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EGY2, a chloroplast membrane metalloprotease, plays a role in hypocotyl elongation in *Arabidopsis*

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Abstract Intramembrane proteases control many important processes in a wide variety of organisms through regulated intramembrane proteolysis (RIP). However, very few intramembrane proteases have been characterized in plants. Intriguingly, EGY2 in *Arabidopsis* belongs to the Site-2 protease (S2P) family that performs RIP. It contains the conserved catalytic motifs, HExxH and NPDG on its multiple transmembrane helices. Four *egy2* knockout mutants have significantly shorter hypocotyls and accumulate lower levels of fatty acids in seedlings. Accumulation of fatty acid biosynthesis enzymes in seedlings are also decreased in *egy2* knockout mutants. EGY2 protein resides in the chloroplast and *EGY2* transcripts are found throughout the plant except root. Recombinant EGY2 protein cleaves β-casein in an ATP-independent manner. These results together suggest that EGY2 metalloprotease plays a role in hypocotyl elongation likely through a RIP dependent process to regulate the coordinated expression of nuclear- and plastid-encoded genes.

Keywords EGY2 · *Arabidopsis* · Hypocotyl elongation · Chloroplast · Membrane metalloprotease · Fatty acid

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Introduction

Hundreds of proteases have been identified in plant genomes. For example, There are about 600 to 800 known or putative proteases in *Arabidopsis* and *Oryza sativa* respectively [1]. In addition to their well-known housekeeping function of degrading nonfunctional proteins into amino acids, more and more proteases have been identified as key regulators in many diverse biological processes [2]. For example, PCS1 (promotion of cell survival) is an endoplasmic reticulum-resident protease that prevents cell death in reproduction and embryogenesis [3]. CDR1 (constitutive disease resistance) participates in disease resistance signaling through systemic acquired resistance [4]. VAR2 (yellow variegated), an ATP-dependent metalloprotease, are critical for chloroplast biogenesis and photosystem II repair [5].

During the past decade, in addition to proteolysis occurs in soluble regions, intamembrane proteolysis has been observed in the transmembrane domain of many integral membrane proteins. So far intramembrane proteolysis is carried out by four types of proteases: rhomboid proteases, which are serine proteases, such as GlpG [6]; signal peptide peptidases (SPPs) and Presenilin1 as the catalytic subunit of γ-secretase, both of which are aspartyl proteases [7, 8]; and a large family of metalloproteases, Site-2 proteases [9]. Site-2 protease (S2P) was first identified in human and later in other animals. It is involved in the feedback regulation of mammalian sterol and fatty acid synthesis and uptake by controlling the activity of transcription factors known as sterol regulatory element binding proteins (SREBPs) [10]. Thus far, S2P homologs have been identified in different organisms ranging from eukaryotes to bacteria and archaea (reviewed in [11]). They are involved in intramembrane proteolysis that controls stress responses [12, 13], cell

division [14], bacterial mating [15], pathogenesis [16] and polar organelle biogenesis [17]. Intramembrane proteolysis of regulatory proteins to release effector domains is a well-characterized mechanism in animals and bacteria [11]; however, their function in photosynthetic organisms has received little investigation.

The plant S2P homologs were first characterized in *Arabidopsis*. Their conserved motifs suggest that they may execute intramembrane proteolysis in plastids. EGY1 is a chloroplast membrane-associated metalloprotease, which is essential for plastid development and shoot gravitropism stimulated by ethylene [18, 19]. Yellow-green *egy1* mutants contained defective chloroplasts with fewer stromal thylakoids and fewer starch grains, and they also accumulate significantly less chlorophyll a/b binding proteins of the light-harvesting complexes I and II and smaller and less numerous endodermal plastids. Endogenous expression of EGY1 is found throughout the plant, but expression is most prominent in leaf and stem tissue and scarce in root. Similarity searches identified several other S2P homologs in *Arabidopsis* [18]. AraSP (At2g32480) is localized to the chloroplast inner envelope membrane and has been found to be essential for plant development [20]. In contrast, the function of the homolog At1g01540, which is highly similar to AraSP, has not been elucidated from its T-DNA insertion line [20].

At the onset of our studies, other S2P homologs had not been characterized in *Arabidopsis*, and we were curious about their biological functions and biochemical properties. Thus, we conducted experiments to characterize the knockout phenotype of *EGY2* (At5g05740), investigate its expression and localization as well as its proteolytic activity.

Materials and methods

Arabidopsis

Seeds of wild type Columbia (Col) and the mutants *egy1-2 (SALK_134931), egy2-1 (SALK_001991), egy2-2 (SALK_110403), egy2-3 (SALK_028514)* and *egy2-4 (SALK_142694)* were obtained from Arabidopsis Biological Resource Center (Columbus, OH, USA). Seedlings were grown on water soaked paper towels, and adult plants were grown in soil as described previously [21].

Fatty acid analysis

Fatty acid content and composition were measured by gas chromatography mass spectrometry (GCMS) of fatty acid methyl esters. Fresh seedlings were placed in 1 ml of 0.5 M NaOH in methanol. After incubation for 10 min at 90°C, 2 ml of boron trifluoride/methanol (1/2) was added. After incubation for another 1 h at 90°C, 0.2 ml of saturated NaCl solution was added. Esterified fatty acids were extracted twice in 1 ml of hexane and analyzed by GCMS (Agilent 6890 N-5975i) on a DB-23 capillary column. For quantification, nonadecanoic acid was added as internal standard before derivatization.

Antibodies

Antibodies against ACP1, biotin and α-tubulin were purchased from Santa Cruz biotechnology or Sigma.

Recombinant binary vectors and *Arabidopsis* transformation

To make a full-length EGY2-GFP fusion construct, *EGY2* was amplified using the primers EGY2-*Sal*I-F and EGY2-*Kpn*I-R. GFP was amplified from pEGFP with the primers EGFP-F-*Kpn*I and EGFP-*Pac*I-R. Purified EGY2 PCR product was digested with *Sal*I and *Kpn*I, and the GFP fragment was digested with *Kpn*I and *Pac*I; ligation was then performed with the *Sal*I/*Pac*I-cut pBI-hyg-d35S-UTR-HBH binary vector. To make the Pro_{EGY2}::GUS construct, the EGY2 promoter was amplified using the primers EGY2-5′UTR-F and EGY2-5′UTR-R. The resulting fragment was digested with *BamH*I and *Nco*I and cloned into *BamH*I/*Nco*I-cut pCambia1301, which fuses upstream of the GUS reporter gene. Constructs were introduced into *Arabidopsis* via floral dip [22]. Transgenic seedlings were selected on MS medium with 50 mg/l hygromycin. Sequences of the primers are described in Table 1.

Histological GUS staining and confocal microscopic examination

GUS staining followed a modified method described previously [23]. GFP fluorescence analysis was performed via confocal microscopic examination with a Leica DMIRE2 confocal laser scanning microscope (Leica, Bensheim, Germany).

RT-PCR and authentification of T-DNA insertion lines

In RT-PCR, primers for *EGY2* are EGY2F and EGY2R, primers for *EGY1* are EGY1L and EGY1R, and primers for *ACTIN* (At3g18780) are ACTINL and ACTINR. Semi-quantitative PCR were conducted based on prior RT-PCR using different cycles to ensure that the obtained band intensities were within the linear range. T-DNA insertion lines were authenticated with primers 740-5, 740-6 and LB.

Table 1 Primers used in experiments

Name of primer	Sequence of primer
EGY2-*Sal*I-F	5′-GGG GTCGAC AAT CTC GCG GTG GCT AGT-3′
EGY2-*Kpn*I-R	5′-GGG GGTACC CAA GCC AAT CAT CAT GGC-3′
EGFP-F-*Kpn*I	5′-AAA GGTACC ATG GTG AGC AAG GGC GAG GAG C-3′
EGFP-*Pac*I-R	5′-GCG TTAATTAA TTA CTT GTA CAG CTC GTC-3′
EGY2-5′UTR-F	5′-ATA GGATCC AAC AAT ACC ATT TTA TCT C-3′
EGY2-5′UTR-R	5′-TAT CCATGG GTC CTT AAG AAA GCG-3′
EGY2F	5′-AAT CTC GCG GTG GCT AGT-3′
EGY2R	5′-CAA GCC AAT CAT CAT GGC-3′
EGY1L	5′-AAT GGG GAC TCT CAC CAG CGT-3′
EGY1R	5′-GAT TGG CCC TAA CTA CAA CGT C-3′
ACTINL	5′-GAA GTA CAG TGT CTG GAT CGG-3′
ACTINR	5′-GAG TTT TGA AAG AAA TTA TAG-3′
740-5	5′-ATG AGG AGG AAA ACA AAG CTC A-3′
740-6	5′-CAG GAT TCG TGA GAA GGA AAA G-3′
LB	5′-ATT TTG CCG ATT TCG GAA C-3′
740cDNA-F	5′-CGC GGATCC ATG AAT CTC GCGG TGG CTA GT-3′
740cDNA-R	5′-TCC CCCGGG AGC AAA ATA TTA GTT CAC ATT-3′

The restriction enzyme cleavage sites are underlined

Expression of recombinant EGY2 and proteolytic activity assay

The coding sequence of EGY2 was amplified using primers 740cDNA-F and 740cDNA-R. This cDNA fragment was cloned into pGEX-2T cut with BamHI and SmaI to generate a glutathione S-transferase::EGY2 fusion protein (GST-EGY2). This construct was expressed in *E. coli* strain BL21(DE3)pLysS, and recombinant EGY2 was purified through GST affinity column. The proteolytic activity assay was conducted at 37°C in a reaction buffer containing 50 mM Tris–acetate (pH 8.0), 80 mM NaCl, 5 mM Mg-acetate and 12.5 μM Zn-acetate. When indicated, 5 mM ATP was supplemented. Quantification of *β*-casein was determined by ImageJ (http://rsb.info.nih.gov/ij/) on the Coomassie brilliant blue R 250-stained gel.

Results

EGY2, a novel S2P homolog in *Arabidopsis*

EGY2 (At5g05740) was identified through BLAST searching for EGY1 homologs in *Arabidopsis* [18]. The 1584 bp coding sequence of EGY2 revealed by RT-PCR encodes a protein of 527 amino acids. EGY2 shares 48% similarity with EGY1 and belongs to the membrane metalloprotease family M50 (MEROPS the peptidase database: http://merops.sanger.ac.uk/) [1]. This family contains at least five members in *Arabidopsis*, namely EGY1, EGY2, AraSP, At1g05140 and At4g20310. They share the distinct motifs HEXXH and NPDG (Fig. 1a), which are conserved in S2P and S2P-like proteases [11, 24]. The His in the HEXXH motif and the Asp in the NPDG motif are the zinc ligands. In the characterized S2P homologs, the conserved motifs were predicted to be located either within the membrane or very close to the membrane surface [25]. Seven strong transmembrane helices were predicted in the C-terminus of EGY2 (Fig. 1b). The HEXXH motif is located at the end of the second TM helix, while the NPDG motif lies near the fourth TM helix, which places both motifs very close to the membrane surface. Alignment of the amino acid sequences around the HEXXH motif indicates that EGY2 is more similar to EGY1 than to AraSP. Similar to EGY1, EGY2 does not contain the PDZ-domain that is present in AraSP and At1g05140, and EGY2 contains the GNLR motif specific to EGY1 and some of its homologs in rice and cyanobacteria [18].

EGY2 plays a role in hypocotyl elongation

To explore the role of the EGY2 metalloprotease in plants, we characterized the phenotype in knockout plants. Four independent T-DNA insertion lines of *EGY2*, namely *egy2-1*, *egy2-2*, *egy2-3* and *egy2-4* were ordered, and homozygous mutant plants were obtained after they were selfed for several generations. T-DNA insertions are situated in the third intron, the fifth exon and sixth exon respectively and were authenticated by PCR and sequencing (Fig. 2a). RT-PCR confirmed that the EGY2 transcripts were absent in these knockout mutants (Fig. 2b).

Under the experimental conditions we used, adult plants of mutants showed no difference with wild type in

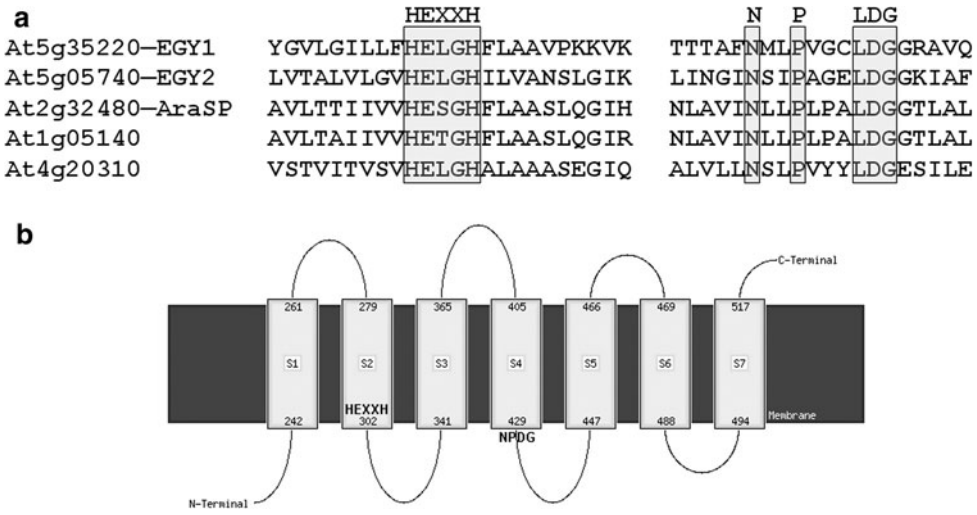

Fig. 1 Alignment and transmembrane topology of EGY2. **a** Alignment of EGY2 proteases and S2P homologs in *Arabidopsis*. Conserved motifs are highlighted in *grey*. **b** Transmembrane topology predicted by MEMSAT3 [31] (http://bioinf.cs.ucl.ac.uk/psipred/)

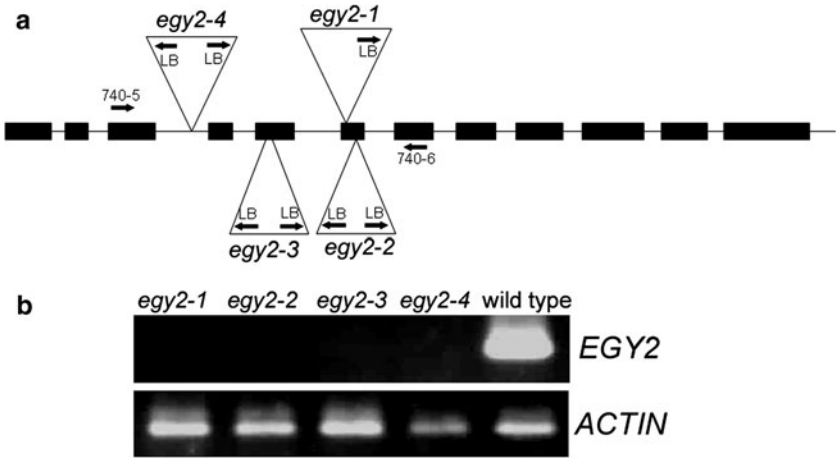

Fig. 2 Identification of *egy2* mutants. **a** Location of T-DNA inserts in the *EGY2* gene. The *black boxes* indicate exons, the *lines* indicate introns and 3′-untranslated regions. The triangles represent T-DNA inserts in the four independent mutants. *Horizontal arrows* indicate the primer used in RT-PCR. **b** *EGY2* mRNA accumulation detected by RT-PCR in *egy2-1*, *egy2-2*, *egy2-3*, *egy2-4* and wild type in the upper panel. Expression of ACTIN was used as control in the lower panel

chloroplast number in cotyledons, chlorophyll content of leaves, length of inflorescent stem, and weight of seeds (data not shown). However, differences were found in the seedlings. Though the cotyledons of the *egy2* seedlings were as green as wild-type (Fig. 3), their hypocotyls were significantly shorter than wild-type ($P < 0.001$) (Table 2). Since mammalian S2Ps play important roles in regulation of sterol and fatty acid biosynthesis, we measured the fatty acid content in egy2 mutants by GCMS. Using nonadecanoic acid as an internal standard, the fatty acid composition and overall accumulation were compared between mutants and wild type. The composition of fatty acids was similar in mutants and wild-type. C18:1, C18:2 and C18:3 were the most prominent fatty acids, and C16:0, C20:1 and C18:0 were less, little C16:1, C16:2, C16:3, C20:0, C20:2 and C22:0 were also present. However, the overall fatty acid contents were slightly less in mutants seedlings ($0.05 > P > 0.001$) (Table 2) which suggests that EGY2 may influence the overall synthesis of fatty acids. To examine the in vivo function of EGY2 in seedlings further, total cellular proteins of seedlings were resolved by SDS-PAGE and probed with antibodies. Interestingly, in *egy2-1*, there was a decreased accumulation of several proteins involved in fatty acid biosynthesis, namely ACP1 (acyl carrier proteins), CAC2 (biotin carboxylase subunit of the plastidic acetyl-coenzyme A carboxylase (ACCase)) and BCCP1 (biotin carboxyl carrier protein, subunit of ACCase) (Fig. 4). These results suggest that the reduced amount of fatty acids in *egy2* seedlings may result from a deficiency in fatty acid biosynthesis.

EGY2 is localized in chloroplast membranes

According to the ChloroP software [26], EGY2 is a chloroplast protein with a N-terminal transit peptide of 64 amino acids. To corroborate its subcellular localization, a full-length EGY2-GFP fusion construct was generated under the control of the CaMV35S promoter and introduced into plants. Stable transgenic plants were obtained and examined with confocal laser microscopy. GFP

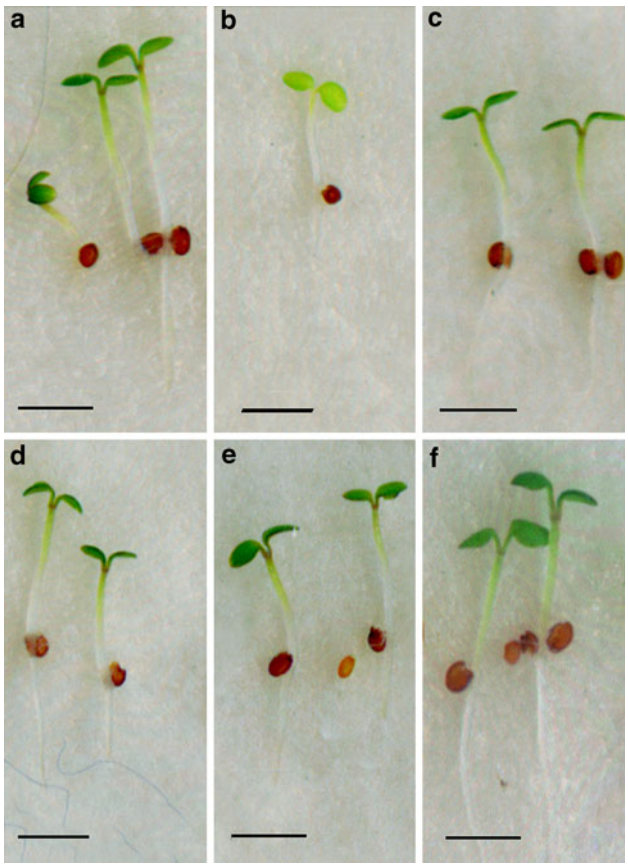

Fig. 3 Seedlings morphology of wild type (**a**), *egy1-2* (**b**), *egy2-1* (**c**), *egy2-2* (**d**), *egy2-3* (**e**), *egy2-4* (**f**). *Bars* represent 1 cm

fluorescence co-localized with red fluorescence from chlorophyll in the chloroplasts, confirming the chloroplast localization of EGY2 (Fig. 5a–c). In the control plants with the GFP construct, green fluorescence was found mainly at the plasma membrane and nucleus (Fig. 5d–f). A search of the Plant Proteome Database (PPDB, http://ppdb.tc.cornell.edu/) found that EGY2 was identified in the thylakoid membrane proteome in their experiments and supported by a previously reported study [27]. Thus, it was suggested that EGY2 resides in the chloroplast thylakoid membrane.

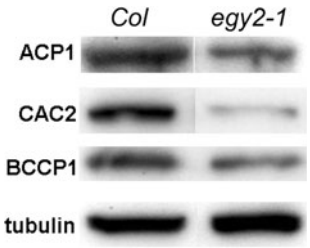

Fig. 4 Accumulation of enzymes involved in fatty acid biosynthesis in wild type and *egy2-1* mutants. Western blot detection of total protein extracts are from light-grown seedlings of wild type and *egy2-1*, for which equal amounts of protein were loaded. The blots were probed with anti-ACP1, anti-biotin and anti-α-tubulin antibodies. α-tubulin was used as the loading control

EGY2 is expressed throughout the plant except root

To investigate the expression pattern of *EGY2*, we conducted RT-PCR and GUS reporter-aided analysis of the promoter activities. *EGY2* transcripts were detected in seedlings, leaves, inflorescent stem and flowers, but few transcripts were detected in roots (Fig. 6). The semi-quantitative RT-PCR indicated that more *EGY2* transcripts were accumulated in the light-grown seedlings than in the dark-grown seedlings, which suggests that EGY2 may play a role in the light-induced seedling development. When compared with *EGY1*, *EGY2* shows similar expression patterns in the organs analyzed, and both transcripts were up-regulated in the presence of light (Fig. 6).

The *EGY2* promoter::*β*-glucuronidase (GUS) construct was introduced into wild-type *Arabidopsis*. The Pro_{EGY2}::GUS transformed plants show clear GUS staining in their cotyledons, hypocotyls, leaves, stems and flowers, while staining was barely detectable in roots and siliques (Fig. 7a). These results are consistent with the accumulation of transcripts that was detected by RT-PCR. The Pro_{EGY1}::GUS transformed plants show similar GUS staining patterns to the Pro_{EGY2}::GUS transformed plants (Fig. 7b). These results suggest that, similar to *EGY1*, the *EGY2* gene is constitutively and ubiquitously expressed in plant tissues, except little in roots.

Table 2 Characterization of *egy2* mutants

	Wild type	*egy2-1*	*egy2-2*	*egy2-3*	*egy2-4*
Hypocotyl length (cm)	2.14 ± 0.05	1.83 ± 0.03	1.86 ± 0.03	1.80 ± 0.03	1.89 ± 0.03
	(n = 104)	(n = 225)	(n = 185)	(n = 86)	(n = 130)
P-value		2.07×10^{-8}	2.71×10^{-7}	3.31×10^{-7}	4.30×10^{-5}
Fatty acid content (μg/mg fresh weight)	2.76 ± 0.21	1.71 ± 0.09	1.81 ± 0.14	1.84 ± 0.07	1.80 ± 0.09
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)
P-value		0.021	0.038	0.029	0.028

Hypocotyl length of 6-day-old light-grown seedlings on paper towels was measured. Fatty acid content of 6-day-old light-grown seedlings on paper towels was measured. Measurements were mean ± SE; *P*-value is *t*-test probability between wild-type and mutants

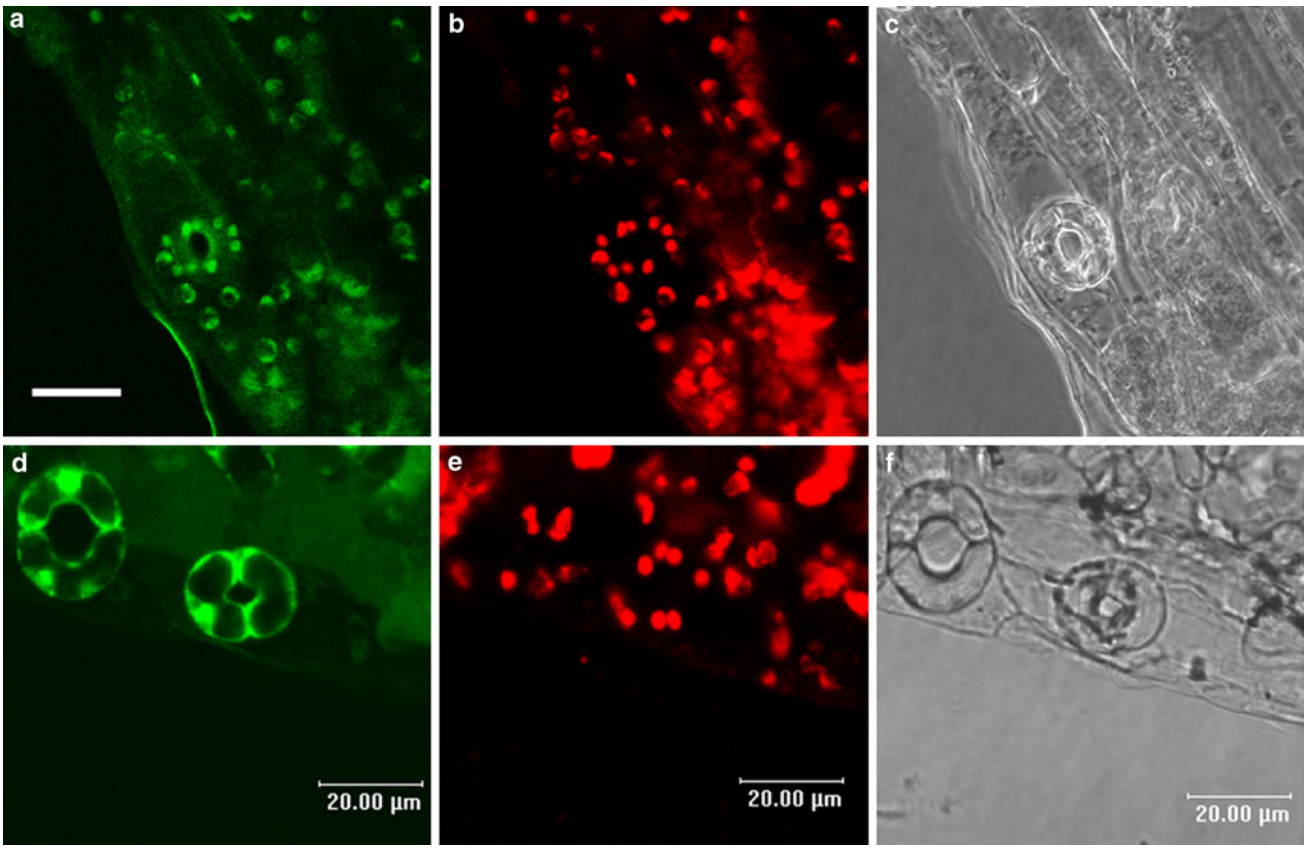

Fig. 5 Targeting of EGY2-GFP fusion protein to chloroplasts. **a–c** Show the subcellular localization of EGY2-GFP fusion protein in transgenic *Arabidopsis*. **d–f** Show the GFP localization in the control plant. The epidermal cells from the seedlings were used for confocal laser microscopy examination. Images of GFP (**a**, **d**) and chlorophyll (**b**, **e**) are shown in *green* and *red*, respectively. Bright field images (**c**, **f**) are in *black* and *white*. *Bars* represent 20 µm. (Color figure online)

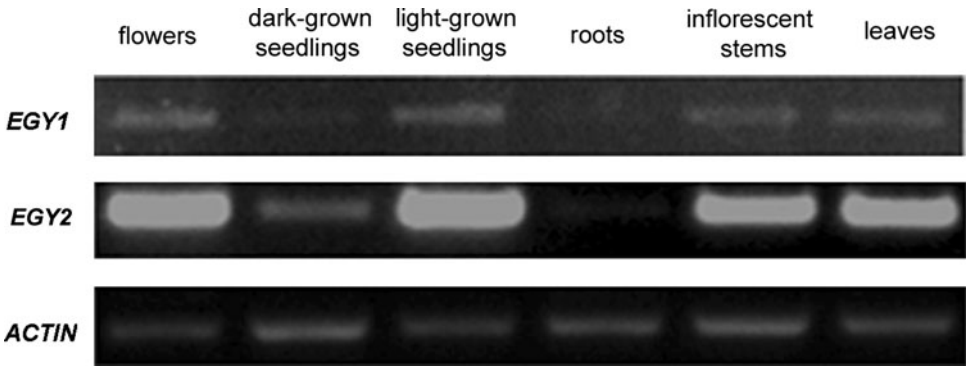

Fig. 6 Expression of *EGY1* and *EGY2*. RNA transcripts accumulation of *EGY1* and *EGY2* were analyzed by semi-quantitative RT-PCR. The light-grown seedlings exhibit higher levels of transcripts of both genes than dark-grown seedlings. *EGY1* and *EGY2* have similar distribution patterns in the organs analyzed. Transcripts of these two genes were found in leaves, inflorescent stem and flowers, but were seldom found in root. Expression of *ACTIN* was used as control

Recombinant EGY2 displays metalloprotease activity in vitro

To investigate the metalloprotease activity of EGY2, a GST-EGY2 construct was overexpressed in *E. coli*, and the expressed fusion protein was tested against β-casein. Recombinant EGY2 cleaved β-casein into smaller fragments in an ATP-independent manner (Fig. 8). And the caseinolytic activity was not inhibited by the serine and cysteine protease inhibitors, but was blocked by *o*-phenanthroline, a metalloprotease inhibitor (Fig. 8). As a control, GST expressed and purified through the same

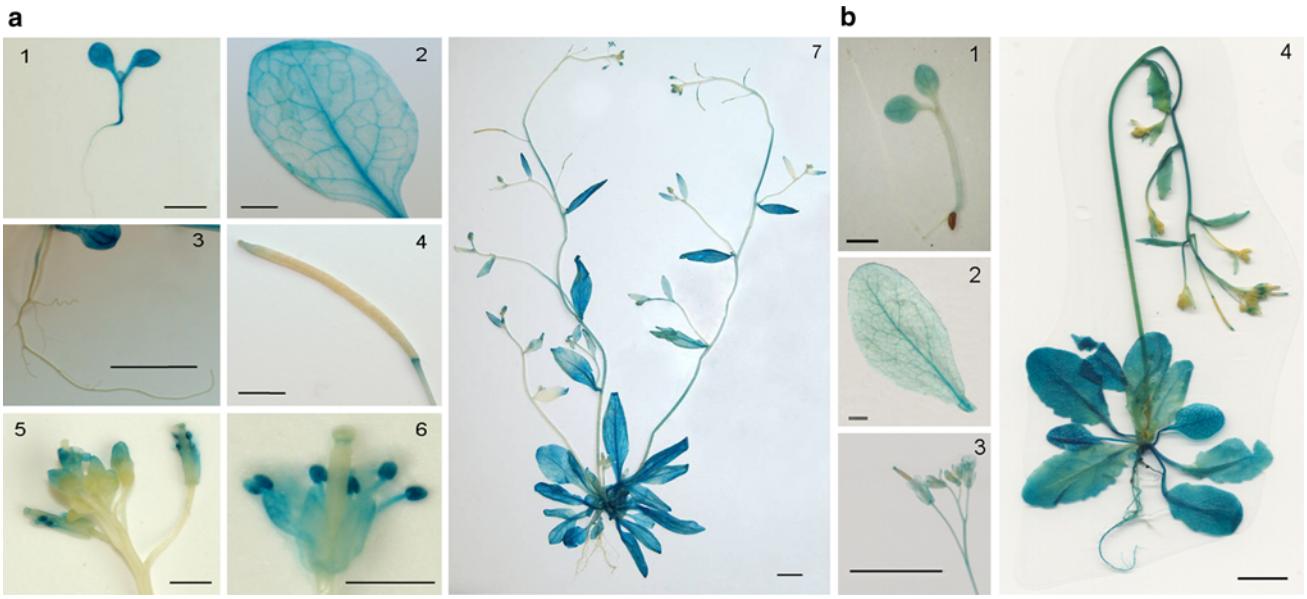

Fig. 7 Histological study of *EGY1* and *EGY2* promoter activity by promoter-GUS fusion construct transformed *Arabidopsis*. **a** In the transformed plants, when GUS expression was driven by the *EGY2* promoter, GUS staining was clearly found in cotyledons and hypocotyls of seedlings (*1*), leaves (*2, 7*), flowers (*5, 6*) and inflorescent stems (*7*), but little staining was found in roots (*3*) and siliques (*4*). *Bars* represent 2 mm in 1, 2, 4, 5 and 6, and bars represent 10 mm in 3 and 7. **b** When GUS expression was driven by the *EGY1* promoter in the plants, GUS staining was similarly found in the cotyledons and hypocotyls of seedlings (*1*), leaves (*2, 4*), flowers (*3*) and inflorescent stems (*4*). *Bars* represent 1 mm in 1, 2 mm in 2 and 10 mm in 3 and 4

process could not cleave β-casein, which precludes the possibility that this metalloprotease activity is resulted from contamination of *E. coli* proteases. When the GST-EGY2 fusion protein was processed, intermediate products and GST were also detectable (Fig. 8).

Discussion

Data from molecular, cellular, genetic and physiological experiments have elucidated that a novel nuclear-encoded plastid-resident metalloprotease, EGY2, plays a role in hypocotyl elongation. EGY2 was identified by its primary sequence similarity to EGY1[18]. EGY2 does show higher structural similarity to EGY1 with multiple transmembrane helices as well as the localization of conserved motifs HEXXH and NPDG (Fig. 1). The EGY2 protein is localized in the chloroplast thylakoid membrane, and transcript accumulation parallels EGY1 (Figs. 5, 6, 7). An in vitro assay against β-casein confirmed its metalloprotease activity (Fig. 8). However, four independent *egy2* knockout plants display different phenotypes from *egy1* mutants, most obviously as the green cotyledons (Fig. 3), which indicate that EGY2 has its own unique biochemical properties in plant cells.

In seedlings, the reduced amount of enzymes involved in fatty acid biosynthesis (Fig. 4) may partly explain the reduced accumulation of fatty acids in *egy2* knockout plants (Table 2). As construction blocks, slightly less fatty acid content in seedlings may contribute to the significantly shorter hypocotyls, though it may not be the only contributor. How is the decrease in enzymes involved in fatty acid synthesis related to the absence of EGY2 metalloprotease activity in plastids? Plastids are known to be an important site for lipid biosynthesis [28]. S2P identified in human and hamster was found to regulate the cholesterol and fatty acid biosynthesis. In response to low levels of cellular cholesterol, SREBPs are cleaved by S1P and S2P sequentially so that their amino-terminal transactivation domain are released and translocated into the nucleus, where they induces expression of genes that control synthesis and uptake of sterol and fatty acids [10]. If a similar situation exists for EGY2, then its absence in seedlings of a knockout mutant would result in a reduced amount of active transcription factors available to activate downstream genes controlling fatty acid synthesis. However, this speculation needs to be verified by further experiments.

Though EGY2 RNA transcripts were detected throughout the plant except in the root (Figs. 6, 7), no obvious phenotype has been observed in the adult plants of knockout mutants thus far. This observation indicates that other proteases in adult plants may compensate for the missing EGY2, or EGY2 has condition-specific roles in

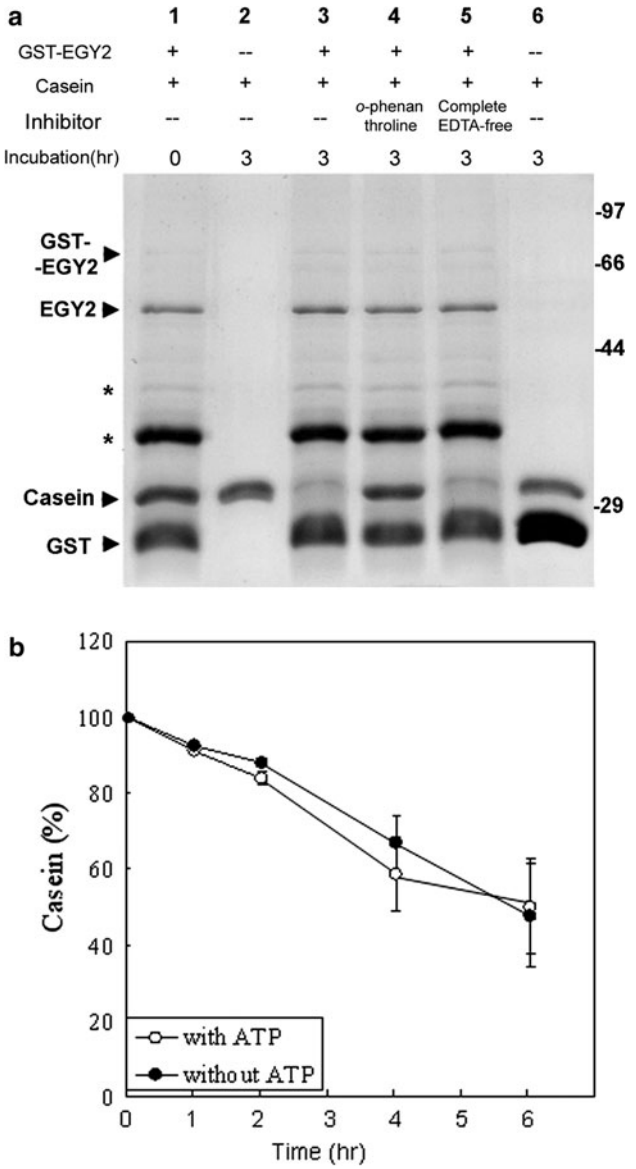

Fig. 8 Metalloprotease activity assay. **a** Assay for the in vitro metalloprotease activity of EGY2. *Lane 1*, reaction mixture of GST-EGY2 and *β*-casein prior to incubation; *lane 2*, in the absence of GST-EGY2, the substrate *β*-casein remained intact in the reaction buffer after 3 h of incubation; *lane 3*, with GST-EGY2, the substrate *β*-casein was cleaved significantly in the reaction mixture after 3 h of incubation; *lanes 4 and 5* indicate the reaction mixture as in *lane 3*, but the mixture includes 10 mM *o*-phenanthroline and Complete EDTA-free (a cocktail of serine and cysteine protease inhibitors), respectively; and *lane 6* shows a control reaction mixture of casein and GST, which is expressed and purified from the same systems as GST-EGY2. The initial amount of casein was 1.25 μg. The *short arrows* indicate the locations of GST-EGY2, EGY2, casein and GST, and the *asterisks* indicate partially cleaved GST-EGY2. **b** Quantification of casein remnants during the 6 h of incubation with GST-EGY2 in the presence (*open circle*) or absence (*closed circle*) of ATP. The initial amount of casein was 2.5 μg. The initial amount of casein was defined as 100%, and the means plus the standard deviations from three independent experiments are shown

plant growth and development. It is also possible that EGY2 is post-transcriptionally regulated.

Further questions arising from this study concern the relationship between EGY1 and EGY2, as well as other S2P homologs. Similar spatial expression patterns of *EGY1* and *EGY2* (Figs. 6, 7) and their localization in chloroplast thylakoid membranes suggest that EGY1 and EGY2 may have a close relationship in plant growth and development which remains to be clarified. Another interesting question for further study is to identify substrates of EGY2. Substrates of EGY1 and AraSP are still elusive. A genome-scale screening of membrane-bound transcription factors (MTF) identified at least 85 MTFs in *Arabidopsis*, which are proposed to be regulated by controlled proteolytic activation in response to various environmental changes [29]. These MTFs provide a good reservoir for substrate candidates of EGY1 and EGY2, as well as other intramembrane proteases. When we were preparing this manuscript, one S2P homolog, At4g20310 were reported to cleave transcription factors bZIP17 and bZIP28 in Golgi. The proteolytically released transcription factors then translocate to the nucleus to active Brassinosteroids (BR) signaling and promote acclimation to stress [30]. Since EGY1 and EGY2 reside in chloroplasts, MTF of plastid RNA polymerases would be the better candidates of their substrates. The exact substrates of EGY1 and EGY2 remain the most important and hardest question to be resolved.

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