

Distinct expression patterns of two *Arabidopsis* phytolectatin genes, *AtCYS1* and *AtCYS2*, during development and abiotic stresses

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Abstract The phytolectatins of plants are members of the cystatin superfamily of proteins, which are potent inhibitors of cysteine proteases. The *Arabidopsis* genome encodes seven phytolectatin isoforms (*AtCYSs*) in two distantly related *AtCYS* gene clusters. We selected *AtCYS1* and *AtCYS2* as representatives for each cluster and then generated transgenic plants expressing the *GUS* reporter gene under the control of each gene promoter. These plants were used to examine *AtCYS* expression at various stages of plant development and in response to abiotic stresses. Histochemical analysis of *AtCYS1* promoter- and *AtCYS2* promoter-*GUS* transgenic plants revealed that these genes have similar but distinct spatial and temporal expression patterns during normal development. In particular, *AtCYS1* was preferentially expressed in the vascular tissue of all organs, whereas *AtCYS2* was expressed in trichomes and guard cells in young leaves, caps of roots, and in connecting regions of the immature anthers and filaments and the style and stigma in flowers. In addition, each *AtCYS*

gene has a unique expression profile during abiotic stresses. High temperature and wounding stress enhanced the expression of both *AtCYS1* and *AtCYS2*, but the temporal and spatial patterns of induction differed. From these data, we propose that these two *AtCYS* genes play important, but distinct, roles in plant development and stress responses.

Keywords Abiotic stress · Cysteine protease inhibitor · Gene expression · *GUS* staining · Promoter · Transgenic plant

Introduction

Cystatins are a group of proteins that inhibit cysteine (*Cys*) proteases and are widespread in eukaryotes (Turk and Bode 1991; Margis et al. 1998). Plant cystatins are called phytolectatins (*PhyCYSs*) and form an independent subfamily in the cystatin superfamily (Margis et al. 1998). Several *PhyCYSs* have been well characterized and take part in a variety of physiological processes, including programmed cell death (Belenghi et al. 2003), fruit development (Ryan et al. 1998; Neuteboom et al. 2009), and seed development and germination (Abe et al. 1987; Bolter 1993; Misaka et al. 1996; Gaddour et al. 2001; Hong et al. 2007; Hwang et al. 2009). Most studies have focused on the roles played by *PhyCYS* proteins in defense mechanisms against pathogens and insect attack (Koiwa et al. 1997; Delledonne et al. 2001; Soares-Costa et al. 2002; Urwin et al. 2003; Outchkourov et al. 2004; Yang and Yeh 2005; Christova et al. 2006; Wang et al. 2008). Recently, studies have also suggested that *PhyCYSs* are involved in responding to abiotic environmental stresses (Gaddour et al. 2001; Diop et al. 2004; Zhang et al. 2008). The expression levels of soybean *PhyCYS* change during wounding

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(Botella et al. 1996), and a chestnut *PhyCYS* is strongly induced in the roots and leaves of plantlets subjected to cold and salt stress, and in the roots after heat stress (Pernas et al. 2000). A cDNA from developing barley endosperm encoding the *PhyCYS* Hv-CPI (gene *Icy*) has been reported that its expression in vegetative tissues is affected by cold and anaerobiosis (Gaddour et al. 2001), and its expression in aleurone layers is regulated by two DOF transcription factors (Martínez et al. 2005a). Corn *PhyCYS* genes are rapidly expressed in response to cold stress and drought (Massonneau et al. 2005). In *Arabidopsis*, seven *PhyCYS* genes (*AtCYS1–AtCYS7*) have been identified (Martínez et al. 2005b). The expression of *AtCYSa* and *AtCYSb* (synonymous with *AtCYS3* and *AtCYS6*, respectively) increases resistance to salt and drought stresses, as well as oxidative and cold stresses (Zhang et al. 2008).

Despite the important roles played by *PhyCYS* proteins in biotic and abiotic stress responses, tissue-specific *PhyCYS* gene expression patterns or regulation with respect to developmental and environmental cues have not been determined. We examined these facets of two *Arabidopsis PhyCYS* genes, *AtCYS1* and *AtCYS2*, using β -glucuronidase (GUS; *uid* gene) expression analysis. Cell- and tissue-specific expression driven by the *AtCYS1* and *AtCYS2* promoters was monitored at several developmental stages and in response to different abiotic stresses. Our study has determined the specific expression patterns of *AtCYS1* and *AtCYS2* and establishes a framework for further examination of the physiological roles played by these proteins.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana L. Heynh. ecotype Columbia (Col-0) plants were grown in soil or MS medium (Murashige and Skoog 1962) containing 3% sucrose and 0.25% phyta-gel (pH 5.8), under long-day conditions (16 h of 100 $\mu\text{E s}^{-1}\text{ m}^{-2}$ light and 8 h darkness) at 22°C. To induce synchronous germination, seeds were incubated at 4°C for 3 days in the dark, and then transferred to a growth chamber, as previously described (Lim et al. 2007).

Generation of transgenic *Arabidopsis*

DNA fragments encompassing the promoter regions of the *PhyCYS* genes *AtCYS1* (At5g12140; –1381 to +30 relative to the ATG translation start codon) and *AtCYS2* (At2g31980; –1392 to +30) were PCR-amplified from *Arabidopsis* genomic DNA using the following primers: *AtCYS1* promoter forward (5'-GAA TTC GAG CAA CTG CAA GCT GAG AG-3'), *AtCYS1* promoter reverse (5'-GAT

CCG ACG ATT GTT CCT GCT TGT TG-3'); *AtCYS2* promoter forward (5'-GAA TTC GAG ACT CTT ACG CTT AGG G-3'), and *AtCYS2* promoter reverse (5'-GGA TCC TAC AAG AGA GAC CTT CAA CAT GG-3'). The PCR products were cloned into pMD18-T (Takara, Tokyo, Japan) using the TA overhang, and the integrity of the constructs was verified by sequencing. Cloned DNA was digested with *EcoRI* and *BamHI*, and the fragments were then inserted into the pCAMBIA1381-GUS plasmid (<http://www.cambia.org.au>). Each construct was designated *AtCYS1P::GUS* for *AtCYS1* promoter and *AtCYS2P::GUS* for *AtCYS2* promoter. Recombinant plasmids were introduced into *Agrobacterium tumefaciens* GV3101 and then transferred into *Arabidopsis* plants using the floral dip method (Clough and Bent 1998). Homozygous T₃ lines containing a single T-DNA insertion were used for the analyses, and transgenic plants were maintained under the previously described long-day conditions.

Histochemical GUS assays

Histochemical localization of GUS activity was performed as described by Jefferson et al. (1987). Briefly, wild-type or transgenic *Arabidopsis* seedlings, organs, and tissues were vacuum-infiltrated in 50 mM sodium phosphate buffer (pH 7.0), 2 mM potassium ferrocyanide (Sigma, St. Louis, MO, USA), 2 mM potassium ferricyanide (Sigma), and 0.2% Triton X-100 (Sigma) containing 1 mM X-GlcA (Duchefa, Haarlem, The Netherlands). The samples were incubated in the dark at 37°C for 12 h and, subsequently, transferred to 70% ethanol to remove the chlorophylls. Digital images were obtained using an Olympus SZX12 stereoscope (Olympus, Tokyo, Japan). GUS staining data are the representatives of at least ten independent transgenic lines for each construct.

Stress treatments for RT-PCR analysis

Arabidopsis plants grown on MS medium at 22°C for 10 days were subjected to various abiotic stresses. Plants were exposed to air (22°C) on filter paper for rapid induction of drought conditions, or placed in a 4 or 37°C chamber in the dark (EYELA, Tokyo, Japan) for thermal stress induction. Mechanical wounding was performed by punching holes in rosette leaves and then incubating the plants in a dark chamber at 22°C. Materials were collected at 0, 1, 3, 6, 12, 24, or 48 h after treatment. Harvested plants were immediately frozen in liquid nitrogen and stored at –80°C for RNA extraction.

Total RNA was extracted from 100 mg whole plant tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from 2 μg total RNA using the Revert AidTM M-MuLV

Reverse Transcriptase (Fermentas, Glen Burnie, MD, USA). Each cDNA sample was diluted tenfold, and 1 μ l of the diluted cDNA was used for PCR amplification with gene-specific primer sets (*AtCYS1* forward, 5'-TCT AGA ATG GCG GAT CAA CAA GCA GG-3', *AtCYS1* reverse, 5'-GGA TCC TTA AAC ATC GTG AAG GTG GTT G-3'; *AtCYS2* forward, 5'-TCT AGA ATG GCT ACC ATG TTG AAG GTC-3', *AtCYS2* reverse, 5'-GGT ACC TTA GTA GAC AGG ACT GAC AAC AGG-3'). To minimize inherent RT-PCR errors, each comparison was performed at least three times, using independently isolated RNA samples. The resulting PCR products were sequenced and analyzed by electrophoresis.

Heat and wounding stress treatments of transgenic plants

Heat and wounding stress treatments of transgenic plants carrying an *AtCYS*::*GUS* construct were conducted using plants grown on plates for 10 days. At least three independent transgenic T₃ lines of *AtCYS1P*::*GUS* and *AtCYS2P*::*GUS* were analyzed. For heat stress treatments, plants were placed in a temperature-controlled chamber (EYELA) at 37°C for 48 h in the dark. Wounding experiments were conducted using ticket punches to punch a single hole in each of the rosette leaves. Plants were then placed in a growth chamber in the dark at 22°C. Plant materials were collected 48 h after treatment.

For transverse sections of rosette leaf tissues, GUS-stained samples were dehydrated using a graded ethanol series, stained with 0.1% Eosin Y (Sigma) followed by incubation in a *tert*-butanol series, and then embedded in paraffin. Embedded tissue sections (12 μ m thick) were fixed on poly-lysine-treated slides (Polysciences, PA,

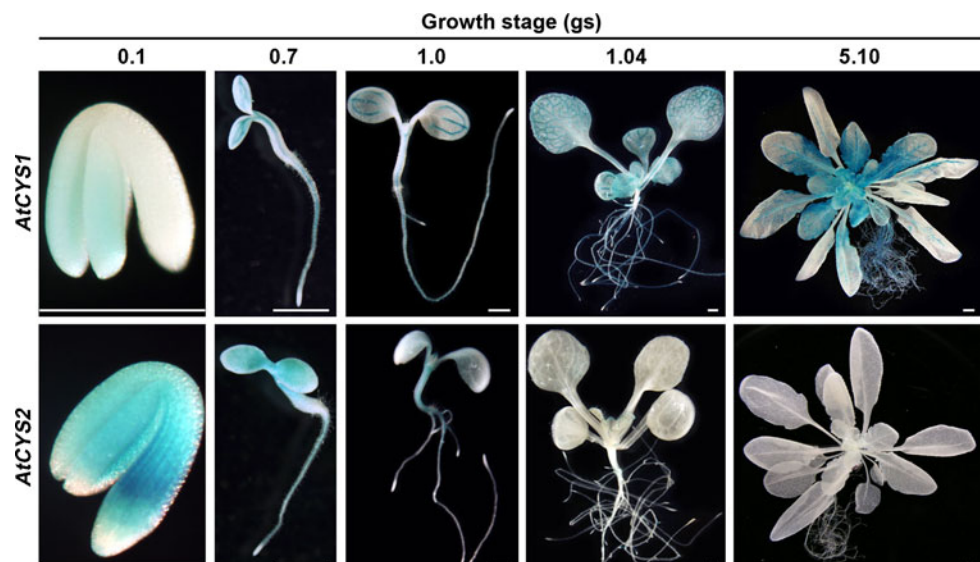
USA) at 42°C overnight. Slides were deparaffinized using a clearing agent (Amresco, OH, USA; Yang et al. 2006) and digital images were collected using an Olympus AX-70 microscope (Olympus).

Results

Expression of *AtCYS1* and *AtCYS2* during plant growth

To monitor the activity of *AtCYS* promoters during plant development, T₃ *AtCYS1P*::*GUS* and *AtCYS2P*::*GUS* transgenic plants were grown to various stages and then subjected to histochemical staining for GUS activity. In the hypogeal seedling stage [growth stage (gs) 0.1; the gs numbers follow the descriptions of Boyes et al. (2001)], *AtCYS1* expression was observed in the cotyledons, whereas *AtCYS2* was expressed in the whole seedling (Fig. 1). In epigeal seedlings (gs 0.7–1.0), *AtCYS1* expression was restricted to the vasculature, whereas *AtCYS2* was expressed in the non-vascular tissue of the cotyledon, hypocotyl and root (gs 0.7). *AtCYS2* expression in the cotyledons decreased dramatically as the seedlings matured (gs 1.0). In fully grown plants, *AtCYS1* was continuously expressed in the rosette leaves and root vasculature, but *AtCYS2* was almost undetectable in whole plants (gs 1.04). *AtCYS2* was detected only in unextended young leaves and some root tips. When the transgenic plants started to bolt (gs 5.10), GUS activity was observed in both leaves and roots in *AtCYS1P*::*GUS* transgenic plants, but GUS activity was almost undetectable in *AtCYS2P*::*GUS* plants. These GUS activity assays clearly show that *AtCYS1* expression was low in the seedling stages, but strong in the mature plants, especially in the vasculature. In

Fig. 1 Developmentally regulated GUS expression in transgenic *Arabidopsis* plants carrying the GUS coding region fused to the *AtCYS1* (*AtCYS1P*::*GUS*) or *AtCYS2* (*AtCYS2P*::*GUS*) promoter. Histochemical assays show *AtCYS1P*::*GUS* and *AtCYS2P*::*GUS* expression patterns in representative GUS expression from 1- (gs 0.1), 3- (gs 0.7), 5- (gs 1.0), 10- (gs 1.04), and 28- (gs 5.10) day-old plants grown under normal growth conditions. Scale bars are 500 μ m in gs 0.1, 0.7, 1.0, 1.04, and 5 mm in gs 5.10



contrast, *AtCYS2* was expressed in the seedling stages, but expression was almost absent in the mature plant stages.

Expression of *AtCYS1* and *AtCYS2* in leaves and roots

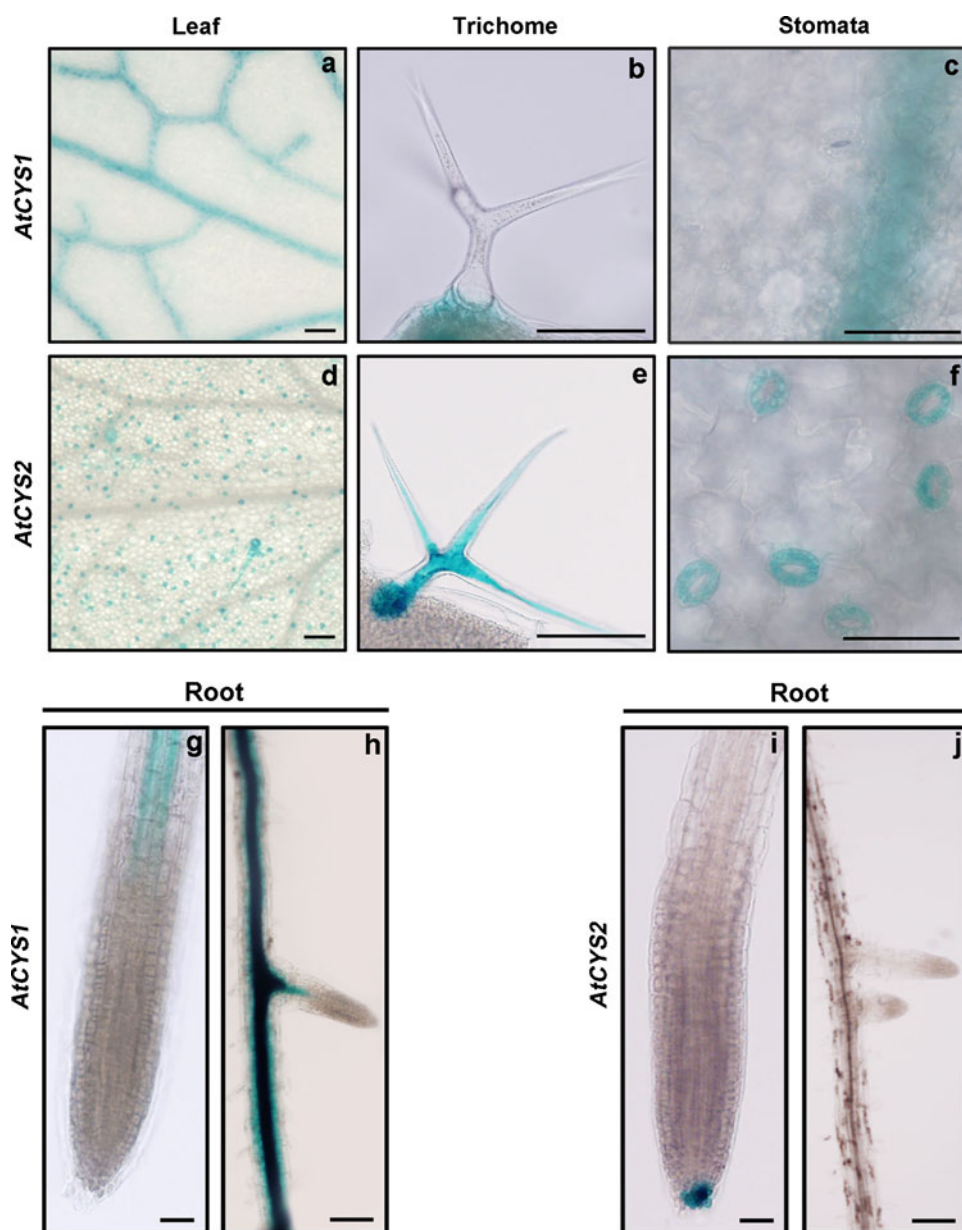
To explore the tissue-specific expression driven by the *AtCYS1* and *AtCYS2* promoters, we examined GUS expression in the leaves and roots of *gs 1.04* transgenic plants (Fig. 2). In the young rosette leaves of plants, strong expression of *AtCYS1* was detected in the vascular tissues and the trichome basal cells, but not in stomata (Fig. 2a–c), whereas *AtCYS2* was expressed in the single-celled leaf trichomes and the guard cells (Fig. 2d–f). The expression patterns of *AtCYS1* and *AtCYS2* in the roots also differed.

AtCYS1 expression was observed in the vascular tissue of the primary and lateral roots (Fig. 2g, h), but *AtCYS2* expression was restricted to the root cap of the primary root (Fig. 2i, j). These observations indicate that the two *AtCYS* gene promoters are responsible for the precise transcriptional regulation that determines the tissue-specific and developmental-stage-specific expression of the two *AtCYS* genes in the leaves and roots of the transgenic *Arabidopsis* (Hwang et al. 2009).

Expression of *AtCYS1* and *AtCYS2* during flowering

Sequential expression of the *AtCYS1* and *AtCYS2* promoters was observed during flower development (Fig. 3).

Fig. 2 Histochemical localization of *AtCYS1P::GUS* and *AtCYS2P::GUS* expression in 10-day-old (*gs 1.04*) plants. **a–f** Representative expression patterns in leaf tissues. *AtCYS1P::GUS* and *AtCYS2P::GUS* expression in the vascular tissues (**a, d**), the trichomes (**b, e**) and the stomata (**c, f**). Scale bars are 50 μ m. **g–j** Representative expression patterns in root tissues. *AtCYS1P::GUS* expression in the vascular tissue of the primary (**g**) and the lateral roots (**h**). The *AtCYS2P::GUS* expression in the root caps of the primary roots (**i, j**). Scale bars are 10 μ m in **g** and **i**, 50 μ m in **h** and **j**



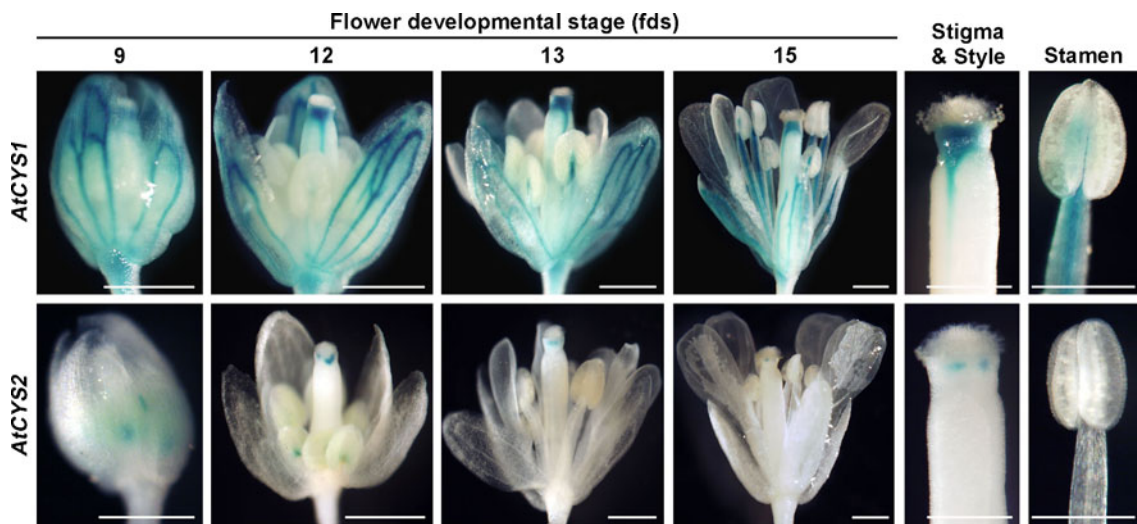


Fig. 3 Histochemical localization of *AtCYS1P::GUS* and *AtCYS2P::GUS* expression in flowers. The numbers indicate flower development stages (fds). Bars are 500 μm in the main panels and 200 μm in the enlarged images of the carpel and stamen

AtCYS1 was expressed at all flower developmental stages in the vascular tissues of the anther filaments and the sepal, as well as in the regions connecting the stigma and style. *AtCYS2* expression was weaker overall than *AtCYS1* expression and *AtCYS2P::GUS* activity was observed in the regions connecting the immature anthers and filaments and the stigma and style at flower development stages (fds) 9–12. After fds 12, *AtCYS2* expression declined and was almost undetectable by fds 15. These differences in *AtCYS1* and *AtCYS2* expression in the floral organs indicate that the *AtCYS1* and *AtCYS2* promoters control both the developmental and tissue specificity of expression.

Semi-quantitative RT-PCR analysis of *AtCYS1* and *AtCYS2* expression by abiotic stresses

To examine the expression of the *AtCYS1* and *AtCYS2* genes in relation to abiotic stress, *Arabidopsis* plants (gs 1.04) were subjected to cold, drought, heat, or wounding stress under dark conditions. By 48 h after the stress treatment, the *AtCYS1* expression had increased following cold, drought, heat, and wounding stress. *AtCYS2* expression was also induced by drought, heat, and wounding stress, but not by cold stress (Fig. 4a). Additionally, we examined the expression patterns of each gene at various times after heat stress or wounding. As shown in Fig. 4b,

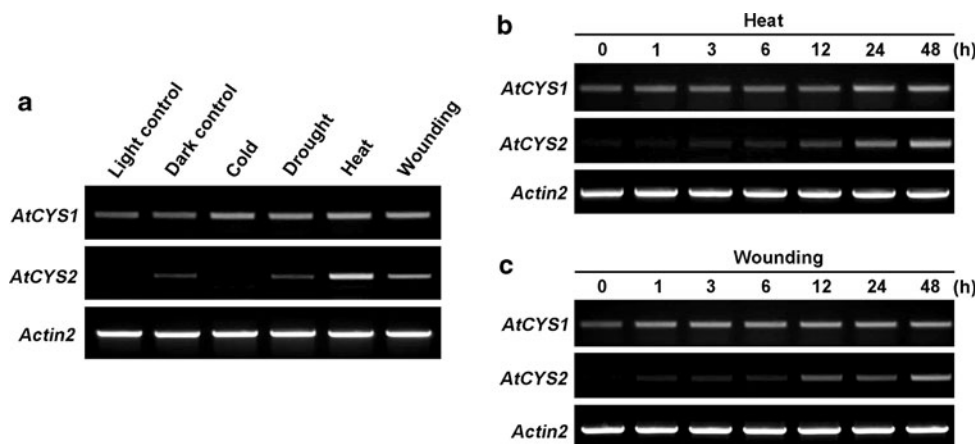


Fig. 4 Expression patterns of *AtCYS1* and *AtCYS2* in response to abiotic stresses. Semi-quantitative RT-PCR was performed on total RNA using gene-specific primer sets. **a** Transcript abundance was determined after 10-day-old plants were exposed for 48 h to darkness, heat (37°C), cold (4°C), drought, or wounding. **b** Transcript levels of

AtCYS1 and *AtCYS2* genes in response to heat stress (37°C) for the indicated times. **c** Effects of wounding on *AtCYS1* and *AtCYS2* transcript accumulation. Total RNA was isolated from whole plants at the indicated time points. The *Arabidopsis Actin2* gene (At3g18780) served as a loading control

AtCYS1 expression started to increase by 1 h after heat stress treatment and reached a maximum at 24 h. However, *AtCYS2* expression started to increase 3 h after heat stress treatment and then gradually increased for 12–48 h. To explore the expression of these genes after a wounding stress, hole was punched in the rosette leaves of plants (gs 1.04) and the plants were then incubated in the dark at 22°C. RNA was collected at various times from whole plants as in the heat stress experiments. As shown in Fig. 4c, *AtCYS1* and *AtCYS2* were both expressed in response to wounding and exhibited expression patterns similar to the heat stress treatments.

Computational analysis of the *AtCYS1* and *AtCYS2* promoter sequences

Generally, stress-responsive *cis*-acting elements are found in the promoter regions of stress-inducible genes. Since *AtCYS1* and *AtCYS2* were induced by abiotic stresses (Fig. 4), we performed a motif search using PLACE (Higo et al. 1999; <http://www.dna.affrc.go.jp/PLACE/>) and PlantCARE (Lescot et al. 2002; <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) to identify putative *cis*-elements in the promoter regions of *AtCYS1* and *AtCYS2*. We identified potential regulatory elements associated with stress-related transcription factor-binding sites including ABA-response elements (ABREs), CCAAT-boxes, heat shock elements (HSEs), stress response elements (STREs) and W-boxes (Fig. 5). The promoter regions of *AtCYS1* (−242 to −233) and *AtCYS2* (−428 to −420 and −356 to −348) both contain HSEs (Amin et al. 1988; Xiao and Lis 1988). The *AtCYS1* promoter contains two perfect CCAAT sequences (−650 to −646 and −607 to −603), while five CCAAT-box sequences were identified in the *AtCYS2* promoter (−1293 to −1289, −997 to −993, −524 to −520, −272 to −268, and −143 to −139). These CCAAT enhancer sequences represent binding sites for CCAAT enhancer binding proteins (C/EBP) and act co-operatively with HSEs to increase promoter activation under heat stress conditions (Rieping and Schöffl 1992). STRE elements are important for transcriptional activation in response to a variety of stress conditions and, in particular, heat stress (Siderius and Mager 1997). Two STREs (consensus sequence AGGGG) were identified in the *AtCYS2* promoter (−910 to −906 and −903 to −899). The W-box (consensus sequence TTGAC) binds WRKY factors and is responsive to heat and wounding (Ülker and Somssich 2004; Levée et al. 2009; Li et al. 2009). Multiple copies of this element were found in both the *AtCYS1* (−1285 to −1282, −1105 to −1102, −779 to −776, −463 to −460, −433 to −430, and −68 to −65) and *AtCYS2* (−1341 to 1338, −1303 to −1229, −697 to −693, −550 to 546, and −315 to 311) promoters. Since the promoter

regions of these genes contain many *cis*-elements correlated with responses to heat and wounding stress, the expression of *AtCYS1* and *AtCYS2* may be controlled by complex regulatory mechanisms that respond to heat and wounding stresses.

AtCYS1P::GUS and *AtCYS2P::GUS* expression in response to heat stress

To precisely define the spatio-temporal expression pattern of *AtCYS1* and *AtCYS2* under heat stress, we examined the pattern of GUS expression in *AtCYS1P::GUS* and *AtCYS2P::GUS* transgenic plants (gs 1.04). As shown in Fig. 6a, heat stress triggers a strong increase in *AtCYS1* and *AtCYS2* expression in several tissues. However, the pattern of induction differs in roots and leaves between *AtCYS1* and *AtCYS2* (Fig. 6b, c). In the roots, the expression of *AtCYS1* was induced in the vascular bundles, whereas *AtCYS2* was detected in the elongation and maturation zone (Fig. 6b). In the lateral roots, *AtCYS2* expression increased in the elongation and maturation zones, similar to the primary root, but it was not expressed in the root caps of the lateral roots under heat stress. These data suggest that *AtCYS2* may modulate the elongation and maturation of roots during heat stress.

Expression of both *AtCYS1* and *AtCYS2* was induced in the leaf blade in response to heat stress, with the strongest increase in young leaves in *AtCYS2P::GUS* expressing plants (Fig. 6c). Transverse sections using the same stage (gs 1.04) leaves used in Fig. 6a were used to examine the expression patterns in detail. As shown in Fig. 6d, *AtCYS1* and *AtCYS2* expression was consistent with the expression patterns shown in Fig. 6c. Interestingly, *AtCYS2* expression was induced in the leaf blade and vascular bundles, where it is not expressed under normal growth conditions. Taken together, the comparisons of *AtCYS1* and *AtCYS2* expression in response to heat stress have revealed not only differences in expression pattern, but also common expression in some cells and tissues.

AtCYS1P::GUS and *AtCYS2P::GUS* expression in response to wounding stress

AtCYS1 and *AtCYS2* transcripts levels increase in response to wounding stress (Fig. 4a, c), and these data are consistent with previous findings (Belenghi et al. 2003), suggesting that the expression of *AtCYS* mediates the wounding defense response. In this study, to compare the spatial expression pattern of *AtCYS1* and *AtCYS2* in response to wounding stress, leaves from *AtCYS1P::GUS* or *AtCYS2P::GUS* transgenic plants (gs 1.04) were mechanically wounded with ticket punches. GUS staining patterns were analyzed 48 h after wounding (Fig. 7).

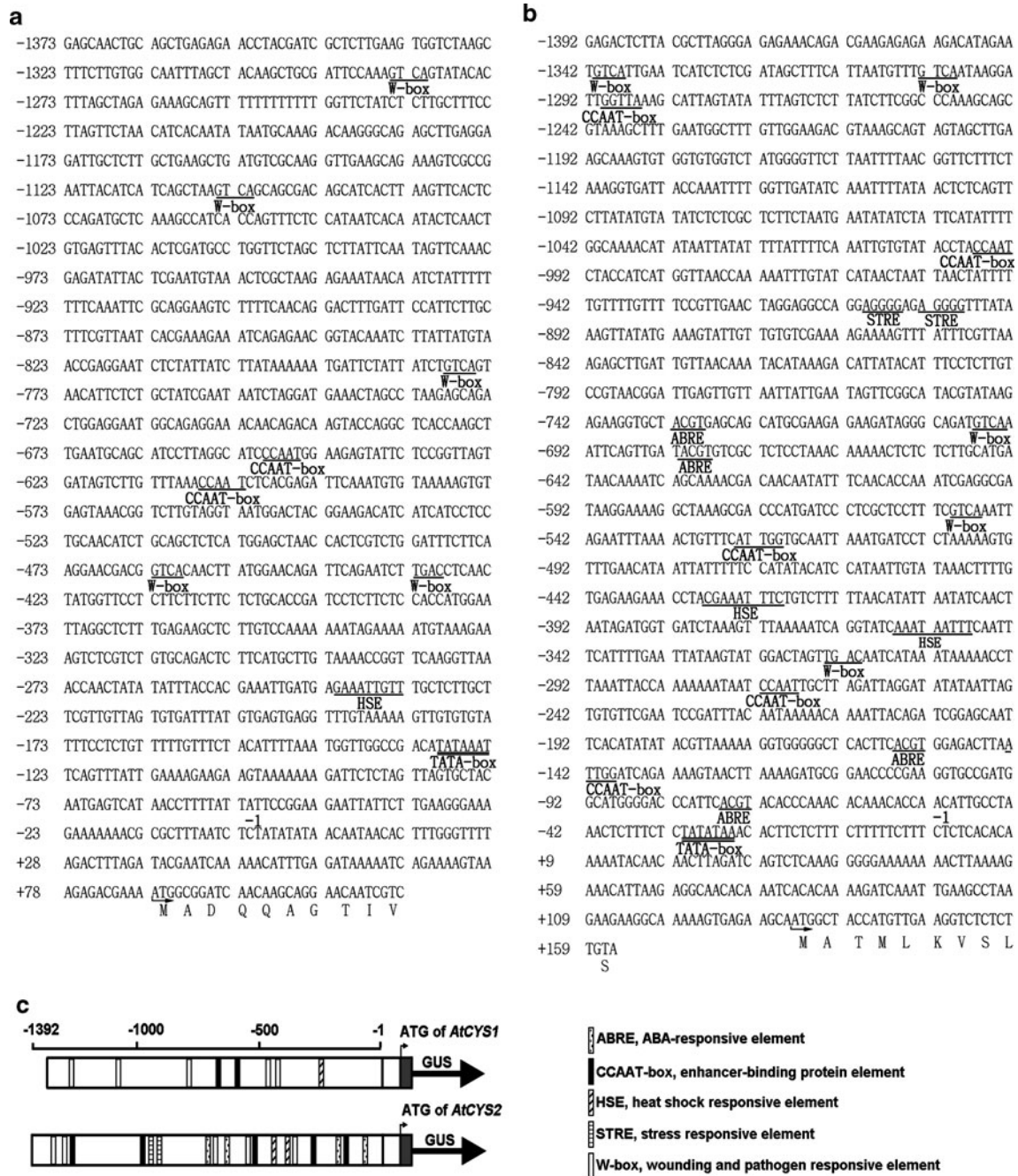


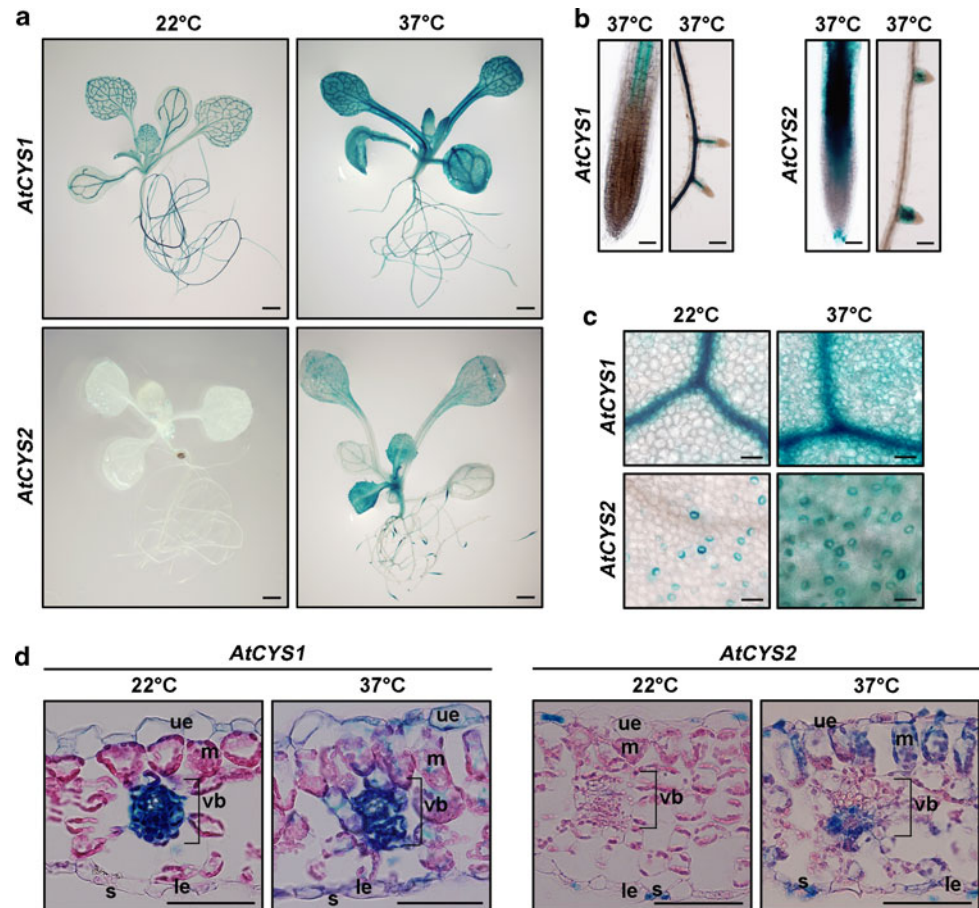
Fig. 5 Sequences and structural features of the *AtCYS1* and *AtCYS2* promoters. The sequences of the 5'-flanking region of *AtCYS1* (a) and *AtCYS2* (b) are shown together with a partial amino acid sequence. The numbering of nucleotides relative to the putative transcriptional initiation site (-1) is shown above the sequences. The putative TATA box is identified by *double underlined*. The deduced amino acid

Increased GUS activity was detected around the wound sites in both *AtCYS1P::GUS* and *AtCYS2P::GUS* transgenic plants (Fig. 7a), but GUS staining was stronger in *AtCYS1* transgenic plants. Transverse sections were also cut using the same leaves as in Fig. 7a to examine the expression patterns in detail. As shown in Fig. 7b, *AtCYS1*

sequences of the first exon are indicated by *single letter codes in bold*. Putative *cis*-regulatory promoter sequences are *underlined*. c Schematic representation of the *AtCYS1* and *AtCYS2* promoters. The locations of abiotic responsive *cis*-elements of interest were identified using the PLACE and PlantCARE databases. The *symbols* of each *cis*-element are annotated below

expression was greater in the vascular bundles around the wound and *AtCYS2* increased in the lamina and vasculature surrounding the wound. These findings indicate that *AtCYS1* and *AtCYS2* mediate the wounding defense response in different cells and tissues surrounding the wounded region.

Fig. 6 Elevation of GUS expression in transgenic plants following heat stress. **a** GUS expression after 10-day-old plants was subjected to heat treatment for 48 h at 37°C. Scale bars are 1 mm. **b, c** GUS activity in the roots (scale bars 15 µm) and leaves (scale bars 12.5 µm). **d** Transverse sections of leaves from heat-treated seedlings. Scale bars are 50 µm. The abbreviations indicate upper epidermis (*ue*), mesophyll cells (*m*), vascular bundle (*vb*), stoma (*s*), and lower epidermis (*le*)



Discussion

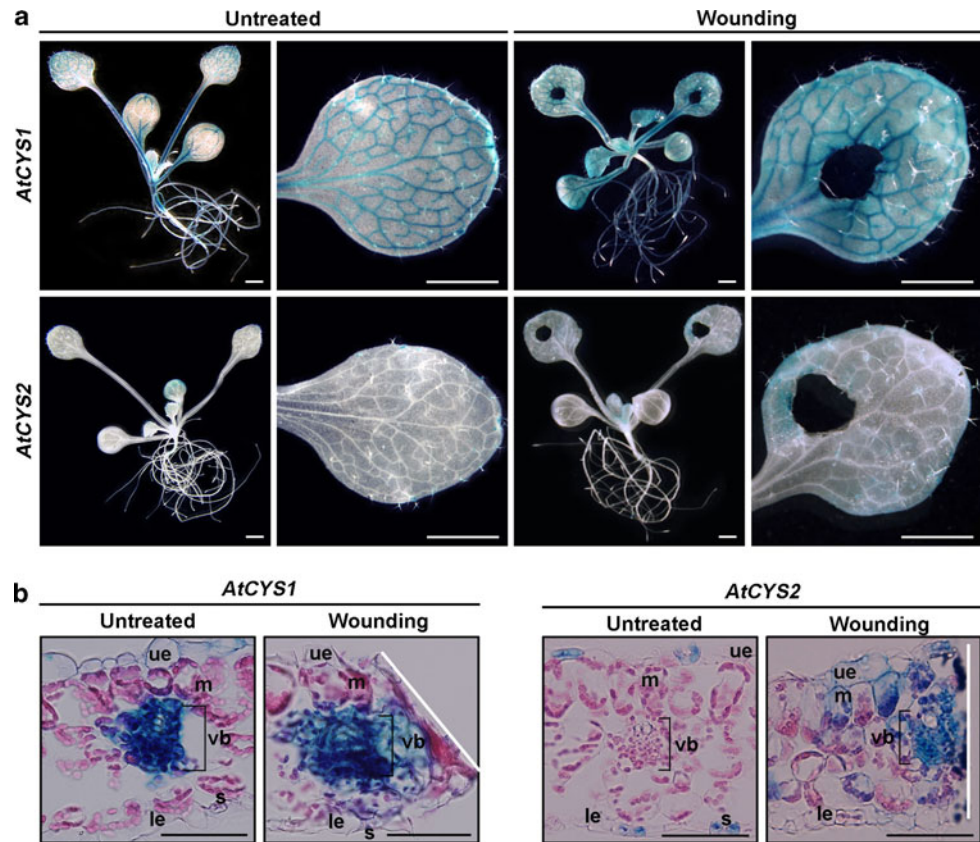
Cys protease and PhyCYS interactions have been repeatedly implicated in the control of plant development and defense mechanisms (Otlewski et al. 2005; Neuteboom et al. 2009). Therefore, to gain a better understanding of the regulatory mechanisms controlling *Arabidopsis* *PhyCYS* gene expression, constructs consisting of the *GUS* reporter gene under the control of either the *AtCYS1* or *AtCYS2* promoter were introduced into *Arabidopsis* via *Agrobacterium* mediated transformation.

In the leaves, *AtCYS1* expression was found in the vasculature, whereas *AtCYS2* expression was restricted to the stomata and trichomes (Figs. 1, 2). In the roots, *AtCYS1* expression was also restricted to the vascular tissues of the primary and lateral roots in transgenic plants. Therefore, *AtCYS1* may be involved in regulating vascular development and/or function in roots and leaves, whereas *AtCYS2* may play a role in the development of epidermal cells such as trichomes and stomata in young, rapidly growing leaves by interaction with Cys proteases (Reeves et al. 2002). Interestingly, *AtCYS2* expression was restricted to the root cap of the primary root (Fig. 2). The root cap is the first point of contact between the growing root system and the

soil environment, programmed cell death, and it functions as an important sensory organ (Huh et al. 2002; Jiang et al. 2006). Therefore, we hypothesize that the specific expression of *AtCYS2* in the root cap may reflect the involvement of *AtCYS2* in root growth and/or cell death in the root cap. Further studies using Cys proteases, which interact with the PhyCYS proteins in the root tips, will be needed to address this possibility. We also identified differences between the tissue-specific expression patterns driven by the *AtCYS1* and *AtCYS2* promoters in the flower (Fig. 3), based on accurate sampling of floral materials (Smyth et al. 1990). *AtCYS1* was strongly expressed in the vasculature of all floral tissues, as in the seedlings and mature plants; however, *AtCYS2* was only detected at minimal levels, in the regions connecting the anther and stamen, and the carpel and stigma. The level of *AtCYS2* expression rapidly decreased as the anthers matured. These data suggest that *AtCYS1* may play roles in development of the floral vasculature in the carpel, sepal, and stamen, whereas *AtCYS2* may be involved in regulating growth of the carpel and stamen via interactions with Cys proteases.

Expression of PhyCYS is induced by several abiotic stresses (Pernas et al. 2000; Gaddour et al. 2001; Diop et al. 2004; Massonneau et al. 2005; Zhang et al. 2008) and

Fig. 7 Changes in expression of *AtCYS1* and *AtCYS2* by wounding stress. **a** GUS expression 48 h after 10-day-old plants was subjected to wounding. Scale bars are 1 mm. **b** Transverse sections of wounded and control leaves. Scale bars are 50 μ m. White lines indicate the wound sites. The abbreviations indicate upper epidermis (*ue*), mesophyll cells (*m*), vascular bundle (*vb*), stoma (*s*), and lower epidermis (*le*)



PhyCYS over-expressing transgenic plants exhibit increased abiotic stress tolerance (Belenghi et al. 2003; Van der Vyver et al. 2003; Huang et al. 2007). These observations support the hypothesis that *PhyCYS* proteins play a crucial role in plant abiotic stress defense mechanisms. Hence, we assessed the response of *AtCYS1* and *AtCYS2* to abiotic stresses using RT-PCR (Fig. 4). *AtCYS1* expression is induced by cold, drought, heat, and wounding stresses. *AtCYS2* expression is induced by drought, heat, and wounding stresses, but not by cold stress. These findings suggest that the selective induction of these genes may be due to the presence of specific promoter elements. Therefore, we analyzed the upstream promoter sequences of the *AtCYS1* and *AtCYS2* genes to determine whether they contain stress-related *cis*-elements (Fig. 5). This analysis revealed that *AtCYS1* and *AtCYS2* contain several interesting putative heat and wounding stress-related *cis*-elements, including ABREs, CCAAT-boxes, HSEs, STREs, and W-boxes. The ABRE, HSE, and W-box motifs have been identified as binding sites for ABRE-binding factors (ABFs) (Choi et al. 2000), heat shock factors (Hsfs) (Nover et al. 1996), and WRKY transcription factors (Eulgem and Somssich 2007), respectively. ABREs control ABA- and/or heat stress-responsive gene expression, as determined by numerous studies (Giraudat et al. 1994; Gong et al. 1998; Larkindale and Knight 2002). Several

WRKY transcription factors are involved in mechanical wounding stress (Levéé et al. 2009; Li et al. 2009). The CCAAT-box sequences act cooperatively with HSEs to increase promoter activity (Rieping and Schöffl 1992; Haralampidis et al. 2002). Both motifs were found in the *AtCYS1* and *AtCYS2* promoter regions and may modulate expression of these genes in response to heat and/or wounding stress.

The *cis*-elements identified in the *AtCYS1* and *AtCYS2* promoter sequences prompted us to analyze the transgenic plants carrying the *GUS*-promoter fusions in heat and wounding stress. The expression of *GUS* driven by the *AtCYS1* and *AtCYS2* promoters in transgenic *Arabidopsis* was induced by heat and wounding stresses (Figs. 6, 7). *AtCYS1* expression was induced in most plant tissues, with the exception of root tips, under heat stress (Fig. 6a, b). Heat treatment induced a significant increase in *AtCYS2* in young leaves (Fig. 6a), especially in the guard cells (Fig. 6c). This result suggests that *AtCYS2* may specifically participate in defense against heat stress via stomatal regulation. In response to wounding stress, both genes exhibited expression patterns similar to heat stress (Fig. 7). *AtCYS1* expression was induced in most plant tissues, especially in vasculature around the wounding sites, and *AtCYS2* was induced in the mesophyll cells and vasculature around the wounding sites (Fig. 7a, b). In a previous study,

wounding caused extensive changes in the synthesis of defense proteins, leading to localized resistance at the site of the lesion (McConn et al. 1997). Expression of both Cys protease inhibitors and Cys proteases is also induced by wounding stress (Kinoshita et al. 1999; Belenghi et al. 2003; Yamada et al. 2004). These observations support the hypothesis that the differential expression of *AtCYS1* and *AtCYS2* may indicate the presence of different defense mechanisms against wounding, and distinct interactions with target Cys proteases in specific tissues and organs.

The data from our study underscore the importance of comparative analysis in identifying the activities of *AtCYS1* and *AtCYS2* in each tissue and cell type, as well as in response to different stresses, in order to understand the complexity underlying multiple signaling systems. However, the detailed functions of the entire *AtCYS* gene family remain to be elucidated, and further experiments are required to define their complete biochemical and physiological functions in response to abiotic stresses.

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