of repression functionally downstream of CRF6. Importantly, this repression also seems to be necessary for wild-type response to stress under these conditions, as crf6 mutants (in which the downregulation of these genes does not occur) shows significantly decreased oxidative stress response in both whole seedlings and detached leaves (Fig. 4). Collectively, the results presented in this study demonstrate a mechanistic link between cytokinin and oxidative stress: a set of cytokinin associated genes are repressed downstream of CRF6 in response to H2O2 and the resulting low levels of expression modulate the response to oxidative stress.

**DISCUSSION**

Plant responses to oxidative stress provoking conditions are crucial for survival. As such, these responses are highly regulated and must be specific to conditions both external, such as the environment, as well as internal, such as developmental status. This regulation can be achieved through cross-talk between various signaling pathways, including several plant hormones.
known to have a role in the regulation of stress responses; such a role for cytokinin has recently gained support (O’Brien and Benková, 2013; Argueso et al., 2009; Ha et al., 2012; Zwack and Rashotte, 2015). Expression of the Arabidopsis transcription factor CRF6 is induced by both cytokinin and oxidative stress, indicating a possible connection between these pathways (Zwack et al., 2013; De Clercq et al., 2013; Ng et al., 2013). In response to cytokinin, CRF6 has been shown to play a role in the inhibition of leaf senescence; however, the functional relevance of its induction by oxidative stress was unknown. Interestingly, transcriptional induction of CRF6 in response to stress appears to be independent of cytokinin; a comparison of transcriptional changes as a result of salt stress in wild-type and cytokinin-deficient mutant Arabidopsis plants showed that expression of CRF6 was up-regulated in both backgrounds (Nishiyama et al., 2012). Here, we demonstrated that expression of CRF6 partially mediates responses to H₂O₂, and that plants constitutively over-expressing CRF6 are able to maintain both photosynthetic efficiency and root growth better than wild-type plants when exposed to oxidative stress-inducing conditions (Fig. 1).
To examine the mechanism by which this transcription factor regulates response to oxidative stress, we analyzed genome-wide differences in transcriptional response to H₂O₂ of crf6 knockout mutants and wild-type plants. From this analysis, genes requiring CRF6 for their responsiveness to H₂O₂ or CRF6-dependent genes, were identified. A much greater proportion of CRF6-dependent genes was repressed (28%) than induced (8%) in wild type in response to H₂O₂ stress, suggesting that CRF6 functions largely as a transcriptional repressor under these conditions (Fig. 2B; Supplemental Tables S1 and S2). Interestingly, we identified a set of six genes involved in various fundamental aspects cytokinin function (biosynthesis, transport, and signal transduction) that are repressed downstream of CRF6 during oxidative stress. We further investigated these genes as a potential functional module of cytokinin-associated genes that is repressed during oxidative stress (Fig. 3). We demonstrated oxidative stress response phenotypes for the arr6, arr9, arr11, log7, and abcg14 loss-of-function mutants that are similar to plants overexpressing CRF6. In addition, we found crf6 loss-of-function plants have the opposite oxidative-stress-response phenotype to the overexpression lines. Together, this strongly supports these genes as being repressed by CRF6 in response to oxidative stress and the notion that this repression is part of the mechanism by which oxidative stress response is altered in CRF6oe plants (Fig. 4). More detailed analyses of CRF6-target-promoter interactions indicated regulation by CRF6 may be indirect for most genes in the module (Fig. 3). Nonetheless, our transcriptional analyses in conjunction with the mutant phenotype results clearly indicate that CRF6 is involved in the oxidative-stress-triggered repression of these genes. Importantly, we identified an interaction between CRF6 and the promoter of ARR6, suggesting that CRF6 may act to directly regulate some of its targets (Fig. 3, C and D).

Our findings provide strong evidence that CRF6 mediates a crucial interaction between oxidative stress responses and cytokinin. Cytokinin is involved in a wide range of indispensable processes throughout the plant, yet it is known to have a negative impact on stress tolerance (O’Brien and Benková, 2013; Úrbez-Torres et al., 2009; Ha et al., 2012; Zwack and Rashotte, 2015). Additionally, increased cytokinin levels have been found to increase endogenous ROS production (Wang et al., 2015). If and by what means a plant could overcome the antagonistic effects of cytokinin has until now remained unclear. Our results provide a clear mechanism by which this effect can occur: in response to oxidative stress, CRF6 down-regulates the expression of genes involved in various aspects of the biological function of cytokinin (biosynthesis, transport, and signaling), possibly allowing for improved stress response. Here, we have used oxidative stress to demonstrate this mechanism, but we speculate that a similar response to other stresses may also occur, as a wide range of stress conditions can lead to both ROS production and increased expression of CRF6.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All transgenic lines were generated in the Col-0 background. Both CRF6ox3 and crf6−/− (Zwack et al., 2013) and CRF6ox19.3 and 19.7 (Inzé et al., 2012) were previously described. The CRF6 overexpression line 19.3 was kindly provided by Frank Hoeberichts. T-DNA insertional mutants arrr9, arr11-3 (SALK_006544), log7 (SALK_113173C), and abcg14 (SK_15918) were obtained from the Arabidopsis Biological Resources Center. Unless otherwise stated, plants were sterilized and sown on plates containing full-strength Murashige and Skoog (MS) medium and 1% Suc buffered with MES at pH 5.7. After 2 d at 4°C, plates were moved to a controlled environmental chamber and grown under diurnal conditions of 16 h light (100 µE at 22°C) and 8 h dark at 18°C. For root growth, seedlings were transferred at day 4 to new plates either as described above (MS) or supplemented with 5 mM AA and allowed to grow until day 9 when the day 4 to 9 growth was measured as previously described (Rashotte et al., 2006). Root growth was measured in three independent experiments with 10 or more samples per replicate (±) per treatment. For extended growth (>10 d after germination), plants were moved to soil (safelight mix no. 8) and grown under similar conditions but at approximately 150 µE light.

Chlorophyll Fluorescence Analyses

Treatments with H₂O₂, AA, or 3-AT were carried out on either whole seedlings (3 d old) or from leaves 3/4 or 5/6 excised from 14- to 20-d-old plants as noted in the text. Leaves were cut and immediately floated abaxial side down on deionized water buffered with 3 mM MES (pH 5.7). Cut leaves were dark-adapted for 30 min before illumination. Measurement of chlorophyll fluorescence was measured using a standard setting from FluoroCam7 software on a Handy FluorCam, Photon Systems Instruments: Fv/Fm = (Fm-Fo)/Fm as maximum PSII quantum efficiency or Rhd = (Fp - Ft_Lss) / Fp, the fluorescence decline ratio using peak fluorescence (Fp) and steady-state fluorescence in the terminal light-adapted phase (Ft_Lss). Leaves were then transferred to individual wells of 6- to 24-well plates containing a similarly buffered solution of either 20 mM H₂O₂, 5 mM AA, or 10 mM 3-AT. The plates were covered (not sealed) and returned to the growth chamber under normal conditions. After 24 h, leaves were again dark-adapted and chlorophyll fluorescence was measured. For chlorophyll measurements, cut leaves treated as above were weighed, then placed in methanol 4C overnight and examined using a spectrophotometer to yield chlorophyll levels (nmol/mg fresh weight) as previously described (Zwack et al., 2013). All experiments were performed three to six times with three to ten samples per replicate (±) for each treatment.

For the photosynthetic-promoting conditions, plants were grown on MS medium for 2 weeks at 21°C and 100 µE light intensity in a 16-h-light/8-h-dark photoperiod. Then, plates were sealed with two layers of parafilm. Root growth was measured in three independent experiments with 10 or more samples per replicate (±) for each treatment. For the photosynthetic-promoting conditions, plants were grown on MS medium for 2 weeks at 21°C and 100 µE light intensity in a 16-h-light/8-h-dark photoperiod. Then, plates were sealed with two layers of parafilm. Root growth was measured in three independent experiments with 10 or more samples per replicate (±) for each treatment.

Expression Analyses

Ten-day-old plants were removed from plates and floated on 3 mM MES buffer pH 5.7. After 1 h treatment, sample had H₂O₂ added to a final concentration of 20 mM. Treatment and control solutions both contained 0.2% dimethyl sulfoxide. Plants were treated for 6 h then immediately frozen in liquid nitrogen. For each treatment, approximately 10 individual plants were pooled for analysis. RNA from two independent experiments was isolated using the Qiagen RNeasy Plant Mini-kit according to the manufacturer’s instructions. Hybridization to Affymetrix Arabidopsis Gene 1.0 ST arrays, scanning, and raw data preprocessing were performed as a service by the Hefflin Center for Genomic Science at the University of Alabama-Birmingham.

Normalization and differential expression analyses were performed using the FlexArray 1.6 software package from McGill University and Genome Quebec. Normalization was performed using algorithms provided by Affymetrix. Fold change in expression was calculated by Cyber-T analysis, and
adjusted P values were determined using the Benjamini Hochberg method of False Discovery Rate.

qRT-PCR verification was carried out using Sybr-Green and sequence specific primers. Reactions were carried out as previously described (Zwack et al., 2013).

Yeast One-Hybrid

The enhanced yeast one-hybrid assay was performed as previously reported (Gaudinier et al., 2011). Briefly, the promoters of ABCG14, AHP1, ARR6, ARR9, ARR11, and LOC7 were amplified via PCR (5x Phire reaction buffer, 0.25 μM of each of the primers, 0.2 mM dNTPs, Phire Hot Start II DNA Polymerase [Thermo Scientific], and Arabidopsis genomic DNA) using primers designed to introduce the Gateway attP4 and attP1 cassette sequences. The initial denaturation step occurred at 94°C for 5 min followed by 40 cycles of 18 s at 94°C, 30 s at 58°C, and 1 min at 72°C. The final elongation step was 7 min at 72°C. The PCR products were transferred into the pDONR P4-P1R vector (Invitrogen) using Gateway BP recombination (2 μL 5x BP Clonase II enzyme mix, 150 ng PCR product, 150 ng empty P4-P1R vector). P4-P1R promoter Entry clones were transferred into the pMWH2 vector containing the reporter gene HIS3 by Gateway LR recombination (1 μL 5x LR Clonase II enzyme mix, 150 ng P4-P1R Entry clone, and 150 ng empty pMW2 vector). Additionally, CRF6 was transferred to a pDEST-AD vector by LR cloning to create a CRF6-Gal4 activating domain fusion protein.

The promoter HIS3 sequences were integrated into the genome of the haploid yeast (Saccharomyces cervisiae) strain YM4271, and the pDEST-AD-CRF6s was transformed into the strain Ya1867 as previously described to create the bait and prey strains, respectively (Deplancke et al., 2006; Reece-Hoyes et al., 2011). The transformed haploid baits were grown on minimal selective media containing all necessary amino acids except His (synthetic dropout [SD-HT]) and the transformed haploid preys were selected on SD-T (Trp). The haploid baits and preys were mated pairwise overnight in liquid yeast extract peptone dextrose. The resulting diploid yeast cells were grown overnight in liquid yeast extract peptone dextrose. The resulting diploid yeast cells were plated on solid SD-HT media containing varying concentrations (from 0 to 100 mM) of 3-AT. The pDEST-AD vector was also transformed into Ya1867 and mated with each bait to serve as a negative control.

Transient Expression in Root Suspension Culture Protoplasts

The luciferase assays were performed on 4-d-old Arabidopsis (Arabidopsis thaliana) root suspension culture by PEG-mediated transformation. Protoplasts were isolated in enzyme solution (1% cellulase; Serva, 0.2% Macerozyme; Yakult in B5-0.34M Glc-mannitol solution; 2.2 g MB with vitamins, 15.25 g Glc, 15.25 g mannitol, H2O to 500 mL, pH to 5.5, with KOH) with slight shaking for 3 to 4 h, centrifuged at 800 g for 5 min. The pellet was washed with B5-0.34M Glc-mannitol solution and resuspended in B5-0.34M Glc-mannitol solution to a final concentration of 2 x 105 protoplasts per 50 μL. Protoplasts were cotransfected with 1 μg of a reporter plasmid that contained Firefly luciferase (fLUC), 1 μg of effector construct, and 2 μg of normalization plasmid expressing the Avenille luciferase (rLUC) under the control of the 35S promoter. The total amount of DNA was equalized in each experiment with the p2GW7-GLUS mock effector plasmid. DNAs were gently mixed together with 50 μL of protoplast suspension and 60 μL of PEG solution [0.1M Ca(NO3)2, 0.45M mannitol, 25% PEG 6000] and incubated in the dark for 30 min. Then 140 μL of 0.25M Ca(NO3)2 solution was added to wash off PEG and waited for sedimentation of protoplasts and removed 240 μL of supernatant. The protoplast pellet was resuspended in 200 μL ofB5-0.34M Glc-mannitol solution and incubated for 16 h in the dark at room temperature. After transfection, protoplasts were centrifuged at 1200 g for 5 min and analyzed; fLUC and rLUC activities were determined with the Dual-Luciferase reporter assay system (Promega). Variations in transfection efficiency and technical errors were corrected by normalization of fLUC by the rLUC activities. The mean value was calculated from six measurements and experiment was repeated two times.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GSE844770.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Chlorophyll fluorescence responses to photorespiratory-inducing conditions of plants overexpressing CRF6.

Supplemental Figure S2. Graph of ratio of expression H2O2/mock in the wild-type and crf6 backgrounds as determined by qRT-PCR.

Supplemental Figure S3. Schematic representation of promoter sequences used in luciferase transactivation and yeast one-hybrid assays.

Supplemental Figure S4. Results of yeast one-hybrid screen.

Supplemental Figure S5. Effects of oxidative stress on chlorophyll levels of cytokinin-related targets of CRF6.

Supplemental Table S1. Expression of CRF6-dependent-induced genes.

Supplemental Table S2. Expression of CRF6-dependent-repressed genes.

Supplemental Table S3. Expression of genes showing enhanced regulation in the absence of CRF6.

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LITERATURE CITED


CRF6 Represses Cytokinin Genes during ROS


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