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# Serine racemases from barley, Hordeum vulgare L., and other plant species represent a distinct eukaryotic group: gene cloning and recombinant protein characterization

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**Serine racemases from barley, *Hordeum vulgare* L., and other plant species represent a distinct eukaryotic group: gene cloning and recombinant protein characterization** ¶

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(Footnotes)

*Abbreviations:* PLP, pyridoxal 5-phosphate; PCR, polymerase chain reaction; IPTG, isopropyl-ß-D-thiogalactopyranoside.

¶ The sequence reported in this paper has been deposited in the DDBJ, EMBL, and GenBank data banks under the accession no. **AB271213**.

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**Abstract**

Several D-amino acids have been identified in plants. However, the biosynthetic pathway to them is unclear. In this study, we cloned and sequenced a cDNA encoding a serine racemase from barley which contained an open reading frame encoding 337 amino acid residues. The deduced amino acid sequence showed significant identity to plant and mammalian serine racemases and contained conserved pyridoxal 5-phosphate (PLP)-binding lysine and PLP–interacting amino acid residues. The purified gene product catalyzed not only racemization of serine but also dehydration of serine to pyruvate. The enzyme requires PLP and divalent cations such as $Ca^{2+}$, $Mg^{2+}$, or $Mn^{2+}$, but not ATP, whereas mammalian serine racemase activity is increased by ATP. In addition to the results regarding the effect of ATP on enzyme activity and the phylogenetic analysis of eukaryotic serine racemases, the antiserum against *Arabidopsis* serine racemase did not form a precipitate with barley and rice serine racemases. This suggests that plant serine racemases represent a distinct group in the eukaryotic serine racemase family and can be clustered into monocot and dicot types.

*Keywords: Hordeum vulgare* L.; *Oryza sativa*; Gramineae; Pyridoxal 5-phosphate; Serine racemase; Serine dehydratase; D-Amino acid

## 1. Introduction

Serine racemase, which catalyzes racemization of L- and D-amino acids, is found in mammals as a biosynthetic enzyme for D-serine (**1a**), with the latter acting as an agonist at the glycine site of the N-methyl-D-aspartate receptor in the mammalian nervous system (Dunlop et al., 1986; Nagata et al., 1989; Hashimoto et al., 1992; Hashimoto et al., 1993; Nagata et al., 1994; Hashimoto et al., 1995). Mammalian serine racemase is an unique enzyme that catalyzes not only serine (**1a**, **1b**) racemization but also dehydration of **1a** and **1b** to pyruvate (**2**) in the presence of divalent cations; this distinguishes it from bacterial amino acid racemases (Cook et al., 2002; De Miranda et al., 2002; Strisovsky et al., 2003; Foltyn et al., 2005) (Fig. 1). In plants, several D-amino acids have been discovered in pea seedlings (Ogawa et. al., 1977), barley grain, hops blossoms (Erbe and Bruckner, 2000), and tobacco leaves (Kullman et al., 1999). Recently, a serine racemase gene was cloned from *Arabidopsis thaliana* and expressed to produce a protein that has both racemase and dehydratase activities (Fujitani et al., 2006). This gene also has 46 and 45% identity to human and mouse serine racemases, respectively, and the pyridoxal 5-phosphate (PLP)-binding domain is conserved as with mammalian serine racemases. The PLP-dependent *A. thaliana* serine racemase also requires divalent cations but not ATP, whereas there is synergy between $Mg^{2+}$ and ATP in the activity of mammalian serine racemases. These reports suggest that this plant serine racemase is distinct from mammalian serine racemases. However, there has been no report on the cloning and characterization of plant serine racemases except for that in *A. thaliana*.

In this report, we describe the isolation and characterization of a cDNA encoding serine racemase from barley. In addition, we obtained a rice serine racemase by cloning a cDNA encoding a homolog of barley serine racemase and compared the structure-function relationships of plant and mammalian serine racemases.

## 2. Results

### *2.1. Sequence analysis of barley serine racemase*

The resulting nucleotide sequence of the barley serine racemase cDNA, except for the poly(A) sequence, is 1380 bp in length and contains an open reading frame encoding a polypeptide of 337 amino acid residues with a calculated molecular mass of 35.7 kDa (HvSR, **AB271213**). The deduced amino acid sequence of HvSR

showed 89% identity with that of a putative rice serine racemase in data banks (OsSR, **AL606647**), and 68, 46, 45, and 28% identity with a serine racemase from *A. thaliana* (AtSR, **AB206823**, Fujitani et al., 2006), as well as human (hSR, **AF169974**, De Miranda et al., 2000) and mouse (mSR, **AF148321**, Wolosker et al., 1999) serine racemases, and catalytic domain of L-threonine dehydratase from *Escherichia coli* (EcTD, Swiss-Plot no. **P04969**, Gallagher, et al., 1998), respectively. The amino acid sequence alignment (Fig. 2) shows that plant and mammalian serine racemases belong to the fold type II PLP enzymes and that Lys62 which binds to PLP, Phe61 and Gly241, which sandwich the PLP ring, Ser315, the side-chain of which is hydrogen-bonded to the pyridium nitrogen of PLP, and Asn89, which stabilizes the 3' oxygen of PLP by a hydrogen bond in the PLP-binding domain of *E.coli* threonine dehydratase, a typical fold type II PLP enzyme, correspond to Lys67, Phe66, Gly246, Ser322, and Asn94 of HvSR, respectively, and all of these residues were conserved in rice, *A. thaliana*, human, and mouse serine racemases. The glycine-rich loop which coordinates the phosphate group of PLP is composed of a triglycine loop (Gly194–196 of HvSR) in the three plant serine racemases known it contains a tetraglycine loop in human and mouse serine racemases. The phylogenetic tree presented shows that plant serine racemases represent a distinct group from mammalian serine racemases, and that the monocot serine racemases of barley and rice are distinct from the dicot serine racemase of *A. thaliana* (Fig. 3).

*2.2. Purification and enzyme activity of recombinant barley serine racemase*

SDS-PAGE analysis of *E. coli* cells harboring pHvSR contained an extra protein with the expected molecular mass of ca. 36 kDa, the expression level of which reached ca. 10% of the total protein after 8 h of isopropyl-ß-D-thiogalactopyranoside (IPTG) induction. Purification of recombinant HvSR by Ni-NTA column chromatography yielded ~95% pure preparation (Fig. 4). The purified HvSR was incubated with 5 μM PLP and 1 mM $CaCl_2$ which activates AtSR and mammalian serine racemases (Cook et al., 2002; De Miranda et al., 2002; Strisovsky et al., 2003), and the formation of **1a** and **2** was determined. The specific activities for racemization of L-serine (**1b**) to **1a,** and dehydration of **1b** to **2,** were 14 and 228 nmol/min/mg, respectively, and those for racemization of **1a** to **1b,** and dehydration of **1a** to **2,** were 2.2 and 89 nmol/min/mg, respectively

The purified rice serine racemase expressed in *E.coli* cells harboring pOsSR, had a molecular mass ~36 kDa, as estimated by both the deduced amino acid sequence and SDS-PAGE, respectively. It was also purified by Ni-NTA column chromatography (Fig. 4), and displayed racemase and dehydratase activities for **1b** of 10 and 214 nmol/mg/min, respectively, and those for **1a** of 1.5 and 77 nmol/mg/min, respectively

*2.3. Enzymatic characterization of recombinant barley serine racemase*

A maximum at 420 nm in the absorption spectrum of the purified HvSR disappeared after dialysis against a buffer containing 10 mM sodium borohydride with a loss of enzyme activity (Fig. 5). Additionally, the enzyme activity of HvSR was inhibited completely by 0.1 mM hydroxylamine (data not shown), indicating that HvSR depends on PLP and is bound to PLP through a Schiff base (Grishin et al., 1995).

The optimum pH and concentration of $CaCl_2$ in the racemization and dehydration of **1b** were examined in a reaction mixture containing 10 μM PLP. HvSR has maximum activities for both racemization and elimination at pH 8.5 in the presence of 1mM $CaCl_2$.

The racemase activities for converting various **1b** to **1a** acids were examined in a reaction mixture containing 1 mM $CaCl_2$ at pH 8.5. HvSR showed high substrate specificity with regard to **1b**, 14 nmol/mg/min, whereas L-alanine, L-glutamine, and L-arginine were poorer substrates: the activities were 1.9, 0.74, and 1.7 nmol/mg/min, respectively. The $K_m$ and $V_{max}$ were estimated in a reaction mixture containing 1 mM $CaCl_2$, 10 μM PLP at pH 8.5, with corresponding values for converting **1b** to **1a** being 2.6 mM and 21 nmol/mg/min, and for **1a** to **1b** of 9.0 mM and 455 nmol/mg/min, respectively. Additionally the $K_m$ and $V_{max}$ for dehydratase activity from **1b** to **2** were 2.7 mM and 2.8 nmol/mg/min, whereas those of **1a** to pyruvate were 8.3 mM and 161 nmol/mg/min, respectively.

*2.4 Effects of divalent cations and ATP*

The effects of divalent cations and ATP on **1b** racemization and dehydration activities were analyzed by adding 1 mM divalent ion to a reaction mixture at pH 8.5 (Fig. 6). Racemase activity was increased 2.9-, 3.8-, and 3.8-fold by addition of $Mg^{2+}$, $Ca^{2+}$, and $Mn^{2+}$, whereas EDTA, $Fe^{2+}$, or $Ni^{2+}$ inhibited this activity. The addition of $Mg^{2+}$, $Ca^{2+}$, and $Mn^{2+}$ also increased dehydratase activity 13-, 11-, and 13-fold, respectively, whereas $Fe^{2+}$ showed only a 2.8-fold increase and $Ni^{2+}$ inhibited the activity. HvSR was not activated by 1mM ATP, and the activity with the combination of 1mM $Mg^{2+}$and 1mM ATP was slightly decreased compared to that in the presence of 1mM $Mg^{2+}$.

*2.5 Immunological properties*

An immunological comparison among plant serine racemases was performed by Ouchterlony double diffusion (Fig. 7). Anti-HvSR serum formed one line not only with purified recombinant HvSR but also with purified recombinant OsSR and AtSR; however, anti-AtSR serum formed a precipitate with AtSR and no line was detected with HvSR and OsSR.

## 3. Discussion

We demonstrated that barley serine racemase, HvSR, is a bifunctional PLP-dependent enzyme that catalyzes not only racemization of **1a** and **1b** but also dehydration of **1a** and **1b** to **2**. The enzyme activity of HvSR and *A. thaliana* serine racemase, AtSR, are enhanced by addition of divalent cations such as $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$. $Fe^{2+}$ inhibits racemase activity of HvSR whereas that of AtSR is slightly increased by addition of $Fe^{2+}$ (Fujitani et al., 2006). While the combined effect of $Mg^{2+}$ and ATP on mouse serine racemase leads to an increase in **1a** production, there was no synergy between $Mg^{2+}$ and ATP on HvSR activity, as with AtSR. These results suggest that the protein structure interacting with ATP might be different between plant and mammalian serine racemases. The amino acid residues that interact with PLP (Phe61, Lys62, Asn89, Gly241, Ser315) are conserved in HvSR, with other plant and mammalian serine racemases, which belong to the fold type II group of PLP enzymes (Gallagher et al., 1998), except for the glycine-rich loop, which consists of a triglycine and a tetraglycine in plant and mammalian serine racemases, respectively. These results show that plant serine racemase represents a distinct group in the eukaryotic serine racemase family.

The amino acid sequence identities of HvSR with OsSR and AtSR (89 and 68%, respectively) and the phylogenetic tree suggest that plant serine racemases can be clustered into monocot and dicot types. To show the structural differences between serine racemases of monocot and dicot plants, we prepared anti-HvSR and anti-AtSR sera, and then purified recombinant rice serine racemase with both racemase and dehydratase activities: the latter was obtained by expression of an open reading frame of the putative rice serine racemase gene in DDBJ, EMBL, and GenBank data banks. The specific cross-reactivity of the anti-AtSR serum against AtSR by the Ouchterlony test supports the possibility that there may be monocot and dicot clusters in plant serine racemase based on a comparison of the three plant enzymes examined so far.

Recently, an aspartate racemase gene was cloned from a bivalve mollusk and expressed to characterize its

enzyme properties (Abe et al., 2006). The aspartate racemase is a PLP-dependent enzyme and has both racemase and dehydratase activities. The amino acid sequence shows 38, 39, 32, 30, and 36 % identities to human, mouse, barley, rise, and *A. thaliana* serine raceases, respectively, and contains conserved PLP-interacting amino acid residues, corresponding to Phe61, Lys62, Asn89, Gly241, and Ser315 of *E. coli* threonine dehydratase. Interestingly, its enzyme activity was decreased by ATP but not by EDTA, whereas mammalian serine racemases was activated by both divalent cations and ATP. In contrast to the eukaryotic racemases, plant serine racemase activity was increased by divalent cations but not by ATP. This suggests that the interaction of the enzyme with ATP and the divalent cation restricts the substrate specificity of eukaryotic racemases.

A novel mechanism of mammalian serine racemase has been proposed, whereby dehydratase activity of serine racemase regulates the intracellular **1a** level. This effect occurs especially in brain areas, since a D-amino acid oxidase, which degrades D-amino acids in mammalian cells, is restricted to the cerebellum and brainstem and a dehydratase activity-deficient serine racemase mutant accumulates **1a** (Foltyn et al., 2005). Several D-amino acids and D-amino acid-containing peptides have been identified in plants. However, both **1a** and D-alanine inhibit plant growth at low concentrations (Erikson et at., 2004), indicating that D-amino acid level should be strictly regulated in plant cells. There is no previous report on a plant D-amino acid oxidase, and the serine racemase is the only known enzyme that degrades **1a** in plants. The specific activities of racemization and dehydration for **1b** were as follows: 14 and 228 nmol/min/mg with regard to HvSR, 10 and 214 nmol/min/mg with regard to OsSR, and 4.1 and 86 nmol/min/mg with regard to AtSR (Fujitani et al., 2006), respectively: the corresponding dehydratase activities were about 20-fold higher than racemase activity. This is as contrast to the dehydration/racemization ratio of about 0.7 for mouse serine racemase (De Miranda et al., 2002). These results thus suggest that the main physiological function of plant serine racemase might be to degrade **1a** rather than produce **1a**.

## 4. Experimental

### *4.1. Plant material and growth conditions*

The seeds of barley, *Hordeum vulgare* L., cv. Haruna nijo, were germinated and cultured in a hydroponic

solution that consisted of 4 mM $KNO_3$, 1 mM $NaNO_3$, 1 mM $NaH_2PO_4$, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 45 μM Fe-citrate, 18 μM $H_3BO_3$, 4.6 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1.5 μM $CuSO_4$, and 1.5 μM $Na_2MoO_4$. The pH of the culture solution was adjusted to 5.5 with NaOH. After one week of cultivation, the roots were harvested, frozen in liquid $N_2$, and stored at -80 ˚C.

*4.2. Rapid amplification of cDNA ends PCR (RACE PCR)*

Total RNA was isolated from the storage roots using the RNeasy Plant Mini Kit (Qiagen) and adaptor-ligated cDNA was synthesized from 1 μg of total RNA using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacture's instructions. The primers for 3- and 5-RACE, 5-GCCTTGAACTTTTGGAGCAAGTCCCTG-3 and 5-TCCCTATTTTGCTGCTCTCATGCCAAG-3, respectively, were synthesized based on the conserved nucleotide sequences from truncated barley genes (Accession nos. **BJ450541** and **CA023779**) and PCR was performed on the cDNA with a synthesized primer and the universal primer mixture ("UPM") supplied in the kit. Fragments of about 280 and 800 bp amplified by 3- and 5-RACE PCR, respectively, were cloned into a pGEM-T vector (Promega) and subjected to nucleotide sequencing on both strands using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems) with a series of synthetic primers.

*4.3. Cloning of serine racemase gene*

Based on the putative open reading frame of the barley serine racemase gene predicted by the RACE PCR experiment, primers were designed as follows: sense primer, 5-GCCATATGGGAAGCAGAGATGACGATG- 3, which creates an *Nde*I site (underlined), and antisense primer, 5-GACTCGAGTTTATACAAGGAATC- CCAT-3, which creates an *Xho*I site (underlined). The open reading frame was amplified by reverse-transcription PCR (RT-PCR) using the OneStep RT-PCR kit (Qiagen) with total RNA from the barley roots. The PCR product of 1026 bp was cloned into the pGEM-T vector and the *Nde*I- and *Xho*I-digested fragment of the plasmid was cut out and subcloned into an *Nde*I- and *Xho*I-digested pET20b(+) vector (Novagen), in which the endogenous stop codon of the serine racemase gene was replaced by a polyhistidine tag gene. The resulting plasmid, pHvSR, was transformed into *E. coli* BL21(DE3) pLysS cells.

The open reading frame of the rice serine racemase gene was amplified with total RNA from the rice, *Oryza sativa*, cv. Nipponbare, and the primers were designed based on the putative gene (**AL606647**), as follows: sense primer, 5- CCCATATGGGGAGCAGAGGTGGAAGTGG-3, which creates an *Nde*I site (underlined), and antisense primer, 5-CCCTCGAGACGTTTATAGAGAGACTCCC-3, which creates an *Xho*I site (underlined) by OneStep RT-PCR. The *Nde*I- and *Xho*I-digested fragment of the 1032 bp PCR product was subcloned into an *Nde*I- and *Xho*I-digested pCold I vector (Takara), in which a polyhistidine tag gene is fused upstream from the start codon. The resulting plasmid, pOsSR, was transformed into *E. coli* BL21 cells.

*4.4. Expression and purification of serine racemase*

*E. coli* cells harboring pHvSR were grown at 25 ˚C in Luria-Bertani (LB) medium containing 50 μg/ml of ampicillin. When the $OD_{600}$ became 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM. After cultivation at 25 ˚C for 6 h, the cells were harvested by centrifugation and frozen at -80 ˚C for at least 2 h. In the case of *E. coli* cells harboring pOsSR, the culture of 0.5 $OD_{600}$ was incubated at 15 ˚C for 30 min and cultured at 15 ˚C for 8 h with 0.5mM IPTG. The frozen cell pellets were suspended in a BugBuster™ HT protein extraction reagent (Novagen) according to the manufacturer's instructions. The resulting recombinant protein was purified using an Ni-NTA CC (Qiagen) initially equilibrated in 20 mM Tris-HCl buffer (pH 7.9) containing 0.5 M NaCl and 5 mM imidazole (Buffer A). The column was next washed with Buffer A, followed by 60 mM imidazole in Buffer A, with the absorbed protein finally eluted with 1 M imidazole in Buffer A. The enzyme solution was collected, dialyzed against a 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT and 10 μM PLP, and concentrated by an Ultracent-30 (Tohso).

*4.5. Enzyme and protein assays*

The serine racemase and dehydratase activities were assayed by calculating the hydroxyperoxide production with amino acid oxidase-peroxidase-OPA and by calculating the pyruvate production with 2,4-dinitrophenylhydrazine, respectively, according to the method described previously (Fujitani et al., 2006). The **1b** solution used in the enzyme assay was pre-treated with D-amino acid oxidase and catalase to remove any contaminating **1a** according to De Miranda et al. (2002). L-Amino acid oxidase was used to remove contaminating **1b** in the **1a** solution. Protein concentrations were quantified according to Bradford (1976) with

bovine serum albumin as the standard.

*4.6. Antiserum preparation*

The purified recombinant serine racemase of barley or *A. thaliana* was mixed with either Freund's complete adjuvant (for the first injection) or incomplete adjuvant (for following injections). The emulsified mixture containing protein (1 mg) was used to immunize a male Japanese white rabbit by subcutaneous injection followed by four subsequent injections, once every two weeks. Titration of the antiserum was performed by standard enzyme-linked immunosorbent assay (ELISA).

*4.7. Computer analysis*

Analysis and translation of the nucleotide sequences were performed using GENETYX-MAC (Software Development). The homology searches, alignments, and phylogenetic analyses of the amino acid sequences were performed using BLAST (Altschul et al., 1990; Karlin and Altschul, 1990), CLUSTAL W (Tompson et al., 1994), and TreeView (Page, 1996) programs, respectively.

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**References**

Abe, K., Takahashi, S., Muroki, Y., Kera, Y., Yamada, R., 2006. Cloning and expression of the pyridoxal 5-phosphate-dependent aspartate racemase gene from the bivalve mollusk *Scapharca broughtonii* and characterization of the recombinant enzyme. J. Biochem. 139, 235-244.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248-254.

Cook, S.P., Galve-Roperh, I., Martinez, d.P., Rodriguez-Crespo, I., 2002. Direct calcium binding results in activation of brain serine racemase. J. Biol. Chem. 277, 27782-27792.

De Miranda, J., Santoro, A., Engelender, S., Wolosker, H., 2000. Human serine racemase: molecular cloning, genomic organization and functional analysis. Gene 256, 183-188.

De Miranda, J., Panizzutti, R., Foltyn, V.N., Wolosker, H., 2002. Cofactors of serine racemase that physiologically stimulate the synthesis of the *N*-methyl-D-aspartate (NMDA) receptor coagonist D-serine. Proc. Natl. Acad. Sci. USA. 99, 14542-14547.

Dunlop, D.S., Neidle, A., McHale, D., Dunlop, D.M., Lajtha, A., 1986. The presence of free D-aspartic acid in rodents and man. Biochem. Biophys. Res. Commun. 141, 27-32.

Erbe, T., Bruckner, H., 2000. Chromatographic determination of amino acid enantiomers in beers and raw materials used for their manufacture. J. Chromatogr. A 881, 81-91.

Erikson, O., Hertzberg, M., Nasholm, T., 2004. A conditional marker gene allowing both positive and negative selection in plants. Nature biotechnol. 22, 455-458.

Foltyn, V.N., Bendikov, I., De Miranda, J., Panizzutti, R., Dumin, E., Shleper, M., Lu, P., Toney, M.D., Kartvelishvily, E., Wolosker, H., 2005. Serine racemase modulates intracellular D-serine levels through an α,β-elimination activity. J. Biol. Chem. 280, 1754-1763.

Fujitani, Y., Nakajima, N., Ishihara, K., Oikawa, T., Ito, K., Sugimoto, M., 2006. Molecular and biochemical characterization of a serine racemase from *Arabidopsis thaliana*. Phytochemistry 67, 668-674.

Gallagher, D.T., Gilliland, G.L., Xiao, G., Zondlo, J., Fisher, K.E., Chinchilla, D., Eisenstein, E., 1998. Structure and control of pyridoxal phosphate dependent allosteric threonine deaminase. Structure 6, 465-475.

Grishin, N.V., Phillips, M.A., Goldsmith, E.J., 1995. Modeling of the spatial structure of eukaryotic ornithine decarboxylases. Protein Sci. 4, 1291-1304.

Hashimoto, A., Nishikawa, T., Hayashi, T., Fujii, N., Oka, T., Takahashi, K., 1992. The presence of free D-serine in rat brain. FEBS Lett. 296, 33-36.

Hashimoto, A., Kumashiro, S., Nishikawa, T., Oka, T., Takahashi, K., Mito, T., Takashima, S., Doi, N., Mizutani, Y., Yamazaki, T., Kaneko, T., Ootomo, E., 1993. Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. J. Neurochem. 61, 783-786.

Hashimoto, A., Oka, T., Nishikawa, T., 1995. Anatomical distribution and postnatal changes in endogenous free

D-aspartate and D-serine in rat brain and periphery. Eur. J. Neurosci. 7, 1657-1663.

Karlin, S., Altschul, S.F., 1990. Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. Proc. Nalt. Acad. Sci. USA. 87, 2264-2268.

Kullman, J.P., Chen, X., Armstrong, D.W., 1999. Evaluation of the enantiomeric composition of amino acids in tobacco. Chirality 11, 669-673.

Nagata, Y., Konno, R., Yasumura, Y., Akino, T., 1989. Involvement of D-amino acid oxidase in elimination of free D-amino acids in mice. Biochem. J. 257, 291-292.

Nagata, Y., Horiike, K., Maeda, T., 1994. Distribution of free D-serine in vertebrate brains. Brain Res. 634, 291-295.

Ogawa, T., Bando, N., Sasaoka, K., 1977. Occurrence of D-α-amino-*n*-butyric acid in legume seedlings. Agric. Biol. Chem. 41, 1811-1812.

Page, R.D.M., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357-358.

Strisovsky, K., Jiraskova, J., Barinka, C., Majer, P., Rojas, C., Slusher, B.S., Konvalinka, J., 2003. Mouse brain serine racemase catalyzes specific elimination of L-serine to pyruvate. FEBS Lett. 535, 44-48.

Tompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Wolosker, H., Blackshaw, S., Snyder, S.H., 1999b. Serine racemase, a glial enzyme synthesizing D-serine to regulate glutamate-*N*-methyl-D-aspartate neurotransmission. Proc. Natl. Acad. Sci. USA. 96, 13409-13414.

**Figure legends**

Fig. 1. Reaction pathways of D-serine (**1a**) and L-serine (**1b**) by mammalian serine racemase.

Fig. 2. Alignment of the deduced amino acid sequences of barley and rice serine racemases with those of *A. thaliana*, human, and mouse serine racemases, as well as *E. coli* biosynthetic L-threonine dehydratase. Barley, rice, *A. thaliana*, human, and mouse serine racemases (HvSR, OsSR, AtSR, hSR, and mSR) correspond to GenBank accession nos. **AB271213**, **AL606647**, **AB206823**, **AF169974**, and **AF148321**, respectively, whereas

the *E. coli* biosynthetic L-threonine dehydratase (EcTD) corresponds to Swiss-Plot accession no. **P04968**. Gaps, indicated by dashes, are introduced in the sequences to maximize the homology. Identical amino acid residues among either serine racemases or all six of the enzymes are represented by black boxes. The closed arrowhead, open arrowheads, and asterisks indicate the PLP-binding Lys, amino acid residues that interact with PLP, and the glycine-rich loop, respectively.

Fig. 3. Phylogenetic analysis of eukaryotic serine racemases. The scale bar represents 0.1 amino acid substitutions per site.

Fig. 4. Analysis of the expression of serine racemase genes from barley and rice in *E. coli* cells by SDS-polyacrylamide gel. Total cell lysate (lane 1), soluble protein (lane 2), purified enzyme following Ni-NTA column chromatography (CC) (lane 3) from *E. coli* cells harboring pHvSR, soluble protein (lane 4) and purified enzyme following Ni-NTA CC (lane 5) from *E. coli* cells harboring pOsSR. Gels were obtained using 15% SDS-PAGE with molecular mass marker series (lane M) for calibration, with detection by Coomassie Brilliant Blue R-250 staining.

Fig. 5. Absorption spectra of purified HvSR before and after $NaBH_4$ incubation. The spectra of the purified HvSR (1mg/ml) before and after dialyzing against 500 volumes of 20 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 10μM PLP, and 10 mM sodium borohydride are shown as solid and dashed lines, respectively.

Fig. 6. Effects of divalent cations and ATP on racemase and dehydratase activities of barley serine racemase. Racemase (black columns) and dehydratase (white columns) activities of the purified recombinant enzyme were assayed in the presence of 1mM EDTA, 1mM metal chloride, or 1mM ATP. The results represent the average ± s.e. of three experiments.

Fig. 7. Ouchterlony double diffusion of the purified recombinant plant serine racemases. Ab1, anti-HvSR serum; Ab2, anti-AtSR serum; 1, OsSR; 2,HvSR; 3, AtSR.