A mutation in the cytosolic O-acetylserine (thiol) lyase induces the genome-dependent old3-I early leaf death phenotype in Arabidopsis

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Abstract

The Arabidopsis onset of leaf death3-1 (old3-1) mutant shows a temperature dependent premature leaf death phenotype. The old3-1 mutation segregates as a monogenic co-dominant trait when backcrossed to its wild type accession Landsberg erecta (Ler-0). In contrast, F2 progeny of old3-1 backcrossed to Col-0 segregates in an 11:4:1 (wild type: co-dominant: mutant) ratio demonstrating that the early leaf death phenotype depends on two loci. The second locus that determines the old3-1 early leaf death phenotype is referred to as ODD (for old3 determination). The old3-1 mutation is caused by a point mutation in the cytosolic O-acetylserine (thiol) lyase OAS-TL gene causing a single amino acid change from Gly\textsuperscript{162} to Glu\textsuperscript{162}. OAS-TL enzymes catalyse the formation of cysteine and mitochondrial and plastidic OAS-TL enzymes are believed to contribute primarily to cysteine biosynthesis as well. The old3-1 protein has no detectable in vitro OAS-TL activity. In order to distinguish the direct effects of the mutated OAS-TL we isolated a T-DNA insertional OAS-TL mutant and designated as old3-2. The cytosolic OAS-TL was found to function in providing resistance against cadmium-induced stress and for maintaining sulphur and thiols levels, independent of the ODD gene. The old3-1 early leaf death phenotype is associated with high expression of defence response and oxidative stress marker genes. Further analysis placed the ODD gene within a ~ 50-kb genome region on chromosome 3 which carries a recently integrated copia-like retrotransposon gene that is present in the Ler-0, but not in the Col-0 genome. The insertion is associated with a high genetic diversity including SNPs, insertions/deletions and altered gene structures. Thus, the old3-1 mutation causes genome dependent and independent phenotypes.
Introduction

Programmed cell death (PCD) is essential for growth and development of multicellular organisms. In plants, it occurs at all stages of the life cycle, from fertilization of the ovule to death of the whole plant (Van Doorn and Woltering, 2005). The final stage of leaf development is marked by a PCD process and allows for the recycling of accumulated nutrients from senescing leaves to young leaves and/or developing seeds (Lim et al., 2007). Activation of PCD could be dependent on phytohormones such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), ethylene, lipid-related signals, ion fluxes and reactive oxygen species (ROS) pathways (Kotchoni and Gachomo, 2006; Khurana et al., 2005; Guo et al., 2003). Thus, PCD is a vital and dynamic process for plant survival that involves many signalling pathways.

ROS, as one of the key regulators of PCD in plants, is associated with aging, cellular and molecular alteration in plant cells and considered as the most essential by-product of energy-generating processes such as photosynthesis and respiration (Van Breusegem and Dat, 2006; Kotchoni and Gachomo, 2006). Moreover, the ROS network is involved in disease resistance and even to mediate resistance to multiple stresses in plants (Gadjev et al., 2006; Kotchoni and Gachomo, 2006). Under normal conditions, ROS are quickly metabolised (Kotchoni and Gachomo, 2006), while excessive ROS accumulation under various conditions such as ozone fumigation, cold stress, UV radiation and senescence lead to PCD (Hoeberichts and Woltering, 2003). The levels of ROS therefore need to be tightly regulated to avoid cell damage (Mittler et al., 2004; Neill et al., 2002). Plant adaptive responses to increased levels of ROS are mediated in part through changes in cellular glutathione (GSH) levels (Youssefian et al., 2001). Cysteine is the building block for sulphur-containing organic components including GSH that have been implicated in the adaptation of plants to a wide range of biotic and abiotic stresses (May et al., 1998). Moreover, cysteine availability was found to be a major factor controlling GSH biosynthesis (Noctor et al., 1996; Strohm et al., 1995). GSH not only serves as the main storage form of reduced sulphur, but it also plays an important role in the control of thiol-disulphide status of the cell (Blaszczyk and Brodzik, 1999). Cysteine-derived GSH and other secondary organic sulphur components are involved in the scavenging of free radicals and hence are implicated in...
the oxidative damage-induced cell death processes including leaf senescence (Navabpour et al., 2003).

Cysteine synthesis is a point of convergence for nitrogen and sulphur assimilation. Sulphur interacts with nitrogen in such a way that lack of one reduces the uptake and assimilation of the other (Hesse et al., 2004). Sulphur uptake and assimilation has been shown to be dependent upon the constant supply of the precursor of cysteine, O-acetylserine (OAS), which is dependent on adequate nitrogen and carbon availability (Kopriva et al., 2002; Koprivova et al., 2000). In the presence of OAS-TL, OAS is generated from serine and acetyl-CoA in a reaction catalysed by sulphur acetyltransferase (SAT) (Hesse et al., 2004). Sulphur assimilation starts with the uptake of anionic sulphate into plant cells by a protein family of plasma membrane associated proton/sulphate co-transporters (Buchner et al., 2004). Through serial enzymatic reactions, sulphate is converted into sulphide, which, together with O-acetylserine (OAS), forms cysteine in a reaction catalysed by O-acetylserine (thiol) lyase (OAS-TL) (Leustek et al., 2000; Saito, 2000). Detailed biochemical studies have shown that OAS-TL and SAT form an enzymatic complex or dissociate from each other, depending on the sulphur, OAS and cysteine status inside the cell (Hell et al., 2002; Noji and Saito, 2002; Saito, 2000). SAT is only active when it is associated with OAS-TL, whereas OAS-TL is only active when it is dissociated from SAT (Hell et al., 2002). The SAT to OAS-TL protein expression ratio suggests that SAT is the limiting factor for the reaction (Wirtz et al., 2004). In a number of species, both OAS-TL and SAT were shown to have three subcellular isoforms in the cytosol, chloroplasts and mitochondria (Hesse et al., 1999; Noji et al., 1998), but their contribution to the overall cysteine biosynthesis and plant metabolism is unclear. Interestingly, the cytosolic SAT/OAS-TL complex is subject to cysteine feedback control and this is postulated to be important for sensing the sulphur nutrition status and fine-tuning cysteine biosynthesis in plants (Hell et al., 2002; Noji and Saito, 2002).

In this study, we show that the old3-1 gene (Jing et al., 2002) encodes a mutated cytosolic OAS-TL that induces an early leaf death phenotype through an interaction with an unknown gene designated as ODD. The mutated OAS-TL is unable to catalyze cysteine biosynthesis in vitro, but this is not the cause for the leaf death phenotype. The
results show that the cytosolic OAS-TL is involved in the maintenance of sulphur balance and thiols contents and for resistance against cadmium-induced stress.

Results

The *old3-1* early leaf death phenotype is a temperature dependent trait

The *old3-1* (onset of leaf death3-1) mutant was isolated from an EMS-mutagenized *Arabidopsis* Landsberg erecta (Ler-0) population as a co-dominant trait (Jing et al., 2002). As figure 1a and 1b show, the early leaf death syndrome in homozygous *old3-1* plants occurs in cotyledons of air-grown seedlings after 12 days concomitant with a rapid drop in chlorophyll content and an increase in ion leakage, followed by death of the plant after approximately 4 weeks. Plants heterozygous for the *old3-1* mutation show premature leaf yellowing but continue to grow further and set seeds. The *old3-1* phenotype was found to be a temperature-dependent trait, and growth at 28°C completely abolished the cell-death phenotype (Figure 2a). Subsequent transfer of the plants to 21°C after 2, 4 or 6 weeks resulted in the cell death phenotype within 10 days (Figure 2b), showing that the temperature dependence is independent of the developmental stage. We examined expression of several marker genes involved in developmental senescence, defence response, and programmed cell death in *old3-1* and wild type plants. A constitutive expression of PR1, SAG14, SAG21 and SAG13 was detected in 16- and 24-day-old *old3-1* plants, while no signs of SAG12 expression were found (Jing et al., 2002). Marker genes of PR-1 and SAG13 are associated with programmed cell death as well as oxidative stress signals (Brodersen et al., 2002). Therefore, relative transcription levels of a general oxidative stress marker gene *DEFL* (*AT2G43510*) (Gadjev et al., 2006) were measured, and a ~160-fold increased signal was detected in 12-day-old *old3-1* plants, as compared to wild type plants (Figure 3). The absence of SAG12 expression, concomitant with the strong expression of SAG13, SAG21 and SAG14 in association with over expression of the PR-1 gene and oxidative stress marker gene *DEFL* suggest that early leaf death in *old3-1* seedlings does not result from activation of a developmental senescence program, but rather via a cell death pathway that is related to the defence system.

The *old3-1* phenotype is a genome dependent trait in *Arabidopsis thaliana*

It was shown previously that the F2 generation obtained from the cross between
heterozygous old3-1 and Ler-0 plants segregates for the old3-1 phenotype as a monogenic trait, while the same phenotype segregates in a ratio of one to four to eleven (old3-1 homozygous : heterozygous : wild type) when crossed to Col-0 (Jing et al., 2002; Table 1). Thus, two genes control the old3-1 phenotype. The presence of the Ler-0 ODD allele in old3-1 plants, rather than the absence of the same allele in Col-0, is required for the manifestation of the old3-1 phenotype. The old3-1 heterozygous plants were crossed to two additional accessions. The old3-1 phenotype segregated in D1-2 as in Ler-0, while the Ws-0 accession behaved similar to Col-0 (Table 1). Therefore, different Arabidopsis genomes resulted in two distinct segregation patterns for old3-1 phenotype.

Initial mapping revealed that old3-1 phenotype is linked to two locations on the genome, a CAPS marker called G4539a on chromosome 4, and a SSLP marker called K11J14 on chromosome 3 (Jing et al., 2002). Therefore, one of the genome locations carries the old3-1 mutation, and the other gene, determining old3-1 phenotype, was designated as ODD (old3-1 determinant). The Ler-0 and Col-0 alleles were called ODD and odd, respectively. To clarify the position of the old3-1 and ODD loci on the Ler-0 genome, heterozygous plants for old3-1 mutation were crossed to fca-1 and abil-1 (both having the Ler-0 background). The old3-1 mutation was found to co-segregate
with *abi1-1* and *fca-1* alleles (Data not shown). Thus, the *old3-1* mutation is located on chromosome 4 and the *ODD* gene on chromosome 3. Thus, the *old3-1* early leaf death phenotype is a genome dependent trait in *Arabidopsis* and it requires the *ODD* gene.

**Table 1.** Phenotypes and segregation of F1 and F2 progeny of crosses between heterozygous *old3-1* plants and various wild type *Arabidopsis* accessions

<table>
<thead>
<tr>
<th>Male Female</th>
<th>F1 phenotype</th>
<th>F2 phenotype and segregation</th>
<th>Insertion size</th>
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<tbody>
<tr>
<td></td>
<td>Wild type old3-1</td>
<td>Wild type old3-1 old3-1</td>
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<tr>
<td></td>
<td>Het</td>
<td>Het Hom</td>
<td>χ² Segregation</td>
</tr>
<tr>
<td><em>old3-1</em></td>
<td>Ler-0</td>
<td>13 12</td>
<td>115 241 123 0.197 1:2:1</td>
</tr>
<tr>
<td>Het</td>
<td>Di-2</td>
<td>29 24</td>
<td>183 382 166 0.74 1:2:1</td>
</tr>
<tr>
<td></td>
<td>Bu-18</td>
<td>15 17</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>Wa-1</td>
<td>7 8</td>
<td>- - - -</td>
</tr>
<tr>
<td><em>old3-1</em></td>
<td>Col-0</td>
<td>49 0</td>
<td>237 88 19 0.337 1:4:11</td>
</tr>
<tr>
<td>Het</td>
<td>Ws-0</td>
<td>53 0</td>
<td>341 119 32 0.18 1:4:11</td>
</tr>
<tr>
<td></td>
<td>Wil-2</td>
<td>30 0</td>
<td>- - - -</td>
</tr>
</tbody>
</table>

1 In the crosses between Het-*old3-1* and Ler-0, Di-2 and Bu-18 accessions, only the F1 progeny with the Het-*old3-1* phenotypes was selected and continued for F2 segregation analyses. In the crosses between Het-*old3* and Col-0 and Ws-0, at least ten F1 plants were selected and allowed to set seed and the phenotypes of F2 populations were observed. The segregation analyses were done with at least 500 plants in the F2 populations containing the mutant phenotypes.

The *OLD3* gene encodes the cytosolic O-acetylserine (thiol) lyase (OAS-TL)

Further mapping placed the *old3-1* mutation into a 14-kb region on the bacterial artificial chromosome clone FCA2 (accession number Z97337) spanning 8 open reading frames. The sequence data analysis revealed a G to A substitution in AT4G14880, encoding the cytosolic O-acetylserine (thiol) lyase (cytosolic OAS-TL), and the mutation resulted in a Gly\(^{162}\) to Glu\(^{162}\) substitution. The entire *old3-1* gene including its promoter region was cloned and transformed into wild-type Ler-0 plants. The transformants homozygous for *old3-1* mutation displayed the same phenotypes as the *old3-1* mutants (Figure 4a). Thus, the *old3-1* gene is responsible for the *old3-1* phenotype.
Figure 2. The effect of temperature on the old3-1 phenotype
a) Wild type (Ler-0) and homozygous 21-day-old old3-1 plants growing at 28°C. Bar represents 0.5 cm.
b) Representative soil-grown old3-1 mutants were grown either in 50 µmol cm⁻² s⁻¹ cool white fluorescent light and 28 °C for 32 days (left) or in 50 µmol cm⁻² s⁻¹ cool white fluorescent light and 28 °C for 16 days, and then for another 16 days in 65 µmol cm⁻² s⁻¹ cool white fluorescent light and 21 °C (right). Bar represents 0.5 cm.

Figure 3. Relative transcript abundance of the DEFL gene
Relative transcript levels of the general oxidative stress-related marker DEFL in 12-day-old old3-1 and Ler-0 plants were examined by quantitative RT-PCR analysis, using ACTIN2 as internal control. The values shown are the means of three repeats ± SD (indicated by error bars).

OAS-TL uses pyridoxal 5’-phosphate (PLP) as a cofactor to synthesize cysteine from O-acetylserine (OAS) and sulfide (Leustek et al., 2000; Saito, 2000; Bogdanova and Hell, 1997). According to the amino acid sequence alignment of three OAS-TL isomers in Arabidopsis, the old3-1 amino acid substitution is located at the fifth α-helix in the middle of highly conserved area (TTGPEIW) (Figure 4b) (Bonner et al., 2005). The fifth α-helix, including His157 that interacts with the pyridoxal 5’-phosphate (PLP) binding site, is in close proximity to a mostly conserved region at the sixth α-helix (GAGKYLK) including Ala189, Tyr192 and Leu193 (Figure 4c). The Gly162 to Glu162 substitution results in a size difference that may cause a change in the protein structure and activity.
Figure 4. Complementation of the old3-1 phenotype and amino acid sequence alignment and secondary structure of the cytosolic OAS-TL.

a) The old3-1 genomic region was cloned and transformed to Ler-0 wild type plants. A representative 14-day-old Ler-0 plant carrying the homozygous old3-1 transgene is shown.

b) Amino acid sequence alignment of the area surrounding G_{162} of three Arabidopsis OAS-TL proteins. The position of the G_{162} to E_{162} mutation in the cytosolic OAS-TL is indicated in the figure. Amino acids shown in green boxes are highly conserved amino acids; amino acid residues shown in red boxes are involved in interacting with pridoxal 5'-phosphate (PLP), the schematic drawing indicates location of amino acids in helix-5 and helix-6. P (AT2G43750 - plastid OAS-TL); M (AT2G59760 - mitochondria OAS-TL); C (AT4G14880 - cytosol OAS-TL).

c) The location of PLP, Lys^{46}, which is the only residue forming a covalent bond (the Schiff base) with cofactor PLP, and His^{157} that is involved in interacting with PLP are shown by stick drawings (highlighted black on orange). The position of Gly^{162} (gold) in helix-5 is shown as a CPK model. This residue is in close proximity with residues in helix-6 including Ala^{189} (green), Tyr^{192} (white), and Leu^{193} (rose). Figure made using Pymol, http://pymol.sourceforge.net/.

The old3-1 protein has no in vitro OAS-TL activity

The *E.coli* Cys auxotrophic strain NK3 (van der Ploeg et al., 2001) lacks the cysteine synthase gene and consequently is unable to grow on medium without supplemental cysteine. The cytosolic OAS-TL is able to complement the *E. coli* NK3 strain (Hesse et al., 1999) and we determined whether the old3-1 gene was able to complement the *E.coli* strain as well. The old3-1 and the wild type OLD3 cDNAs were...
cloned into an expression vector. Subsequently, the *E. coli* NK3 strain was transformed with the expression vector harbouring the cDNAs and the empty vector and grown on minimal medium M9 with and without cysteine. *E. coli* NK3 transformed with the OLD3 cDNA could grow on the medium lacking cysteine, while *E.coli* NK3 carrying the old3-1 cDNA or empty vector were unable to grow further (Figure 5). These results confirm that the cytosolic OAS-TL is able to complement the *E. coli* Δcys NK3 strain and suggest that the cytosolic old3-1 protein has either a reduced or no OAS-TL enzyme activity in *E.coli* NK3.

**Figure 5. Genetic complementation of the Cys^- E.coli strain NK3**

The empty vector (pTrc99A), and vector harbouring the wild type cytosolic OAS-TL (pTrc99A:OLD3) and mutated old3-1 OAS-TL (pTrc99A:old3-1) cDNAs were transformed to the *E.coli* NK3 strain and plated onto M9 medium with (a) or without (b) 0.5 mM cysteine and incubated at 37°C.

**Figure 6. Phenotypes of wild type and old3 mutant plants**

Representative 27-d-old soil-grown plants of Ler-0, Col-0, old3-1odd (carrying the homozygous old3-1 mutation and homozygous odd allele in a Col-0/Ler-0 mixed background) and old3-2 (carrying a homozygous T-DNA insertion in the OLD3 gene) grown in 250 µmolcm^-2 s^-1 cool white fluorescent light at 21°C. Bars represent 0.5 cm.
For further old3-1 OAS-TL activity analysis, the entire open reading frames of OLD3 and old3-1 genes were cloned into the expression vector pET15b, and subsequently old3-1 OAS-TL and OAS-TL OLD3 proteins over-expressed in E.coli BL21 (DE3) strain. The protein purification was performed through a Ni$^{2+}$-NTA-agarose column, and molecular masses on a SDS-PAGE gel were found to be in agreement with the expected molecular mass. We applied 3.1 nM purified OLD3 and old3-1 OAS-TL enzymes to the cysteine precursors OAS and Na$_2$S, and subsequently OAS-TL activity was measured according to Gaitonde (1967). A specific activity of 84 ±4 µmol min$^{-1}$ mg$^{-1}$ (48 ±2 s$^{-1}$) was found for the OLD3 OAS-TL, but for the same enzyme concentration no activity was detected for old3-1 OAS-TL. The obtained value in this experiment lies within those values already published (906 ±39 µmol min$^{-1}$ mg$^{-1}$ (Wirtz et al., 2004), 2 µmol min$^{-1}$ mg$^{-1}$ (Burandt et al., 2002), and 225 µmol min$^{-1}$ mg$^{-1}$ (Jost et al., 2000)). Together, the data shows that old3-1 cytosolic OAS-TL has no in vitro activity at the enzyme concentrations tested and suggests that the old3-1 mutation results in a loss-of-function protein.

The cytosolic OLD3 OAS-TL affects sulphur balance and thiols levels

Under sulphur-sufficient conditions, metabolites such as cysteine and glutathione act as the regulators of sulphur uptake and assimilation (Hesse et al., 2004). In order to clarify if the non-functional cytosolic OAS-TL has some effects on availability of down-stream and up-stream compounds in the cysteine biosynthesis pathway, we measured the contents of total and ion sulphate as direct measurements of plant sulphur balance as well as thiols levels as the representative parameter involved in plant secondary metabolism (Gotor, 2003; Cobbett, 2000; Rauser, 1993). To be able to distinguish between direct effects of the mutated OAS-TL and indirect effects as a result of the early leaf death phenotype we isolated the old3-2 mutant. This mutant carries a homozygous T-DNA insertion in the cytosolic OAS-TL and has the Col-0 (odd) background. Similar to the old3-1odd line (carrying the homozygous old3-1 mutation and odd allele in a Col-0/Ler-0 mixed background) the old3-2 mutant did not show the early leaf death phenotype (Figure 6). Figure 7a and 7b show the total and ion sulphur contents as well as thiols levels of the old3 mutants and their respective wild types. Total and ion sulphur contents were increased in old3-1 and old3-2 lines as compared to their wild types, while the thiols contents were decreased. The data shows that the
cytosolic OAS-TL, independently from the *ODD* gene affects sulphur balance as well as thiols levels in *Arabidopsis*.

**old3 mutant lines show an increased sensitivity to cadmium stress**

The cytosolic OAS-TL gene expression is up regulated in response to cadmium-induced stress and this may allow for enhanced cadmium tolerance (Dominguez-Solis et al., 2004). The effect of cadmium on seedling growth of the *old3-1* and *old3-2* mutants and their wild types was investigated. Figure 7c shows the effect of various cadmium concentrations on plant growth. At 150 µM cadmium all tested genotypes showed a considerable decrease in fresh weight as compared to growth without cadmium. The *old3-1* and *old3-2* lines showed significant reductions in fresh weight from 50 µM cadmium onwards. Thus, the cytosolic OAS-TL is involved in cadmium resistance, independent of the presence of the *ODD* or *odd* gene.

**Figure 7.** Effect of a mutated cytosolic OAS-TL on sulphur metabolism and cadmium-induced stress

a) Total sulphur and sulphur anions were measured in dry materials obtained from the grown plants in soil saturated with 25% Hoagland solution after 27 days.

b) Total water-soluble non-protein sulphydryl (thiols) compounds were measured in fresh materials obtained from 27-day-old plants. Aboveground parts of 27-day-old plants were harvested for total sulphur, sulphur anions and thiols measurements. Data are shown as mean ± standard deviation of four replicates each includes 17 plants. Bars with the same letters are not significantly different from each other after Duncan multiple variant significance test at a significance level of p < 0.05.

c) Effect of CdCl₂ on fresh weight of *old3-1*, *old3-2* and their respective wild types. Fresh weight was measured after 16 days of growth on the indicated concentration of CdCl₂. Fresh weight is expressed as percentage of the fresh weight when grown without CdCl₂ (set at 100). Data are shown as mean ± standard deviation of three replicates (20 seedlings for each replicate).
Early leaf death *old3-1*

![Diagram showing the structure of the ODD genome region](image)

**Figure 8.** A diagram showing the structure of the *ODD* genome region

- **a)** The location of the copia-like retrotransposon insertion in *Ler*-0 genome is based on the Col-0 DNA sequence. The exact position of the insertion is indicated by the triangle. DNA sequence from the left and right sides of the retrotransposon are shown.
- **b)** The genome region carrying a DNA fragment in which Col-0 and *Ler*-0 are different in size and sequence. Flanking sequence of left and right sides of the 685 bp in Col-0 genome, and the DNA sequence of left and right sides of the 859 bp in *Ler*-0 genome are shown.
- **c)** Predicted promoter region for the *AT3G44630* gene is available at Agris database (http://Arabidopsis.med.ohio-state.edu).

The *old3-1* early leaf death phenotype is associated with a recent copia-like retrotransposon insertion

Map-based cloning placed the *ODD* locus within a ~50-kb region on chromosome 3 designated as the *ODD* genome region. The *ODD* region encompasses 5 genes including *AT3G44600, AT3G44605, AT3G44610, AT3G44620* and *AT3G44630*. Analysis of the *ODD* region revealed that a ~10-kb PCR fragment could be amplified in the inter-genic region between genes *AT3G44610* and *AT3G44620* from the *Ler*-0 *ODD* region, while Col-0 DNA template resulted in a ~ 5-kb PCR fragment, consistent with the available genome sequence. Examination of the insertion polymorphism in several *A.thaliana* accessions showed that the accessions Di-2, Bu-18 and Wa-1 that carry an *ODD* allele, have the ~ 10-kb fragment, while Ws-0 and Wil-2 that carry the *odd* allele harbour the ~ 5-kb fragment (Table 1). Thus, the insertion seems to be associated with the *old3-1* leaf death phenotype. The insertion was present in the *Ler*-0 genome.
between the position 16203698 and 16203718 according to the Col-0 genome sequence (Figure 8a). The insertion from Ler-0 was sequenced and showed a high homology with the copia-like retrotransposon gene family AT2g14930 in A.thaliana (GenBank accession number EU982550), and was placed in the long-terminal-repeat (LTR) retrotransposon category. Retrotransposons have played a significant role in evolution of plant genomes (Kumar and Bennetzen, 2000; Kumar and Bennetzen, 1999) and are activated through various biotic and abiotic stresses (Hirochika, 1997; Grandbastien et al., 1997; Wessler, 1996) and induce alteration in transcription levels of adjacent genes (Kashkush et al., 2003). Sequencing of 3500 bp on the left side of insertion, and 2700 bp from the right side resulted in detection of, on average, one polymorphism every 60 bp and 24 deletions/insertions over the sequenced region (Supplementary table 1 and 2 available at http://www.rug.nl/gbb/research/researchgroups/molecularBiologyOfPlants). Interestingly, a DNA structure rearrangement was found 300 bp away from the copia-like retrotransposon insertion between genome position of 16204018 and 16204674, 5’of AT3G44620 (Figure 8b) (GenBank accession number EU982550).

![Figure 9. Relative transcript abundance of the AT3G44630 gene](image)

Relative transcript levels of the AT3G44630 gene of cotyledons of 9 and 11 day-old Col-0, Ler-0 and old3-1 plants were examined by quantitative RT-PCR analysis, using ACTIN2 as internal control. The values shown are the means of three repeats ± SD (indicated by error bars).

The effect of the retrotransposon insertion itself and/or the sequence variation in the region on the relative gene expression of the three genes AT3G44610, AT3G44620 and AT3G44630 was tested. Gene expression was measured in 9 and 11 day-old air-grown seedlings of the wild type accessions Col-0 and Ler-0 and the old3-1 mutant, using real time PCR. No significant difference between expression levels of AT3G44610, AT3G44620 in Col-0, Ler-0 and old3-1 plants was found (Data not
shown). However, the relative transcription levels of AT3G44630, a NB-LRR gene was ~12 and ~5 times higher in 9- and 11-day-old Col-0 seedlings, respectively compared to Ler-0 seedlings. The same gene reached a ~5 and ~11 fold higher expression in 9- and 11-day-old old3-1 seedlings, respectively when compared with their own wild type Ler-0 (Figure 9). The data suggests that the altered expression levels of AT3G44630 gene in Ler-0 and Col-0 seedlings may be caused by changes in activity of the copia-like retrotransposon gene and/or mutations in the region. The higher expression in old3-1 seedlings as compared to its own wild type Ler-0 may be caused by the specific interaction between the old3-1 and ODD genes.

The polymorphism found in the ODD region between Arabidopsis accessions furthermore suggests that this region may have a haplotype structure in Arabidopsis.

**Discussion**

**The old3-1 early leaf death phenotype depends on ODD**

Here we show that the co-dominant old3-1 mutation causes a Gly\(^{162}\) to Glu\(^{162}\) substitution in the cytosolic OAS-TL. The presence of this mutation causes an early leaf death phenotype in combination with a natural variant gene, ODD. The ODD gene is present in Arabidopsis accessions Ler-0, Di-2, Bu-18 and Wa-1 but not in Col-0, Ws-0 or Wil-2. Interestingly, the presence of odd/ODD alleles does not correlate with the geographic origins of the accessions, or with their phylogeny relationships as illustrated with molecular markers (Barth et al., 2002). Therefore the early leaf death phenotype is a genome dependent trait and the activity of the mutated old3-1 allele, rather than the absence of the wild type OLD3 allele is responsible for the phenotype. The leaf death phenotype coincides with increased expression of an oxidative stress marker and with other marker genes commonly associated with the activation of the plant immune system. The old3-1/ODD interaction resembles that of bon1-1 with the natural modifier gene SNC1 (Ichimura et al., 2006; Yang and Hua, 2004). The growth defect of bon1-1 plants was reported to be a genome dependent phenotype. The BON1 gene negatively regulates the haplotype-specific resistance gene SNC1 that is present in the Col-0, but not in the Ws genome. Activation of SNC1 by bon1-1 leads to constitutive defence responses and subsequent compromised plant growth (Yang and Hua, 2004). Interestingly, the old3-1 cell death phenotype strictly depends on temperature and
growth at 28°C abolishes the phenotype. Constitutive defence responses in the cell death mutants have previously been reported to be suppressed by high temperature and/or high humidity (Bomblies et al., 2007; Noutoshi et al., 2005; Xiao et al., 2003; Hua et al., 2001; Yoshioka et al., 2001; Whitham et al., 1994). Thus, the old3-1 early leaf death phenotype is consistent with a constitutive activation of the plant immune system through the interaction of the old3-1 and ODD genes.

The ODD genomic region includes a big insertion in the inter-genic area between the genes of AT3G44610 and AT3G44620. The insertion is a copia-like retrotransposon gene, with high similarity in sequence with the AT2g14930, AtRE2-1 and Ty1-copia-element genes. LTR-retrotransposons are located mainly in inter-genic regions and are the single largest components of most plant genomes (Kumar and Bennetzen, 1999). Retrotransposons are a major element of most eukaryotic genomes representing approximately 50-80% of some of the grass genomes (Kashkush et al., 2003; Feschotte et al., 2002). Retrotransposons can affect genome structure by inserting into and/or excising from loci and as such generate a great variety of mutations in plant genomes (Casacuberta and Santiago, 2003; Kashkush et al., 2003; Hirochika et al., 1996). Transposons are often transcriptionally silent but can be activated under certain stresses, external changes and plant hybridization (Kashkush et al., 2003; Liu and Wendel, 2000; Grandbastien et al., 1997). Subsequently active retrotransposons can affect transcription of adjacent genes by producing sense or antisense transcripts of those genes (Kashkush et al., 2003). It is tempting to speculate that the copia-like retrotransposon is the cause for the presence of the ODD allele. The transposon is associated with a more than two-fold increase in SNPs between the Col-0 and Ler-0 accessions. A recent finding reported on average one SNP every 166 bp (Buckler and Gore, 2007), while the genomic region flanking the transposon contains an average of one SNP polymorphism every 60 bp. In addition, several small and one large InDels were found. Moreover the presence of the transposon insertion may be the direct or indirect cause of the decreased expression of the AT3G44630 gene in Ler-0, as the copia-like retrotransposon integrated next to the predicted promoter region of the AT3G4430 gene (Figure 8c), according to the prediction of promoter regions of Arabidopsis genes (Davuluri et al., 2003) (Agris database available at (http://Arabidopsis.med.ohio-state.edu). The increased AT3G4430 expression in old3-1 mutants may be the results of the high level
of stress caused by the interaction between old3-1 and ODD. Interestingly, AT3G4430 encodes a TIR NBS-LRR putative resistance gene. Its altered expression in old3-1 mutants, together with the increased expression of immune system marker genes justifies the suggestion that AT3G4430 may be identical to ODD, but this requires further study. Incidentally, the AT3G44630 gene was designated as a candidate genes for the DM2 interaction with DM1 in a cross between the Arabidopsis accessions Uk-3 and Uk-1 that results in a hybrid necrotic phenotype (Bomblies et al., 2007). NB-LRR resistance genes are often associated with regions that carry high levels of polymorphism (Clark et al., 2007; Cork and Purugganan, 2005; Noel et al., 1999). The presence of the retrotransposon, the putative resistance gene and the increased number of SNPs allows for the possibility that the ODD genomic region segregates as a haplotype allele. However, further mapping would need to be pursued to confirm this. Thus, the old3-1 phenotype is caused by a specific interaction between a mutated cytosolic OAS-TL and a genome region encompassing a copia-retrotransposon gene.

The cytosolic OAS-TL is involved in cadmium resistance and sulphur metabolism

The old3-1 mutation causes a Gly162 to Glu162 substitution in the cytosolic OAS-TL and this results in a dysfunctional enzyme in vitro and likely in vivo as well. The 3D structure of the cytosolic OAS-TL has been resolved (Bonner et al., 2005) and this allows for an explanation for the compromised enzyme activity: the small and neutral Gly162 residue is substituted with a much larger charged amino acid Glu162 that is in close proximity to the sixth α-helix, therefore likely causing a positional movement in the α-helices 5 and 6, and subsequently shifts of the associated residues in these helices. Consequently most residues involved in the positioning of the substrate and the pyridoxal 5'-phosphate (PLP) cofactor, which is ~16 Å away from Gly162, might be affected by these helical shifts (Francois et al., 2006; Bonner et al., 2005). The effect of the mutation on the formation of the cysteine synthase complex and SAT activity is unclear.

There are three OAS-TL isomers in different cell compartments the cytosol, the plastids and the mitochondria with the same capacity for cysteine synthase (Lunn et al., 1990) suggesting that the availability of cysteine is essential for all cell compartments. Heeg et al. (2008) have recently studied the effects of T-DNA insertions in each of the OAS-TLs. The cytosolic isoform was found to be completely dispensable. Here we
confirm and extend their analysis on the specific function of the cytosolic OAS-TL on plant metabolism. Compromising OAS-TL function by a T-DNA insertion (old3-2) or by the old3-1 mutation, in combination with the homozygous odd allele resulted in plants that were indistinguishable from the wild type, when grown under standard conditions. However, a significant increase was found in both total and ion sulphate content in the old3-2 mutant. Heeg et al. (2008) did not find a similar increase. This may be due to differences in the growth conditions and/or plant age. In addition a decrease in non-protein thiols content was found. These results are consistent with a decreased sulphate assimilation resulting in lower organic sulphur, as a result of compromised cytosolic OAS-TL activity. However, the changes are small and may have little, if any, impact on plant growth when grown under standard growth conditions (Heeg et al., 2008).

Plants have developed defence systems to adapt with environmental stresses such as heavy metal pollution. Cysteine is required for the synthesis of glutathione (GSH) and is as such involved in plant responses to toxic levels of heavy metals (Cobbett, 2000). The cytosolic OAS-TL has been suggested to be important for cadmium-induced stress responses as heavy metal stress induced its mRNA levels by 3 to 7 fold (Dominguez-Solis et al., 2001; Barroso et al., 1999). Moreover, its overexpression resulted in increased cadmium tolerance (Dominguez-Solis et al., 2001; Dominguez-Solis et al., 2004). Thus, increased OAS-TL activity increases cadmium resistance. Here we show that plants with compromised cytosolic OAS-TL function are more sensitive to cadmium stress. Thus, the cytosolic OAS-TL is required for coping with cadmium-induced stress and the mitochondrial and/or the plastidic OAS-TL cannot fully compensate for the lack of cytosolic OAS-TL activity. The function of the cytosolic OAS-TL may therefore be to quickly respond to environmental stress, while its function during growth under environmentally ideal conditions may be limited.

Our data have allowed us to allocate a specific function for the cytosolic OAS-TL in heavy metal stress. In addition, the old3-1 mutation causes a specific, genome dependent interaction causing early leaf death. The nature of this interaction is still obscure, but the further identification of the ODD allele together with analysis of the leaf death pathways will allow us to unravel this unique death pathway.
Experimental procedures

Plant material and growth conditions

*Arabidopsis thaliana* accessions Landsberg erecta (Ler-0), Colombia (Col-0), Bu-18, Di-2, Wa-1, Wil-2 and Ws-0 were used in this study and several accessions were obtained from the NASC (Nottingham Arabidopsis Stock Centre). The old3-1 mutant has been described by Jing et al. (2002). A T-DNA line carrying a T-DNA insertion in the cytosolic OAS-TL gene in Col-0 background was obtained from ABRC under number N572213 (Alonso et al., 2003). A plant line homozygous for the insertion was designated as old3-2. The T-DNA insertion was recognized by a PCR product from the primer pair PrRuG659: TGGTTCACGTAGTGGGCCATCG / PrRuG761: TACACCAATGGAGTGTTCCCAATCA. Surface-sterilized seeds of Ler-0, Col-0, old3-1 and old3-2 genotypes were sown on MS media (Murashige and Skoog, 1962), pH 5.6, 1% sucrose, 1% agar, and CdCl2 at various concentrations. Seeds were sown and kept at 4°C for 3 days and subsequently transferred to a growth chamber (21°C, 16 h light/8 h dark) for 16 days before harvesting. Soil-grown plants were sown on an organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) as described by Jing et al. (2002).

Map-based cloning and complementation test

To indentify the old3-1 mutation, approximately 5000 old3-1old3-1ODDDODD F2 seedlings were selected from a mapping population generated by crossing old3-1OLD3ODDDODD plants with Col-0. DNA was isolated using the SHORTY quick preparation method (http://www.biotech.wisc.edu/Arabidopsis). By comparing the genomes of Col-0 (TAIR database) and Cereon Ler-0 (Monsanto SNPs and Ler-0) (http://www.Arabidopsis.org; Jander et al., 2002), potential SNPs were selected. Primers were designed, using the Web SNAPER program, and used for amplification of Col-0 DNA fragments (Drenkard et al., 2000; http://ausubellab.mgh.harvard.edu/resources). The mutation was mapped onto a 14 kb region spanning 8 open reading frames. Sequence analyses revealed a single nucleotide change inside the cytosolic OAS-TL. Agrobacterium-mediated transformation (Clough and Bent, 1998; Hellens et al., 2000) was performed to confirm that the old3-1 mutation causes the early leaf death phenotype.
Sequencing of the ODD region

The above-described mapping population was used to map the ODD region within an ~50kb region. The primers PrRuG2221 (ACACTACACTATCGTGCACATCA) and PrRuG2135 (TGAGGATAGACCGTTGACGAGT) were used to amplify a ~10 kb PCR fragment including the copia-like retrotransposon and AT3G44620. The obtained PCR products were purified and sequenced with the Solexa genome system (Bennett, 2004). DNA sequence was confirmed using Sanger sequencing.

Cloning, enzyme activity assay and complementation of ΔCys E.coli

OLD3 and old3-1 cDNA was amplified from cDNA of Ler-0 and old3-1 plants, respectively, using primers as described by Bonner et al. (2005). The PCR products were subcloned into a pGEM-T Easy vector (Promega) and sequenced. The DNA fragments were cut with NdeI and BamHI and ligated into NcoI/BamHI digested pTrc99A (Amann et al., 1988) and pET15b (Novagen) to create the pTrc99A:OLD3, pTrc99A:old3-1, pET15b:OLD3 and pET15b:old3-1 constructs.

For protein purification, E. coli BL21 (DE3) cells were transformed with pET15b:OLD3 and pET15b:old3-1 constructs as well as the empty vector pET15b. Transformed BL21 (DE3) cells were subsequently grown at 37°C until A₆₀₀ ~ 0.8. Cells were induced with 1mM 1-thio-β-D-galactopyranoside and grown for 4h. Following sonication, the cells were resuspended in lysis buffer (50 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, 10% (v/v) glycerol and 1% Tween 20). The supernatant was passed over an equilibrated Ni²⁺-nitrilotriacetic (NTA)-agarose column and washed with lysis buffer without Tween 20. Bound proteins were eluted with wash buffer containing 150 mM imidazole. The eluted proteins were dialyzed against 100mM Mopso (pH 7.0) and stored at -20°C. OAS-TL enzyme activity was determined as described by Bonner et al. (2005) using the Gaitonde method (Gaitonde, 1967). Briefly, the reaction was started with the addition of 26.7ng (3.1 nM) of OLD3 and old3-1 protein and incubated for 5, 10 and 15 min and subsequently stopped in 50 µl trichloroacetic acid. After centrifugation, 0.25 ml supernatant was added to 0.25ml acid-ninhydrin reagent (Gaitonde, 1967), and placed in a boiling water bath for 5min. After cooling, 0.5 ml 100% cool ethanol was added and the extinction at
560 nm was measured. The standard cysteine curve was made according to Nakamura et al. (1987).

For *E. coli* NK3 (ΔtrpE5 leu-6 hsdR hsdM<sup>+</sup> cysK cysM) (van der Ploeg et al., 2001) complementation, *E. coli* cells were transformed with the empty vector pTrc99A, pTrc99A:OLD3 and pTrc99A:old3-1 and grown on 5× M9 Minimal Media containing 1.5% agar plates, supplemented with 0.2g L⁻¹ leucine and tryptophan, 50 µg ml⁻¹ ampicilene, and 0.5 mM cysteine where indicated.

**Physiological analyses**

Chlorophyll content and ion leakage was measured as described by Jing et al. (2002). For total sulphur, anions and thiols measurement, plants were grown in soil saturated with 25% Hoagland solution for 27 days at 25 °C and 70% relative humidity and at a light intensity of 200 µmolm⁻²s⁻¹. The light cycle was set at a 16-h white light/8-h dark photoperiod. The above ground parts of plants were harvested for fresh weight and dry weight measurement. Dry plant materials were used for total sulphur measurement using the barium sulphate precipitation method (Jones, 1995) and for anion measurement using HPLC-based methods (Maas et al., 1986). Parts of the fresh samples were used for total water-soluble non-protein sulphhydril (SH) compounds following the method described by de Kok and Graham (1989). For the total and anion sulphate content data of old3-1old3-1ODDODD mutants, one replicate of 50 plants was used for analysis due to the small size of plants and for other data points four replicates of 17 plants were used.

**RNA-isolation and RT-PCR**

Total RNA was isolated and purified using the Qiagen RNeasy plant mini kit according to the manufacturer's protocol. Fifteen hundred nanograms of RNA were used as template for first-strand cDNA synthesis using 200U of RevertAid H-minus MMuLV reverse transcriptase (Fermentas, USA) and an oligo (dT) primer. Primer pairs for real-time PCR were designed with open-source PCR primer design program PerlPrimer v1.1.10 (Marshall, 2004). Real-time PCR amplification was performed using the Applied Biosystems power SYBR Green PCR Master Mix kit according to the manufacturer's protocol. The primers used for amplification of the genes At3g44610, At3g44620, At3g44630, ACTIN2 and DEFL (AT2G43510) are available on
request. The PCR program was 2’ at 94, 40x (94-10”/58-10”/72-25”), meltcurve. The obtained data was analysed with Bio-Rad software and Bio-Rad excel sheet.

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