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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*, *RRTF1*, *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.

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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 embryo development, nucellar degeneration, maturation of tracheal elements and epidermal trichomes, formation of lace 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].

Abbreviations: AT, aminotriazole; AAL-toxin, *Alternaria alternata* f.sp. *lycopersici* toxin; GSH, reduced glutathione; GSSG, oxidized glutathione; PQ, paraquat; PCD, programmed cell death; H₂O₂, hydrogen peroxide; O₂^{•−}, superoxide radical; ROS, reactive oxygen species; TF, transcription factor(s).

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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* had 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 (H_2O_2) and superoxide radical ($O_2^{\cdot-}$) by treating the plants with AT and PQ, respectively. Catalases are the main H_2O_2 -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 H_2O_2 accumulation and subsequent cell death [12]. PQ accepts electrons from photosystem I or ferredoxin and transfers them to molecular oxygen, producing the destructive $O_2^{\cdot-}$ [16]. The biological effect of both herbicides is light-dependent. Application of either 7 μ M AT or 1 μ M 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. 2A 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.

Fig. 2. Growth inhibition and chlorophyll loss in *loh2* and *atr7* mutants exposed to oxidative stress. *Loh2* and *atr7* were grown on MS media or MS supplemented with 7 μ M AT or 1 μ M PQ. Seedlings were sampled one week after germination. A, growth inhibition assessed by fresh weight (FW) measurements. B, chlorophyll content. The differences between unstressed *loh2/atr7* and AT/PQ-treated *loh2/atr7* plants were statistically significant ($P < 0.05$, Student's test), as were the differences between stressed *loh2* and *atr7* plants ($P < 0.05$). Data are means of three independent biological repetitions $\pm$ SD.

Supplemental Table 1 contains four groups: (i) genes known to primarily respond to H_2O_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*, *ERF5*, *RRTF1*, *STZ*, *WRKY6*, *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 [12,20,21], so their induction will be an indication for H_2O_2 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 [19,22]. This platform was utilized previously for the functional analysis of transgenic Arabidopsis lines over- and underexpressing the NAC TF gene *JUB1/ANAC042* [23].

The genes of the newly designed antioxidant platform were selected based on previous studies defining the ROS network in *Arabidopsis* [19,24] and included 217 genes for antioxidant and ROS-related enzymes. The list contains superoxide dismutases (SOD), ascorbate peroxidases (APX), mono- and dehydroascorbate

reductases (MDHAR, DHAR), catalases, glutathione peroxidases (GPX), ferritins, copper-binding proteins, NADPH oxidases, alternative oxidases (AOX), peroxiredoxins, a ferredoxin-thioredoxin reductase, a phosphoadenylyl-sulfate reductase, thioredoxins, glutaredoxins, and many guaiacol peroxidase genes (**Supplemental Table 2**).

The two multi-parallel qRT-PCR platforms were used to study gene expression during AT- and PQ-induced oxidative stress in *loh2* and *atr7* in four- and seven-day-old seedlings germinated either on AT- or PQ- containing Murashige and Skoog (MS) medium. Log2 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_2O_2 -, O_2^- -, 1O_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_2O_2 -responsive, 19 of the 22 1O_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

Fig. 3. Glutathione content in *loh2* and *atr7* mutants exposed to oxidative stress. Reduced (GSH) and oxidised (GSSG) glutathione was determined in plants grown on plain MS media or MS supplemented with 7 μ M AT or 1 μ M PQ. Samples for the measurements were collected four (A) and seven (B) days after germination. Asterisks indicate statistically significant differences between unstressed *loh2* and *atr7* plants (for GSH), as well as between unstressed *loh2* and PQ-treated *loh2* plants (for GSSG) ($P < 0.05$, Student's test). Data are means of three independent biological repetitions $\pm$ SD.

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 μ M AT or 1 μ M 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.)

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. 5. Transcript level changes of hydrogen peroxide-, superoxide-, singlet oxygen- and common ROS-responsive genes in *loh2* and *atr7* mutants during AT- and PQ-induced oxidative stress. *Loh2* and *atr7* plants were grown on MS media or MS supplemented with 7 μ M AT or 1 μ M PQ. Seedlings were sampled on the fourth day (first five columns in each panel) or on the seventh day (columns 6–10) after germination. The number of transcripts with a 2- to 5-fold, 5- to 10-fold, and a more than 10-fold increase or decrease (FC) in expression in treated plants compared to untreated control plants as well as in unstressed *atr7* relative to unstressed *loh2* (columns 5 and 10) are represented white light gray, gray, and black bars, respectively.

induction of ¹O₂-responsive genes suggests that additional mechanisms could be activated, leading to ¹O₂ in *loh2* 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 *atr7* compared to *loh2* 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 *atr7* compared with *loh2* included the transcription factor *HSAF2* and the entire heat shock regulon, other transcription factors related to abiotic and oxidative stress (*ZAT11*, *RRTF1*, *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 *atr7* relative to *loh2* under non-stress conditions (Fig. 6; Supplemental Table 2). The same genes were induced by AT and PQ in *loh2*, but not in *atr7* (Figs. 4 and 6; Supplemental Table 1; Supplemental Table 2). These data collectively indicate higher basal levels of ROS in *atr7* 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 *atr7* 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 *loh2* four days after germination, including all AOX genes (Fig. 6). Another surprising observation was that oxidative

stress actually decreased the transcript levels of most antioxidant genes in *atr7* 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 H₂O₂ and O₂⁻ 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, *loh2* developed cell death symptoms under both, AT and PQ treatment, while *atr7* 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, *loh2* 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 *atr* mutants, as evidenced by their reduction of fresh weight and repression of growth-related genes [11,25]. However, the *atr7* mutant eventually overcomes the oxidative stresses and

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 μ M AT or 1 μ M 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 $\log_2$ 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

continues to develop. *Atr7* is therefore an appropriate model mutant to study processes associated with oxidative stress and programmed cell death.

Under optimal growth conditions, the glutathione pool is predominantly in the reduced GSH form [26]. 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_2O_2 , is complicated by several interfering factors like ascorbate and polyphenols [31,32]. Not surprisingly, there is high variability in reported H_2O_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

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.)

Table 1
Transcription factors that regulate stress responses and development are induced by oxidative stress and have elevated expression levels in *atr7* under non-stress conditions.

AGI code	Gene name	loh2		atr7		atr7/loh2	Biological process / stress response	Reference
		MS + AT	MS + PQ	MS + AT	MS + PQ	MS		
AT1G77450	ANAC032	2.0	14.7	1.9	3.34	1.29	induced by oxidative stress	[14]
AT2G43000	ANAC042	3.6	330.6	1.94	4.4	2.17	senescence, camalexin biosynthesis	[23,59]
AT3G04070	ANAC047	–1.4	2.9	–1.29	–1.23	1.45	induced by AAL-toxin and H ₂ O ₂	[5,10]
AT3G15500	ANAC055	2.2	2.3	2.47	1.18	–1.16	regulator of drought stress responses	[49]
AT5G63790	ANAC102	2.0	23.3	1.3	–1.12	2.4	seed germination, hypoxia	[38]
AT5G51190	AP2 TF	1.2	53.5	–2.5	1.2	8.22	ethylene responses	[44]
AT2G33710	AP2 TF	2.67	30.99	2.27	1.22	1.86	ethylene responses, induced by AAL-toxin and oxidative stress	[5,10]
AT2G38250	DNA-binding	5.1	271.8	1.56	4.93	7.78	induced by H ₂ O ₂ and pathogens	[41]
AT5G05410	DREB2A	1.9	15.1	–1.53	–2.02	1.29	drought stress	[46]
AT2G38340	DREB19	9.2	316.7	2.49	1.85	12.07	drought stress	[46]
AT5G47230	ERF5	–2.2	2.4	–1.36	–2.99	1.2	pathogen responses, ethylene pathway	[48]
AT4G17490	ERF6	1.0	2.5	1.23	–1.84	1.31	pathogen responses, ethylene pathway	[48]
AT3G23230	ERF	4.2	13.1	1.93	9.03	2.7	pathogen responses, ethylene pathway	[35]
AT2G26150	HSFA2	10.0	41.2	–1.68	–5.89	13.19	heat, osmotic shock and anoxia	[57]
AT4G18880	HSFA4A	1.3	20.7	2.38	2.87	1.72	activator of heat shock genes	[51]
AT3G02550	LOB domain 41	1.54	9.62	1.19	–2.61	1.87	induced by oxidative stress	[14]
AT3G24500	MBF1c	1.81	11.29	–1.65	–3.98	7.86	thermotolerance, osmotic stress	[61–63]
AT1G18570	MYB51	–1.7	13.3	–1.33	–3.55	3.43	pathogen response, glucosinolate biosynthesis	[53]
AT1G43160	RAP2.6	–47.22	1.81	–252.46	–95.25	3.18	osmotic stress	[54,65]
AT4G34410	RRTF1	29.59	920.77	–2.02	–1.57	104.51	redox regulation, light stress	[42]
AT3G55980	SZF1	1.9	35.4	2.33	4.6	2.97	salt stress	[60]
AT1G10585	TF	2.1	41.4	–1.33	–3.19	3.38	induced by H ₂ O ₂ , abiotic and biotic stresses	[41]
AT1G62300	WRKY6	1.5	21.4	1.96	1.44	1.86	senescence, herbivory, low phosphate stress	[47,37,68]
AT5G24110	WRKY30	1.6	12.0	2.45	2.4	1.88	induced by superoxide; may be involved in senescence	[35,45]
AT2G38470	WRKY33	1.6	48.0	1.05	2.97	2.74	plant defense, autophagy, thermotolerance	[43,55]
AT1G80840	WRKY40	1.5	36.2	1.15	1.21	3.44	pathogen response	[64]
AT5G13080	WRKY75	–1.5	32.1	–1.28	–1.9	1.98	root development; phosphate stress	[39]
AT5G04340	ZAT6	2.1	22.9	2.5	8.03	1.24	repressor of primary root growth	[40]
AT1G27730	ZAT10	5.4	123.0	1.78	3.78	6.71	systemic acquired acclimation, oxidative and abiotic stresses	[56,58]
AT2G37430	ZAT11	–1.4	15.3	1.01	3.64	1.26	induced by oxidative stress	[5,10,19]
AT5G59820	ZAT12	5.0	35.9	2.34	7.27	3.32	oxidative, osmotic, and temperature stresses	[50,66,67]
AT3G46080	ZF TF	10.34	37.7	2.9	2.13	3.58	responds to arsenic, oxidative stress and biotic stress elicitors	[10,12,34]

loh2 and *atr7* were grown on Murashige-Skoog (MS) medium or MS medium supplemented with 7 μ M AT or 1 μ M 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.

apparent differences in the transcriptomes of *atr1* and *atr7* under AT-induced oxidative stress confirm the notion that different 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 the 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 (*RRTF1*), 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 *ANAC102* mediates low oxygen stress responses and is important for protection against hypoxia during seed germination; *DREB2A* and *DREB19* are well-established regulators of drought 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

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 unstressed *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 (*ANAC102*), 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/PQ as the wild-type *A. thaliana* ecotype *Was-silewskija*. 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 A_{412} was monitored for 1 min. GSSG was measured by the same principle after incubation of neutralized extract with 2 μl 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'-TTTTTGGCCCCCTTCGAATC-3' and Reverse: 5'-ATCTTCGCCA CCACATTGTAC-3'). RNA integrity was checked on 1% (w/v) agarose gel and concentration measured with 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'- TTCCTCA GCACATTCCAGCAGAT-3' and Reverse: 5'-AACGATTCCTGGACCTG CCTATC-3'), *GAPDH*, 5' region (At1g13440; Forward: 5'-TCTCGATCTCAATTCGCAAAA-3' and Reverse: 5'-CGAAACCG TTGATTCCGATTC-3') and *GAPDH*, 3' region (At1g13440; Forward: 5'-TTGGTGACAACAGGTCAAGCA-3' and Reverse: 5'-AAACTTGTG GCTCAATGCAATC-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 $2^{-\Delta\Delta Ct}$ 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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