Journal of Experimental Botany, Vol. 61, No. 10, pp. 2683–2692, 2010
doi:10.1093/jxb/erq103 Advance Access publication 25 April, 2010
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Two solanesyl diphosphate synthases with different subcellular localizations and their respective physiological roles in *Oryza sativa*

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Received 1 December 2009; Revised 24 March 2010; Accepted 26 March 2010

Abstract

Long chain prenyl diphosphates are crucial biosynthetic precursors of ubiquinone (UQ) in many organisms, ranging from bacteria to humans, as well as precursors of plastoquinone in photosynthetic organisms. The cloning and characterization of two solanesyl diphosphate synthase genes, *OsSPS1* and *OsSPS2*, in *Oryza sativa* is reported here. OsSPS1 was highly expressed in root tissue whereas OsSPS2 was found to be high in both leaves and roots. Enzymatic characterization using recombinant proteins showed that both OsSPS1 and OsSPS2 could produce solanesyl diphosphates as their final product, while OsSPS1 showed stronger activity than OsSPS2. However, an important biological difference was observed between the two genes: OsSPS1 complemented the yeast *coq1* disruptant, which does not form UQ, whereas OsSPS2 only very weakly complemented the growth defect of the *coq1* mutant. HPLC analyses showed that both OsSPS1 and OsSPS2 yeast transformants produced UQ9 instead of UQ6, which is the native yeast UQ. According to the complementation study, the UQ9 levels in OsSPS2 transformants were much lower than that of OsSPS1. Green fluorescent protein fusion analyses showed that OsSPS1 localized to mitochondria, while OsSPS2 localized to plastids. This suggests that OsSPS1 is involved in the supply of solanesyl diphosphate for ubiquinone-9 biosynthesis in mitochondria, whereas OsSPS2 is involved in providing solanesyl diphosphate for plastoquinone-9 formation. These findings indicate that *O. sativa* has a different mechanism for the supply of isoprenoid precursors in UQ biosynthesis from *Arabidopsis thaliana*, in which SPS1 provides a prenyl moiety for UQ9 at the endoplasmic reticulum.

Key words: Coenzyme Q, *Oryza sativa*, plastoquinone, solanesyl diphosphate synthase, ubiquinone.

Introduction

Ubiquinone (UQ) is a lipid-soluble electron carrier required in the mitochondria of higher organisms, and also in some bacteria for the respiratory chain (Ernster and Dallner, 1995). The physiological roles of UQ have mostly been investigated using mutants of unicellular organisms such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Escherichia coli*, which lack biosynthetic enzymes for UQ (Kawamukai, 2002). For instance, UQ-deficient *S. cerevisiae* and *S. pombe* mutants showed less tolerance for oxidative stress than wild-types (Suzuki *et al.*, 1997), and they did not grow when grown solely in a non-fermentable carbon source such as glycerol (Ashby *et al.*, 1992). In higher plants, such as in the *Arabidopsis thaliana* mutant which lacks the orthologue of *S. cerevisiae COQ2*, arrested

Abbreviations: DMAPP, dimethylallyl diphosphate; DPS, decaprenyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; GFP, green fluorescent protein; GPPS, geranyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; IPP, isopentenyl diphosphate; PHB, *p*-hydroxybenzoic acid; PPT, PHB polyprenyltransferase; SPP, solanesyl diphosphate; SPS, solanesyl diphosphate synthase; UQ, ubiquinone.

embryonic development was shown at an early stage of zygotic embryogenesis (Okada *et al.*, 2004). In comparison, for transgenic tobacco, the overproduction of UQ conferred tolerance to oxidative stress caused by methyl viologen, as well as tolerance to salinity (Ohara *et al.*, 2004).

Long chain polyprenyl diphosphates are produced by consecutive condensation of isopentenyl diphosphate (IPP, C5) units with allylic diphosphates in the *trans*-configuration and are catalysed by polyprenyl diphosphate synthases (PPS). Long chain polyprenyl diphosphates are crucial biosynthetic precursors of important quinone compounds, such as UQ in mitochondria and plastoquinone in plastids (Fig. 1). Particularly in UQ, whose prenyl chain length is species-specific in the range between C20–C55, the primary determinant of the side-chain length of UQ is the product specificity of PPS in each organism (Okada *et al.*, 1996). In *S. cerevisiae*, the hexaprenyl diphosphate synthase (gene name *COQ1*) is responsible for the biosynthesis of UQ6 and is localized to the mitochondrial inner-membrane; there is only a single biosynthetic pathway to UQ in the mitochondria (Gin and Clarke, 2005). An exception was reported in *Trypanosoma cruzi*, which had its PPS in glycosomes (Ferella *et al.*, 2006). In higher plants, such as in *A. thaliana*, solanesyl diphosphate (SPP) synthases (AtSPS1) were shown to provide a C45 prenyl chain for UQ biosynthesis, and the subcellular localization has been reported to be in the endoplasmic reticulum (ER) (Hirooka *et al.*, 2003, 2005; Jun *et al.*, 2004). It is worth noting that although the localization of AtSPS1 is in the ER for *A. thaliana*, the localization of *p*-hydroxybenzoate prenyltransferase (AtPPT1) is in the mitochondria where the aromatic intermediate *p*-hydroxybenzoic acid (PHB) is condensed with SPP leading to UQ9 formation. In addition, most UQ biosynthetic enzymes are still unidentified in plants, and thorough characterizations are necessary to understand the whole UQ biosynthetic pathway of plants. To clarify whether or not the ER-localization of SPS for UQ biosynthesis is common among plants, two *SPSs* have been cloned from *Oryza sativa* and their functions, as well as their subcellular localizations, have been characterized.

Materials and methods

Plant materials and isolation of two OsSPS *cDNAs*

Japonica rice cultivars (*Oryza sativa* L. cv. Kinmaze) were cultivated as described previously (Isshiki *et al.*, 2001). Total RNA was extracted from mature leaves of *O. sativa* with an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Reverse transcription was performed using Superscript III RNase H- reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) with 2.0 μg of total RNA. To isolate candidate genes encoding SPSs, the full-length cDNA database of *O. sativa* (http://cdna01.dna.affrc.go.jp/cDNA/) were searched and two genes were found encoding polypeptides similar to yeast hexaprenyl diphosphate synthase, which were designated *OsSPS1* and *OsSPS2*. The coding region of the *OsSPS1* gene was amplified by nested PCR with KOD plus DNA polymerase (TOYOBO, Tokyo, Japan) and

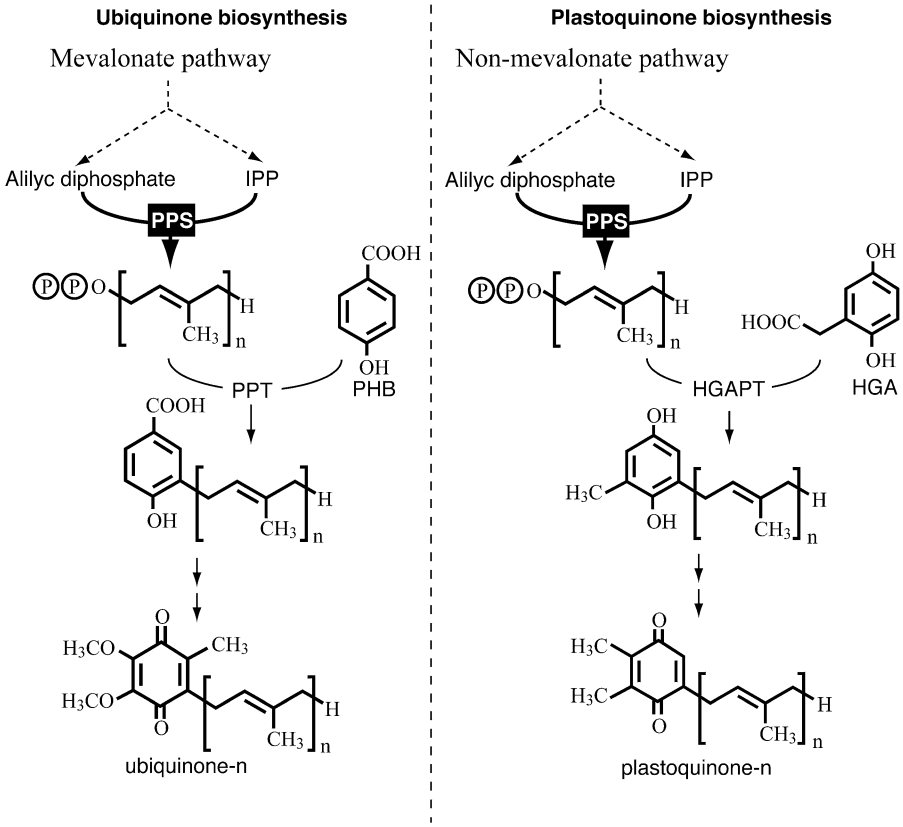

Fig. 1. Biosynthetic routes of ubiquinone and plastoquinone. Polyprenyl diphosphates for ubiquinone and plastoquinone biosynthesis are provided by polyprenyl diphosphate synthases. IPP, isopentenyl diphosphate; PPS, polyprenyl diphosphate synthase; PHB, *p*-hydroxybenzoic acid; PPT, PHB polyprenyltransferase; HGA, homogentisic acid; HGAPT, HGA polyprenyltransferase.

the DNA-RNA hybrid as the template. The primer pair for the first PCR was Os11stfw and Os11strv (Table 1), and the second PCR was carried out using the primer pairs Os1fullKpn1fw and Os1fullXhoIrv (Table 1). The PCR product (1293 bp) was subcloned in pENTR3C (Invitrogen) via *Kpn*I and *Xho*I sites to yield pENTR3C-OsSPS1. The isolation of the *OsSPS2* cDNA was done in the same manner as applied to *OsSPS1* with the following modifications. The primer pair for the first PCR to isolate *OsSPS2* was Os21stfw and Os21strv (Table 1). The second PCR was carried out using the primer pairs Os2fullEcoRIfw and Os1fullXhoIrv (Table 1). The PCR product (1212 bp) was subcloned in pENTR3C (Invitrogen) via *Eco*RI and *Xho*I sites to yield pENTR3C-OsSPS2. For yeast expression of *OsSPS1* and *OsSPS2*, pDR196GW, was used with a Gateway™ Cloning System Reading Frame Cassette A (Invitrogen Corp., Carlsbad, CA, USA) in the *Sma*I site of the yeast shuttle vector pDR196 (Rentsch *et al.*, 1995). The pDR196GW was used for the LR recombination with pENTR3C-OsSPS1 or pENTR3C-OsSPS2 to yield pDR196GW-OsSPS1 and pDR196GW-OsSPS2, in which these genes were inserted downstream of the strong constitutive promoter, PMA1 of the pDR196GW vector.

Heterologous expression of OsSPSs *and their SPS activity*

The plasmids pDR196GW-OsSPS1 and pDR196GW-OsSPS2 were introduced into the *S. cerevisiae* strain W303-1A-Δcoq1, which is a disruptant of the *COQ1* gene (Gin and Clarke, 2005). The yeast strain harbouring pDR196GW-OsSPS1 and pDR196GW-OsSPS2 were cultured in SD (-ura) liquid media to reach the mid-log phase. The harvested yeast cells were used to extract UQ.

For the expression in *E. coli*, coding sequences of *OsSPS1* and *OsSPS2* without stop codons were amplified with KOD plus DNA polymerase (TOYOBO) using the following primer pair: Os1BglIIfw and Os1deltastopXhoIrv or Os2fullEcoRIfw and Os2deltastopXhoIrv (Table 1) to subclone into pET22b (Novagen, Darmstadt, Germany). *E. coli* origami B (DE3) (Novagen) harbouring pET-SPS1 or pET-SPS2, was used for expression, and the recombinant proteins were expressed by adding 10 mM (final concentration) isopropyl β-D-thiogalactoside at 37 °C for 6 h. The bacteria were sonicated in an extraction buffer (50 mM TRIS-HCl, pH 8.0, containing 5% glycerol, 1 mM DTT) and the supernatant, which was centrifuged at 9200 *g*, was used for purification. TALON Metal Affinity Resin (Clontech, CA, USA) was used for purification of the His-tag fusion protein. The SPS assay was followed according to the protocol described by Hirooka *et al.* (2003) and Fujii *et al.* (1982). Prenyltransferase activity was measured by determination of the radioactivity from ^{14}C-IPP incorporated into 1-butanol extractable polyprenyl diphosphates. The standard assay mixture contained, in a final volume of 200 µl, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 15 µg ml^{-1} Tween 80, 2.5 mM DTT, 20 µM allylic substrate (DMAPP, GPP, FPP or GGPP), 1.8 µM ^{14}C-IPP (55 mCi mmol^{-1}), 41 mM TRIS-HCl pH 7.5 and a suitable amount of enzyme. The incubation was carried out at 30 °C for 10 min. The reaction was stopped with 200 µl of NaCl-saturated water. The reaction products were extracted with 1 ml 1-butanol saturated with NaCl-saturated water, and the radioactivity in the 1-butanol extract was measured in the dpm mode with 3 ml of liquid scintillation detector cocktail (Aquasol-2 No. 6NE9529, Perkin Elmer, Winter Street Waltham, MA, USA) by Liquid scintillation analyser (Tri-Carb 2800TR, Perkin Elmer). For product analysis of the enzymatic reaction, the standard assay mixture contained, in a final volume of 200 µl, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 15 µg ml^{-1} Tween 80, 2.5 mM DTT, 20 µM allylic substrate (DMAPP, GPP, FPP or GGPP), 1.8 µM ^{14}C-IPP, 80 µM IPP, 41 mM TRIS-HCl pH 7.5 and a suitable amount of enzyme. The incubation was carried out at 37 °C for 12 h. After enzymatic reaction, 100 µl acid phosphatase solution (5 units potato acid phosphatase, 50 mM phosphate buffer pH 5.6) and 450 µl methanol (0.17% TritonX-100) were added to hydrolyse the resulting polyprenyl diphosphates. This hydrolysis was carried out at 37 °C for 12 h. The hydrolysates were extracted with 100 µl of hexane, and analysed by reversed-phase thin-layer chromatography (LKC-18, Whatman, Kent ME14 2LE UK) that was developed with acetone/water (19:1 v/v). Authentic standard alcohols were visualized with iodine vapour, and the distribution of radioactivity in the products was detected and quantified with BAS1800 analyser (Fuji Film, Tokyo, Japan).

Table 1. Oligonucleotides used in this study

Underline indicates non-native restriction sites or non-native *attB* site for subcloning, respectively.

Os11stfw	5′-CTCGGGAGCCATGTCGTG-3′
Os11strv	5′-CCACCTTGTGAATGCTAACAGTTG-3′
Os1fullKpn1fw	5′-gcggtaccATGTCGTGGCGGTGGGCTC-3′
Os1fullXholrv	5′-ggctcgagCTATTTTGTTCTAGTGATGACTCTCTC TGTG-3′
Os21stfw	5′-GGAAGAGGTTCTTGGTGTCCAAAG-3′
Os21strv	5′-CTTCCCAAGATCGACATTATTCTAGCAAG-3′
Os2fullEcoRIfw	5′-gcgaattcATGTTGTCTGTGAGCTGCCCG-3′
Os1fullXholrv	5′-cgctcgagTCAGTCAATCCTCTGAAGATTATATTT CACC-3′
Os1BglIIfw	5′-gcagatctATGTCGTGGCGGTGGGCTC-3′
Os1deltastopXholrv	5′-ggctcgagTTTTGTTCTAGTGATGACTCTCTCTGTG-3′
Os2fullEcoRIfw	5′-gcgaattcATGTTGTCTGTGAGCTGCCCG-3′
Os2deltastopXholrv	5′-cgctcgagGTCAATCCTCTGAAGATTATATTTCACC– 3′
Os1GFPfwBP	5′-ggggacaagtttgtacaaaaaagcaggcttcATGTCGTGGCGG TGGGCTC-3′
Os1GFPrvBP	5′-ggggaccactttgtacaagaaagctgggtcTTTTGTTCTAGTGA TGACTCTCTCTGTG-3′
Os2GFPfwBP	5′-ggggacaagtttgtacaaaaaagcaggcttcATGTTGTCTGTGA GCTGCCCG-3′
Os2GFPrvBP	5′-ggggaccactttgtacaagaaagctgggtcGTCAATCCTCTGA AGATTATATTTCACC-3′
OsSPS1probe2ndfw	5′-CAACTGTTAGCATTCACAAGGTG-3′
OsSPS1probe2ndrv	5′-GCGCCTAATCTTGGAAGATTGAAAAC-3′
OsSPS23UTRfw	5′-CAGAGGATTGACTGATGGATTCAC-3′
OsSPS23UTRrv	5′-GTCGTTCTCCAATGACAAATGAAATG-3′

Transient expression of GFP fusion protein

The coding sequences of *OsSPS1* and *OsSPS2* without stop codons were amplified with KOD plus DNA polymerase (TOYOBO) using the primer pairs Os1GFPfwBP and Os1GFPrvBP, or Os2GFPfwBP and Os2GFPrvBP (Table 1) to subclone into pDONR221 (Invitrogen Corp., Carlsbad, CA, USA) by a BP reaction. Subsequently, pDONR-OsSPS1 and pDONR-OsSPS2 were used for LR reaction with psmRSGW-GFP, which had a cauliflower mosaic virus 35S promoter upstream of the cloning site, to yield psmRSGW-OsSPS1GFP and psmRSGW-OsSPS2GFP, respectively. The resulting plasmids (10 µg) were precipitated onto spherical gold beads (Bio-Rad, CA, USA). Onion peel or tobacco leaves were bombarded using a particle gun (PDS-1000, Bio-Rad) according to the manufacturer's instructions. After 24 h, the GFP fluorescence in onion and tobacco cells were observed with an Axioskop 2 (Carl Zeiss, Jena, Germany) which had an excitation filter of 470 nm and a barrier filter of 500 to 530 nm (band path). Mito Tracker Red CMXRos (Molecular Probes Inc., OR, USA) was used to stain mitochondria.

Semi-quantitative reverse transcription-PCR analysis

Total RNA was extracted from mature leaves, stems, and roots of *O. sativa* as described above. Reverse transcription was performed using Superscript III RNase H- reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) with 2.0 µg of total RNA. The 3′-untranslated regions of *OsSPS1* (445 bp) and *OsSPS2* (340 bp)

were amplified using GoTaq DNA Polymerase (Promega, WI, USA) with the primer pairs OsSPS1probe2ndfw and OsSPS1probe2ndrv, or OsSPS23UTRfw and OsSPS23UTRrv (Table 1).

HPLC analysis of UQ6 and UQ9 in yeast

UQ extraction from yeast cells and HPLC analysis were carried out according to the method described by Uchida *et al.* (2000), with slight modifications. HPLC was conducted with the following conditions on a Shimadzu LC-10A system: column, TSK-gel ODS-80TM (Tosoh, Tokyo, Japan; 4.6 mm i.d. ×250 mm); solvent system, ethanol/H_2O (97.5:2.5 v/v); temperature, 40 °C; flow rate, 1.0 ml min^{-1}; detection, absorbance measured at 275 nm with a SPD6A photodiode array detector. UQ9 and UQ6 were identified by direct comparison with standard specimens.

Results

Isolation of two SPS *genes from* O. sativa

To identify the genes encoding the SPSs responsible for UQ or plastoquinone biosynthesis in *O. sativa*, the full-length

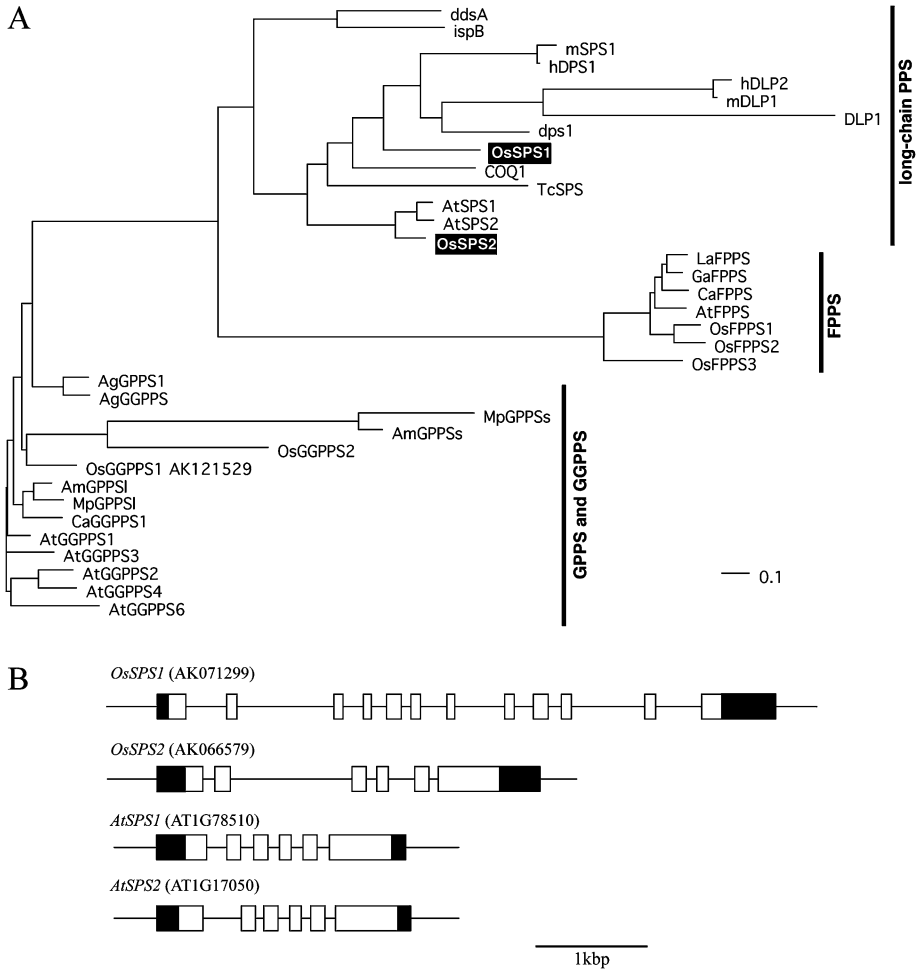

Fig. 2. Phylogenetic relationship and exon-intron structure of prenyl diphosphate synthases. (A) Phylogenetic tree of polypeptide sequences of prenyl diphosphate synthases made by the ClustalW program. Polypeptide sequences (accession number) used for the analysis are listed as follows: AgGPPS1, *Abies grandis* GPPS1 (AF513111); MpGPPSl, *Mentha×piperita* GPPS large subunit (AF182828); MpGPPSs, *Mentha×piperita* GPPS small subunit (AF182827); AmGPPSl, *Antirrhinum majus* GPPS large subunit (AAS82860), AmGPPSs, *Antirrhinum majus* GPPS small subunit (AAS82859); LaFPPS, *Lupinus albus* FPPS (U15777); GaFPPS, *Gossypium arboreum* FPPS (Y12072); AtFPPS, *A. thaliana* FPPS (Q09152); CaFPPS, *Capsicum annuum* FPPS (Q42658); OsFPPS1, *O. sativa* FPPS1 (D85317); OsFPPS2, *O. sativa* FPPS2 (AB021979); OsFPPS3, *O. sativa* FPPS3 (AK072747); AgGGPPS, *A. grandis* GGPPS (AF425235); AtGGPPS1, *A. thaliana* GGPPS1 (NM_119845); AtGGPPS2, *A. thaliana* GGPPS2 (NM_127943); AtGGPPS3, *A. thaliana* GGPPS3 (NM_112315); AtGGPPS4, *A. thaliana* GGPPS4 (NM_127420); AtGGPPS6, *A. thaliana* GGPPS6 (NM_103841); CaGGPPSl, *Capsicum annuum* GGPPS1 (P80042); OsGGPPS1, *O. sativa* GGPPS1 (AK121529); OsGGPPS2, *O. sativa* GGPPS2 (AK100459); AtSPS1, *A. thaliana* SPS1 (AB188497); AtSPS2, *A. thaliana* SPS2 (AB188498), ispB; octaprenyl diphosphate synthase of *E. coli* (NP417654); ddsA, DPS of *Gluconobacter suboxydans* (AB006850); COQ1, hexaprenyl diphosphate synthase of *S. cerevisiae* (J05547); dps1, a component of DPS encoded by *dps1* from *S. pombe* (D84311); DLP1, a component of DPS from *S. pombe* (AB118853); mSPS1, a component of SPS from *Mus musculus* (AB210841); mDLP1, a component of SPS from *M. musculus* (AB210840); hDPS1, a component of DPS from human (AB210838); hDLP2, a component of DPS from human (AI742294); TcSPS, *Trypanosoma cruzi* SPS (AAK69519). (B) Exon–intron structures of plant solanesyl diphosphate synthase genes. Black boxes and white boxes indicate untranslated regions and coding regions, respectively.

cDNA database of *O. sativa* (http://cdna01.dna.affrc.go.jp/cDNA/) was searched, and two candidate genes were found whose products exhibited significant similarities with the yeast COQ1 polypeptide, otherwise known as hexaprenyl diphosphate synthase (identities 41% and 39%, respectively). These *O. sativa* genes, designated *OsSPS1* and *OsSPS2*, code for polypeptides of 430 and 403 amino acids, and both possess two conserved aspartate-rich motifs (DDxxD motif) among the Mg^{2+}-dependent prenyltransferase family, which are responsible for prenyl substrate binding. As predicted by web programs such as TargetP, ChroroP, and SignalP, OsSPS1 showed a putative mitochondrial signal peptide sequence (23 amino acids), while OsSPS2 possessed a putative transit peptide (57 amino acids) for plastidal localization at the N-terminus. As shown in Fig. 2, phylogenetic analysis revealed that PPS proteins for UQ and plastoquinone biosynthesis formed an independent clade from other prenyltransferases involved in prenyl chain elongation, such as geranylgeranyl diphosphate (C20) synthase (GGPPS), farnesyl diphosphate (C15) synthase (FPPS), or geranyl diphosphate (C10) synthase (GPPS). Two rice paralogues of SPS, OsSPS1 and 2, clearly belonged to the long chain PPS family involved in UQ or plastoquinone biosynthesis (Fig. 2A), whereas OsSPS1 was classified as a separate branch from AtSPS1, AtSPS2, and OsSPS2 in the phylogenetic analysis. The rice genome analysis on the exon–intron structure revealed that *OsSPS2* had a very similar structure to *AtSPS1* and *AtSPS2*, whereas the structure of *OsSPS1* was completely different from them (Fig. 2B). This difference in the exon–intron structure also suggests that *OsSPS1* evolutionarily branched very early on to form a different subfamily of plant *SPS* genes. For subsequent functional analyses, the coding regions of these two genes were isolated by nested reverse-transcription PCR.

Enzyme assay of OsSPS1 and OsSPS2

To confirm the enzyme activities of OsSPS1 and OsSPS2, an *in vitro* enzyme assay was carried out using recombinant proteins. For the *in vitro* SPS assay, two OsSPSs were heterologously expressed as a His-tagged fusion protein in *E. coli* cells, and purified with TALON Metal Affinity Resin (Clontech, CA, USA). The substrate specificity was studied using ^{14}C-labelled IPP as a prenyl donor, and DMAPP, GPP, FPP, and GGPP as prenyl acceptors. The results in Table 2 clearly showed that OsSPS1 and OsSPS2 could utilize all prenyldiphosphates that were tested. The reaction products with GPP or FPP from OsSPS1 or OsSPS2 were then dephosphorylated and analysed by reverse-phase TLC. When either GPP or FPP was used as the primer substrate, solanesol (C45) was detected as the final product in the TLC analysis (Fig. 3; Table 3). These results indicated that both OsSPS1 and OsSPS2 were functional as SPSs.

Yeast complementation test of OsSPS1 and OsSPS2

The direct involvement of OsSPS1 and OsSPS2 in the UQ biosynthesis was investigated in the yeast complementation assay. The *coq1* gene disruptant of *S. cerevisiae* (strain: Δcoq1) was found to be unable to produce UQ due to a lack of hexaprenyl diphosphate synthase activity (Gin and Clarke, 2005). This strain showed a clear growth defect on the minimum medium containing glycerol as the sole carbon source, since UQ, which is required in order to utilize this non-fermentable carbon source, was not present. *OsSPS1* and *OsSPS2* cDNAs were subcloned into the yeast expression vector pDR196GW, to yield pDR196GW-OsSPS1 and

Table 2. Substrate specificity of recombinant OsSPS1 and OsSPS2

The absolute activities of OsSPS1 and OsSPS2 were 13.7±1.03 pkat mg^{-1} protein (for FPP), 0.17±0.03 pkat mg^{-1} protein (for GPP), respectively.

Alylic substrate	Relative activity (%)	
	OsSPS1	OsSPS2
DMAPP	6.00±5.08	18.1±6.59
GPP	59.3±30.8	100
FPP	100	44.0±31.1
GGPP	8.23±4.26	34.6±9.80

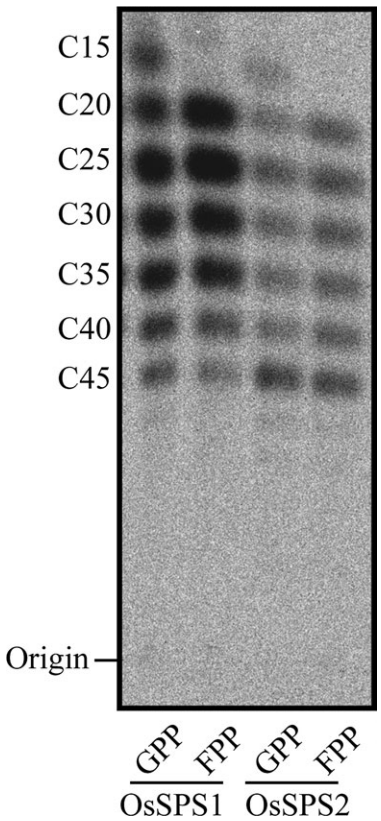

Fig. 3. Enzyme assays of OsSPS1 and OsSPS2. Enzyme assays were carried out using ^{14}C-IPP and GPP or FPP as substrates, and the reaction products were analysed by reverse-phase TLC. Purified recombinant enzymes were used for the assays as described in the experimental section. Signals were detected by BAS1800 (Fuji Film).

Table 3. Composition of enzymatic reaction products by OsSPS1 and OsSPS2

	OsSPS1		OsSPS2	
Alyllic substrate	**GPP**	**FPP**	**GPP**	**FPP**
Product distribution (%)				
C15	9.66	–	8.04	–
C20	15.1	24.5	11.7	15.2
C25	27.0	32.5	19.6	19.8
C30	18.0	20.1	14.8	17.3
C35	16.4	14.3	12.8	15.0
C40	8.71	5.90	10.6	14.2
C45	5.11	2.76	22.6	18.5

pDR196GW-OsSPS2, respectively, for the constitutive expressions. As shown in Fig. 4A, the functional expression of OsSPS1 successfully complemented the growth of Δcoq1 yeast on a glycerol plate in almost the same manner as in pDR196GW-COQ1 expression in the Δcoq1 strain, whereas no growth was observed in the Δcoq1 harbouring the empty vector that was used as a negative control. In addition, UQ9 was detected in HPLC analysis of the extract of the Δcoq1 transformant harbouring pDR196GW-OsSPS1, instead of the native UQ6 of yeast. Neither UQ9 nor UQ6 were detected in the yeast transformant of the empty vector control (Fig. 4B). This result clearly indicated that OsSPS1 is a functional SPS involved in the UQ production of yeast *in vivo*. The chain-length of the UQ9 molecule produced

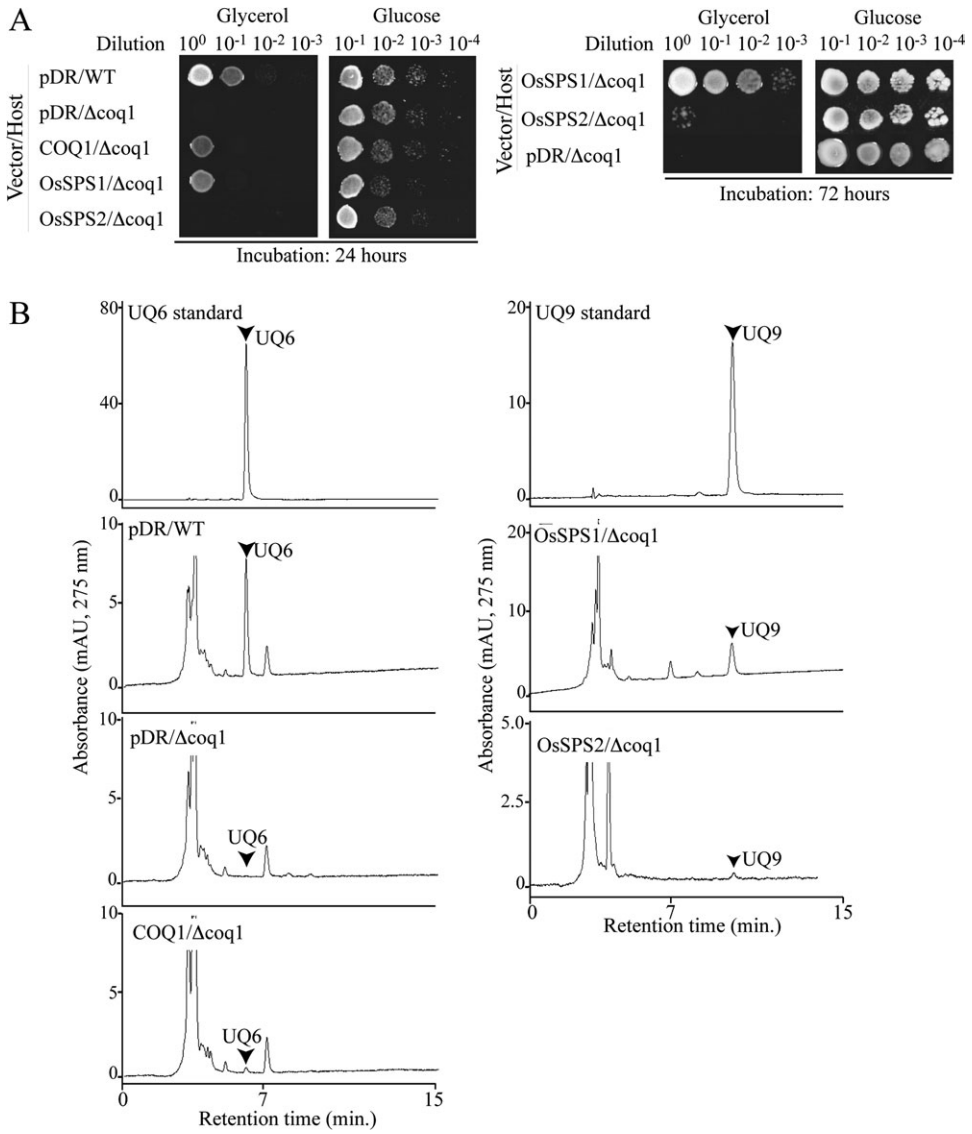

Fig. 4. Yeast complementation analyses of *OsSPS1* and *OsSPS2*. (A) Full-length cDNAs of *OsSPS1* and *OsSPS2* were introduced into yeast *coq1* disruptant, and the transformants were grown on an SD-plate with either glucose or glycerol as the sole carbon source. The yeast *COQ1* gene was used as a positive control. (B) UQ9 detection in yeast *coq1* disruptants harbouring pDR196GW-OsSPS1 and pDR196GW-OsSPS2. Arrowheads indicate the retention time of UQ6 or UQ9 in HPLC chromatograms. WT, wild-type yeast (W303-1A); Δcoq1, *coq1* disruptant; pDR, empty vector control; COQ1, expression vector for COQ1; OsSPS1, expression vector for OsSPS1; OsSPS2, expression vector for OsSPS2; UQ6, ubiquinone-6; UQ9, ubiquinone-9.

by the expression of OsSPS1 coincided with the native UQ9 in *O. sativa*.

In addition to OsSPS1, pDR196GW-OsSPS2 was also introduced into the yeast Δcoq1 strain. The yeast complement study showed that the growth of the Δcoq1 mutant on the glycerol plate was recovered by OsSPS2 expression, and, in addition, UQ9 was detectable in the extract of the Δcoq1 transformant harbouring pDR196GW-OsSPS2. However, the complementation of the growth defect with OsSPS2 was much weaker than that with OsSPS1, and the UQ9 level in OsSPS2 was also lower than in the OsSPS1 transformant (Fig. 4A, B).

Expression analysis of OsSPS1 and OsSPS2 and the intracellular localization of their gene products

Organ-specific mRNA accumulations of *OsSPS1 and OsSPS2* were investigated by reverse transcription-PCR analysis using total RNA prepared from leaves, stems, and roots. *OsSPS1* and *OsSPS2* mRNAs were detected in all *O. sativa* organs that were tested, but the expression of *OsSPS1* was highest in roots (4.8 times and 8.6 times higher than that of leaves and stems, respectively, Fig. 5). By contrast, high expression of *OsSPS2* mRNA was observed in both leaves and roots, which were 5.7-fold and 6.1-fold stronger than in stems (Fig. 5).

To determine the subcellular localization of OsSPS1 and OsSPS2, two plasmids were constructed in which the coding regions of OsSPSs without stop codons were fused to green fluorescent protein (GFP) under the control of the cauliflower mosaic virus 35S promoter. These plasmids were then introduced into onion peels by particle bombardment. In the transient expression experiment, GFP fluorescence of OsSPS1–GFP was mainly localized in dotted organelles, and the green fluorescence completely matched the red fluorescence of mitochondria stained with a marker dye Mito Tracker (Fig. 6). These data indicate that OsSPS1 localized to mitochondria, in conformity with the prediction made by the TargetP program. Contrary to this, GFP fluorescence of OsSPS2–GFP fusion revealed larger sized dots than that of OsSPS1–GFP in the cells, which did not matched the Mito Tracker staining (Fig. 6). To confirm the intracellular localization of OsSPS2, OsSPS2–GFP plasmids were also introduced into tobacco leaves by particle bombardment. The green fluorescence of OsSPS2–GFP was also observed in dotted organelles, which completely matched the chlorophyll fluorescence of chloroplasts, indicating that OsSPS2 localized at plastids (see Supplementary Fig. S1 at *JXB* online).

Discussion

In this report, two functional SPS cDNAs, *OsSPS1* and *OsSPS2* were isolated from *O. sativa*. Long-chain-producing PPSs were divided into two classes, a homodimeric-type PPS found in *S. cerevisiae* or *A. thaliana* (Hirooka *et al.*, 2003, 2005; Jun *et al.*, 2004), as well as a heterotetramer-type PPS reported in *S. pombe* (Saiki *et al.*, 2003), *Homo sapiens* or *Mus musculus* (Saiki *et al.*, 2005). In the *O. sativa* genome, no homologue encoding the subunit of heterotetramer-type decaprenyl diphosphate synthase (DPS) of *S. pombe* was observed. The expression of a single gene, either of *OsSPS1* or *OsSPS2*, could complement UQ production in *S. cerevisiae* Δcoq1 mutant, in which homodimeric-type hexaprenyl diphosphate synthase gene (*COQ1*) was disrupted. These results suggested that both OsSPS1 and OsSPS2 show the enzymatic function as a single gene product as observed in the hexaprenyl diphosphate synthase of *S. cerevisiae*, which were proposed to form a homodimer to have the enzyme activity.

To complement the growth defect of *coq1* disruption in *S. cerevisiae*, an additional mitochondrial signal peptide was not necessary for OsSPS1. In comparison, AtSPS1 required the addition of a mitochondrial signal peptide at the N-terminus for the complementation of *S. pombe dlp1* and *dps1* mutants (Jun *et al.*, 2004). These data strongly suggest that OsSPS1 is sorted to mitochondria in *S. cerevisiae* and functions to provide SPP for UQ formation. This coincided with the microscopic observation that the GFP–fusion protein of OsSPS1 localized to mitochondria in plant cells (Fig. 6).

Recently, it was reported that the mitochondrial expression of *Gluconobacter suboxydans* DPS in *O. sativa* yielded the generation of UQ10 in addition to endogenous UQ9; however, the enforced localization of this foreign protein to

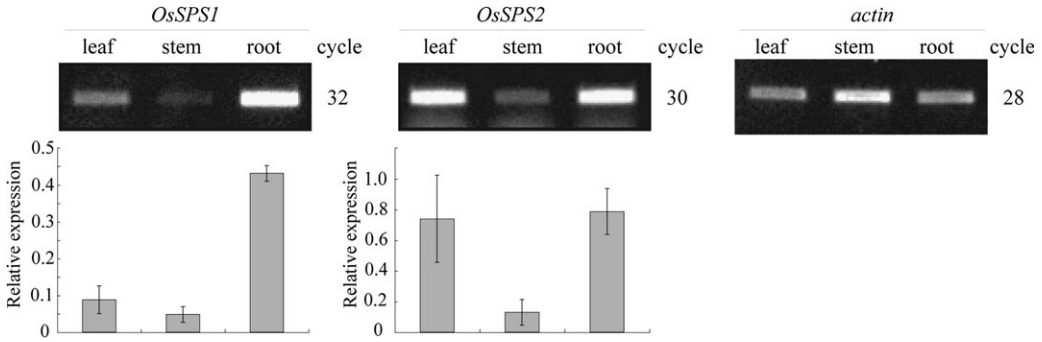

Fig. 5. Organ-specific expression of *OsSPS1 and OsSPS2* mRNAs in rice plants. Adult plants were divided into mature leaves, stems, and roots, and total RNA was extracted. Organ-specific mRNA expression levels of *OsSPS1* and *OsSPS2* in rice plants were estimated by reverse transcription-PCR. Values indicate data ±SD, normalized by *actin* as a control.

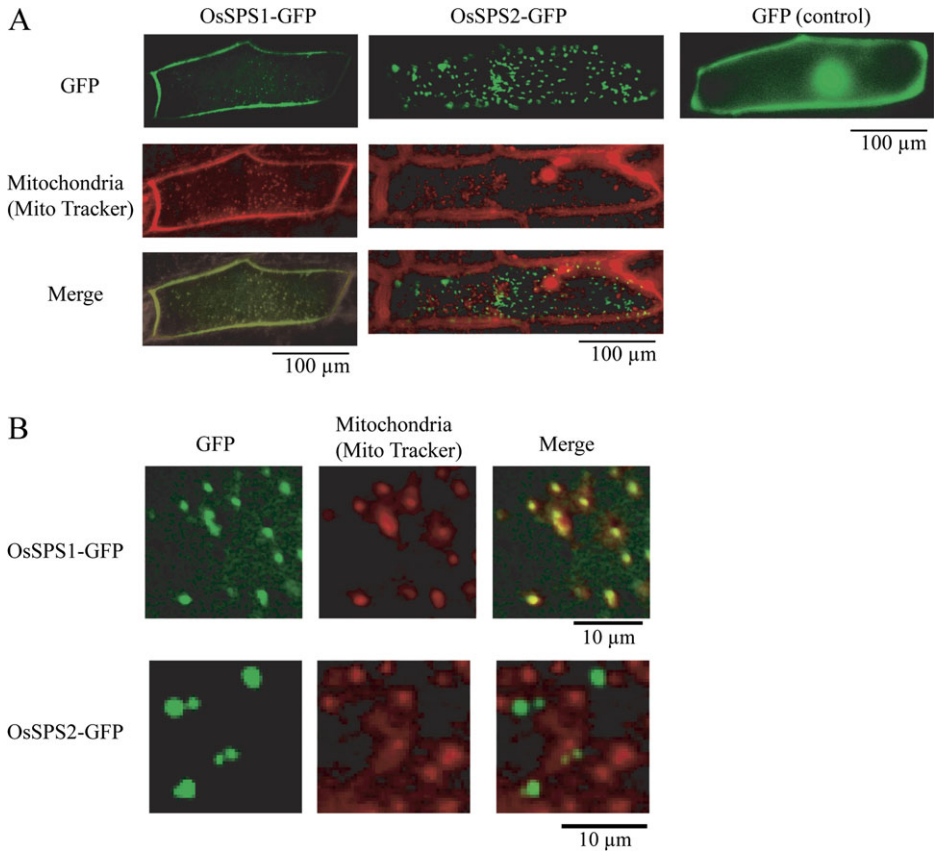

Fig. 6. Transient expression of GFP fusion constructs of OsSPS1 and OsSPS2. The GFP-fusion constructs of OsSPS1 and OsSPS2 were introduced into onion peels by particle bombardment. These images were obtained 24 h after bombardment. (A) The GFP fluorescence in onion and tobacco cells were observed with an Axioskop 2 (Carl Zeiss, Jena, Germany) with an excitation filter of 470 nm and a barrier filter of 500–530 nm (band path). (B) Enlargement of fluorescent images.

the ER was unsuccessful in producing UQ10 in rice (Takahashi *et al.*, 2006). The present study also indicated that the native compartment of SPS for UQ biosynthesis in *O. sativa* was in the mitochondria. Moreover, the mitochondrial localization of OsSPS1 seemed to be advantageous for UQ production, since the next biosynthetic enzyme OsPPT1a, which was responsible for condensation of SPP and PHB, was also localized in the mitochondria (Ohara *et al.*, 2006).

Contrary to OsSPS1, it was shown that *A. thaliana* AtSPS1 was localized to the ER, whereas the enzyme catalysing the subsequent prenylation step, AtPPT1, was localized in the mitochondria of *Arabidopsis*. ER localizations of PPS may be reasonable in terms of substrate availability in UQ production because the prenyl-side-chain of UQ was supplied from the mevalonate pathway located in the cytosol. Thus, the polyprenyl diphosphate synthesized at the ER is proposed to move into the mitochondria to form UQ. Our previous report also suggested that biosynthetic intermediates of UQ can move from one organelle to another inside the cell (Ohara *et al.*, 2004). The different subcellular localization of SPS among plant species suggests that the UQ biosynthetic route is organized in a different manner depending on the plant species (Swiezewska *et al.*, 1993).

In addition to OsSPS1, OsSPS2 expression could also complement the growth defect of the *coq1* disruption in yeast (Fig. 4). However, the ability of OsSPS2 to complement the growth of Δcoq1 was low. In other words, the growth rate of the OsSPS2 transformant on the glycerol plate was estimated to be approximately 1000-fold lower than that of the OsSPS1 transformant. This weak complementation by OsSPS2 in Δcoq1 may be due to the non-mitochondrial localization of OsSPS2 in yeast cells, and therefore could not efficiently compensate the function of the native hexaprenyl diphosphate synthase (COQ1) in yeast, which is localized in the mitochondria. In addition to intracellular localization, lower catalytic activity of OsSPS2 may be another reason of this weak complementation. In plant cells, GFP fusion protein analyses clearly indicated that OsSPS2 is localized to plastids. Since there is higher mRNA expression of *OsSPS2* than of *OsSPS1* in rice leaves, and since OsSPS2 is localized in the plastids, it is strongly suggested that OsSPS2 is involved in plastoquinone biosynthesis by supplying SPP in plastids, as was proposed for the AtSPS2 protein (Jun *et al.*, 2004; Hirooka *et al.*, 2005). The different preference in substrate specificity for both OsSPS1 and OsSPS2 may reflect this difference in their intracellular localization to either the mitochondria or plastid. For example, FPP, which is supplied from the cytosol-localized mevalonate pathway, is the most preferable

substrate for OsSPS1, whereas GPP, which is mainly provided from the non-mevalonate pathway in plastids, is preferable for OsSPS2 (Table 2). In future, reverse genetic studies on these SPS genes, such as over-expression and/or knockout of OsSPS1 and 2 in rice, for example, utilizing TOS17 mutants (http://www.rgrc.dna.affrc.go.jp/stock.html), would bring additional information of their detailed physiological functions *in planta*.

Recently, UQ has been used widely in cosmetics and food supplements worldwide, since new biological activities of UQ have been reported (Grundman and Delaney, 2002; Muller *et al.*, 2003). Thus, from a biotechnological point of view, the increase in UQ content in crops and vegetables is an attractive target for genetic engineering of UQ biosynthesis. For such metabolic engineering, an increase in the prenyl chain supply using the *SPS* gene and enforcing rate-limiting reaction steps, such as the prenylation of PHB mediated by PPS, will be an efficient way to raise the UQ content in transgenic plants (Takahashi *et al.*, 2006). Determining unidentified biosynthetic genes involved in the biosynthetic pathway of UQ, as well as discovering the intracellular transport mechanism of UQ and its intermediates in plants still remains to be completed.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. 1. Transient expression of the GFP fusion protein of OsSPS2 in tobacco leaves.

Acknowledgements

We thank Dr Catherine F Clarke and Dr Peter Gin of the University of California, Los Angeles for the generous gift of the *S. cerevisiae coq1* disruptant. We also thank Dr Ko Shimamoto and Dr Masayuki Isshiki of the Nara Institute of Science and Technology for providing us with the plant material. We also thank Dr Atsuhiro Oka, Dr Takashi Aoyama, and Dr Tomohiko Tsuge of Kyoto University for their technical assistance with operating the particle gun. The yeast shuttle vector pDR196 was a generous gift from Dr W Frommer of the Carnegie Institution. We also thank Dr Kazuyoshi Terasaka of the Nagoya City University for pDR196GW. Analysis of DNA Sequencing was conducted with the Life Research Support Center at Akita Prefectural University. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 17310126 and No. 21310141 to KY), a Grant from the Research for the Future Program: 'Molecular mechanisms on regulation of morphogenesis and metabolism leading to increased plant productivity' (No. 00L01605 to KY) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists (No. 17·2011 to KO).

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