In vitro assays using recombinant enzymes enabled three cDNAs encoding ent-copalyl diphosphate synthases to be identified in wheat (Triticum aestivum): TaCPS1, TaCPS2, and TaCPS3. The phylogenetic tree and expression analyses suggest that TaCPS3 is responsible for gibberellin biosynthesis, while TaCPS1 and TaCPS2 are possible functional homologs of diterpene cyclase genes OsCPS2 and OsCPS4 involved in phytoalexin biosynthesis in rice.

Key words: biosynthesis; diterpene; ent-copalyl diphosphate synthase; wheat

Rice (Oryza sativa L.) produces a variety of cyclic diterpenoids, including the phytohormone gibberellin and phytoalexins. A series of diterpene cyclase genes has been identified in rice. OsCPS1 encodes an ent-CDP synthase that acts in gibberellin biosynthesis, while OsCPS2/OsCyc2 and OsCPS4/OsCyc1 encode ent- and syn-CDP synthase, respectively, both of which act in the biosynthesis of phytoalexins (e.g., momilactones, phytocassanes, and oryzalexins). Both ent- and syn-CDP are converted from GGDP (Fig. 1A). Either ent- or syn-CDP is converted into specific cyclic hydrocarbons by secondary cyclization. OsKS1 encodes ent-kaurene synthase, which acts in gibberellin biosynthesis, while OsKS4, OsKSL7/OsDTC1, OsKSL8/OsDTC2, and OsKSL10 encode 9β-pimara-7,15-diene synthase, ent-cassa-12,15-diene synthase, stemar-13-ene synthase, and ent-sandaracopimaradiene synthase, respectively, all of which act in phytoalexin biosynthesis. In comparison, the Arabidopsis genome contains one set of CPS- and KS-type gene homologs, AtCPS and AtKS, which are responsible for gibberellin biosynthesis. A BLAST search using the Lotus japonicus genome database (http://www.kazusa.or.jp/lotus/) revealed the presence of a single set of homologs, as in Arabidopsis. Furthermore, in a phylogenetic tree based on amino acid sequence information, OsCPS2/OsCyc2 and OsCPS4/OsCyc1 were grouped in a different clade from the clade including OsCPS1 and ent-CDP synthases in other plant species. This information prompted us to question whether homologs of OsCPS2/OsCyc2 and OsCPS4/OsCyc1 are present in plant species other than rice. To address the question, we isolated and characterized CPS-type genes in wheat (Triticum aestivum), a member of the Gramineae family.

We searched a wheat expressed sequence tag (EST) database by BLASTn from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome-j.html) by using the nucleotide sequences of OsCPS1, OsCPS2/OsCyc2, and OsCPS4/OsCyc1, and found three representative EST clones: CN010657, CN011974, and CF133726. Their full-length sequences in T. aestivum cv. Nourin-61-gou were then determined by rapid amplification of the cDNA ends and end-to-end RT-PCR as described previously. The cDNAs corresponding to CN010657, CN011974, and CF133726 are named TaCPS1 (accession no. AB439588), TaCPS2 (accession no. AB439589), and TaCPS3 (accession no. AB439590), respectively. It was anticipated that the open reading frames of TaCPS1, TaCPS2, and TaCPS3 would be 2,394, 2,274, and 2,496 bp in length, and encode 797, 757, and 831 amino acid residues, respectively. These three CPS-type genes are probably not homoeologs of each other, considering that their homologies are 68% (TaCPS1/TaCPS2), 59% (TaCPS1/TaCPS3), and 57% (TaCPS2/TaCPS3) at the nucleotide level, and 56% (TaCPS1/TaCPS2), 46% (TaCPS1/TaCPS3), and 44% (TaCPS2/TaCPS3) at the amino acid sequence level. A previous report has identified two CPS-type genes on the short arm of chromosome 2 and on the long arm of chromosome 7 by a DNA-blot analysis.

A phylogenetic tree constructed by using primary sequence information grouped TaCPS1, TaCPS2, OsCPS2/OsCyc2 and OsCPS4/OsCyc1 into a separate clade from that including TaCPS3 and ent-CDP synthases in other monocotyledonous plant species (Fig. 1B). These data suggest that TaCPS3 encodes an ent-CDP synthase responsible for gibberellin biosynthesis, while TaCPS1 and TaCPS2 encode CPS-type cyclases that act in the biosynthesis of diterpenoids other than gibberellins. Two cDNAs encoding ent-CDP synthases in pumpkin (Cucurbita maxima), which act
in gibberellin biosynthesis, were isolated in a previous study.\textsuperscript{7} In maize (\textit{Zea mays}), \textit{Anther ear 1} (\textit{An1}) encodes an \textit{ent}-CDP synthase that acts in gibberellin biosynthesis,\textsuperscript{8,9} while its homolog \textit{An2}, the expression of which was found to be up-regulated after the UV treatment, is silencing.\textsuperscript{10} Therefore, we performed an expression analysis to characterize the three wheat diterpene cyclase genes. Leaf sheaths were obtained from wheat plants that had been cultivated in a growth chamber for 9 d at 25°C and exposed to UV light for 10 min according to a previously described method.\textsuperscript{10} Then harvested 1, 2, or 3 d later. To confirm the reproducibility, we also prepared two batches of shoot samples: 9-d-old wheat plants were exposed to UV light for 10 min and then successively cultivated in a growth chamber at 25°C. Total RNA was extracted from frozen samples using SDS-phenol, and poly (A)\textsuperscript{+} RNA was purified by using Dynabeads (Dynal) as previously described.\textsuperscript{5} cDNA was synthesized from 1-μg aliquots of poly (A)\textsuperscript{+} RNA by using a QuantiTect reverse transcription kit (Qiagen). Real-time QRT-PCR, using SYBR Green II, was carried out in a TP800 thermal cycler for 10 min and then harvested 1, 2, or 3 d later. To confirm the reproducibility, we also prepared two batches of shoot samples: 9-d-old wheat plants were exposed to UV light for 10 min and then successively cultivated in a growth chamber at 25°C. Total RNA was extracted from frozen samples using SDS-phenol, and poly (A)\textsuperscript{+} RNA was purified by using Dynabeads (Dynal) as previously described.\textsuperscript{5} cDNA was synthesized from 1-μg aliquots of poly (A)\textsuperscript{+} RNA by using a QuantiTect reverse transcription kit (Qiagen). Real-time QRT-PCR, using SYBR Green II, was carried out in a TP800 thermal cycler (Takara). The mean values from two replicates were normalized by using \textit{TEFI} (M90077, the \textit{α}-subunit of wheat translation elongation factor 1) as an internal control. The following primers were used: for \textit{TaCPS1}, 5'-GGATTCCGACGTAACAGGTTGAGTC-3' (BamHI sense) and 5'-CAGGGGTATGACCTTTCCAAT-3' (Smal, antisense); for \textit{TaCPS2}, 5'-CCCGGAGGATAAGTCTG-3' (Smal, sense) and 5'-CGCGGCAT-3' (NotI, antisense); and for \textit{TaCPS3}, 5'-GATTAGGATCCGACGTCACCTTTTCC-3' (NotI, antisense). Each cDNA was subcloned into pGEX-4T-3 (GE Healthcare, Piscataway, NJ, USA). Heterologous expression in \textit{Escherichia coli}, extraction and purification of the recombinant enzymes, and enzyme assays were performed by previously described methods.\textsuperscript{10} The reaction products were identified by full-scan GC-MS as their respective alcohol derivatives, after dephosphorylation using alkaline phosphatase.\textsuperscript{10} The retention times and mass spectra of peaks A, B, and C, representing the reaction products derived from the incubation of GGDP with GST-\textit{TaCPS1}, GST-\textit{TaCPS2}, and GST-\textit{TaCPS3}, respectively, were identical with those of authentic \textit{ent}-copalol derived from OsCPS2/OsCyc2\textsuperscript{10} (Fig. 2). These results suggest that \textit{TaCPS1}, \textit{TaCPS2} and \textit{TaCPS3} catalyzed the conversion of GGDP into \textit{ent}-CDP (Fig. 1A). However, we cannot completely exclude the possibility that the product produced in each case was (+)-copalol, because it was impossible to distinguish between (+)- and (−)-\textit{ent}-copalol by using our GC-MS system.

A previous study has shown that UV irradiation increased the transcription of OsCPS2/OsCyc2 and OsCPS4/OsCyc1, which promote phytodalexin production, while that of OsCPS1 was decreased.\textsuperscript{10} Therefore, we performed an expression analysis to characterize the three wheat diterpene cyclase genes. Leaf sheaths were obtained from wheat plants that had been cultivated in a growth chamber for 9 d at 25°C and exposed to UV light for 10 min according to a previously described method,\textsuperscript{10} and then harvested 1, 2, or 3 d later. To confirm the reproducibility, we also prepared two batches of shoot samples: 9-d-old wheat plants were exposed to UV light for 10 min and then successively cultivated in a growth chamber at 25°C. Total RNA was extracted from frozen samples using SDS-phenol, and poly (A)\textsuperscript{+} RNA was purified by using Dynabeads (Dynal) as previously described.\textsuperscript{5} cDNA was synthesized from 1-μg aliquots of poly (A)\textsuperscript{+} RNA by using a QuantiTect reverse transcription kit (Qiagen). Real-time QRT-PCR, using SYBR Green II, was carried out in a TP800 thermal cycler (Takara). The mean values from two replicates were normalized by using \textit{TEFI} (M90077, the \textit{α}-subunit of wheat translation elongation factor 1) as an internal control. The following primers were used: for \textit{TaCPS1}, 5'-GGATTCCGACGTAACAGGTTGAGTC-3' (BamHI sense) and 5'-CAGGGGTATGACCTTTCCAAT-3' (Smal, antisense); for \textit{TaCPS2}, 5'-CCCGGAGGATAAGTCTG-3' (Smal, sense) and 5'-CGCGGCAT-3' (NotI, antisense); and for \textit{TaCPS3}, 5'-GATTAGGATCCGACGTCACCTTTTCC-3' (NotI, antisense). Each cDNA was subcloned into pGEX-4T-3 (GE Healthcare, Piscataway, NJ, USA). Heterologous expression in \textit{Escherichia coli}, extraction and purification of the recombinant enzymes, and enzyme assays were performed by previously described methods.\textsuperscript{10} The reaction products were identified by full-scan GC-MS as their respective alcohol derivatives, after dephosphorylation using alkaline phosphatase.\textsuperscript{10} The retention times and mass spectra of peaks A, B, and C, representing the reaction products derived from the incubation of GGDP with GST-\textit{TaCPS1}, GST-\textit{TaCPS2}, and GST-\textit{TaCPS3}, respectively, were identical with those of authentic \textit{ent}-copalol derived from OsCPS2/OsCyc2\textsuperscript{10} (Fig. 2). These results suggest that \textit{TaCPS1}, \textit{TaCPS2} and \textit{TaCPS3} catalyzed the conversion of GGDP into \textit{ent}-CDP (Fig. 1A). However, we cannot completely exclude the possibility that the product produced in each case was (+)-copalol, because it was impossible to distinguish between (+)- and (−)-\textit{ent}-copalol by using our GC-MS system.

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![Fig. 1. Synthesis of \textit{ent}- or \textit{syn}-Copalyl Diphosphate from Geranylgeranyl Diphosphate (A) and a Phylogenetic Tree Constructed from the Deducible Amino Acid Sequences of \textit{ent}- and \textit{syn}-Copalyl Diphosphate Synthases in Higher Plants (B).](image-url)
that of TaCPS3 gradually decreased (Fig. 3). The UV-inducible patterns of TaCPS1 and TaCPS2 were confirmed by two other independent experiments using wheat shoots (data not shown). The expression patterns of TaCPS1 and TaCPS2 after UV irradiation were similar to those of OsCPS2/OsCyc2 and OsCPS4/OsCyc1, although the expression of TaCPS1 and TaCPS2 was lower than that of TaCPS3. We were unable to amplify TaCPS1 or TaCPS2 by QRT-PCR when using total RNA as the template. The transcript levels of OsCPS2/OsCyc2 and OsCPS4/OsCyc1 after UV irradiation were higher than that of OsCPS1. This is probably one reason why we did not detect any cyclic diterpene hydrocarbons in the UV-irradiated wheat leaves, whereas we did detect a series of diterpene hydrocarbons in UV-irradiated rice leaves prepared under the same experimental conditions (data not shown). The accumulation of these hydrocarbons in rice leaves confirmed the reproducibility of our previous data. These results suggest that the expression of TaCPS1 and TaCPS2 was extremely low in the younger-stage shoots, despite their up-regulation after UV irradiation, unlike OsCPS2/OsCyc2 and OsCPS4/OsCyc1.

Notably, all partial draft sequences for the EST clones of TaCPS1 (11/11 clones) and TaCPS2 (5/5 clones) were found in a cDNA library derived from the floral tissues of wheat plants infected with Fusarium graminearum or from salt-stressed wheat plants, while those of TaCPS3 (4/4 clones) were found in a cDNA library derived from the healthy floral tissues of wheat or from imbibed seeds, according to a BLASTn search of the wheat EST database (http://www.tigr.org/tdb/e2k1/tae1f/). The frequency of EST clones also suggests that TaCPS1 and TaCPS2 are responsible for the anti-pathogen effects, while TaCPS3 functions in gibberellin biosynthesis. It is possible that TaCPS1 and TaCPS2 mRNA might accumulate at the flowering stage to produce substances that protect the reproductive tissues against pathogens. Additional studies should be carried out to elucidate the physiological roles of TaCPS1 and TaCPS2. It remains unexplained whether wheat can produce anti-pathogenic diterpenes similar to rice. We did not detect phytocassanes or momilactones in our UV-irradiated wheat leaves (data not shown). We found at least six independent KS-like genes in the wheat EST database; however, we have not yet identified a gene encoding syn-CDP synthase in wheat. The isolation and
characterization of all CPS- and KS-type genes in the wheat genome will enable us to identify the possible diterpene phytoalexins in wheat. Our present study nevertheless indicates that at least two homologs of OsCPS2/OsCyc2 and OsCPS4/OsCyc1 are present in the wheat genome, and suggests the presence of ancestral genes of OsCPS2/OsCyc2 and OsCPS4/OsCyc1 in Gramineae. These data provide an important insight into the evolution of rice phytoalexin biosynthetic genes.

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