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Research article

Oxidative stress provokes distinct transcriptional responses in the stress-tolerant atr7 and stress-sensitive loh2 Arabidopsis thaliana mutants as revealed by multi-parallel quantitative real-time PCR analysis of ROS marker and antioxidant genes

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Abstract

The Arabidopsis thaliana atr7 mutant is tolerant to oxidative stress induced by paraquat (PQ) or the catalase inhibitor aminotriazole (AT), while its original background loh2 and wild-type plants are sensitive. Both, AT and PQ, which stimulate the intracellular formation of H2O2 or superoxide anions, respectively, trigger cell death in loh2 but do not lead to visible damage in atr7. To study gene expression during oxidative stress and ROS-induced programmed cell death, two platforms for multi-parallel quantitative real-time PCR (qRT-PCR) analysis of 217 antioxidant and 180 ROS marker genes were employed. The qRT-PCR analyses revealed AT- and PQ-induced expression of many ROS-responsive genes mainly in loh2, confirming that an oxidative burst plays a role in the activation of the cell death in this mutant. Some of the genes were specifically regulated by either AT or PQ, serving as markers for particular types of ROS. Genes significantly induced by both AT and PQ in loh2 included transcription factors (ANAC042/JUB1, ANAC102, DREB19, HSFA2, RRF1, ZAT10, ZAT12, ethylene-responsive factors), signaling compounds, ferritins, alternative oxidases, and antioxidant enzymes. Many of these genes were upregulated in atr7 compared to loh2 under non-stress conditions at the first time point, indicating that higher basal levels of ROS and higher antioxidant capacity in atr7 are responsible for the enhanced tolerance to oxidative stress and suggesting a possible tolerance against multiple stresses of this mutant.

1. Introduction

The increase in cellular levels of reactive oxygen species (ROS) known as oxidative stress is a consequence of many adverse abiotic conditions such as drought, salinity, extreme temperatures, high light, pollutants, as well as biotic interactions triggering a hypersensitive response [1,2]. In most cases, ROS levels are elevated as a result of both, enhanced ROS production and impaired detoxification. Furthermore, two or more adverse conditions often act simultaneously, enhancing the production of ROS and complicating the ROS response [3]. Plants have evolved an elaborate enzymatic and non-enzymatic antioxidant system, which together with the various ROS-producing enzymes constitute a highly sophisticated and redundant network [2]. By developing such a complex network, plants can manage with ROS toxicity and use ROS as signaling molecules to modulate growth, important developmental processes, and plant responses to the environment [2].

Programmed cell death (PCD) occurs during several developmental processes, such as embryonic development, nucellar degeneration, maturation of tracheal elements and epidermal trichomes, formation of lobe leaf shape, and leaf senescence, as well as during the hypersensitive response to avirulent pathogens [4]. On the other hand, PCD can be a destructive consequence of extreme abiotic and biotic stress [4]. In many cases, ROS together with other signalling molecules can modulate PCD [4,5].
The necrotrophic fungus Alternaria alternata f.sp. lycopersici triggers PCD in tomato by secreting AAL-toxin, which in turn inhibits sphinganine N-acyltransferase (a key enzyme in sphingolipid biosynthesis), perturbs sphingolipid metabolism, and induces oxidative burst [6,7]. The sensitivity of tomato to the AAL-toxin is due to a mutation in the Asc gene that encodes sphinganine N-acyltransferase [8,9]. Knocking out one of the Arabidopsis thaliana Asc homologues resulted in the loh2 mutant, which is more sensitive to the AAL-toxin than the wild type [10]. However, loh2 has the same sensitivity as the wild type to oxidative stress induced by the catalase inhibitor aminotriazole (AT) or by the herbicide paraquat (PQ), agents that cause the endogenous accumulation of hydrogen peroxide or superoxide radicals, respectively. It has the same growth rate and similar phenotype as its original background A. thaliana ecotype Wassilewskija under optimal growth conditions [10]. In order to find mutants perturbed in oxidative stress-induced cell death, a large-scale screen of chemically mutagenized loh2 seeds was carried out. The screening recovered nine atr (AAL-toxin resistant) mutants, five of which were more tolerant to AT- and PQ-Induced PCD, with one of them, atr7, exhibiting the strongest oxidative stress tolerance [11]. The atr7 mutant tends to have slightly less biomass expressed by fresh weight. Some of the other atr mutants have more striking phenotypes in absence of stress. For example, atr2 and atr6 are much smaller than both loh2 and atr7; furthermore, atr2 has delayed senescence and atr6 has altered leaf shape [11]. Crosses between the different atr lines and rough mapping indicated that the mutations in all these lines are in different genes. As atr7 has the strongest oxidative stress-tolerant phenotype on AT- or PQ-supplemented media, it was our obvious choice for further analysis.

This paper reports on the employment of two platforms for multi-parallel quantitative real-time PCR (qRT-PCR) analysis of antioxidant and ROS marker genes. Due to their sensitivity and the presence of gene markers specific for particular types of ROS (hydrogen peroxide, superoxide radicals, and singlet oxygen), the two platforms are particularly helpful for studying gene expression of low-abundant transcripts and for detecting fluctuations in specific types of ROS. The platforms are used in this paper as a tool to study gene expression during AT- and PQ-induced oxidative stress in loh2 and atr7 mutants under conditions that trigger oxidative stress-dependent PCD in loh2 but no visible cell damage in atr7.

2. Results

2.1. Atr7 is tolerant to AT- and PQ-induced oxidative stress

ROS levels can be modulated by either perturbing the antioxidant system or by treating plants with specific ROS generators [12–14]. We used both approaches to generate hydrogen peroxide (H2O2) and superoxide radical (O2−) by treating the plants with AT and PQ, respectively. Catalases are the main H2O2-detoxifying antioxidant enzymes in plants with paramount importance for stress tolerance [15]. AT is a potent catalase inhibitor; when applied exogenously by spraying or when added to the plant growth media, it causes H2O2 accumulation and subsequent cell death [12]. PQ accepts electrons from photosystem I or ferredoxin and transfers them to molecular oxygen, producing the destructive O2− [16]. The biological effect of both herbicides is light-dependent. Application of either 7 μM AT or 1 μM PQ in plant growth media caused reduction in growth, chlorophyll loss, and eventually cell death of loh2 (Figs. 1 and 2). At the conditions used, growth inhibition and chlorophyll loss were more prominent under PQ than AT stress; in both cases the loh2 plants were severely affected, in contrast to atr7 seedlings that retained chlorophyll under stress and were less strongly impaired at the level of biomass accumulation (Fig. 2 A and B), demonstrating its tolerance to oxidative stress. Cell death in the presence of PQ was evident 7 days after germination (Fig. 1), while only growth inhibition but no cell death was observed in 4-day-old seedlings (not shown). The sensitivity of loh2 to AT and PQ was similar to that of its original A. thaliana, ecotype Wassilewskija wild-type background (data not shown).

Glutathione is a multifunctional metabolite in plants that is involved in maintenance of cellular redox homeostasis and transmitting oxidative stress signals, as well as in defense reactions [17]. It is also part of the intricate plant antioxidant system [18]. The levels of reduced (GSH) and oxidised (GSSG) glutathione were determined in both mutants (Fig. 3). Interestingly, atr7 had higher reduced and total glutathione levels than loh2 under normal growth conditions, both in four- and seven-day-old seedlings. In loh2, AT reduced the GSH level at the two time points while PQ elevated oxidized glutathione in seven-day-old loh2 seedlings (Fig. 3). In contrast, none of the treatments increased the GSSG in atr7.

2.2. Gene expression of ROS marker and antioxidant genes under AT- and PQ-induced oxidative stress

In order to understand more about the molecular mechanisms that may contribute to the stress tolerance in atr7, two platforms for multi-parallel qRT-PCR analysis of (1) ROS marker genes and (2) antioxidant genes were employed to compare gene expression in loh2 and atr7 under normal conditions and under oxidative stress, generated by AT and PQ. The ROS marker gene platform contained 180 genes, selected on the bases of our own ROS-related experiments and on literature surveys [10,14,19]. The list of genes given in

Fig. 1. Atr7 is tolerant to oxidative stress-induced programmed cell death. Loh2 and atr7 plants were grown on MS plant media supplemented or not with either aminotriazole (AT) or paraquat (PQ), two ROS inducing agents that elevate the endogenous hydrogen peroxide or superoxide radical levels, respectively. The picture was taken 7 days after germination.
**Supplemental Table 1** contains four groups: (i) genes known to primarily respond to H$_2$O$_2$, (ii) genes inducible by superoxide radicals, (iii) genes responsive to singlet oxygen, and (iv) genes commonly regulated by all types of ROS. In the platform included were genes known to encode important players of ROS and abiotic stress signaling such as various protein kinases and phosphatases (ATMPK11, ATPK19, OXI1, one of the PP2Cs), signaling peptides (PROPEP3), and transcription factors and co-regulators (JUB1/ANAC042, ANAC047, ANAC055, ANAC102, DREB2A, DREB19, ERFS, RRTF1, STZ, WRKY30, WRKY33, WRKY40, WRKY75, ZAT6, ZAT10, ZAT11, ZAT12). Downstream players of signaling cascades as well as many heat shock and disease-related genes were also included. The gene selection not only enables the evaluation of responses towards different oxidative and abiotic stresses (drought, salinity, cold etc.) but also an indirect assessment of the presence of particular types of ROS during the investigated stress responses. For example, the heat shock regulon and the 17.4 kDa class III heat shock protein in particular are markers for hydrogen peroxide burst during the process or stress response studied. Other genes such as At1g64160 and At5g64905, which respectively encode a disease resistance-responsive protein and an elicitor peptide 3 precursor, are markers for the presence of superoxide radicals, while At2g21640 (unknown protein) is an established hallmark gene for oxidative stress [12,20,21], so their induction will be an indication for H$_2$O$_2$ burst in four- and seven-day-old seedlings germinated either on AT- or PQ-containing Murashige and Skoog (MS) medium. Log$_2$ ratios of expression changes were calculated for both mutants by comparing gene expression levels in seedlings grown on AT- or PQ-supplemented MS medium with those in plants grown on MS medium without any ROS-inducing agents. Additionally, atr7 and loh2 grown on plain MS medium were compared with each other to evaluate the differences between these two mutants when developing in non-stress conditions. The ROS marker genes from the four individual groups (consisting of H$_2$O$_2$-responsive, O$_2^-$- and $^{1}$O$_2^-$- and common ROS-responsive genes) were subjected to hierarchical average clustering using the MultiExperiment Viewer program (Fig. 4). Fig. 5 graphically presents the intensity of the transcriptomic responses within the individual ROS marker groups.

Both, AT and PQ invoked massive transcriptional reprogramming in loh2 and atr7 at the two developmental time points. The strongest response was observed in loh2 on PQ, especially on the fourth day after germination (Fig. 4A, Supplemental Table 1). At this time point all O$_2^-$-responsive, 50 of the 53 H$_2$O$_2$-responsive, 19 of the 22 $^{1}$O$_2^-$-responsive, and 85 of the 87 common ROS marker genes were upregulated by at least 2-fold. AT also predominantly induced most of the ROS marker genes at this time point (Fig. 4A). In contrast, AT and PQ did not induce so many genes in atr7; instead, downregulated and upregulated genes were similar in numbers. At
the second time point, however, the AT- and PQ-induced genes predominated in both mutant lines (Fig. 4; Supplemental Table 1). Nevertheless, genes in atr7 were induced to a lesser extent compared with loh2. Genes known as H2O2-responsive were induced by both AT and PQ, probably because PQ-generated superoxide radicals are eventually converted to H2O2. Genes known as superoxide responsive are induced only by PQ and can therefore serve as marker genes for superoxide accumulation. The

Fig. 4. Heat map of ROS-regulated transcripts in loh2 and atr7 plants subjected to AT- and PQ-induced oxidative stress. Loh2 and atr7 were grown on MS media or MS supplemented with 7 μM AT or 1 μM PQ. Seedlings were collected on the fourth and seventh day after germination. Expression levels were determined by multi-parallel qRT-PCR analysis. The ROS marker genes from the four individual groups, encompassing H2O2-, O2*--, 1O2*- and common ROS-responsive genes, were subjected to hierarchical average clustering using the MultiExperiment Viewer program. Red and green indicate higher and lower expression values, respectively. Intensity of the colors is proportional to the absolute value of log2 of the fold difference in expression. Black indicates no change in gene expression. Two biological repetitions with two technical replicates in each repetition were performed. For the gene orders in the heat maps please refer to Supplemental Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
induction of \(^{1}\text{O}_2\)-responsive genes suggests that additional mechanisms could be activated, leading to \(^{1}\text{O}_2\) in \(\text{loh}_2\) after PQ treatment at this time point. Genes induced or repressed by many ROS are indeed regulated by both, AT and PQ.

One-hundred-and-two of the 180 ROS marker genes were induced more than 2-fold in \(\text{atr}_7\) compared to \(\text{loh}_2\) under normal conditions at the first developmental time point (Fig. 4; Supplemental Table 1). The group of ROS-responsive genes that are more abundantly expressed in \(\text{atr}_7\) compared with \(\text{loh}_2\) included the transcription factor \(\text{HSFA2}\) and the entire heat shock regulon, other transcription factors related to abiotic and oxidative stress (\(\text{ZAT11}, \text{RRTF1}, \text{DRE2B}\)), and several genes known as general markers for oxidative stress (Table 1; Supplemental Table 1). Interestingly, ferritins, alternative oxidases, and other antioxidant genes were also upregulated in \(\text{atr}_7\) relative to \(\text{loh}_2\) under non-stress conditions (Fig. 6; Supplemental Table 2). The same genes were induced by AT and PQ in \(\text{loh}_2\), but not in \(\text{atr}_7\) (Figs. 4 and 6; Supplemental Table 1; Supplemental Table 2). These data collectively indicate higher basal levels of ROS in \(\text{atr}_7\) and constant transcription of these genes. The fact that there are a number of transcription factors associated with oxidative and abiotic stress in this group indicates that other genes that are controlled by these transcription factors (but not represented by the platforms) might also have been induced and that the constant expression of those genes in \(\text{atr}_7\) might additionally provide tolerance to abiotic stresses other than AT and PQ.

Regarding the antioxidant gene platform, the most striking observation was that all antioxidant gene families had members induced by PQ in \(\text{loh}_2\) four days after germination, including all \(\text{AOX}\) genes (Fig. 6). Another surprising observation was that oxidative stress actually decreased the transcript levels of most antioxidant genes in \(\text{atr}_7\) at the first developmental time point. However, many of these genes became induced at the second time point (Fig. 6).

3. Discussion

Chemically distinct ROS are produced simultaneously within plant cells under stress conditions, making it virtually impossible to attribute visible stress symptoms to particular types of ROS. Application of the catalase inhibitor AT or/and the superoxide generator PQ in growth media is an ideal approach to examine the consequences of increased levels of endogenous \(\text{H}_2\text{O}_2\) and \(\text{O}_2^-\) because of the non-invasive nature of the treatment and the sustained disturbance in ROS homeostasis over a prolonged period of time. Furthermore, desired high or low levels of ROS can be obtained by adjusting the concentrations of the ROS inducing agents. Under the conditions used, \(\text{loh}_2\) developed cell death symptoms under both, AT and PQ treatment, while \(\text{atr}_7\) was clearly tolerant to the two ROS inducing agents. The phenotype of AT-dependent cell death was different from the PQ-induced cell death, as seen by the morphological, biochemical, and gene expression differences. PQ caused much more pronounced growth inhibition in both mutants. When grown on PQ, \(\text{loh}_2\) seedlings were unable to fully develop their cotyledons, while when grown on AT they expanded their cotyledons but then started yellowing and eventually died without developing any further. The oxidative stress-induced growth inhibition is apparently a common response of all plants and also occurs in \(\text{atr}\) mutants, as evidenced by their reduction of fresh weight and repression of growth-related genes [11,25]. However, the \(\text{atr}_7\) mutant eventually overcomes the oxidative stresses and
plants during AT- and PQ-induced oxidative stress. Oxidative stress, as well as various types of abiotic stresses, can stimulate glutathione synthesis and induce a shift from this highly reduced state towards a more oxidized GSSG form [26–28]. On the other hand, ROS such as O$_2^•$ and hydroxyl radical are able to directly oxidize GSH and thus produce GSSG at high rates [17,18]. GSH not only serves as a direct scavenger of ROS, preventing excessive oxidation of sensitive cellular components, but also as a substrate for various antioxidant enzymes, including glutathione reductases, glutathione peroxidases, and glutathione-S-transferases [17,18]. The slight reduction in GSH content of AT-treated plants is in contrast with the generally observed increase in total glutathione of AT-treated and catalase2 (cat2) antisense plants [26,27,29,30]. Interestingly, atr7 had more GSH than loh2 when plants were grown on normal medium, which may be explained by a stimulation of GSH synthesis.

Because of their superior sensitivity, the two multi-parallel qRT-PCR platforms for ROS marker and antioxidant genes are a valuable tool for studying gene expression under various types of oxidative or abiotic/biotic stresses. Expression analysis by microarrays is widely used for expression profiling to study transcriptional effects under different growth conditions and stress treatments. However, weakly expressed genes are difficult to evaluate with microarrays because signal intensities of their transcript levels are often not much above background. For example, according to microarrays studies [25], the transcription factor genes DREB2A and HSFA2 have low levels of expression and are only weakly induced by AT in loh2. The induction of these genes by AT in loh2 is much more evident in the current study where we used the qRT-PCR technology to measure transcript abundance (Table 1). Furthermore, the presence of many genes specific for particular types of ROS in the qRT-PCR platforms allows an indirect but accurate assessment of endogenous levels of particular ROS (e.g., hydrogen peroxide, superoxide radicals, singlet oxygen). Direct measuring ROS in plants, particularly H$_2$O$_2$, is complicated by several interfering factors like ascorbate and polyphenols [31,32]. Not surprisingly, there is high variability in reported H$_2$O$_2$ contents in the literature, even in the absence of stress and between studies using the same technique [33]. In addition, the representation of many signaling components, genes associated with various types of abiotic/biotic stresses, and the most important antioxidant genes in the two platforms makes them useful for many plant biologists.

Induction of so many ROS-responsive genes in loh2 undoubtedly indicates AT- and PQ-provoked oxidative stress. A massive transcriptional reprogramming is evident already at the first time point, which precedes the cell death symptoms. For example, the heat shock transcription factor HSFA2 and many genes of the heat shock regulon are massively induced by oxidative stress in loh2. This result is consistent with the previous observations of AT-induced Hsp17 in loh2, but not in atr7 [11]. A comparison with results from the previous microarray profiling of loh2 and atr1 under AT stress confirmed the AT responsiveness of these and most of the other genes in the loh2 background [25]. Comparison of AT-regulated gene expression in atr7 (current study) with atr1 [25] revealed that some genes like ANAC102, ZAT6, ZAT11, and WRKY75 have similar expression patterns in both mutants, while other genes like SZF1 and ZAT10 are induced in atr7, but not in atr1. These

Fig. 6. Heat map of 217 antioxidant genes differentially regulated in loh2 and atr7 plants during AT- and PQ-induced oxidative stress. Loh2 and atr7 were grown on MS media or MS supplemented with 7 μM AT or 1 μM PQ. Seedlings were collected on the fourth and seventh day after germination. Expression levels were determined by multi-parallel qRT-PCR analysis. Red and green indicate higher and lower expression values, respectively. Intensity of the colors is proportional to the absolute value of log2 of the fold difference in gene expression. Black indicates no change in gene expression. Two biological repetitions with two technical replicates in each repetition were performed. The gene order in the heat maps is the same as in Supplemental Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
apparent differences in the transcriptomes of atr7 and atr7 under AT-induced oxidative stress confirm the notion that changes in the activity of different stress mechanisms may be involved in the acquired stress tolerance of the two mutants. The heat shock genes are believed to be good markers for oxidative stress and H2O2 in particular [12, 20, 21]. The induction of these genes by AT as well as by PQ implies that superoxide radicals produced by the PQ treatment are converted to H2O2, most likely swiftly by the action of superoxide dismutases. PQ-induced many genes encoding chloroplast-targeted antioxidant enzymes, as expected by the mode of action of this herbicide. However, it affected also the expression of genes encoding enzymes localized in other cellular compartments, most probably because H2O2 can affected also the expression of genes encoding enzymes localized in expected by the mode of action of this herbicide. However, it many genes encoding chloroplast-targeted antioxidant enzymes, as likely swiftly by the action of superoxide dismutases. PQ-induced germination. Expression levels were determined by multi-parallel qRT-PCR and expressed as fold changes of stressed for oxidative stress and H2O2 in particular [12, 20, 21]. The induction of these genes by AT as well as by PQ implies that superoxide radicals produced by the PQ treatment are converted to H2O2, most likely swiftly by the action of superoxide dismutases. PQ-induced many genes encoding chloroplast-targeted antioxidant enzymes, as expected by the mode of action of this herbicide. However, it affected also the expression of genes encoding enzymes localized in other cellular compartments, most probably because H2O2 can easily cross membranes. The AT- and PQ-dependent induction of several transcription factors such as the redox responsive transcription factor 1 (RRF1), the drought-responsive DREB19, the abiotic stress-responsive ZAT12, as well as many other abiotic stress- and disease resistance-related genes, provides a crosslink between oxidative stress and abiotic/biotic stress responses. Interestingly, these genes are not induced by AT or PQ in atr7; however, a comparison of loh2 and atr7 seedlings indicates much higher transcript levels of these genes in unstressed atr7 plants. These two facts collectively indicate an elevated basal level of ROS in atr7, which subsequently leads to constitutive expression of the genes and maintenance of elevated transcript levels comparable to those of oxidative stress-treated loh2 plants. This idea is further supported by the elevated GSH levels in atr7 seedlings. The constitutive high levels of expression of the oxidative- and abiotic stress-related transcription factor-encoding genes in unstressed atr7 (Table 1) provides a primed defence system ready to face AT- or PQ-induced oxidative stress. Some of these genes were previously shown to be induced by oxidative, biotic, and abiotic stresses [34–50]. The transcription factors in turn may regulate more genes, not represented by our platforms, to orchestrate the protective responses. As some of these transcription factor-encoding genes are related to heat, drought, cold, osmotic stress, and disease resistance, atr7 might be tolerant to a variety of abiotic or biotic stresses. For example, the NAC domain-containing gene ANAC012 mediates low oxygen stress responses and is important for protection against hypoxia during seed germination; DREB2A and DREB19 are well-established regulators of abiotic stress responses; HSFA2 is an inducer of heat shock genes and confers tolerance to several abiotic stresses; WRKY33 and WRKY40 are important players in pathogen stress tolerance, as well as JUB1/ANAC042, but are also implicated in several abiotic stresses such as heat and salinity; ZAT10 and ZAT12 are well-known players at the crossroads of oxidative and several abiotic stresses; SZF1 modulates the response to salinity; MYB51 governs glucosinolate biosynthesis and is important for protection against herbivores; MBF1c is a master

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<td>1.81</td>
<td>11.29</td>
<td>–1.65 –3.98</td>
<td>7.86 thermotolerance, osmotic stress</td>
<td>[61,63]</td>
</tr>
<tr>
<td>AT1G43160</td>
<td>RAP2.6</td>
<td>–47.22</td>
<td>1.81</td>
<td>–252.46 95.25</td>
<td>3.18 osmotic stress</td>
<td>[54,65]</td>
</tr>
<tr>
<td>AT4G34410</td>
<td>RRF1</td>
<td>29.59</td>
<td>920.77</td>
<td>–2.02</td>
<td>–1.57 104.51 redox regulation, light stress</td>
<td>[42]</td>
</tr>
<tr>
<td>AT3G55980</td>
<td>SZF1</td>
<td>1.9</td>
<td>35.4</td>
<td>2.33 4.6</td>
<td>2.97 salt stress</td>
<td>[60]</td>
</tr>
<tr>
<td>AT1G10585</td>
<td>TF</td>
<td>2.1</td>
<td>41.4</td>
<td>–1.33</td>
<td>–3.19 3.38 induced by H2O2, abiotic and biotic stresses</td>
<td>[41]</td>
</tr>
<tr>
<td>AT1G62300</td>
<td>WRKY6</td>
<td>1.5</td>
<td>21.4</td>
<td>1.96</td>
<td>1.44 1.86 induced by superoxide; may be important for protection against herbivores; MBF1c is a master</td>
<td>[41]</td>
</tr>
<tr>
<td>AT5G24110</td>
<td>WRKY30</td>
<td>1.6</td>
<td>12.0</td>
<td>2.45 2.4</td>
<td>1.88 induced by oxidative stress</td>
<td>[35,45]</td>
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<tr>
<td>AT2G38470</td>
<td>WRKY33</td>
<td>1.6</td>
<td>48.0</td>
<td>1.05</td>
<td>2.97 2.74 plant defense, autophagy, thermotolerance</td>
<td>[43,55]</td>
</tr>
<tr>
<td>AT1G10580</td>
<td>WRKY40</td>
<td>1.5</td>
<td>36.2</td>
<td>1.15</td>
<td>1.21 3.44 pathogen response</td>
<td>[64]</td>
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<td>AT5G13080</td>
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<td>1.5</td>
<td>32.1</td>
<td>–1.28</td>
<td>1.98 1.98 root development; phosphate stress</td>
<td>[39]</td>
</tr>
<tr>
<td>AT5G04340</td>
<td>ZAT6</td>
<td>2.1</td>
<td>22.9</td>
<td>2.5</td>
<td>8.03 1.24 repressor of primary root growth</td>
<td>[40]</td>
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<tr>
<td>AT1G27730</td>
<td>ZAT10</td>
<td>5.4</td>
<td>123.0</td>
<td>1.78</td>
<td>3.78 6.71 systemic acquired acclimation, oxidative and abiotic stresses</td>
<td>[56,58]</td>
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<td>AT2G37430</td>
<td>ZAT11</td>
<td>–1.4</td>
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<td>1.01</td>
<td>3.64 1.26 induced by oxidative stress</td>
<td>[5,10,19]</td>
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<td>ZAT12</td>
<td>5.0</td>
<td>35.1</td>
<td>2.34</td>
<td>7.27 3.32 oxidative, osmotic, and temperature stresses</td>
<td>[50,66,67]</td>
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<tr>
<td>AT2G46080</td>
<td>ZF TF</td>
<td>10.34</td>
<td>37.7</td>
<td>2.9</td>
<td>2.13 3.58 oxidative stress</td>
<td>[10,12,34]</td>
</tr>
</tbody>
</table>

loh2 and atr7 were grown on Murashige-Skoog (MS) medium or MS medium supplemented with 7 μM AT or 1 μM PQ. Seedlings were sampled on the fourth day after germination. Expression levels were determined by multi-parallel qRT-PCR and expressed as fold changes of stressed loh2 or atr7 mutants compared with unstressed loh2 or atr7 controls, or unstressed atr7 compared with unstressed loh2. Two biological repetitions with two technical replicates in each repetition were performed. The data are means of the two biological repetitions. TF, transcription factor; ZF, zinc finger. WT, wild type. Bold and italics indicate higher and lower expression values, respectively.
regulator of thermotolerance, interacting with trehalose-6-phosphate synthase 5 and ethylene signalling pathways to counteract heat and osmotic stresses [38,43,51–67]. The constantly elevated levels of these genes in unstimulated atr7 plants may provide protection against a wide range of abiotic and biotic stressors but at the same time could be responsible for the slightly smaller size of atr7, relative to its loh2 background. Furthermore, a number of these transcription factor genes are involved in the regulation of developmental processes such as seed germination under hypoxic conditions (ANAC0102), root development (WRKY6, WRKY75), and senescence (JUB1/ANAC042, WRKY6), suggesting a possible development-related phenotype of atr7 under certain growth conditions [23,38–40,68].

In conclusion, the two platforms for multi-parallel qRT-PCR designed for the analysis of ROS marker and antioxidant genes proved to be a valuable tool for the molecular analysis of the oxidative stress-tolerant mutant atr7 and its original background, the stress-sensitive loh2. The expression patterns of the ROS marker and antioxidant genes indicated that atr7 has higher levels of important ROS-related and antioxidant genes than loh2 under non-stress conditions, most likely due to constitutive expression of these genes. This conclusion is further supported by the fact that most of these genes are induced by oxidative stress in loh2, but not in atr7. The broad spectrum of these genes (drought-, osmotic-, heat-shock-, hormone-related transcription factors; cold-regulated and disease-related genes) demonstrates that ROS-related genes are involved in abiotic and biotic stress responses and indicates that the oxidative stress-tolerant atr7 mutant may exhibit reduced sensitivity to abiotic and biotic stress factors.

4. Materials and methods

4.1. Plant materials and growth conditions

The Arabidopsis thaliana loh2 mutant, a knockout of ceramide synthase (sphinganine N-acyltransferase, AT3G19260), was described previously [10]. The loh2 mutant has similar growth rate and sensitivity to AT/PO as the wild-type A. thaliana ecotype Wassilewskija. The atr7 mutant, obtained by chemical mutagenesis of loh2 plants, was genetically characterized to be recessive [11]. Crosses of atr7 with the other atr mutants and preliminary mapping studies confirmed that the atr7 mutation lies in a gene different from that of the other oxidative stress-tolerant mutants (Sujeeth N, Qureshi MK, Gechev T, and Hille J, unpublished data).

The loh2 and atr7 mutants were grown in plant growth chambers (Percival Scientific, USA) at 21 °C, 80 μmol m⁻² s⁻¹ light intensity and 16 h light/8 h dark photoperiod. Assessment for tolerance to ROS-induced cell death was done by germinating and growing the two mutants on Murashige and Skoog (MS) agar plates containing either 7 μM aminotriazole (AT) or 1 μM paraquat (PQ) and comparing them with plants grown on MS media without any ROS-inducing supplements. Seedlings from four- and seven-day-old plants were collected, ground with liquid nitrogen, aliquoted and used for all subsequent measurements.

4.2. Determination of fresh weight and chlorophyll content

Fresh weight data were recorded from seven-day-old seedlings and presented as mg weight per 10 seedlings. Leaf chlorophyll content was determined spectrophotometrically after extraction of fresh plant material with 80% acetone for overnight. Absorption of the extracts was measured at 663 and 645 nm and chlorophyll content was calculated as microgram chlorophyll per milligram fresh weight.

4.3. Glutathione measurements

Total and oxidized glutathione were measured by an enzymatic assay using Ellman’s Reagent (5,5'-dithiobis-2-nitrobenzoic acid; DNTB) and glutathione reductase (GR), essentially as described by Gechev et al. [69] with some modifications. The method relies on the GR-dependent reduction of DTNB, monitored at 412 nm. Briefly, 100 mg ground plant material was homogenized in 1 ml 5% sulfosalicylic acid (Sigma–Aldrich) made in 0.1 M potassium phosphate buffer (pH 7.6)/5 mM EDTA) on ice. The suspension was centrifuged for 10 min at 10.000 g and the supernatant was aliquoted. To measure total glutathione, an aliquot of neutralized extract was added to a cuvette containing 0.1 M potassium phosphate buffer (pH 7.6)/5 mM EDTA), 1.2 mM DNTB (Sigma–Aldrich), and 0.3 mM NADPH. The reaction was started by the addition of 1 U glutathione reductase (Sigma–Aldrich). After mixing by pipetting, the increase in A₄₁₂ was monitored for 3 min. GSSG was measured by the same principle after incubation of neutralized extract with 2 μM 2-vinylpyridine (Sigma–Aldrich) for 1 h at room temperature to complex GSH. To remove excess 2-vinylpyridine, the derivatized solution was treated with diethyl ether. Reduced glutathione was determined as the difference between total glutathione and GSSG.

4.4. RNA extraction, DNA digestion and cDNA synthesis

RNA from frozen four- and seven-day-old loh2 and atr7 seedlings was extracted with Trizol Reagent (Invitrogen) according to the manufacturer’s recommendations. Ten μg total RNA was treated with DNA-free™ Kit (Ambion) and absence of genomic DNA contamination was subsequently confirmed by qRT-PCR using intron-specific primers for the gene At5g65080 (Forward: 5'-TTTTTTCCTCCGTCAATC-3' and Reverse: 5'-ATTTCTCCGCA CCACATTGTAC-3'). RNA integrity was checked on 1% (w/v) agarose gel and concentration measured using a Nanodrop ND-1000 Spectrophotometer before and after DNase I digestion. cDNA was synthesized from 2 μg of total RNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) with oligo-dT primers, according to the manufacturer’s instructions. The efficiency of cDNA synthesis was determined by qRT-PCR amplifications of control transcripts of the genes ACTIN2 (At3g18780; Forward: 5'- TTCTCTA GCACATTCCGCAGAT-3' and Reverse: 5'-AACGATTCGTGACCT CCTCCT-3'), GAPDH, 5' region (At1g13440; Forward: 5'-TTCTGATCTCAATTTCGCAAAA-3' and Reverse: 5'-CGAGACCC TTGATTCCGATTC-3') and GAPDH, 3' region (At1g13440; Forward: 5'-TTTCTCCGCAACGCGTCAACAGA-3' and Reverse: 5'-AACTTTGTC GCCTAATGGAATC-3'). For further analysis only cDNA preparations that gave similar Ct values for ACTIN2 (i.e., 18 ± 1) and no more than 3 cycles difference between GAPDH (3') and GAPDH (5') were selected.

4.5. Primer design, quantitative RT-PCR and data analysis

Primers were designed using QuantPrime [70]. Primer sequences of antioxidant genes are given in Supplemental Table 3. Primer sequences of the ROS marker genes are given in [23] and some further genes are included in Supplemental Table 3. PCR reactions were conducted in an ABI PRISM 7900 HT (Applied Biosystems) sequence detection system using an optical 384-well plate and SYBR Green (Applied Biosystems) to monitor dsDNA synthesis. All reactions contained 2.5 μl of SYBR Green Master Mix, 0.5 μl of cDNA (1.5 ng/μl) and 200 nM of each gene-specific primer in a final volume of 5 μl. An electronic pipette was used to load cDNA/SYBR Green-containing master mix into individual wells, while primers were aliquoted by a robot. The qRT-PCR reactions were executed using the following program: 50 °C for 2 min, 95 °C for 10 min,
followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After 40 cycles, amplification specificity was tested by melting curve analysis. The efficiencies of the polymerase chain reactions were estimated using the LinRegPCR software. Only PCR runs which gave R2 value between 0.995/1.000, corresponding to linear amplification, were selected for analysis. Data analysis was performed using SDS 2.2.1 software (Applied Biosystems). The reference control gene (ACTIN2) was measured with four replicates in each PCR run, and its average Ct value was used for relative expression analysis. Relative mRNA abundance was calculated using the comparative 2^(-ΔΔCt) method and normalized to the corresponding reference gene levels [71]. Fold changes in gene expression were calculated for both, four- and seven-day-old loh2 and atr7 plants germinated on AT- or PQ-supplemented growth media. Resulting data sets were log transformed and visualized by MEV (MultiExperiment Viewer)-created heat maps. Two biological and two technical repetitions were performed for each platform and time point.

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Appendix A. Supplemental material

Supplemental material related to this article can be found online at doi:10.1016/j.plaphy.2012.05.024.

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