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ORIGINAL PAPER

Isolation and characterization of *Arabidopsis* mutants with enhanced tolerance to oxidative stress

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Abstract We have previously reported a method for isolation of mutants with enhanced tolerance to the fungal AAL toxin and given a detailed characterization of *atr1* (AAL toxin resistant, Gechev et al. in Biochem Biophys Res Commun 375:639–644, 2008). Herewith, we report eight more mutants with enhanced tolerance to the AAL toxin. Phenotypic analysis showed that six of the mutants were reduced in size compared with their original background *loh2*. Furthermore, *atr2* showed delayed flowering and senescence. The mutants were also evaluated for oxidative stress tolerance by growing them on ROS-inducing media supplemented with either aminotriazole or paraquat, generating, respectively, H_2O_2 or superoxide radicals. Oxidative stress, confirmed by induction of the marker genes, *HIGH AFFINITY NITRATE TRANSPORTER* At1G08090 and *HEAT SHOCK PROTEIN 17* At3G46230, inhibited growth of all lines. However, while the original background *loh2* developed necrotic lesions and died rapidly on ROS-inducing plant growth media, *atr1, atr2*, *atr7* and *atr9* remained green and viable. The tolerance against oxidative stress-induced cell death was confirmed by fresh weight and chlorophyll measurements. Real-time PCR analysis revealed that the expression of the *EXTENSIN* gene At5G46890, previously shown to be downregulated by aminotriazole in *atr1*, was repressed in all lines, consistent with the growth inhibition induced by oxidative stress. Taken together, the data indicate a complex link between growth, development and oxidative stress tolerance and indicates that growth inhibition can be uncoupled from oxidative stress-induced cell death.

Keywords AAL toxin · Programmed cell death · Aminotriazole · Paraquat · Hydrogen peroxide · Oxidative stress

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Introduction

Oxidative stress-induced programmed cell death (PCD) can occur under many unfavorable environmental conditions as well as in biotic interactions (Apel and Hirt 2004). Furthermore, reactive oxygen species (ROS)-induced cell death is also observed during several developmental processes (Gadjev et al. 2008). Oxidative stress-induced PCD is a genetically controlled process triggered mostly by hydrogen peroxide (H_2O_2) and also by other types of ROS, including superoxide radicals and singlet oxygen (Gadjev et al. 2008; Gechev et al. 2006; Gechev and Hille 2005).

In addition to the abiotic stress factors mentioned earlier, elevated levels of H_2O_2 and subsequently H_2O_2-induced cell death can be triggered by catalase deficiency, especially under conditions that promote photorespiration such as high light intensity (Gechev et al. 2005; Vanderauwera et al. 2005). Catalase deficiency can be induced by either silencing the catalase gene(s) or inhibiting catalase activity by the catalase inhibitor aminotriazole (AT) (Gechev et al. 2005; Vanderauwera et al. 2005). Moreover, AT can be

used as a screening agent for identifying mutants more tolerant to oxidative stress (Gechev et al. 2008).

The fungal AAL toxin causes PCD through perturbations in sphingolipid metabolism (Brandwagt et al. 2000; Spassieva et al. 2002). The toxin inhibits ceramide synthase, a key enzyme in sphingolipid biosynthesis, which leads to accumulation of precursors and depletion of complex sphingolipids. In tomato, the sensitivity to AAL toxin is conferred by a mutation in the *Asc* gene that is most likely a component of the ceramide synthase (Brandwagt et al. 2000). Likewise, the *loh2* mutant, knockout of the *Asc* homologous gene in *Arabidopsis thaliana,* has increased sensitivity to AAL toxin (Gechev et al. 2004). The AAL toxin-induced PCD in *Arabidopsis* is associated with elevated levels of H_2O_2 that precede cell death (Gechev et al. 2004). The burst of ROS was confirmed by microarray analyses of AAL toxin-induced cell death in *loh2*. This analysis revealed induction of H_2O_2-responsive genes and genes that are involved in the oxidative burst at early time points preceding visible cell death symptoms (Gechev et al. 2004). The oxidative burst in AAL toxin-treated plants was in agreement with previous studies demonstrating accumulation of ROS in *Arabidopsis* plants treated with fumonisin B1 (FB1), an AAL toxin analog, and by the recently identified FB1 resistant mutant compromised in serine palmitoyl transferase, a key enzyme of de novo sphingolipid synthesis. This mutant failed to generate ROS and to initiate cell death upon FB1 treatment (Asai et al. 2000; Shi et al. 2007).

We have previously reported a new method for isolation of mutants with enhanced tolerance to the fungal AAL toxin as well as a detailed characterization of one such mutant, named *atr1* (AAL toxin resistant, Gechev et al. 2008). In this paper, we report eight more mutants with enhanced tolerance to the AAL toxin. The new mutants are phenotyped and their tolerance toward ROS-induced cell death evaluated. In addition, we discuss the link between oxidative stress and plant development.

Materials and methods

Plant growth conditions, mutagenesis and mutant screening

Plants were grown in a greenhouse under standard conditions (14-h light/10-h dark period, photosynthetic photon flux density 400 μmol m^{-2} s^{-1}, 22°C and relative humidity 70%) or in a climate room (14-h light/10-h dark period, photosynthetic photon flux density 100 μmol m^{-2} s^{-1}, 22°C and relative humidity 70%). Seeds from the AAL toxin-sensitive *A. thaliana loh2* mutant were mutagenized with 0.1, 0.2 and 0.3% ethane methyl sulfonate for 8 h, washed extensively and planted on soil in the greenhouse to self-pollinate and the progeny collected (M2). Isolation of AAL toxin-resistant *atr* mutants was done by plating seeds from M2 plants on Petri dishes with Murashige and Skoog (MS) media containing 40 nM of AAL toxin and grown in a climate room. The independent AAL toxin-resistant survivors from different pools were transferred to the greenhouse and seeds collected for further analysis.

Stress treatments and evaluation of stress tolerance

Oxidative stress was applied by plating the seeds on media containing either AT or paraquat at concentrations of 5, 7 or 9 μM for AT and 0.5, 1 or 1.5 μM for paraquat. Plants were grown for 2 weeks; fresh weight and chlorophyll content were assessed 7 and 10 days after germination. Chlorophyll content was measured photometrically as previously described (Gechev et al. 2002, 2003). Briefly, the pigments were extracted with 80% acetone at 4°C overnight, samples centrifuged to remove solid particles, and absorbtion of chlorophyll *a* and *b* measured at 663 and 647 nm. Chlorophyll content was calculated as microgram per milligram fresh weight.

Isolation of RNA and real-time PCR measurements

Total RNA was isolated using TRIZOL® reagent (Invitrogen), following the manufacturer's guidelines. RNA was extracted from 4 days old seedlings planted on MS media with or without 7 μM AT. RNA was quantified at 260 nm and its quality was checked on gel. The following genes and corresponding primer pairs were used in the real-time PCR analysis: *HIGH AFFINITY NITRATE TRANSPORTER* (At1g08090), primer pairs CCATGGGAGTT GAGTTGAGC and AAAGTCAGATGCGTAGCCTCC; *HSP17* (At3g46230), GTATGGGATCCGTTCGAAGG and TCTTCCTTCTTAAGCCCAGGC; *EXTENSIN* (At5g4 6890), CAAGAGCTACCACAAGAAGCC and GAGCG CAACAGTTGGACG; *PROFILIN 1* (At2g19760), AGAG CGCCAAATTTCCTCAG and CCTCCAGGTCCCTT CTTCC. Primer pairs were designed to cross exon–intron boundaries to minimize genomic DNA amplification. Reverse transcription products were obtained by using the RevertAid™ First strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions. Real-time PCR was performed with 7500 Real-Time PCR machine (Applied Biosystems). Reaction mixture per well contained: 12.5 μl Maxima™ SYBR Green/ROX qPCR Master Mix (2x) (Fermentas), 1 μl of each primer, 9.5 μl nuclease free water and 50 ng (1 μl) cDNA. Profilin 1 was used as internal standard. The annealing temperature selected was 60°C and the program was run for 40 cycles. Melting curves did not show any unspecific products and primer dimers. Relative gene expression levels were

obtained using the $\Delta\Delta C_t$ method (Winer et al. 1999). All samples were run in three independent repetitions.

Protein isolation and enzyme assays

Total protein was isolated and protein concentration quantified by the method of Bradford with a kit supplied by Bio-Rad as previously described (Gechev et al. 2002, 2003). Catalase was determined photometrically following the decrease in absorbance of H_2O_2 at 240 nm and in gel assays (Gechev et al. 2002, 2003).

Results and discussion

Isolation and phenotypic characterization of mutants more tolerant to cell death

The method for screening AAL toxin-tolerant mutants and isolation of *atr1* has previously been described in detail (Gechev et al. 2008). Briefly, ethane methyl sulfonate-mutagenized seeds from the AAL toxin-sensitive line *loh2* plants were germinated on soil, self-pollinated and 40,000 batches of progeny screened on 40 nM AAL toxin-containing media, lethal to *loh2*. Nine *atr* mutants were isolated. Genetic studies by crossing *atr* with the wild type and studying the progeny indicated that *atr* mutants behaved as recessive mutants (data not shown). Wild-type plants are tolerant to as much as 200 nM AAL toxin, which is more than tenfold higher than *loh2*, and can survive without any visible cell death symptoms. The tolerance of *atr* mutants to AAL toxin ranged between 40 and 100 nM. It is unclear why full resistance to the AAL toxin was never achieved. Sphingolipids are essential regulators not only of cell death, but also of development in both animal and plant cells (Teufel et al. 2009; Chen et al. 2008). This notion is supported by the various developmental defects in mutants with compromised very long-chain fatty acids that are components of sphingolipids and in mutants with impaired hydroxylation of long-chain bases (Bach et al. 2008; Zheng et al. 2005; Chen et al. 2008). It could be that all mutants, being in *loh2* background that lacks certain complex sphingolipids, are unable to proceed into mature plants when AAL toxin is present.

Some of the *atr* mutants were smaller in size than their original background *loh2* (Fig. 1). *Loh2* has the same size as *A. thaliana* ecotype *Wassilewskija* or *A. thaliana* ecotype *Columbia* (data not shown). However, *atr1, atr2, atr6, atr7,*

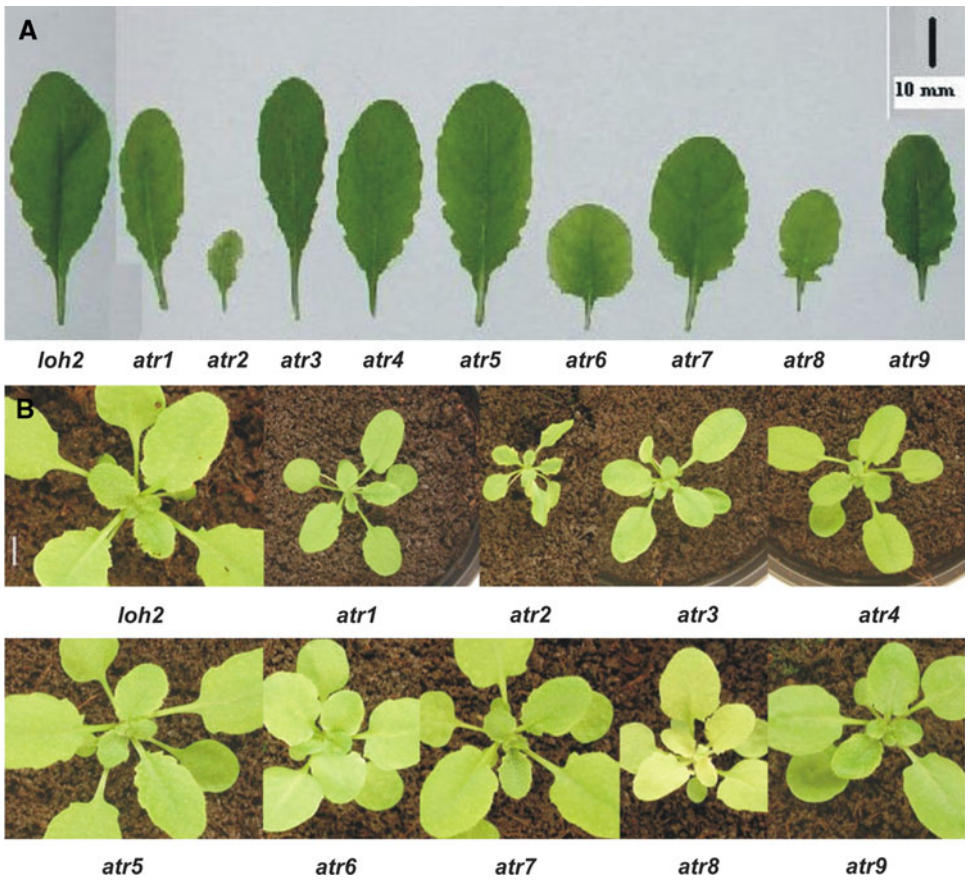

Fig. 1 Phenotypes of *loh2* and *atr* mutants. *Loh2* and *atr* mutants were grown on soil under standard greenhouse conditions (14-h light/10-h dark periods, PPFD 400 µmol m^{-2} s^{-1}, 22°C, and relative humidity 70%) and representative pictures taken at inflorescence emergence, 30 days after germination. **a** Sixth leaves of 1-month-old plants. **b** Phenotypes of different mutant lines

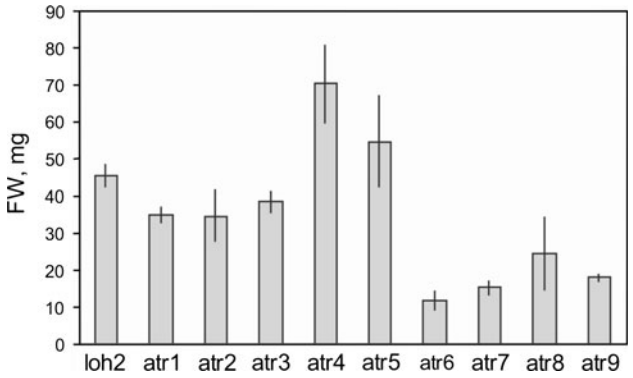

Fig. 2 Fresh weight (*FW*) of *loh2* and *atr* mutants. Seedlings were grown for 7 days on MS media under normal growth conditions (PPFD 100 μmol m^{-2} s^{-1}, 22°C) and fresh weight of the seedlings calculated from three independent biological repetitions. Data are means ± SD

atr8 and *atr9* had smaller leaves and were reduced in size to different extents when grown on soil (Fig. 1). Furthermore, some of the mutants had altered leaf shape (Fig. 1). *Atr6* had smaller adult leaves rounded in shape; it remained dwarfed throughout its life cycle as compared to *loh2* and other mutants. In addition, these mutant lines were also smaller when grown in vitro (Fig. 2). On the other side, *atr4* and *atr5* had slightly higher fresh weight when grown in vitro. To see if this was due to delayed growth and/or development, we analyzed the entry into different developmental stages of all *atr* mutants and compared the data with *loh2*. No statistically significant differences were observed during the earlier stages, from germination to rosette leaf stages (data not shown). However, *atr2* had late emergence of inflorescence, late flowering and delayed senescence (Table 1). These findings suggest that some of the genes involved in stress tolerance and PCD also play roles in plant growth and development/senescence.

Atr mutants exhibit enhanced tolerance to oxidative stress

Earlier studies have indicated a clear link between AAL toxin and oxidative stress (Gechev et al. 2004). Furthermore, the first mutant isolated and characterized exhibited enhanced tolerance toward ROS-induced cell death (Gechev et al. 2008). To evaluate the oxidative stress tolerance of other *atr* mutants, *loh2* and the nine *atr* mutants, including *atr1* as a positive control, were germinated and grown on plant media supplemented with 5, 7 and 9 μM AT. The sensitivity of *loh2* to AT was comparable to that of its original wild-type background *A. thaliana* ecotype *Wassilewskija*. Under these conditions, all lines were clearly inhibited in growth, which was confirmed by fresh weight measurements (Fig. 3). However, while *loh2* developed necrotic lesions, bleached, lost its chlorophyll and eventually died within 2 weeks after germination (with first necrotic lesions and chlorophyll loss already visible 5 days after germination), some of the *atr* mutants did not exhibit such severe yellowing characteristic of *loh2* on media with AT. In particular, *atr1, atr2, atr7, atr8* and *atr9* stayed much greener and did not die even 1 month after AT treatment. This was confirmed by measurements of fresh weight and chlorophyll content (Fig. 3a, b). The mutants, *atr1, atr2, atr7, atr8* and *atr9*, had much less pronounced reduction of fresh weight and chlorophyll loss compared to *loh2* on all three concentrations of AT. In addition to H_2O_2, oxidative stress and subsequent cell death can be imposed by other types of ROS as superoxide radicals or singlet oxygen (Op Den Camp et al. 2003; Vranova et al. 2002). For example, paraquat generates superoxide radicals by accepting electrons from PS I and transferring them to oxygen (Gechev et al. 2006). We tested *atr* mutants on media supplemented with 0.5, 1 or 1.5 μM paraquat and found that *atr1, atr2, atr7* and *atr9* had enhanced tolerance to paraquat on all three concentrations. For example, the oxidative stress-tolerant mutants have much less fresh weight loss and retain more of their chlorophyll compared with *loh2* on media with 1.5 μM paraquat (Fig. 3c, d). However, there was a gradation in the tolerance of the mutants to paraquat. Paraquat-induced growth inhibition and fresh weight loss was much more pronounced than AT-induced growth inhibition. According to the fresh weight data, *atr7* and *atr9* suffered less, followed by *atr1* and *atr2*. As for the

Table 1 The *atr2* mutant exhibits late flowering and delayed senescence

	loh-2	*atr-1*	*atr-2*	*atr-3*	*atr-4*	*atr-5*	*atr-6*	*atr-7*	*atr-8*	*atr-9*
IE	29.2 ± 2.1	30 ± 2.3	36.1 ± 1.9	30.7 ± 2.5	28.3 ± 1.4	30.1 ± 2	32.4 ± 1.7	31.5 ± 2.9	30.4 ± 1.3	32.5 ± 1.9
FI	37.9 ± 2.3	38.7 ± 2.3	48.3 ± 2.3	41.1 ± 3.3	37.7 ± 2.1	39.5 ± 2.9	42.8 ± 1.4	42 ± 2.7	38.4 ± 1.9	41.8 ± 1.8
FC	62.6 ± 3.2	61.1 ± 2.7	78.5 ± 4	71.1 ± 4.7	66.2 ± 3.7	62.9 ± 2.7	72 ± 4.2	64.4 ± 5.8	65.6 ± 3.9	67.9 ± 3.9
S	77.9 ± 2.2	79.8 ± 1.3	89.5 ± 2.9	81.1 ± 1.9	80.9 ± 1	80.7 ± 1.6	85.1 ± 2.8	80.1 ± 2.1	79.9 ± 2	80.6 ± 1.6

Loh2 and *atr* mutants were grown on soil under standard greenhouse conditions and the days of appearance of the late developmental stages recorded. Data are means ± SD of three biological repetitions

IE inflorescence emergence, *FI* flower initiation, *FC* flowering completed, *S* senescence

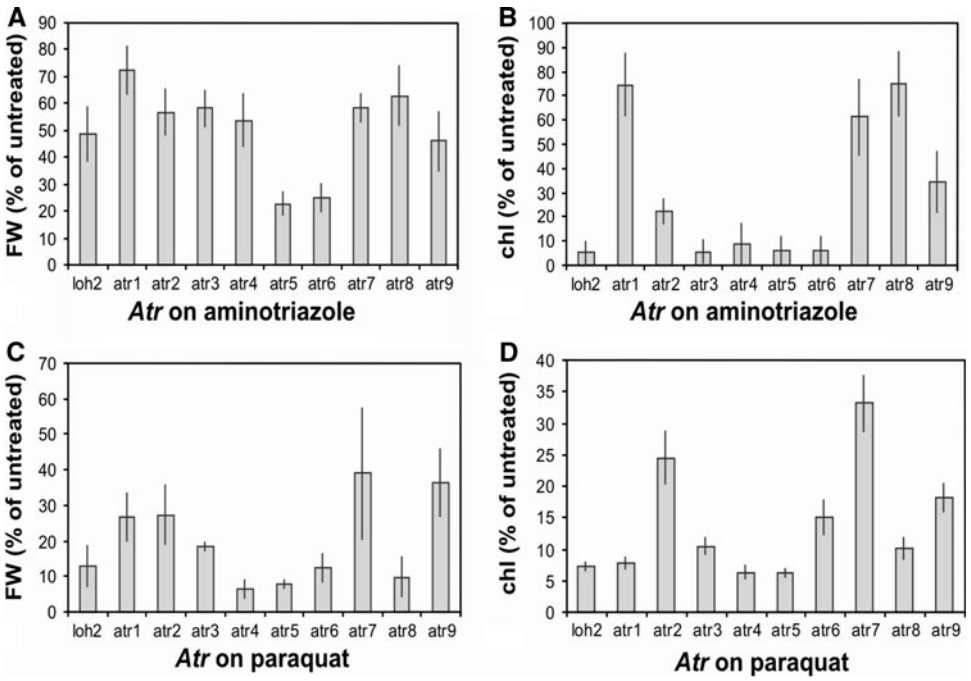

Fig. 3 *Atr* mutants and their tolerance to ROS-induced cell death. Seeds of nine *atr* mutants initially identified as more tolerant to AAL toxin were plated on Murashige and Skoog (*MS*) media supplemented either with 7 μM aminotriazole (*AT*) (**a**, **b**) or with 1.5 μM paraquat (**c**, **d**) to assess their tolerance to cell death induced by superoxide radicals or H_2O_2, respectively. Data on the loss of fresh weight (*FW*) or chlorophyll (*chl*) of 10-day-old *loh2* and *atr* seedlings grown on media supplemented with paraquat or aminotriazole were compared with plants grown without paraquat and aminotriazole (percentage from untreated). Samples for the measurements were collected 1 week after germination. Data are means of three measurements ± SD

Table 2 Gene expression analysis of *loh2* and *atr* mutants exposed to AT-induced oxidative stress

Gene name	TAIR locus	*loh2*	*atr1*	*atr2*	*atr3*	*atr4*	*atr5*	*atr6*	*atr7*	*atr8*	*atr9*
High-affinity nitrate transporter	At1g08090	7.29	2.95	6.37	50.16	27.39	11.72	44.88	45.25	1.11	5.75
Hsp17	At3g46230	4.27	−2.17	130.82	6.42	8.41	11.17	57.39	−9.09	41.65	524.98
Extensin like	At5g46890	−2.08	−16.67	−25	−2.38	−9.09	−10	−1.92	−2.13	−14.29	−2.63

Loh2 and *atr* mutants were grown in vitro on MS media supplemented or not with 7 μM AT and samples for real-time PCR analysis were taken 4 days after germination before any visible stress symptoms. Positive values indicate higher expression in AT-treated plants (fold change, genes induced by oxidative stress), while negative values indicate lower expression in AT-treated plants (fold change, genes repressed by oxidative stress)

chlorophyll content, *atr2*, *atr7* and *atr9* retained more pigments than the other mutants.

The nine *atr* mutants can be divided into two groups: mutants tolerant to AAL toxin only and mutants more tolerant to both AAL toxin and ROS generated by AT or paraquat. The first group may reflect genes with specific roles in AAL toxin stress responses, whereas the second group may contain genes situated in a more general cell death pathway, probably the converging path of AAL toxin, H_2O_2 and superoxide radical-induced PCD.

Catalase deficiency has previously been shown to induce oxidative stress and subsequently cell death in a number of species, including tobacco and *Arabidopsis* (Dat et al. 2003; Vanderauwera et al. 2005). Using a reverse genetics approach, an oxoglutarate-dependent dioxygenase was implicated as a player in AT-induced cell death (Gechev et al. 2005). Because H_2O_2-induced cell death is believed to be a complex process involving many genes, the *atr* mutants can serve as a starting point for further exploring the complexity of oxidative stress-induced cell death.

Molecular analysis of mutants exposed to AT-induced oxidative stress

To further gain understanding of the oxidative stress tolerance, we performed real-time PCR analysis of gene expression with selected genes in the mutant lines exposed to AT-induced oxidative stress (Table 2). A gene

Table 3 Catalase activity in *loh2* and *atr* mutants under normal growth conditions and AT-induced oxidative stress

	loh2	*atr1*	*atr2*	*atr3*	*atr4*	*atr5*	*atr6*	*atr7*	*atr8*	*atr9*
MS	0.229 ± 0.025	0.263 ± 0.053	0.245 ± 0.062	0.183 ± 0.029	0.211 ± 0.028	0.233 ± 0.044	0.193 ± 0.036	0.202 ± 0.066	0.248 ± 0.037	0.169 ± 0.029
MS + 7 µM AT	0.097 ± 0.018	0.081 ± 0.021	0.089 ± 0.015	0.102 ± 0.024	0.086 ± 0.018	0.114 ± 0.032	0.107 ± 0.017	0.061 ± 0.013	0.095 ± 0.027	0.078 ± 0.016

Seedlings were grown for 4 days under normal growth conditions (PPFD 100 µmol m^{-2} s^{-1}, 22°C) on MS media or on MS supplemented with 7 µM AT. Catalase activity is expressed in µmol H_2O_2 min^{-1} µg $protein^{-1}$. Data are means ± SD of three biological repetitions

encoding HIGH AFFINITY NITRATE TRANSPORTER (At1G08090) was previously shown to be highly induced in both *loh2* and *atr1* in microarray experiments of AT-treated plants (Gechev et al. 2008). Indeed, this gene was induced in all mutant lines (Table 2), indicating that AT induced oxidative stress resulting in growth inhibition. The growth inhibition was confirmed by the repression of the *EXTENSIN* gene At5G46890 (Table 2). Extensins are known to play an essential role in cell expansion and their repression is consistent with growth inhibition. Earlier microarray analysis of AT-induced gene expression in *loh2* and *atr1* also revealed downregulation of the extensin gene (Gechev et al. 2008). It seems that oxidative stress results in a common response of all lines exhibited as a cessation of growth. However, the oxidative stress-tolerant lines, *atr1, atr2, atr7* and *atr9*, are able to somehow circumvent the growth inhibition and avoid AT-induced cell death. The heat shock encoding gene *HSP17* is not expressed under normal conditions at this stage of development, but is highly upregulated in *loh2* on AT-induced oxidative stress, as revealed by microarray analysis (Gechev et al. 2008). Interestingly, this upregulation was evident only for *loh2* and not *atr1*. Herewith, we confirm the induction of *HSP17* in *loh2* and the absence of induction in *atr1*. Furthermore, we show that this gene is induced in all other lines except *atr7* (Table 2). The numbers even show downregulation of *HSP17* in *atr7*; however, taking into account the very low basal levels of expression, we can conclude that this gene is actually not expressed. As *atr7* is one of the mutants with high level of tolerance to AT, it seems that the mechanisms conferring oxidative stress tolerance may be different in *atr7* compared with other mutants.

Because AT inhibits catalase and this leads to subsequent elevation of H_2O_2 levels, we determined the catalase activity in *loh2* and *atr* mutants. Catalase activity was inhibited by AT in all lines, demonstrating that the oxidative stress tolerance of the *atr* mutants was not due to inability of AT to inhibit the calatase (Table 3). This means that an initial accumulation of H_2O_2 serving as a signal for oxidative stress and cell death is given. This conclusion is indirectly supported also by the expression of the nitrate transporter and the extensin genes. In the mutants most tolerant to oxidative stress, catalase activities were significantly lowered to an extent comparable to *loh2*. The inhibition of catalase activity by AT in both *loh2* and *atr* mutants suggests that the mutations conferring oxidative stress tolerance may be interfering with the perception or/and transduction of the redox signal rather than H_2O_2 accumulation. Alternatively, the mutation may inactivate a gene essential for regulation or execution of the cell death program that is situated below the H_2O_2 perception and transduction, downstream in the signaling cascade. Interference with other signaling molecules such as reactive nitrogen

species also cannot be excluded. ROS and nitric oxide (NO) interact to modulate together many cellular processes, including PCD (Gechev et al. 2006). H_2O_2-stimulated NO accumulation has recently been shown to be executed by the prohibitin gene *PHB3* (Wang et al. 2010). Mutation in PHB3 abort NO accumulation but does not affect H_2O_2 signaling. Further substantiating the link between ROS and NO during cell death is the recent positional cloning of *PARAQUAT RESISTANT2* gene in Arabidopsis, which encodes an *S*-nitrosoglutathione reductase (Chen et al. 2009). Positional cloning of the *atr* mutants will help to understand the intricate mechanisms of cell death tolerance in *Arabidopsis*.

Summary

Nine mutants with enhanced tolerance to AAL toxin have been isolated. Some of the mutants also exhibit enhanced tolerance to ROS-induced cell death triggered by AT or/and paraquat. Mutants with increased tolerance to all cell death stimuli may represent genes from a converging or downstream cell death pathway, whereas mutants that exhibit enhanced tolerance to the AAL toxin only may represent genes involved specifically in AAL toxin stress responses. Six of the mutants were smaller in size both when grown in vitro and on soil. Furthermore, some of them had altered leaf shape and one of them exhibited delayed senescence. Taken together, these results indicate that some of the genes responsible for oxidative stress tolerance and cell death may also be involved in modulating plant growth, development and senescence. Evaluation of oxidative stress tolerance together with expression analyses of gene markers for oxidative stress and cell expansion reveal that AT-induced oxidative stress leads to growth inhibition in all mutants, but AT-induced cell death is overcome in *atr1, atr2, atr7* and *atr9*. The different gene expression pattern of *atr7* suggests a different mechanism of stress tolerance.

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