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ORIGINAL PAPER

Vascular-specific activity of the *Arabidopsis* carotenoid cleavage dioxygenase 7 gene promoter

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Abstract Carotenoid cleavage dioxygenases (CCDs) are involved in the production of diverse apocarotenoids including phytohormones, the visual molecules and the aromatic volatile compounds derived from carotenoids. Here, we examined the spatial expression of four of the CCD genes (*AtCcd1*, *4*, *7* and *8*) among the nine members of this family in *Arabidopsis* by RT-PCR. We found that the *AtCcd7* gene showed strong expression in seeds. However, the promoter activity of the 1,867-bp 5′-upstream region of this gene exhibited a vascular specificity at all developmental stages throughout the transgenic *Arabidopsis* plants tested. The strength of the *AtCcd7* promoter was also found to be lower than that of the 35S promoter by about 60%. The whole body expression of the β-glucuronidase (GUS) reporter gene driven by the *AtCcd7* promoter in *Arabidopsis* plants was confirmed in different organs by RT-PCR and GUS enzymatic assays. Histochemical GUS staining further revealed that the *AtCcd7* promoter has utility in limiting the expression of target genes to the vascular tissues in all plant organs such as the leaf, stem, root, flower and seed.

Keywords *Arabidopsis* · Carotenoid cleavage dioxygenase · Promoter · Vascular-specific

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Introduction

Carotenoid cleavage dioxygenases (CCDs) catalyze the production of diverse apocarotenoids that serve a range of biological functions in plants as hormones, pigments, flavors and defense compounds (Giuliano et al. 2003; Auldridge et al. 2006b). The CCDs that cleave diverse carotenoid substrates at specific double bond positions are encoded by a multi-gene family in plants (Tan et al. 2003; Rubio et al. 2008). Nine CCD genes have been identified in the *Arabidopsis* whole genome: four CCDs (1, 4, 7 and 8) and five 9-*cis*-epoxycarotenoid dioxygenases (NCED 2, 3, 5, 6 and 9) (Tan et al. 2003). The NCEDs are characterized by their role in abscisic acid (ABA) biosynthesis, i.e. the oxidative cleavage of 9-*cis*-epoxycarotenoids at the 11, 12 double bond position. The first *Arabidopsis* NCED gene was identified through its high homology to the causative gene in the maize ABA-deficient viviparous mutant, *vp14* (Schwartz et al. 1997).

Among the four enzymes in *Arabidopsis* that were designated as CCDs, i.e. differing in function to the NCEDs, CCD1 generates C_{13} and C_{14} apocarotenoids from multiple carotenoid substrates and thereby synthesizes both flavor and aroma volatiles in a similar manner to CCD1 in bean, tomato and petunia (Schwartz et al. 2001; Simkin et al. 2004). CCD7 and CCD8 in *Arabidopsis* produce novel signaling molecules that regulate lateral branching through the consecutive cleavage of carotenoid substrates (Schwartz et al. 2004). This process was elucidated in studies of the more axillary branching mutant (*max*) *3* and *max4* that were identified as the equivalent alleles to *ccd7* and *ccd8*, respectively (Sorefan et al. 2003; Booker et al.

2004). The cleavage activity of *Arabidopsis* CCD4 has recently been found to produce β-ionone from 8′-apo-β-carotene-8′-al when expressed in *E. coli*, but its function in vivo has not yet been elucidated (Huang et al. 2009).

The spatial expression of the five *Arabidopsis* NCED genes (*AtNced 2*, *3*, *5*, *6* and *9*) have been examined previously in diverse *Arabidopsis* tissues by TaqMan real-time PCR and their promoter activities have been analyzed using the GUS reporter system (Tan et al. 2003). In the case of *Arabidopsis* CCD genes, the root specificity of *AtCcd8* expression was demonstrated by real-time PCR analysis in an earlier report (Auldridge et al. 2006a). Another study has shown that the spatial expression of the *AtCcd7* gene is higher in roots than in other organs (mature seeds were not examined) by real-time PCR (Booker et al. 2004).

Since *Arabidopsis* microarray data have become publicly available (http://www.arabidopsis.org/info/), the expression profiles of whole sets of genes involved in a particular metabolic pathway can be employed to screen candidates and thereby identify useful tissue-specific promoters. Most carotenogenic genes, including the nine CCD genes, show different expression profiles during development in diverse plant tissues (Liang et al. 2009). This suggests that some of these genes could be strong candidates for promoter analysis.

In our current study, we further examined the spatial expression of four CCD genes (*AtCcd1*, *4*, *7* and *8*) by semi-quantitative RT-PCR to validate the *Arabidopsis* microarray data. The promoter activities of the 5′-upstream regions of three of these genes (*AtCcd1*, *4* and *7*) were then investigated to evaluate potential new promoters that would have utility in dicot transgenesis.

Materials and methods

RNA analysis

Total RNAs were isolated from various *Arabidopsis* tissues using Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. RNA aliquots of 1 μg were simultaneously reverse transcribed to first-strand cDNAs and amplified using the mRNA Selective PCR Kit (Takara, Tokyo, Japan). RT-PCR was performed for 25 cycles using the gene-specific primer sets for four *Arabidopsis* CCD genes (*AtCcd1*, *4*, *7* and *8*) listed in Table 1. The *AtEF1α* (5′-GTTCAC ATTAACATTGTGGTCATT-3′/5′-CAGGTACCAGTGAT CATGTTCTTG-3′) and *Gus* (5′-ACCTGCGTCAATGT AATGTTCTGC-3′/5′-CTCCCTGCTGCGGTTTTTCA-3′) genes are predicted to generate 305- and 478-bp amplified products, respectively.

Vector construction and *Arabidopsis* transformation

The putative promoter regions located 5′-upstream of the *AtCcd1*, *4* and *7* genes were amplified with primer sets specific to *Arabidopsis* genomic DNA (Table 2). A transit peptide (TP) sequence of 93-bp encoding 31 amino acids (MSLPIPPKFLPPLKSPPIHHHQTPPPLAPPR) as predicted by ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) was attached to the putative *AtCcd7* promoter using the specific primer set indicated in Table 2. The four PCR products were subcloned into the Gateway® destination vector pBGWFS7 (VIB-Ghent University, Ghent, Belgium) as previously described (Chung et al. 2008) followed by sequencing verification. These constructs were

Table 1 RT-PCR primers used in this study

Gene	Accession	RT-PCR analysis	
		Forward/reverse primer (5′ → 3′)	PCR product/full length (bp)
AtCcd1	At3g63520	GGGTACGCATCAAAGATGGG/CATTTCACCCGTAACCGGG	383/1,617
AtCcd4	At4g19170	TTCTTCGCCGCCGATCTT/AACGGAAGGACGTGAAGGTG	257/1,788
AtCcd7	At2g44990	CCACGAGCCGCAATATCAA/AACAAAGCAATCGCCCAGC	496/1,857
AtCcd8	At4g32810	AACCTCAAGTCCCTCACCGG/CGAATATACGACCGCCATCG	321/1,713

Table 2 Promoter cloning primers used in this study

Promoter	Additional transit peptide	Promoter analysis	
		Forward/reverse primer (5′ → 3′)	Size (bp)
AtCcd1-P	–	AAAAAGCAGGCTGAAGAATCTCGCTAGAC/AGAAAGCTGGGTGATTGTTTGTTTTGCTG	1,997
AtCcd4-P	–	AAAAAGCAGGCTCCTTTGTAGTAGGAGAC/AGAAAGCTGGGTTGCTCTCCTTTTGTTTTTAC	1,815
AtCcd7-P	–	AAAAAGCAGGCTCAGACAAGTGGTGTGG/AGAAAGCTGGGTTTTGGTCAACATTTTTG	1,867
AtCcd7-P:TP	31aa	AAAAAGCAGGCTCAGACAAGTGGTGTGG/AGAAAGCTGGGTTTTCGTGGAGGTGCAA	1,960

then separately transformed into *Arabidopsis* ecotype Columbia (Col-0) using the *Agrobacterium* strain (GV3101)-mediated method. As a reference control for promoter strength, a previously generated *Arabidopsis* transgenic line harboring a 35S dual promoter was used (Liang et al. 2009).

Fluorometric GUS assay

For the 4-methyl umbelliferyl β-D-glucuronide (MUG) assay of GUS activity, various organs from transgenic *Arabidopsis* plants were homogenized in extraction buffer (50 mM sodium phosphate buffer pH 7.0, 10 mM dithiothreitol, 1 mM disodium EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). The protein concentration of the supernatants was then measured using a Qubit fluorometer (Invitrogen). The GUS enzyme activity of the supernatants was subsequently assayed with buffer containing 1 mM MUG as the fluorescent substrate at 37°C using the FluorAce™ β-glucuronidase Reporter Assay Kit (Bio-Rad, Hercules, CA). Fluorometric values were calculated using a 4-methyl umbelliferone (4 mU) dilution series. Each assay was repeated three times for each independent transgenic line.

Histochemical GUS assay

Histochemical GUS assays were performed using a 1% 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) solution in 20 mM sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, 10 mM EDTA, and 5 mM potassium ferricyanide/5 mM potassium ferrocyanide overnight at 37°C (Chung et al. 2008). GUS staining was observed under both light and differential interference contrast microscopy (Olympus, Tokyo, Japan). All images were recorded digitally (Olympus).

Results

Spatial expression of endogenous genes encoding four *Arabidopsis* CCDs

The expression patterns of four *Arabidopsis* CCD genes (*AtCcd1*, *4*, *7* and *8*) in several organs of *Arabidopsis* were compared by semi-quantitative RT-PCR using gene-specific primer sets (Table 1). Figure 1 shows the ubiquitous expression of *AtCcd1*, *4* and *8* at similar levels in all of the organs tested including seedling (Sd), rosette leaf (RL), cauline leaf (CL), stem (S), root (R), flower (F) and seed (Se). *AtCcd1* exhibited the higher expression levels in most organs but the lowest expression in seed.

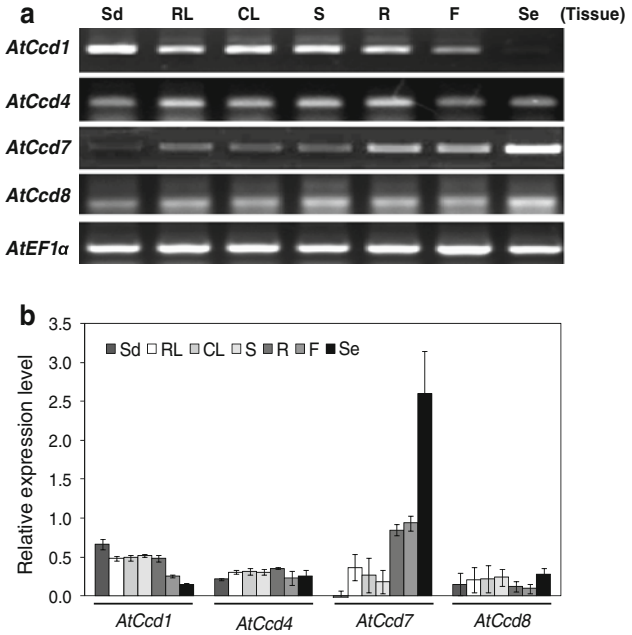

Fig. 1 Spatial expression patterns of four *Arabidopsis* CCD genes (*AtCcd1*, *4*, *7* and *8*) in various tissues of wild-type *Arabidopsis*. **a** Semi-quantitative RT-PCR analyses in seedling (Sd), rosette leaf (RL), cauline leaf (CL), stem (S), root (R), flower (F) and seed (Se) of wild-type *Arabidopsis*. *Arabidopsis* elongation factor 1-α (*AtEF1α*) was used as a normalization control for the RNA levels. **b** Relative expression levels measured using the Quantity One program (version 4.4.1, Bio-Rad). *Error bars* indicate the standard deviations (SDs) of three replicates

The *AtCcd7* gene was also expressed in all tissues examined at different levels (Fig. 1). It was noteworthy that the *AtCcd7* transcripts were found to be more abundant in seeds than in the seedling, leaf, stem, root or flower. The strong expression of *AtCcd7* in mature seeds contrasts with the previous finding that it shows its highest expression in the roots (including siliques but not mature seeds; Booker et al. 2004) but consistent with the public *Arabidopsis* microarray data (http://www.arabidopsis.org/info/).

On the basis of these expression profiles, we performed promoter analyses using the 5′-upstream regions of *AtCcd1*, *4* and *7*. We excluded *AtCcd8* as its promoter activity was expected to be insufficient to drive foreign gene expression.

Organ-specific activities of the *AtCcd1*, *4* and *7* gene promoters

Putative promoter regions of about 2 kb located 5′-upstream of the *AtCcd1*, *4* and *7* genes were PCR amplified from *Arabidopsis* leaf genomic DNA (primers are listed in Table 2). To additionally examine whether chloroplast targeting exerted any influence on the *AtCcd7* promoter, a 1,962-bp fragment (AtCcd7-P:TP) incorporating the

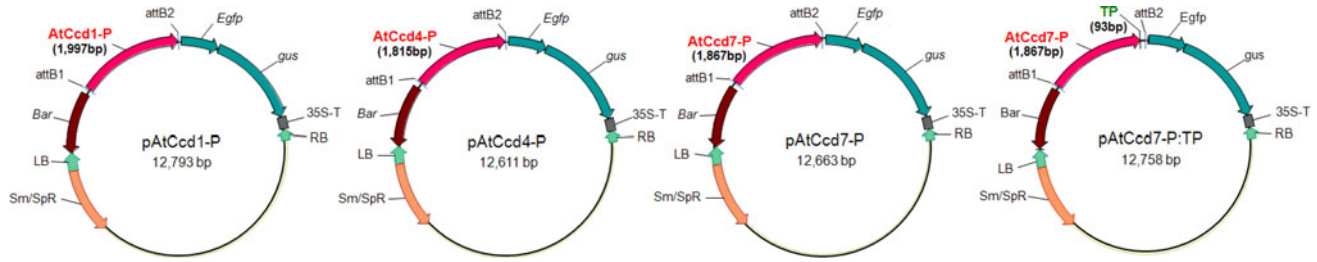

Fig. 2 Schematic representation of the binary vectors used for *Arabidopsis* transformation. DNA fragments for AtCcd1-P, AtCcd4-P, AtCcd7-P and AtCcd7-P:TP extracted from *Arabidopsis* leaf DNA were introduced into the pBGWFS7 vector using Gateway cloning procedures. These maps were drawn using Vector NTI software (Invitrogen). *AtCcd1-P*, *AtCcd1* promoter; *AtCcd4-P*, *AtCcd4* promoter; *AtCcd7-P*, *AtCcd7* promoter; *attB1/B2*, bacterial regions used for attachment; *Bar*, Bialaphos resistant gene; *Egfp*, the enhanced green fluorescent protein gene; *gus*, *β*-glucuronidase gene; *35S-T*, cauliflower mosaic virus 35S terminator; *LB*, left border; *RB*, right border; *Sm/SpR*, streptomycin/spectinomycin resistant gene; *TP*, transit peptide

1,867-bp *AtCcd7* promoter (AtCcd7-P) with a trailing 93-bp TP, encoding the N-terminal 31 amino acids (as predicted using the ChloroP 1.1 program), was simultaneously generated using *Arabidopsis* leaf genomic DNA (Table 2). The four PCR products were, respectively, introduced into a promoter-less pBGWFS7 vector harboring the EGFP-GUS fusion reporter gene (*Egfp:gus*; Fig. 2). Through *Agrobacterium*-mediated *Arabidopsis* transformation, a total of 83 transgenic lines (20 for *AtCcd1-P:Gus*, 17 for *AtCcd4-P:Gus*, 27 for *AtCcd7-P:Gus*, 19 for *AtCcd7-P:TP:Gus*) were obtained via 0.3% Basta® selection.

To visualize the organ-specific expression of the GUS reporter gene driven by the different CCD promoters, histochemical GUS staining was performed using 3-week-old transgenic seedlings (Fig. 3). The *AtCcd1-P:Gus* plants showed whole body expression of blue GUS signals at higher levels than *AtCcd4-P:Gus* plants (Fig. 3a, b). This result was consistent with the ubiquitous expression found in earlier experiments for the *AtCcd1* gene, which was at a higher level than that of the *AtCcd4* gene (Fig. 1).

It was interesting to note that the *AtCcd7-P:Gus* plants exhibited vascular-tissue specificity at the whole body level in 3-week-old seedlings (Fig. 3c). This result was unexpected given the strong specificity found for the expressed *AtCcd7* gene in mature seeds by RT-PCR (Fig. 1). The vascular-specific expression of *AtCcd7* was found to be limited to the primary and secondary veins of *AtCcd7-P:Gus* transgenic leaves. In addition to *AtCcd7* promoter activity, the *AtCcd7-P:TP:Gus* plants used to test the influence of a TP upon the exogenous expression of the protein product displayed a quite different pattern of vascular-specific expression. The GUS expression in the primary and secondary veins driven by the *AtCcd7* promoter was weakened by the presence of the TP sequence on the *AtCcd7* gene product. However, under these conditions, GUS expression was newly detected in higher order tertiary and quaternary veins (Fig. 3d), suggesting that the TP sequence alters the pattern of vascular specificity without affecting the overall promoter activity levels.

Constitutive and ubiquitous expression of the *AtCcd7* promoter

Twenty-seven *AtCcd7:Gus* transgenic plants were tested by histochemical staining and found to display similar levels of GUS expression in the rosette leaves of 3-week-old seedlings. Three representative lines showing average GUS enzyme activities were selected following a MUG assay for further promoter analyses (data not shown). Another representative line, *35S-P:Gus*, which was described in a previous study (Liang et al. 2009), was used as a control to compare promoter strength. Semi-quantitative RT-PCR analyses of *Gus* revealed that the *AtCcd7* promoter drives expression at a 0.3–0.6-fold lower transcriptional level than the *35S* promoter (Fig. 4).

To next examine the activity of the *AtCcd7* promoter at different developmental stages in the plants, histochemical GUS staining was performed in the whole bodies of three *AtCcd7:Gus* transgenic lines at 2-week intervals (Fig. 5). The GUS expression levels driven by the *AtCcd7* promoter were found to be ubiquitously maintained in the vascular tissues of the whole body at all developmental stages in 1-, 3- and 5-week-old seedlings.

Vascular specificity of the *AtCcd7* promoter in transgenic *Arabidopsis*

To more closely examine the vascular specificity of the *AtCcd7* promoter, histochemical GUS staining was performed in diverse organs of the *AtCcd7:Gus* transgenic *Arabidopsis* plant. Signals were detected in the mid-vein of 5-day-old young leaves (Fig. 6a) and in the primary and secondary veins of 5-week-old mature leaves (Fig. 6b, c). The vascular specificity of the *AtCcd7* promoter was

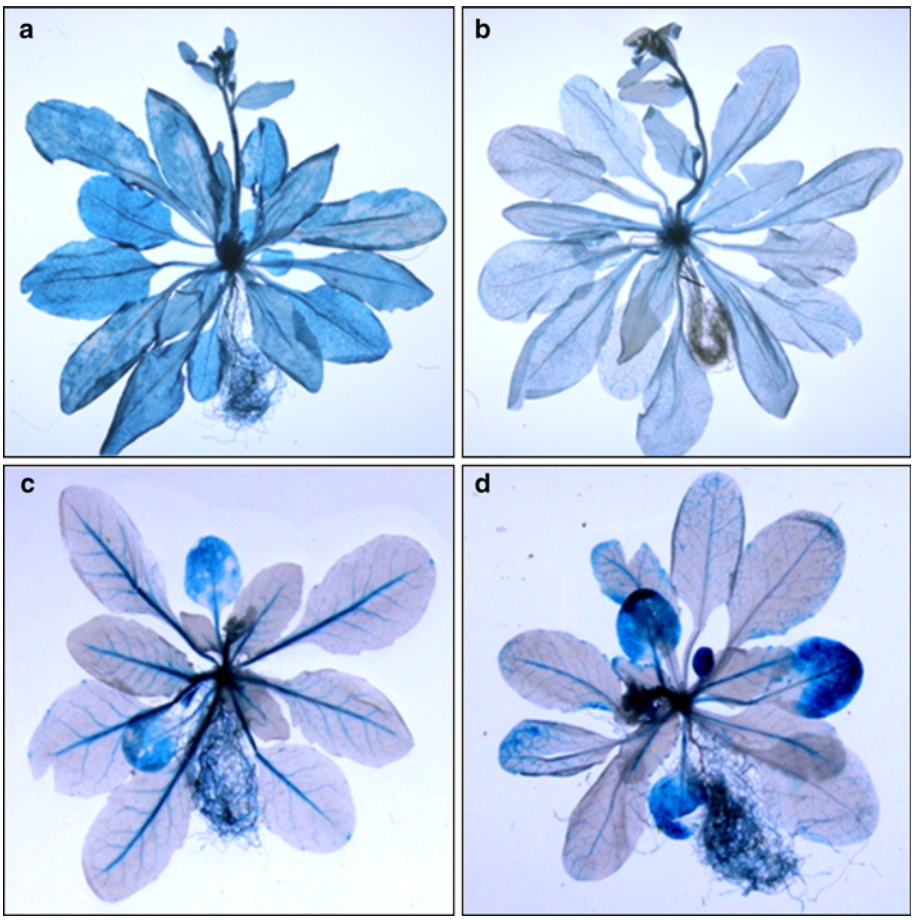

Fig. 3 Histochemical GUS expression profile for the CCD promoters in 3-week-old transgenic seedlings. The organ-specific expression driven by four different CCD promoters was examined in each case using the whole bodies of transgenic plants that had been transformed separately with the plasmids pAtCcd1-P (**a**), pAtCcd4-P (**b**), pAtCcd7-P (**c**) and pAtCcd7-P:TP (**d**)

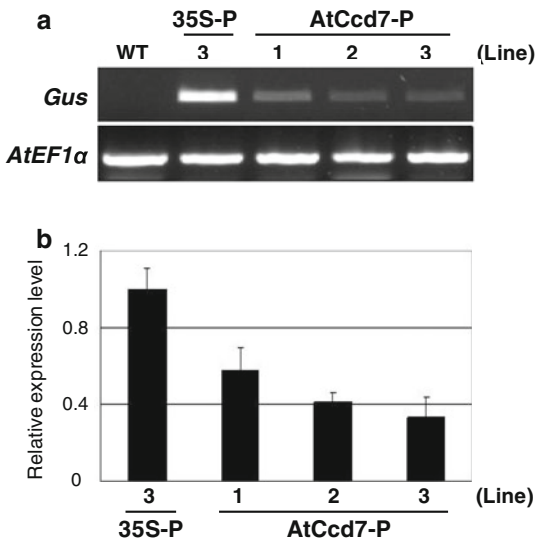

Fig. 4 Comparison of the *35S* and *AtCcd7* promoter strengths. **a** Semi-quantitative RT-PCR analysis using *Gus*-specific primers. **b** Relative expression levels of *Gus* measured using the Quantity One program (version 4.4.1, Bio-Rad). *Error bars* indicate the standard deviations (SDs) of three replicates

clearly evident in the stem with strong GUS signals present in both cross- and longitudinal sections (Fig. 6d, e). Further observations in other individual organs including the root, flower (sepal and pistil), green silique (placenta) and seed revealed GUS expression to be limited to specific vascular tissues (Fig. 6f–i).

To next compare the activity of the *AtCcd7* promoter in diverse *Arabidopsis* organs, semi-quantitative RT-PCR and fluorometric GUS activity analyses were each performed in the RL, CL, S, R, F and Se of *AtCcd7-P:Gus* transgenic *Arabidopsis* plants. *Gus* transcripts were detectable in all organs of the *AtCcd7-P:Gus* plants with marginally higher levels observed in the cauline leaf and stem (Fig. 7a). In addition, the GUS enzyme activities driven by the *AtCcd7* promoter were measured at equivalent levels among all of the organs examined apart from the stem which showed higher levels (Fig. 7b).

Discussion

Our current data demonstrate the vascular-specific activity of the *AtCcd7* promoter among the promoter activities that were predicted from the spatial expression patterns of four *Arabidopsis* CCD genes (*Atccd1*, *4*, *7* and *8*). Through the analysis of plant *cis*-acting regulatory DNA elements in the PLACE database (http://dna.affrc.go.jp, Higo et al. 1999), basal regulatory elements typically present in plant

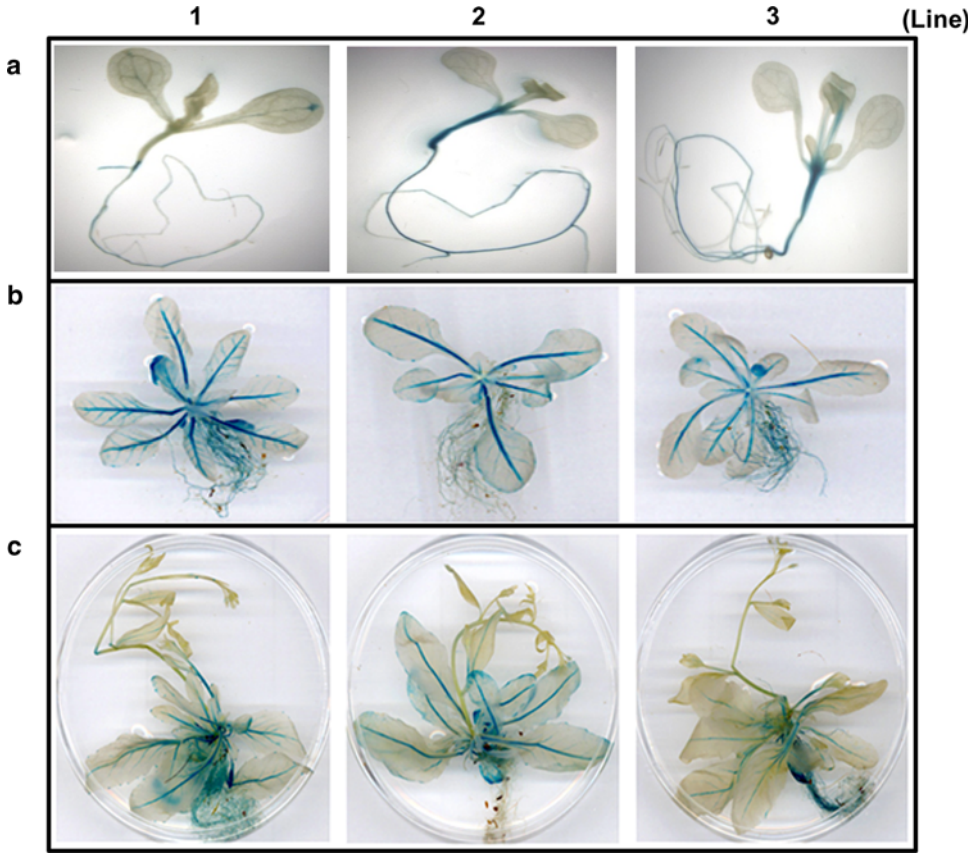

Fig. 5 Histochemical GUS staining of three independent *AtCcd7:Gus* transgenic lines at different developmental stages. GUS expression in most organs was detected in vascular tissues at all developmental stages in 1-week-old (**a**), 3-week-old (**b**) and 5-week-old (**c**) seedlings

promoters including a TATA box (TATATAA) and CCAAT box (CCAAT) were found at positions −74 to −67 and −348 to −343 within a 1,867-bp *AtCcd7* 5′-flanking region. Several conserved elements for seed specificity are also present in this region: positions −1,014 to −1,007 for a SEF1 binding site (ATATTTA), −325 to −319 for an E-box (CATGTG) and −838 to −832 for a Sh1 box (TGAATG) (Allen et al. 1989; Stålberg et al. 1996; D'Aoust et al. 1999). Moreover, an element required for phloem-specific expression (Saha et al. 2007), including two potential *cis*-elements, i.e. an ASL box (−1,837 to −1,823) and repeated GATA box (−1,307 to −1,298 and −798 to −777), is also present in the *AtCcd7* promoter region.

The biochemical functions of AtCCD7 include the inhibition of lateral branching through the synthesis of a novel carotenoid-derived plant-signaling molecule along with a successive reaction with AtCCD8 (Schwartz et al. 2004). Through a map-based cloning of the *max3* mutant that shows a bushy phenotype and a dysregulated shoot branching, *AtCcd7* was identified as the causative gene (Booker et al. 2004). This previous study also demonstrated by real-time PCR that the spatial expression of the *AtCcd7* gene is higher in the roots than in other organs. In contrast, however, our current semi-quantitative RT-PCR and the *Arabidopsis* microarray data (http://www.arabidopsis.org/info/) indicate that strongest expression of this gene is in the seeds.

The vascular-tissue specificity of the *Ccd7* gene was first described in rice plants (Zou et al. 2006). The *OsCcd7* gene, an ortholog of *AtCcd7*, was previously cloned through the fine mapping of an *htd-1* rice mutant which shows high tillering and dwarf phenotypes (Zou et al. 2005). The *GUS* reporter expression driven by the *OsCcd7* promoter has been detected in most rice organs including the leaf, stem, panicle and root (Zou et al. 2006). Furthermore, this expression was mainly observed in vascular-associated tissues through the analysis of sheath and stem cross-sections in rice. This is consistent with the findings for the *AtCcd7* promoter in *Arabidopsis* plants. A recent report proposing strigolactones as a new plant hormone class has now elucidated that OsCCD7 is almost certainly required for the production of normal levels of strigolactones in seedlings through analysis of the shoot and root branching phenotype of the *htd-1*/*d17*/*max3* mutant (Umehara et al. 2008). In addition, as the *AtCcd8* gene is causative for the *MAX4* phenotype, the *OsCcd8* gene has also been found to be involved in control lateral branching from positional cloning analysis of *d10*/*max4*, a rice dwarf mutant (Arite et al. 2007). This *D10*/*OsCcd8* promoter also drives the *GUS* reporter gene in the vascular tissues of most

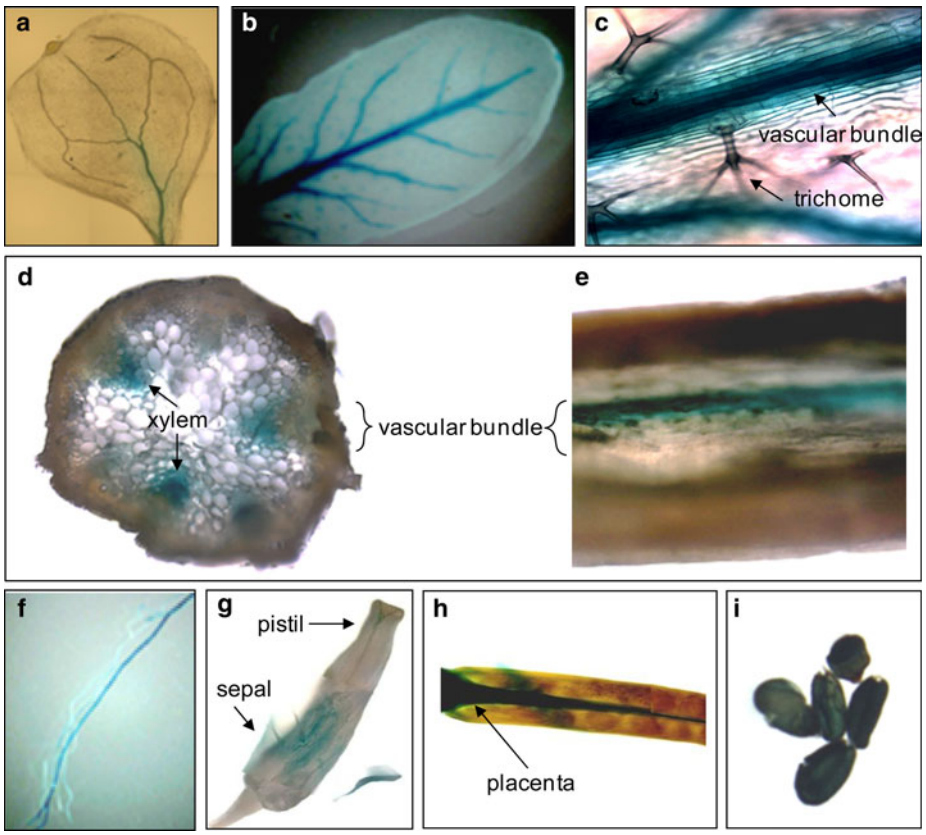

Fig. 6 Vascular specificity of the *AtCcd7* promoter in various organs of transgenic *Arabidopsis*. Histochemical GUS staining was performed using a 5-day-old young leaf (**a**), a 5-week-old mature leaf (×20 in **b**, ×100 in **c**), and cross- and longitudinal sections of stem (**d**, **e**), root (**f**), flower (**g**), green silique (**h**) and seed (**i**)

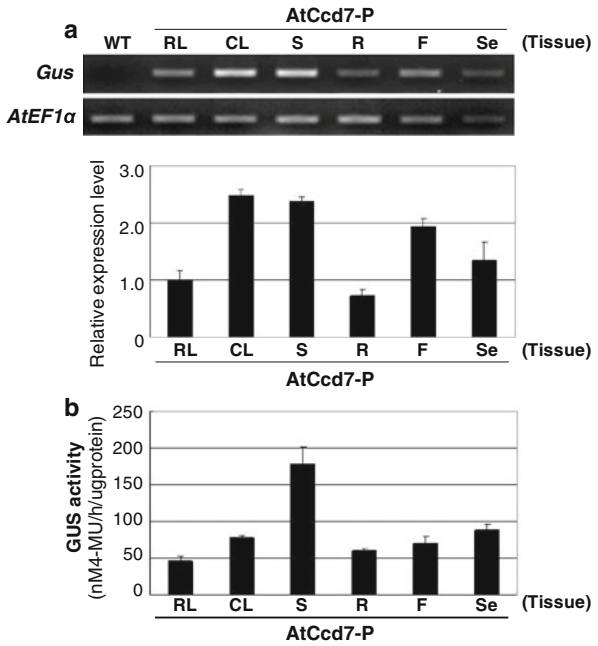

Fig. 7 Activity of the *AtCcd7* promoter in driving GUS expression in different organs. **a** Detection of *Gus* transcripts by semi-quantitative RT-PCR. **b** Enzyme activity of GUS measured by MUG assay. Both experiments were performed in triplicate with the same samples of rosette leaf (RL), cauline leaf (CL), stem (S), root (R) and flower (F) from 5-week-old *AtCcd7* promoter transgenic plants. Mature seeds (Se) harvested at 6 weeks post-flowering in *AtCcd7* promoter transgenic plants were used in both experiments

rice organs including roots, nodes, internodes and the inflorescence, suggesting similarities with the *AtCcd7* promoter described herein as well as *OsCcd7* promoter.

Vascular tissues are essential for the transport of water, nutrients and signaling molecules (Sieburth and Deyholos 2006). They support higher plants through the formation of structural molecules including lignin and can act as reservoirs of invading fungi and bacteria, resulting in yield loss and quality reduction in commercial crops. Hence, several vascular-specific promoters have been developed from lignin biosynthetic genes including the phenylalanine ammonia-lyase (PAL) gene from the loblolly pine (Gray-Mitsumune et al. 1999), the cinnamyl alcohol dehydrogenase (CAD2) and cinnamoyl-CoA reductase (CCR) genes from *Eucalyptus gunnii* (Lacombe et al. 2000; Lauvergeat et al. 2002). In addition, some sucrose transport genes such as the *Cucumis* phloem protein 2 (PP2) gene (Dinant et al. 2003), the soybean sucrose binding protein (SBP) gene (Freitas et al. 2007) and the rice sucrose synthase 1 (*RSs1*) gene (Saha et al. 2007) have been used as targets for vascular-cell specific promoters. These promoters have been characterized for their potential utilization in genetic engineering applications to enhance the woody properties that are desirable for the paper and fiber industries (Lauvergeat et al. 2002) and also to develop resistance to pathogen and insect pests (Saha et al. 2007). In a similar

vein, our current data indicate that the *AtCcd7* promoter may well contribute to the range of vascular-specific promoters available for commercial applications in plants.

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