J Plant Biotechnol (2019) 46:88–96
DOI:https://doi.org/10.5010/JPB.2019.46.2.088

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Physiological and molecular analysis of *OsTPS30* by gamma irradiation

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Received: 1 February 2019 / Revised: 29 March 2019 / Accepted: 29 March 2019
ⓒ Korean Society for Plant Biotechnology

Abstract Terpenes constitute a large class of secondary metabolites in plants. The *Oryza sativa terpene synthase* is a vital gene in plant defense response. In this study, the molecular and physiological functions of *Oryza sativa terpene synthase 30* (*OsTPS30*, LOC_Os08g07080) were investigated after exposure of the seeds and plants to gamma-rays. The *OsTPS30* expression was slightly induced at 200 Gray (Gy), but was significantly induced at 400 Gy. The total terpenoid was synthesized more in *OsTPS30*-overexpressing (OX-*OsTPS30*) *Arabidopsis thaliana* plants than in wild-type (WT) plants. The OX-*OsTPS30* plants exhibited resistance to gamma-rays, as compared to WT. The OX-*OsTPS30* plants had significantly increased height and weight after gamma irradiation. Additionally, the activity of antioxidant enzymes was increased more in OX *OsTPS30* plants than in WT plants after gamma irradiation. Furthermore, the OsTPS30-GFP fusion protein was mostly localized in the chloroplast, suggesting that OsTPS30 is putative MEP pathway-related terpene synthase.

Keywords Terpene synthase, gamma-ray, chloroplast, MEP pathway

Introduction

Terpenes, which constitute a large class of secondary metabolites in plants, have been divided into the following classes: hemiterpenes (C5); monoterpenes (C10); sesquiterpenes (C15); diterpenes (C20); sesterterpenes (C25); triterpenes (C30); tetraterpenes (C40) and polyterpenes (> C40) (Tholl 2006; Singh and Sharma 2015). Most plant genomes include a medium-sized family of genes encoding terpene synthases (TPSs) (Chen et al. 2011). There are two terpene biosynthesis pathways in plants, namely the chloroplast 2-C-methylerythritol-4-phosphate (MEP) pathway and cytosolic mevalonate (MEV) pathways, both of which require a 5-carbon isoprenoid diphosphate precursor for the production of all terpenes. Based on their phylogenetic relationships, the plant TPS genes are distributed in seven different clades. The angiosperm clades consist of TPS-a, TPS-b, and TPS-g. The TPS-a clade comprises the sesquiterpene synthases, while the TPS-b and TPS-g clades consist mainly of monoterpene synthases and sesquiterpene synthases, respectively. The TPS-d clade includes monoterpene, sesquiterpene, and diterpene synthases. Meanwhile, the TPS-h clade consists of diterpene synthases (Bohlmann et al. 1998; Dudareva et al. 2006; Chen et al. 2011; Li et al. 2012).

The most studied TPS gene family has been that of *Arabidopsis thaliana* (Sun et al. 1994; Yamaguchi et al. 1998; Bohlmann et al. 2000; Chen et al. 2003; Fäldt et al. 2003; Chen et al. 2004; Tholl et al. 2005; Herde et al. 2008), with only a few TPSs having been isolated in cultivated plants, including rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), grape (*Vitis vinifera*), poplar (*Populus trichocarpa*), and sorghum (*Sorghum bicolor*) (Falara et al. 2011; Irmisch et al. 2014; Lee et al. 2015; Yoshitomi et al. 2016; Zhang et al. 2016). However, a detailed analysis of the presence and role of terpenes in these plants has not been published. Monoterpene synthases generally comprise 600-650 amino acid residues, while sesquiterpene synthase have 50-70 fewer amino acids (Martin et al. 2004). All TPS C-terminals contain an aspartate-rich DDxxD domain, which helps coordinate divalent metal ions for a subsequent binding of a substrate (Lesburg et al. 1997; Peterd et al. 2003; Tholl 2006; Tholl et al. 2011). All plant terpenoids are biosynthesized by TPSs, with monoterpene and diterpene synthases localized to the chloroplast *via* the MEP pathway, and the sesquiterpene synthases present in the cytoplasm *via* MEV pathway (Aharoni et al. 2003; Singh and Sharma 2015; Chen et al. 2011;

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Tholl et al. 2011). Tomato TPS are localized in mitochondria, but the *in vivo* substrates are currently unknown (Falara et al. 2011).

Some plant TPSs effect primary metabolism [e.g., gibberellin biosynthesis], but most TPSs are involved in the biosynthesis of secondary metabolites in response to environmental stimuli (Chen et al. 2011; Cheng et al. 2007). There has been recent progress in the characterization of the biochemistry and molecular genetics underlying terpenoid biosynthesis. Additionally, the biological functions of many terpene compounds associated with phytohormone biosynthesis have been determined. For example, jasmonate-responsive volatile terpenoids are important for defense responses in rice and help protect plant from UV radiation and photo-oxidative stress (Jenkins et al. 2009; Yohitomi et al. 2016). Moreover, terpenes provide protection from heat stress and also contribute to membrane stabilization, resistance to insects and microorganisms, and plant-plant signaling (Loreto et al. 1998; Copolovici et al. 2005; Baldwin et al. 2006; Keeling et al. 2006).

The rice genome has 33 TPS genes. OsTPS18 has localized in the cytoplasm and synthesized sesquiterpenes (*E*)-nerolidol and (*E*)-β-farnesene (Kiryu et al. 2018). OsTPS19 acts as an (*S*)-lemonine synthetase in rice and defends against *Magnaporthe oryzae* by inhibiting spore germination (Chen et al. 2018). OsTPS20 plays a major role in producing terpene volatiles during abiotic stress (Lee et al. 2015). OsTPS46 plays an important role in rice defense against *Rhopalosiphum padi.* (Sun et al. 2017). Additionally, a previous study revealed that the gamma irradiation of rice seedlings upregulated the expression of *OsTPS19*, *OsTPS20*, *OsTPS25*, *OsTPS26*, and *OsTPS30*, although the effect on *OsTPS30* was inconclusive (Lee et al. 2015). In this study, we analyzed molecular and physiological function of OsTPS30 by gamma irradiation.

Materials and methods

Plant materials and gamma irradiation

Rice plants (*Oryza sativa* spp. *japonica* cv. Ilpoom) were grown in half-strength Murashige and Skoog (MS) agar medium (Duchefa Biochemie, Haarlem, Netherlands) and cultured aseptically in a growth chamber at 28°C under a 16-h light/8-h dark cycle. The WT and OX *OsTPS30* plants in *A. thaliana* were sown in plates containing half-strength MS agar medium with 1.5% of sucrose (Sigma-Aldrich, St. Louis, USA). The plates were incubated for 3 days at 4°C in darkness, and then transferred to a growth chamber for a subsequent incubation at 22°C with a 16-h light/8-h dark cycle and 70% of humidity. For the gamma-ray treatment, rice seeds were exposed to 100, 200, or 400 Gy for 24 h using a ^{60}Co gamma irradiator (150 TBq capacity; Atomic Energy of Canada Limited, Ottawa, Canada) at the Korea Atomic Energy Research Institute. *A. thaliana* plants during the vegetative stage and plants with fully expanded rosette leaves were independently irradiated 18 days after seeding. Plants were exposed to gamma-rays at 100, 200, 300, 400, or 500 Gy for 24 h, and the resulting phenotypes were evaluated after 14 days.

RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the leaves using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), after which the concentration and quality were assessed with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The RNA was then used as the template to synthesize first-strand cDNA using the ReverTra Ace-α- kit (Toyobo, Osaka, Japan). The qRT-PCR was conducted using the iQ ™ SYBR® Green Supermix (Bio-Rad, Hercules, USA), and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, USA). Details regarding the qRT-PCR primers are listed in Table 1.

Table 1 Primers used for the qRT-PCR analysis

Gene	Primer name	Sequence (5‘ to 3‘)
OsTPS30	OsTPS30-F	ATGAAAGAACGGGTTCAAGTTGTGA
	OsTPS30-R	AATGTTATACGGCTCCATATAGAGCGAT
	OsTPS30-qRT-F	ACCACCACCATGCTTCTACA
	OsTPS30-qRT-R	ACTCCTTTATCCCAACGCAT
OsNAC10	OsNAC10-qRT-F	TACACAACACCTCATCCAA
	OsNAC10-qRT-R	ATGATCTAGGCGTGACTC
OsActin	OsACT-qRT-F	TGAAGTGCGACGTGGATATTAG
	OsACT-qRT-R	CAGTGATCTCCTTGCTCATCC
AtActin2	AtACT2-qRT-F	GCCATCCAAGCTGTTCTCTC
	AtACT2-qRT-R	GCTCGTAGTCAACAGCAACAA

Cloning of OsTPS30 and the production of transgenic plants

The *OsTPS30* gene was amplified by PCR using gene-specific primers. The PCR product was inserted into the pCR™8/GW/TOPO® vector (Invitrogen, Carlsbad, USA) and sequenced. After the sequence was verified, it was integrated into the pMDC83 destination vector using the recombination-based Gateway Cloning System (Invitrogen, Carlsbad, USA). The CaMV35S:*OsTPS30*-green fluorescence protein (GFP) construct was inserted into *A. thaliana* cells using an *Agrobacterium tumefaciens*-mediated (strain GV3101) floral dip method (Clough et al. 1998).

Total terpenoids of transgenic plants

The harvested plant material was immediately frozen in liquid nitrogen. To homogenize the tissue, one glass bead and cold 95% methanol was added to each sample tube and homogenized with QIAGEN. Remove glass beads and incubate the sample at room temperature for 48 h in dark. Samples were centrifuged to collect the supernatant. 1.5 mL chloroform was added to each and 200 µl sample supernatant was added to each. The Remark standard curve was prepared by diluting 200 µl of Linalool (Sigma-Aldrich, St. Louis, USA) solution in 1.5 ml of chloroform. The sample was vortexed and 100 µl of sulfuric acid (Sigma-Aldrich, St. Louis, USA) was added. The tubes were incubated in the dark for 2 h at room temperature for analysis. The supernatant was discarded and the precipitate was completely dissolved in 95% methanol. The sample was transferred to a colorimetric cuvette and absorbance was measured at 538 nm on EVOLUTION 260 BIO (Thermo Fisher Scientific, Waltham, USA) (Ghorai et al. 2012).

Measurement of antioxidant enzymes activity

For all antioxidant enzyme assays, the proteins from 18-day-old leaves samples (0.1g) were homogenized in 200 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C. After homogenization, the samples were centrifuged at 15,000 rpm for 15 min at 4°C. Total protein content was determined by the Bradford assay using BSA as a standard (Bradford 1976). Peroxidase (POD) activity was determined with a modified Pütter (Pütter et al. 1974) method and Ascorbate peroxidase (APX) activity was analyzed according to the method of Mittler and Zilinskas (1993). Superoxide dismutase (SOD) activity was assayed using the sigma SOD determination kit (#19160) (Sigma-Aldrich, St. Louis, USA) following the manufacturer's instructions. Catalase (CAT) activity was assayed using the Amplex® Red Catalase Assay kit (A22180) (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions.

Subcellular localization of OsTPS30

A. thaliana protoplasts were isolated from the leaves of 14-days-old WT and OX-*OsTPS30* plants (CaMV35S: *OsTPS30-GFP*). All plants were incubated at 22 °C for 2 weeks under a 16-h light/8-h dark cycle. The fluorescence of the GFP and red chlorophyll autofluorescence was detected using the LSM800 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) at an excitation wavelength of 488 nm.

Results

OsTPS30 expression following gamma irradiation

To investigate the effect of gamma-ray on the expression of *OsTPS30* expression, rice seeds were exposed to gamma-ray. A subsequent qRT-PCR revealed that *OsTPS30* expression levels were upregulated in response to gamma-ray. Increasing the gamma-ray dosage from 0 to 400 Gy resulted in a 3.4-fold increase in the *OsTPS30* transcript abundance (Fig. 1A). These observations were consistent with previously reported RT-PCR results (Lee et al. 2015). Moreover, the *OsNAC10* expression was increased with increasing intensity of gamma-rays (Fig. 1B), which is reportedly upregulated by gamma irradiation (Park et al. 2014), was used as a positive control.

Overexpression of *OsTPS30* in *A. thaliana*

The gamma-ray treatment clearly upregulated *OsTPS30* expression. To assess the physiological function of OsTPS30 in plants irradiated with gamma-rays, we constructed OX-*OsTPS30* plants. The *OsTPS30* gene was PCR-amplified from rice. The pMDC83-*OsTPS30:GFP* vector was inserted into *A. tumefaciens* GV3101 cells for subsequent transformation of *A. thaliana* using a floral dip method (Fig. 1C). The T_3 homozygous transgenic plant lines, which were resistant to hygromycin, were produced by the self-pollination of T_2 transgenic plant lines. The *OsTPS30* expression levels were evaluated by qRT-PCR (Fig. 1D). Three homozygous transgenic plant lines (#2-1, #4-6, #9-17) with different expression levels were selected to analyze the *OsTPS30* expression levels and the effects of the gamma-ray

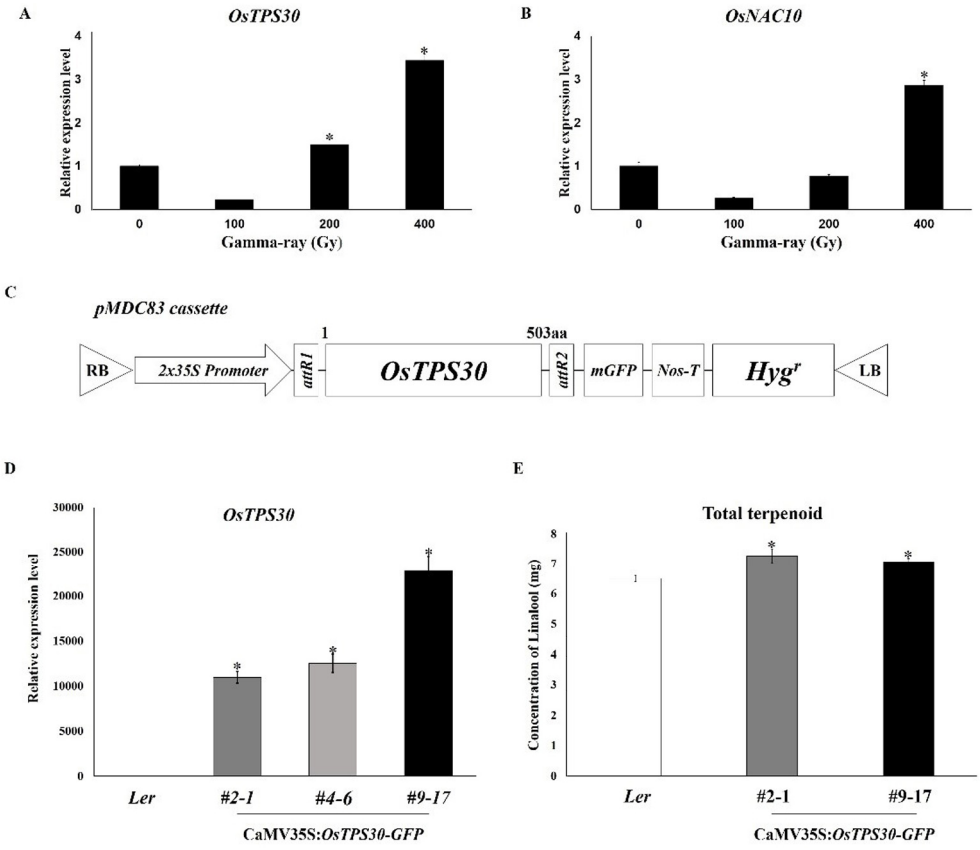

Fig. 1 Expression of *OsTPS30* in rice plants exposed to gamma irradiation and overexpression of *OsTPS30* in transgenic *A. thaliana* plants. (A) Rice seeds irradiated with gamma-rays at 100, 200, or 400 Gy. (B) *OsNAC10* was used as a positive control for the gamma irradiation. The *OsTPS30* transcript levels were analyzed using qRT-PCR, and normalized against those of *OsACT*. (C) Schematic diagram of the CaMV35S:*OsTPS30-GFP* plasmid construct used to generate the *OsTPS30*-overexpressing transgenic plant lines. (D) *OsTPS30* transcript levels were analyzed using qRT-PCR. Transcript levels were standardized against those of *AtACT2*. (E) A linalool Analysis of total terpenoid of OX-*OsTPS30* (#2-1, #9-17) and WT plants. Data is presented as the mean ± standard deviation for the three biological replicates. Asterisks indicate significant differences in the data for non-irradiated plants (0 Gy) or for the WT plants (*Ler*) at $P < 0.05$ (One-way ANOVA)

treatment.

Identification of OsTPS30 as terpene synthase

Linalool, a monoterpene, was used to compare the synthesis of total terpenoid from the OX-*OsTPS30* plants (#2-1, # 9-17) and WT plants. As a result, OX-*OsTPS30* plants synthesized more total terpenoid than WT plants (Fig. 1E).

Effect of gamma irradiation on *OX-OsTPS30* plants

To confirm rice OsTPS30 can increase the tolerance of other plants to gamma irradiation, we generated transgenic *A. thaliana* lines producing the GFP-tagged OsTPS30 protein. The 18-day-old plants exposed to gamma-rays at doses of 100-500 Gy for 24 h were transferred to normal growth conditions for a recovery period. After 14 days, the results showed that the height and weight of OX-*OsTPS30* plants (#2-1, #4-6, #9-17) were significantly higher than those of WT plants. The phenotypes of the OX-*OsTPS30* and WT plants were not significantly different under normal growing conditions (Fig. 2). These results indicate that OsTPS30 contributes to gamma irradiation resistance.

Antioxidant enzyme activity of *OX-OsTPS30* plant

Reactive oxygen species (ROS) scavenging is a common protective response to gamma irradiation. Activity of antioxidant enzymes, POD, APX, CAT and SOD, are induced by gamma irradiation (Moussa 2008). In order to determine the activities of the antioxidant enzyme of different dosages of gamma irradiation on OX- *OsTPS30* (#2-1, #9-17) and WT plants, we measured the enzymatic activity of POD, CAT, APX and SOD (Fig. 3). At 200 Gy, the POD activity showed OX-*OsTPS30* plants (#2-1, #9-17) were

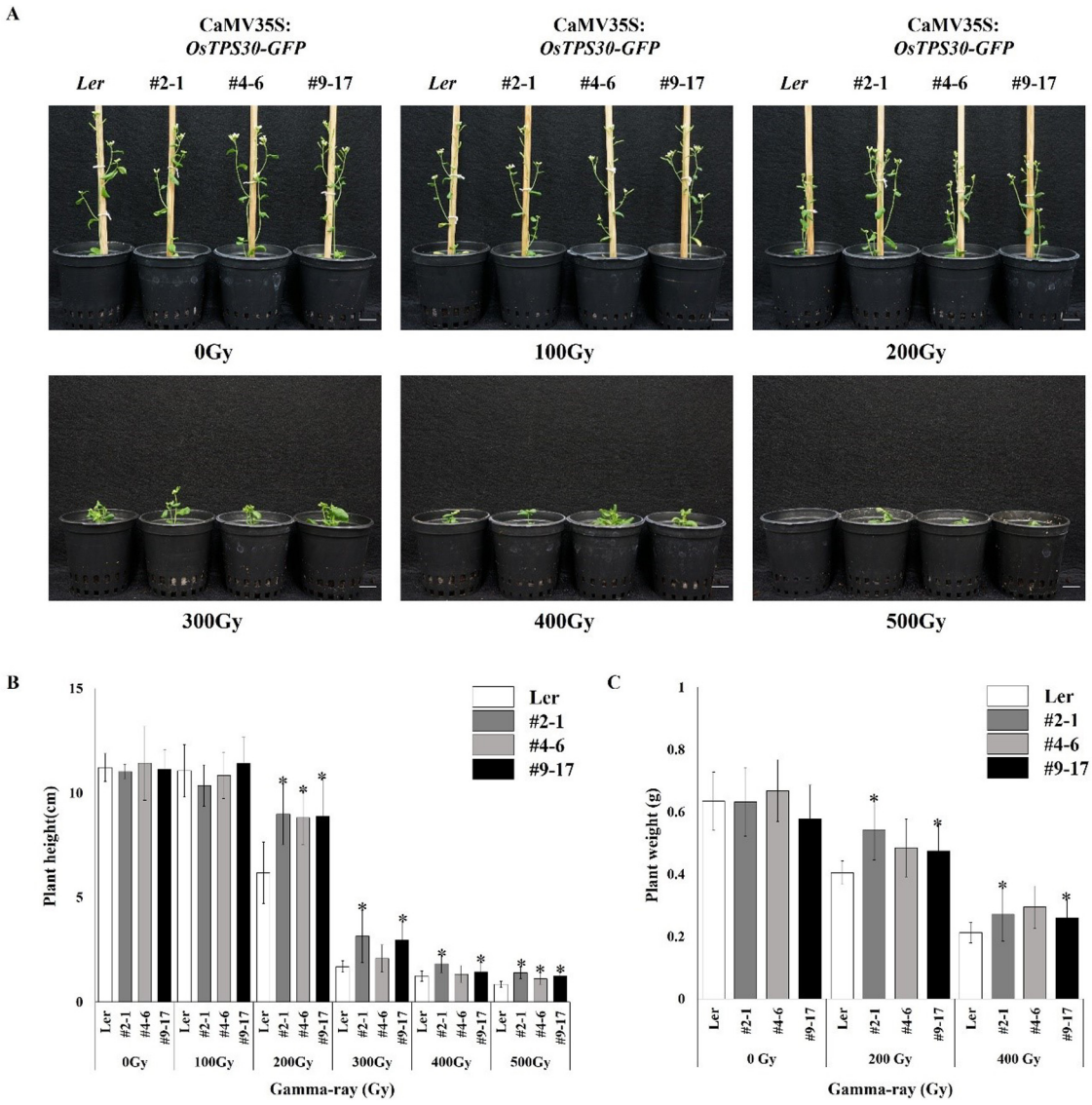

Fig. 2 The OX-*OsTPS30* plants enhances tolerance to gamma irradiation. (A) The OX-*OsTPS30* and WT plants were grown for 18 days after seeding and then irradiated for 24 h at 0, 100, 200, 300, 400, and 500 Gy. The irradiated plants were then analyzed after 2 weeks. White bar = 1 cm. (B) The height of OX-*OsTPS30* and WT plants were also evaluated 2 weeks after the gamma irradiation. (C) The weight of OX-*OsTPS30* and WT plants were evaluated 2 weeks after the gamma irradiation. The data is presented as the mean ± standard deviation for the three biological replicates. Asterisks indicate significant differences in the data for the WT plants (*Ler*) at $P < 0.05$ (One-way ANOVA)

significantly higher than those of WT plants. However, when OX-*OsTPS30* plants (#2-1, #9-17) were exposed to 400 Gy, POD activity was lower than WT plants (Fig. 3A). Regardless of gamma irradiation, CAT activity was higher OX-*OsTPS30* plants (#2-1, #9-17) than WT plants (Fig. 3B). APX activity levels were increased in the OX-*OsTPS30* plants (#2-1, #9-17) at 200 Gy gamma irradiation. However, there was no difference at 400 Gy gamma irradiation (Fig. 3C). At 0, 200 and 400 Gy gamma irradiation, SOD activity inhibition rate was 5 ~ 10% lower in the OX-*OsTPS30* plants (#2-1, #9-17) than WT plants (Fig. 3D). These results suggested that OsTPS30 was positively regulated by regulating the activity of antioxidant enzyme involved in ROS induced by gamma irradiation.

Subcellular localization of OsTPS30

In general, monoterpene and diterpene synthases contain a transit peptides for translocation to the plastids (Tholl 2006; Arimura et al. 2009). OsTPS30 gene consists of 503 amino acid with a calculated molecular mass of 58.5 kDa. The amino acid sequence of OsTPS30 includes a DDxxD motif that has been implicated in binding with a divalent metal

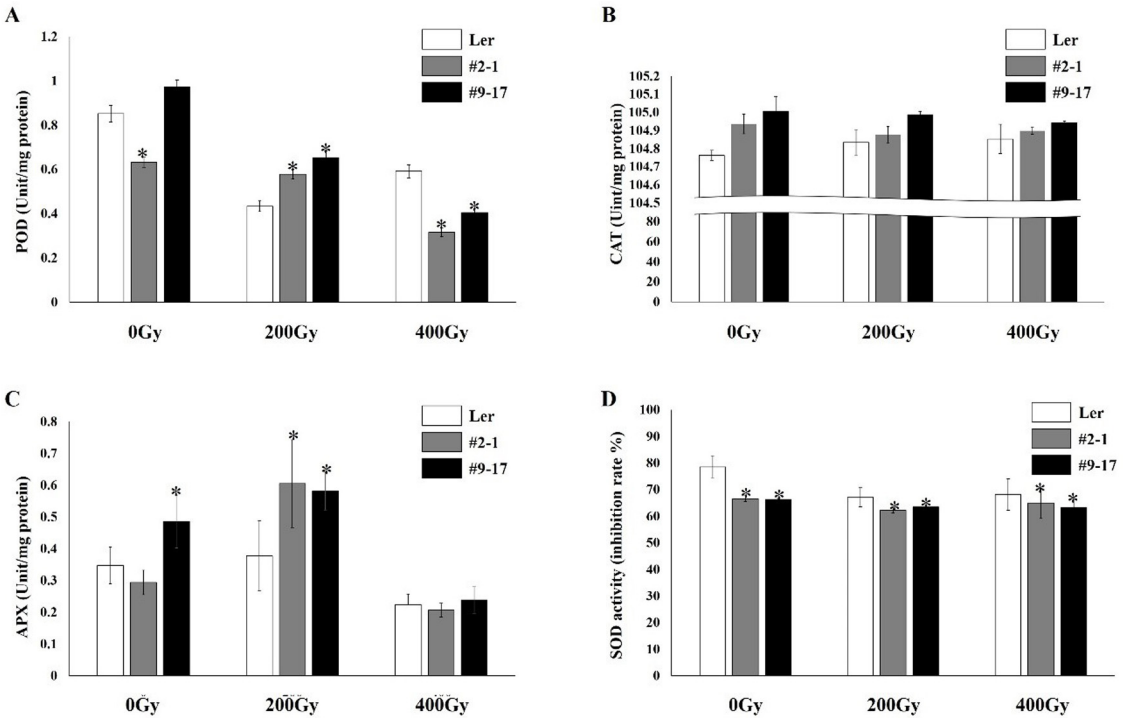

Fig. 3 Antioxidant enzyme activity of OX-*OsTPS30* and WT plants after gamma irradiation. (A) POD activity. (B) CAT activity. (C) APX activity. (D) SOD activity inhibition rate (%). Values are expressed as activity units per mg enzyme. Data is presented as the mean ± standard deviation for the three biological replicates. Asterisks indicate significant differences in the data for the WT plants (*Ler*) at $P < 0.05$ (One-way ANOVA)

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  1 MKERVQVVKEEVRKVVKGSSEVPEILDLVITLQRLGLDSYYETEINDLLC 50
 51 IVYNTDYNDKDLHLVSLRFYLLRKNGYDVSSDMFQHFKDKEGSFVADDVR 100
101 SLLSLYNAAFLRTHGEKVLDEAIVFTTNRLRSELEHLKSPAADEVSLALN 150
151 TPLFRRVRILEIRNYIPIYESATTRNESILEFAKLNFNLLQLIYCEELKS 200
201 ITGWWKELNVESNLSFIRDRIVEMHFWMIGACSEPHYSLSRIILTKMIAF 250
251 ITILDDIFDTYATTEESMMLAKAIYMCNETATVLLPKYMKDFYLYYLKTF 300
301 DSFEEALCPNKSYRVCYLKELFKRLVQEFSQEIKWRDDHYIPKTIEEHLE 350
351 LSRKTVGAFELACASFVGMGDLVAKETLDCLLTYPELLKSFTTCVRLSND 400
401 IASTKREQAGDHHHASTIQSYMLQHGATAHEACVGIKELIEDSWKDMMKE 450
451 YLTPTDLQPKIVARTVIDFARTGDYMYKQVDSFTISHTIKDMIASLYMEP 500
501 YNI-
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Fig. 4 Amino acid sequences of OsTPS30 (Os08g07080). The gene consists of 503 amino acids with a calculated molecular mass of 58.5 kDa. The putative conserved DDxxD motif is underline

cofactor (Fig. 4). Sequence analysis using ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicted the localization of OsTPS30 to the chloroplast, suggest that OsTPS30 functions as a monterpene or diterpene synthases. However, as there was no direct experimental evidence for this behavior, we isolated *A. thaliana* protoplasts from the leaves of 14-day-old WT and OX-*TPS30* plants. The GFP fluorescence was monitored, which revealed that OsTPS30-GFP

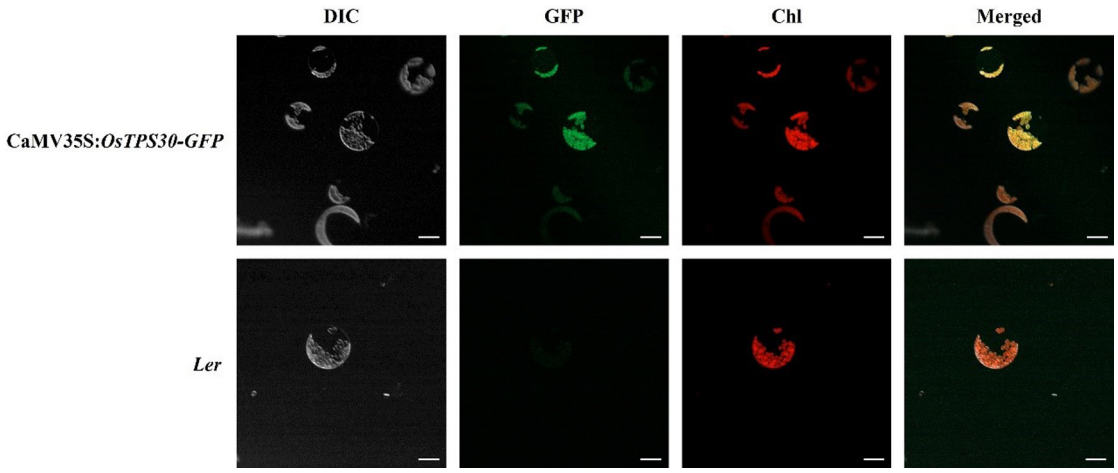

Fig. 5 Subcellular localization of the OsTPS30-GFP fusion protein in *A. thaliana* protoplasts. Isolated protoplasts from the leaves of OX-*OsTPS30* and WT plants (*Ler*). Isolated protoplasts were analyzed using confocal microscopy and photographed. DIC, Differential interference contrast image; GFP, Green fluorescence protein; Chl, Red chlorophyll autofluorescence utilized as a chloroplast marker. The white bar represents 10 μm

fusion protein was localized in chloroplasts, suggest that OsTPS30 is a putative MEP pathway related terpene synthase (Fig. 5).

Discussion

Terpenes are defense compounds present in diverse plant species. Terpenoids play a large role in plant development and stress responses. Terpene production may be induced by abiotic stresses, including gamma-rays, UV-rays, and high temperature, ultimately generating ROS (Jenkins 2009; Esnault et al. 2010). Several aspects regarding the genetic basis of plant-responses to ionizing radiation remain unknown. Lee et al. (2015) reported that the expression levels of five *OsTPS* genes (i.e., *OsTPS19*, *OsTPS20*, *OsTPS25*, *OsTPS26*, and *OsTPS30*) are induced by gamma irradiation. However, *OsTPS30* was not functionally characterized. In the current study, we observed that *OsTPS30* transcript abundance increased following exposures to gamma irradiation (Fig. 1A). These results imply that *OsTPS30*, encodes a putative rice TPS and gamma irradiation-inducible genes.

To characterize the physiological function of OsTPS30 following gamma irradiation, we generated Ox-*OsTPS30* plants. Ghorai et al. (2012) reported, linalool, monoterpene, can indirectly measure total terpenoid. As result, total terpenoid were slightly increased in Ox-*OsTPS30* plants (#2-1, # 9-17) than WT plants (Fig. 1E). We hypothesize that the increase in the total terpenoid of Ox-*OsTPS30* plants may be a reason for resistance to gamma irradiation.

Plants height and weight are a major parameter influencing responses to gamma irradiation. The height and weight of OX-*OsTPS30* plants higher than the WT plants after the gamma irradiation (Fig. 2). Previous studies concluded that *OsTPS30* expression is upregulated by gamma irradiation (Fig. 1A). Moreover, we observed that OX-*OsTPS30* plants were more resistant to the gamma irradiation than the WT plants (Fig. 2). There results, the increased expression of OsTPS30 in response to gamma irradiation and greater tolerance of plants that overexpressed OsTPS30, suggest that OsTPS30 plays an important role in resistant to gamma irradiation.

Produced ROS induce damages of protein, membrane and nucleic acids and negatively influence plant growth and development. The antioxidant role of monoterpenes under oxidative stress have been experimentally elucidated to directly mitigate the ozone level leading to decreased oxidative damage (Fares et al. 2008). The POD, CAT, APX, SOD activity showed OX-*OsTPS30* plants (#2-1, #9-17) were significantly higher than the WT plants (Fig. 3). Thus, OsTPS30 increased the activity of antioxidant enzymes, thereby eliminating ROS increased by gamma irradiation.

Terpene synthases are involved in the MEP pathway in chloroplast. Monoterpene and diterpene synthases generally contain a transit peptide that enables them to translocate to plastids (Tholl 2006; Arimura et al. 2009). Amino acid sequence analysis using ChloroP 1.1, we could predict that OsTPS30 would be present in the chloroplast. However, chloroplast transit peptide was not found. We determined that OsTPS30-GFP fusion protein is mainly localized to the chloroplast (Fig. 5), implying this OsTPS30 is a monoterpene or diterpene synthase associated with MEP pathway.

In conclusion, transcription of *OsTPS30* was strongly

upregulated in rice plants after gamma irradiation. OX-*OsTPS30* plants synthesized more total terpenoid than WT plants. OX-*OsTPS30* plants tended to be more tolerant to gamma irradiation than WT plants. Following gamma irradiation, these OX-*OsTPS30* plants showed significant differences in plant height and weight when compared to WT plants. The OsTPS30 increased the activity of antioxidant enzymes. Subcellular localization analysis of OsTPS30-GFP showed abundant fluorescent signals accumulated in the chloroplast, suggesting that OsTPS30 is putative MEP pathway-related terpene synthase.

Acknowlegements

This research was supported by grants from the Nuclear R&D Program by the Ministry of Science and ICT (MSIT), and the research program of KAERI, Republic of Korea.

Author's Contributions

S. W. Kim designed the experimental plan and wrote the manuscript. S. W. Kim and I. J. Jung performed the experiments. J. -B. Kim, S. -Y. Kang, S. H. Kim and H. -I. Choi supervised the project. All of the authors were involved in data analysis and interpretation.

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