Regulation of seed germination and seedling growth by an *Arabidopsis* phytocystatin isoform, *AtCYS6*

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**Abstract** Phytocystatins are cysteine proteinase inhibitors in plants that are implicated in the endogenous regulation of protein turnover and defense mechanisms against insects and pathogens. A cDNA encoding a phytocystatin called *AtCYS6* (*Arabidopsis thaliana* phytocystatin6) has been isolated. We show that *AtCYS6* is highly expressed in dry seeds and seedlings and that it also accumulates in flowers. The persistence of *AtCYS6* protein expression in seedlings was promoted by abscisic acid (ABA), a seed germination and post-germination inhibitory phytohormone. This finding was made in transgenic plants bearing an *AtCYS6* promoter–β-glucuronidase (*GUS*) reporter construct, where we found that expression from the *AtCYS6* promoter persisted after ABA treatment but was reduced under control conditions and by gibberellin 4,7 (GA4,7) treatment during the germination and post-germinative periods. In addition, constitutive over-expression of *AtCYS6* retarded germination and seedling growth, whereas these were enhanced in an *AtCYS6* knock-out mutant (*cys6-2*). Additionally, cysteine proteinase activities stored in seeds were inhibited by *AtCYS6* in transgenic *Arabidopsis*. From these data, we propose that *AtCYS6* expression is enhanced by the germination inhibitory phytohormone ABA and that it participates in the control of germination rate and seedling growth by inhibiting the activity of stored cysteine proteinases.

**Keywords** Cysteine proteinase inhibitor · GUS expression · Knock-out mutant · Promoter · Transgenic plant

**Introduction**

Phytocystatin (PhyCys) are potent inhibitors of cysteine proteinases (CPs) of the papain- (family C1A; MEROPS peptidase database, [http://merops.sanger.ac.uk](http://merops.sanger.ac.uk)) and legumain-like families (family C13) in plants (Martínez and Díaz 2008). Most PhyCys have a molecular mass in the 12–16 kDa range and contain no disulphide bonds (Gaddour et al. 2001). However, several PhyCys with a molecular mass of ≈23 kDa have a carboxy (C)-terminal extension that has been shown to be involved in the inhibition of legumains (Martínez et al. 2007). During seed germination and seedling growth, these legumains act as processing enzymes and contribute to the activation of papain-like CPs to degrade seed storage proteins (Okamoto and Minamikawa 1999; Kato et al. 2003; Zakharov et al. 2004) that serve as precursors for the synthesis of new proteins and other nitrogen-containing compounds in seedlings (Zakharov et al. 2004). PhyCys have also been described as regulators of papain-like CPs due to their ability to inhibit endogenous proteolytic activity during seed germination and seedling growth. The reversible tight binding of PhyCys to papain-like CPs is one of the possible mechanisms by which they control the activity of these peptidases.
T-DNA insertion in the AtCYS6 subject to PCR genotyping using the following primer sets: AtCYS6 P1 primer (5′-GCGTTACCCAACTTAAT-3′) and T-DNA left border P2 primer (5′-TGGGAAAACCTG-3′) and T-DNA right border P3 primer (5′-TGGGAAAACCTG-3′). PCR amplicons were cloned into the vector pGEM-T Easy (Promega, Madison, WI, USA) and sequenced to confirm the fidelity of amplification. The sequence was then analyzed with the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.afirc.go.jp/PLACE) databases. The putative promoter sequence was digested from the pGEM-T Easy vector with HindIII/XbaI and subcloned into the same sites of pBI121 (Clontech, Palo Alto, CA, USA). The construct (PAtCYS6:GUS) was transformed into Agrobacterium tumefaciens strain GV3101 containing the plasmid pSoup vector (Hellens et al. 2000) by electroporation and then into A. thaliana Col-0 by the floral dip method (Clough and Bent 1998).

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh ecotype Columbia (Col-0) plants were grown in soil or in vitro on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 0.25% phyta-gel (pH 5.8), under 16 h of 100 μE m⁻² s⁻¹ light at 22°C. To induce synchronous germination, seeds were vernalized at 4°C for 3 days in the dark, as previously described (Lim et al. 2007).

A T-DNA insertional mutant line containing a single T-DNA insertion in the AtCYS6 gene was identified in the SALK T-DNA collection (SALK_027847). To identify mutants homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings and is responsive to the phytohormone ABA. Moreover, constitutive over-expression of AtCYS6 slowed germination and inhibited CP activity in transgenic Arabidopsis. Additionally, an Arabidopsis knock-out mutant (cys6-2) harboring a T-DNA insertion in the AtCYS6 gene initiated germination earlier than wild-type plants. These findings indicate that AtCYS6 plays a role in the control of seed germination and seedling growth through its ability to regulate CP activity.

Promoter–GUS fusion and histochemical analysis

For promoter analysis in transgenic plants, the putative AtCYS6 promoter sequence (~1,268 to +25 bp from the ATG translation start codon) was amplified by PCR from Arabidopsis genomic DNA using EX taq polymerase (Takara, Shiga, Japan) and the forward primer 5′-ACCAATCAAGGAACTTACGAATATTCCAG-3′ and the reverse primer 5′-ATTCTCACCTTATTTG7TGTT CGGTGCCTG-3′. PCR amplicons were cloned into the vector pGEM-T Easy (Promega, Madison, WI, USA) and sequenced to confirm the fidelity of amplification. The sequence was then analyzed with the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.afirc.go.jp/PLACE) databases. The putative promoter sequence was digested from the pGEM-T Easy vector with HindIII/XbaI and subcloned into the same sites of pBI121 (Clontech, Palo Alto, CA, USA). The construct (PAtCYS6:GUS) was transformed into Agrobacterium tumefaciens strain GV3101 containing the pSoup vector (Hellens et al. 2000) by electroporation and then into A. thaliana Col-0 by the floral dip method (Clough and Bent 1998).

Histochemical staining to detect GUS expression in PAtCYS6:GUS transgenic plants was performed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; Duchefa, Haarlem, The Netherlands) as a substrate (Jefferson et al. 1987). Tissue was harvested and immediately fixed for 30 min in ice-cold 90% acetone (Vanderbeld and Snedden 2007), rinsed with water, and incubated in GUS staining solution (100 mM sodium phosphate buffer [pH 7.0], 0.5 mM EDTA, 0.1% Triton X-100 and between 0 [high sensitivity] and 1 mM [standard sensitivity] potassium ferrocyanide and potassium ferricyanide). The histochemical reaction was performed in the dark at 37°C for 12 h. The plant materials were cleared by washing with several changes of 80% ethanol.

Western blot analysis

The AtCYS6 expression pattern was determined by western blot analysis using the anti-BrCYS1 polyclonal antibody (1:10,000 dilution) as previously described (Hong et al. 2007), followed by the addition of peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution) according to the manufacturer’s guidelines. Hybridization to protein bands was detected using the ECL Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK).

To confirm AtCYS6 expression during germination or post-germinative growth in response to germination-related phytohormones, dry seeds were placed into 15-cm Petri-dishes containing filter papers moistened with or without 10 μM ABA or GA₄⁺₇ (Sigma, St. Louis, MO, USA). Seeds were primed at 4°C for 3 days in the dark and incubated for 12 days under normal conditions (16 h of 100 μE m⁻² s⁻¹ light at 22°C). Samples were collected throughout this period (Kim et al. 2008), and western blot analysis was performed as described above.
Reverse transcription (RT)-PCR

For quantitative RT-PCR analysis, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 2 μg total RNA using SuperScript II RNase H-reverse transcriptase (Invitrogen). Each cDNA sample was diluted 1:10, and 1 μl of the diluted cDNA was used for PCR amplification with AtCYS6-specific primer sets (forward primer P1 and reverse primer P4, Fig. 4b). PCR amplification of Arabidopsis Actin2 (At3g18780) using gene-specific primers (forward primer: 5'-TCGGTGTTCCATTTGCT-3'; reverse primer: 5'-GCTTTTTAAGCCCTTTGATCTTGAGAG-3') was performed as a loading control (Lim et al. 2007).

Generation of AtCYS6 over-expressing plants and rescue of the cys6-2 allele

AtCYS6 (At3g12490) cDNA was synthesized using RNA extracted from Arabidopsis flowers. Two micrograms of total RNA was reverse transcribed in a 30 μl reaction volume with 1 μg oligo (dT)18 primer using MMLV RTase according to the manufacturer’s protocol (Toyobo, Osaka, Japan). The gene-specific primers P1 and P4 were used for amplification of AtCYS6 cDNA (Fig. 4b). A PCR product of the predicted size was cloned into pGEM-T Easy and confirmed by DNA sequencing. A 0.74 kb fragment of AtCYS6 cDNA was inserted in the sense orientation between the cauliflower mosaic virus (CaMV) 35S promoter (35S-P) and the nopaline synthase terminator (nos-ter) in pBI121 (Fig. 4a). Arabidopsis was transformed by the floral dip method, and homozygous T3 lines containing a single T-DNA insertion were used for analyses. To verify the presence of the transgenes and proteins in transformed lines and progeny, RT-PCR was performed using the P1 and P4 primer set, and protein bands were detected by western blotting as described above.

To complement the cys6-2 allele, a genomic fragment (Fig. 4b) of the AtCYS6 locus encoded by a 3,666 bp genomic PCR amplicon was cloned in the vector pCAMBIA1301 (http://www.cambia.org). This plasmid was transformed into a cys6-2 T-DNA insertional mutant using the floral dip method.

Germination assay and measurement of CP activity

To compare germination rates, transgenic and untransformed wild-type Arabidopsis seeds were harvested on the same day. Seeds were surface-sterilized and sown on 0.25% (w/v) phyta-gel plates containing half-strength MS medium (1/2MS, pH 5.8). The plates were placed at 4°C for 3 days and then moved to 22°C under a long day photoperiod, and germination was scored by microscopy based on radicle emergence. In each experiment, approximately 100 seeds were used, and triplicate experiments were carried out using independent seed lots (Tatematsu et al. 2008; Zheng et al. 2008). CP activity measurements were performed following the methods described in Hong et al. (2007).

Results

Sequence characterization of the AtCYS6 promoter region

An upstream region including the putative promoter sequence of the AtCYS6 gene was isolated by PCR of genomic DNA, and subsequent sequence analysis with the PLACE (Higo et al. 1999) and PlantCARE (Rombauts et al. 1999) databases revealed several motifs that regulate gene expression and are commonly found in most eukaryotic promoters (Fig. 1). Potential regulatory elements associated with hormone- and stress-related responses that are found in other plant promoters were identified within the AtCYS6 promoter. These include two copies of the ABA-responsive element (ABRE; PyACGTGGGC), which is involved in abscisic acid responsiveness (Washio 2003), and pyrimidine boxes (P-box; CTTTT), which are involved in gibberellin responsiveness (Huang et al. 1990). One copy of the MYB binding site (MBS; CAACCTG), involved in drought inducibility, together with two MYB recognition elements (MRE; AACCTAA) that mediate response to light (Feldbrügge et al. 1997) were also found in the AtCYS6 promoter sequence. The presence of these motifs indicates that AtCYS6 may be regulated by various cis-acting elements within the promoter as well as by corresponding trans-acting factors (Hong and Hwang 2009).

Spatial and temporal expression of AtCYS6 in Arabidopsis

To precisely define the spatio-temporal expression pattern of AtCYS6, we studied the expression levels of AtCYS6 protein by western blot analysis. As shown in Fig. 2a, AtCYS6 was detected in most plant tissues examined and was particularly prevalent in dry seeds, seedlings and flowers. In 5-day-old seedlings, AtCYS6 was more strongly expressed in root tips than in cotyledons. In mature plants grown in soil, AtCYS6 was barely detectable in rosettes and cauline leaves. These results are in agreement with the expression patterns of several other PhyCys that accumulate more extensively in seeds and seedlings than in fully grown vegetative tissues (Kondo et al. 1990; Abe et al. 1995; Lim et al. 1996; Kuroda et al. 2001; Hong et al. 2007).
To further explore this expression pattern, we characterized transgenic plants harboring a transcriptional fusion of the *GUS* reporter gene and the *AtCYS6* promoter (*P*<sub>*AtCYS6* :*GUS*). The 10 transgenic lines that were analyzed revealed similar patterns of *GUS* expression. These patterns were highly consistent among the transgenic lines, but minor variations in *GUS* staining intensity were observed among lines with different *P*<sub>*AtCYS6* :*GUS* constructs, suggesting mild positional effects of transgene insertion (Vanderbeld and Snedden 2007). *P*<sub>*AtCYS6* :*GUS* transgenic Arabidopsis seedlings displayed *GUS* activity in both cotyledons and root tips (Fig. 2b–c), which decreased dramatically in cotyledons as the seedlings matured and was difficult to detect in rosette and cauline leaves in fully grown plants (Fig. 2d–e), with the exception of root tips. *GUS* expression in the root tip was prominent in the root apical meristem and root cap (Fig. 2f). These results indicate that *AtCYS6* likely plays important roles in seedling growth and that its promoter possesses a complex regulatory mechanism.

After the emergence of the primary inflorescence, relatively weak levels of *GUS* expression were visible during the early stages of floral development, at stage 9 (Fig. 2g) and stage 12 (Fig. 2h). The growth stages of *Arabidopsis* have been carefully defined, which allows for an accurate sampling of materials for comparative analysis (Boyes et al. 2001). In open flowers (stage 15), *GUS* expression was strongly detected in pollen grains in the anthers (Fig. 2i). In weakly expressing transgenic lines, *GUS* expression in pollen grains was only slightly visible by stage 15 (Fig. 2j), but in more strongly expressing lines was easily visible in pollen grains until dehiscence occurred (Fig. 2k). The increased expression pattern of *GUS* in pollen grains in anthers during flower development suggests that the *AtCYS6* gene might be engaged in microsporogenesis (Charbonnel-Campaa et al. 2000). The observation that *P*<sub>*AtCYS6* :*GUS* was particularly abundant in the developing stigmatic papillae of immature siliques (Fig. 2l) suggests that *AtCYS6* also modulates the activity of CPs during silique development. The *GUS* staining patterns in transgenic Arabidopsis were similar to the expression patterns of the *AtCYS6* protein (Fig. 2a), and *GUS* expression analysis revealed more detailed expression patterns of *AtCYS6* in plant tissues than was possible to detect by western blotting. All of these results indicate that a 1,268 bp fragment of the *AtCYS6* promoter drives expression in seeds, seedlings, meristematic tissues in roots, pollen grains in anthers and stigmatic papillae in
young siliques. This finding means that the AtCYS6 promoter can modulate the precise transcriptional regulation of specific and developmental expression of AtCYS6 in the seedling, root, flower and siliques of transgenic Arabidopsis. Taken together, these distribution characteristics suggest that AtCYS6 is likely to have a variety of roles and functions in plants in response to complex developmental cues.

Expression of AtCYS6 during seed germination

The AtCYS6 promoter sequence contains several motifs identical or similar to many well-characterized motifs present in inducible promoters that respond to germination-related phytohormones (Fig. 1). The GUS reporter gene driven by the ArCYS6 promoter was expressed primarily in germinating seedlings, especially in organs of embryonic origin, such as cotyledons and primary root tips (Fig. 2). This suggests that regulation of the ArCYS6 promoter may be controlled by germination-related phytohormones, such as ABA and/or GAs. To test this hypothesis, we examined the expression levels of AtCYS6 during seed germination and seedling growth, either under standard growth conditions or following treatment with 10 μM ABA or GA$_{4+7}$, using the anti-BrCYS1 polyclonal antibody (Hong et al. 2007). AtCYS6 expression decreased within 3 days after imbibition on 1/2MS medium without any phytohormones (1/2MSO) or on 1/2MS medium containing GA$_{4+7}$ (1/2MSGA$_{4+7}$). Conversely, in response to ABA treatment (1/2MSABA), AtCYS6 expression strongly persisted throughout the sampling period and continued to be detected at day 12 (Fig. 3a).

Expression of P$_{AtCYS6}$:GUS was also examined under the same conditions to evaluate the effect of germination-related phytohormones on AtCYS6 expression (Fig. 3b). Like the expression patterns obtained by western blotting, GUS levels essentially disappeared after 7 days in 1/2MSO or 1/2MSGA$_{4+7}$ medium. In contrast, robust GUS expression was detected throughout the sampling period in 1/2MSABA medium. Interestingly, in response to treatment with ABA, GUS was strongly expressed in the apical hook, cotyledon and root tip of seedlings. Taken together,
these findings reveal that AtCYS6 expression is continuously maintained by ABA treatment during germination and seedling growth, suggesting that AtCYS6 may play an inhibitory role in these processes.

Over-expression and knock-out mutants in Arabidopsis

To define the in vivo function of AtCYS6 in seed germination and seedling growth, we transformed Arabidopsis seedlings with a vector carrying a fusion of the CaMV35S promoter and AtCYS6 cDNA (P35S:AtCYS6; Fig. 4a). From T1 plants, we selected 15 independent lines using selection medium containing kanamycin. PCR analysis revealed the presence of both AtCYS6 and nptII in each plant selected (data not shown). To determine the number of AtCYS6 copies in the transgenic plants, T1 plants were self-pollinated and the progeny (T2) were allowed to segregate on selection media. Following self-pollination of the T2 lines, three T3 homozygous lines (OX1–OX3) that contained a single T-DNA insertion were selected (Fig. 4c). The presence of AtCYS6 genomic DNA (1.44 kb) and cDNA sequences (0.74 kb) was verified by genomic DNA PCR using gene-specific primers. In addition, the in vivo function of AtCYS6 was analyzed using a T-DNA insertion mutant that disrupts the AtCYS6 locus. A T-DNA insertion of AtCYS6 was identified in the Salk collection (Columbia background; donor stock number SALK_027847) and was designated cys6-2 (Fig. 4b). The DNA sequence of the T-DNA flanking region of cys6-2 indicated that the insertion was in the third intron of AtCYS6. To confirm these data, genomic fragments adjacent to the left border of the T-DNA insertion were sequenced. Sequence analysis confirmed that the T-DNA insertion was located at nucleotide 969 of the AtCYS6 gene (numbering begins at the ATG start codon; Fig. 4b). To identify plants homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant cys6-2 seedlings and subjected to PCR analysis (Fig. 4d). Genomic PCR analysis detected a 1.44 kb AtCYS6 fragment in wild-type (WT) plants; however, PCR analysis using the T-DNA left border primer (P2) or right border primer (P3), did not detect an amplicon. For the cys6-2 mutant allele, PCR amplicons that encompass the T-DNA insertion site were not detected; however, when the P1 primer and the T-DNA left border primer (P2), or the P4 primer and right border primer (P3), were used, amplicons were evident.

Fourteen-day-old untransformed WT plants, the over-expression lines (OX1–OX3), the cys6-2 mutant line and a rescued cys6-2 line (CM) were selected for examination of AtCYS6 transcripts levels by RT-PCR (Fig. 4e). AtCYS6
cDNA was strongly detectable only in OX1–OX3 plants. Protein expression in seeds and in 14-day-old progeny of homozygous transgenic lines was also analyzed by immunoblotting (Fig. 4f). A single 22.4 kDa band corresponding to AtCYS6 was detected in WT, OX1–OX3 and CM seeds, but not in cys6-2 seeds. Both AtCYS6 transcripts and protein were undetectable in 14-day-old plants (Fig. 4e–f), which is in agreement with the finding that AtCYS6 accumulates more in seeds and seedlings than in mature plants. RT-PCR and western blot analysis revealed that the cys6-2 mutation resulted in the complete loss of AtCYS6 expression at the transcription (Fig. 4e) and translation levels (Fig. 4f), respectively. The 5 kb T-DNA insertion potentially disrupts splicing or affects the stability of the AtCYS6 transcript. Therefore, the cys6-2 mutant likely contains a null allele of AtCYS6.

Over-expression of AtCYS6 retards seed germination and seedling growth

We germinated seeds from OX2, cys6-2 and CM transgenic lines as well as from WT plants on 1/2MSO medium. As shown in Fig. 5a, OX2 seeds germinated at a slower rate than WT and CM seeds, whereas cys6-2 seeds germinated slightly faster. This raises the possibility that AtCYS6 may inhibit stored CP activity in seeds and during seedling growth, since transgenic plants that over-express AtCYS6 displayed retarded seed germination. Therefore, we measured and quantified endogenous CP activity in transgenic seeds and during seed germination (Fig. 5b). In OX2 seeds, endogenous CP activity was lower than in WT or cys6-2 seeds. Following germination, endogenous CP enzymatic activity increased slightly in OX2 seedlings at 8 h.
however, this enzymatic activity was lower than that of WT and cys6-2 seedlings. The lower CP enzymatic activity might be a result of the delayed germination of OX2 seeds due to the accumulation of AtCYS6. This suggests that over-expression of AtCYS6 caused the decrease in stored CP activity during germination.

As AtCYS6 over-expressing transgenic seedlings grew slowly, we sought to explore the function of AtCYS6 in post-germination growth. We compared seedling growth rates in transgenic plants and found that the primary root elongation pattern of OX2 plants was delayed compared to that of WT and CM plants, whereas cys6-2 roots grew slightly faster (Fig. 6a–b). The fresh weights of transgenic Arabidopsis seedlings paralleled root length patterns (Fig. 6c). These data indicate that the over-expression of AtCYS6 also retards seedling growth. This evidence strongly implicates a key role for AtCYS6 both in the regulation of stored CPs in vivo and in the control of seedling growth.

Discussion

Cysteine proteinase and PhyCys interactions have been repeatedly implicated in the control of germination and seedling growth (Shutov and Vaintraub 1987; Müntz 1996; Okamoto and Minamikawa 1999; Kato et al. 2003; Zakharov et al. 2004). In light of this, we considered the possibility that AtCYS6 might be regulated by germination-related phytohormones and play crucial roles in the regulation of germination and seedling growth. As an initial step toward understanding the regulatory mechanisms that control AtCYS6 gene expression, we analyzed the upstream promoter sequences of the AtCYS6 gene (Fig. 1). This analysis revealed that AtCYS6 contains several interesting putative cis-elements. Among them, we observed important cis-acting elements that are responsive to germination-related phytohormones and may direct seed germination and seedling growth, including ABREs and P-boxes. The ABRE motif and the P-box motif have been identified as binding sites for ABRE binding proteins (AREBs) (Lopez-Molina and Chua 2000) and Dof (DNA with one finger) transcription factors (Yanagisawa 2002), respectively. The distinct cis-elements identified in the AtCYS6 promoter sequences prompted us to analyze the 5′-upstream regions of AtCYS6 using transgenic Arabidopsis plants carrying a GUS fusion to the putative promoter region. Analysis of transgenic Arabidopsis carrying the AtCYS6 promoter–GUS construct revealed that AtCYS6 is expressed predominantly in seedling cotyledons and root meristematic tissues (Fig. 2). Additionally, the levels of AtCYS6 and GUS were rapidly decreased by treatment with GA_4 or water, whereas expression strongly persisted following ABA treatment (Fig. 3). These results concur with the findings of Martínez et al. (2003) who showed that ABA represses the steady-state levels of the cathepsin B-like CP gene (CatB) and induces PhyCys-encoding gene (Icy) expression. Thus, it is tempting to speculate that the two putative ABREs in the AtCYS6 promoter (Fig. 1) may be responsible for controlling AtCYS6 expression by ABA (Fig. 3). It is possible that AtCYS6 is regulated by transcriptional regulators that are activated in response to ABA signaling. A more detailed functional analysis is necessary to determine which ABRE is responsible for ABA regulation of the AtCYS6 promoter.

We further investigated the inhibitory activities of AtCYS6 during germination and seedling growth using AtCYS6 over-expressing (P_35S:AtCYS6) and knock-out

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**Fig. 5** Comparison of germination and post-germination growth in transgenic AtCYS6 plants. **a** Time course of germination for freshly harvested seeds of untransformed wild-type (WT), over-expression (OX2), and knock-out (cys6-2) plants, and rescued lines (CM). **b** Analysis of endogenous CP activity during seed germination and post-germination growth. Endogenous CP activity was measured in protein extracted from seeds and seedlings at different stages. Seeds used in each experiment originated from the same seed batch. Data are means ± S.E. from at least three independent experiments.
transgenic plants (Fig. 4). Transgenic Arabidopsis lines (T₃) that constitutively express AtCYS6 displayed weak growth inhibition in seedlings, whereas seedling growth was enhanced by suppression of AtCYS6 (Figs. 5, 6). Additionally, CP activity stored in seeds was inhibited by AtCYS6 in P₃S:AtCYS6 transgenic plants. These findings reconfirmed that persistent AtCYS6 expression modulates the activity of papain-like CPs in transgenic Arabidopsis seeds and seedlings. Several PhyCys with a molecular mass of ≈23 kDa have a C-terminal extension that has been shown to be involved in the inhibition of legumains (Zakharov et al. 2004). We observed that AtCYS6 (22.4 kDa) has an extended C-terminus and modulates the activity of commercial papaya latex papain. This suggests that AtCYS6 might inhibit not only the activity of papain-like CPs but also that of legumains, which are generally considered to be the major endopeptidases responsible for the degradation of seed storage proteins during early seedling growth (Zakharov et al. 2004).

The data from our study indicate that AtCYS6 plays a critical role not only in seed germination but also in seedling growth. It is possible that AtCYS6 also suppresses the action of endogenous papain-like CPs during germination and seedling growth. To confirm this, it will be necessary to define the interactions of various endogenous papain-like CPs with PhyCys in Arabidopsis. Further investigation of Arabidopsis PhyCy isoforms should improve our understanding of the biological roles and functions of PhyCys in seed germination and seedling growth.

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