Expression of a mouse selenocysteine lyase in *Brassica juncea* chloroplasts affects selenium tolerance and accumulation

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Selenium is an essential nutrient for many organisms, as part of certain selenoproteins. However, selenium is toxic at high levels, which is thought to be due to non-specific replacement of cysteine by selenocysteine leading to disruption of protein function. In an attempt to prevent non-specific incorporation of selenocysteine into proteins and to possibly enhance plant selenium tolerance and accumulation, a mouse selenocysteine lyase was expressed in *Brassica juncea* (Indian mustard) chloroplasts, the site of selenocysteine synthesis. This selenocysteine lyase specifically breaks down selenocysteine into elemental selenium and alanine. The transgenic cpSL plants showed normal growth under standard conditions. Selenocysteine lyase activity in the cpSL transgenics was up to 6-fold higher than in wild-type plants. The cpSL transgenics contained up to 40% less selenium in protein compared to wild-type plants, indicating that Se flow in the plant was successfully redirected. Surprisingly, the selenium tolerance of the transgenic cpSL plants was reduced, perhaps due to interference of produced elemental selenium with chloroplastic sulphur metabolism. Shoot selenium levels were enhanced up to 50% in the cpSL transgenics, but only during the seedling stage.

Introduction

Sulphur (S) and selenium (Se) are chemically similar elements. Most enzymes involved in S metabolism can catalyse the analogous reaction with the corresponding Se substrates (Stadtman 1990). Non-specific incorporation of Se into S-containing molecules is thought to lead to toxicity, especially when selenocysteine (SeCys) randomly replaces cysteine (Ohlendorf et al. 1986). On the other hand, many organisms require Se as an essential element. They contain selenoenzymes, which often play a role in free radical scavenging (Stadtman 1996). Selenoenzymes require a selenocysteine (SeCys) in their active site for activity. The specific incorporation of Se into selenoproteins it thought to start with the production of elemental Se (Se⁰) from SeCys by selenocysteine lyase (SL), also producing alanine. Se⁰ is the substrate for selenophosphate synthase (Lacourciere and Stadtman 1998, Lacourciere et al. 2000). The resulting selenophosphate is used as a substrate to convert Ser-tRNA to SeCys-tRNA, which is then used in the translation of UGA opal (stop) codons in specific mRNAs encoding SeCys-containing enzymes (Bock et al. 1991). The question whether Se is essential for plants is still unanswered. No selenoenzymes have been reported in higher plants, only in the unicellular alga *Chlamydomonas reinhardtii* (Fu et al. 2002, Novoselov et al. 2002). There is, however, some evidence that the machinery for incorporation of Se into selenoproteins is present in plants: a SeCys-tRNA has been found in *Beta vulgaris* that recognizes the UGA anticodon (Hatfield et al. 1992).

Assimilation of selenate to SeCys and selenomethionine (SeMet) is mediated by the sulphate assimilation pathway (Stadtman 1990, Anderson 1993, Pilon-Smits et al. 1999) which is localized in the chloroplast in plants (Leustek and Saito 1999). As mentioned above, non-specific incorporation of SeCys into proteins causes disruption of protein structure and function, and thus toxicity. Different biological mechanisms have evolved that provide enhanced selenium tolerance. Many of these are based on prevention of incorporation of SeCys into proteins. Se can be volatilized as dimethyl (di)selenide (Ganther et al. 1966, Lewis et al. 1966), accumulated as
non-protein selenoaminoacids (e.g. methyl-SeCys, Neuhierl et al. 1999), or accumulated as Se⁰, which is insoluble and has a relatively low toxicity (Wilber 1980). Se⁰ can be formed from selenite via a reduction reaction, or from SeCys by SL activity. It is not known whether the latter reaction plays a role in Se tolerance, in addition to its proposed role in essential Se metabolism.

In this study, the hypothesis was tested that introduction of SL activity in plants will reduce non-specific incorporation of Se into proteins and thus lead to enhanced Se tolerance and perhaps accumulation. This is based on the reasoning that when SL activity is introduced in plants, the flow of Se may be redirected toward accumulation as relatively low-toxic elemental Se, rather than into proteins. To test this hypothesis, the mouse SL enzyme was expressed in *Brassica juncea* (Indian mustard), a plant with a very active sulphur/selenium metabolism. The mouse SL enzyme was chosen because of its reported high activity toward SeCys, but negligible activity toward cysteine (Mihara et al. 2000). Therefore, by introducing the mouse SL gene in the plant it may be possible to manipulate Se fluxes without interfering with S metabolism. The mouse SL enzyme was targeted to the chloroplast, because this is the site of selenocysteine formation in plants, as well as an important site of translation. The transgenic cpSL plants were compared with untransformed wild-type plants with respect to Se incorporation into proteins, Se tolerance and Se accumulation.

**Materials and methods**

*B. juncea* seeds (Indian mustard, accession no. 173874) were obtained from the North Central Regional Plant Introduction Station, Ames, IA, USA. The mouse selenocysteine lyase gene (Mihara et al. 2000) was cloned into cloning vector pWA1, under the control of the CAMV 35S promoter and the nos terminator sequence.

This vector also contains the ferredoxin transit sequence into cloning vector pWA1, under the control of the selenocysteine lyase gene (Mihara et al. 2000) was cloned by introducing the mouse SL gene in the plant it may be possible to manipulate Se fluxes without interfering with S metabolism. The mouse SL enzyme was targeted to the chloroplast, because this is the site of selenocysteine formation in plants, as well as an important site of translation. The transgenic cpSL plants were compared with untransformed wild-type plants with respect to Se incorporation into proteins, Se tolerance and Se accumulation.

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Five kanamycin-resistant lines were confirmed by PCR to be transgenic SL lines. The following primers were used: the forward primer was directed against the 35S promoter, with sequence 5'-CCTTCGCAAAGCCCTTCTC-3'. The reverse primer was directed against the mouse SL gene and had sequence 5'-TGATCTCGAGACAGGCATGA-3'. The plants were selfed and seeds from the T₂ generation were used for further experiments.

Leaf samples for selenocysteine lyase enzyme analysis were collected from 1-week-old seedlings grown on half-strength MS agar medium (Murashige and Skoog 1962). The samples were ground in liquid nitrogen, and extracted with 1 ml g⁻¹ FW of extraction buffer containing 50 mM Tris pH 8, 20% glycerol, 2 mM EDTA and 0.1 mM phenylmethylsulphonylfluoride (PMSF). SL enzyme activity was measured at 37°C in 0.12 M Tricine-NaOH pH 7.9, 10 mM l-selenocysteine, 50 mM DTT and 0.2 mM pyridoxal phosphate (PLP). Elemental selenium produced was measured with lead acetate as described by Esaki et al. (1982). The specific activities are expressed in units mg⁻¹ protein with one unit of enzyme defined as the amount that catalyses the formation of 1 μmol of product in 1 min.

Chloroplast isolation was performed as described by Rensink et al. (1998). Phosphoenolpyruvate carboxylase activity in total plant extracts and chloroplast fractions was measured as described by Pilon-Smits et al. (1990). Proteins from total plant extracts and chloroplast fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes. The mouse SL protein was visualized by immunodetection using polyclonal antibodies raised against the mouse SL protein.

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**Fig. 1.** Representation of the chloroplastic selenocysteine lyase (cpSL) gene construct used in the transformation of *Brassica juncea*. LB, left border; RB, right border; nptII, coding sequence of the neomycin phosphotransferase gene; mouseSL, coding sequence of the mouse selenocysteine lyase gene; TrFd, chloroplast transit sequence of ferredoxin; 35SCAMV, cauliflower mosaic virus promoter; NOS, promoter of the nopaline synthetase gene; AMV, translational enhancer sequence. Forward (a) and reverse (b) primers used for PCR are shown with arrows.
To determine selenium content in protein, seedlings were grown for 1 week on half-strength MS medium with 40 \( \mu \)M sodium selenite, or 6-week-old mature plants were treated with 20 \( \mu \)M sodium selenite for 1 week in half-strength Hoagland’s solution (Hoagland and Arnon 1938). The plant material was harvested, washed, and ground in liquid nitrogen. One-gram FW aliquots were extracted in 5 ml \( g^{-1} \) of buffer containing 100 mM NaCl, 50 mM Tris/HCl pH 7.5, 0.5% Triton X-100, 1 mM DTT and 1 mM PMSF. The homogenate was cleared by centrifugation (10 min 7500 \( g \)). A small sample was taken for protein determination, and the volume of the extract measured. The protein in the extract was precipitated by adding trichloroacetic acid (TCA) to a final concentration of 15% (w/v). The extract was incubated on ice for 30 min, and then centrifuged for 20 min at 7000 \( g \) at 4°C. The pellet was washed with ice-cold acetone, dried, and dissolved in 1 ml of concentrated nitric acid. After acid digestion, the Se concentrations in these samples were determined by ICP-AES (Pilon-Smits et al. 1999).

Selenium tolerance in seedlings was measured as root length after 1 week of growth on agar medium with or without Se, as described by Pilon-Smits et al. (1999). Selenium tolerance in mature plants was analysed using a nutrient film technique set-up (Zhu et al. 1999). Five-week-old plants were transferred to the NFT set-up and treated for 14 days with half-strength Hoagland’s solution (Hoagland and Arnon 1938), with or without 40 \( \mu \)M sodium selenite. Selenium accumulation in seedling and mature plant tissue was analysed by ICP-AES as described by Pilon-Smits et al. (1999).

Statistical analyses were performed using the software package JMP-IN from the SAS institute (Cary, NC, USA). T-tests were done to statistically compare pairs of means. Statistically significant \( (\alpha = 0.05) \) differences are indicated in the text.

### Results

In order to express the mouse SL gene in plant chloroplasts, a gene construct was made that contains the mouse SL coding sequence under the control of the 35S CaMV promoter with translational enhancer, and coupled to the ferredoxin chloroplast transit sequence (Fig. 1). *B. juncea* hypocotyls were transformed by *Agrobacterium tumefaciens*-mediated transformation. The resulting kanamycin-resistant regenerant lines were screened by PCR for the presence of the mouse SL gene. Five transgenic lines were identified: cpSL A1, A2, B2, C3, and E7. The transgenics did not show any phenotypic differences compared to untransformed plants, when grown under standard conditions on soil or agar medium. \( T_2 \) generation seeds from these transgenic lines were used for further experiments.

The expression level of the mouse SL gene was analysed using immunoblotting and enzyme activity measurements. Using antiserum against the mouse SL protein, an approximately 43-kDa protein was detected in seedling (shoot) extracts of the five cpSL transgenics, but not in the wild-type extract (Fig. 2A). The protein in the transgenics corresponded in size with the mouse SL protein. Therefore it was concluded that all cpSL lines express the mouse SL protein, and that the chloroplast transit sequence was removed. Judging from the intensities on the immunoblot, line cpSL A1 is the highest SL expressor, followed by cpSL C3 and E7, then B2 and A2. The transgenic seedling extracts showed levels of SL activity that were 2- to 6-fold higher than wild-type *B. juncea* (Fig. 2B), indicating that the mouse SL enzyme is active in the cpSL transgenics. Three lines with different expression levels were selected for further physiological characterization: cpSL A1, A2 and C3.

The chloroplastic location of the mouse SL protein was investigated via immunoblotting, comparing homogenates with isolated chloroplast fractions. The chloroplast fractions were checked and found to be free from cytosolic contamination, based on activity of the cytosolic enzyme phosphoenolpyruvate carboxylase (results not shown). As shown in Fig. 3, the mouse SL-specific band was equally intense in the chloroplast fractions compared to the corresponding homogenate fractions on a chlorophyll-basis. It was therefore concluded that the mouse SL protein is indeed targeted to the chloroplasts in the transgenics. In addition to the mouse SL-specific band, there was a second, slightly larger endogenous protein that reacted with the
antiserum. Based on its abundance, size and chloroplast location, this may be the large subunit of Rubisco.

Since the cpSL transgenics are expected to shuttle Se from SeCys into elemental Se (Se⁰), thus preventing non-specific incorporation of SeCys into proteins, the concentration of Se in protein was expected to be lower in the transgenics than in the wild-type. To test this, total protein fractions were isolated from selenite-treated seedlings of transgenics and wild type, and analysed for Se content. All three transgenic lines contained approximately 40% less Se in proteins than wild-type seedlings (P < 0.05, Fig. 4A). It therefore appears that the Se flow in the transgenic seedlings is successfully redirected, at least in part, away from incorporation into proteins. When mature plants were treated with selenite for 1 week and analysed for Se in protein, a similar trend was visible, but no significant differences were observed between the transgensics and wild-type (Fig. 4B).

The Se that is not stored in proteins in the cpSL transgenics is presumably accumulated as Se⁰ in the chloroplast. If so, these transgenics can be considered to contain a new Se ‘sink’. Since Se⁰ is relatively inert and less toxic than SeCys (Wilber 1980) the cpSL transgenics may be more tolerant to Se. To investigate this, the cpSL and wild-type plants were compared with respect to Se tolerance, both at the seedling level and as mature plants. Tissue Se concentrations were also measured. Contrary to expectation, seedlings of all three cpSL lines were significantly less tolerant to Se than wild-type seedlings, both when supplied as selenate or selenite, at two concentrations each (Fig. 5). As for Se accumulation, lines cpSL A2 and C3 contained higher levels of Se than wild-type (Fig. 6; P < 0.05), both when supplied with selenate (+15–30%) or selenite (+35–50%). Line cpSL A1, despite having the highest SL expression level, showed no significant difference in Se accumulation from selenite or selenate, relative to wild-type.

The cpSL mature plants showed reduced Se tolerance (Fig. 7A, P < 0.05), similar to seedlings. However, the shoot Se levels in the cpSL transgenics were not different between wild-type and cpSL plants (Fig. 7B). There were some differences in shoot concentrations of other elements, however, with the general tendency for the cpSL plants to have enhanced levels of Cu, Mg and S.

Fig. 3. Western blot immunostained with mouse SL-specific antibodies, showing localization of the mouse SL protein in the chloroplasts of B. juncea cpSL transgenic lines. H: homogenate; CP: chloroplasts. Equal amounts of chlorophyll were loaded in each lane. WT: wild-type.
compared to wild-type (Table 1). There were no significant differences in the levels of Fe, Mn, or Zn (results not shown).

Thus, expression of the mouse SL gene in *B. juncea* chloroplasts reduces Se tolerance and enhances Se accumulation at the seedling stage.

**Discussion**

Expression of the mouse selenocysteine lyase in chloroplasts of *B. juncea* was shown to lead to reduced incorporation of Se into proteins at the seedling level; at the mature plant level there were no significant differences with respect to Se incorporation into proteins, although the same trend was visible. The finding that cpSL plants incorporated less Se in protein is in agreement with the hypothesis that breakdown of SeCys into Se⁰ and alanine will prevent non-specific translational incorporation of SeCys into proteins. Since replacement of Cys by SeCys in proteins is thought to be toxic, and Se⁰ is relatively inert, it was expected that the transgenic SL plants would be more tolerant to Se. However, the transgenic cpSL plants showed reduced Se tolerance, both at the seedling and mature plant stage. Moreover, the cpSL transgenics showed enhanced shoot Se concentrations at the seedling stage, but not at the mature plant stage. This difference in the effect of SL expression on shoot Se accumulation between seedling and mature plant stage may in part be due to the more pronounced differences in Se incorporation in protein at the seedling stage, compared to the mature plant stage.

The adverse effect of expression of the SL enzyme in *B. juncea* chloroplasts appears to be related to produced Se⁰ since the transgenics showed a normal phenotype in the absence of Se. It is feasible that the produced Se⁰ interferes with chloroplast S metabolism. The chloroplast is a site of iron-sulphur (Fe–S) cluster formation, as well as for biosynthesis of biotin, thiamine and molybdenum cofactor. Elemental S (S⁰) is thought to be one of the substrates for these processes (Mihara et al. 2002). It is feasible that Se⁰ can compete with S⁰ and disrupt the formation or functioning of these compounds, especially since enzymes involved in S metabolism typically have an equal or higher affinity for the Se analogue compared to their S substrate (Anderson 1993).

If the reduced Se tolerance of the cpSL plants is due to interference of the produced Se⁰ with Fe–S cluster formation, then perhaps this could be alleviated by expressing the SL enzyme in the cytosol instead of the chloroplast, the cytosol not being a major site of Fe–S cluster formation. This may still prevent non-specific incorporation of SeCys into proteins since the cytosol is an important site of translation. Recent results from studies with the model plant *A. thaliana* have shown that expression of mouse SL in the cytosol indeed leads to enhanced Se tolerance as well as accumulation (Pilon et al. 2003).

At the seedling level, the introduction of the mouse SL enzyme led to enhanced shoot Se levels. It can only be speculated what could be the mechanism behind this phenomenon. Perhaps a reduced availability of S⁰ for Fe–S cluster formation, due to competition with Se⁰, is sensed as a sulphur deficiency, triggering the induction of enzymes involved in uptake and reduction of sulphate and selenate. An effect of SL expression on S uptake is supported by the observation that the cpSL plants contained higher levels of S than wild-type plants (Table 1). The fact that the cpSL plants also contained more S when grown in the absence of Se is somewhat surprising, and suggests that the mouse SL enzyme directly affects S metabolism, in spite of its reported very low activity toward cysteine (Mihara et al. 2000); similarly, the reason for the elevated levels of Mg and Cu in the cpSL plants is not clear. The cpSL transgenics did not show enhanced Se levels at the mature plant level, in contrast to the results obtained with seedlings. This may be due to physiological differences between the two developmental stages, or to differences in the experimental set-up such as supplied Se and S concentrations, agar versus liquid medium, and the timing and duration of the Se treatment.

It was found earlier that the reduction of selenate to selenite is a rate-limiting step for selenium assimilation to organic compounds. *B. juncea* plants supplied with sodium selenate showed enhanced Se uptake in the presence of the SL enzyme (Feng et al. 2002). It is feasible that Se⁰ can compete with S⁰ and disrupt the formation or functioning of these compounds, especially since enzymes involved in S metabolism typically have an equal or higher affinity for the Se analogue compared to their S substrate (Anderson 1993).

Table 1. Shoot concentrations of Cu, Mg, and S in mature wild-type (WT) and cpSL transgenic *B. juncea* plants grown with or without selenate (40 µM, 2 weeks). Shown are the means and standard errors of 10 replicate plants each. Stars (*) indicate a statistically significant difference from wild-type (*P < 0.05).

<table>
<thead>
<tr>
<th>Shoot concentration (mg kg⁻¹ DW)</th>
<th>Cu</th>
<th>Mg</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13 ± 0.3</td>
<td>5190 ± 138</td>
<td>11945 ± 340</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpSL A1</td>
<td>14 ± 0.5</td>
<td>5970 ± 272*</td>
<td>14676 ± 1025*</td>
</tr>
<tr>
<td>cpSL A2</td>
<td>15 ± 0.4</td>
<td>6132 ± 272*</td>
<td>15170 ± 1282*</td>
</tr>
<tr>
<td>cpSL C3</td>
<td>17 ± 1.7*</td>
<td>6057 ± 211*</td>
<td>15724 ± 675*</td>
</tr>
<tr>
<td>+Na₂SeO₄</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>10.2 ± 0.5</td>
<td>4918 ± 134</td>
<td>19042 ± 890</td>
</tr>
<tr>
<td>cpSL A1</td>
<td>13.8 ± 0.6*</td>
<td>5332 ± 68*</td>
<td>21608 ± 424</td>
</tr>
<tr>
<td>cpSL A2</td>
<td>12.4 ± 0.9</td>
<td>5347 ± 170*</td>
<td>20740 ± 1089</td>
</tr>
<tr>
<td>cpSL C3</td>
<td>15.5 ± 1.0*</td>
<td>5766 ± 174*</td>
<td>22685 ± 929*</td>
</tr>
</tbody>
</table>
selenate accumulated selenate, whereas plants supplied with selenite accumulated organic Se (de Souza et al. 1998); also, overexpression of ATP sulphurylase, involved in reduction of selenate to selenite, led to enhanced reduction of selenate and accumulation of organic Se (Pilon-Smits et al. 1999). In spite of the slower conversion of selenate to SeCys relative to selenite–SeCys conversion, overexpression of SL affected Se tolerance and accumulation very similarly for selenate and selenite. This may in part be due to the fact that selenate is taken up and translocated to a larger extent than selenite (Fig. 6; de Souza et al. 1998). It is also possible that even a low level of Se⁰ production already results in the observed phenotype. The latter is suggested by the fact that the three transgenic cpSL lines showed similar results with respect to Se incorporation in proteins and Se tolerance, despite their different expression levels.

Transgenic plants with enhanced Se tolerance and/or accumulation may be useful for phytoremediation of Se-contaminated soil or water, or may have enhanced nutritional value with respect to the essential element Se. The cpSL transgenics show limited promise in this respect: although they have higher Se levels as seedlings, they are less tolerant to toxic Se levels. The tendency of the cpSL plants to accumulate more Se than wild-type may be more pronounced under optimal experimental conditions and developmental stage. Moreover, their reduced growth in the presence of Se may be less severe or absent at lower Se concentrations, as was observed when these cpSL plants were grown side-by-side with wild-type plants on Se-containing environmental soil (unpublished results). Still, the best approach to alleviate the adverse effects of expression of the mouse SL enzyme in plants while maintaining the positive effects will likely be to express the enzyme in the cytosol, as indicated by results from A. thaliana (see above).

In conclusion, it was shown that it is possible to redirect Se flow in the plant, away from incorporation of SeCys into proteins, and towards accumulation of Se⁰. The expression of SL (and presumably the production of Se⁰) in the chloroplast leads to reduced Se tolerance, but also to enhanced Se accumulation at the seedling level. Future experiments will focus on expressing the mouse SL enzyme in the cytosol of B. juncea, and exploring the effects on Se metabolism.

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