Regulation of leaf senescence in Arabidopsis
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Chapter 5
*Arabidopsis onset of leaf death 3* encodes a mutated cytosolic O-acetylserine (thiol) lyase that results in enhanced cadmium tolerance and early leaf senescence

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Abstract
The Arabidopsis onset of leaf death (old) 3 is a co-dominant mutation that causes seedling-lethal, early senescence symptoms. The phenotype segregated as a monogenic trait in the parental line Ler-0 and 4 other accessions but as a two-gene controlled trait in Col-0, Ws-0 and Wil-2. This resulted from the specific interaction between old3 and a Ler-0 gene, named ODD (for old3 determinant). Our data implied that the corresponding Col-0 odd allele was either different or not present at all. Map-based cloning showed that old3-1 causes a gly162 to glu162 substitution in the cytosolic O-acetylserine (thiol) lyase (OAS-TL). Leaf senescence and sulphur metabolism was examined in old3-1ODD mutants, in old3-1odd mutants with a Ler-0 and Col-0 mixed background but with the odd allele from the Col-0 accession, and in a Col-0 old3-Todd mutant containing a T-DNA insertion in the OAS-TL gene. Leaf senescence was only enhanced in old3-1ODD mutants. Altered sulphur balances (the contents of the total, ionic and organic sulphur) and lower thiol levels were observed in old3-1ODD and old3-1odd mutants, but not in old3-1odd mutants. Thus, the old3-1 allele requires the ODD gene for the altered sulphur metabolism and early leaf senescence phenotypes. Cadmium tolerance was enhanced in old3-1ODD and old3-1odd mutants but reduced in old3-Todd mutants, showing that the ODD gene is not required for the enhanced cadmium tolerance phenotype. Together, the old3 OAS-TL gene has novel features of enhancing cadmium tolerance and leaf senescence.

Keywords: Arabidopsis, cadmium tolerance, cysteine synthesis, leaf senescence, old3, O-acetylserine thiol lyase (OAS-TL), sulphur metabolism

Introduction
Sulphur is essential for life and plants play an important role in the biological sulphur cycle. Cysteine is the building block for sulphur-containing organic components including glutathione that have been implicated in the adaptation of plants to a wide range of biotic and abiotic stresses (May et al., 1998). Cysteine synthesis creates a link between sulphur reduction and amino acid metabolism and therefore is a point of convergence for nitrogen and sulphur assimilation.

Sulphur assimilation starts with the transportation of anionic sulphate into plant cells by a gene 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). OAS is synthesised from serine and acetyl-CoA and this reaction is catalysed by sulphur acetyltransferase (SAT). Detailed biochemical studies have shown that OAS-TL and SAT physically interact with each other and can form an enzymatic complex or dissociate from each other depending on the status of sulphur, OAS and cysteine inside the cell (Saito, 2000; Hell et al., 2002; Noji and Saito, 2002). 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 cytosol, chloroplasts and mitochondria, but their contribution to the overall cysteine biosynthesis is unclear. Interestingly, the cytosolic SAT/OAS-TL complex is subject to cysteine feedback control and...
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is postulated to sense the sulphur nutrition status and fine-tune the overall cysteine synthesis in plants (Hell et al., 2002; Noji and Saito, 2002).

Organisms are not programmed for senescence or death but are programmed for survival (Kirkwood and Austad, 2000). It has been recognised that plant general metabolic and developmental processes are important for senescence regulation (Woo et al., 2002; Gan, 2003; Jing et al., 2003). In Arabidopsis, several senescence regulation genes have been identified through mutational analyses and were shown to have general functions in plant growth and development (Woo et al., 2000; He et al., 2002; Woo et al., 2003). Our study showed that CPR5/OLD1 might be a senescence regulation gene with multiple functions throughout plant development (Chapter 4). Cysteine-derived glutathione 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). Nonetheless, little molecular genetic evidence is available to show a causal link between leaf senescence and cysteine biosynthesis or sulphur metabolism in general.

We studied the interaction between leaf age and ethylene and isolated several classes of onset of leaf death (old) mutants with altered leaf senescence (Jing et al., 2002). Among the mutants isolated, a single old3-1 mutant line was isolated from the Landsberg erecta (Ler-0) accession. To elucidate the molecular basis of old3-1 induced early leaf senescence, cloning of old3 was initiated and revealed that old3-1 results in an amino acid substitution in the cytosolic O-acetylserine (thiol) lyase. The cysteine synthesis, sulphur balance and cadmium tolerance were examined in old3 mutants and their relationships with old3-induced senescence discussed.

Results

Arabidopsis accessions varied in the control of old3-induced phenotypes

A single old3-1 allele was isolated from an EMS-mutagenised Arabidopsis Ler-0 population in screen for mutants with altered ethylene-induced senescence. old3-1 was shown to be a co-dominant trait (Figure 1A; Jing et al., 2002). The senescence syndrome of old3-1 occurred in air-grown plants but was not further enhanced by exogenously applied ethylene. A rapid drop in cotyledon chlorophyll content occurred in old3-1 between 12-14 days after germination, and was followed by an increase in ion leakage (Figure 1B). Similar changes were not observed in wild type plants during this period. When old3-1 mutants were grown in a light intensity of 250µmol/s/cm², the lethal phenotype of homozygous old3-1old3-1 plants were rescued and the early senescence phenotypes of heterozygous old3-1OLD3 plants delayed (Figure 1C). This implies that the early senescence phenotype of old3 mutants is not the result of a general growth defect.

Plants heterozygous for the old3-1 mutations were crossed to Col-0 and all the F₁ progeny showed the wild type phenotype, instead of a 1: 1 ratio of wild-type: mutant in Ler-0 (Table 1; Jing et al., 2002). The segregation pattern of F₂ progeny also deviated from 1: 2: 1 of wild-type: intermediate: mutant. These results revealed that Col-0 and Ler-0 differ in the control of old3-1 phenotypes. Initial mapping showed that old3-1 phenotypes were linked to SSLP marker K11J14 on chromosome 3 and to CAPS marker G4539a on chromosome 4 (Jing et al., 2002). Thus, in addition to old3-1, a second gene is involved in the control of old3 phenotypes. The gene that determines the old3 phenotype was designated as ODD (old3 determinant). The Ler-0 allele was called ODD and the Col-0 allele odd. old3-1ODD was crossed to fca-1 and abi1-1 (both having the Ler background) to clarify which of the loci represents old3. old3-1
Figure 1. Phenotypes, chlorophyll content and ion leakage of old3-1 mutant lines and wild types.
(A). Representative 21-d-old soil-grown plants of Ler-0, heterozygous (old3-1OLD3ODD) and homozygous (old3-1ODD) mutants, grown in 60µmolcm⁻²s⁻¹ cool white fluorescent light.
(B). Chlorophyll content and ion leakage of cotyledons from Ler-0 plants and old3 mutants grown in 60µmolcm⁻²s⁻¹ cool white fluorescent light. Each data point is shown as mean ± sd deviation from 4 replicates.
(C). Representative 27-d-old soil-grown plants of Ler-0, heterozygous (old3-1OLD3ODD) and homozygous (old3-1ODD) mutants, Col-0, old3-1odd, and old3-Todd, grown in 250µmolcm⁻²s⁻¹ cool white fluorescent light. Bars represent 0.5cm.

Figure 2. Phenotypes of old3 and its various double mutants in comparison with wild type, hormonal mutants and the nahG transgenic plant.
Double mutants were isolated as described in Experimental procedures and were homozygous for both the old3-1 and the ODD gene. Plants were grown on soil in 60µmolcm⁻²s⁻¹ cool white fluorescent light for 17 days and representative plants were photographed. Bars represent 0.5cm.
was found to co-segregate with *abi1-1* and *fca-1* (data not shown). Thus, *old3-1* is located on chromosome 4 and *ODD* on chromosome 3.

**old3** is a downstream regulator of leaf senescence

*old3* was proposed to work later in a senescence regulatory pathway (Jing et al., 2002). To further understand the position of *old3* in the senescence regulatory network, we performed a detailed epistatic analysis to clarify the roles of several senescence-promoting hormones in *old3*-induced senescence.


table 1 The genotypes, phenotypes and segregation of F1 and F2 progeny of the *old3* heterozygously crossed to the parental lines Ler-0 and several other *Arabidopsis* accessions

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>F1 genotype</th>
<th>F1 phenotype</th>
<th>WT He:Ho</th>
<th>PCR Marker</th>
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<tr>
<td>old3-1</td>
<td>Bu-1, Di-2,</td>
<td>old3-1OLD3</td>
<td>Wild type</td>
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<td></td>
<td>Lc-0, Wa-1,</td>
<td>OLD3ODD</td>
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<td>old3-1-ODD3</td>
<td>He-old3</td>
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1: The pollen from *He-old3* plants was used to individually pollinate various *Arabidopsis* accessions indicated in the female column. The possible genotypes and corresponding phenotypes of the resultant F1 progeny are shown. In the crosses between *He-old3* and Ler-0 and its similar accessions, only the F1 progeny with the mutant phenotypes was selected and continued for F2 segregation analyses. In the crosses between *He-old3* and Col-0 and its similar accessions, at least 10 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 PCR marker was examined in the female *Arabidopsis* accessions as described in Experimental procedures.

2: Among these accessions similarly containing the 2.5kb PCR fragments, Col-0, Wil-2 and Ws-0 were crossed to *He-old3* for genetic segregation analyses.
seedling-lethal phenotype of old3-1 plants. Although the double mutants could survive and finish the whole life cycle under an elevated light intensity similar to the single old3-1 mutant, they showed early leaf senescence (data not shown). These results suggest that the early senescence phenotype of old3-1ODD mutants does not depend on these hormonal pathways.

**Figure 3.** A diagram showing map-based cloning of old3-1 and ODD. The BACs spanning the mapped regions are shown. The tested SNP markers are shown by their positions on the Lister & Dean RI map and on the physical map. The number of recombination events between old3-1 (A) or ODD (B) and a particular marker is shown below the marker. The OLD3 gene and the predicted genes in the ODD region are indicated.

**Figure 4.** Molecular characterisation of the old3 mutation. (A). An agarose gel photograph showing the polymorphism between wild type Ler-0 gene and old3-1ODD at the nucleotide sequence level. PCR-products were amplified from Ler-0, old3-1ODD (Ho) and old3-1OLD3ODD (He) mutants and restricted with Sau96I. (B). Generation of old3-1 phenotypes in Ler-0 wild type plants. The genomic sequence spanning the old3-1 region was cloned and transformed to wild type as described in Experimental procedures. A representative 14-day old ler-0 plant and a Ler-0 plant transformed with the old3-1 gene are shown.
**OLD3 encodes the cytosolic O-acetylserine (thiol) lyase (OAS-TL)**

Further mapping placed *old3-1* into a 14-kb region on bacterial artificial chromosome clone FCA2 (accession number Z97337) spanning 8 open reading frames (Figure 3A). The *old3-1* genome of this region was sequenced and a GGA to GAA codon change was found at the fifth exon of the gene encoding the cytosolic O-acetylserine (thiol) lyase (OAS-TL, *AT4G14880*). The *old3-1* mutation generated a Sau96I restriction polymorphism between *old3-1* and the wild type (Figure 4A). At the protein level, it changes a glycine at position 162 to glutamic acid. The amino acid sequences of the OAS-TL isoforms are highly conserved among plant species and share very high homologies to those in unicellular organisms (e.g. the wild type OAS-TL shares 49% identities with that of *Salmonella typhimurium*, for a review, see Leustek et al., 2000). According to the crystallised structure of the enzyme from *S. typhimurium*, the substitution caused by *old3-1* is located at the sixth α-helix of the encoded protein (Burkhard et al., 1998).

A complementation test was conducted to further confirm the isolation of *old3-1*. Because of the co-dominant nature, the mutated *old3-1* genomic sequence was cloned and transformed into wild-type *Ler*-0 plants. The transformants were found to display the same phenotypes as the *old3-1* mutants (Figure 4B). Thus, the product of the mutated OAS-TL gene is responsible for the phenotypes generated by *old3-1*.

**old3 works in pair with the Ler-0 ODD allele to induce early leaf senescence**

Our initial genetic evidence indicated that the *Ler*-0 and Col-0 *ODD/odd* alleles function different in *old3*-induced early leaf senescence. Using a mapping population, the gene was fine mapped to a 52-kb region containing 5 predicted open reading frames on chromosome 3 spanning parts of the bacterial artificial chromosome clones F14L2 and T18B22 (Figure 3B). We took advantage of the availability of the genomic sequences of TAIR Col-0 and Cereon Ler-0 databases and performed in silico comparison of the five predicted genes. No differences were found in the coding sequence of *AT3G44600*, which is predicted to have high similarity to *homo sapiens* cyclophilins. *AT3G44603* has a high similarity with a retroelement transposon fragment and was therefore not believed to encode ODD. The remaining region in the Col-0 genome sequences contains three predicted open reading frames encoding a protein kinase (*AT3G44610*), a phosphotyrosine protein phosphatase (*AT3G44620*), and a putative TIR-NBS-LRR class disease resistance protein (*AT3G44630*), respectively. However, large variation was found in this area between Col-0 and Ler-0 genome and no direct genic synteny was possible. A 10-kb Ler-specific PCR fragment could be amplified from the region between *AT3G44610* and *AT3G44620*. When Col-0 DNA was used as a template, a 2.5 kb fragment was amplified, consistent with the genome sequence. The polymorphism for this marker was further examined in additional 6 accessions and the results showed that these accessions split into two groups, either containing the 10-kb fragment as in *Ler*-0, or the 2.5-kb fragment as in Col-0 (Table 1). The *old3-1OLD3ODD* mutants were crossed to several accessions, and the results showed that in the accessions containing the 10-kb fragment (Di-2, Bu-18, Rsch-0 and Wa-1), the *old3-1* phenotypes behaved as in *Ler*-0, but in those (Ws-0 and Wil-2) containing the 2.5-kb fragment behaved as in Col-0. Sequencing of the 10-kb fragment did not reveal an intact open reading frame.

The co-dominant nature of the *odd* allele suggested that, similar to *old3-1*, the activity of the *Ler ODD* gene, rather than the absence of the Col-0 *odd* allele, is required for the
manifestation of the \textit{old3-1} phenotypes. To validate this hypothesis, a \textit{Ler}-0 binary BAC library (Chang et al., 2003) was probed with the 10-kb fragment as described in Experimental procedures. \textit{old3-1odd} plants (containing \textit{old3-1} allele in a \textit{Col}-0 and \textit{Ler}-0 mixed background and the \textit{Col}-0 \textit{odd} allele) harbouring the positive BAC clones exhibited \textit{old3-1ODD} phenotypes (data not shown). Thus, the \textit{Ler}-0 \textit{ODD} allele is essential and the specific interaction between \textit{old3-1} and the \textit{ODD} gene is responsible for the observed early leaf senescence phenotype.

\textbf{Sulphur metabolism and physiology is altered in \textit{old3} mutants}

The OAS-TL gene family works at the last step of sulphur assimilation and synthesis of cysteine is the key step for the formation of other organic sulphur-containing compounds (Saito, 2000). We examined sulphur-associated physiology in \textit{old3-1} mutants (Figure 5). Two more mutant lines were included for a better comparison. One SALK knock-out line containing a T-DNA insertion inside the \textit{OAS-TL} gene was isolated as described in Experimental procedures and designated as \textit{old3-Todd}. In an F$_2$ population generated from an \textit{old3-1OLD3ODD} x \textit{Col}-0 (\textit{OLD3OLD3odd}) cross, plants with \textit{old3-1odd} genotypes were selected. Observations on the identified \textit{old3-1odd} lines showed that they performed similarly in a number of growth conditions and hence only one line was used for further study. Under our growth conditions, differences in leaf senescence were not observed among \textit{old3-Todd}, \textit{old3-1odd} and wild type plants (Figure 1C). This further confirmed that the \textit{ODD} allele is required for the early leaf senescence phenotype.

As shown in Figure 5A, the \textit{old3-1ODD} and \textit{old3-1OLD3ODD} mutants in the \textit{Ler}-0 background exhibited reduced average dry weight as compared to wild type, concomitant with the early onset of leaf senescence. \textit{old3-1odd} plants did not differ from \textit{Col}-0 in the average dry weight, but a higher average dry weight was found in \textit{old3-Todd}. The contents of total, ion and organic sulphur are direct measurements of plant sulphur balance and hence were quantified in the mutants and wild type plants. In \textit{old3-Todd}, the total sulphur content was increased as compared to \textit{Col}-0. This increase was associated with an increase in the ion sulphur contents. Compared to \textit{Ler}-0, \textit{old3-1OLD3ODD} and \textit{old3-1ODD} exhibited higher contents of total and ion sulphur, but \textit{old3-1odd} did not differ from \textit{Ler}-0 or \textit{Col}-0. The organic sulphur contents could be measured by the ratio between the total and ion sulphur contents. All the examined lines had a similar level of organic sulphur. Among plant organic sulphur compounds, the water-soluble non-protein thiols are related to various stress responses (Rauser, 1993; Cobbert, 2000; Gotor et al., 2003) and therefore were examined in the mutants. \textit{old3-Todd} contained a lower thiol content than \textit{Col}-0. In comparison with \textit{Ler}-0, the thiol content was lower in \textit{old3-1ODD}, but higher in \textit{old3-1OLD3ODD}. \textit{old3-1odd} displayed a thiol content similar to \textit{Col}-0, but lower than \textit{Ler}-0. Thus, the \textit{old3} mutation caused different effects on the total sulphur, ion sulphur and thiols contents.

We further performed two bioassays related to sulphur physiology. Cysteine can be added into the culture media as an instant organic sulphur source in plant growth assays (e.g. Herschbach and Rennenberg, 1995; Dominguez-Solis et al., 2001). Hence, the \textit{old3-1OODD} mutant seedlings were grown in cysteine-containing media to examine whether cysteine feeding could alleviate the \textit{old3-1} mutant phenotype. Figure 5B shows that the mutant phenotypes did not change, indicating that cysteine deficiency was not the cause of the \textit{old3-1} phenotypes.

The cytosolic OAS-TL has been shown to be important for cadmium tolerance in \textit{Arabidopsis} (Dominguez-Solis et al., 2001), we tested the growth of the \textit{old3} mutant lines
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together with wild type plants on various cadmium concentrations (Figure 5C). No differences were observed among all the tested lines when the cadmium chloride concentration was 0 to 20µM, except that the old3-1ODD seedlings were smaller and showed signs of visible yellowing on cotyledons. At 50µM CdCl₂, a reduced growth occurred. Clear differences were observed at 100µM CdCl₂. Under this condition, old3-Todd seeds showed only the protrusion of radicles and post-germination growth was completely inhibited. The development of wild type seedlings was arrested at the cotyledon stage. However, both old3-1ODD and old3-1odd were still able to grow showing elongated roots and growth of the first pairs of rosette leaves. Interestingly, early senescence was still evident in old3-1ODD plants. Thus, old3-Todd is hypersensitive to cadmium ions but old3-1ODD and old3-1odd mutants exhibit enhanced tolerance.

 Taken together, these results indicated that the old3-1 together with the ODD causes an altered sulphur balance. In contrast, old3-T shows an altered sulphur balance, independent of ODD. Further, old3-T causes a reduced cadmium tolerance, while the old3-1 mutation enhances cadmium tolerance independently of the ODD mutation.
Discussion

In this study, the previously isolated old3-1 mutant was characterised. old3-induced leaf senescence is controlled by two genetic loci, old3 and ODD. Our epistatic analyses of double mutants between old3-1 and mutants with a defect in ET, SA, JA, or ABA/sugar signalling suggested that old3-induced leaf senescence does not involve these senescence signalling molecules. Thus, old3 may act downstream in the senescence regulatory network as previously proposed (Jing et al., 2002). Map-based cloning showed that old3-1 caused an amino acid substitution in the cytosolic OAS-TL, an enzyme implicated in cysteine synthesis. The results generated here allowed us to dissect the genetic basis of old3-induced leaf senescence and to exploit a causal link between regulation of leaf senescence and a general metabolic process.

Genetic basis for old3-induced early leaf senescence

Two genes are involved in the regulation of old3-induced leaf senescence. Apart from old3-1, a second gene ODD is required, which is present in the Ler genetic background. Among 8 accessions tested, 5 contain an ODD allele, with similar properties as the Ler-0 ODD allele, while 3 contain an odd allele, similar to the one found in the Col-0 accession. Interestingly, the presence of odd/ODD alleles does not correlate with the geographic origins of the accessions, or with their phylogenetic relationships as illustrated with molecular markers (Barth et al., 2002).

The results show that old3-1 encodes a functional gene as transgenic presence of the old3-1 allele in otherwise wild type Ler plants causes the old3-1 phenotype. Similarly, the activity of the Ler ODD allele is required for the old3-1 senescence phenotype. Thus, the senescence phenotype depends on the presence of both the old3-1 allele and the Ler ODD gene, implying that the two genes may directly interact. At present, the identity of the ODD gene is unknown. Although ODD is mapped to a region encompassing AT3G44610 and AT3G44620 in Col-0, we were unable to pinpoint the gene since the genome sequence is highly polymorphic between the Ler-0 and Col-0 accessions in this part of the genome. A recent thorough experimental analysis of transcriptional activity in Col-0 genome has identified over 3000 new genes (Yamada et al., 2003). Thus, in silico prediction of genes will not detect all genes and odd may not be an annotated gene yet. Our data showed the situation may be even more complex when functional genes are compared among different accessions. Indeed, the Col-0 odd allele is either different or not present since leaf senescence is manifested only when both old3-1 and ODD are present. At the moment, the ODD gene allele was mapped to a 52-kb region and further molecular analysis is required to reveal the true identity of the ODD gene.

Plants showing old3-1 phenotypes were homozygous for both old3-1 and ODD alleles. Those with intermediate phenotypes contained either two copies of old3-1 and one copy of ODD, or two copies of ODD and one copy of old3-1. Thus, to generate early senescence phenotype, a specific interaction between old3-1 and ODD appears to be required and their effects are gene-dosage dependent. Such a pattern resembles the naturally occurring ecotype-specific interactions between FLOWERING LOCUS D (FLD) and FLOWERING LOCUS C (FLC) in the control of flowering time, although the proposed old3-ODD interaction is generated by mutagenesis. fld is a recessive allele causing delayed flowering, which segregated in a typical 1.3 ratio in Col-0, but in Ler-0 fld generates minimal phenotype, suggesting the involvement of another Col-0 gene in the control of fld-induced delayed flowering (Sanda and
The fld-interacting gene turned out to be FLC, which is truncated in Ler-0 due to the insertion of a nonautonomous Mutator-like transposon in the first intron that is required for normal FLC regulation (Gazzani et al., 2003). Thus, such ecotype-specific interaction may well be preserved in senescence regulation as well.

The relationship between sulphur metabolism and leaf senescence in old3 mutants

Plants carrying a mutation in the old3 gene showed a changed sulphur metabolism and physiology, including altered sulphur balance, changed thiol levels, and cadmium tolerance. However, old3-1ODD, old3-1OLD3ODD, old3-Todd and old3-1odd mutants displayed different alterations. The sulphur balance as monitored by the total, ionic and organic sulphur content, was altered in old3-1ODD, old3-1OLD3ODD and old3-Todd, but was not changed in old3-1odd mutants; the thiols were lower in old3-1ODD, higher in old3-1OLD3ODD, but not changed in old3-1odd. These results show that in addition to the old3-1, the ODD gene is required to cause alterations in sulphur metabolism. Thus, in old3 mutants, early leaf senescence occurs concomitant with alterations in sulphur metabolism.

At present it is not clear what is the causal relationship between the senescence phenotype and the alterations in sulphur metabolism. It is possible that early leaf senescence is the cause of the altered sulphur metabolism. During senescence, ionic sulphate contents are expected to increase due to the remobilisation of nutrients (Himelblau and Amasino, 2001), and this could be partially the reason why the ionic sulphate contents increased in old3-1ODD and old3-1OLD3ODD. Furthermore, it has been shown that in Arabidopsis leaves, the thiol levels initially increased at the onset of senescence, but decreased at late senescence (de Kok and Graham, 1989). old3-1OLD3ODD mutants exhibited less obvious senescence symptoms and a higher than wild-type thiol level. Therefore, the thiol levels in old3-1OLD3ODD and old3-1ODD may mimic the changes of thiol levels in wild type plants during senescence. On the other hand, the altered sulphur metabolism can be the cause of the accelerated leaf senescence in old3 mutants. Sulphur depletion results in up-regulation of genes involved in jasmonic acid biosynthesis and genes induced by jasmonic acid or methyl jasmonic acid (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003). Changed sulphur nutrition may affect senescence through the action of jasmonic acid, whose role in leaf senescence has been substantiated (He et al., 2002). The expression of nitrilase, genes which are involved in auxin biosynthesis, were up-regulated and free IAA levels were increased upon sulphur starvation (Hirai et al., 2003; Nikiforova et al., 2003). Similar changes were reported during senescence (Quirino et al., 1999). Recently, sulphurtransferases have been found to be one type of senescence-associated genes (Meyer et al., 2003). These studies provide circumstantial evidence that sulphur alteration may affect leaf senescence. However, the effect of the changes in sulphur balance on leaf senescence in the old3-1ODD mutants remains unclear.

Several lines of evidence suggest that the old3-1 mutation may not result in the complete elimination of OAS-TL activity. First, the senescence symptoms were not changed when old3-1 mutants were grown in the presence of exogenously applied cysteine. Second, in contrast to the effect of the old3-1 mutation, the old3-1 mutation did not affect sulphur metabolism in the absence of ODD. Finally, the old3-T and old3-1 mutation have opposite effects on cadmium tolerance. Clearly, the mutation caused by the T-DNA insertion has a different effect than the amino acid substitution caused by old3-1, suggesting that old3-1 may encode a functional OAS-TL.
Intriguingly, the enhanced cadmium tolerance in plants containing the old3-1 allele did not depend on ODD: cadmium tolerance was similarly enhanced in old3-1ODD and old3-1odd mutants, while tolerance was reduced in old3-Todd mutants. The molecular mechanism of the enhanced cadmium tolerance is unclear. Plant thiols are predominantly present in the form of glutathione, which is implicated in plant responses to various stresses including heavy metal toxicity (Cobbert, 2000). Cysteine is the precursor for glutathione synthesis and the pools of the non-protein thiols might be important for cadmium tolerance. However, no correlation has been found between the thiol level and the cadmium tolerance in the various old3 mutants. Consistently, it has been shown that the absolute levels of thiols do not correlate with freezing tolerance in wheat (Stuiver et al., 1995). De novo synthesis of cysteine and glutathione was proposed to be essential for the coupling reaction with cadmium (Dominguez-Solis et al., 2001). Significant increases in the mRNA level and the enzymatic activity of the cytosolic OAS-TL have been observed within 1 hour after cadmium treatment, and transgenic plants overexpressing the cytosolic OAS-TL display enhanced tolerance to cadmium. Therefore, it is possible that the old3 mutation may cause an enhanced OAS-TL activity allowing a rapid response upon cadmium stress. The three-dimensional structure of the Salmonella typhimurium OAS-TL has been resolved (Burkhard et al., 1998). The proposed OAS-binding pocket includes four essential residues, Ser<sup>69</sup>, Gly<sup>70</sup>, Asn<sup>71</sup>, and Thr<sup>72</sup>, which are conserved in all the plant OAS-TL characterised up to date. Plant OAS-TLs are expected to have a structure that is similar to the one from Salmonella, owing to the high sequence similarity. The Gly<sup>162</sup> to Glu<sup>162</sup> substitution in the old3-1 mutated OAS-TL is located at the sixth α-helix and is outside the substrate-binding site. Thus, it is possible that the old3-1 OAS-TL may have a changed three-dimensional conformation with an increased cysteine synthetic activity. Alternatively, the Arabidopsis cytosolic OAS-TL has been shown to play a central regulatory role (Saito, 1999; Leustek et al., 2000; Hell et al., 2002) and the old3-1 mutation may enhance the overall control on cysteine biosynthesis and/or cysteine coupling with cadmium. No matter what the exact molecular mechanism are, our results show that old3-T and old3-1 mutations appear to have different effects on the cytosolic OAS-TL activity as envisaged by their effects on cadmium tolerance.

In summary, the cloning and characterisation of old3-1 revealed that OLD3 encodes a cytosolic OAS-TL and that the old3-1 gene is functional with novel features of enhancing cadmium tolerance. Furthermore, old3-1 discloses a novel link between sulphur metabolism and leaf senescence in combination with the ODD gene. Future cloning of the ODD gene and characterisation of its molecular interaction with old3-1 will allow the regulation of leaf senescence to be viewed from a fresh perspective.

**Experimental procedures**

**Plant material and growth conditions**

Arabidopsis thaliana accessions Ler-0 and Col-0 were the wild types. The Arabidopsis accessions Bu-18, Di-2, Wa-1, Rsch-0, Wil-2 and Ws-0 were obtained from the NASC. The mutant alleles and transgenic plants used were old3-1 (Jing et al., 2002), npr1-1 (Cao et al., 1994), ein2-1 (Guzman and Ecker, 1990), jar1-1 (Staswick et al., 1992), abi1-1 (Koornneef et al., 1984), fca-1 (Koornneef et al., 1991), abi4-1 (Finkelstein et al., 1998), and Arabidopsis plants expressing the bacterial nahG gene (Bowling et al., 1997). Depending on phenotypes and identities of the particular mutations or insertions, a variety of screening methods employing
hormonal response and PCR-based marker assays were used to isolate double mutants. SALK line N572213 contains a T-DNA insertion inside the cytosolic OAS-TL and was ordered from ABRC (Alonso et al., 2003). The insertion line has the Col-0 background. To isolate plants homozygous for the T-DNA insertion, DNA samples were prepared from young leaf tissues and PCR amplification was done using two sets of oligonucleotide primers. The T-DNA left border primer (PrRuG659 TGGTTCAGTGATGGCCATCG) was combined with a gene specific primer PrRuG761 (TACACAAATGGGAGTCCCAATCA) to check the presence of the T-DNA insertion inside the targeted gene. PrRuG761 was combined with PrRuG760 (CTATGATCCCTCCGTTTGAGAA) to check the presence of the wild type gene. PCR was performed using standard procedures. Plants containing the PrRuG659/761 PCR product but not the PrRuG760/761 PCR product, were homozygous for the T-DNA insertion and were designated as old3-T and used in the experiments described.

Unless otherwise indicated, plants were grown in an organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) under the conditions described by Jing et al. (2002). For cysteine culture and cadmium tolerance assay, seeds were surface-sterilized and kept at 4 °C for 3 days and germinated on solid MS medium containing 0.8% agar with and without cysteine or cadmium chloride at the indicated concentrations.

**Map-based cloning and complementation test**

Map-based cloning was carried out as described in Chapter 4. DNA was isolated as described above. SNPs were selected, and primers were designed according to Drenkard et al. (2000). In total 5000 recombinants were used for fine mapping. Agrobacterium-mediated transformation was performed to further confirm the mutation (Clough and Bent, 1998). The transformants were selected based on basta herbicide resistance.

Oligonucleotide primers PrRuG760 and PrRuG775 (GATGGAAGCAAAGACGCAATGTAACTAA) were used to amplify the cytosolic OAS-TL gene in Ler-0, old3-1ODD and old3-1OLD3ODD spanning the nucleotide change caused by old3-1. This primer combination gave a PCR product of 2928bp. After Sau96I restriction, the Ler-0 copy gave a restriction pattern of three bands consisting of 1813, 718 and 319bp, whereas old3-1ODD gave 2210 and 718bp bands. The primers PrRuG848 (ACCTCGTGATAATCAATTGTTCCAGCGGAAT) and PrRuG849 (GAATGAAACCGTGAGCTGTGGTGGTGTTA) were designed to span the inter-gene region between AT3G44610 and AT3G44620 and were used for PCR amplification and identification of the ODD polymorphism between different Arabidopsis accessions.

**BAC library screen**

An Arabidopsis Ler-0 binary BAC library (010-ATL-BI) equivalent to 14.6x the genome was screened. The features of the library were described by Chang et al. (2003). The 10-kb PrRuG848/849 PCR fragment was 32P labelled and hybridised to the filters containing the binary BAC library clones as described (Jing et al., 2002). Twelve clones 5O18, 7N10, 8L11, 9O19, 10F6, 10G16, 20O12, 24H10, 26N21, 27C3, 29B24, and 30G9 showed the strongest hybridisation signals. Two independent clones (10G16 and 24H10) were transformed to Agrobacteria, which were subsequently used to transform old3-1odd plants via the floral dip method. Seeds were harvested and transformants were selected on agar plates containing 50µg/ml kanamycin.
The survivors were transferred to soil and the transgenic presence of the kanamycin gene was confirmed by PCR amplification. The transformants exhibited old3-1 phenotypes.

**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 250µmolm⁻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. For each line four replicates of 17 plants were used for quantification. For the total and anion sulphate content data of old3-IODD mutants, one replicate of 50 plants was used for analysis due to the small size of plants. The dry plant material was used for total sulphur measurement using the barium sulphate precipitation method (Jones, 1995) and for anion measurement using HPLC-based methods (Tauze et al., 1996). Parts of the fresh samples were used for total water-soluble non-protein sulfhydryl (SH) compounds following the method described by de Kok and Graham (1989).

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