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The rice *ent-KAURENE SYNTHASE LIKE 2* encodes a functional *ent*-beyerene synthase

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ABSTRACT

The rice genome contains a family of kaurene synthase-like (*OsKSL*) genes that are responsible for the biosynthesis of various diterpenoids, including gibberellins and phytoalexins. While many *OsKSL* genes have been functionally characterized, the functionality of *OsKSL2* is still unclear and it has been proposed to be a pseudogene. Here, we found that *OsKSL2* is drastically induced in roots by methyl jasmonate treatment and we successfully isolated a full-length cDNA for *OsKSL2*. Sequence analysis of the *OsKSL2* cDNA revealed that the open reading frame of *OsKSL2* is mispredicted in the two major rice genome databases, IRGSP-RAP and MSU-RGAP. *In vitro* conversion assay indicated that recombinant OsKSL2 catalyzes the cyclization of *ent*-CDP into *ent*-beyerene as a major and *ent*-kaurene as a minor product. *ent*-Beyerene is an antimicrobial compound and *OsKSL2* is induced by methyl jasmonate; these data suggest that OsKSL2 is a functional *ent*-beyerene synthase that is involved in defense mechanisms in rice roots.

1. Introduction

Rice (*Oryza sativa* L.) produces a variety of labdane-related diterpenoids, including gibberellins (GAs), phytocassanes, momilactones, oryzalexins, and oryzadions. GAs are phytohormones that regulate various aspects of plant growth and development throughout the life cycle, such as stem elongation, germination, and flowering [1,2]. Phytocassanes A-E [3–5], momilactones A and B [6,7], oryzalexins A-F [8–10], and oryzalexin S [11] are phytoalexins, which are low-molecular-weight antimicrobial compounds produced in response to biotic stress (e.g. pathogen attack) or abiotic stress (e. g. UV irradiation) [12]. Momilactone B also functions as an allelopathic substance [13]. Oryzadions are antimicrobial diterpenoids isolated from healthy rice leaves [14]. During biosynthesis, the intermediate cyclic hydrocarbons of these rice diterpenoids are converted from geranylgeranyl diphosphate (GGDP), a common prenyl substrate, via *ent*-copalyl diphosphate (*ent*-CDP) or *syn*-CDP. Both *OsCPS1* and *OsCPS2* encode *ent*-CDP synthases, but the former is responsible for GA biosynthesis while the latter functions in the biosynthesis of phytocassanes A-E and oryzalexins A-F [17–20]. *OsCPS4* encodes *syn*-CDP synthase which participates in the biosynthesis of momilactones A and B and oryzalexin S [18,21]. Conversion of *ent*-CDP or *syn*-CDP to diterpenoids is catalyzed by kaurene synthase (KS) or by kaurene synthase-like proteins (KSLs). *OsKS1* encodes an *ent*-kaurene synthase for GA biosynthesis [17,21], while *OsKSL4*, *OsKSL7*, *OsKSL8*, and *OsKSL10* encode a *syn*-pimara-7,15-diene synthase, an *ent*-cassadiene synthase, a stemar-13-ene synthase, and an *ent*-sandaracopimaradiene synthase, respectively, for the biosynthesis of labdane-related phytoalexins [22–26]. *OsKSL5* and *OsKSL6* encode *ent*-pimara-8(14),15-diene synthase and *ent*-isokaurene synthase, respectively, and it has been suggested that *OsKSL6* is responsible for oryzadione biosynthesis [16,22,27]. *OsKSL3* is located next to *OsKS1* in the genome and is considered to be a pseudogene. The nucleotide sequences of *OsKSL3* cDNA isolated from the leaf blades of cv. Nipponbare suggested that translation would be terminated at 10th exon by frame

Abbreviations: GA, gibberellin; GGDP, geranylgeranyl diphosphate; CDP, copalyl diphosphate; KS, kaurene synthase; KSL, kaurene synthase-like; IRGSP-RAP, International Rice Genome Sequencing Project-Rice Annotation Project; MSU-RGAP, Michigan State University Rice Genome Annotation Project; MeJA, methyl jasmonate; RACE, Rapid amplification of cDNA ends; cTP, chloroplast transit peptide.

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shift, resulting in the production of a truncated protein having no KSL activity [22,27]. The functionality of *OsKSL2* is also ambiguous since no *OsKSL2* full-length cDNA has been isolated. This may be due to the relatively low abundance of the transcript in tissues [17,22]. The putative *OsKSL2* sequences, Os04g0612000 (NP_001053842) and LOC_Os04g52240 provided by the International Rice Genome Sequencing Project-Rice Annotation Project (IRGSP-RAP) and the Michigan State University Rice Genome Annotation Project (MSU-RGAP), respectively were produced by *ab intio* prediction and were therefore not directly supported by a cDNA sequence. Recently, Ji et al. [28] reported that a GA-responsive dwarf mutant of rice cultivar Dongjin, *dwarf2*, contains a mutation within the *OsKSL2* coding region that results in silencing of *OsKSL2* gene expression. Although the study suggested the possibility that *OsKSL2* encodes an *ent*-kaurene synthase that is involved in GA biosynthesis, it lacked direct biochemical analysis for its activity and genetic complementation. In addition, their study did not take into account the functionality of *OsKS1*, which encodes an authentic *ent*-kaurene synthase. It is therefore necessary to determine the enzymatic activity of OsKSL2 to clarify this point.

In this study, we have successfully isolated a full-length cDNA for *OsKSL2* and provided evidence that OsKSL2 mainly converts *ent*-CDP into *ent*-beyerene. Our data suggest that OsKSL2 is involved in phytoalexin biosynthesis rather than GA biosynthesis.

2. Materials and methods

2.1. Plant material and growth condition

Rice seeds (cv. Yukihikari) were surface-sterilized with 70% ethanol for 5 min and with 2% sodium hypochlorite for 20 min. Sterilized seeds were germinated for 1 d at 25 °C in the dark. Germinated seeds were then evenly placed onto a plastic mesh grid supported by a plastic container filled with sterilized tap water just to the base of the mesh grid, and kept in a growth chamber at 25 °C under continuous illumination. Methyl jasmonate (MeJA)-treatment was performed by transferring the mesh grid to a new container filled with 100 μM MeJA solution.

2.2. cDNA synthesis and RT-PCR

Total RNA was extracted from MeJA-treated rice roots and shoots using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed with High Capacity RNA-to-cDNA kit (Life Technologies, CA, USA). Amplification of a partial *OsKSL2* sequence was performed using primers 5′-CTGGGTGAAA-GAGAGCAGG-3′ and 5′-TCTGCCTCCACTCTACCTC-3′. The PCR cycling protocol consisted of 5 min at 98 °C, followed by 35 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 30 s.

2.3. 5′- and 3′- Rapid amplification of cDNA ends

5'- and 3′-Rapid amplification of cDNA ends (RACE) was performed using a GeneRacer kit (Life Technologies, CA, USA). Total RNA was ligated to the GeneRacer RNA oligo adapter: 5′-CGACUGGAG CACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′. Synthesis of single-stranded cDNA was carried out with SuperScript III Reverse Transcriptase (Life Technologies, CA, USA) using a GeneRacer oligo dT primer: 5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{18}$-3′. The 5′ ends of *OsKSL2* cDNA were amplified with the GeneRacer 5′ nested primer: 5′-GGACACTGACATGGACTGAAGGAGTA-3′, and a gene-specific primer: 5′-AGAGGTCGCGCTCGGTGACGATCT-3′. The amplified fragments were sequenced. The same procedure was utilized to perform 3′-RACE using the GeneRacer 3′ nested primer: 5′-CGCTACGTAACGGCATGACAGTG-3′, and a gene specific primer: 5′-GAGGCGCCGTTCCAAGGCCATGCA-3′. A full length cDNA was isolated by RT-PCR with the primers, 5′-CGCTACGTAACGGCATGACAGTG-3'and 5′-GCCTCTGTCGTTAGTTAGTCAC-3′. The PCR cycling protocol consisted of 5 min at 98 °C, followed by 35 cycles of 98 °C for 10 s, 60 °C for 15 s and 68 °C for 2.5 min.

2.4. Recombinant protein purification and functional assay

For recombinant protein production, the full-length *OsKSL2* cDNA fragment was isolated from pGEM-OsKSL2 by *Eco*RI digestion and was ligated into the *Eco*RI-digested pGEX-4T-3 (GE Healthcare Japan). The resulting plasmid pGEX-OsKSL2 was introduced into *E. coli* BL21. The recombinant OsKSL2 protein was purified by glutathione-affinity chromatography according to the method described previously [18]. *ent*-CDP and *syn*-CDP were enzymatically prepared through conversion of GGDP by recombinant OsCPS2 and OsCPS4, respectively, and were used as substrates for functional assay. The conditions of the conversion assay and GC–MS analysis using an Agilent 6890N GC-5973N MSD mass selective detector system were the same as those described previously [18]. A mass spectral data library (Wiley Registry 7th) was used for determination of putative product structure.

2.5. Subcellular localization using GFP-fused proteins

The transit peptide region of the *OsKSL2* cDNA was isolated by PCR by incorporating *Sal*I and *Nco*I restriction sites into the 5′- and 3′- termini, respectively. The primers used were: 5′-GTCGACGATGCTGCCGTGCTTGTTCC-3′ (*Sal*I site is underlined) and 5′-CCATGGAGCTGCAGTGCCGCATGGC3′ (*Nco*I site is underlined). The amplified fragment was first cloned into the pGEM-TEasy vector (Promega, WI, USA) and then introduced into the expression vector, pUC-sGFP [29], after digestion with *Sal*I and *Nco*I. The resulting plasmid, pUC-KSL2tpGFP, contains the putative transit peptide sequence OsKSL2 fused in-frame to the N-terminus of sGFP and allows expression under the control of the CaMV 35S promoter. Rice protoplasts were prepared as described by Yoo et al. [30]. The 3rd and 4th leaf sheaths from hydroponically grown 12-day-old seedlings were cut into small pieces and digested by cellulose RS and macerozyme R-10 (Yakult, Nishinomiya, Japan). After digestion, the protoplasts were filtered through a 77-μm nylon mesh. Plasmid was introduced into the protoplast cells using a polyethylene glycol method [30]. Subsequently, the transformed cells were incubated overnight at 24 °C in the dark. The GFP signals and chlorophyll autofluorescence were monitored using a fluorescent microscope (Leica DM6000B) with GFP and Texas Red filters, respectively.

3. Results

3.1. Isolation of a full-length cDNA for OsKSL2

Since the *OsKSL2* cDNA sequence (DQ823350) registered in NCBI GenBank is a partial clone [22], we first tried to isolate a full-length cDNA for *OsKSL2*. Our initial approach to amplify *OsKSL2* cDNA from rice shoots was not successful as reported by previous researchers due to low-level expression of the gene. However, we found that *OsKSL2* mRNA is detectable in root tissue following treatment with MeJA. Expression analysis using semi-quantitative RT-PCR revealed biphasic induction of *OsKSL2* in response to 100 μM MeJA in roots (Fig. 1A). By contrast, no induction of *OsKSL2* in response to MeJA was observed in shoot tissue (Fig. 1A). Using primers designed from the predicted *OsKSL2* sequence (NM_001060377), a cDNA fragment that covers the putative coding region of *OsKSL2* was first isolated from the MeJA-treated root tissue. To determine the 5′- and 3′- ends of the mRNA, 5′- and 3′- RACE was performed using the GeneRacer kit (Invitrogen). With the 5′- and 3′- ends information, we successfully

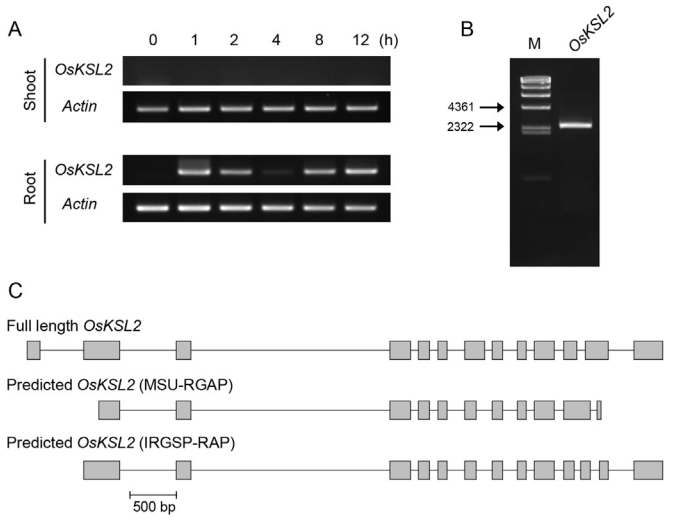

Fig. 1. Isolation of full-length *OsKSL2*. (A) Expression of *OsKSL2* during MeJA treatment. Total RNA was extracted from shoot and root tissues of 14-d-old seedlings. Semi-quantitative RT-PCR was performed with primers designed from partial cDNA sequence. (B) Isolation of a full length *OsKSL2* cDNA. MeJA-treated root RNA was used as a template. (C) Schematic models for *OsKSL2* sequences, two predicted sequences, and this study's sequence. Gray boxes indicate exons and black lines denote introns.

isolated a full-length cDNA for *OsKSL2* (Fig. 1B). The full-length clone contained a 2460 bp open reading frame that spans over 13 exons in rice genome and encodes a 91 kD protein. A full-length cDNA was also isolated from cv. Nipponbare and the sequence was completely identical (data not shown). Comparison of the deduced amino acid sequences from the full-length cDNA and the predicted coding sequences from the IRGSP-RAP (Os04g0612000) and the MSU-RGAP (LOC_Os04g52240) identified critical differences in the OsKSL2 sequences. The full length cDNA revealed an additional exon at the 5′-teminus, which extends the predicted Os04g0612000 and LOC_Os04g52240 sequences by 48 and 100 residues, respectively (Fig. 1C). In addition, we found that splicing sites were mis-predicted in the Os04g0612000 and LOC_Os04g52240 sequences, and thus the length of 7th exon and the structure of 11th to 12th exons were perturbed (Fig. 1C). We also found two discrepancies between the Os04g0612000 and LOC_Os04g52240 nucleotide sequences: insertion of a single G at 5392 nt and a C to G substitution at 5402 nt (nucleotide numbers are for LOC_Os04g52240) (Fig. S1). The full-length cDNA sequence supported the LOC_Os04g52240 sequence.

3.2. *OsKSL2 encodes a diterpene cyclase*

The amino acid sequence of OsKSL2 was compared with that of other known rice diterpene cyclases (Fig. 2). OsKSL2 showed a high similarity (45–63% amino acid identity) to other KSL proteins, and contained SAYDTAW and QXXDGSW motifs, which are conserved in

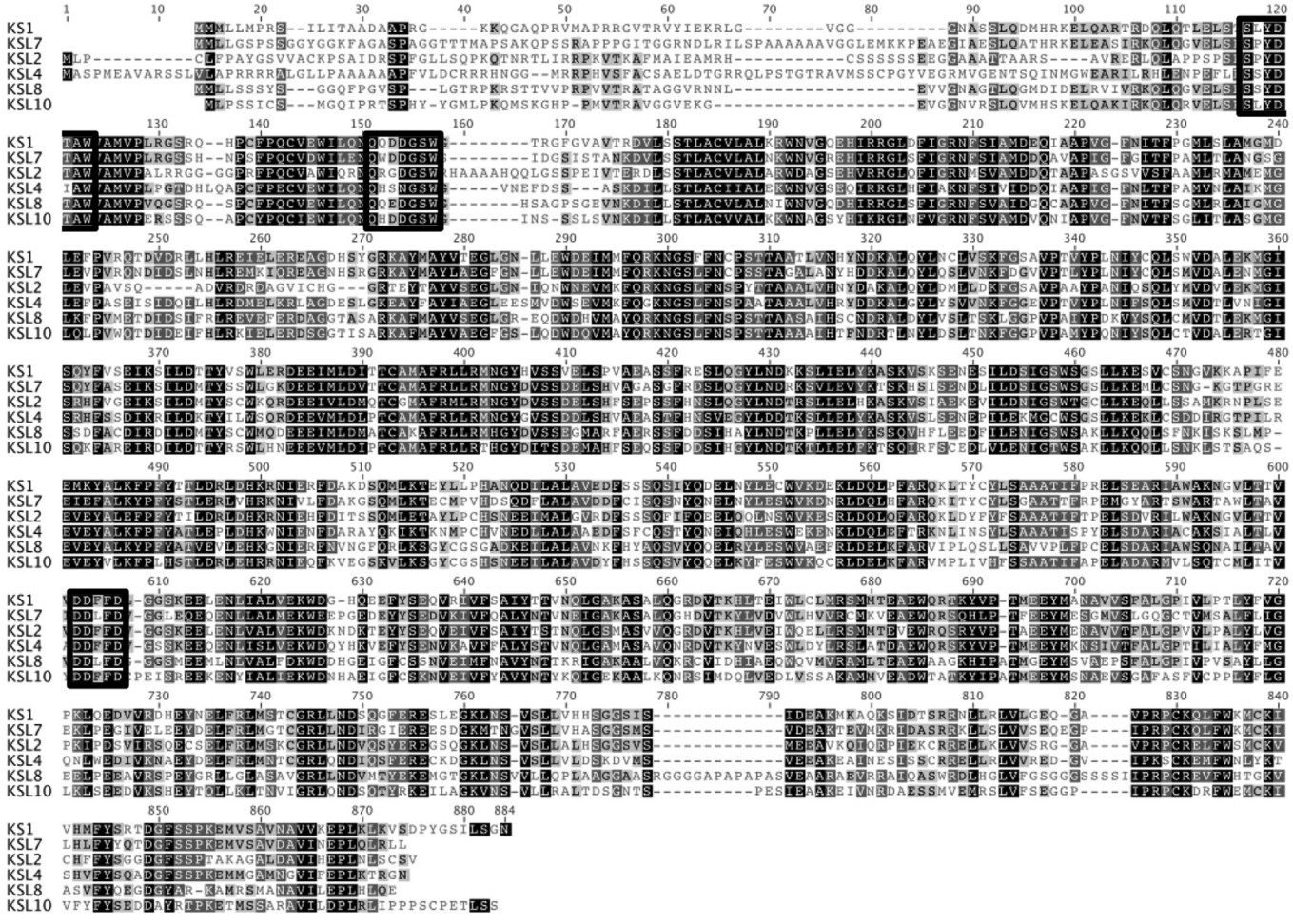

Fig. 2. Alignment of OsKSL2 and representative rice KSL sequences. Amino acid sequences of OsKS1(AB126933.1), OsKSL4(Q0JEZ8), OsKSL7 (Q0E088), OsKSL8 (Q6BDZ9), OsKSL10 (Q2QQJ5), and OsKSL2 (LC033788) were aligned using ClustalW software (http://clustalw.ddbj.nig.ac.jp/). The regions conserved in diterpene cyclases, SAYDTAW, QXXDGSW, and DDXXD motifs, are boxed.

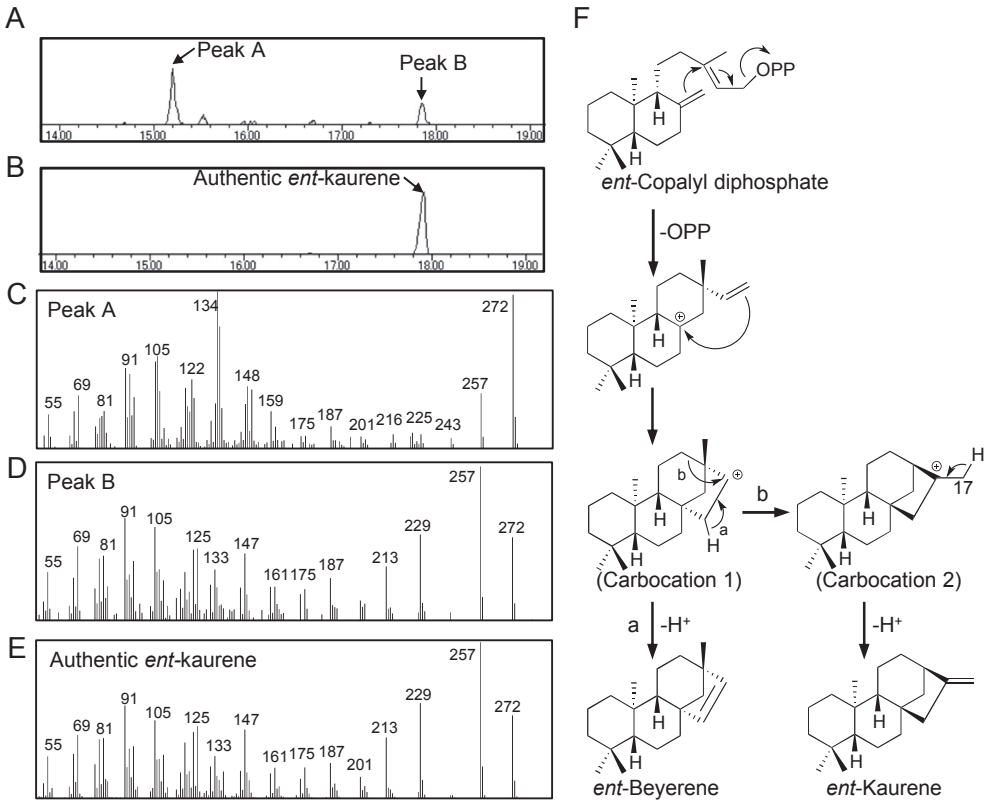

Fig. 3. Functional analysis of OsKSL2. Gas chromatograms (*m/z* 272) of the products converted from *ent*-CDP by recombinant GST-OsKSL2 (A) and authentic *ent*-kaurene (B). Mass spectra of peak A (C), peak B (D), and authentic *ent*-kaurene (E). Scheme of biosynthetic pathway for synthesis of *ent*-beyerene and *ent*-kaurene from *ent*-CDP (F).

plant diterpene cyclases [31–33]. The aspartate-rich motif, DDXXD, is the binding site of a diphosphate-divalent metal complex and is responsible for KS-type activity [34,35]. The DDXXD motif was also found in OsKSL2 at the same location in the alignment with other KSL proteins. These data suggested that the isolated cDNA encodes a KS-type diterpene cyclase.

3.3. Enzymatic function of recombinant OsKSL2

To investigate the function of OsKSL2, we prepared a recombinant GST-OsKSL2 protein using a bacterial expression system, and carried out *in vitro* conversion assay using *ent*-CDP or *syn*-CDP as substrates. In case of *ent*-CDP, two product peaks (peak A at 15.2 min and peak B at 17.8 min) were detected by GC–MS analysis (Fig. 3A), whereas no diterpene-like peak from *syn*-CDP was detected (data not shown). The retention time and mass spectrum of peak B were almost identical to those of *ent*-kaurene, suggesting that peak B is indeed *ent*-kaurene (Fig. 3A, B, C, E). On the other hands, the mass spectrum of peak A (Fig. 3D) was almost identical to that of 17-norkaur-15-ene 13-methyl (beyerene) in the Wiley mass spectral data library. Considering that the products were derived from *ent*-CDP, it was suggested that the peak A is *ent*-beyerene, enantiomer of beyerene, although we did not obtain an authentic sample for comparison. *ent*-Beyerene is produced by deprotonation of H-16 in carbocation 1, derived from *ent*-CDP, whereas *ent*-kaurene is produced by the rearrangement of carbocation 1 and successive deprotonation of H-17 in carbocation 2 (Fig. 3F). Thus, recombinant OsKSL2 possibly catalyzes alternative reactions from carbocation 1.

3.4. OsKSL2 is localized to the chloroplast

As a diterpene cyclase that utilizes *ent*-CDP as a substrate, OsKSL2 is expected to localize to the plastid where this substrate is available [36]. The public database ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/), which predicts chloroplast transit peptides, predicted that OsKSL2 is localized to plastids and that the first 58 residues of OsKSL2 function as a putative chloroplast transit peptide (cTP). To confirm the predicted subcellular localization, we constructed a recombinant gene where GFP is fused to the putative cTP sequence of OsKSL2. The fusion gene was transiently expressed in rice leaf protoplasts. As shown in Fig. 4A–C, GFP signals from the transformed cells overlapped with autofluorescence from chloroplasts, indicating that OsKSL2 is localized to the plastid. By contrast, GFP signal was detected in cytoplasm and nucleus when GFP alone is expressed in the protoplast cells (Fig. 4D–F). We also determined the subcellular localization of a recombinant protein where GFP was fused with the putative transit peptide predicted from Os04g0612000 (NP_001053842). Transformed protoplast cells exhibited GFP signals that are similar to GFP alone (Fig. 4G–I), indicating this shorter sequence failed to function as a transit peptide. These data confirmed that the *OsKSL2* sequence obtained in this study is a full-length clone.

4. Discussion

Rice produces a variety of diterpenoids that are synthesized from GGDP, including the GAs and phytoalexins. In the rice genome, there are seven A-type diterpene cyclases, which are denoted as KS or KSL. Biochemical analyses of KSLs have identified their functions in the diterpenoid biosynthetic pathway [15,16]. OsKSL2 is one of the few KSLs whose function is still unclear. One reason for this is the lack of a full length cDNA. Several groups have tried to isolate the full length *OsKSL2* cDNA but were unsuccessful, possibly because the expression level of *OsKSL2* in leaf tissue is very low, and its expression is not induced by either UV irradiation or spray treatment of MeJA. Moreover, the transcripts of *OsKSL2* are also

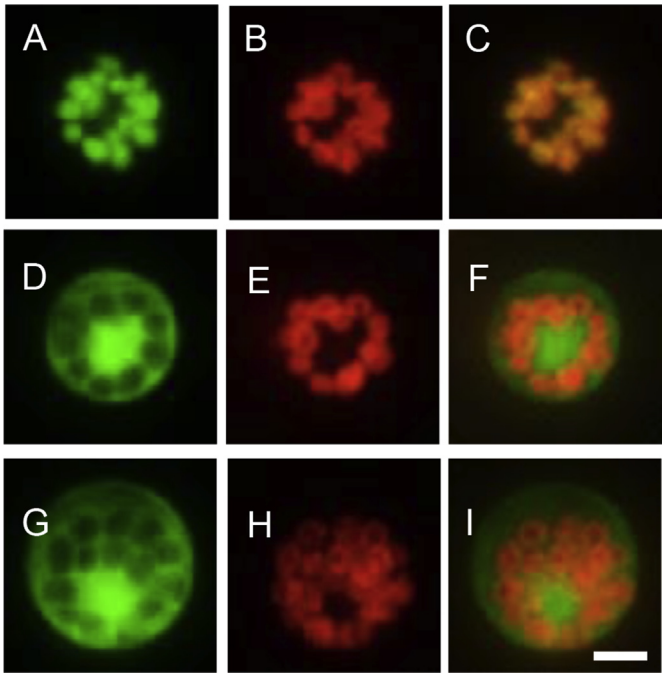

Fig. 4. Subcellular localization of KSL2 in rice protoplasts Fluorescence microscopic images of transformed rice protoplast cells. Images of a cell expressing GFP fused with a putative transit peptide from the full length OsKSL2 (A to C), free GFP (D to F), and GFP fused to a putative transit peptide from IRGSP-RAP-based OsKSL2 (NP_001053842) (G to I). Fluorescent signals from GFP (A, D, and G) and chlorophylls (B, E, and H) are merged (C, F, and I). Scale bar is 5 μm.

undetectable in elicitor-treated culture cells [17,22]. Therefore, *OsKSL2* has been considered to be a pseudo-gene [37]. Here, we isolated a full-length cDNA of *OsKSL2* for the first time. The isolated cDNA encodes a 91 kD protein that shows a high sequence homology to known KSLs, and catalyzes the cyclization of *ent*-CDP to the major product, *ent*-beyerene *in vitro*.

Recently, a function of OsKSL2 in GA biosynthesis has been proposed [28]. An Ac/Ds-induced mutant of rice (*dwarf2*) shows a GA-responsive dwarf phenotype. Since the mutant contained a one-base deletion in the putative *OsKSL2* gene sequence, the authors suggested that *OsKSL2* is a novel kaurene synthase gene for GA biosynthesis. However, in their study, the enzymatic activity of OsKSL2 was not determined and complementation of the dwarf phenotype with the wild type *OsKSL2* gene was not tested. It has been shown that OsKS1 is a functional kaurene synthase that is involved in GA biosynthesis. The recombinant OsKS1 protein converts *ent*-CDP into *ent*-kaurene [18,19]. The *osks1-1* mutant is GA deficient and exhibits a severe dwarf phenotype, suggesting that OsKS1 is the only functional kaurene synthase for GA biosynthesis in rice [17]. Therefore, the hypothesis that OsKSL2 is involved in GA biosynthesis needs to be carefully elucidated. The amino acid sequence of OsKSL2 predicted by Ji et al. [28] contains an extremely long C-terminal region that was not found in the OsKSL2 sequence determined in this study or in other known OsKSLs. In addition, this "unusual" C-terminus has not been detected as a transcript so far. Importantly, a single nucleotide deletion, which could cause a frame shift and possible loss of function of OsKSL2 in their model, exists within the "unusual" C-terminal region. This region is present downstream of the 3′UTR in comparison with the cDNA we isolated in this study. Therefore, the mutation is less likely to affect OsKSL2 activity. It is possible to speculate that the dwarf phenotype of the mutant is caused by a mutation not in *OsKSL2* but in another GA biosynthesis gene.

ent-Beyerene, the major product of OsKSL2, is one of the diterpenoids isolated as a natural substance in various plants [38–40]. Recent studies have suggested that KSL proteins are responsible for the biosynthesis of *ent*-beyerene in wheat and castor bean [41,42]. In rice, on the other hand, there have been no reports of beyerene and its derivative as an internal substance, nor about the possibility of its biosynthesis. The derivatives of *ent*-beyerene; *ent*-beyene-19-ol, *ent*-beyerene-19-oic acid, *ent*-beyeren-18-O-oxalate, and *ent*-beyeren-18-O-succinate have been shown to exhibit anti-microbial activities [43–45], and *ent*-beyerene itself also has anti-bacterial and anti-fungal activities [46].

Function of OsKSL2 in phytoalexin biosynthesis is supported by the expression pattern of the gene. Expression of *OsKSL2* is almost undetectable at normal condition but highly inducible within 1 h in response to MeJA (Fig. 1A). *OsCPS2*, *OsCPS4*, *OsKSL4*, *OsKSL7*, and *OsKSL8*, which are involved in phytoalexin biosynthesis, are inducible when treated with elicitor or MeJA [19,21,23,24,26]. In comparison, *OsCPS1* and *OsKS1*, which are involved in GA biosynthesis, are not induced under same condition [17,19]. As *OsKSL2* is specifically induced in root tissue, the functions of *ent*-beyerene-related phytoalexins in the root defense mechanism will be an interesting subject for future research.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.104.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.104.

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