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SHORT COMMUNICATION

Localization and expression of serine racemase in *Arabidopsis thaliana*

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Abstract *Arabidopsis* plants transformed by promoter of *A. thaliana* serine racemase fused with β-glucuronidase (GUS) reporter gene showed strong GUS staining in elongating and developing cells such as tip regions of primary and lateral roots, developing leaves, and shoot meristems. RT-PCR and digital northern hybridization showed that expression of the serine racemase gene was not induced by L- and D-serine, light irradiation, biotic and abiotic stresses.

Keywords Serine racemase · D-amino acid · *Arabidopsis thaliana* · GUS · Promoter

Introduction

Mammalian and plant serine racemases are the bifunctional enzymes that catalyze not only the racemization of serine but also the dehydration of serine to pyruvate, showing the difference from bacterial amino acid racemase (Wolosker et al. 1999; de Miranda et al. 2000, 2002; Strisovsky et al. 2003; Foltyn et al. 2005; Fujitani et al. 2006, 2007). Mammalian serine racemase is distributed in neurons of the brain (Kartvelishvily et al. 2006; Dumin et al. 2006; Yoshikawa et al. 2007), and controls the level of D-serine, which acts as an agonist at the glycine site of *N*-methyl-D-aspartate receptor in the mammalian nervous system (Dunlop et al. 1986; Nagata et al. 1994; Hashimoto et al. 1992; Mothet et al. 2000). Plant serine racemases from *Arabidopsis*, barley, and rice showed both racemase and dehydratase activities, but reaction specificity of dehydration was about 20-fold higher than that of racemization in contrast to the dehydration/racemization ratio of about 0.7 for mouse serine racemase (Fujitani et al. 2006, 2007). In plants, a number of D-amino acids have been detected (Robinson 1976; Bruckner and Weshauser 2003), but growth of *Arabidopsis* was inhibited by 0.5 mM D-serine and 1 mM D-alanine but not by 15 mM D-valine and 30 mM D-isoleucine, suggesting that D-amino acid levels, especially showing toxicity on organisms, should be severely regulated in plants (Erikson et al. 2004). Furthermore, there is no report of a plant D-amino acid oxidase, and serine racemase is the only known enzyme that degrades D-serine in plants, suggesting that the main physiological function of plant serine racemase might be to degrade serine to reduce D-serine levels. In this study, we show that *Arabidopsis thaliana* serine racemase (AtSR) gene is expressed in elongating and developing cells without regulation of biotic and abiotic stresses in *Arabidopsis* plants transformed by AtSR promoter-GUS reporter gene, as the first report on the localization and expression of plant serine racemase.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana*, ecotype Columbia, and transgenic *A. thaliana* were soaked in water at 4°C

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overnight and sterilized by incubation for 10 min in 2% hypochlorite containing 0.01% Tween-20 followed by rinsing five times with sterilized water. The sterilized seeds were germinated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962), of which the composition is 440 mg/l $CaCl_2$, 0.025 mg/l $CoCl_2$, 0.025 mg/l $CuSO_4$, 36.7 mg/l FeNa-EDTA, 6.2 mg/l H_3BO_3, 170 mg/l KH_2PO_4, 0.83 mg/l KI, 1.9 g/ml KNO_3, 370 mg/l $MgSO_4$, 22.3 mg/l $MnSO_4$, 0.25 mg/l Na_2MoO_4, 1.65 g/l NH_4NO_3, 8.6 mg/l $ZnSO_4$, 30 g/l sucrose, 0.5 mg/l nicotinic acid, 0.1 mg/l thiamin, and 0.5 mg/l pyridoxine, pH 5.8, and grown in a growth chamber under a light/dark cycle of 16 h/8 h at 23°C.

Plasmid construction

A DNA fragment comprising the promoter and the 5′ end of the coding region of AtSR gene was amplified from genomic DNA by PCR with specific sense and antisense primers, 5′-ACGTATGCGTCGACGTGTTTTTCCACC-3′, which creates a *Sal*I site (underlined), and 5′-TCGGATCCATAC TTCTCTCTATTTGCT-3′, which creates a *Bam*HI (underlined), respectively, designed on the basis of the *Arabidopsis* genome sequence (accession no. AL049500). The amplified fragment of 1,488 bp was cloned into the pGEM-T vector (Promega) and the *Sal*I- and *Bam*HI-digested fragment of the plasmid was cut out and subcloned into a *Sal*I- and *Hin*dIII-digested pBI101 vector. The resulting plasmid, pAtSRPro:GUS, was transformed into *Arabidopsis* plants by vacuum infiltration using *Agrobacterium tumefaciens* culture, and transgenic plants were selected and cultured on MS medium containing 50 μg/ml of kanamycin.

Identification of GUS activity

Plant staining for β-glucuronidase (GUS) activity was performed with 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) as a substrate (Jefferson et al. 1987). Transgenic plants harboring pAtSRPro:GUS were soaked in X-Gluc solution (2 mM X-Gluc in 50 mM sodium phosphate buffer, pH 7.2) containing 0.5 mM potassium ferrocyanide and 0.5 mM potassium ferricyanide for accelerating the formation of intensely blue product (indigo) and minimizing the diffusion of indigo, incubated for 1 day at room temperature, and followed in 99.5% ethanol.

Semi-quantitative RT-PCR

Total RNA was isolated from *A. thaliana* samples with the RNeasy Plant Mini Kit (Qiagen) and first strand cDNA was synthesized from 1 μg of total RNA in a volume of 20 μl with the PrimeScript first strand cDNA Synthesis Kit (Takara) according to the manufacture's instruction. PCR was performed in a mixture of 50 μl containing 1 μl of first strand cDNA, 0.4 mM dNTPs, 1×PCR buffer, 2.5 U Ex-Taq HT (Takara), and 10 pmol each of 5′-TCAGGTA TGGTGGTAAGGTTATATGGA-3′ and 5′-TCCACTTGG TTCCACAGAGACCTTCAG-3′ for AtSR gene and 5′-AC ATCACCGAGTTCCAGACCAACCTTG-3′ and 5′-TCAG AGAACTCTCCTTCTTCCATACCC-3′ for α-tubulin gene. The thermal cycle profile was 1cycle of 94°C for 1 min and followed by 30 and 25 cycles for AtSR and α-tubulin genes, respectively, of 94°C for 15 s, 60°C for 15 s, 68°C for 1 min. Reaction mixture (3 μl) was loaded on agarose gel electrophoresis and amplified fragments of 502 and 510 bp, which correspond to nucleotide 356–855 of AtSR gene (accession no. AB206823) and nucleotide 752–1259 of α-tubulin gene (accession no. AY091372), respectively, were detected by ethidium bromide staining. The concentration of first strand cDNA from each sample was adjusted after calibration of the α-tubulin gene level by PCR.

Results and discussion

We have shown previously that AtSR gene was expressed in root, rosette, shoot, and inflorescence of *A. thaliana* by northern hybridization (Fujitani et al. 2006). In order to investigate the tissue-specific expression profile of AtSR gene in detail, the transgenic *Arabidopsis* plants were generated by the plasmid pAtSRPro:GUS, in which 1,488-bp fragment covering from the end of the upstream gene (AT4g11630) to the start codon of AtSR gene (AT4g11640) was fused with the GUS reporter gene. In 14-day-old plants of kanamycin-resistant lines from T2-generated seeds, strong GUS staining was detected in tip regions of primary and lateral roots and in developing leaves (Fig. 1a, b). In 19-day-old plants, GUS stains remained substantially in root tips and shoot meristems, whereas in leaves they were not as intense as in that in 14-day-old plants and limited to leaf vein and petioles (Fig. 1c). These results show that AtSR gene is predominantly expressed in meristematic cells. We have demonstrated that dehydration catalyzed by serine racemase from *Arabidopsis*, barley, and rice was about 20-fold higher than racemization (Fujitani et al. 2006, 2007), suggesting that plant serine racemase dehydrates serine to produce pyruvate rather than produce the other serine enantiomer. Preferential expression of AtSR in meristem area suggests that plant serine racemase might play a role in supplying additional energy material to cells by producing pyruvate from L-serine in such highly deviding cells.

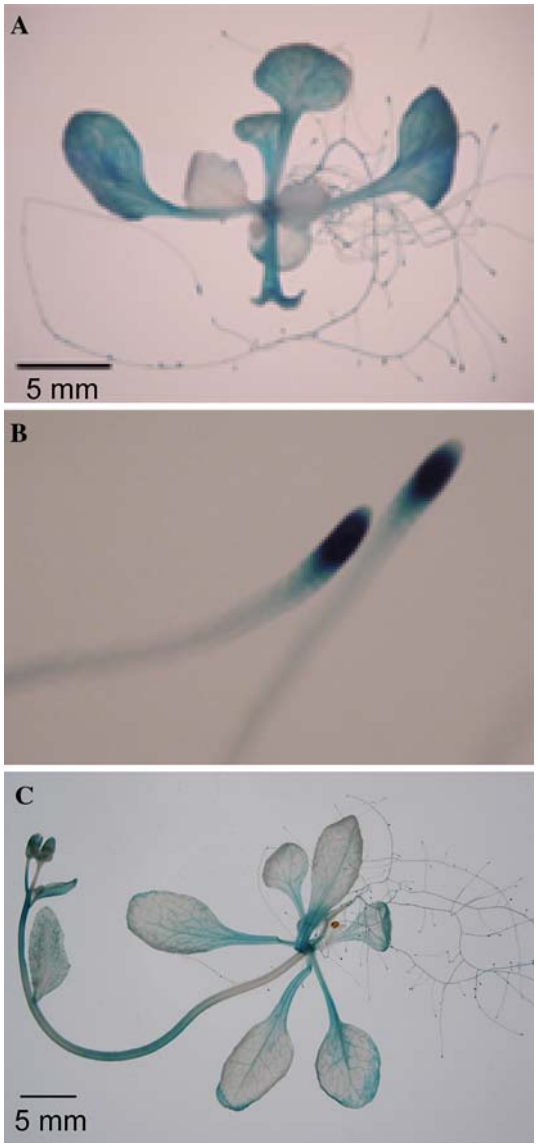

Fig. 1 GUS staining of transgenic *Arabidopsis* plants harboring pAtSRPro:GUS. Transgenic plants were grown on MS medium containing kanamycin for 14 days (**a** whole seedling, **b** lateral root) and 19 days (**c** whole seedling)

It has been reported that D-alanine was incorporated into D-alanylglycine when D-alanine was fed to rice leaf blades during the day and D-alanylglycine content in rice leaf blades increased by light irradiation but not in the dark (Manabe and Ohira 1980, 1983). In order to investigate the effect of D-amino acid and light irradiation on the expression level of AtSR gene, semi-quantitative RT-PCR was performed to obtain expression profiles. *Arabidopsis* seeds were germinated and cultured for 18 days on MS medium with 0.1 mM L- or D-serine, of which the concentration is not affected on the *Arabidopsis* growth. As shown in Fig. 2a, the expression level of AtSR mRNA by supplying L-serine was similar to that by D-serine in roots, and similar expression levels were observed between in leaves grown under L- and D-serine. To demonstrate whether AtSR gene expression is under control of light irradiation, *Arabidopsis* seeds were germinated and grown in the dark and the expression level was compared to that from *Arabidopsis* plants grown under a light/dark cycle of 16 h/8 h. Semi-quantitative RT-PCR showed that the AtSR gene level in shoots under the light was the same as that in the dark, showing no effect on the expression by light irradiation (Fig. 2b). Digital northern hybridization by *Arabidopsis* microarray database, GENEVESTIGATOR (Zimmermann et al. 2004), showed that the expression level of AT4g11640 gene was not changed significantly not only by light irradiation but also light quality such as blue, red, far red, white, UV-A, and UV-AB, of which the ratios with/without treatment were 1.06, 1.09, 0.96, 1.12, 1.02, 0.99, and 1.08, respectively. Furthermore, signal strength of AT4g11640 gene on any anatomy and development stages of *A. thaliana* was not changed significantly by plant hormones such as ABA, GA3, IAA, BL, MJ, and environment stresses such as cold, drought, heat, osmotic, oxidative, and salt stresses, of which the ratios with/without treatment were 0.78, 1.13, 1.04, 1.04, 0.89, 0.81, 0.97, 0.92, 0.84, 0.92, and 0.94, respectively.

In summary, this study demonstrates that AtSR gene was expressed significantly in younger leaves, root cap, shoot apices and inflorescence, suggesting that AtSR might contribute to cell proliferation and cell differentiation. Our results are the first clue to clarify the physiological function of plant serine racemase. It has been reported that D-aspartate level was increased transiently during

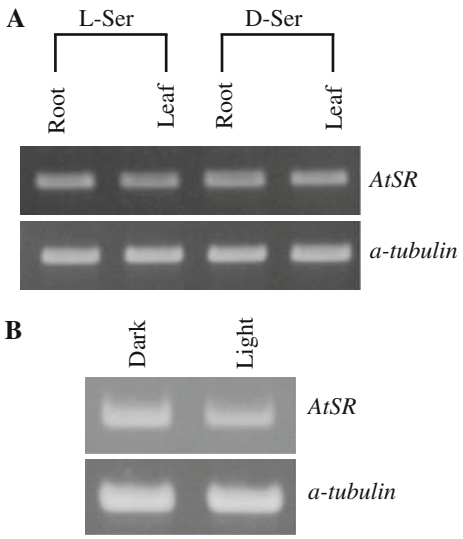

Fig. 2 Expression of AtSR gene in *A. thaliana*. Total RNAs isolated from root and leaf of *A. thaliana* grown on MS medium containing 0.1 mM L- or D-serine (**a**) and from shoot grown under 16 h lighting or dark (**b**) were subjected to semi-quantitative RT-PCR

germination and early stage of growth in *A. thaliana*, exogenous D-aspartate was taken up into *A. thaliana* to be converted to D-glutamate and D-alanine, and D-amino acid aminotransferase (D-AAT) from *A. thaliana* catalyzes transamination between D-amino acids, suggesting the presence of D-amino acid metabolism in plants (Funakoshi et al. 2008). However, the physiological roles of D-aspartate, D-glutamate, D-alanine, and D-AAT in plants are unknown. D-Serine and D-aspartate are the components related to the neural and endocrine function in mammals and D-alanine is the most potent osmolyte for intracellular isosmotic regulation in crustaceans and several bivalve mollusks (Abe et al. 1999; Fujimori and Abe 2002). Construction of transgenic *Arabidopsis* plants, in which AtSR gene is overexpressed or destroyed, is in progress to find not only the influence of AtSR on growth phenotype, root and shoot phenotype, flower morphology, and fertility of plant but also the physiological and biological function of D-serine in plants.

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