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## Identification of three shikimate kinase genes in rice: characterization of their differential expression during panicle development and of the enzymatic activities of the encoded proteins

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**Abstract** The shikimate pathway is common to the biosynthesis of the three aromatic amino acids and that of various secondary metabolites in land plants. Shikimate kinase (SK; EC 2.7.1.71) catalyzes the phosphorylation of shikimate to yield shikimate 3-phosphate. In an attempt to elucidate the functional roles of enzymes that participate in the shikimate pathway in rice (*Oryza sativa*), we have now identified and characterized cDNAs corresponding to three SK genes—*OsSK1*, *OsSK2*, and *OsSK3*—in this monocotyledonous plant. These SK cDNAs encode proteins with different NH<sub>2</sub>-terminal regions and with putative mature regions that share sequence similarity with other plant and microbial SK proteins. An in vitro assay of protein import into intact chloroplasts isolated from pea (*Pisum sativum*) seedlings revealed that the full-length forms of the three rice SK proteins are translocated into chloroplasts and processed, consistent with the assumption that the different NH<sub>2</sub>-terminal sequences function as chloroplast transit

peptides. The processed forms of all three rice proteins synthesized in vitro manifested SK catalytic activity. Northern blot analysis revealed that the expression of *OsSK1* and *OsSK2* was induced in rice calli by treatment with the elicitor *N*-acetylchitoheptaose, and that expression of *OsSK1* and *OsSK3* was up-regulated specifically during the heading stage of panicle development. These results suggest that differential expression of the three rice SK genes and the accompanying changes in the production of shikimate 3-phosphate may contribute to the defense response and to panicle development in rice.

**Keywords** Defense response · Gene expression · *Oryza sativa* · Shikimate kinase · Shikimate pathway

**Abbreviations** *cTP*: Chloroplast transit peptide · *DAHP*: 3-Deoxy-D-arabino-heptulosonate · *SK*: Shikimate kinase · *EPSP*: 5-Enolpyruvylshikimate 3-phosphate · *S3P*: Shikimate 3-phosphate · *PCR*: Polymerase chain reaction · *EPSPS*: EPSP synthase · *ORF*: Open reading frame · *PAGE*: Polyacrylamide gel electrophoresis · *TLC*: Thin-layer chromatography

The nucleotide sequences of *OsSK1*, *OsSK2*, and *OsSK3* cDNAs are available in GenBank under the accession numbers AB188834, AB188835, and AB188836, respectively.

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### Introduction

The shikimate pathway is responsible for the biosynthesis of aromatic compounds in bacteria, yeasts and other fungi, plants, and apicomplexan parasites such as *Plasmodium falciparum* (Roberts et al. 1998; Herrmann and Weaver 1999). The pathway begins with two intermediates of carbohydrate metabolism, phosphoenolpyruvate (from glycolysis) and erythrose 4-phosphate (from the pentose phosphate pathway), which are eventually converted to chorismate, the last common intermediate in the synthesis of the three aromatic amino acids (phenylalanine, tyrosine, tryptophan). Plant genes for enzymes that function in the shikimate pathway have been isolated; the proteins encoded by all of these genes possess a chloroplast transit peptide (cTP) at their NH<sub>2</sub>-termini, indicating that the shikimate pathway takes

place in plastids (Weaver and Herrmann 1997). In higher plants, the aromatic amino acids produced by this pathway further serve as precursors for various secondary metabolites, such as indole 3-acetic acid, indole alkaloids, phenylpropanoids, and flavonoids (Herrmann 1995). In contrast, more than 90% of these amino acids are used for protein synthesis in bacteria (Dixon and Paiva 1995). Characterization of the shikimate pathway (pre-chorismate pathway) and the post-chorismate pathway is thus important for elucidation and engineering of secondary metabolism in plants (Rippert et al. 2004).

Certain secondary metabolites, including phenylpropanoids and flavonoids, play important roles in plant defense systems (Dixon and Paiva 1995; Dixon and Steele 1999). Pathogens and elicitors have also been found to affect the expression of plant genes for proteins in the pre- or post-chorismate pathways (Keith et al. 1991; Görlach et al. 1995; Tozawa et al. 2001; Kanno et al. 2004). Expression of DHS2, which encodes 3-deoxy-D-arabino-heptulosonate (DAHP) synthase, is thus induced by wounding or pathogen invasion in *Arabidopsis thaliana* (Keith et al. 1991). Furthermore, expression of genes encoding DAHP synthase, shikimate kinase (SK; EC 2.7.1.71), 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19), chorismate synthase (EC 4.6.1.4), and phenylalanine ammonia-lyase (EC 4.3.1.5) is induced in cultured tomato cells by elicitor treatment (Görlach et al. 1995). We have also shown that expression of *OASA2* and *OASB1*, which encode  $\alpha$  and  $\beta$  subunits, respectively, of anthranilate synthase (EC 4.1.3.27), is induced by elicitor treatment in rice suspension culture (Tozawa et al. 2001; Kanno et al. 2004).

In the shikimate pathway, SK catalyzes the phosphorylation of shikimate to produce shikimate 3-phosphate (S3P), which is then converted to EPSP by EPSP synthase. The *aroL* gene of *Escherichia coli* encodes an SK enzyme that is conserved widely among eubacteria (Minton et al. 1989; Whipp and Pittard 1995; Oliveira et al. 2001). In contrast, in yeasts and fungi, SK exists as a domain of a multifunctional enzyme, AROM, that catalyzes five consecutive biosynthetic steps of the shikimate pathway from DAHP to EPSP (Giles et al. 1967; Kinghorn and Hawkins 1982; Duncan et al. 1988). An SK gene of higher plants was isolated and characterized for tomato (Schmid et al. 1992). The tomato enzyme possesses a plastid-targeting transit peptide at its NH<sub>2</sub>-terminus, with the putative mature region exhibiting high sequence similarity to bacterial SK proteins and it manifests SK catalytic activity in vitro. As far as we are aware, the tomato gene is the only SK gene described to date for land plants and no information has been available for SK genes of monocot plants.

We now describe the isolation and characterization of three distinct SK genes—*OsSK1*, *OsSK2*, and *OsSK3*—and their encoded proteins in rice. These proteins are imported into chloroplasts and exhibit SK catalytic activity in vitro, and the genes are expressed differen-

tially in rice plants. Our observations suggest that these three distinct SK enzymes contribute to the defense response and to floral organ development in rice.

## Materials and methods

### Plant materials

Rice (*Oryza sativa* cv. Nipponbare; National Institute of Crop Science, Tsukuba, Japan) plants and calli were grown as described previously (Tozawa et al. 2001). Rice suspension culture and elicitor treatment of the cultured cells were also performed as described previously (Nojiri et al. 1996; Tozawa et al. 2001). Pea (*Pisum sativum*, cv. TOYONARI; Sakata's seeds) plants were grown at 25°C under a 16-h-light, 8-h-dark cycle with artificial light (150–170  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### Gene isolation and analysis

Standard recombinant DNA techniques were performed basically as described (Sambrook et al. 1989). Rice shoot cDNAs were prepared as described (Tozawa et al. 2001). The polymerase chain reaction (PCR) was performed in a reaction mixture (50  $\mu\text{l}$ ) containing 1 $\times$ Ex Taq buffer (Takara, Tokyo, Japan), 20 ng of rice cDNA or 10 ng of *E. coli* genomic DNA, 1 mM of each deoxynucleoside triphosphate, 0.2  $\mu\text{M}$  of each oligonucleotide primer, and 2.5 U of Ex Taq DNA polymerase (Takara). Amplification was achieved with a PCR Thermal Cycler system (Takara) with a protocol comprising an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min.

To amplify rice SK cDNA fragments, we performed PCR with rice template cDNAs and the primers OsSK1-f and OsSK1-r for *OsSK1*, OsSK2-f and OsSK2-r for *OsSK2*, and OsSK3-f and OsSK3-r for *OsSK3* (Table 1). PCR was also carried out with the primers OsEPSPDN67-f and OsEPSP-r (Table 1) to amplify a portion of rice EPSP synthase (EPSPS) cDNA (Sato et al. 1999) that encodes the putative mature enzyme, lacking the 67 NH<sub>2</sub>-terminal amino acids that are presumed as the cTP (Gasser et al. 1988). For amplification of the open reading frames (ORFs) of the *E. coli* genes *aroL* and *aroA*, genomic DNA from *E. coli* strain JM109 was used as the template for PCR with the primer sets Ec-aroL-f and Ec-aroL-r as well as Ec-aroA-f and Ec-aroA-r, respectively (Table 1).

For determination of the sequences of the three rice SK cDNAs, the PCR products were purified with the use of a QIAquick PCR Purification Kit (Qiagen) and were directly sequenced with a DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech) and an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

**Table 1** Oligonucleotide primers used in the present study

Primer	Sequence	Note
OsSK1-f	5'-tgtatactagtATGGAGGCGGGCGTGGGGCTGG-3'	Sense primer for <i>OsSK1</i> , Spe I
OsSK1-r	5'-atatgtcgacTCTACAAGGTCTGTATCCTCTGAGC-3'	Reverse primer for <i>OsSK1</i> , Sal I
OsSK2-f	5'-tgtatactagtATGGAGGCTAGAGCGGGCTGGCG-3'	Sense primer for <i>OsSK2</i> , Spe I
OsSK2-r	5'-atatgtcgacTCTACAAGGCCCTTGTCCCTTCGATTC-3'	Reverse primer for <i>OsSK2</i> , Sal I
OsSK3-f	5'-tgtatactagtATGGACGCCGGTGTGGGTCTCC-3'	Sense primer for <i>OsSK3</i> , Spe I
OsSK3-r	5'-atatgtcgacGTTAGTTTCTGATCATTGCCTTC-3'	Reverse primer for <i>OsSK3</i> , Sal I
Ec-aroL-f	5'-acgccgactagtATGACACAACCTCTTTTCTG-3'	Sense primer for <i>aroL</i> , Spe I
Ec-aroL-r	5'-aaagtctagaTCAACAATTGATCGTCTGTG-3'	Reverse primer for <i>aroL</i> , Xba I
OsEPSPDN67-f	5'-aaaactagt ATGAAGGCGGAGGAGATCGTGCT-3'	Sense primer for <i>OsEPSPS</i> , Spe I
OsEPSP-r	5'-aaagtctagaTCAGTTCTTGACGAAAGTGCTTAG-3'	Reverse primer for <i>OsEPSPS</i> , Xba I
Ec-aroA-f	5'-aaaactagtATGGAATCCCTGACGTTACAACC-3'	Sense primer for <i>E. coli aroA</i> , Spe I
Ec-aroA-r	5'-aaagtcgacTCAGGCTGCCTGGCTAATCC-3'	Reverse primer for <i>E. coli aroA</i> , Sal I
OsSK1DN62	5'- <b>cccttccagggeccca</b> ATGGCCAAGAAATCGTCCGG-3'	For split-primer method, <i>OsSK1</i>
OsSK2DN60	5'- <b>cccttccagggeccca</b> ATGTACTGCCTCAAGGCGTC-3'	For split-primer method, <i>OsSK2</i>
OsSK3DN57	5'- <b>cccttccagggeccca</b> ATGTGTTCCAGGAAACCG-3'	For split-primer method, <i>OsSK3</i>
PEU-anti3	5'-GGAGAAAGGCGGACAGGTAT-3'	For split-primer method, plasmid-specific
OsSK1-3'-f	5'-ttctgaattcAGCATGCTCGCGGAGCAAAG-3'	Sense primer for 3' region of <i>OsSK1</i> , Eco RI
OsSK1-3'-r	5'-acgaagcttTTCTGATTAGCTCCATTATTC-3'	Reverse primer for 3' region of <i>OsSK1</i> , Sal I
OsSK2-3'-f	5'-aaaggaattcTGCTTATGCAAATGCTGATG-3'	Sense primer for 3' region of <i>OsSK2</i> , Eco RI
OsSK2-3'-r	5'-aaacaagcttGCAACGATTCTTATTCCAG-3'	Reverse primer for 3' region of <i>OsSK2</i> , Sal I
OsSK3-3'-f	5'-atctggaattcCTTATGCAAAGGCTTATGC-3'	Sense primer for 3' region of <i>OsSK3</i> , Eco RI
OsSK3-3'-r	5'-acaacacaagcttTTCTTTCAATTGAAAGC-3'	Reverse primer for 3' region of <i>OsSK3</i> , Sal I
OsEPSP-3'-f	5'-aaaggatccCTGGGAGCATCGGTTGAAGAAGG-3'	Sense primer for 3' region of <i>OsEPSPS</i> , Bam HI
OsEPSP-3'-r	5'-aaagaattcCAAGGAGAAATTTAGTGAGATGGCAA-3'	Reverse primer for 3' region of <i>OsEPSPS</i> , Eco RI

Normal lowercase letters indicate linker sequences for generation of recognition sites of restriction enzymes; bold lowercase letters indicate linker sequences required for the split-primer method (Kanno et al. 2004). Artificially introduced initiation codons for the design of NH<sub>2</sub>-terminally truncated proteins are underlined.

### Cell-free protein synthesis

The PCR products described above were digested with restriction enzymes for the recognition sites generated by the linker sequences shown in Table 1 and were then cloned into the corresponding sites of the pEU3b expression vector (Sawasaki et al. 2002b). The resulting plasmids were designated pEUOSK1 (*OsSK1*), pEUOSK2 (*OsSK2*), pEUOSK3 (*OsSK3*), pEUOEPDN67 (*OsEPSPS*), pEUaroL (*E. coli aroL*), and pEUaroA (*E. coli aroA*). After verification of the inserted sequences, the plasmids were used as templates for in vitro transcription with SP6 RNA polymerase as described (Madin et al. 2000). A series of cDNAs encoding NH<sub>2</sub>-terminally truncated forms of *OsSK1*, *OsSK2*, and *OsSK3* was constructed by the split-primer PCR method, as described previously (Kanno et al. 2004), with plasmids harboring the corresponding full-length cDNAs as templates together with appropriately designed oligonucleotide primers (Table 1).

Wheat embryo extracts were kindly provided by T. Shibui and colleagues at Zoe Gene (Yokohama, Japan). Cell-free protein synthesis with these extracts was performed by two different methodologies. For the synthesis of <sup>35</sup>S-labeled proteins, the “batch method” was performed as described (Madin et al. 2000) in the presence of [<sup>35</sup>S]methionine (14.8 MBq ml<sup>-1</sup>). For preparation of small amounts of protein for enzyme assays, the “bilayer method” (Sawasaki et al. 2002a) was performed with [<sup>14</sup>C]leucine (0.074 MBq ml<sup>-1</sup>, 289 MBq mmol<sup>-1</sup>). The solubility of synthesized proteins

was assessed by SDS-polyacrylamide gel electrophoresis (PAGE), both of the total reaction mixture and of the supernatant obtained after centrifugation at 22,000 g for 20 min at 4°C. The gel was dried and exposed to a BAS-III imaging plate (Fuji Film), and protein-associated radioactivity was detected with a BAS-2500 analyzer (Fuji Film) and quantitated with Image Gauge version 3.41 software (Fuji Film).

### In vitro assay of protein import into chloroplasts

Chloroplasts were isolated as described previously (Perry et al. 1991) from 7 to 8-day-old pea seedlings. The import assay was performed with <sup>35</sup>S-labeled precursor proteins, synthesized with the cell-free system described above. Chloroplasts were suspended in import buffer [300 mM sorbitol, 50 mM Hepes-KOH (pH 8.0)] at a density of 1 mg of chlorophyll per milliliter. The translation products were diluted fourfold in import buffer supplemented with 25 mM methionine. The assay was performed at 25°C for 20 min in a 150-μl reaction mixture consisting of 45 μl of diluted translation products, 100 μl of chloroplasts, and 3 mM ATP (Mg<sup>2+</sup> salt). After the addition of thermolysin (final concentration, 100 μg ml<sup>-1</sup>; Nacalai Tesque, Kyoto, Japan) and CaCl<sub>2</sub> (final, 1 mM), the reaction mixture (153 μl) was incubated for 30 min on ice. Proteolysis was terminated by the addition of 3 μl of 0.5 mM EDTA. Intact chloroplasts were separated by centrifugation of the reaction mixture through 35% Percoll

(Amersham Pharmacia Biotech) in import buffer, washed by suspension and centrifugation in import buffer containing 1 mM EDTA, and finally suspended in 50  $\mu$ l of 2 $\times$ SDS sample buffer. After incubation for 10 min at 67°C, portions (20  $\mu$ l) of the chloroplast extract were subjected to SDS-PAGE through a 12% gel. The gel was dried and the associated radioactivity was analyzed as described above. The molecular sizes of proteins were estimated from the positions of molecular size standards (Precision Plus Protein Standards, Bio-Rad).

#### Assay of SK and EPSP synthase activities

A crude cell-free reaction mixture containing SK or EPSP synthase labeled with [<sup>14</sup>C]leucine was subjected to gel filtration on a MicroSpin G-25 column (Amersham Biosciences) that had been equilibrated with SK buffer [100 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl<sub>2</sub>]. The column was centrifuged at 22,000 *g* for 20 min at 4°C to remove insoluble forms of the synthesized proteins before assay of enzyme activity. For determination of the amount of the soluble form of each synthesized protein, 5  $\mu$ l of each treated reaction mixture was applied to a filter paper, which was then dried, heated for 10 min at 100°C in 10% trichloroacetic acid, washed for 3 min twice with 5% trichloroacetic acid and once with ethanol, and dried again. The radioactivity associated with each filter disk was then measured with a liquid scintillation counter. The protein concentration (nM) was calculated from the amount of [<sup>14</sup>C]leucine incorporated (dpm).

The assay of SK activity was performed as described (Vinella et al. 1996), with some modifications. The reaction was performed at 30°C in a 10- $\mu$ l reaction mixture containing 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 5.7 mM shikimate, 2 mM ATP, [ $\gamma$ -<sup>32</sup>P]ATP (0.925 MBq  $\mu$ mol<sup>-1</sup>), and 20 nM synthesized SK enzyme. After incubation (2, 4, 6, 8, 10 or 60 min), portions (2  $\mu$ l) of the reaction mixture were spotted onto polyethyleneimine-cellulose thin-layer chromatography (TLC) plates (Merck) and subjected to ascending chromatography with 0.8 M LiCl.

The coupled SK and EPSP synthase assay was performed with the reaction mixture used for the SK assay with the exception that it also contained 2 mM phosphoenolpyruvate and 20 nM EPSP synthase. The reaction was performed for 1 h at 30°C, after which 2- $\mu$ l portions of the reaction mixture were analyzed by TLC on polyethyleneimine-cellulose plates with 1.0 M LiCl as the solvent.

#### RNA gel-blot hybridization

Isolation of total RNA and RNA gel-blot hybridization were performed as previously described (Tozawa et al. 2001). For preparation of riboprobes, ~400-bp fragments of the 3' regions of each of the three rice SK

cDNAs and of the rice EPSP synthase cDNA were amplified by PCR with primers listed in Table 1. The PCR products were cloned into the corresponding restriction sites of pSPT19 (Roche) and digoxigenin-labeled antisense riboprobes were produced by run-off in vitro transcription with T7 or SP6 RNA polymerase (Roche). A riboprobe specific for rice *actin2* mRNA as an internal marker was also synthesized and tested as described (Tozawa et al. 1998).

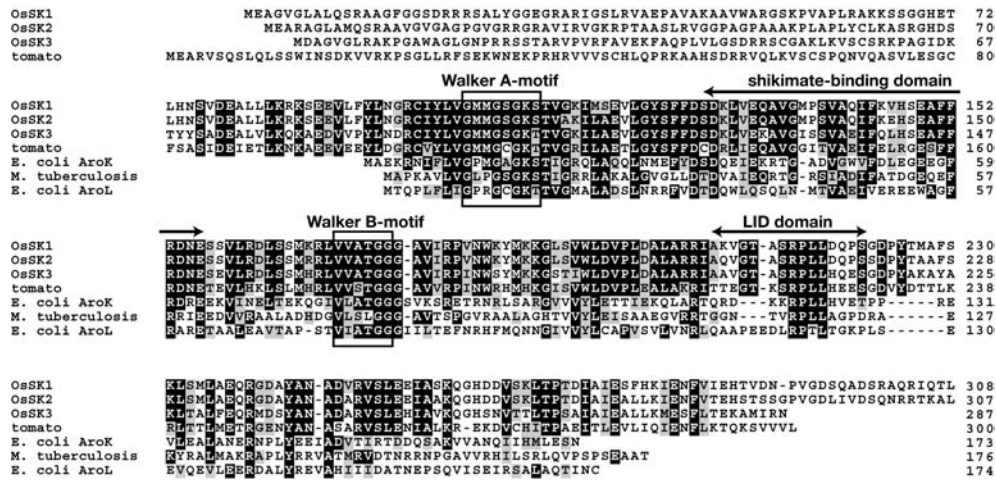
## Results

### Isolation of cDNAs corresponding to three rice SK genes

To identify rice genes for SK, we performed a database search with TBLASTN software (Altschul et al. 1997) and the amino acid sequence of tomato (*Lycopersicon esculentum* L.) SKI (GenBank accession no. CAA45121) as the query. The search identified four rice DNA sequences (AK066687, AK109730, AK070318, AK067948) in a full-length cDNA database (<http://cdna01.dna.affrc.go.jp/cDNA/>). A further search of rice genome sequences revealed that the genes corresponding to the AK066687 and AK109730 sequences are located on chromosomes 2 and 6, respectively, and that the AK070318 and AK067948 sequences correspond to the same locus on chromosome 4. The four cDNAs contained multiple intron-like spacer sequences, however, with the result that the ORFs could not be assigned. To determine the complete sequences of the ORFs of the three identified genes, we amplified the ORF regions by reverse transcription and PCR with polyadenylated RNA isolated from rice seedlings. A single DNA fragment was obtained for each gene and the nucleotide sequences of these three fragments were determined. Comparison of the nucleotide sequences of the three amplified cDNAs revealed that they shared 54–77% identity and that they encoded full-length proteins that are highly homologous to SKs of other species. The corresponding genes were designated *OsSK1* (*Oryza sativa* shikimate kinase 1), *OsSK2*, and *OsSK3* (Fig. 1).

Alignment of the predicted amino acid sequences of SK family members revealed that the three rice and the tomato proteins possess dissimilar NH<sub>2</sub>-terminal regions that likely correspond to cTPs (Schmid et al. 1992; Emanuelsson et al. 2000). The amino acid sequences of the putative mature *OsSK1*, *OsSK2*, and *OsSK3* proteins are highly similar both to that of the corresponding mature region of tomato SK (53–57% identity) as well as to each other (86, 69, and 72% identity between *OsSK1* and *OsSK2*, *OsSK1* and *OsSK3*, and *OsSK2* and *OsSK3*, respectively). They also exhibit substantial similarity to microbial homologs and they contain functional motifs that have been assigned to bacterial counterparts as substrate binding and nucleotide binding sites (Fig. 1) (Walker et al. 1982; Krell et al. 1998; Gu et al. 2002).





**Fig. 1** Alignment of the predicted amino acid sequences of rice SKs with those of other SK proteins. The sequences shown include those of rice OsSK1, OsSK2, and OsSK3 (GenBank accession nos. AB188834, AB188835, and AB188836, respectively), SK of tomato (LeSK, CAA45121), AroK of *Mycobacterium tuberculosis* (MtAroK, AAK46924), and AroK (EcAroK, CAA56448) and AroL (EcAroL, AAA83833) of *E. coli*. Hyphens indicate gaps introduced

to optimize alignment. Identical or similar residues among the various proteins are indicated by *dark* and *light shading*, respectively, and *residue numbers* are shown on the right. Walker A and B motifs (boxed) correspond to regions that resemble the functional motifs identified in other nucleotide binding enzymes (Walker et al. 1982). *Two-headed arrows* indicate the putative shikimate binding domain and LID domain (Gu et al. 2002)

To examine the evolutionary relations among members of the SK family, we constructed a phylogenetic tree (Fig. 2). The three rice SKs formed a cluster that was separated slightly from a cluster of dicotyledonous SKs. Among the rice enzymes, OsSK1 and OsSK2 were most closely related to each other, with OsSK3 being the next closest relative of each. In turn, the plant SK proteins were more closely related to SKs of cyanobacteria than to those of eubacteria or fungi. This observation is consistent with the endosymbiotic theory based on the putative evolutionary relation between plastids and cyanobacteria (Douglas 1994).

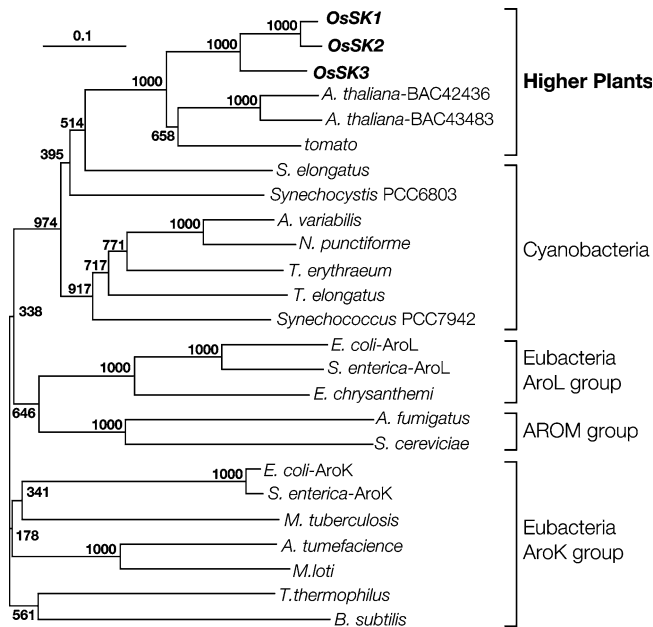
#### Import of rice SK precursor proteins into chloroplasts in vitro

Tomato SK localizes to plastids (Schmid et al. 1992) and the deduced amino acid sequences of the three rice SKs suggested that these proteins might also be targeted to plastids (Fig. 1). To determine whether the putative cTPs of rice SKs are functional, we performed an in vitro protein import assay with pea chloroplasts. The full-length SK proteins labeled with [<sup>35</sup>S]methionine were incubated with the intact chloroplasts, after which the reaction mixture was treated with the protease thermolysin in order to digest unincorporated protein. Analysis by SDS-PAGE and autoradiography revealed that the full-length OsSK1 (32.6 kDa), OsSK2 (31.7 kDa), and OsSK3 (29.5 kDa) proteins were proteolytically processed to products of 25.9, 25.0, and 23.7 kDa, respectively, during incubation with pea chloroplasts (Fig. 3). Subsequent treatment with thermolysin resulted in digestion of the full-length pro-

teins but not of the processed products, indicating that the latter had been translocated into chloroplasts. These results thus support the notion that the three rice SKs are targeted to chloroplasts and are subsequently processed to their mature forms on translocation into these organelles.

#### Catalytic activity of in vitro-synthesized rice SK proteins

We next examined whether the predicted mature forms of the three rice SK proteins possess SK activity. The truncation sites for deletion of the cTP region from each precursor protein were selected on the basis of comparisons of their amino acid sequences with those of other SK family members (Fig. 1). The sizes of the truncated proteins are very similar to that of the processed proteins observed in the chloroplast import assay (Fig. 3). With the cell-free protein synthesis system, we thus prepared [<sup>14</sup>C]leucine-labeled forms of OsSK1, OsSK2, and OsSK3 that lacked the NH<sub>2</sub>-terminal 62, 60, or 57 amino acids of the corresponding full-length proteins, respectively (Fig. 4a). Protein synthesis mixtures containing equal molar amounts (20 nM) of the soluble truncated SK proteins were then assayed for SK activity by incubation for 10 or 60 min at 30°C with the substrates shikimate and [ $\gamma$ -<sup>32</sup>P]ATP. As a positive control, we also synthesized *E. coli* AroL (EcAroL) and subjected it to the SK activity assay. Production of S3P was detected by TLC and autoradiography (Fig. 4b). A radioactive spot corresponding to S3P was generated by each of the three truncated rice SK proteins (OsSK1ΔN62, OsSK2ΔN60, OsSK3ΔN57). On the basis of the amounts of <sup>32</sup>P-labeled S3P generated in the reaction



**Fig. 2** Phylogenetic relations among SK proteins. The amino acid sequences of OsSK1 (residues 105–252), OsSK2 (residues 103–250), OsSK3 (residues 100–247), tomato SK (LeSK, residues 113–260), two putative *A. thaliana* SKs (BAC42436, residues 107–256; BAC43483, residues 111–258), *Synechococcus elongatus* PCC7942 SK (ZP\_00164039, residues 15–153), *Synechococcus* sp. WH8102 AroK (CAE08717, residues 23–162), *Thermosynechococcus elongatus* BP-1 AroK (BAC08434, residues 18–156), *Trichodesmium erythraeum* IMS101 SK (ZP\_0032725, residues 20–158), *Nostoc punctiforme* SK (ZP\_00111870, residues 16–154), *Synechocystis* sp. PCC6803 AroK (BAA16811, residues 21–159), *Anabaena variabilis* ATCC29413 SK (ZP\_00160042, residues 2–140), *Bacillus subtilis* AroK (CAA57123, residues 20–160), *Thermus thermophilus* HB27 SK (AAS81361, residues 19–159), *Agrobacterium tumefaciens* AroK (AAL44438, residues 1–139), *Mesorhizobium loti* MAFF303099 SK (NP\_104650, residues 31–169), *Erwinia chrysanthemi* SK (S09613, residues 11–152), MtAroK (residues 11–149), EcAroK (residues 13 to 153), EcAroL (residues 11–152), *Salmonella enterica* AroK (AAL44438, residues 13–153), *S. enterica* AroL (CAD08843, residues 11–152), *Saccharomyces cerevisiae* AROM (P08566, residues 896–1037), and *Aspergillus fumigatus* AROM (CAD29607, residues 872–1010) were analyzed. The phylogenetic tree was constructed from evolutionary distance data derived by the neighbor-joining method (Saitou and Nei 1987). The bar indicates the distance corresponding to 10 changes per 100 amino acid positions. Numbers at nodes indicate bootstrap values out of  $1000 \times$  resamplings

mixtures, we calculated the specific activities of the rice proteins and *E. coli* AroL under the initial velocity conditions (0 to 6 min; Fig. 4c). The OsSK3 $\Delta$  N57 protein exhibited the highest catalytic activity ( $293 \mu\text{mol min}^{-1} \text{mg}^{-1}$  protein) followed in order by OsSK1 $\Delta$  N62 (261), OsSK2 $\Delta$  N60 (122), and AroL (35).

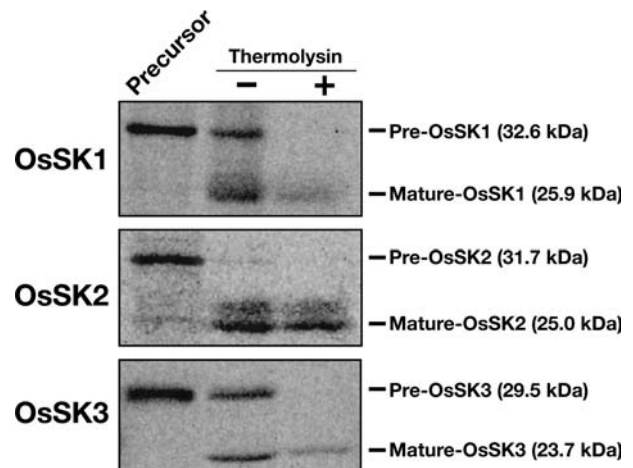
The catalytic activities of the truncated rice SK proteins were further examined by a coupled enzyme assay with EPSP synthase of rice (OsEPSPs) or *E. coli* (AroA), which catalyzes conversion of S3P to EPSP. In combination with either EPSP synthase, the three rice SK proteins and *E. coli* AroL each generated a radioactive spot presumed to be EPSP (Fig. 4d). No such spot was generated from shikimate in the presence of EPSP

synthase alone. The rate of EPSP generation was lower for OsSK2 $\Delta$  N60 than for the other two truncated rice proteins, consistent with the results of the direct SK activity assay (Fig. 4c) and indicative of a measure of functional diversity among the rice SK isoforms.

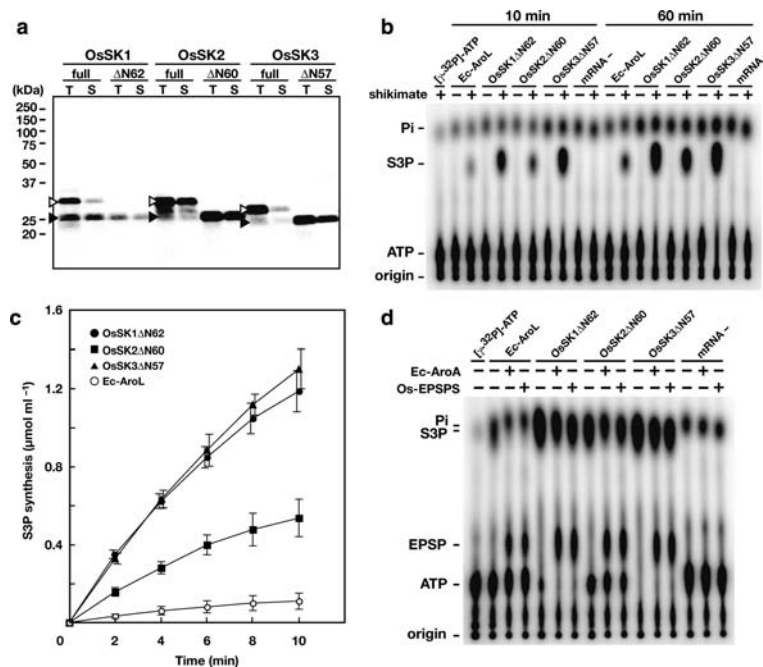
### Expression of rice SK genes

All of the four SK-related cDNAs, initially identified in the rice cDNA database, contained intron-like sequences, indicating that they were products of either incomplete or alternative splicing. We therefore performed Northern blot analysis to investigate the expression of the three rice SK genes. Each of the three genes was expressed in shoots and roots with a transcript size of  $\sim 1.4$  kb (Fig. 5a). The transcript abundance decreased in the order *OsSK1* > *OsSK2* > *OsSK3* in both tissues. These results do not support the notion that the rice SK genes undergo alternative RNA splicing. The original sequences identified in the database were thus likely the products of incomplete splicing.

Given that pathogens and elicitors have been shown to affect the expression of genes for shikimate pathway enzymes (Keith et al. 1991; Görlach et al. 1995), we examined the effect of *N*-acetylchitoheptaose, a well-characterized rice elicitor, on expression of the three SK genes in rice cells in suspension culture. Callus induction from seed embryo and the preparation of the suspension culture were performed as previously described (Tozawa et al. 2001). Northern blot analysis revealed that induction of *OsSK1* and *OsSK2* expression was apparent 30-min after exposure to the elicitor,



**Fig. 3** Import of rice SK proteins into pea chloroplasts in vitro. The full-length (precursor) forms of OsSK1, OsSK2, and OsSK3 were synthesized with a cell-free system in the presence of [ $^{35}$ S]methionine, and were then assayed for import into plastids by incubation with intact pea chloroplasts in vitro. The reaction mixtures were subsequently treated (or not) with thermolysin before analysis by SDS-PAGE and autoradiography. The input of precursor proteins into the assay is shown in the leftmost lanes. The positions and sizes of the precursor and mature proteins are indicated on the right



**Fig. 4** Catalytic activity of mature forms of the three rice SK proteins. **a** Synthesis of full-length and NH<sub>2</sub>-terminally truncated forms of rice SK proteins with a cell-free system in the presence of [<sup>14</sup>C]leucine. The protein products were detected by SDS-PAGE and autoradiography. *T*, total translation mixture; *S*, supernatant fraction prepared by centrifugation of the reaction mixture at 22,000 *g* for 20 min at 4°C. *Open* and *closed arrowheads* indicate the positions of the full-length protein and a cleavage product, respectively. The positions of molecular size standards are indicated on the left. **b** Direct assay of SK activity. The assay was performed at 30°C for 10 or 60 min with OsSK1Δ N62, OsSK2Δ N60, OsSK3Δ N57, or *E. coli* AroL (EcAroL), each at a concentration of 20 nM, in the presence of [ $\gamma$ -<sup>32</sup>P]ATP and in the absence or presence of shikimate. As a negative control, a cell-free

reaction mixture without template mRNA (mRNA<sup>-</sup>) was analyzed. The leftmost lane contains only the SK assay reagents. The origin and migration positions of orthophosphate (Pi), shikimate 3-phosphate (S3P), and ATP are indicated on the left. **c** Quantitation of relative SK activity. The amounts of [<sup>32</sup>P]S3P ( $\mu\text{mol ml}^{-1}$ ) generated in each time course reaction (0, 2, 4, 6, 8 or 10 min) was determined by densitometric analysis by using standard [ $\gamma$ -<sup>32</sup>P]ATP. Results are representative of experiments performed with at least three independent reactions, and standard deviations are indicated by bars. **d** Coupled assay of SK and EPSP synthase activities. The assay was performed in a manner similar to that in (b) with the addition of phosphoenolpyruvate and of in vitro-synthesized EPSP synthase (20 nM) of *E. coli* (EcAroA) or rice (OsEPSPS; lacking the 67-residue cTP)

whereas induction of *OsEPSPS* expression was not apparent until 2 h (Fig. 5b). *OsSK3* transcripts were barely detected in calli, and were not increased by elicitor treatment (data not shown).

In young panicles, all three rice SK genes were expressed in a stage-dependent manner (Fig. 5c, d). The abundance of *OsSK1* and *OsSK3* mRNAs was greater during the heading stage (P2) than before (P1) or after (P3) it. In contrast, although the level of *OsSK2* expression in panicles was lower than that of the other two genes, it increased slightly during progression from stage P1 to P3. In contrast to the SK genes, the expression level of *OsEPSPS* remained unchanged during panicle development.

## Discussion

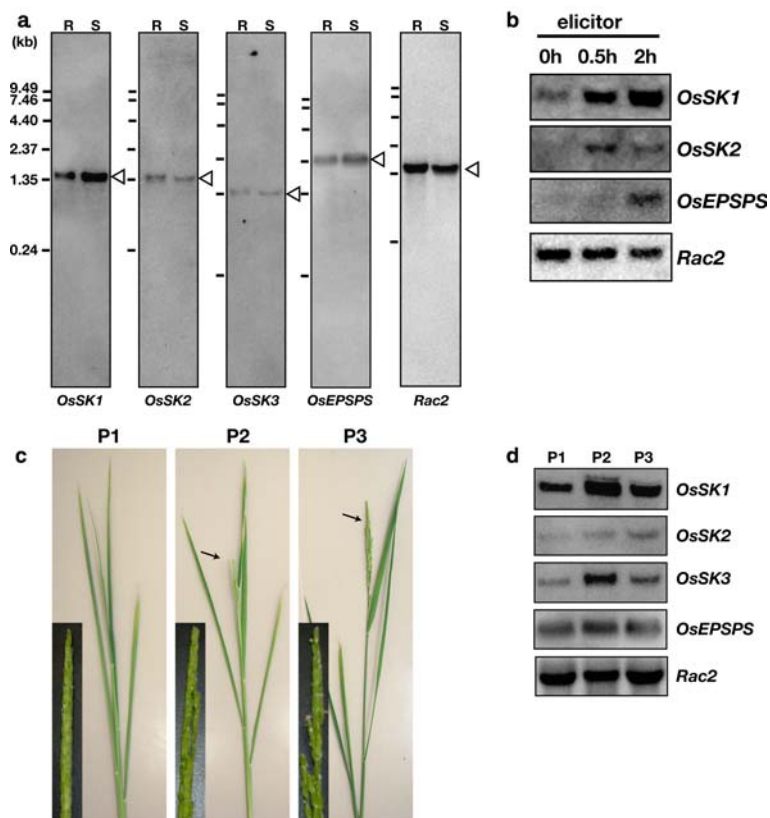
### Identification of three SK genes in rice

We have identified and characterized three distinct SK genes in rice. As far as we are aware, these are the first SK genes isolated from a monocotyledonous plant. A database search also revealed at least two putative SK

genes in the dicot *A. thaliana* (Fig. 2), suggesting that the existence of multiple SK genes is common in land plants. An in vitro assay of protein import revealed that all three rice SK proteins—*OsSK1*, *OsSK2*, and *OsSK3*—translocate into chloroplasts and that this translocation is accompanied by the proteolytic cleavage of an NH<sub>2</sub>-terminal cTP. Biochemical analysis of the putative mature forms of the three rice SK proteins, synthesized with a wheat embryo cell-free system, confirmed that they possess SK catalytic activity. The activity of *OsSK2* was lower than that of *OsSK1* or *OsSK3*, indicative of functional diversity among the isozymes.

SDS-PAGE analysis revealed that the full-length forms of the rice SK proteins, synthesized by the bilayer method for enzyme activity assays, were accompanied by smaller products similar in size to the predicted mature forms of the enzymes (Fig. 4a). We previously found that two  $\alpha$  subunits (OASA1, OASA2) and two  $\beta$  subunits (OASB1, OASB2) of rice anthranilate synthase were also processed at the predicted cTP cleavage sites in the wheat embryo cell-free system (Kanno et al. 2004). In contrast, the smaller forms of the rice SK isozymes were not observed when synthesis was performed by the





**Fig. 5** Expression of SK genes in rice tissues. **a** Tissue distribution of transcripts. Total RNA (20  $\mu$ g) isolated from rice roots (R) or green shoots (S) was subjected to gel-blot analysis with digoxigenin-labeled riboprobes specific for *OsSK1*, *OsSK2*, *OsSK3*, *OsEPSPS*, or rice actin2 gene (*Rac2*) transcripts. Arrowheads indicate the positions of the corresponding transcripts. The molecular sizes of RNA standards are indicated on the left. **b** Effect of an elicitor on transcript abundance. Rice calli were incubated with *N*-

acetylchitoheptaose (1  $\mu$ g ml<sup>-1</sup>) for 0, 0.5, or 2 h, after which total RNA was isolated and analyzed as in (a). **c** Stages of rice panicle development corresponding to before (P1), during (P2), and after (P3) heading. Arrows indicate heading panicles. The magnified picture of the sampled panicle is shown at the left side in each panel. **d** Transcript abundance during stages of panicle heading. Total RNA isolated from panicles shown in (c) was analyzed as in (a)

batch method for the chloroplast import assay (Fig. 3). The incubation time for the batch method of protein synthesis was 1 h compared with 16 h for the bilayer method. It is thus likely that the longer incubation time of the latter method allows proteolytic processing at the cTP cleavage site by peptidases present in the wheat embryo extract. The NH<sub>2</sub>-terminally truncated proteins OsSK1 $\Delta$  N62, OsSK2 $\Delta$  N60, and OsSK3 $\Delta$  N57, synthesized by the bilayer method, appeared as single bands on SDS-PAGE gels (Fig. 4a), indicating that no additional processing of proteins that lack the putative cTP occurred. The solubility (Fig. 4a) and catalytic activity (data not shown) of these truncated proteins were greater than were those of the corresponding full-length proteins, suggesting that removal of the cTP promotes protein folding stabilizes enzyme structure and function.

#### Differential expression of rice SK genes during panicle development

The gene for EPSP, one of the enzymes that function in the shikimate pathway, is present in single copy in rice

(Sato et al. 1999), and we recently confirmed that chorismate synthase is also encoded on a single gene in rice (data not shown). In contrast, there are three SK genes in rice and at least two in *Arabidopsis*. We found that the expression of *OsSK1* and *OsSK2* was induced by elicitor treatment in cultured rice cells. Similar results have been described previously for shikimate pathway genes, including that for SK, in tomato (Görlach et al. 1995), implicating SK genes in a defense response system common to higher plants. We also showed that the SK genes responded more rapidly to elicitor treatment than did the EPSP synthase gene in rice calli. We therefore suggest that SK plays an important role in the contribution of the pre-chorismate pathway to the defense response.

We found that the three rice SK genes are differentially expressed during development of the panicle. Whereas *OsSK3* was expressed at the lowest level among the three SK genes in shoots and roots, its expression, like that of *OsSK1*, was specifically increased at the P2 stage of panicle development. The catalytic activity of OsSK3 was the highest among the three SK isozymes. The heading stage-specific accu-



mulation of *OsSK3* transcripts is therefore suggestive of a specific requirement for S3P production during the development of the panicle in rice. Transcripts of the tomato SK gene were also previously shown to be abundant in the floral organ (Görlach et al. 1994). Plant SKs may thus play important roles in control of metabolic flux through the shikimate pathway with regard to both the defense response and floral organ development.

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## References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402
- Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* 7:1085–1097
- Dixon RA, Steele CL (1999) Flavonoids and isoflavonoids: a gold mine for metabolic engineering. *Trends Plant Sci* 4:394–400
- Douglas SE (1994) The molecular biology of cyanobacteria (chap 5). Kluwer Academic Publishers, Amsterdam
- Duncan K, Edwards RM, Coggins JR (1988) The *Saccharomyces cerevisiae ARO1* gene. An example of the co-ordinate regulation of five enzymes on a single biosynthetic pathway. *FEBS Lett* 241:83–88
- Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* 300:1005–1016
- Gasser CS, Winter JA, Hironaka CM, Shah DM (1988) Structure, expression, and evolution of the 5-enolpyruvylshikimate-3-phosphate synthase genes of petunia and tomato. *J Biol Chem* 263:4280–4287
- Giles NH, Case ME, Partridge CW, and Ahmed SI (1967) A Gene Cluster in *Neurospora Crassa* coding for an aggregate of five aromatic synthetic enzymes. *Proc Natl Acad Sci USA* 58:1453–1460
- Görlach J, Schmid J, Amrhein N (1994) Abundance of transcripts specific for genes encoding enzymes of the prechorismate pathway in different organs of tomato (*Lycopersicon esculentum* L.) plants. *Planta* 193:216–223
- Görlach J, Raesecke HR, Rentsch D, Regenass M, Roy P, Zala M, Keel C, Boller T, Amrhein N, Schmid J (1995) Temporally distinct accumulation of transcripts encoding enzymes of the prechorismate pathway in elicitor-treated, cultured tomato cells. *Proc Natl Acad Sci USA* 92:3166–3170
- Gu Y, Reshetnikova L, Li Y, Wu Y, Yan H, Singh S, Ji X (2002) Crystal structure of shikimate kinase from *Mycobacterium tuberculosis* reveals the dynamic role of the LID domain in catalysis. *J Mol Biol* 319:779–789
- Herrmann KM (1995) The shikimate pathway: early steps in the biosynthesis of aromatic compounds. *Plant Cell* 7:907–919
- Herrmann KM, Weaver LM (1999) The shikimate pathway. *Annu Rev Plant Physiol Plant Mol Biol* 50:473–503
- Kanno T, Kasai K, Ikejiri-Kanno Y, Wakasa K, Tozawa Y (2004) In vitro reconstitution of rice anthranilate synthase: distinct functional properties of the alpha subunits OASA1 and OASA2. *Plant Mol Biol* 54:11–22
- Keith B, Dong X, Ausubel FM, Fink GR (1991) Differential induction of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase genes in *Arabidopsis thaliana* by wounding and pathogenic attack. *Proc Natl Acad Sci USA* 88:8821–8825
- Kinghorn JR, Hawkins AR (1982) Cloning and expression in *Escherichia coli* K-12 of the biosynthetic dehydroquinase function of the arom cluster gene from the eucaryote, *Aspergillus nidulans*. *Mol Gen Genet* 186:145–152
- Krell T, Coggins JR, Laphorn AJ (1998) The three-dimensional structure of shikimate kinase. *J Mol Biol* 278:983–997
- Madin K, Sawasaki T, Ogasawara T, Endo Y (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc Natl Acad Sci USA* 97:559–564
- Minton NP, Whitehead PJ, Atkinson T, Gilbert HJ (1989) Nucleotide sequence of an *Erwinia chrysanthemi* gene encoding shikimate kinase. *Nucleic Acids Res* 17:1769
- Nojiri H, Sugimoto M, Yamane H, Nishimura Y, Yamada A, Shibuya N, Kodama O, Murofushi N, Omori T (1996) Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. *Plant Physiol* 110:387–392
- Oliveira JS, Pinto CA, Basso LA, Santos DS (2001) Cloning and overexpression in soluble form of functional shikimate kinase and 5-enolpyruvylshikimate 3-phosphate synthase enzymes from *Mycobacterium tuberculosis*. *Protein Exp Purif* 22:430–435
- Perry SE, Li HM, Keegstra K (1991) In vitro reconstitution of protein transport into chloroplasts. *Methods Cell Biol* 34:327–334
- Rippert P, Scimemi C, Dubald M, Matringe M (2004) Engineering plant shikimate pathway for production of tocotrienol and improving herbicide resistance. *Plant Physiol* 134:92–100
- Roberts F, Roberts CW, Johnson JJ, Kyle DE, Krell T, Coggins JR, Coombs GH, Milhous WK, Tzipori S, Ferguson DJ, Chakrabarti D, McLeod R (1998) Evidence for the shikimate pathway in apicomplexan parasites. *Nature* 393:801–805
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sambrook J, Maniatis T, Fritsch EF (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Sato T, Wu LH, Nagano H, Kishima Y, Sano Y (1999) Rice EPSP synthase gene resides 45-kb downstream of the Waxy gene. *Rice Genet Newslett* 16:108
- Sawasaki T, Hasegawa Y, Tsuchimochi M, Kamura N, Ogasawara T, Kuroita T, Endo Y (2002a) A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett* 514:102–105
- Sawasaki T, Ogasawara T, Morishita R, Endo Y (2002b) A cell-free protein synthesis system for high-throughput proteomics. *Proc Natl Acad Sci USA* 99:14652–14657
- Schmid J, Schaller A, Leibinger U, Boll W, Amrhein N (1992) The in-vitro synthesized tomato shikimate kinase precursor is enzymatically active and is imported and processed to the mature enzyme by chloroplasts. *Plant J* 2:375–383
- Tozawa Y, Tanaka K, Takahashi H, Wakasa K (1998) Nuclear encoding of a plastid sigma factor in rice and its tissue- and light-dependent expression. *Nucleic Acids Res* 26:415–419
- Tozawa Y, Hasegawa H, Terakawa T, Wakasa K (2001) Characterization of rice anthranilate synthase alpha-subunit genes *OASA1* and *OASA2*. Tryptophan accumulation in transgenic rice expressing a feedback-insensitive mutant of *OASA1*. *Plant Physiol* 126:1493–1506
- Vinella D, Gagny B, Joseleau-Petit D, D'Ari R, Cashel M (1996) Mecillinam resistance in *Escherichia coli* is conferred by loss of a second activity of the AroK protein. *J Bacteriol* 178:3818–3828

- Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* 1:945–951
- Weaver LM, Herrmann KM (1997) Dynamics of the shikimate pathway in plants. *Trends Plant Sci.* 2:346–351
- Whipp MJ, Pittard AJ (1995) A reassessment of the relationship between *aroK*- and *aroL*-encoded shikimate kinase enzymes of *Escherichia coli*. *J Bacteriol* 177:1627–1629