Plant Cell Rep (2009) 28:1623–1632
DOI 10.1007/s00299-009-0762-7

ORIGINAL PAPER

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

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Received: 25 May 2009 / Revised: 27 July 2009 / Accepted: 3 August 2009 / Published online: 19 August 2009

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$_{4+7}$ (GA_{4+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

Communicated by J. R. Liu.

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Introduction

Phytocystatin (PhyCys) are potent inhibitors of cysteine proteinases (CPs) of the papain- (family C1A; MEROPS peptidase database, 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

(Corre-Menguy et al. 2002; Hong et al. 2007). Numerous studies have attempted to better understand how germination and post-germination are controlled by various hydrolases and inhibitors. However, very little is known about the processes by which the embryo emerges from the seed and the seedling completes growth (Koornneef et al. 2002).

In the present study, we describe the molecular characteristics of *Arabidopsis thaliana* PhyCys6 (AtCYS6), which has a molecular mass of 22.4 kDa and a C-terminal extension. Our results show that AtCYS6 accumulates in seeds and 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.

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 μEs^{-1} m^{-2} 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 subjected to PCR genotyping using the following *AtCYS6* primer sets: *AtCYS6* P1 primer (5′-ATACAGGGCAC CGAACCAAATAAAG-3′) and T-DNA left border P2 primer (5′-GTGATGGTTCACGTAGTGGGCCATCG-3′) or T-DNA right border P3 primer (5′-TGGGAAAACCTG GCGTTACCCAACTTAAT-3′) and *AtCYS6* P4 primer (5′-AGGGACTAGTCATGGTGTTGCTCCG-3′) (Fig. 4b).

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′-ATTCTCACCTTTATTTGGTT CGGTGCCCTG-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.affrc.go.jp/PLACE) databases. The putative promoter sequence was digested from the pGEM-T Easy vector with *Hin*dIII/*Xba*I and subcloned into the same sites of pBI121 (Clontech, Palo Alto, CA, USA). The construct (P_{AtCYS6}*: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 P_{AtCYS6}*: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 GA_{4+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 μEs^{-1} m^{-2} 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′-TCGGTGGTTCCATTCTTGCT-3′; reverse primer: 5′-GCTTTTTAAGCCTTTGATCTTGAGAG-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 T_3 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; PyACGTGGC), 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; CAACTG), 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).

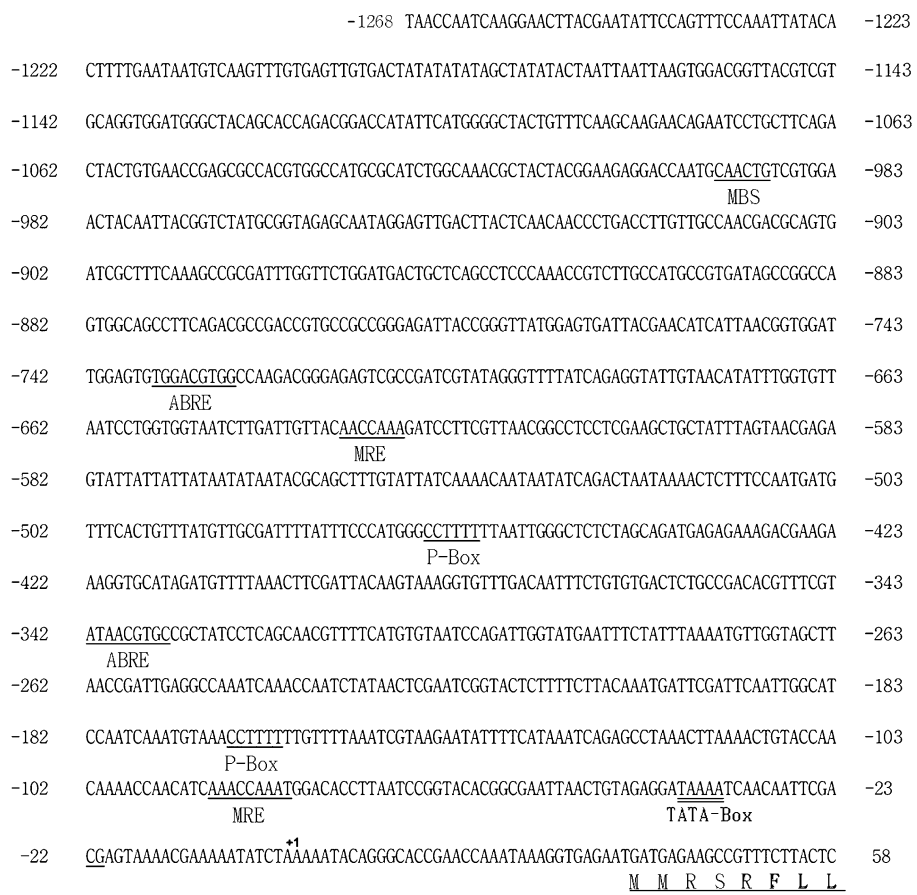

Fig. 1 The nucleotide sequence of the 5′-flanking promoter region and putative *cis*-acting elements of the *AtCYS6* gene. The sequences of the 5′-flanking region and the first exon of *AtCYS6* are shown together with a partial amino acid sequence specified by the 5′-end of the AtCYS6 coding region. The numbering of nucleotides relative to the putative transcription initiation site (+1) is shown above. The putative TATA box is identified by *a double underline*. The deduced amino acid sequences of the first exon are indicated by *single initial bold letter codes*. ABRE, ABA-responsive element; MBS, MYB binding site; MRE, MYB recognition element; P-box, pyrimidine box

To further explore this expression pattern, we characterized transgenic plants harboring a transcriptional fusion of the *GUS* reporter gene and the *AtCYS6* promoter (P_{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_{AtCYS6}:*GUS* constructs, suggesting mild positional effects of transgene insertion (Vanderbeld and Snedden 2007). P_{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_{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

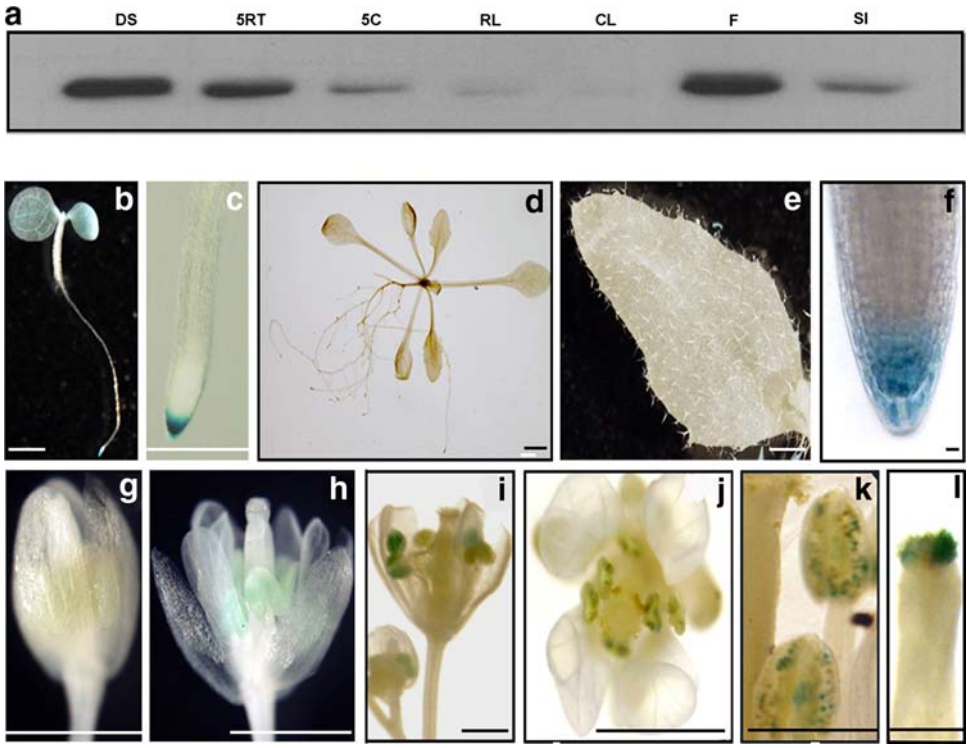

Fig. 2 Western blot analysis and histochemical localization of GUS activity in various organs of transgenic *Arabidopsis*. **a** Tissue-specific expression of AtCYS6 in different tissues. Total proteins from dry seeds (DS), root tips from 5-day-old seedlings (5RT), cotyledons from 5-day-old seedlings (5C), rosette leaves (RL), cauline leaves (CL), flowers (F) and siliques (SI) were isolated and subjected to western blot analysis. **b** Seedling grown on 1/2MSO medium at day 5. **c** Primary root from a 5-day-old seedling. **d** Eighteen-day-old transgenic plant grown on soil. **e** Cauline leaf from a 5-week-old transgenic plant. **f** Close-up of a primary root tip with predominant GUS expression in the meristematic tissues. **g** Floral bud (stage 9). **h** Young flower (stage 12). **i** Mature flower (stage 15) in a strongly expressing transgenic line. **j** Mature flower (stage 15) in a weakly expressing transgenic line. **k** Close-up of anthers with predominant GUS expression in pollen grains from a strongly expressing transgenic line. **l** Young silique. Bars are 1 mm in (**b**), (**d–e**), 500 μm in (**c**), (**g–k**), 10 μm in (**f**) and 200 μm in (**l**)

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 *AtCYS6* 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 *AtCYS6* 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,

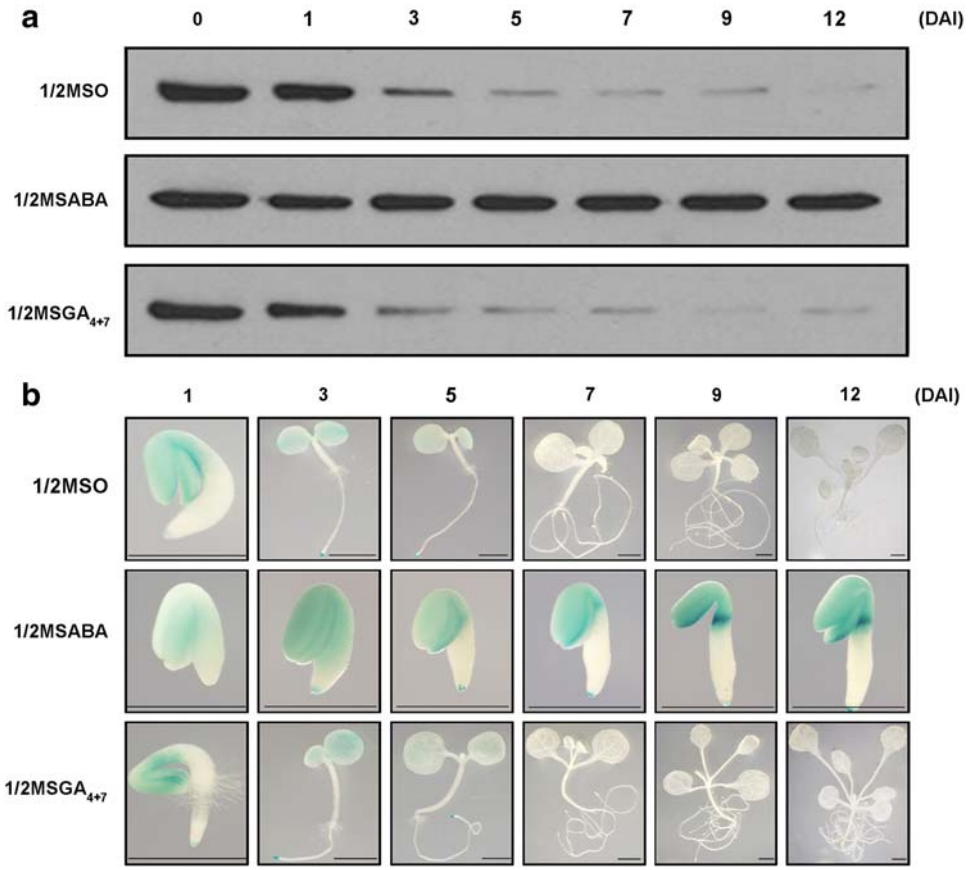

Fig. 3 AtCYS6 is expressed in seeds prior to germination and seedling growth. Western blot analysis (**a**) and P_{AtCYS6}:*GUS* expression (**b**) in response to phytohormones during germination and post-germination growth of *Arabidopsis*. Total proteins were prepared from control (1/2MSO), 10 μM ABA (1/2MSABA), or GA_{4+7} (1/2MSGA_{4+7}) -treated seeds at the indicated day after imbibition (DAI). *Bars* are 1 mm in (**b**)

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 (P_{35S}:*AtCYS6*; Fig. 4a). From T_1 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, T_1 plants were self-pollinated and the progeny (T_2) were allowed to segregate on selection media. Following self-pollination of the T_2 lines, three T_3 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 insertional 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*

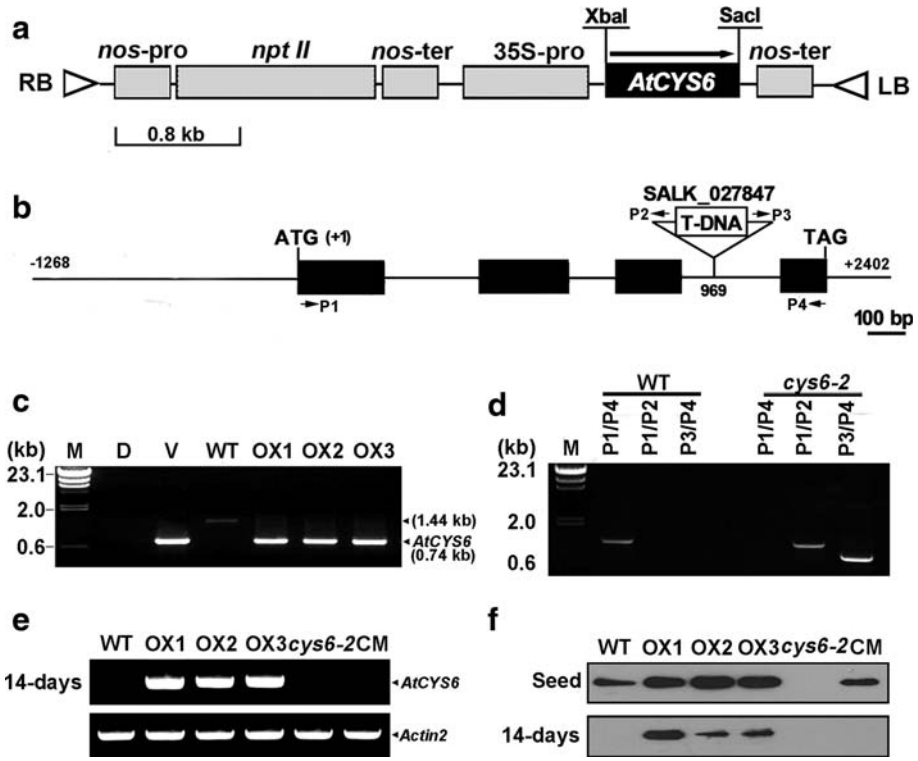

Fig. 4 The expression of *AtCYS6* in transgenic plants. **a** Structure of the P_{35S}*:AtCYS6* construct for *AtCYS6* cDNA expression. The gene encoding bacterial neomycin phosphotransferase II (*npt II*), which is regulated by the nopaline synthase gene promoter (*nos*-pro) and 3′-terminator (*nos*-ter), serves as a selectable marker for *Arabidopsis* transformation. *AtCYS6* is regulated by the *CaMV* 35S promoter (35S-pro). LB and RB indicate left and right T-DNA borders, respectively. **b** Schematic diagram of the *AtCYS6* gene and localization of the T-DNA insertion in the *cys6-2* allele (SALK_027847). Numbering begins at the ATG translation start codon (+1). Exons are represented by *black boxes*. The position of the T-DNA insertion is indicated in a bracket with respect to the ATG start codon. *Numbered arrows* show the positions and orientations of the P1 through P4 primers that were used for genotyping and PCR expression analysis. **c** PCR analysis of untransformed wild-type and transgenic *Arabidopsis* plants. The presence of *AtCYS6* was verified by genomic PCR using the P1 and P4 primers. *M* molecular mass markers; *D* double-distilled water as template; *V* P_{35S}*:AtCYS6* vector as template; *WT* untransformed wild-type plants; OX1–OX3, P_{35S}*:AtCYS6* lines no. 1–3. **d** Diagnostic PCR of the T-DNA inserted in the *AtCYS6* locus. Genomic DNA from WT plants and *cys6-2* homozygotes was used. PCR primers are indicated above each lane. **e** RT-PCR analysis of *AtCYS6* transcripts in 14-day-old WT, OX1–OX3, *cys6-2* and KO transgenic plants rescued with the *AtCYS6* gene (CM). The P1 and P4 primer set used for RT-PCR is shown in (**b**). The *Actin2* gene (At3g18780) served as a control. **f** Western blot analysis of AtCYS6 in seeds (*upper panel*) and 14-day-old progeny of transgenic plants (*lower panel*)

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;

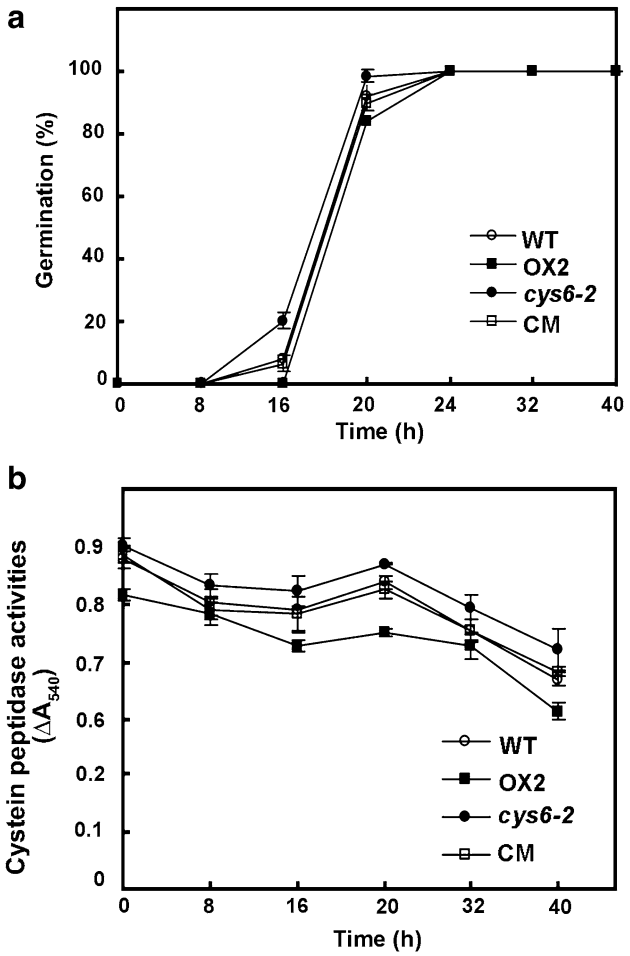

Fig. 5 Comparison of germination and post-germination growth in transgenic *AtCYS6* plants. **a** Time course of germination for freshly harvested seeds of untransformed wild-type (WT), over-expression (OX2), and knock-out (*cys6-2*) plants, and rescued lines (CM). **b** Analysis of endogenous CP activity 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

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+7} 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

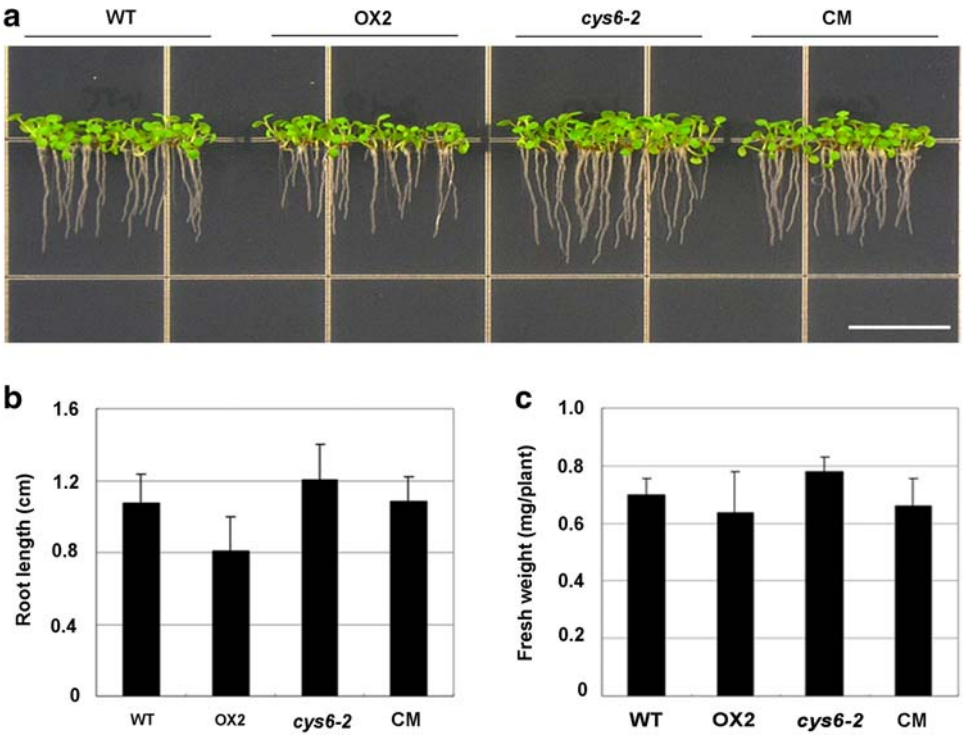

Fig. 6 Comparison of seedling growth in transgenic *AtCYS6* plants. **a** Phenotypes are shown for 5-day-old untransformed wild-type (WT), over-expression (OX2), and knock-out (*cys6-2*) plants, and rescued lines (CM). *Bar* is 1 cm. **b** Quantitative analysis of primary root lengths of 5-day-old seedlings from each transgenic line. **c** Fresh weights of 5-day-old WT and transgenic plant seedlings. The average fresh weight was estimated in three independent experiments using three to five of the 20 seedlings used in each experiment. Data are presented as the means ± SE. from at least three independent experiments

(*cys6-2*) transgenic plants (Fig. 4). Transgenic *Arabidopsis* lines (T_3) 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 P_{35S}*: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.

Acknowledgments This work was supported by the Environmental Biotechnology National Core Research Center Program (R15-2003-012-01001-0). J.E. Hwang and J.H. Je were supported by a BK21 fellowship from the MEST, Republic of Korea.

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