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

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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 characterized. We therefore characterized 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 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 important targets for manipulation of plant growth, development and stress tolerance, and also seed quality traits.

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 has 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 (Pillay *et al.*, 2012; Rivard *et al.*, 2006). 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 (D'Silva *et al.*, 1998; Lohman *et al.*, 1994; Sheokand *et al.*, 2005) and environmental cues (Belenghi *et al.*, 2003; Benchabane *et al.*, 2010; Botella *et al.*, 1996; Diop *et al.*, 2004; Hwang *et al.*, 2010; Pernas *et al.*, 2000; Zhang *et al.*, 2008). The CPs 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). CPs 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

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(Benchabane *et al.*, 2010; Hwang *et al.*, 2010; Van der Vyver *et al.*, 2003; Zhang *et al.*, 2008). 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 the 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 is 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 (Araus *et al.*, 2008; Bray, 1997; Parry *et al.*, 2012). The application of classical breeding approaches in recent decades has increased the productivity of agricultural crops by an average of 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 the 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 (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003). Here, we have compared the effects of ectopic OCI expression on the growth, development and drought tolerance in a crop (soybean; *Glycine max*) and a model plant (*Arabidopsis 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 characterization 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 characterized 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 (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003).). 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 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 seed numbers per plant in the wild-type soybeans and transformed SOCI lines (Figure 3c). While SOCI 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

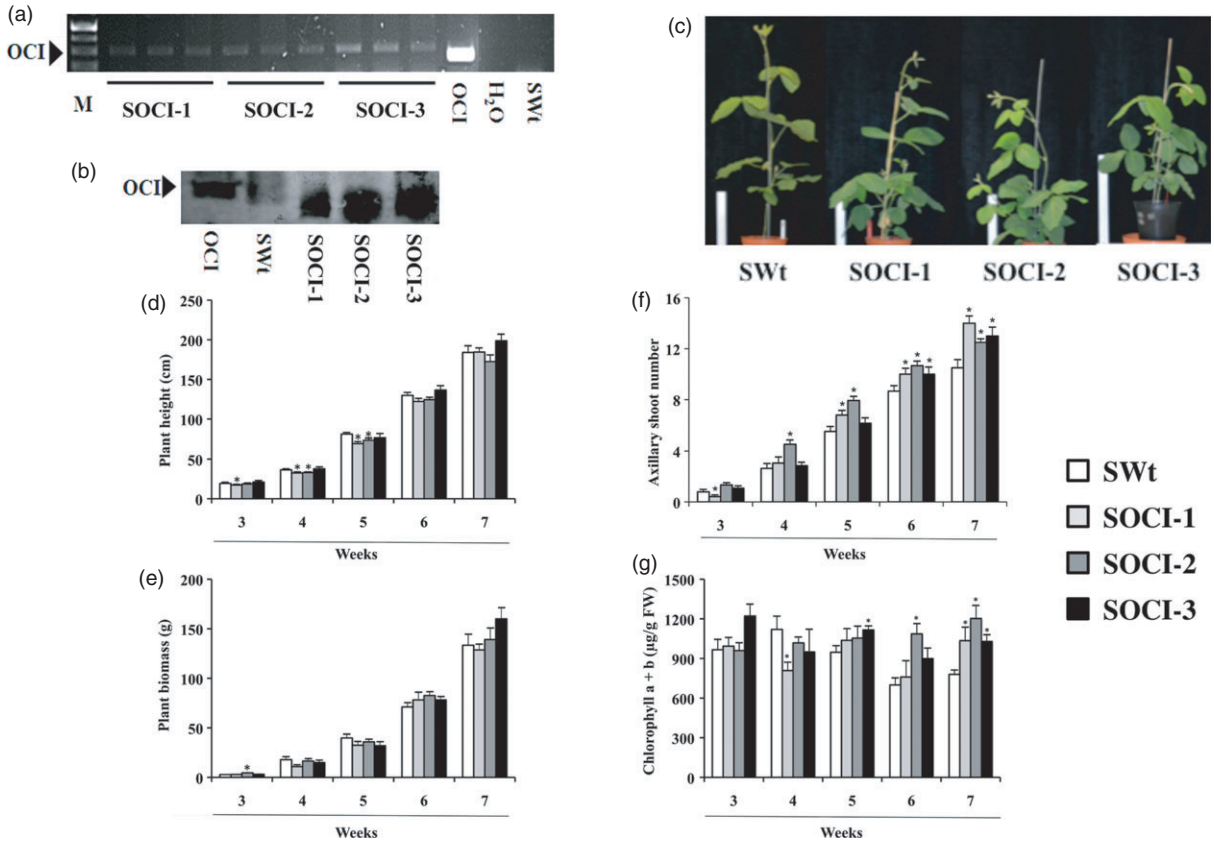

Figure 1 The effects of ectopic OCI expression on soybean. (a) Identification of the presence of OCI sequence in the leaves of three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild type (SWt); (b) Western blot showing the presence of the OCI protein in SOCI-1, SOCI-2 and 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 three different plants per line ± SD. Asterisks (*) denote significant differences between the lines and the wild type at $P < 0.01$.

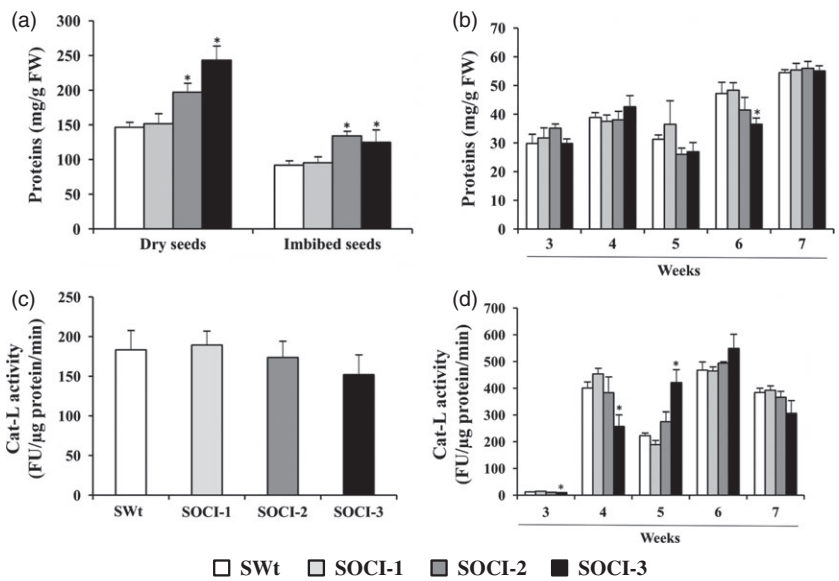

Figure 2 The effects of ectopic OCI expression on seed and leaf protein contents and on cathepsin-like (Cat-L) activities. Comparison of the protein contents of (a) dry and imbibed seeds; (b) leaf protein contents; (c) seed cathepsin L-like (Cat-L) activities and (d) leaf Cat-L activities in the three 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 and the wild type at $P < 0.01$.

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

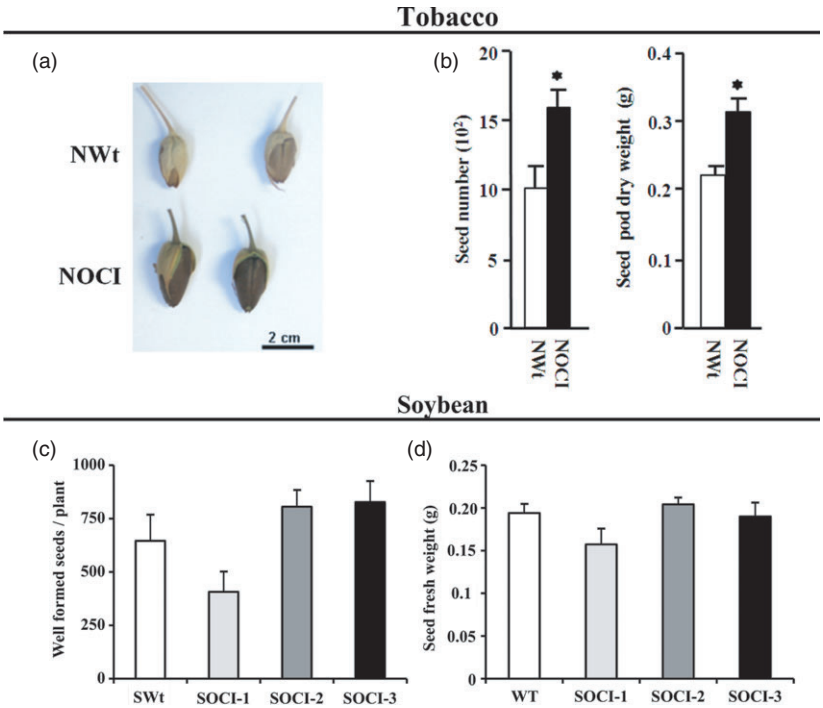

Figure 3 The effects of ectopic OCI expression on seed production and germination in tobacco and soybean. A comparison of (a) seed pod phenotype and (b) seed numbers and dry weights in OCI-expressing tobacco plants (NOCI) plants relative to wild type (NWt) controls; (c) comparison of seed production 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 and the wild type at $P < 0.01$.

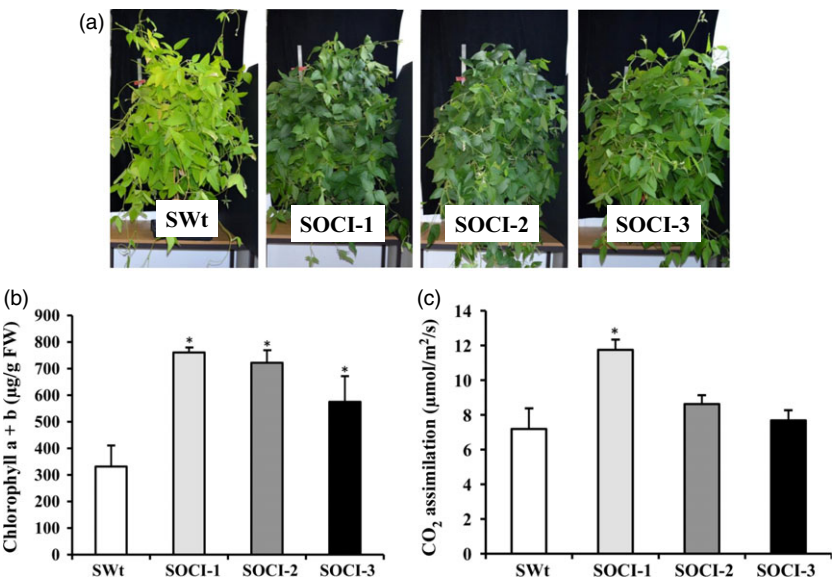

Figure 4 The effects of ectopic OCI expression on senescence in soybean. A comparison of (a) the shoot phenotype at 18 weeks; (b) leaf chlorophyll contents and (c) photosynthetic CO_2 assimilation rates in the three independent transformed soybean lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild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

(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, that is, in the leaves of 3-, 6- and 12-week-old plants (Table 1). Overall, the levels of all the CPs, Cys and VPEs mRNAs measured in leaves in this study were very low and ectopic OCI expression had little effect on transcript levels (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, are similar to phenotypes observed in *A. thaliana* mutants that lack a response to branching inhibition signals, such as strigolactones (Beveridge *et al.*, 2009; Gomez-Roldan *et al.*, 2008; Leyser, 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).

Table 1 The effect of OCI expression on the abundance of transcripts encoding papain-like cysteine proteases (CP), cystatins (Cys) and vacuole-processing enzymes (VPE) in the leaves of 3-, 6- and 12-week-old SOCI-1 soybean plants. The abundance of transcripts in SOCI-1 is expressed relative to the wild type (SWt) plants. Data are the mean ± SD of three replicates per gene

		Relative expression ($2^{-\Delta\Delta ct}$)		
Sequence	Accession	3 weeks	6 weeks	12 weeks
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

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 (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003). 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 6c). 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 leaf area than the wild type, with a smaller but more branched flowering stem (Figure 6f).

Effects of ectopic OCI expression on root architecture in the *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 (Beveridge *et al.*, 2009; Gomez-Roldan *et al.*, 2008). The relationship between CP activities and strigolactone pathways 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 were 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 in 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, AOCI1, AOCI3 and

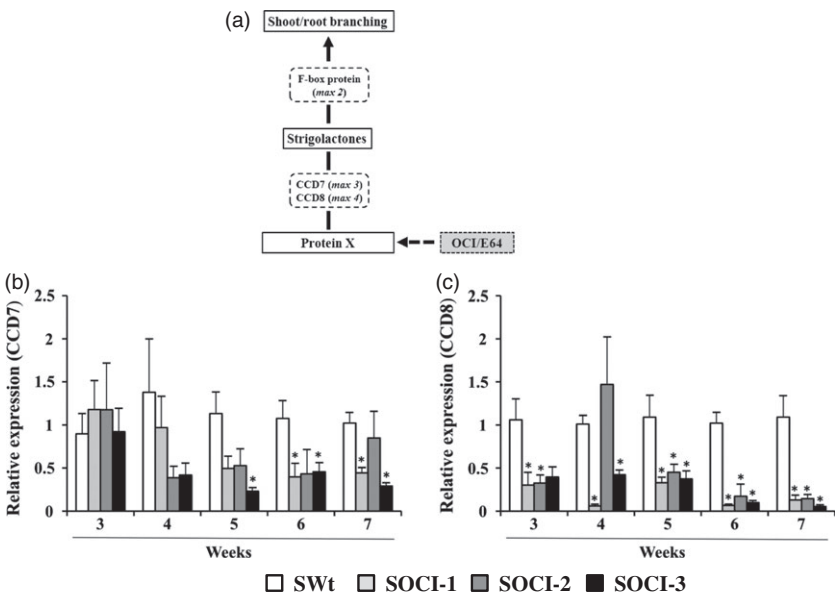

Figure 5 The effects of ectopic OCI expression on the abundance of transcripts encoding carotenoid cleavage dioxygenases 7 (CCD7) and CCD8 in *Arabidopsis thaliana*. (a) A simple depiction of the strigolactone synthesis pathway; the relative abundance of (b) CCD7 mRNAs and (c) CCD8 mRNAs in the leaves of three independent transformed lines (SOCI-1, SOCI-2 and SOCI-3) relative to the wild type (SWt). Values represent the mean from three different plants per line ± SD. Asterisks (*) denote significant differences between the lines and the wild type at each time point at $P < 0.01$.

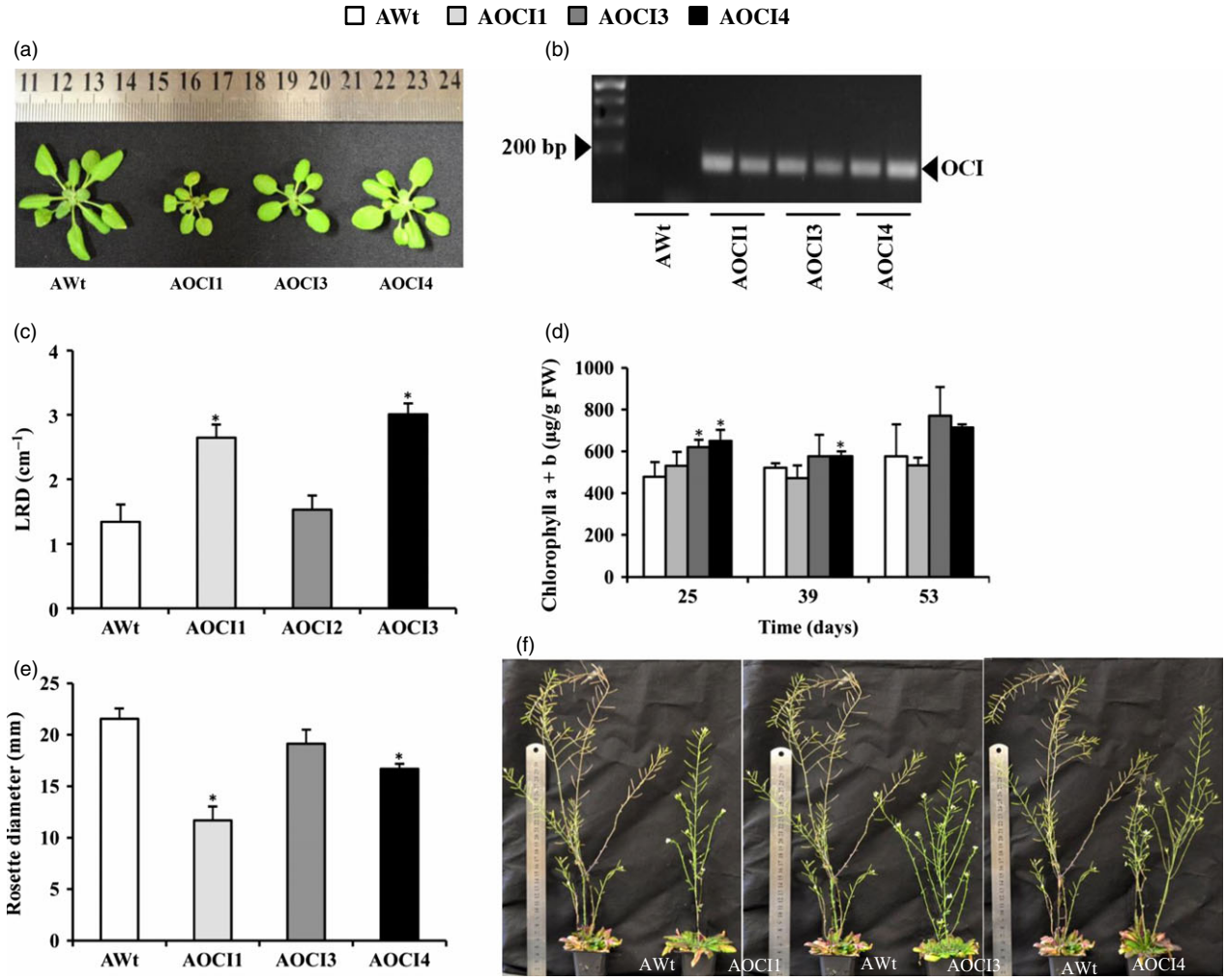

Figure 6 The effects of ectopic OCI expression on the development of *Arabidopsis thaliana* shoots and roots. (a) comparison of rosette phenotype at 4 weeks; (b) identification of the presence of the OCI transgene in the leaves of three independent transformed lines (AOCI1, AOCI3 and AOCI4) relative to the wild type (AWt); (c) lateral root densities (LRD) measured on seedlings at 11 days; (d) chlorophyll content; (e) rosette diameter at 4 weeks and (f) rosette phenotype at 10 weeks. Values represent the mean from three different plants per line ± SD. Asterisks (*) denote significant differences between the lines and the wild type at $*P < 0.01$.

AOCI4, showed no signs of drought-induced senescence (Figure 7a). At this stage, the AOCI1, AOCI3 and AOCI4 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 AOCI1, AOCI3 and AOCI4 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 in soybean at three leaf ranks on the stem (top, middle and 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 resulted in greater shoot branching and significantly higher amounts of leaf chlorophyll at later stages of shoot development in soybean and *A. thaliana*. Moreover, while the OCI-expressing *A. thaliana* rosettes had a slower growth phenotype than the wild type controls, they had enhanced lateral root densities, with significantly greater leaf area at flowering, which is very similar to phenotype we have described for OCI-expressing tobacco lines (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003). Several lines of evidence suggest that these traits are linked to effects on the action of plant hormones, particularly strigolactones, which have pleiotropic effects on plant growth, development and flowering,

Table 2 A comparison of E64-dependent inhibition of primary root length, the number of lateral roots and lateral root densities in 8-day-old wild type *Arabidopsis thaliana* seedlings, the *max-2-1* strigolactone signalling mutants and in the *max-3-9* strigolactone synthesis 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's HSD test. Data are shown as mean ± SE of 40 samples per genotype and condition.

particularly the regulation of root architecture and shoot branching (Rasmussen *et al.*, 2013). 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* (Stirnberg *et al.*, 2002; Woo *et al.*, 2001, 2004). Plants with defects in the strigolactone pathways exhibit delayed senescence (Snowden *et al.*, 2005). The *max2* mutants have delayed leaf senescence and are more tolerant to oxidative stress than the wild type (Stirnberg *et al.*, 2002; Woo *et al.*, 2001, 2004). MAX2 (for MORE AXILLARY GROWTH2) is an F-box protein that functions in strigolactone-mediated regulation of branching, temperature and karrikin signalling, and senescence pathways through the regulation of the turnover of proteins that delay senescence via ubiquitin-dependent pathways (Brewer *et al.*, 2013; Gomez-Roldan *et al.*, 2008; Stirnberg *et al.*, 2002; Umehara *et al.*, 2008; Woo *et al.*, 2001). Secondly, the abundance of transcripts encoding enzymes involved in the strigolactone pathway was decreased in OCI-expressing soybean leaves relative to controls. Strigolactones are predominantly synthesized in both roots and stems and are transported upwards in the xylem (Kohlen *et al.*, 2011; Rasmussen *et al.*, 2013). For example, strigolactones are produced in roots in response to phosphate deficiency, and they are transported to roots to decrease shoot growth (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008, 2010). While strigolactone production in leaves is low, the data in Figure 5 show that *CCD7* and *CCD8* transcripts were detectable in leaves. Moreover, light-dependent processes influence strigolactone levels in roots (Koltai *et al.*, 2011). Thirdly, the OCI-expressing *A. thaliana* lines show enhanced root branching relative to the wild type. Strigolactones play a crucial role in the control of root architecture (Kapulnik *et al.*, 2010; Koltai *et al.*, 2010; Ruyter-Spira *et al.*, 2011; Rasmussen *et al.*, 2013). 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. E64 can also impair proteasome activity as well as that of papain-like CPs, albeit with less sensitivity (Hardin and Huber, 2004). However, if the observed effects of E64 were due to direct inhibition of proteasome activity, the E64-dependent changes in lateral root densities should be observed in the *max3-9* strigolactone synthesis mutants, as well as the wild type, and this was not observed. Taken together, the observation of the enhanced root branching phenotype in the OCI-expressing *A. thaliana* lines and in the wild type in the presence of E64 suggests that CP-regulated steps influence strigolactone synthesis, but not *MAX2*-dependent strigolactone signalling pathways.

The data presented here also show that ectopic OCI expression significantly enhances drought tolerance in soybean and *A. thaliana*. While we have not as yet been able to fully investigate the molecular mechanism that underpin the enhanced drought tolerance traits, the enhanced drought tolerance induced by OCI expression may also be associated strigolactone pathways, which function in the regulation of leaf natural and stress-induced senescence and in drought responses (Stirnberg *et al.*, 2002; Woo *et al.*, 2001, 2004). The *max2* mutants are hypersensitive to drought stress, with a lower sensitivity to abscisic acid than the wild type in terms of the regulation of stomatal closure (Bu *et al.*, 2014). CPs are thought to be important in the turnover of thylakoid membrane proteins, such as the light-harvesting

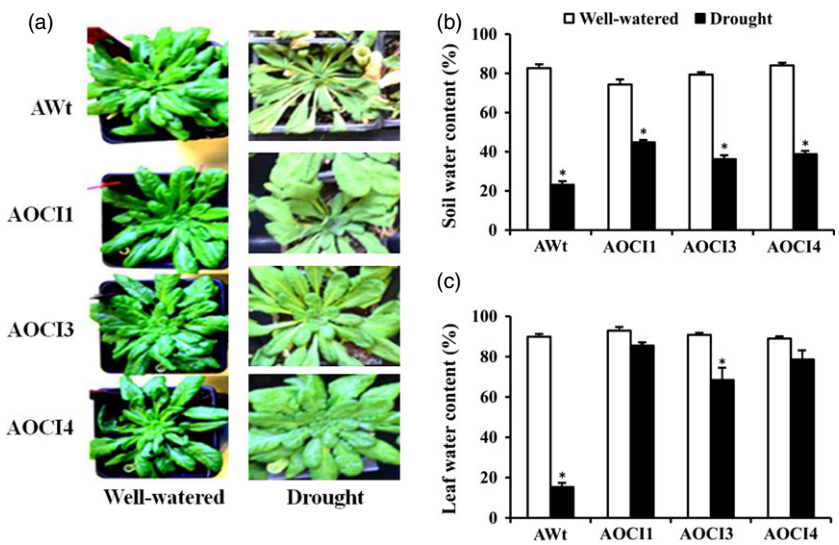

Figure 7 The effects of ectopic OCI expression on drought tolerance in *Arabidopsis thaliana*. A comparison of (a) rosette phenotypes, (b) soil water content and (c) leaf water content in well-watered plants of the transformed lines (AOCI1, AOCI3 and AOCI4) and the wild type (AWt) with plants that had been deprived of water for 15 days (drought). Values represent the mean from three different plants per line ± SD. Asterisks (*) denote significant differences between the lines and the wild type at $P < 0.01$. [Correction added on 02 May 2014, after first online publication: Legend for panel (c) was amended.]

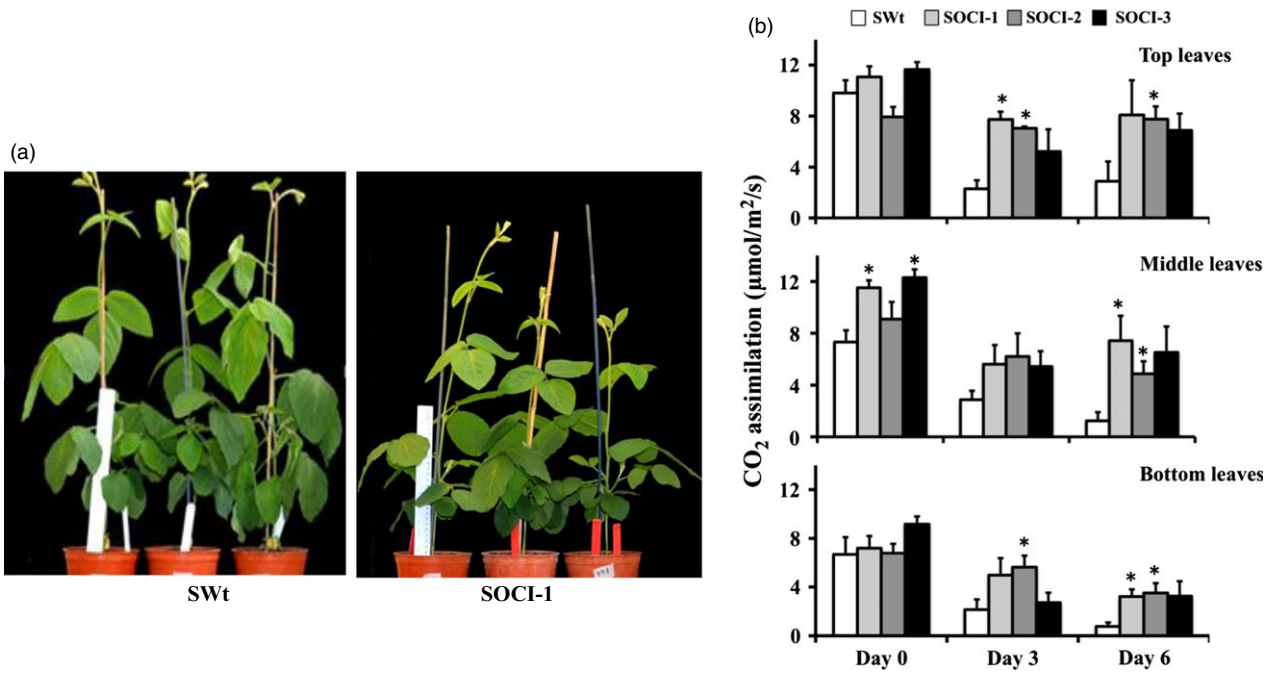

Figure 8 The effects of ectopic OCI expression on drought tolerance in soybean. A comparison of (a) shoot phenotypes after 6 days of drought and (b) photosynthetic CO_2 assimilation rates in three different leaf ranks (top, middle and bottom) of 6-week-old SOCI-1, SOCI-2, SOCI-3 and wild type (SWt) plants under well-watered conditions (day 0) and plants that had been deprived of water for 3 and 6 days; values represent the mean from three different plants per line ± SD. Asterisks (*) denote significant differences between the lines and the wild type at each time point at $P < 0.01$.

chlorophyll proteins (Forsberg *et al.*, 2005). Our previous studies have shown that chloroplast proteins such as ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBiSCO), and RuBiSCO activase are more stable in tobacco plants with ectopic OCI expression (Prins *et al.*, 2008). Taken together, these data suggest that CPs function upstream of strigolactone pathways to regulate defence and senescence pathways in leaves that are important in the drought responses of photosynthesis. The effects of CP inhibition on root branching, as observed in *A. thaliana,* might also have a beneficial influence on drought tolerance.

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 with the wild type having significantly more leaves at flowering, whereas vegetative growth was similar in the OCI-expressing soybean lines 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 in 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 (Badri *et al.*, 2009; Brunelle *et al.*, 2004; Masoud *et al.*, 1993; Rivard *et al.*, 2006), but not in others (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003). Similarly, interspecific variations probably explain why the ectopic 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), produces 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 a crop and model species, providing new information on the mechanism by which CPs exert control over plant growth, development and stress tolerance. The data presented here show that ectopic OCI expression alters key traits such as shoot and root branching, lifespan and senescence. These findings are consistent with the previous observation that CPs, such as strigolactones (Koltai *et al.*, 2011), influence auxin-dependent processes (Chen *et al.*, 2007) and the known role of

CPs in the turnover of proteins that control of plant development and senescence (Schlüter *et al.*, 2010; Vorster *et al.*, 2013).

Taken together, the data suggest as a new result that particularly 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 of endogenous CP activities to regulate plant productivity and stress tolerance. The data are presented here illustrate the central role the CPs play in the control of the growth, development and stress tolerance in soybean and *A. thaliana*, building on our previous studies in tobacco (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003). We have also observed very similar shoot phenotype changes to those reported here for *A. thaliana* and also reported in tobacco (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003) in OCI-expressing cotton lines (Figure S1). 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, complementing improved pest control (Kinney, 2006) and possibly avoiding gene stacking approaches to address multiple stresses.

Experimental procedures

Soybean transformation

The OCI gene was cloned as a SacI–XbaI fragment into the plasmid pTF101 to create plasmid pTF101.1-Cys-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 (2× 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 12-h photoperiod. Pots were watered daily.

Arabidopsis transformation

Wild type *A. 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 16-h photoperiod. Primary inflorescence buds were clipped to allow the 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 10 s 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 (Prins *et al.*, 2008; Van der Vyver *et al.*, 2003), 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 15-h 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 (SOCI-1, SOCI-2 and SOCI-3) and the wild type (SWt) were deprived of water for 3 and 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 sterilized 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 was transferred to new plates and grown for five 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 photoperiod and 22 °C. Three independent biological replicates with five plates per treatment and genotype and eight seeds per plate were used.

Root system architecture measurements in *A. thaliana*

The root length and number of lateral roots formed per treatment were analysed on eight or 11-day-old seedlings. Photographs 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, Little Chalfont, UK), protein detection was conducted using antibodies directed OCI.

Photosynthesis measurements

Photosynthetic gas exchange measurements were performed essentially as described by Soares *et al.* (2008).

Chlorophyll and protein measurements

Leaf chlorophyll and protein contents were determined in leaf samples that had been ground in liquid nitrogen. Pigments extracted in 96% (v/v) ethanol were determined according to Lichtenhaler and Wellburn (1983). The soluble protein content was determined according to the method of Bradford (1976).

RNA extraction and qPCR analysis

Real-time qPCR was performed as described previously (Pellny *et al.*, 2009). Total RNA was extracted from samples using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA reverse transcription and quantitative PCR were performed on an Eppendorf Realplex^2 real-time PCR system by one-step RT-PCR using Quantifast SYBR Green RT-PCR Kit (Qiagen) following the manufacturer's instructions.

The expression of the genes of interest was normalized with an endogenous control, the soybean elongation factor (ELF forward: GTTGAAAAGCCACGGGACA; reverse: TCTTACCCCTTGAGCGT GG). Accession numbers and sequences used for forward and reverse primers used for papain-like CP, vacuole-processing CPs (VPE) and cystains (Cy) are provided in Table S1. The primer sequences used for *CCD7* were as follows: forward: CAC CAAACCCCTCCCTCTAT; reverse: CCTTCCACGGTGCTTAGAGT. The primer sequences used for *CCD8* were as follows: forward: CTTGTTCCTGACATGCCTCA; reverse: CTAGTCCATGCAACGTGGT.

Statistical analysis

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

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Constitutive OC-1 expression leads to increased protein and total chlorophyll content in cotton leaves.

Table S1 Sequences for forward and reverse primers used for papain-like cysteine proteases (CP), vacuole-processing cysteine proteases (VPE) and cysteine protease inhibitors (Cy).