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Ectopic phytocystatin expression leads to enhanced drought stress tolerance and improved seed traits in soybean (Glycine max) and Arabidopsis thaliana through effects on strigolactone pathways

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Running title: Enhancing crop quality by cystatin expression

Summary

Ectopic cystatin expression has long been used in plant pest management but the cysteine protease targets of these inhibitors might also have important functions in the control of plant lifespan and stress tolerance that remain poorly characterised. We therefore characterised the effects of expression of the rice cystatin, oryzacystatin-I (OCI), on the growth, development and stress tolerance of crop (soybean) and model (Arabidopsis thaliana) plants. Ectopic OCI expression in soybean enhanced shoot branching and leaf chlorophyll accumulation at later stages of vegetative development significantly and enhanced seed protein contents and decreased the abundance of mRNAs encoding strigolactone synthesis enzymes. The OCI expressing A. thaliana showed a slow growth phenotype, with increased leaf numbers and enhanced shoot branching at flowering. The OCI-dependent inhibition of cysteine proteases enhanced drought tolerance in soybean and A. thaliana, photosynthetic CO_2 assimilation being much less sensitive to drought-induced inhibition in the OCI expressing soybean lines. Ectopic OCI expression or treatment with the cysteine protease inhibitor E64 increased lateral root densities in A. thaliana. E64 treatment also increased lateral root densities in the max2-1 mutants that are defective in strigolactone signalling, but not in the max3-9 mutants that are defective in strigolactone synthesis. Taken together, these data provide evidence that OCI-inhibited cysteine proteases participate in the control of growth and stress tolerance through effects on strigolactones. We conclude that cysteine proteases are an important target for manipulation not only to control plant growth, development and stress tolerance, but also seed quality traits.

Key Words: soybeans, tobacco, Arabidopsis, drought tolerance, seed quality, cysteine protease, cystatin, strigolactone

Abbreviations: CP: cysteine protease; OCI: orzacystatin-I; SWt wild-type soybean plants

Introduction

Several biotechnological approaches to plant improvement using phytocystatins, particularly in over-expression studies, have been successful in recent years. For example, ectopic over-expression of phytocystatins have been used to deter insect feeding in transformed plants (Christou et al., 2006; Kiggundu et al,. 2010) and to improve the yields of bio-engineered proteins such as vaccines and metabolic enzymes (Rivard et al., 2006, Pillay et al., 2012). Second generation insect-resistant plants containing constructs designed to express multiple protease inhibitors might be important in the future to reduce pesticide usage (Vorster et al., 2010). However, relatively little is known about additional pleiotropic effects arising from ectopic phytocystatin over-expression, particularly with regard to plant development or crop quality.

Like their cysteine protease (CP) targets, endogenous phytocystatins controlling CP activity are regulated by developmental (Lohman et al., 1994; D'Silva et al., 1998; Sheokand et al., 2005) and environmental cues (Botella et al., 1996; Belenghi et al., 2003; Pernas et al., 2000; Diop et al., 2004). The cysteine proteases of seeds are important in the processing and folding of storage proteins during development (Gruis et al., 2002), the remobilization of stored proteins during seed germination, hormone signalling, embryogenesis and morphogenesis (Salas et al., 2008). Cysteine proteases are also responsible for the regulated dismantling of organelles during senescence so that macromolecules can be remobilized and transported to the actively growing parts of the plant (Beers et al., 2000).

The expression of phytocystatins is also considered to be important in the acquisition of abiotic stress tolerance (Van der Vyver et al., 2003; Zhang et al., 2008; Hwang et al., 2010; Benchabane et al., 2010). Transformed tobacco plants expressing the rice cystatin oryzacystatin-I (OCI) were more resistant to the negative impacts of chilling stress on photosynthesis (Van der Vyver et al., 2003). Similarly, over-expression of two cystatins, atCYSa and atCYSb, in transformed Arabidopsis increased resistance to high salt, drought, cold and oxidative stress (Zhang et al., 2008). These beneficial effects of phytocystatin action in enhancing stress tolerance are considered to be the direct result of the inhibition of the cysteine protease targets (Van der Vyver et al., 2003; Zhang et al., 2008). In addition to effects on stress tolerance, OCI expression had marked effects on the growth and development of the transformed tobacco plants (Van der Vyver et al., 2003). For example, all the OCI-expressing transformed tobacco lines were smaller than controls at 16 weeks, the point where the wild-type flowered and the vegetative growth phase ceased. In contrast to the wild-type, the transformed lines flowered at 26-27 weeks, at which time they were much taller than the controls with a greater number of leaves (Prins et al., 2008). The transformed tobacco plants showed delayed senescence characteristics, and had significantly higher protein contents than the wild-type controls at the late senescence stage (26-27 weeks; Prins et al., 2008). Such observations of pleiotropic effects of recombinant protease inhibitors in planta demonstrate that our current knowledge of the range of functions of plant proteolytic processes in incomplete. Cysteine proteases therefore might mediate a range of additional useful traits for crop improvement and productivity.

Enhanced crop productivity is required to meet the needs of a growing world population which is one of the most important challenges in plant biology of our time.

The development of crop varieties with enhanced environmental stress tolerance traits for example is a major target in current plant breeding and improvement strategies (Bray, 1997; Araus et al., 2008; Parry et al., 2012). The application of classical breeding approaches in recent decades has increased the productivity of agricultural crops by an average 1% per year (Kucharik and Ramankutty, 2005). However, the amelioration of tolerance to environmental stresses, such as drought, is complex and involves factors that control plant development and growth as well as senescence (Cleays and Inze, 2013; Lawlor, 2013). Successful breeding involves the recombination of large sets of genes, followed by selection of a whole plant or crop level criterion, often yield. Results obtained to date, suggest that cysteine protease inhibitors might also be an attractive target providing improvement of stress tolerance traits in transformed plants. We have previously shown that ectopic OCI expression alters the growth and stress responses of tobacco (Van der Vyver et al., 2003; Prins et al., 2008). Here, we have compared the effects of ectopic OCI expression on the growth, development and drought tolerance in a crop (soybean; Glycine max) and model plant (A. thaliana), to determine whether this CP inhibitor exerts similar effects in different plant species. Moreover, we have explored the mechanisms by which inhibition of endogenous plant CPs leads to altered plant development and enhanced stress tolerance, demonstrating that, at least in part, the observed changes are linked to effects on strigolactone-mediated regulation. Taken together, these results demonstrate that cystatin technologies can be successfully applied to crops such as soybean, providing beneficial characteristics such as enhanced drought tolerance and improved seed traits, as well as the existing benefits of pest control.

Results

Phenotypic characterisation of transformed soybean lines with ectopic OCI expression

The effects of ectopic OCI expression on plant growth and development were determined in three independent transformed soybean lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild type (SWt). OCI gene insertion was detected in all the leaves of the transformed soybean lines, SOCI-1, SOCI-2 and SOCI-3 (Figure 1a). Similarly, OCI protein expression was detected using specific antibodies on Western blots in the leaves of the SOCI-1, SOCI-2 and SOCI-3 lines (Figure 1b).

The effects of ectopic OCI expression on the shoot phenotype were compared in the three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild-type at different stages of development (Figure 1c-g). Shoot height (Figure 1d) and shoot biomass (Figure 1e) were similar in the three independent transformed lines and the wild-type (Figure 1d). However, the transformed lines had more axillary branches than the wild-type plants at later stages of development (e.g. week 7; Figure 1f) with significantly higher amounts of chlorophyll than the wild type after 7 weeks (Figure 1g).

Seed properties in transformed soybean and tobacco lines with ectopic OCI expression

The dry and imbibed seeds of two of the transformed soybean lines (SOCI-2 and SOCI-3) had significantly more soluble protein than those of the wild-type and SOCI-1 (Figure 2a). In contrast, the soluble protein content of the leaves was similar in all genotypes (Figure 2b). However, there were no consistent differences between the lines in extractable seed (Figure 2c) or leaf (Figure 2d) cathepsin L-like protease activities, which was used here as a measure of leaf CP activity.

We have previously characterised the vegetative growth and development of three independent tobacco lines (NOCI) with ectopic expression of OCI, which showed a marked slow growth phenotype but had greater biomass and leaf numbers at flowering (Van der Vyver, et al., 2003; Prins et al., 2008).). Here, we further characterized the effects of ectopic OCI expression on reproductive development in NOCI tobacco line 4/5. Ectopic OCI in tobacco line had marked effects on reproductive development, resulting in visibly larger pods than those of the wild-type (NWt; Figure 3a). The OCI-expressing tobacco pods also had significantly greater numbers of seeds than the than wild-type with a significantly greater average seed pod dry weight (Figure 3b). The number of seeds per pod was 60% higher in transformed line 4/5 and the average seed weight was increased by 35% relative to the NWt (Figure 3b). As far as possible we have assessed seeds numbers per plant in the wild type soybeans and transformed SOCI lines (Figure 3c). While SOC-I showed a trend to lower seed numbers there were no significant differences between the lines (Figure 3c) and the average seed weight was comparable in all lines (Figure 3d). The germination efficiency was also similar in all lines (data not shown).

Vegetative development in transformed soybean lines with ectopic OCI expression

The phenotype of the shoots of transformed soybean lines SOCI-1, SOCI-2 and SOCI-3 was visibly different from that of the wild type at 18 weeks (Figure 4a). The wild type leaves had started to senesce at 18 weeks, whereas the transformed lines were visibly greener (Figure 4a). The chlorophyll content of the leaves of the transformed soybean lines was significantly greater than that of the wild type at 18 weeks (Figure 4b) confirming the results obtained at 7 weeks (figure 1g). However, only the leaves of the SOCI-1 line had significantly higher rates of photosynthesis than the wild-type (Figure 4b).

The abundance of transcripts encoding two types of endogenous cysteine proteases, various papain-like cysteine proteases (CP) and vacuole processing proteolytic (cysteine protease) enzymes (VPE) and cystatins (Cys) was determined in SOCI-1 leaves at three time points during vegetative growth i.e. in the leaves of 3, 6 and 12 week old plants (Table 1). While the levels of all the CPs, Cys and VPEs mRNAs measured in this study were very low, ectopic OCI expression tended to decrease the abundance of these transcripts (Table 1),

Strigolactone-associated gene expression in soybean leaves

The higher leaf chlorophyll contents and enhanced shoot branching observed in the OCI-expressing soybean lines relative to the wild-type, is similar to phenotypes observed in A. thaliana mutants that lack a response to branching inhibition signals, such as strigolactones (Gomez-Roldan et al., 2008; Beveridge et al., 2009). Two carotenoid cleavage dioxygenases (CCDs), which are called CCD7 and CCD8, are required in the pathway for strigolactone synthesis, as illustrated in Figure 5a. We therefore measured the levels of CCD7 and CCD8 transcripts in the different soybean

lines. The abundance of CCD7 and CCD8 mRNAs was lower in the SOCI-1, SOCI-2 and SOCI-3 leaves than in the wild-type leaves (Figure 5b and c). In particular, the abundance of CCD8 transcripts was significantly lower in the SOCI-1, SOCI-2 and SOCI-3 leaves than in the wild-type at nearly all harvest points (Figure 5c). The levels of CCD7 mRNAs were similar in all lines at the early stages of development but became significantly lower in the leaves of the transformed lines than in the wild-type as the plants grew older (Figure 5b). The branching of soybean shoots and roots was insensitive to addition of the synthetic strigolactone GR24, at a concentration of 2 μM (data not shown). At this concentration, GR24 causes marked changes in shoot and root branching in A. thaliana (Ruyter-Spira et al., 2011; Kapulnik et al., 2011).

Growth and development in A. thaliana lines with ectopic OCI expression in relation to the wild-type

Ectopic OCI expression in A. thaliana resulted in a slow growth phenotype, which was observed in all the homozygous independent transformed lines (Figure 6a). This phenotype was similar to that previously observed in OCI-expressing tobacco lines such as line 4/5 (Van de Vyver et al., 2003; Prins et al., 2008). OCI gene insertion into the Arabidopsis genome was detected in the leaves of the transformed lines (Figure 6b). Ectopic OCI expression in A. thaliana resulted in increased lateral root densities, with values that were significantly higher in AOCI1 and AOCI3 than the wild type (Figure 6 c). Although the increases relative to the wild type were not always significant, the leaves of the OCI-expressing A. thaliana lines tended to have higher chlorophyll levels at similar stages of vegetative development (Figure 6d). The rosettes of the OCI-expressing A. thaliana lines were significantly smaller than those of the wild type

during vegetative development (Figure 6e). However, at flowering, the OCI-expressing A. thaliana lines had double the number of leaves than the wild type, with a smaller but more branched flowering stem (Figure 6f).

Inhibition of CP activity on root architecture in A. thaliana wild type and strigolactone synthesis (max3-9) and signalling (max2-1) mutants

The altered shoot and root branching phenotype observed in the OCI-expressing A. thaliana lines relative to the wild-type, is similar to that of strigolactone synthesis and signalling mutants (Gomez-Roldan et al., 2008; Beveridge et al., 2009). The relationship between changed CP activity and strigolactones was therefore examined further using A. thaliana mutants that are deficient in either strigolactone synthesis (max3-9) or signalling (max2-1) mutants. The effects of the CP inhibitor E64 on root branching was compared in the wild-type and max3-9 and max2-1 mutant genotypes (Table 2). In the absence of E64, lateral root densities were significantly higher in the max2-1 mutants than the wild-type plants and significantly lower in the max3-9 mutants than in the wild-type plants (Table 2). Lateral root densities were significantly increased in the wild-type and max2-1 (strigolactone signalling) mutants in the presence of E64 but not in the max3-9 (strigolactone synthesis) mutants (Table 2).

Responses to drought

The rosette leaves of the wild type A. thaliana plants (AWt) showed signs of senescence after 15 days of drought (Figure 7a). In contrast, the leaves of transformed

lines, AOCI-1, AOCI-3 and AOCI-4, showed no signs of drought-induced senescence (Figure 7a). At this stage, the AOCI-1, AOCI-3 and AOCI-4 had removed less water from the soil than the wild type A. thaliana plants, as indicated by the soil water contents (Figure 7b). Moreover, the leaves of the AOCI-1, AOCI-3 and AOCI-4 plants retained higher leaf water contents than the wild-type (Figure 7c).

The leaves of the wild type soybean plants (SWt) were visibly flaccid after 6 days of drought (Figure 8a). However, the leaves of transformed lines were visibly more turgid after 6 days of drought (Figure 8a). The rates of photosynthesis were measured at three leaf ranks on the stem (top, middle, bottom) of plants of the three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) and the wild-type under well-watered conditions (day 0) and after 3 and 6 days of drought (Figure 8b). Under well-watered conditions (day 0), the rates of photosynthesis measured in the bottom and top leaves of the transformed lines were similar to those at equivalent positions on the stem of the wild-type plants (Figure 8b). However, photosynthesis rates were significantly higher in the middle rank leaves of the SOCI-1 and SOCI-3 plants than those of the wild-type at an equivalent position (Figure 8b). The rates of photosynthesis were significantly decreased after 3 and 6 days of drought in all lines, particularly in the oldest bottom leaves, the drought-induced inhibition of photosynthesis was significantly less marked in transformed lines relative to the wild-type (Figure 8b).

Discussion

The data presented here show that ectopic OCI expression significantly enhances drought tolerance in soybean and A. thaliana. While we have not as yet been able to investigate the molecular mechanism that underpin the enhanced drought tolerance traits particularly the protection of photosynthesis against drought-induced inhibition, our previous studies in tobacco suggests that chloroplast proteins such as ribiluose-1, 5-bisphosphate carboxylase oxygenase (RuBiSCO) and RuBiSCO activase are more stable in plants with ectopic OCI expression (Prins et al., 2008). Moreover, the observed effects of CP inhibition on root branching in A. thaliana, might also have a beneficial influence on drought tolerance.

Although, growth was similar in the OCI-expressing soybeans relative to the wild type controls, OCI expression resulted in greater shoot branching and significantly higher levels of leaf chlorophyll at later stages of shoot development. Moreover, while the OCI-expressing A. thaliana rosettes had a slow growth relative to the wild type controls, they had enhanced lateral root densities, with significantly more leaves at flowering, which is very similar to phenotype we have described for OCI-expressing tobacco lines (Van de Vyver et al., 2003; Prins et al., 2008). Several lines of evidence suggest that these traits are linked to effects on the action of plant hormones, particularly strigolactones. Firstly, the altered branching and stress tolerance characteristics are very similar to those previously described in the A. thaliana max2 mutant, which was initially called ore9 (Woo et al., 2001; Stirnberg et al., 2002; Woo et al., 2004). The max2 mutants have delayed leaf senescence and are more tolerant to oxidative stress than the wild type (Woo et al., 2001; Stirnberg et al., 2002; Woo et al., 2004). Secondly, the abundance of transcripts encoding enzymes involved in the

strigolactone pathway was decreased in OCI-expressing soybean leaves relative to controls. Thirdly, the enhanced root branching phenotype observed in the OCI-expressing A. thaliana lines was also observed in the wild type in the presence of E64. Fourthly, lateral root densities were also significantly increased in the max2-1 strigolactone signalling mutants in the presence of E64 but not in the max3-9 strigolactone synthesis mutants. Taken together, these data demonstrate that CP-regulated steps influence strigolactone synthesis but not MAX2-dependent strigolactone signalling pathways and hence ectopic OCI expression alters key traits such shoot and root branching, lifespan and senescence. These findings are consistent with the known role of CPs in the turnover of proteins involved in the control of plant development and senescence (Schlüter et al., 2010; Vorster et al., 2013).

While a number of the traits described here in the OCI-expressing lines result from common effects on strigolactone-dependent processes in soybean and A. thaliana, the phenotypes produced by OCI expression do not precisely mirror each other in the two species. For example, like the OCI-expressing tobacco lines (Van der Vyver et al., 2003), the OCI-expressing A. thaliana lines had a slow growth phenotype compared to the wild type having significantly more leaves at flowering, whereas vegetative growth was similar in the OCI-expressing soybean likes to the wild type. These interspecific variations may arise from differences in the interactions of the cystatin with the various CP forms present in each species, together with differences in the affinities of the CPs for OCI-binding. Moreover, the CP/cystatin balance may also vary between species. In these studies, OCI was not targeted to a specific cellular location using an appropriate peptide targeting signal, and this lack of targeting may also lead to variations between

species. Regardless of species to species variations of OCI-interacting partners, the data presented here strongly suggest that interventions that impair or modify specific CP functions can be targeted to modify plant growth and development and also to improve seed quality traits. For example, seed numbers were greatly increased in OCI-expressing tobacco lines relative to the wild type. While seed production was not greatly increased in OCI-expressing soybean lines relative to the wild type, OCI-expressing soybean seeds accumulated significantly more protein. In the absence of a full analysis of the composition of soybean seed CPs, we can only speculate that CP action in soybean limits seed protein accumulation. While we are still in the process of identifying the exact CP targets of OCI in soybean, the observed increases in seed protein contents in the OCI-expressing soybean lines are commercially interesting because no adverse effects of OCI expression on seed germination were observed.

Variations in the affinities of ectopically expressed protease inhibitors, such as OCI, for endogenous CPs may also explain previous observations of the absence of strong phenotypic effects arising from the ectopic expression of protease inhibitors in transformed plants (Masoud et al., 1993; Brunelle et al., 2004; Rivard et al., 2006; Badri et al., 2009) but not in others (Van der Vyver et al., 2003; Prins et al., 2008). Similarly, interspecific variations probably explain why the ecotopic expression of other protease inhibitors, such as cereal cystatin in potato (Munger et al., 2012) and a trypsin inhibitor in Nicotiana attenuate (Zavala et al., 2004) produce large differences in effects on growth phenotype and stress responses

Conclusions and perspectives

In this study, we have compared the effects of ectopic cystatin (OCI) expression in model and crop species, providing new information on the mechanism by which CPs exert control over plant growth, development and stress tolerance. Taken together, the data suggest that strigolactone synthesis is influenced in vivo by CPs, a process that can be controlled by phytocystatins. These data therefore complement studies on the proteasome, which is known to play a key role in the regulation of the abundance of proteins and transcription factors that mediate hormone-dependent growth (Eckardt, 2001). The results presented here highlight the potential of using phytocystatins, such as OCI, in the control endogenous CP activities in order to regulate plant productivity and stress tolerance. While, data are presented here for three plant species to illustrate the central role the CPs play in the control of plant growth, development and stress tolerance, we have also observed very similar shoot phenotype changes to those reported here for tobacco and *A. thaliana*, in OCI-expressing cotton lines (Supplemental Fig. 1). Taken together, these findings demonstrate the potential of ectopic OCI expression as a useful tool for plant improvement, particularly with regard to drought tolerance, together with other agronomically useful traits such as delayed leaf senescence and enhanced nutrition characteristics, in addition to improved pest control (Kinney, 2006).

Experimental procedures

Soybean transformation

The OCI gene was cloned as a SacI-XbaI fragment into the plasmid pTF101 to create plasmid pLBRPRKCys-I. This vector has a spectinomycin resistant marker gene (aadA) for bacterial selection. The plant selectable marker gene cassette consists of a double 35S promoter (2x P35S) of the cauliflower mosaic virus (CaMV) and the phosphinothricin acetyl transferase gene from Streptomyces hygroscopicus that confers resistance to the herbicide phosphinothricin and its derivatives. Soybean transformation was performed at the Iowa State University Plant Transformation Facility for providing soybean transformation service following the method by Paz et al. (2006). Seeds of the wild type (SWt) and the T3 generation of three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) were sown in pots in Levington's compost (Levington F2 plus Scotts Professional, Ipswich, UK) and grown in controlled environment chambers, at day/night temperatures of 28°C/20°C and an irradiance of 400 μmol $m^{-2}s^{-1}$ with a 12h photoperiod. Pots were watered daily.

Arabidopsis transformation

Wild type Arabidopsis thaliana (ecotype Col-0) plants were grown in controlled environment chambers, at day/night temperatures of 25°C/20°C and an irradiance of 400 μmol $m^{-2}s^{-1}$ with a 16h photoperiod. Primary inflorescence buds were clipped to allow formation of secondary inflorescence buds and increase the transformation process by obtaining more flower buds per plant. Plants were grown for 8 weeks before floral dip transformation with Agrobacterium tumefaciens strain GV3101 carrying the plasmid pTF101.1-Cys-I (Clough and Bent, 1998). Secondary inflorescences were

submerged into the A. tumefaciens cell suspension for 10s and then allowed to grow in the greenhouse until seed maturity. The following studies were performed on three independent transformed homozygous lines (AOCI1, AOCI3 and AOCI4) and the wild-type (AWt)

Tobacco

Wild type tobacco (Nicotiana tabacum L.; NWt) and transformed line 4/5 (NOCI), as described previously (Van der Vyver, et al., 2003; Prins et al., 2008) were grown in compost in pots for 6 weeks in controlled environment chambers, at day/night temperatures of 26°C/20°C and an irradiance of 600 μmol $m^{-2}s^{-1}$ with a 15h photoperiod for 28 weeks. Seed pods were photographed and weighted at 28 weeks. Forty seed pods were weighed and then seed numbers were counted.

Drought stress treatments

For studies on A. thaliana, watered plants of the transformed lines (AOCI1, AOCI3 and AOCI4) and the wild-type (AWt) were deprived of water for 15 days before analysis. For studies on soybean, well-watered plants of the transformed lines (SOCI1, SOCI3 and SOCI4) and the wild-type (SWt) were deprived of water for 6 days before analysis.

Effects of E64 on A. thaliana seedling growth

Seeds of wild-type A. thaliana (ecotype Columbia; Col-0), the strigolactone synthesis mutant (max3-9) and the strigolactone signalling mutant (max2-1) were surface-sterilized, immersed in ethanol 75% for 1 min, then in sodium hypochlorite 4% for 5 min and then rinsed three times with sterilised water. They were then placed on 12 cm

square plates with ½ strength MS medium solidified with 0.8% agar and supplemented with 0.01% myo-inositol, 0.05% MES buffer (pH 5.7) and 1% sucrose and grown vertically for 3 days. Then seedling were transferred to new plates and grown for 5 more days supplemented with E64 (10 μM). All plates were cold stratified for 2 days and then placed to a plant growth cabinet with 16 h day photoperiod and 22°C. Three independent biological replicates with 5 plates per treatment and genotype and 8 seeds per plate were used.

Root system architecture measurements in A. thaliana

The root length and number of lateral roots formed per treatment was analyzed on 8 days old seedlings. Photos were taken and the root length was measured using ImageJ software. Lateral root density was calculated as the division between the number of visible lateral roots and the main root length for each root analysed.

Cathepsin-like CP activity measurements

Cathepsin-like (Cat-L) activities were measured in extracts from leaf discs prepared in citrate phosphate buffer (0.1 M, pH 6.5) as previously described (Salvesen and Nagase, 1989).

Western blot analysis

Leaf discs were extracted in buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 3 mM DTT, 6 mM PMSF and 30 mg insoluble PVPP. Proteins were separated by standard SDS-PAGE procedures. After transfer to nitrocellulose membranes (Hybond

C-extra, Amersham Pharmacia Biotech, UK) protein detection was conducted using antibodies directed OCI.

Statistical analysis

The gas exchange data were analyzed by ANOVA. Data for all other physiological parameters was analyzed by the Student's t-test or LSD test comparing directly wild-type plants with transgenic plants.

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Figure legends

Figure 1. The effects of ectopic OCI expression on soybean. a. Identification of the presence of OCI sequence in the leaves of three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild-type (SWt); b. Western blot showing the presence of the OCI protein in SOCI-1, SOCI-2 and SOCI-3 leaves; c, comparison of shoot phenotypes at 3 weeks, d, stem height, e, shoot biomass (fresh weight), f, number of axillary branches and g, chlorophyll content in the three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild-type.. Values represent the mean from 3 different plants per line ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Figure 2. The effects of ectopic OCI expression on seed and leaf protein contents and on cathepsin-like, (Cat-L) activities. Comparison of the protein contents of dry and imbibed seeds; b, leaf protein contents; c, seed cathepsin L-like, (Cat-L) activities and d, leaf Cat-L activities in the three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild-type (SWt); Values represent the average of 20 repetitions ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Figure 3. The effects of ectopic OCI expression on seed production and germination in tobacco and soybean. A comparison of a. seed pod phenotype and b, seed number s and dry weights in OCI-expressing tobacco plants (NOCI) plants relative to wild-type (NWt) controls: c. comparison of a. seed production and .d. seed biomass (fresh weight) in the three independent transformed lines (SOCI-1, SOCI-2 and SOCI-

3) relative to the wild-type (SWt). Values represent the average of 20 repetitions ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Figure 4. The effects of ectopic OCI expression on senescence in soybean. A comparison of a, the shoot phenotype at 18 weeks, b, leaf chlorophyll contents and c, photosynthetic CO_2 assimilation rates in the three independent transformed soybean lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild type (SWt). Values represent the mean from 3 different plants per line ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Figure 5. The effects of ectopic OCI expression on the abundance of transcripts encoding carotenoid cleavage dioxygenases 7 (CCD7) and CCD8 in Arabidopsis thaliana. a. A simple depiction of the strigolactone synthesis pathway. The relative abundance of b. CCD7 mRNAs and c. CCD8 mRNAs in the leaves of three independent transformed lines (AOCI1, AOCI3 and AOCI4) relative to the wild-type (AWt). Values represent the mean from 3 different plants per line ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Figure 6. The effects of ectopic OCI expression on the development of Arabidopsis thaliana shoots and roots. a. comparison of rosette phenotype at 4 weeks, b. identification of the presence of the OCI transgene in the leaves of three independent transformed lines (AOCI1, AOCI3 and AOCI4) relative to the wild-type (AWt) , lateral root densities measured on seedlings at 8 days, c. rosette diameter at 4 weeks and f

rosette phenotype at 16 weeks. Values represent the mean from 3 different plants per line ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Figure 7. The effects of ectopic OCI expression on drought-tolerance in Arabidopsis thaliana. A comparison of a. rosette phenotypes, b. soil water, c leaf water contents and d. leaf protein contents in well-watered plants of the transformed lines (AOXI1, AOCI3 and AOCI4) and the wild-type (AWt) with plants that had been deprived of water for 15 days (Drought). Values represent the mean from 3 different plants per line ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Figure 8. The effects of ectopic OCI expression on drought-tolerance in soybean soybean. A comparison of a. shoot phenotypes after 6 days of drought and b. photosynthetic CO_2 assimilation rates in three different leaf ranks (top, middle, bottom) of six week-old SOCI-1 and wild type (SWt) plants under well watered conditions (day 0) and plants that had been deprived of water for 3 and 6 days; Values represent the mean from 3 different plants per line ± SD. Asterisks denote significant differences between the lines at *P<0.01.

Table 1: The effect of OCI expression on the abundance of transcripts encoding leaf papain-like cysteine proteases (CP), cystatins (Cys) and vacuole processing enzymes (VPE) in plants grown for 3, 6 and 12 weeks. The relative abundance of transcripts in SOCI-1 is expressed relative to the wild-type (SWt) plants

Sequence	**Accession**	**Relative expression ($2^{-\Delta\Delta ct}$)**		
		3 wks	6 wks	12 wks
CP				
SWtCP1	Glyma04g04400	1.01±0.11	1.00±0.10	1.00±0.06
SOCICP1		0.56±0.06	1.01±0.41	1.87±0.17
SWtCP2	Glyma17g05670	1.00±0.05	1.01±0.15	1.00±0.05
SOCICP2		1.85±0.18	1.22±0.46	0.84±0.07
SWtCP4	Glyma14g40670	1.02±0.12	1.04±0.15	1.01±0.13
SOCICP4		1.22±0.11	0.57±0.03	0.54±0.04
SWtCP5	Glyma04g03090	1.01±0.10	1.01±0.08	1.00±0.06
SOCICP5		1.39±0.17	0.67±0.04	0.51±0.05
Cystatin				
SWtCy1	Glyma15g36180	1.02±0.16	1.04±0.20	1.01±0.11
SOCICy1		1.54±0.14	0.42±0.06	1.17±0.12
SWtCy2	Glyma14g04250	1.00±0.00	1.03±0.18	1.03±0.18
SOCICy2		1.85±0.16	1.17±0.18	1.17±0.18
SWtCy4	Glyma05g28250	1.03±0.19	1.0±0.06	1.01±0.10
SOCICy4		1.23±0.16	1.5±0.11	0.53±0.05
VPE				
SWtVPE1	Glyma17g14680	1.04±0.21	1.08±0.30	1.0±0.00
SOCIVPE1		2.05±0.44	1.52±0.20	2.28±0.42
SWtVPE2	Glyma05g04230	1.00±0.02	1.02±0.14	1.00±0.04
SOCIVPE2		1.65±0.19	2.08±0.38	1.48±0.1
SWtVPE3	Glyma14g10620	1.03±0.16	1.00±0.04	1.00±0.04
SOCIVPE3		1.31±0.17	1.92±0.14	0.50±0.06

Table 2: E64-dependent inhibition of primary root length, the number of lateral roots and lateral root densities in wild-type Arabidopsis seedlings and in max-2-1 and in max-3-9 Arabidopsis mutants measured at 8 days in wild-type Arabidopsis seedlings and in max-2-1 and in max-3-9 Arabidopsis mutants

Line	Primary root length (cm)	Lateral roots (number/plant)	Root density (roots/cm)
Wild-type-control	3.63 ± 0.11b	1.90 ± 0.23c	0.52 ± 0.07
+ E64	2.12 ± 0.05d	1.48 ± 0.17c	0.70 ± 0.08
Fold-change	1.71	1.28	
max-2-1-control	3.60 ± 0.08b	3.95 ± 0.22a	1.09 ± 0.05
+ E64	1.96 ± 0.08d	2.55 ± 0.17b	1.36 ± 0.10
Fold-change	1.83	1.55	
max-3-9-control	4.23 ± 0.06a	1.47 ± 0.19c	0.34 ± 0.04
+ E64	2.44 ± 0.06c	0.77 ± 0.17e	0.32 ± 0.07
Fold-change	1.73	1.90	

Letters indicate significant differences for either primary root length or later root number ($p<0.01$). Comparisons were made for each genotype in control and E64 treatment conditions, as well as between genotypes. Statistics were performed using Anova and Tukey post hoc test. Data are shown as mean ± standard error of 40 samples per genotype and condition.