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Constitutive expression of *CaPLA1* conferred enhanced growth and grain yield in transgenic rice plants

Ki Youl Park[1] · Eun Yu Kim[1] · Young Sam Seo[1,2] · Woo Taek Kim[1]

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Abstract Phospholipids are not only important components of cell membranes, but participate in diverse processes in higher plants. In this study, we generated *Capsicum annuum* phospholipiase A1 (*CaPLA1*) overexpressing transgenic rice (*Oryza sativa* L.) plants under the control of the maize ubiquitin promoter. The T4 *CaPLA1*-overexpressing rice plants (*Ubi:CaPLA1*) had a higher root:shoot mass ratio than the wild-type plants in the vegetative stage. Leaf epidermal cells from transgenic plants had more cells than wild-type plants. Genes that code for cyclin and lipid metabolic enzymes were up-regulated in the transgenic lines. When grown under typical paddy field conditions, the transgenic plants produced more tillers, longer panicles and more branches per panicle than the wild-type plants, all of which resulted in greater grain yield. Microarray analysis suggests that gene expressions that are related with cell proliferation, lipid metabolism, and redox state were widely altered in *CaPLA1*-overexpressing transgenic rice plants. *Ubi:CaPLA1* plants had a reduced membrane peroxidation state, as determined by malondialdehyde and conjugated diene levels and higher peroxidase activity than wild-type rice plants. Furthermore, three isoprenoid synthetic genes encoding terpenoid synthase, hydroxysteroid dehydrogenase and 3-hydroxy-3-methyl-glutaryl-CoA reductase were up-regulated in *CaPLA1*-overexpressing plants. We suggest that constitutive expression of *CaPLA1* conferred increased grain yield with enhanced growth in transgenic rice plants by alteration of gene activities related with cell proliferation, lipid metabolism, membrane peroxidation state and isoprenoid biosynthesis.

Keywords Enhanced growth · Grain yield · Transgenic rice plant · Membrane peroxidation · Phospholipase A1 · *Oryza sativa*

Introduction

Phospholipids are important components of cellular membranes. Phospholipids and their derivatives are also important participants in developmental and physiological processes in high plants, including seedling establishment, seed viability, cell elongation, gravitropism, pollen development, stomatal closure, anther dehiscence, leaf senescence and the response to environmental stress (Chen et al. 2013; Ishiguro et al. 2001; Kim et al. 2011a, b; Lee et al. 2003; Ryu 2004; Scherer 2002; Seo et al. 2011; Wang 2001, 2005; Zhang et al. 2004). Phospholipids are regulated by different phospholipases, which are divided into three groups, phospholipase A (PLA), phospholipase C (PLC) and phospholipase D (PLD), depending on where they hydrolyze the phospholipids.

PLAs catalyze hydrolysis of phospholipid to lysophospholipid and free fatty acid. This group is further divided into PLA1 and PLA2, which cleave the fatty acid from the *sn*-1 or *sn*-2 position of the glycerol molecule of the phospholipid, respectively. In the *Arabidopsis* genome, twelve PLA1 isoforms have been identified and classified into three classes depending on their sub-cellular

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✉ Woo Taek Kim
wtkim@yonsei.ac.kr

[1] Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea

[2] Present Address: Research Institute, Korea Ginseng Corp., Daejeon 305-805, Korea

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localization: class I, class II and class III, which are localized in the chloroplasts, cytosol and mitochondria, respectively (Chen et al. 2011a, b; Hyun et al. 2008; Ryu 2004; Seo et al. 2009). The *DEFECTIVE IN ANTHER DEHISCIENCE1* (*DAD1*) gene encodes a PLA1 that is targeted to the chloroplast. DAD1 regulates anther dehiscence by modulating jasmonic acid biosynthesis (Ishiguro et al. 2001). An *Arabidopsis* cytosolic DAD1-like acylhydrolase, AtDSEL, a class II cytosolic PLA1, is involved in a β-oxidation step during seedling establishment (Kim et al. 2011a, b). The *Arabidopsis sn*-1-mitochondrial-specific acylhydrolase (AtDLAH) is localized in the mitochondria and reduces lipid peroxidation, thereby increasing seed viability (Seo et al. 2011).

Membrane peroxidation and its products affect membrane properties, such as membrane thickness, fluidity and substance permeability (Catalá 2009; Gutteridge 1995; Halliwell and Chirico 1993; Wong-ekkabut et al. 2007; Yin et al. 2011). These changes are deleterious to the membrane's function and membrane peroxidation is often related to human diseases, including Alzheimer's disease, Parkinson's disease, cancer and diabetes (Ayala et al. 2014; Chapple et al. 2013; Cristalli et al. 2012; Niedernhofer et al. 2003; Perluigi et al. 2012). In higher plants, membrane peroxidation is closely correlated with developmental processes and responses to environmental stress (Campo et al. 2014; Gapper and Dolan 2006; Levine et al. 1994; Sundaravelpandian et al. 2013; Taulavuori et al. 2001). Lipid peroxidation occurs in three stages: initiation, propagation and termination. Initially, hydrogen is abstracted from lipid by the attack of a free radical. Next, oxygen is added to generate a peroxyl radical, which extracts hydrogen from an adjacent unsaturated fatty acid of the lipid. These autocatalytic reactions continue until antioxidants, such as vitamin E, react with the peroxyl radicals, forming a non-radical product in the termination step.

CaPLA1 is a class II (cytosolic-localizing) hot pepper (*Capsicum annuum* L.) phospholipase A1. Overexpression of *CaPLA1* significantly enhances growth and biomass in transgenic *Arabidopsis* plants (Seo et al. 2008). It has been proposed that CaPLA1 hydrolyzes phospholipid to lysophospholipid and free fatty acid and that its overexpression enhances metabolic activity by increasing carbon sources. CaPLA1 from hot pepper may have been functionally relevant in the heterologous *Arabidopsis* system and, therefore, may hold promise for developing rapidly growing crop plants of large biomass.

In this study, *CaPLA1* was introduced into rice (*Oryza sativa* L. ssp. *japonica*) plants and its effects on biomass accumulation and growth were assessed. *CaPLA1*-overexpressing transgenic rice plants (*Ubi:CaPLA1*) showed enhanced growth phenotypes and produced more grain yield than wild-type plants. Expression of genes associated with cell proliferation, lipid metabolism and isoprenoid biosynthesis were upregulated in *CaPLA1*-overexpressing transgenic lines as compared to wild-type rice plants. In addition, there was less membrane lipid peroxidation in *Ubi:CaPLA1* plants than in wild-type plants. It may be that CaPLA1 positively regulates growth and grain yield through its effect on membrane peroxidation in the transgenic rice plant.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* L.) japonica variety 'Dong-Jin' was used in this study. Dry rice seeds were soaked in 70 % ethanol, washed with distilled water, treated with 0.3 % NaClO solution for 20 min and rinsed extensively with sterile water. Seeds were germinated on half-strength Murashige and Skoog (MS) medium including vitamins (Duchefa Biochemie, Haarlem, Netherlands), 1 % sucrose and 0.7 % phytoagar or wet filter paper. Seedlings were grown for 7 days at 28 °C under a 16 h light-8 h dark conditions and then transplanted to soil in a greenhouse.

Construction of *Ubi:CaPLA1* transgenic rice plants

Full-length *CaPLA1* cDNA was inserted into the binary vector pGA2897. The resulting gene construct was transformed into rice by an *Agrobacterium*-mediated method (Zeng et al. 2014) with slight modifications as recently described by Byun and Kim (2014). The *Ubi:CaPLA1* transgenic lines were selected based on their resistance to hygromycin (30 μg ml^{-1}). Transcript levels of *CaPLA1* in selected transgenic lines were examined by RT-PCR using gene-specific primers (Supplementary Table S1). Wild-type plants and the independent T4 *Ubi:CaPLA1* transgenic lines (#1, #2 and #3) were evaluated in this study.

Genomic DNA extraction and DNA gel-blot analysis

Total genomic DNA was extracted from mature leaves of wild-type and *Ubi:CaPLA1* transgenic rice plants using cetyl trimethylammonium bromide (CTAB) solution. DNA was digested with *Eco*RI restriction enzyme (Roche, Indianapolis, IN, USA) and separated on 0.7 % agarose gels. The gel was treated sequentially with depurination, denaturation and neutralization solutions and then transferred to Hybond-XL nylon membrane (GE healthcare, Uppsala, Sweden) by capillary transfer method. The blots were hybridized with ^{32}P-labeled hygromycin B phosphotransferase (HPH) probes under high-stringency conditions (65 °C) and visualized using the Bio-Imaging Analyzer (BAS2500; Fuji Film, Tokyo, Japan).

RNA isolation and real-time qRT-PCR

Total RNA was isolated from 7-day-old seedlings or mature leaves of wild-type and *Ubi:CaPLA1* transgenic rice plants using an RNAiso RNA purification kit according to the manufacturer's protocol (Takara, Shiga, Japan). First-strand cDNA was synthesized from 4 μg total RNA with TOPscript RTase (Enzynomics, Daejeon, Korea) and oligo(dT) primer. RT-PCR was performed using 2 μl of the first-strand cDNA and Ex-Taq DNA polymerase (Takara, Shiga, Japan) with gene-specific primer sets (Supplementary Table S1). For real-time qRT-PCR analysis, 1 μl of the diluted (1:10) first-strand cDNA templates and SYBR Premix Ex Taq II (Takara, Shiga, Japan) were used in a total volume of 20 μl. The reaction consisted of 55 cycles of 10 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. The elongation factor 1α (*EF1α*; LOC_Os03g08020; Jain 2009) was used as an internal control. Amplified signals were monitored continuously with the IQ5 light cycler (Bio-Rad, Hercules, CA, USA) as described recently (Shen et al. 2014) and the data were analyzed using the GenesMacro IQ5 conversion template and Genex software (Bio-Rad).

In vivo PLA1 enzyme assay

For preparation of total leaf protein extracts, leaves from wild-type and transgenic plants were ground with a mortar and pestle under liquid nitrogen and suspended in ice-cold protein extraction buffer containing 50 mM Tris–HCl buffer, pH 7.2 and 100 mM NaCl in protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The suspension was centrifuged at 15,000*g* for 15 min at 4 °C and the supernatant was collected. Total protein in the leaf extract was quantified using Bradford reagent. A colorimetric assay for lipase enzyme activity was performed using 4-nitrophenyl butyrate as described by Seo et al. (2008). Briefly, 10 μg of the soluble protein extracts were mixed with 0.75 mM 4-nitrophenyl butyrate (Sigma-Aldrich) solubilized in acetonitrile. The final volume was adjusted to 200 μl with 50 mM sodium phosphate buffer (pH 7.2). The resulting reaction mixtures were incubated for 60 min at 30 °C and the lipase activity was determined from the release of *p*-nitrophenol from 4-nitrophenyl butyrate at 405 nm using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Measurements of parameters of root and shoot tissues

Assays of root and shoot growth of wild-type and transgenic rice seedlings were carried out as described by Kim et al. (2010). Root and shoot tissues were excised from light-grown 6-day-old seedlings and 4-week-old plants and their morphological properties were measured using Multi Gauge v.3.1 software (Fuji Film, Tokyo, Japan).

Microscopy

Vertical sections of leaf sheaths from the epidermis of light-grown, 1-month-old wild-type and *Ubi:CaPLA1* transgenic plants were obtained as described by Wang et al. (2013). The sheath epidermal cells were visualized by light-field microscopy (BX51; Olympus, Tokyo, Japan). The numbers of cells in one square millimeter and their lengths were determined with Image-Pro Plus v.6.0 software (Media Cybernetics, Rockville, MD, USA).

Phenotype evaluation of rice grown in the paddy field

The phenotypic traits of *CaPLA1*-overexpressing transgenic rice plants grown in normal field conditions were evaluated. Plants were grown in a greenhouse until they were 4 weeks old and were transplanted on June 3, 2014 to a rice paddy field at the Kyungpook National University, Gunwi (128:34E/36:15N), Korea. Forty plants of each genotype were assessed: wild-type plants and the three transgenic lines. The following agronomic traits were scored: number of tillers and grain yield per plant, panicle length, number of grains and branches per panicle, number of grains per branch, width and length of grain and the weight of 100 grains.

Microarray

Total RNA was isolated from 4-week-old leaves of wild-type and *Ubi:CaPLA1* (independent transgenic lines #1, #2, and #3) rice plants using RNAiso RNA purification kit according to the manufacturer's protocol (Takara, Shiga, Japan). RNA was quantified by absorbance at 260 nm. The integrity of RNA was checked by Bioanalyzer 2100 (Agilent, CA, USA). Labeled RNA was applied to the microarray (Agilent technologies, 8x60K) according to the manufacturer's protocol. Significance Analysis of Microarrays calculates the fold change and significance of differences in gene expression. Linear Lowess algorithm was used to normalize data. Genes with $P < 0.05$ were considered.

Measurements of malondialdehyde and conjugated diene

The membrane peroxidation state was assessed from the amount of malondialdehyde (MDA) and conjugated diene (CD). MDA content was determined with thiobarbituric acid as described by Hodges et al. (1999), with slight

modifications. Mature leaves from wild-type and transgenic rice plants were homogenized in 80 % (v/v) ethanol and centrifuged at 15,000*g* for 15 min at 4 °C. The supernatants were mixed with an equal volume of 0.65 % (w/v) thiobarbituric acid in 20 % (w/v) trichloroacetic acid solution, boiled for 30 min, cooled on ice and centrifuged at 15,000*g* for 15 min. The absorbance of the supernatant was measured at 532 nm with a spectrophotometer with correction for non-specific absorbance at 600 and 440 nm. The CD content was determined as described by Chowhan et al. (2013). Briefly, wild-type and transgenic leaf extracts were homogenized in 96 % (v/v) ethanol and centrifuged at 15,000*g* for 15 min at 4 °C. Absorbance of the supernatant was measured at 234 nm. The content of both MDA and CD was calculated using their molar extinction coefficients (157 and 26.5 mM^{-1} cm^{-1}, respectively).

Peroxidase enzyme activity assay

The peroxidase enzyme activity assay employed guaiacol as a substrate and was performed as described by Venisse et al. (2001). Leaves from 2-month-old wild-type and *CaPLA1*-overexpressing transgenic rice plants were homogenized in ice-cold protein extract buffer as described earlier and centrifuged at 15,000*g* for 15 min at 4 °C. Ten micrograms of protein extract was added to a solution containing 25 mM guaiacol and 25 mM H_2O_2. The final volume was adjusted to 200 µl with 50 mM sodium acetate buffer, pH 7 and incubated for 5 min at 4 °C. Peroxidase activity was calculated from the increase of absorbance at 470 nm attributed to the formation of tetraguaiacol.

3-Hydroxy-3-methyl-glutaryl-CoA reductase enzyme activity assay

A 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) activity assay was performed as described by Leivar et al. (2011) with modifications. Leaves from light-grown 1-month-old wild-type and transgenic plants were homogenized in extraction buffer [40 mM Tris–HCl, pH 7.2, 100 mM sucrose, 50 mM KCl, 0.2 % (v/v) Triton X-100, 10 mM dithiothreitol (DTT), 10 mM EDTA and protease inhibitor cocktail]. After centrifuging twice at 200*g* for 10 min at 4 °C, the protein concentration in the supernatant was quantified using Bradford reagent. For the assay, the mixture consisted of 5 mM EDTA, 10 mM DTT, 20 mM NADP, 40 mM glucose-6-phosphate, 2 units of yeast glucose-6-phosphate dehydrogenase, 0.5 mg ml^{-1} BSA, 20 mM [3-^{14}C]-HMG-CoA (Perkin Elmer, Boston, MA, USA) and 50 µg of protein extracts adjusted to a final volume of 200 µl with 100 mM Tris–HCl (pH 7.2). The reaction mixtures were incubated at 30 °C for 30 min followed by incubation with 3.5 % (v/v) HCl at 30 °C for 30 min for lactonization. The ^{14}C-labeled mevalonolactone was separated by thin-layer chromatography developed with benzene/acetone (1:1, v/v) and quantified with the Bio-Imaging Analyzer.

Results

Construction and characterization of *CaPLA1*-overexpressing transgenic rice plants

This study was undertaken to assess the possibility that CaPLA1 could improve biomass in crop plants. To this end, a transgenic rice system was employed. The *CaPLA1* gene was introduced into rice under the control of the maize ubiquitin (*Ubi*) promoter by means of *Agrobacterium*-mediated transformation (Fig. 1a). DNA gel-blot analysis identified three independent *Ubi:CaPLA1* T3 transgenic lines (#1, #2 and #3) that possessed a single copy of the *CaPLA1* transgene (Fig. 1b). RT-PCR analysis revealed that the *CaPLA1* transcript was markedly increased in all three transgenic lines (Fig. 1c). Transgenic line #1 contained the highest level of *CaPLA1* mRNA and line #3 contained the lowest level. Lipase enzyme assay using 4-nitrophenyl butyrate indicated that lipase activity was also increased in the three *CaPLA1*-overexpressing progeny as compared to wild-type rice plants (Fig. 1d). These results indicate that *CaPLA1* was expressed at both the transcriptional and translational levels in the transgenic rice plants.

CaPLA1-overexpressing transgenic rice seedlings had enhanced growth compared to wild-type seedling

Growth of wild-type and T4 *Ubi:CaPLA1* transgenic lines were compared in the early vegetative stage. Six days after sowing, transgenic seedlings had longer root and shoot tissues than wild-type seedlings. Primary root and shoot lengths of the transgenic seedlings were approximately 3.5- and 1.5-fold longer, respectively, than of the wild-type seedlings (Fig. 2b). This growth difference was still apparent when the plants were 4 weeks old (Fig. 2c). At this stage, the wild-type rice roots were 5.9 ± 0.9 cm (variability is presented as SD throughout) long and the shoots were 22.5 ± 2.3 cm long, while the same values for the transgenic lines ranged from 7.1 ± 1.2 to 7.8 ± 1.1 cm for roots and from 27.7 ± 4.4 to 27.8 ± 4.0 cm for shoots (Fig. 2d). In addition, *Ubi:CaPLA1* roots contained increased number of adventitious roots and displayed a bush-like morphology as compared to wild-type roots (Fig. 2c). According to these results, *CaPLA1* overexpression affected seedling root growth more than seedling

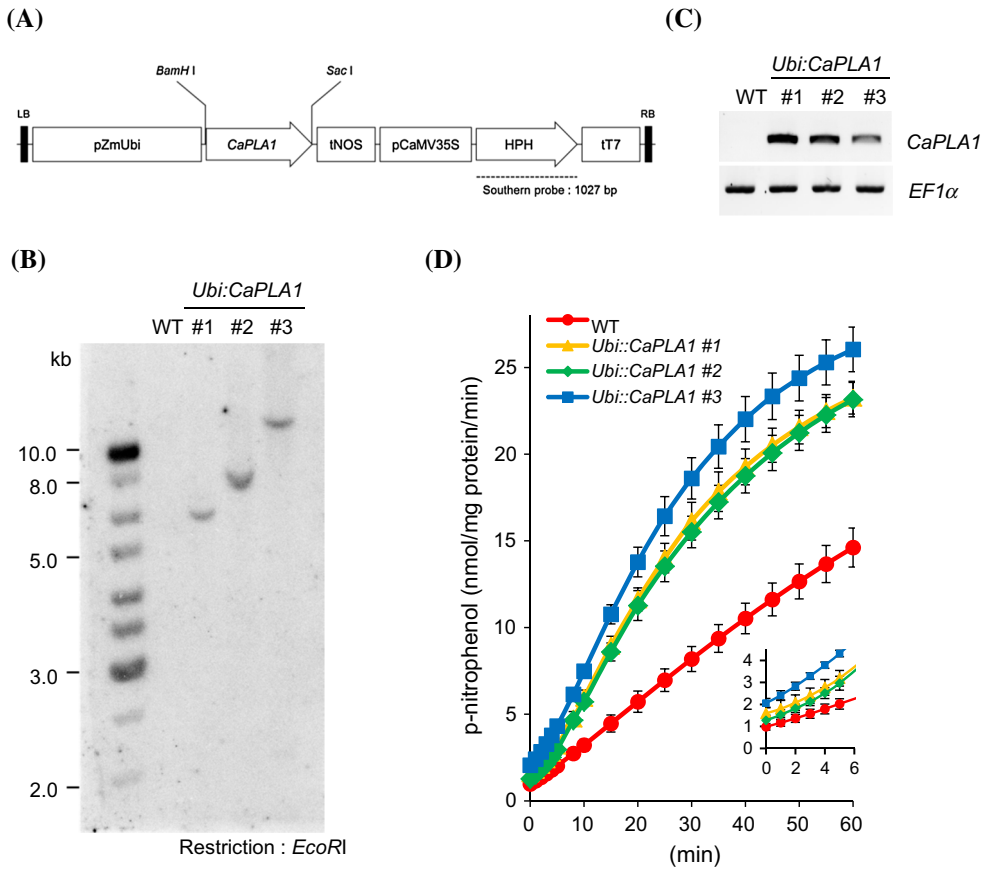


Fig. 1 Generation of *CaPLA1*-overexpressing transgenic rice plants (*Ubi:CaPLA1*). **a** Schematic structure of the binary vector for *CaPLA1* overexpression in rice. LB, left border; pZmUbi, *Zea mays* ubiquitin promoter; tNOS, nopaline synthase terminator; pCaMV35S, cauliflower mosaic virus 35S promoter; HPH, hygromycin B phosphotransferase; tT7, T7 terminator. An HPH probe for DNA gel-blot analysis is represented as a dotted line. **b** DNA gel-blot analysis of wild-type (WT) and *Ubi:CaPLA1* transgenic rice plants. Genomic DNA was digested with *Eco*RI and hybridized with an HPH probe. **c** The *CaPLA1* transcript levels in wild-type (WT) and three independent *Ubi:CaPLA1* transgenic (#1, #2 and #3) rice plants as determined by RT-PCR analysis. The rice *EF1a* gene was used as a loading control. **d** In vivo CaPLA1 enzyme activity assay in wild-type (WT) and *CaPLA1*-overexpressing rice plants as measured by generation of *p*-nitrophenol. Data represent mean ± SD; n = three independent experiments

shoot growth. Consequently, the root-to-shoot mass ratios of the transgenic lines were approximately twice that of the wild-type plants (Fig. 2e). These results suggest that overexpression of *CaPLA1* enhanced vegetative growth in rice.

Enhanced growth of *Ubi:CaPLA1* transgenic rice plants correlated with cell proliferation activity

Leaf sheath cells of the rice plants were examined microscopically (Fig. 3a). Cell number and cell length were measured in vertical sections of 5-week-old sheath tissues. Figure 3b shows that the number of epidermal cells per square millimeter of *Ubi:CaPLA1* leaves was 1.4- to 1.8-fold greater than in the wild-type leaf sheaths, while cells of the *CaPLA1*-overexpressing plants were shorter than wild-type cells. Wild-type epidermal cells were 94.0 ± 8.5 μm long, while transgenic cells were 50.6 ± 7.5, 49.8 ± 3.9 and 52.0 ± 5.4 μm, for lines #1, #2 and #3, respectively (Fig. 3b). Thus, the enhanced growth of *Ubi:CaPLA1* plants may be due to the changes in cell proliferation. Consistent with this notion, real-time quantitative RT-PCR (qRT-PCR) analysis showed that expressions of cell division marker genes, such as the A-type cyclin *CycA2.1* (LOC_Os12g31810), the B-type cyclin *CycB1.1* (LOC_Os01g59120) and the D-type cyclin *CycD3.1* (LOC_Os09g02360; Yang et al. 2013; Meguro and Sato 2014), were elevated in the transgenic plants compared to the wild type. These values ranged from 2.8- to 3.1-fold higher for *CycA2.1*, from 57.6- to 84.0-fold higher for *CycB1.1* and from 61.8- to 184.7-fold higher for *CycD3.1* in the transgenic plants than in the wild-type plants (Fig. 3c). It appears that enhanced growth of *Ubi:CaPLA1* plants correlated with increased cell proliferation activity.

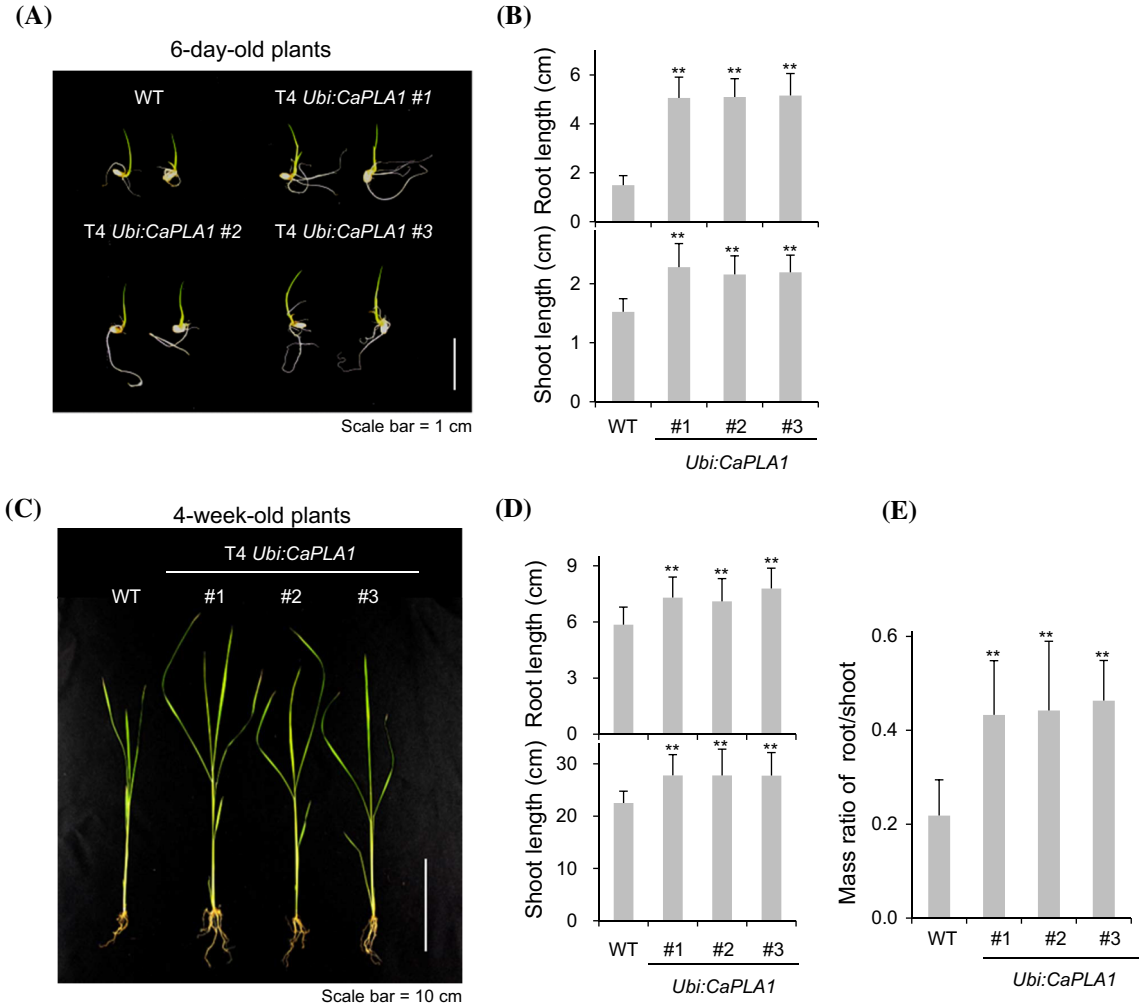


Fig. 2 Phenotype analysis of wild-type and T4 *Ubi:CaPLA1* transgenic rice plants at the vegetative stage. **a** Morphology of 6-day-old wild-type (WT) and *CaPLA1*-overexpressing (independent lines #1, #2 and #3) seedlings. *Scale bar* 1 cm. **b** Root and shoot length of 6-day-old seedlings. **c** Morphology of 4-week-old plants. *Scale bar* 10 cm. **d** Root length and shoot length of 4-week-old plants. **e** Ratio of root mass to shoot mass of 4-week old plants. *Error bars* represent ± SD (n = 60, $^{**}P < 0.005$, Student's *t* test)

Ubi:CaPLA1 transgenic rice plants exhibited an enhanced grain yield

The aforementioned results suggest that overexpression of *CaPLA1* caused an increase in the root-to-shoot mass ratio (Fig. 2e). An increase in root-to-shoot mass ratio exerts positive effects on the harvest yield and stress tolerance through enhanced water uptake from soil (Katsuhara et al. 2003; Shridhar et al. 2012). This led us to analyze the characteristics of T4 *Ubi:CaPLA1* transgenic rice plants grown in the paddy field conditions (Fig. 4a). Mature transgenic plants were approximately 20 % taller (Fig. 4b) and had many more tillers than wild-type plants. Specifically, wild-type plants had 7.0 ± 1.6 tillers per plant, while transgenic lines #1, #2 and #3 had 14.2 ± 1.9, 12.6 ± 2.6 and 8.4 ± 1.9, respectively (Fig. 4c). In addition, grain yields per plant were 1.6- to 2.4-fold higher in *Ubi:CaPLA1* lines than in wild-type plants (Fig. 4d), indicating that tiller number is linked to grain yield in transgenic rice plants.

Panicles from the T4 *Ubi:CaPLA1* lines were compared with those from wild-type rice plants (Fig. 5a). Under the paddy field growth conditions, wild-type panicle length was 15.9 ± 2.6 cm, compared to 21.8 ± 2.9, 17.3 ± 2.6 and 18.4 ± 2.9 cm in *Ubi:CaPLA1* transgenic lines #1, #2 and #3, respectively (Fig. 5b). The *CaPLA1*-overexpressing lines had more grains per panicle (1.2- to 1.9-fold), primary rachis branches per panicle (1.2- to 1.3-fold) and grains per primary rachis branch (1.2- to 1.4-fold) than the wild-type rice plant (Fig. 5c–e). However, width, length and weight of each grain were similar in the transgenic and wild-type plants (Supplementary Fig. S1). The results of these analyses suggest that constitutive expression of *CaPLA1* in rice increased the ratio of root-to-shoot mass

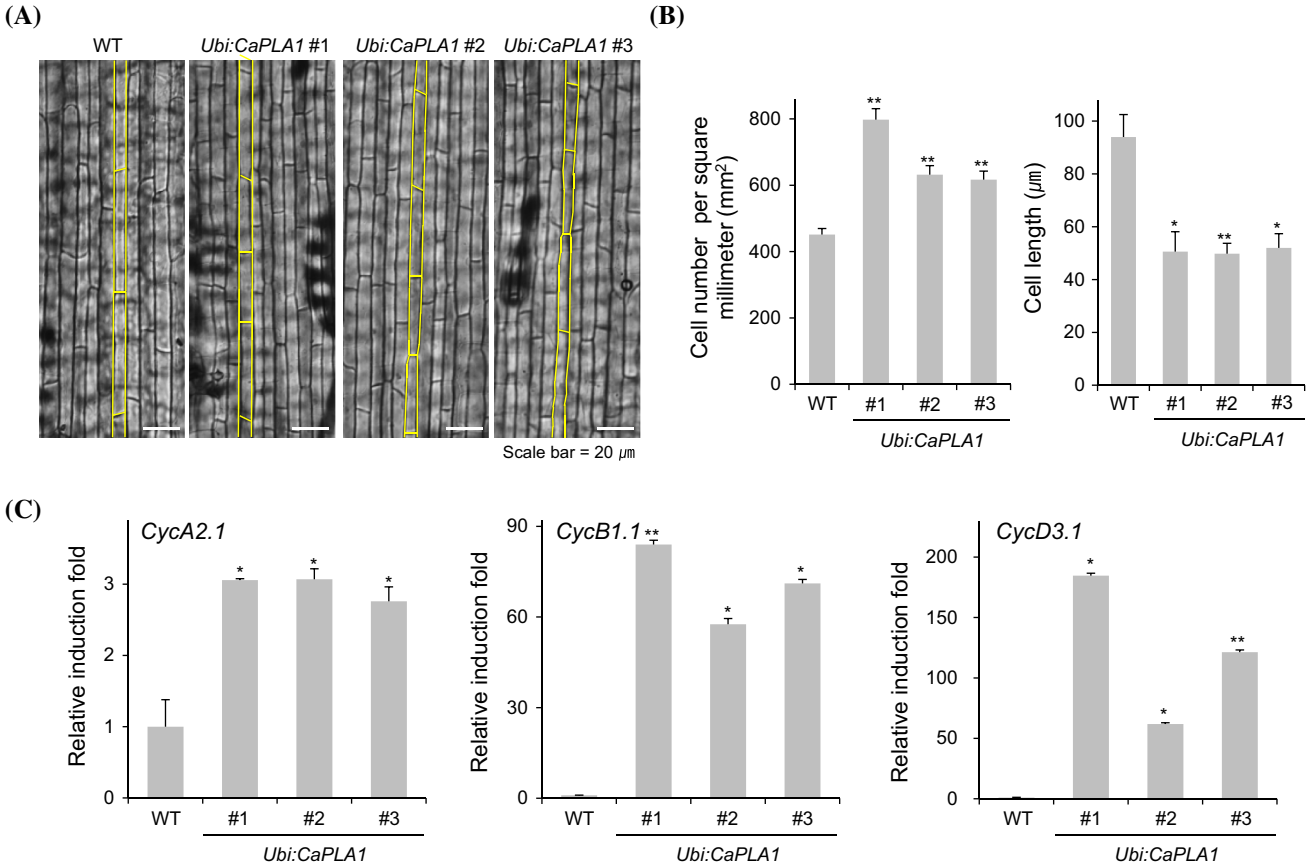


Fig. 3 Characteristics of cells from leaf sheaths and induction of cell-proliferation genes in wild-type and T4 *Ubi:CaPLA1* transgenic rice plants. **a** Vertical sections of sheath epidermal layers from wild-type (WT) and *CaPLA1*-overexpressing lines (#1, #2 and #3). Yellow lines represent cell boundaries; *scale bars* 20 μm. **b** Number of cells per square millimeter (n = 6) and cell length (n = 60). *Error bars* represent ± SD (*$P < 0.05$; **$P < 0.005$, Student's *t* test). **c** Transcript levels of cell-proliferation-related genes as determined by real-time qRT-PCR using gene-specific primer sets (Supplementary Table S1). The graphs indicate the relative expression folds of *CycA2.1*, *CycB1.1* and *CycD3.1* normalized to the expression level of rice *EF1α* gene, used as an internal control. *Error bars* represent ± SD (n = three independent experiments, *$P < 0.05$; **$P < 0.005$, Student's *t* test)

(Fig. 2), cell proliferation activity (Fig. 3) and grain yield (Figs. 4, 5).

Expressions of lipid metabolic genes were elevated in *Ubi:CaPLA1* transgenic rice plants

CaPLA1 was previously identified as a class II phospholipase A1 (Seo et al. 2008). We hypothesized that its overexpression and increased CaPLA1 enzyme activity (Fig. 1c and d) in rice could affect lipid metabolism. To examine this hypothesis, we compared the expression profiles of *acyl-CoA oxidase* (*ACX*; LOC_Os06g01390), *malate synthase* (*MS*; LOC_Os04g40990) and *phospho-enolpyruvate carboxykinase* (*PEPCK*; LOC_Os03g15050) in wild-type and T4 *CaPLA1*-overexpressing rice plants. ACX, MS and PEPCK are key enzymes in the β-oxidation, glyoxylate cycle and gluconeogenesis, respectively; all of these are well-known lipid metabolic processes (Cornah et al. 2004; Hooks et al. 1999; Rylott et al. 2003). The results of qRT-PCR analysis using gene-specific primers revealed that expressions of *ACX*, *MS* and *PEPCK* were enhanced 3.5- to 19.0-fold, 2.6- to 3.4-fold and 1.6- to 6.0-fold, respectively, in *Ubi:CaPLA1* transgenic lines as compared to wild-type rice plants (Fig. 6). These results suggest that the activities of these lipid-metabolism related genes were elevated in *Ubi:CaPLA1* progeny relative to wild-type rice plants.

Expression profiles were changed in *CaPLA1*-overexpressing rice plants

To examine more detailed expression profiles of *CaPLA1*-overexpressing rice plants, microarray analysis was performed using 4-week-old leaves of wild-type and *Ubi:CaPLA1* transgenic (independent lines #1, #2, and #3) plants. The results revealed that transcript levels of 192 annotated genes were up-regulated in *Ubi:CaPLA1* rice as compared to wild-type plants (≥1.5-fold change, $P < 0.05$;

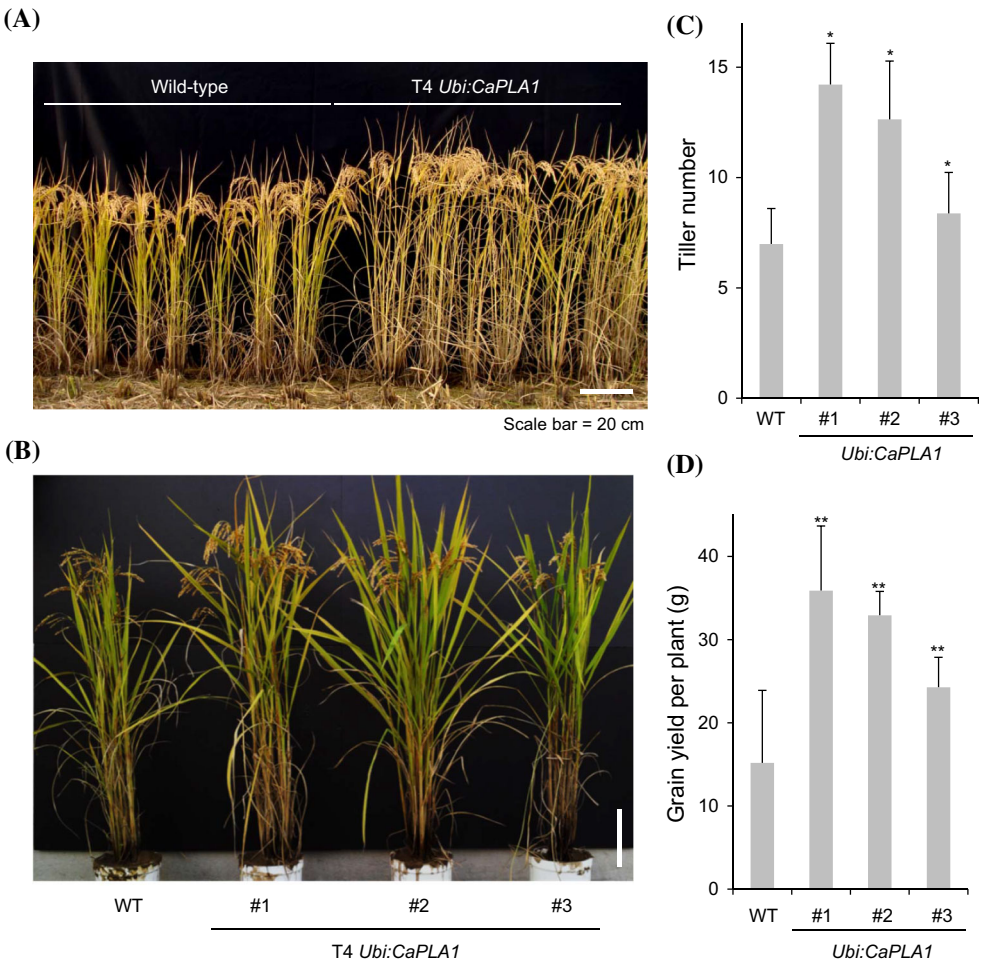


Fig. 4 Phenotypes of wild-type and T4 *Ubi:CaPLA1* transgenic rice at the reproductive stage in paddy field conditions. **a** Morphology of 6-month-old plants. *Scale bar* 20 cm. **b** Morphology of plants at the reproductive stage, transplanted from the field for photographing. *Scale bar* 20 cm. **c** Number of tillers. **d** Grain yield. *Error bars* represent ± SD (n = 40, $*P < 0.05$; $**P < 0.005$, Student's *t* test)

Supplementary Table S2, and S3; Fig. 7a). The genes with up-regulated transcript levels were enriched in the three groups of gene functions; cell proliferation, lipid metabolism, and oxidation/reduction (Fig. 7b). As expected, expression profiles of genes for cell proliferation and lipid metabolism were altered in *Ubi:CaPLA1* progeny relative to wild-type plants. The cyclin family members were up-regulated in *CaPLA1*-overexpressors. Expression levels of *Pectinesterase* (Phan et al. 2007), *Pinoresinol-lariciresinol reductase* (Ayella et al. 2007), *Caffeic acid O-methyl-transferase* (Guo et al. 2001; Tu et al. 2010), and *Cellulose synthase CesA-2* (Endler and Persson 2011), which were reported to regulate cell proliferation via controlling cell wall modification, were increased in *Ubi:CaPLA1* plants. Transcript levels of lipid metabolic proteins, including lipid transfer protein, epoxide hydrolase, and lipolytic enzymes, were also elevated in *CaPLA1*-overexpressing transgenic lines relative to wild-type rice plants. Thus, microarray results are in agreement with qRT-PCR data (Figs. 3, 6) that suggest that cell proliferation and lipid metabolic genes are widely affected by overexpression of *CaPLA1* in transgenic rice plants.

In addition, genes encoding isoprenoid biosynthetic enzymes, such as *Terpenoid synthase* (Chen et al. 2011a, b), *Chalcone synthase J* (Dao et al. 2011), and *3-Beta hydroxysteroid dehydrogenase/isomerase* (*HSD*; Li et al. 2007) were up-regulated. Isoprenoids, also referred to as terpenoids or prenyl lipids, contribute to diverse cellular functions, including antioxidants, membrane structural components, hormones and secondary metabolites (Clouse 2002; Schaller 2003; Umehara et al. 2008). Furthermore, expressions of redox reaction genes were enhanced in *Ubi:CaPLA1* plants (Fig. 7b). For example, reactive oxygen species (ROS)-scavenging genes, including *NADPH HC toxin reductase* (Hayashi et al. 2005), *Cytochrome P450* (Mizutani 2012), and *Glutathione S-transferase* (Edwards et al. 2000) were induced by overexpression of *CaPLA1*. *Peroxidase1*, *Ferrodoxin*, *Quinoprotein amine dehydrogenase*, and *Ascorbate-oxidoreductase* that are involved in the oxidation/reduction reactions were up-regulated in *CaPLA1*-overexpressing plants (Fig. 7b). Collectively, microarray analysis suggests that ectopic overexpression of *CaPLA1* caused the changes in gene expressions that are related with cell proliferation, lipid metabolic activity, and redox state in transgenic rice plants.

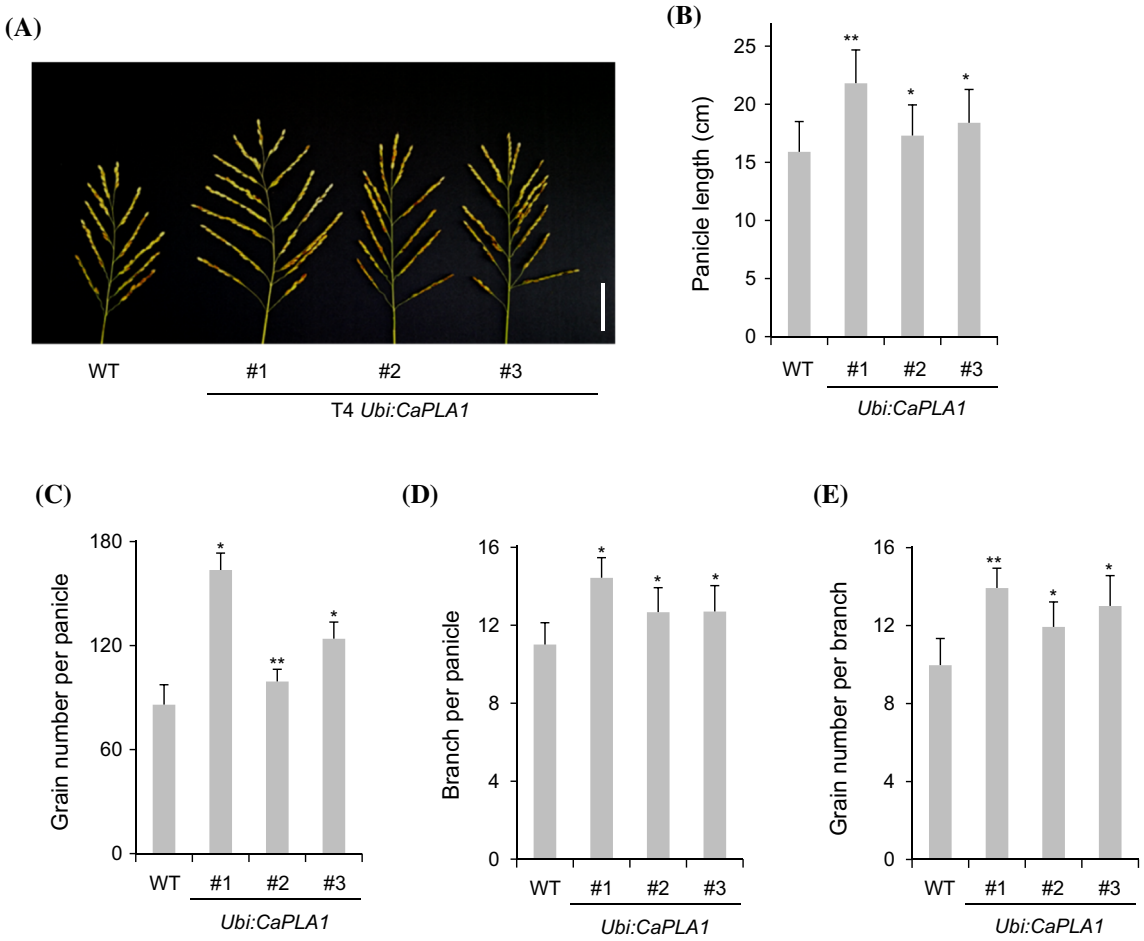


Fig. 5 Panicle phenotypes of wild-type (WT) and T4 *CaPLA1*-overexpressing rice grown in paddy field conditions. **a** Morphology of the main panicles. *Scale bar* 5 cm. **b** Panicle length. **c** Grain number per panicle. **d** Number of primary rachis branches per panicle. **e** Number of grains per primary rachis branch. *Error bars* represent ± SD (n = 40, $*P < 0.05$; $**P < 0.005$, Student's *t* test)

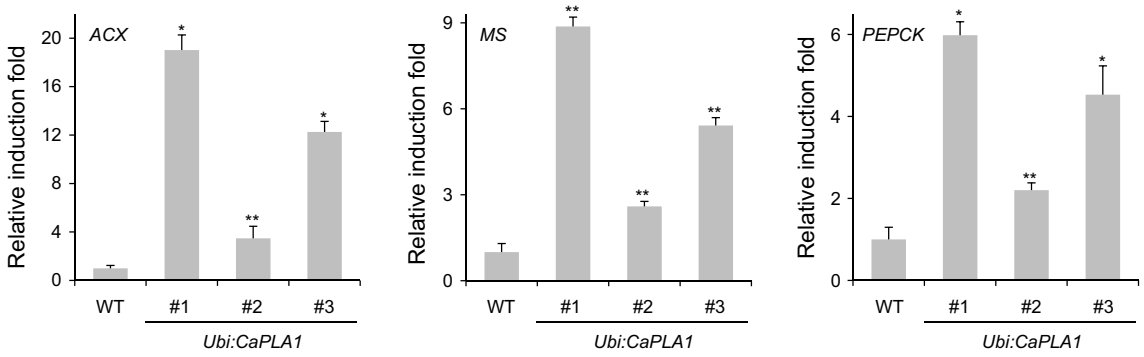
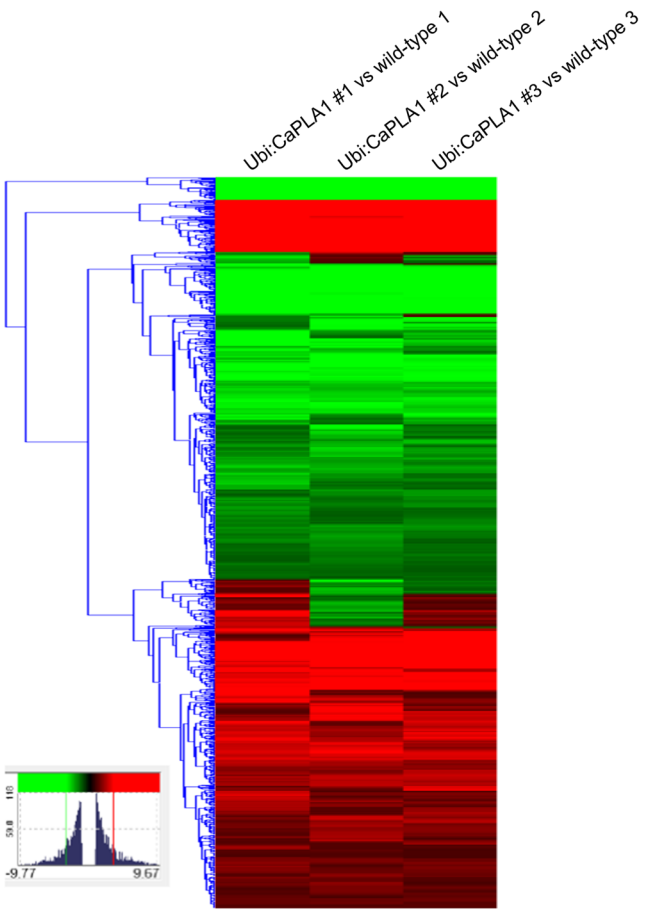


Fig. 6 Relative expression of lipid metabolism-related genes in wild-type (WT) and T4 *Ubi:CaPLA1* transgenic rice plants. Real-time qRT-PCR analysis was performed using RNA from 1-month-old leaves with gene-specific primer sets (Supplementary Table S1). The relative expression levels of acyl-CoA oxidase (*ACX*), malate synthase (*MS*) and phosphoenolpyruvate carboxykinase (*PEPCK*) were normalized to that of rice *EF1α* gene, used as an internal control. *Error bars* represent ± SD (n = three independent experiments, $*P < 0.05$; $**P < 0.005$, Student's *t* test)

(A)



(B)

Gene	Loc No	*Mean	*P*-values
Cell proliferation			
Cyclin	Os02g0825500	7.29	0.0030
Cyclin	Os11g0572800	5.75	0.0073
Cyclin	Os07g0158900	5.24	0.0017
TGF-beta receptor	Os10g0469900	4.35	0.0066
TGF-beta receptor	Os01g0761400	3.68	0.0380
Pectinesterase	Os12g0563700	3.05	0.0062
Cyclin	Os11g0582700	2.60	0.0027
Cyclin	Os10g0139600	2.33	0.0013
Pinoresinol-lariciresinol reductase	Os12g0263800	2.24	0.0024
DMC1	Os12g0143800	2.22	0.0081
Caffeic acid O-methyltransferase	Os04g0175600	2.05	0.0356
Cyclin	Os11g0533800	2.03	0.0124
Prohibitin	Os04g0462900	1.66	0.0404
Expansin S1	Os03g0336400	1.57	0.0269
Cellulose synthase CesA-2	Os01g0750300	1.51	0.0443
Cyclin	Os07g0196300	1.51	0.0060
Lipid metabolism			
Short-chain dehydrogenase/reductase SDR	Os03g0115700	5.62	0.0057
Terpenoid synthase	Os03g0347900	3.62	0.0251
Chalcone synthase J	Os05g0212900	3.30	0.0014
Short-chain dehydrogenase/reductase SDR	Os02g0511400	2.67	0.0070
Nonspecific lipid-transfer protein 3	Os12g0114800	2.64	0.0027
Epoxide hydrolase	Os10g0498000	2.33	0.0087
Lipid transfer protein	Os01g0822900	2.15	0.0200
Soluble epoxide hydrolase	Os03g0829200	1.84	0.0337
Lipolytic enzyme, G-D-S-L	Os06g0694200	1.79	0.0190
Lipolytic enzyme, G-D-S-L	Os01g0216000	1.69	0.0053
3-beta hydroxysteroid dehydrogenase	Os02g0715200	1.62	0.0122
Oxidation/reduction			
NADPH HC toxin reductase	Os07g0601000	6.18	0.0012
Quinoprotein amine dehydrogenase	Os12g0431300	4.39	0.0443
Peroxidase 1	Os07g0639400	2.94	0.0088
Iron/ascorbate-dependent oxidoreductase	Os02g0320800	2.91	0.0023
Cytochrome P450	Os03g0371000	2.43	0.0184
NADPH HC toxin reductase	Os07g0601900	2.41	0.0312
Cytochrome P450 51	Os05g0211100	2.19	0.0048
GST7	Os01g0949900	2.04	0.0442
Copper amine oxidase	Os07g0572100	2.04	0.0031
Glutathione S-transferase GSTF15	Os03g0135100	1.89	0.0354
Ferredoxin	Os03g0659200	1.82	0.0143
Galactose oxidase	Os01g0148500	1.77	0.0010
2OG-Fe(II) oxygenase	Os01g0352100	1.61	0.0028
Cytochrome P450	Os04g0255600	1.54	0.0053

Fig. 7 Global expression profiling analysis of commonly regulated genes in three independent *Ubi:CaPLA1* lines. **a** Heat-map analysis of 1286 transcripts that were commonly expressed in leaves from three independent lines. Scale bar indicates fold changes converted to a log2 scale. Sorting of significantly expressed 1286 genes was obtained from normalized value ≥ 1 and ≤ -1. *Red* and *green colors* indicate upregulation and downregulation of genes, respectively, in *CaPLA1*-overexpressing transgenic rice plants. **b** Up-regulated gene list from microarray in 4-week-old T4 *Ubi:CaPLA1* transgenic rice leaves relative to wild-type rice. Over 1.5-fold induced forty-one genes are enriched in the group of gene functions; cell proliferation, lipid metabolism, and oxidation/reduction. *The mean of three independent biological replicates ($P < 0.05$)

Membrane peroxidation state was reduced in *Ubi:CaPLA1* transgenic plants

The microarray results indicate that constitutive expression of *CaPLA1* affected the redox state in rice (Fig. 7). Oxidative stress and redox regulation are mediated by free radicals, such as ROS, that alter the peroxidation state of the membrane and disrupt its function (Catalá 2009; Gutteridge 1995; Wong-ekkabut et al. 2007). ROS regulate not only the response to stress but also the development, especially root growth (Dunand et al. 2007; Gapper and Dolan 2006; Lin et al. 2007; Singh et al. 2006; Sundaravelpandian et al. 2013). The transgenic plants had an increased vegetative growth and root-to-shoot mass ratio (Fig. 2), suggesting that CaPLA1 enhances growth by regulating the membrane peroxidation state, a hypothesis that led us to analyze the peroxidation of lipids in the transgenic plants. Specifically, we measured the levels of conjugated diene (CD) and malondialdehyde (MDA), which are served as indicators of the membrane peroxidation state (Singh et al. 2006). When the membrane is exposed to free radicals, it is peroxidized and CD is decomposed into toxic aldehydes, such as malondialdehyde (MDA). Monoterpenes, which inhibit growth by peroxidizing lipids, are reported to increase cellular MDA and decrease cellular CD in maize and several weeds (Chowhan et al. 2013; Zunino and Zygadlo 2004). In our study, MDA levels in leaves from 1-month-old *Ubi:CaPLA1*

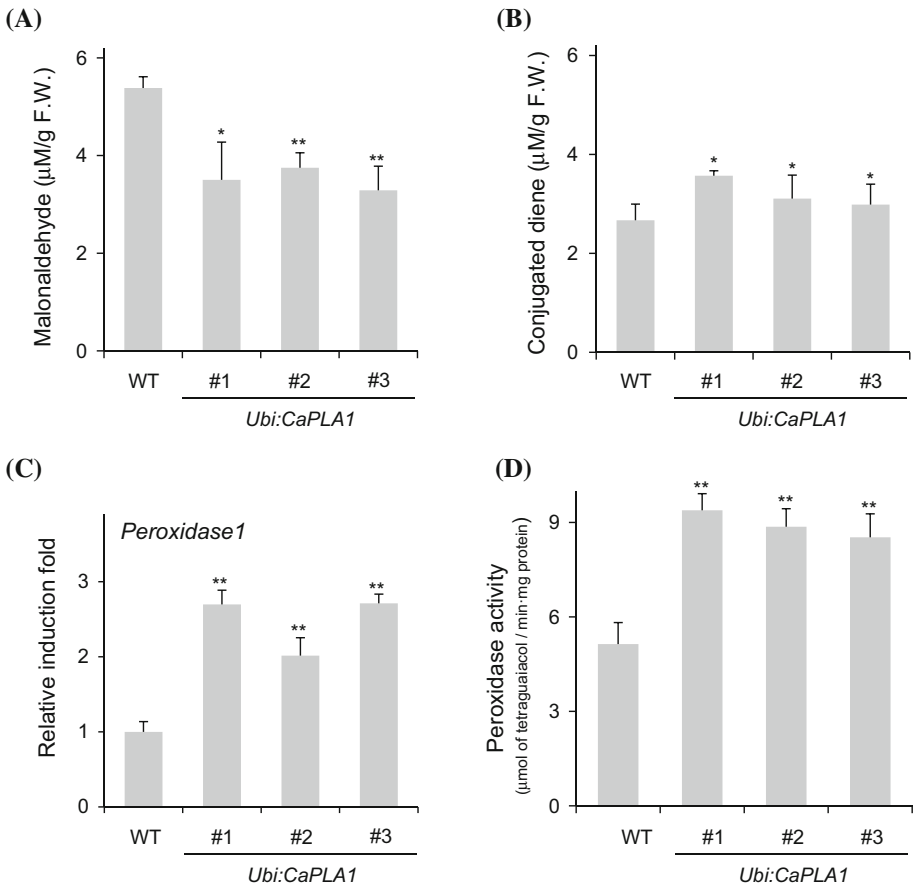


Fig. 8 Membrane peroxidation state in wild-type and T4 *Ubi:CaPLA1* transgenic rice plants. **a** Malondialdehyde (MDA) content as determined by colorimetric assay using thiobarbituric acid. **b** Conjugated diene (CD) content of leaf extracts. **c** Transcript levels of peroxidase as determined by real-time qRT-PCR performed using mRNA from leaf tissues of 1-month-old plants. **d** Peroxidase enzyme activity from extracts of total protein, incubated with guaiacol as substrate. Peroxidase activities are represented as production of tetraguaiacol. *Error bars* represent ± SD (n = three independent experiments, $*P < 0.05$; $**P < 0.005$, Student's *t* test)

plants ranged from 3.3 ± 0.5 to 3.7 ± 0.3 μM g^{-1} FW^{-1}, approximately 35 % lower than the levels in leaves from the wild-type plants (5.4 ± 0.2 μM g^{-1} FW^{-1}; Fig. 8a). In contrast, the CD content of *Ubi:CaPLA1* transgenic leaves were slightly greater (1.1- to 1.3-fold) than in wild-type leaves (Fig. 8b). These results are consistent with our hypothesis that the membrane peroxidation state was reduced in *Ubi:CaPLA1* transgenic rice relative to wild-type plants.

Peroxidase is involved in the ROS scavenging process, resulting in the reduction of membrane peroxidation state (Rhee et al. 2005). We asked if the decreased MDA levels in *Ubi:CaPLA1* transgenic leaves correlated with peroxidase activity. To resolve this question, both the transcript level and enzyme activity of peroxidase were measured. As shown in Fig. 8c, transcript levels of *peroxidase 1* (LOC_Os07g44590) were 2.0- to 2.8-fold higher in *Ubi:CaPLA1* transgenic lines than in wild-type rice plants. Enzyme activity of peroxidase was measured from soluble protein extracts from leaves of *Ubi:CaPLA1* and wild-type plants, with guaiacol as a substrate (Venisse et al. 2001). Soluble peroxidase activities were upregulated to 1.7- to 1.8-fold in the transgenic plants compared to wild-type (Fig. 8d). Overall, these results indicate that overexpression of *CaPLA1* resulted in a reduced membrane peroxidation state and increased peroxidase activity in rice.

Expressions of isoprenoid synthetic genes were increased in *Ubi:CaPLA1* transgenic rice plants

The membrane peroxidation state is regulated by various factors, including antioxidants and ROS scavenging enzymes. Microarray results indicate that isoprenoid synthetic genes were up-regulated in *CaPLA1*-overexpressors (Fig. 7). Isoprenoids, such as sterols and terpenoids, act as antioxidants and stabilize the membrane in higher plants (Affek and Yakir 2002; Kirby and Keasling 2009; Loreto and Velikova 2001; Loreto et al. 2001; Sharkey et al. 2001; Vickers et al. 2009). Isoprenoid biosynthesis may, then, be altered in *Ubi:CaPLA1* transgenic rice. The transcript levels of two isoprenoid biosynthetic genes, *terpenoid synthase* (Chen et al. 2011a, b) and *hydroxysteroid dehydrogenase* (*HSD*; Li et al. 2007), were monitored. qRT-PCR data revealed that transcript levels of both *terpenoid synthase* and *HSD* were 1.3- to 3.5-fold and 2.4- to 11.4-fold higher, respectively, in *Ubi:CaPLA1* progeny than in wild-type plants (Fig. 9a, b). Three-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), which catalyzes HMG-

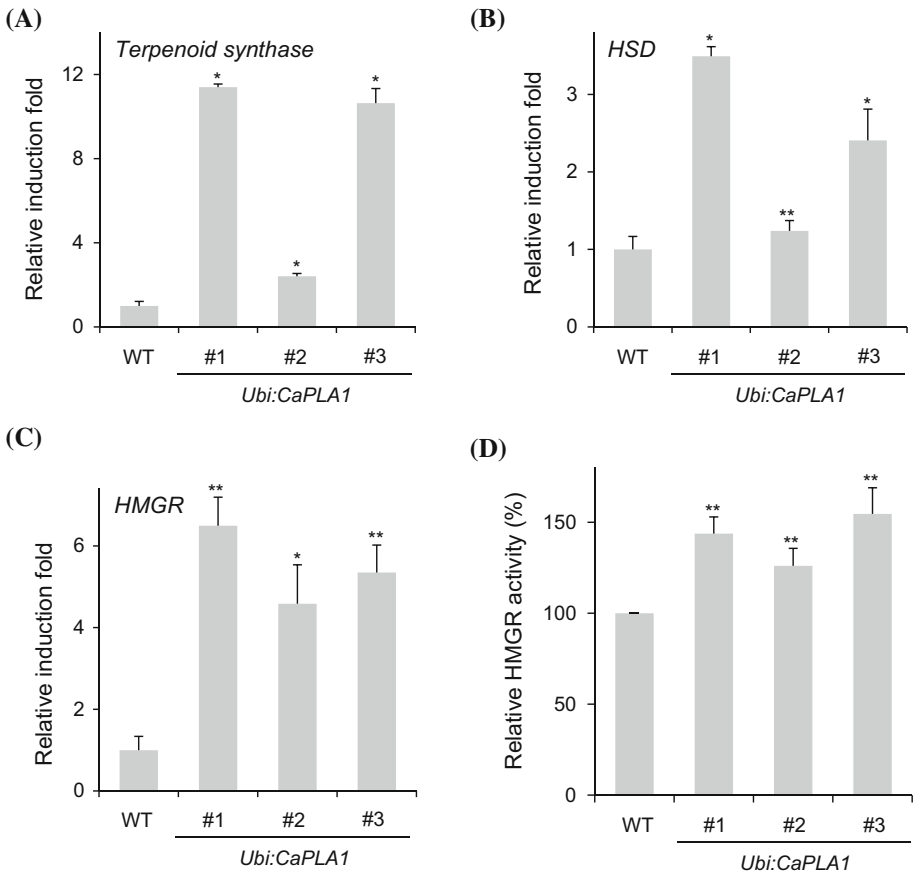


Fig. 9 Expression levels of isoprenoid biosynthesis-related genes and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) enzyme activity in wild-type and T4 *Ubi:CaPLA1* transgenic rice plants. **a** Transcript levels of *terpenoid synthase*. **b** Transcript levels of *hydroxysteroid dehydrogenase* (*HSD*). **c** Transcript levels of *HMGR*. The graphs indicate the relative induction folds normalized to the transcript level of an internal control, *EF1α*. **d** Relative enzyme activity of HMGR in leaf tissues from 1-month-old plants. The HMGR activity was determined using ^{14}C-labeled HMG-CoA and is relative to the activity in wild-type leaves (considered to be 100 %). *Error bars* represent ± SD (n = three independent experiments, $*P < 0.05$; $**P < 0.005$, Student's *t* test)

CoA to mevalonate, is a rate-limiting step in the mevalonate pathway for isoprenoid biosynthesis (Miziorko 2011). As shown in Fig. 9c, the transcript level of *HMGR* was 4.6- to 6.5-fold higher in *Ubi:CaPLA1* lines than in wild-type rice. *HMGR* may be regulated by both transcriptional and post-translational processes (Leivar et al. 2011), leading us to determine if the enhanced transcription of *HMGR* correlated with its post-translational level of HMGR in *Ubi:CaPLA1* transgenic rice plants. HMGR enzyme activity was measured using C^{14}-labeled HMG-CoA as a substrate. Enzyme activity of HMGR in transgenic lines was 1.3- to 1.5-fold higher than in the wild-type plants (Fig. 9d). These results suggest that overexpression of *CaPLA1* enhanced isoprenoid biosynthetic gene expression in rice.

Discussion

In this study, we characterized the effect of CaPLA1, a class II (cytosolic-localizing) phospholipase A1 from hot pepper, on agricultural traits in a heterologous transgenic rice system. CaPLA1 was characterized in *Arabidopsis* because transgenic work was extremely difficult in hot pepper (Seo et al. 2008). Transgenic *Arabidopsis* plants (*35S:CaPLA1*) grew faster and accumulated more biomass than wild-type plants. We wished to explore the potential for CaPLA1 to improve performance of crop plants. To this end, *CaPLA1* was expressed in rice under the control of the maize ubiquitin promoter (Fig. 1). Compared to the wild-type plants, *Ubi:CaPLA1* transgenic plants displayed increased growth phenotypes in the vegetative stage and had a higher root-to-shoot mass ratio (Fig. 2). Increased root-to-shoot mass ratio enhancement of yield and stress tolerance has been reported to act through effective water uptake from soil (Katsuhara et al. 2003; Shridhar et al. 2012). Although *CaPLA1* was expressed in rice by an ectopic promoter (Fig. 1a), root growth was more affected by CaPLA1 than shoot growth in the early seedling stages in transgenic rice plants (Fig. 2e). This is consistent with the previous results that *CaPLA1* was specifically expressed at early stages of root formation in hot pepper (Seo et al. 2008).

Class II PLA1s were reported to be involved in defense signaling, senescence, and seedling establishment (Chen et al. 2013; Hong et al. 2000; Kim et al. 2011a; Lo et al. 2004). CaPLA1 is closely related with *Arabidopsis* AtDSEL PLA1 with 55 % amino-acid sequence identity. Their cellular roles, however, appeared to be different. AtDSEL contributes to seedling establishment by regulating β-oxidation (Kim et al. 2011a), whereas CaPLA1 is involved in

the broad aspects of growth and development in vegetative and reproductive tissues in transgenic rice plants (Figs. 2, 3, 4, 5). On the other hand, a mitochondrial-localized AtDLAH PLA1 plays a role in *Arabidopsis* seed viability (Seo et al. 2011). Thus, we consider the possibility that an as-yet unidentified lipid molecule(s) produced CaPLA1 plays a selective role in the tissue growth and development in rice.

Enhanced growth and greater biomass accumulation of the transgenic rice plants compared to the wild type was accompanied by effects at the cellular level. Epidermal layers in *Ubi:CaPLA1* leaves had more, but smaller, cells than did the wild-type leaves (Fig. 3a, b). Furthermore, overexpression of *CaPLA1* upregulated the cyclins *CycA2.1*, *CycB1.1* and *CycD3.1* as well as the genes involved in lipid metabolism, *ACX*, *MS* and *PEPCK,* compared to expression in the wild-type plant (Figs. 3c, 6). There are few reports on the relationship between cell proliferation and lipid metabolism in plant systems, but in animal cancer cells, cell-cycle progression is closely related to lipid metabolism (Santos and Schulze 2012; Lluis 2013). For example, in the oncogenic signaling pathways, the rate of de novo lipid biosynthesis is highly increased. Also, sterol regulatory element-binding proteins (SREBPs), which regulate fatty-acid and cholesterol biosynthesis, are overexpressed in a number of cancer cells and are involved in downstream of tumor-suppressor pathways (Eberle et al. 2004; Horton 2002; Menendez and Lupu 2007). Furthermore, mice deficient in cyclin D have increased insulin sensitivity, insensitivity to diet-induced obesity and reduced adipocyte size (Lluis 2013). With these findings in mind, we hypothesized that the accelerated cell-cycle progression that we observed in *CaPLA1*-overexpressing rice may be associated with altered lipid metabolism (Fig. 6). This view is consistent with the microarray data that indicate that gene activities for both cell proliferation and lipid metabolic activity are up-regulated in *CaPLA1*-overexpressing transgenic rice plants (Fig. 7).

The transgenic plants also accumulated more biomass in the reproductive stage. Specifically, they produced more tillers and more grains per panicle than the wild-type plants (Figs. 4, 5), but the grain size was not affected (Supplementary Fig. S1). Increased grain yield in *Ubi:CaPLA1* lines may not, then, be due to effects on the developmental process per se (for example, it may not affect meiosis or embryogenesis) but it may enhance the growth of tillers and panicles. In oil seeds, altered lipid metabolism affects seed size (Fatihi et al. 2013; Liu et al. 2014; van Erp et al. 2014). However, glutelin-abundant rice seeds may be less affected by lipid metabolism-mediated embryogenesis than oil seeds. Further detailed phenotypic analysis of *Ubi:CaPLA1* transgenic seeds may unravel the relationship between CaPLA1 and seed development.

The transgenic rice plants had a reduced membrane peroxidation state, based on their lower levels of MDA and greater levels of CD than the wild-type (Fig. 8a, b). Furthermore, transcript levels of peroxidase and its enzyme activities were upregulated in *CaPLA1*-overexpressing lines (Fig. 8c, d). A negative correlation between the membrane peroxidation state and cell growth has been reported in both plant and animal systems. For example, higher membrane peroxidation state inhibits cell growth and induces apoptosis (Dunand et al. 2007; Gapper and Dolan 2006; Jiang et al. 2005; Lin et al. 2007; Singh et al. 2006; Sundaravelpandian et al. 2013), while a reduced membrane peroxidation state increases cell proliferation in colon carcinoma cells (Jonas et al. 2002, 2003; Jones 2006). Expression levels of *CaPLA1* were consistently inversely correlated with the membrane peroxidation state in this study (Fig. 8). The reduction of membrane peroxidation state may account for the increased cell proliferation that we observed in transgenic rice plants (Fig. 3).

Isoprenoid biosynthetic genes were up-regulated in *Ubi:CaPLA1* rice plants based on the microarray data (Fig. 7). Isoprenoids consist of sterols and terpenoids and play diverse roles in cellular metabolism as antioxidants, membrane structural components, hormones and secondary metabolites (Clouse 2002; Schaller 2003; Umehara et al. 2008). Considerable evidence indicates that isoprenoids participate in cell proliferation. In animal prostate cancer cells, the mevalonate pathway is deregulated and cholesterol levels were abnormally high, indicative of accumulation (Hager et al. 2006). Isoprenoids were recently reported to be required for root development in *Arabidopsis* and oat (Kemen et al. 2014; Van Norman et al. 2014). In *CaPLA1*-overexpressing rice plants, three isoprenoid synthetic genes, *terpenoid synthase*, *HSD* and *HMGR*, were up-regulated (Fig. 9). In higher plants, isoprenoid precursors are produced either by the mevalonate pathway in the cytosol or by the 1-deoxy-D-xylulose-5-phosphate pathway in plastids (Kirby and Keasling 2009). Several reports indicate that isoprenoid precursors are exchanged between the cytosol and plastids (Hampel et al. 2005; Kasahara et al. 2002; Yang and Orihara 2002), suggesting plastid-cytosol interactions of isoprenoids (Bouvier et al. 2005; Nagata et al. 2002). Thus, there is a possibility that increased transcription of isoprenoid biosynthetic genes and HMGR enzyme activity influence total isoprenoid content in *Ubi:CaPLA1* transgenic plants, resulting in enhanced growth.

Microarray data indicate that gene expressions for cell proliferation, lipid metabolic activity, and oxidation/reduction state are significantly affected in *CaPLA1*-overexpressing plants (Fig. 7). However, important lipid metabolic genes, such as *ACX* (Fig. 6a) and *HMGR* (Fig. 9c), of witch transcriptional changes were confirmed

by qRT-PCR (Figs. 6, 9c), were excluded in microarray data. This was probably due to the high *P* values in microarray. It is considered as a noise from technical bias of microarray (Jeong et al. 2011).

At this moment, however, molecular mechanism by which CaPLA1 enhances biomass and grain yield in transgenic rice plants remains to be elucidated. As CaPLA1 is a class II cytosolic phospholipase (Seo et al. 2008), constitutive expression of *CaPLA1* may lead to increase in lipid metabolism. Thus, enhanced growth phenotypes of *Ubi:CaPLA1* rice plants are possibly due to as-yet unidentified free fatty acids, which may act as signaling molecules to increase an overall cellular metabolism. In animal systems, the cytosolic lipase-mediated lipid metabolism regulates mitochondrial function via the control of peroxisome proliferator-activated receptors (PPARs; Haemmerle et al. 2011). PPARs are fatty acid binding transcription factors that play major roles in regulation of lipid metabolism, cellular growth, differentiation, and inflammation at the transcriptional level (Poulsen et al. 2012; Varga et al. 2011). Although higher plants lack PPAR homologs, an existence of peroxisome proliferation signaling was reported in *Arabidopsis* (Castillo et al. 2008; León 2008). We speculate that changes in global gene expression profiles including β-oxidation enzymes (Figs. 6, 7), cellular redox states (Fig. 8a, b), and peroxidase activity (Fig. 8c, d) in *CaPLA1*-overexpressing transgenic rice plants may increase mitochondrial and peroxisomal activities that are regulated by free fatty acids produced by cytosolic phospholipase CaPLA1. Further experiments should identify the in vivo substrate of CaPLA1 and products and should provide further insight into the possible functional link between cell proliferation and lipid signaling. In conclusion, our data suggest that constitutive expression of *CaPLA1* and its enzyme activity conferred enhanced growth and increased grain yield in transgenic rice plants through alterations of gene activities related to cell proliferation, lipid metabolism, membrane peroxidation state, and isoprenoid biosynthesis.

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Author contributions KY.P., E.Y.K., and Y.S.S. performed the experiments. K.Y.P., E.Y.K., Y.S.S., and W.T.K. analyzed the data. K.Y.P and W.T.K. planned the project and wrote the article.

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