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Research article

Exogenous proline has favorable effects on growth and browning suppression in rice but not in tobacco

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ABSTRACT

Proline is one of the amino acids that compose proteins and has various roles under non-stress and stress conditions. In this study, we investigated the effect of proline on the growth and browning of two plants, tobacco and rice, by exogenous application and endogenous increase of proline. Exogenous proline had a different effect on the growth and browning between tobacco and rice: proline affected negatively the growth of tobacco seedlings and favorably that of rice seedlings. In addition, proline prevented browning only in rice cultured cells, consistent with the increase of proline contents, but not in tobacco BY-2 cells. These results might be due to the difference of exogenous proline uptake activity in these cells. From the Lineweaver-Burk plots, proline inhibited polyphenol oxidase activity in vitro, which is a major factor of enzymatic browning in plants, by affecting the enzyme-substrate complex. Proline could suppress the browning of the plant callus by inhibition of PPO activity.

1. Introduction

Proline is an amino acid which compose proteins and widely distributed in microbes, animals, and plants (Fichman et al., 2015). In plants, proline acts as an osmolyte, antioxidant, metal chelator, and signaling molecule under various stress conditions (Hayat et al., 2012). In particular, proline is actively synthesized and highly accumulated under stress conditions, such as high salt (Wang et al., 2015), drought (Bandurska et al., 2017), ultra violet (Saradhi et al., 1995), and heavy meal (Schat et al., 1997). Proline is also involved in plant growth and development (Mattioli et al., 2009a; Szabados and Savoure, 2010). Proline contents are extremely different among plant organs. Schwacke et al. (1999) reported that proline is highly accumulated in the tomato flower, especially in pollen, compared with leaf, root, and fruit, suggesting that proline is involved in the development of reproductive organs. It is also suggested that proline is involved in cell wall synthesis (Kavi Kishor et al., 2015), seed development (Szekely et al., 2008), and regulation of cell cycle (Mattioli et al., 2009b; Wang et al., 2014). Proline is mainly synthesized by the glutamate pathway. In this pathway, Δ^1-pyrroline-5-carboxylate (P5C) synthetase (P5CS) converts glutamate to P5C, and P5C reductase (P5CR) then converts P5C to proline in the cytosol and chloroplast (Lehmann et al., 2010). Proline metabolism occurs in mitochondria, where the amino acid is metabolized to glutamate via P5C by proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH). The separation of proline synthesis and metabolism is insured by intracellular proline transport molecules in cells. Because of the protective role of proline under stress condition, the enhancement of proline contents, either by overexpression of biosynthesis enzymes or by suppression of metabolism enzymes as well as by exogenous proline application, presents higher stress tolerance in some plant species (Guan et al., 2018; Saradhi et al., 1995; Siripornadulsil et al., 2002; Tateishi et al., 2005; Wutipraditkul et al., 2015). However, exogenous proline application can result in poor growth in some plant species (Hayat et al., 2012). Therefore, the functions of proline in plants are still unclear.

We have previously reported that the RNAi suppression of pdh in tobacco BY-2 cells showed not only higher proline contents but also diminished the appearance of browning compared with wild type (Tateishi et al., 2005). Other reports show that proline application also suppressed the browning in *Miscanthus* callus (Glowacka et al., 2010; Holme et al., 1997; Takahashi et al., 2017). Browning is often observed in food during processing and storage where it is categorized into either enzymatic or non-enzymatic reactions (Zhang et al., 2015). In plants, browning is mainly caused by enzymatic reaction. In enzymatic browning, phenolic compounds are oxidized to quinones by polyphenol oxidase (EC 1.10.3.1; PPO) and peroxidase (EC 1.11.1.7), and quinones

Abbreviations: MDA, malondialdehyde; MS, Murashige and Skoog; PDH, proline dehydrogenase; PPO, polyphenol oxidase; P5C, Δ^1-pyrroline-5-carboxylate; P5CDH, P5C dehydrogenase; P5CR, P5C reductase; P5CS, P5C synthetase

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spontaneously polymerize to produce dark colored pigments (Tinello and Lante, 2018; Wu and Lin, 2002). PPO is a copper containing enzyme and is widely distributed among plant species (Sullivan, 2015). PPO is normally localized in chloroplasts. However, some processes such as senescence, wounding, pathogens, and mechanical stresses abolish the sub-cellular compartmentalization, allowing PPO to contact with vacuolar phenolic compounds (Taranto et al., 2017). The inhibition of PPO activity results in less browning in potato and apple puree (Sukhonthara et al., 2016).

In this study, we assessed the effect of endogenous enhancement and exogenous application of proline on callus browning. As proline application has different effects on plant growth depending on the plant species, we compared the effect of proline between tobacco and rice. In addition, we also evaluated the effect of proline on PPO activity in vitro.

2. Materials and methods

2.1. Plant materials and culture media

Tobacco seeds (*Nicotiana tabacum* BY-4) were sterilized with diluted commercial bleach containing 3% chlorine for 15 min, then washed with sterilized distilled water. The surface-sterilized tobacco seeds were aseptically germinated on a half-strength Murashige and Skoog (MS) medium containing 1.5% (w/v) sucrose and 0.2% (w/v) gellan gum. The seeds were cultured at 23 °C under 16-h light and 8-h dark conditions for 15 days. The germinated seedlings were transferred to a new half-strength MS medium containing different concentrations of proline (0, 0.5, 1 or 5 mM) and were cultured in the same conditions for 14 days, then the fresh weight of whole seedlings was measured.

Rice seeds (*Oryza Sativa* L ssp Japonica cv Nipponbare) were sterilized with diluted commercial bleach containing 3% chlorine for 30 min, then washed with sterilized distilled water. The surface-sterilized rice seeds were placed on a nylon net floating on water and grown in hydroponic culture at 28 °C in 16-h light and 8-h dark conditions, and tap water was replaced every three days. Eight days after germination, the rice seedlings were treated with different concentrations of proline (0, 3, 9, or 27 mM) for 5 days, then root and shoot length were measured.

2.2. Tobacco BY-2 cells and culture

Tobacco BY-2 cells (*Nicotiana tabacum* cv. Bright Yellow 2) were cultured in a MS medium containing 3% (w/v) sucrose, 0.9 μM 2,4-dichlorophenoxyacetic acid, 0.56 mM *myo*-inositol, and 3 μM thiamine hydrochloride as described previously (Suekawa et al., 2016). For proline treatment, tobacco BY-2 cells were subcultured with different concentrations of proline (0.125, 0.25, 0.5, 1, 2.5, and 5 mM) at 25 °C for 15 days on a rotary shaker at 125 rpm in the dark before measuring proline content. Control cells were subcultured without proline.

2.3. Callus induction and transfection of rice

For callus induction, rice seeds were dehusked and sterilized with 70% (w/v) ethanol for 30 s and diluted commercial bleach containing 3% chlorine for 25 min. The seeds were further washed with sterilized distilled water. The surface-sterilized seeds were placed on a solid N6D medium, which is a modified N6 medium (Kaushal et al., 2014) containing 0.0002% (w/v) glycine, 0.00005% (w/v) nicotinic acid, 0.00005% (w/v) pyridoxine hydrochloride, 0.0001% (w/v) thiamine hydrochloride, 0.001% (w/v) *myo*-inositol, 0.003% (w/v) casamino acid, 0.03% (w/v) L-proline, 0.0002% (w/v) 2,4-dichlorophenoxyacetic acid, 3% (w/v) sucrose and 0.2% (w/v) gelrite (Duchefa Biochemie, Haarlem, Netherlands), at pH 5.8, and cultured at 28 °C for two weeks. The resulting callus was maintained by subculturing part of the callus on a fresh solid N6D medium every month.

The plant binary Ti plasmid vector pGWB2 (Nakagawa et al., 2007) was used to transform the subcultured rice callus. The open reading frame of rice P5CS (Gene Bank Acc. No. AK102633) and P5CR (Gene Bank Acc. No. AK067368) were amplified by PCR using plasmids harboring the full length rice P5CS (clone ID: J033099M14) and P5CR (clone ID: J013104L18), which were obtained from the Rice Genome Resource Center (Tsukuba, Japan). The products of PCR were then cloned into a pDONR221 vector (Invitrogen) followed by cloning into the pGWB2 vector with the Gateway cloning system (Invitrogen). The created expression vectors were designated as pGWB2_P5CS and pGWB2_P5CR, respectively. *Agrobacterium tumefaciens* (strain EHA101) carrying pGWB2_P5CR and pGWB2_P5CS vectors were used to transform rice. *A. tumefaciens* were grown at 28 °C in a liquid YEB medium containing 0.5% (w/v) beef extract, 0.5% (w/v) polypepton, 0.1% (w/v) yeast extract, 0.5% (w/v) sucrose, 0.1% (w/v) $MgSO_4$, 50 mg/L kanamycin, and 50 mg/L hygromycin, to mid-log phase. The culture was collected and the pellet was resuspended in AAM medium (Toki et al., 2006) and co-cultured with rice calli for 3 days in the dark. Then the inoculated calli were washed well with sterilized distilled water containing 500 mg/L carbenicillin and were cultured on solid N6D medium containing 500 mg/L carbenicillin and 25 mg/L hygromycin at 28 °C for one week. The resulting calli were maintained by subculturing part of the callus on a fresh solid N6D medium containing 500 mg/L carbenicillin and 25 mg/L hygromycin every month. Resistant calli were further transferred into liquid R2S selection medium containing R2 (Ohira et al., 1973), 0.02% (w/v) *myo*-inositol, 0.0002% (w/v) nicotinic acid, 0.0001% (w/v) pyridoxine hydrochloride, 0.002% (w/v) thiamine hydrochloride, and 25 mg/L hygromycin to initiate suspension cultures on a rotary shaker at 28 °C in the dark. The calli were maintained in a fresh liquid R2S medium by subculturing every 2 weeks.

2.4. RNA extraction

Total RNA was isolated from rice calli using the Aurum™ Total RNA Mini Kit (BioRad) according to manufacturer's instructions. Rice calli in liquid culture media were collected by vacuum filtration at 7 days after subculture. After the cells were powdered in liquid nitrogen, 100 mg of the resulting powder was used for RNA extraction. The total RNA solution was stored at −80 °C until use.

2.5. Preparation of digoxigenin-labeled cRNA probes

The digoxigenin (DIG)-labeled cRNA probe was prepared with a DIG RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland). The plasmids harboring full length of rice P5CR and P5CS were linearized by *Bgl*II and purified by phenol chloroform extraction. DIG-labeling cRNA probe was produced by in vitro transcription using T7 RNA polymerase and the purified DNA as a template in the presence of DIG-11-dUTP, according to the manufacturer's instructions.

2.6. Northern blot analysis

To investigate the expression levels of rice P5CR and P5CS mRNA in rice calli, northern blot analysis was performed according to the method as described previously (Suekawa et al., 2016), with little modification. In detail, the total RNA (2.5 μg) extracted from rice calli were separated on a 1% (w/v) agarose gel containing formaldehyde and used for northern blot analysis. For hybridization, the RNA transferred nylon membrane was incubated at 63 °C overnight with the DIG-labeled cRNA probes. After hybridization, the membrane was washed with 2 × SSC and 0.1% SDS for 15 min and then six times with 0.2 × SSC and 0.1% SDS for 5 min at 68 °C. After brief wash in buffer 1 (0.1 M maleic acid, pH 7.5; 0.15 M NaCl), the membrane was blocked with 1% blocking reagent (Roche Diagnostics) in buffer 1 for 60 min. The blocked membrane was then incubated with anti-DIG-AP fragments (1:10,000 (v/v); Roche Diagnostics) for 60 min and was washed six

times in buffer 1 containing 0.3% Tween 20 for 5 min. After equilibration in buffer 2 (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl), the membrane was incubated in buffer 2 containing CDP-Star (Roche Diagnostics) for 5 min before exposure to X-ray film (Fujifilm, Tokyo, Japan).

2.7. Determination of proline content

The proline contents of rice tissues and callus extracts were determined according to the method described as previously (Tateishi et al., 2005), with little modification. Rice tissues and calli were grind in liquid nitrogen. The powdered tissue (100 mg) was homogenized in 500 μL of 3% aqueous sulfosalicylic acid and centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant (100 μL) was collected in a new tube and mixed with 100 μL of acid-ninhydrin and glacial acetic acid, then incubated for 1 h at 100 °C. After cooling, the reaction mixture was extracted with 200 μL of toluene and the absorbance was measured at 520 nm. Proline content was calculated using a standard curve.

2.8. Determination of polyphenol oxidase activities

The Laccases (EC 1.10.3.2), which is one of polyphenol oxidase (PPO) (Sullivan, 2015), was used for determination of PPO activity with catechol as a substrate. Commercial laccase Y120 from *Trametes* sp. was kindly supplied by Amano Enzyme JAPAN Co., Ltd and stock laccase solution was prepared in Mcllvaine buffer (pH 5.0). The reaction was initiated by the addition of 100 μL of 0.2 mg/mL laccase solution to 400 μL of Mcllvaine buffer (pH 5.0) in the presence of different concentrations of catechol (0.4, 0.6, 0.8, or 1 mM) with or without proline (0, 20, 22.5, or 25 mM), and the change in the absorbance at 420 nm was monitored for 6 min. PPO activities were determined from the slope of the resulting kinetic curve and were expressed as initial reaction velocity (V_0). The experiments were repeated three times.

2.9. Determination of polyphenol content

For the determination of the polyphenol contents, rice calli were cultured with 4 mM or without proline for 14 days, then collected by vacuum filtration and powdered in liquid nitrogen. The resulting powder (100 mg) was homogenized in 1 mL of dimethyl sulfoxide and incubated for 15 min at room temperature. After centrifugation at 12,000 × g for 1 min, the supernatant was collected in a new tube and mixed with the same amount of 20% (v/v) Folin-Ciocalteu reagent. After incubation for 3 min at room temperature, 10% (w/v) sodium carbonate was added and incubated for 1 h at room temperature. The resulting solution was used to measure the absorbance at 760 nm. The polyphenol contents were calculated using a standard curve by catechin.

2.10. Determination of malondialdehyde content

For the determination of the malondialdehyde (MDA) contents, rice calli were cultured with 4 mM or without proline for 14 days, then collected by vacuum filtration and powdered in liquid nitrogen. The resulting powder (100 mg) was homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant was collected in a new tube and mixed with the same amount of reaction solution containing 0.5% (w/v) thiobarbituric acid and 20% (w/v) trichloroacetic acid. After incubation for 30 min at 95 °C, the reaction was stopped by quick cooling on ice and the tubes centrifuged at 10,000 × g for 10 min at room temperature. The supernatant was used to measure the absorbance at 532 nm and 600 nm. The MDA contents were calculated using a molar extinction coefficient for MDA of $1.55 \times 10^5\ cm^{-1}M^{-1}$.

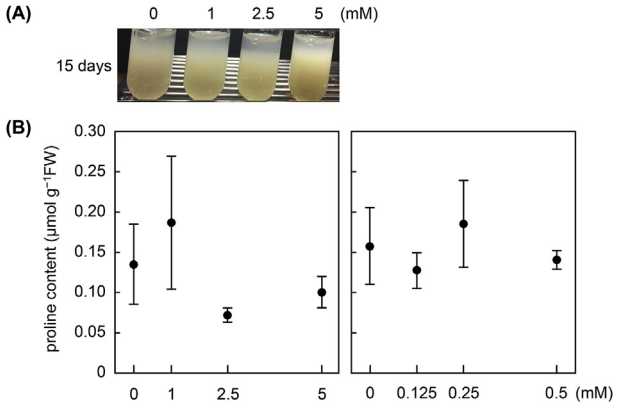

Fig. 1. Effect of exogenous proline on browning and proline contents in tobacco BY-2 cells.
(A) Tobacco BY-2 cells were cultured in MS medium with different concentrations (0, 1, 2.5, and 5 mM) of proline for 15 days. (B) Tobacco BY-2 cells were cultured with high concentrations (0, 1, 2.5, and 5 mM; left panel) or low concentrations (0, 0.125, 0.25, and 0.5 mM; right panel) of proline for 8 days, then proline contents were measured in the BY-2 cells. Bar represents means ± SE (n = 3). Data were analyzed using a *t*-test.

2.11. Statistical analyses

Data points represent the mean of three replications. For comparison of the pairs of means, data were analyzed using a *t*-test and significant differences were calculated ($p < 0.05$ and 0.01). For comparison of the group of means each other, data were analyzed using one-way analysis of variance (ANOVA) and Ryan's method. Significant differences were calculated ($p < 0.05$ and 0.01).

3. Results

3.1. Effect of exogenous proline on the browning and proline contents of tobacco BY-2 cells

To investigate the effect of exogenous proline on the browning of tobacco BY-2 cells in suspension culture, we treated tobacco BY-2 cells with different concentrations of proline for 15 days. However, despite the presence of proline, browning of tobacco BY-2 cells in suspension culture was observed (Fig. 1A). There were no differences in appearance between the proline treated cells at 5 mM and untreated cells at 15 days of culture. The proline treated cells at lower concentrations (1 and 2.5 mM) appeared to have less browning compared to untreated cells, although the differences were unobvious. We next measured the proline contents of tobacco BY-2 cells treated with 0, 1, 2.5 and 5 mM proline at 8 days of culture. As a result, proline contents were not increased by proline treatments and tended to be decreased with more than 2.5 mM proline application (Fig. 1B). We also evaluated the effect of proline application at low levels (0.125, 0.25, and 0.5 mM) on the proline contents of tobacco BY-2 cells. However, there were no differences in the proline contents between proline treated and untreated cells at 8 days of cell culture (Fig. 1B).

3.2. Effect of exogenous proline on the growth of tobacco and rice seedlings

We evaluated the effect of exogenous proline on the growth of rice as well as tobacco. Tobacco and rice seedlings were treated with different concentrations of proline. In tobacco, the fresh weights of seedlings were significantly decreased already with 0.5 mM proline (Fig. 2A). Further fresh weight decreases were observed with 1 mM and 5 mM proline treatment. The tobacco seedlings treated with proline had small leaves with slight chlorosis compared with untreated seedling. On the other hand, the treatments with 9 mM and 27 mM proline improved

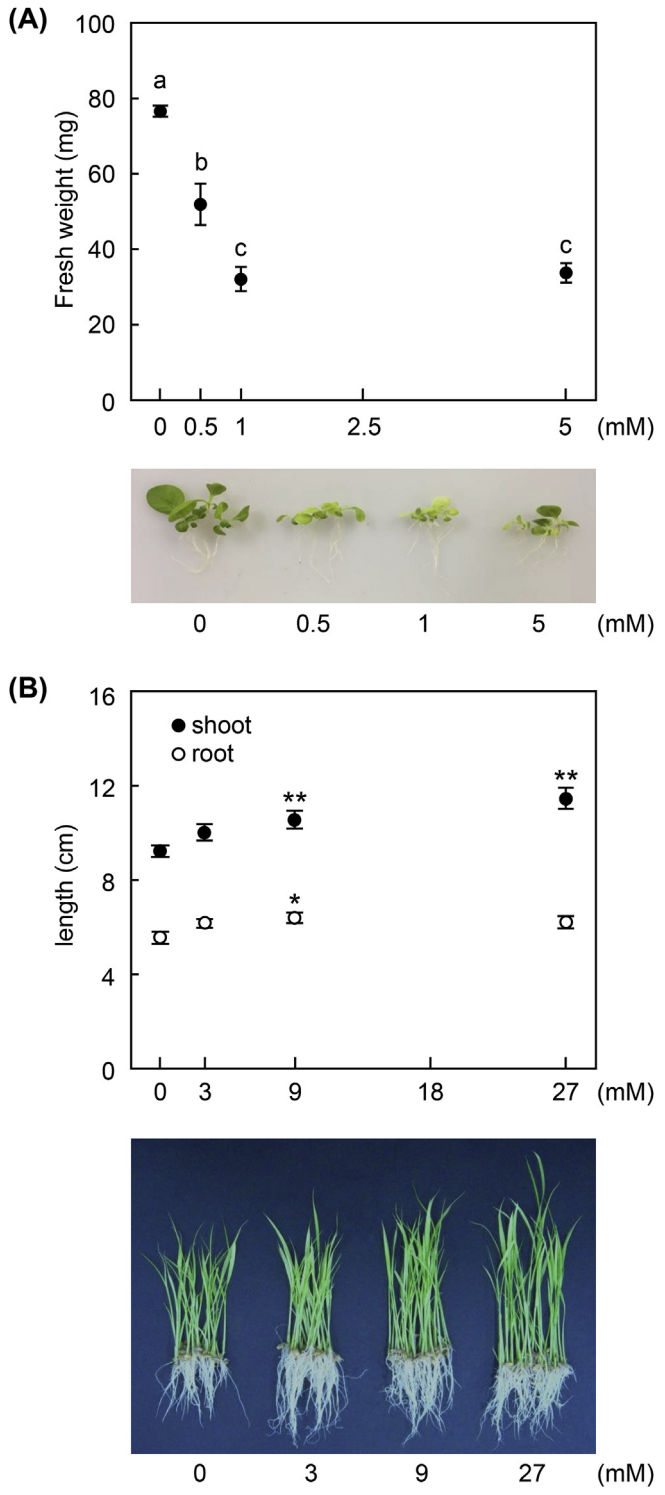

Fig. 2. Effect of exogenous proline on growth of tobacco BY-4 and rice seedlings.

(A) Tobacco BY-4 seedlings were germinated on a half-strength of MS medium with different concentrations (0, 0.5, 1 or 5 mM) of proline. The seeds were cultured at 23 °C under 16-h light and 8-h dark conditions for 14 days (lower panel). The fresh weight of each seedling was measured (upper panel). Bar represents means ± SE (n = 3). Data were analyzed using ANOVA and Ryan's method. Datasets with the same letter indicate no significant differences ($p < 0.05$). (B) Rice seedlings at 8 days after germination were treated with 0, 3, 9, or 27 mM proline at 28 °C under 16-h light and 8-h dark conditions for 5 days (lower panel). The root and shoot length were measured (upper panel). Bar represents means ± SE (n = 3). Data were analyzed using the *t*-test. Single and double asterisks indicate a statistical difference from the untreated (0 mM proline) seedlings ($p < 0.05$ and 0.01, respectively).

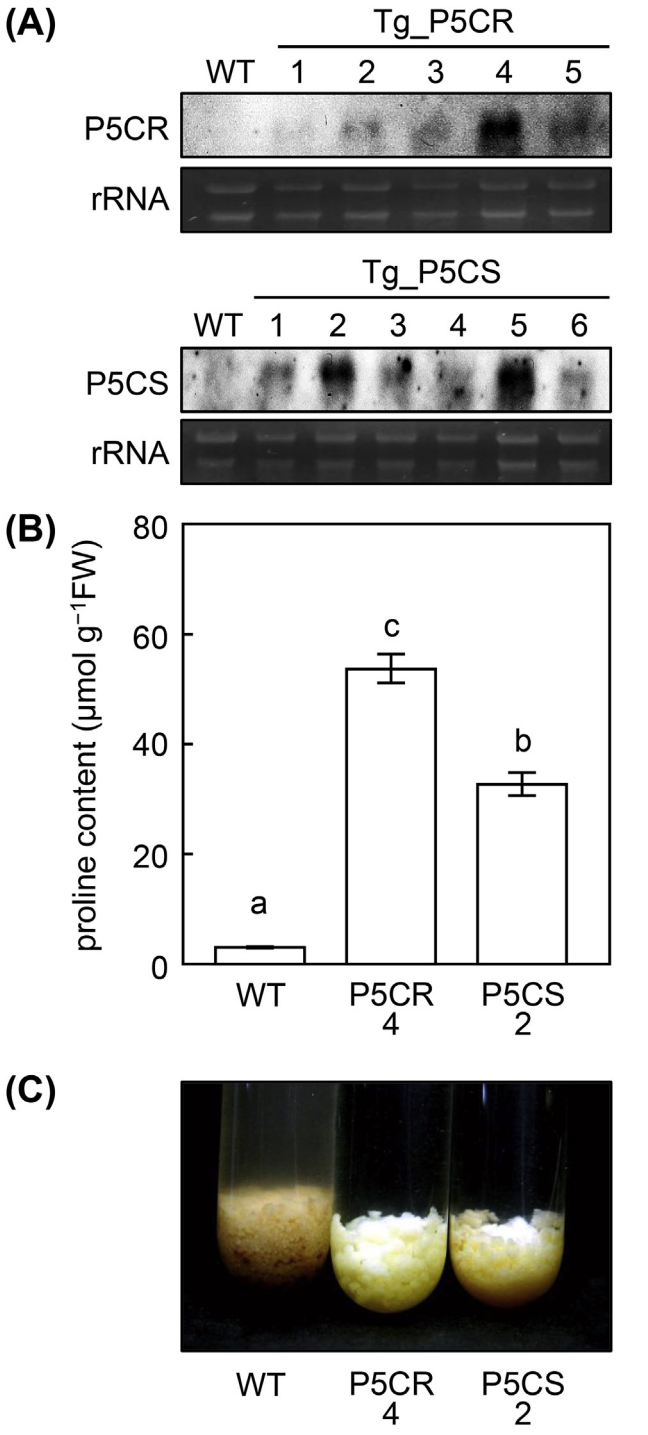

Fig. 3. Proline contents and suppression of browning in transgenic rice cultured cells overexpressing proline biosynthesis enzymes.

Rice cultured cells were transfected with plant gene expression vectors expressing rice P5CS and P5CR. (A) Total RNA was extracted from wild type (WT) and transgenic (Tg) cell lines of P5CR (Tg_P5CR 1, 2, 3, 4, and 5) and P5CS (Tg_P5CS 1, 2, 3, 4, 5, and 6), and analyzed by northern blot analysis with rice P5CR (upper panel) and P5CS (lower panel) cRNA probes. Total RNA was stained with ethidium bromide (rRNA). (B) WT and Tg cell lines overexpressing P5CR (P5CR 4) and P5CS (P5CS 2) were cultured for 25 days and the proline contents were measured. Bar represents means ± SE (n = 3). Data were analyzed using ANOVA and Ryan's method. Datasets with the same letter indicate no significant differences ($p < 0.01$). (C) Photograph of WT and Tg cell lines (P5CR 4 and P5CS 2) cultured for 25 days.

root and shoot length of rice seedlings compared with untreated seedlings, and seemed to induce better growth of seedlings in a concentration-dependent manner (Fig. 2B).

3.3. Effect of enhancement of endogenous proline contents on the browning of transgenic rice cultured cells

To investigate whether higher endogenous proline contents suppress the browning of rice cultured cells, we constructed transgenic cell lines overexpressing the genes of the proline biosynthesis enzymes, P5CR and P5CS. The mRNA expressions of transgenes were analyzed by northern blot analysis. High transgene expressions were observed in the transgenic cell line #4 overexpressing P5CR, and the transgenic cell lines #2 and #5 overexpressing P5CS (Fig. 3A). We measured the proline contents in transgenic cell lines P5CR#4 and P5CS#2 after 25 days of culture and found that P5CR#4 and P5CS#2 exhibited a significantly increase in proline contents up to 18- and 11-fold compared with wild-type (WT), respectively (Fig. 3B). As shown in Fig. 3C, the browning of the transgenic cells was significantly suppressed, compared with WT.

3.4. Effect of exogenous proline on the browning in rice cultured cells

To investigate the effect of exogenous proline on the browning of rice cultured cells, we treated rice cultured cells with different concentrations of proline for 30 days, and then the proline contents were measured. As a result, the rice cultured cells treated with 5, 10, and 15 mM proline contained 117.1, 284.7, and 363.0 μmol g^{-1} fresh weight (FW) of proline, respectively, whereas the control contained 3.1 μmol g^{-1} FW of proline (Fig. 4A). The proline contents in rice cultured cells were dramatically increased by proline application in a concentration-dependent manner. Also, as shown in Fig. 4A, the browning of cells was suppressed in rice cultured cells treated with all concentrations of proline compared with control cells.

3.5. In vitro inhibition of PPO activity by proline

We investigated the inhibition effect of proline on PPO activity that is responsible for the browning in plants by the oxidation of phenolic compounds. We used laccases and catechol as a PPO and a substrate, respectively. As shown in Fig. 5, the Michaelis constant (K_m) and maximum velocity (V_{max}) of PPO without proline were 0.51 mM and 0.037 min^{-1}, respectively. The application of 20 mM or 22.5 mM proline had a small inhibitory effect on PPO activities with the decrease of K_m and V_{max}. PPO activity was significantly inhibited by 25 mM proline, with a K_m of 0.27 mM and a V_{max} of 0.024 min^{-1}. The regression lines of Lineweaver-Burk plots with different concentrations of proline were almost parallel, suggesting that proline interacts with the enzyme-substrate complex and inhibits enzymatic activity.

3.6. Effect of exogenous proline on the polyphenol and MDA contents in rice cultured cells

We investigated the effect of exogenous proline on the polyphenol contents that are substrates of PPO. We treated rice cultured cells with 4 mM proline for 14 days. The proline treated cells showed increased proline contents (Fig. S1A), but no difference of polyphenol contents (Fig. S1B) compared to untreated cells. Also, we measured MDA contents that reflect the lipid peroxidation levels and found that no difference in MDA contents between proline treated and untreated cells (Fig. S1C).

4. Discussion

Proline is a multi-functional amino acid involved in growth and stress tolerance in plants. In our previous study, tobacco BY-2 cells were hypersensitive to exogenously applied proline (Tateishi et al., 2005). In this study, we demonstrated that proline application, even at low concentration (0.5 mM), severely affected the growth of tobacco seedlings (Fig. 2A). Excess cytosolic proline is transported into the

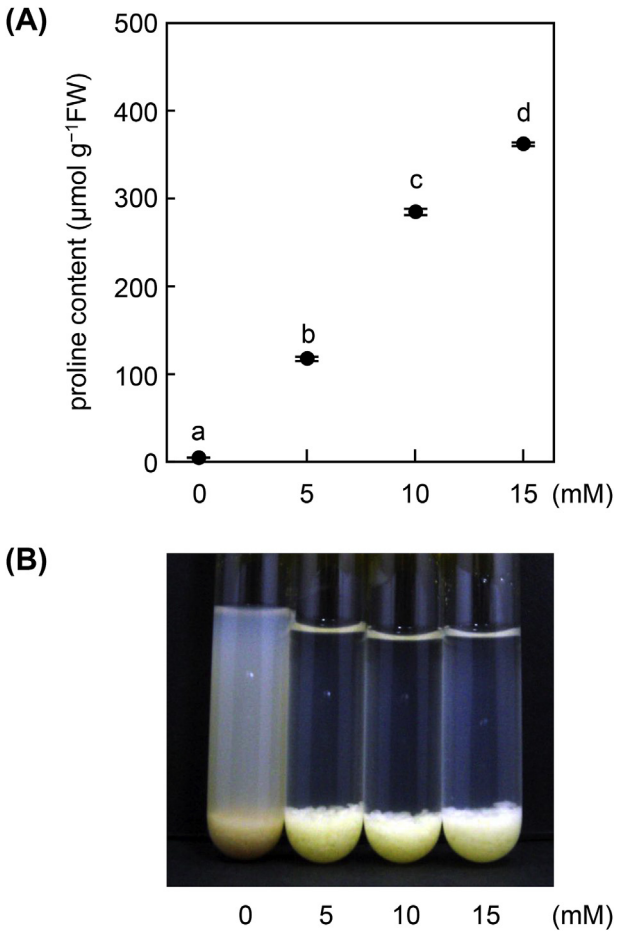

Fig. 4. Effect of exogenous proline on the proline contents and browning of rice cultured cells.
Rice cells were cultured with different concentrations (0, 5, 10, and 15 mM) of proline for 30 days. (A) Proline contents were measured. Bar represents means ± SE (n = 3). Data were analyzed using ANOVA and Ryan's method. Datasets with the same letter indicate no significant differences ($p < 0.01$). (B) Photograph of rice cultured cells.

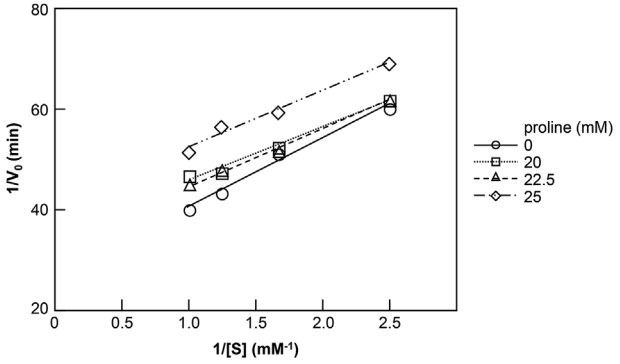

Fig. 5. Lineweaver-Burk plot of PPO activity with proline.
PPO activities against catechol (0.4, 0.6, 0.8, or 1.0 mM) were examined using laccase Y120 (40 μg/mL) in a reaction containing different concentrations (0, 20, 22.5, or 25 mM) of proline. The increase of absorbance per minute as a measure for enzyme activity was plotted in a Lineweaver-Burk diagram. The experiments were repeated three times.

mitochondrion and metabolized to P5C. When the conversion of P5C to glutamine stagnates, P5C is transported to the cytosol and converted back into proline, composing the proline-P5C cycle. The proline-P5C cycle generates reactive oxygen species, which cause oxidative stress in cells (Miller et al., 2009). For this reason, proline application could have a negative effect on the growth of tobacco seedlings. In fact, despite of its function in plant growth, exogenous application of proline has been reported to have an unfavorable effect on growth of some plant species under non-stress condition (Hayat et al., 2012). Rice is frequently studied for the effect of exogenous proline under non-stress and stress conditions (Nounjan et al., 2012; Pawar et al., 2015; Teh et al., 2016; Yu et al., 2017). In this study, the root and shoot growth of rice seedlings tended to be improved by proline application at high concentration (Fig. 2B), suggesting that proline application has different effects on growth depending on the plant species. In Fig. 4A, the exogenous application of proline dramatically increased the proline contents of rice cultured cells, in contrast to tobacco BY-2 cells (Fig. 1B). These results suggest that exogenous proline uptake into cells might differ between tobacco and rice. Plant have proline transporters with various proline affinities (Schwacke et al., 1999; Ueda et al., 2008; Yamada et al., 2011). Exogenous proline also serves as nitrogen source (Santos et al., 1997). In addition, (Teh et al. 2016, 2019) reported that proline application increased the number of roots in rice seedlings and could alter the gene expression of proteins involved in the regulation of reactive oxygen species, stress and defense response, and energy metabolism. The favorable effect of exogenous proline on the growth of rice seedlings observed in this study might be due to high proline uptake activity of rice, and the additional exogenous proline may improve the growth of rice seedlings by the increase of the number of roots and change of the gene expressions.

Proline application suppressed the browning of *Miscanthus* callus (Glowacka et al., 2010; Holme et al., 1997; Takahashi et al., 2017). We have been reported that the pdh suppressed transgenic tobacco BY-2 cells showed less browning appearance than WT cells (Tateishi et al., 2005). In this study, the obvious suppression of browning was not observed in proline treated tobacco BY-2 cells (Fig. 1A), consistent with no increase of proline contents in cells (Fig. 1B). On the other hand, the increase of proline in transgenic rice cell lines overexpressing the proline biosynthesis genes, P5CR and P5CS (Fig. 3B), suppressed the browning of rice cultured cells (Fig. 3C). In addition, proline application also resulted in suppression of browning in rice cultured cells (Fig. 4B), accompanied by the increase of proline contents (Fig. 4A). These results indicate that cell accumulation of proline is effective for suppression of callus browning. Considering that the suppression of browning was already observed in the transgenic rice cell line P5CS#2, which had lower proline content than P5CR#4 (Fig. 3B), it can be assumed that about 30 μmol g^{-1} FW of proline in rice cultured cells may be enough to suppress browning. Then, the exogenous application and endogenous enhancement of proline suppressed the browning of rice cultured cells (Figs. 3C and 4B), whereas only endogenous enhancement suppressed the browning of tobacco BY-2 cells (Tateishi et al., 2005). Some reasons could be conceivable for the different effects of exogenous and endogenous proline on tobacco and rice. One of reasons might be the difference in proline uptake ability between tobacco and rice. Tobacco might be not able to intake enough amount of exogenous proline for suppression of browning. Another reason might be the difference in acceptable amount of proline accumulation between tobacco and rice. The WTs of rice cultured cells (Figs. 3B and 4A) and tobacco BY-2 cells (Fig. 1B) had approximately 3 and 0.15 μmol g^{-1}FW of proline, respectively. In addition, the proline contents in transgenic rice cultured cells were 18- and 11-fold higher than WT (Fig. 3B), although those of transgenic tobacco BY-2 cells were only several times as high as WT (Tateishi et al., 2005). These results suggest that tobacco could only accumulate proline at low level and could not be responsive to the exogenous proline application compared with rice (Figs. 3B and 4A).

In plants, browning results from oxidation of phenolic compounds by polyphenol oxidase and peroxidase (Tinello and Lante, 2018; Wu and Lin, 2002). In terms of preventing foods from browning, suppression of enzymatic browning methods have been studied extensively. Sodium chloride is a well-known inhibitor of PPO as it acts by chelating copper needed for enzyme activity. It was reported that glutamic acid also showed an inhibitory effect on PPO (Dogan et al., 2007). Ozturk and Demir (2002) reported that PPO activity in the extract of spinach leaves was inhibited by proline application in vitro, whereas PPO activity in the *Spinach olerecea* leaves treated with proline was not decreased, rather increased, compared to untreated leaves. In this study, proline appeared to have an uncompetitive inhibitory effect on PPO activity in vitro (Fig. 5). While a little inhibitory effect was observed with 20 mM or 22.5 mM proline, substantial effect was detected with 25 mM proline, suggesting that the inhibitory effect of proline rapidly increases around 22.5 mM–25 mM. It would be difficult to evaluate the in vivo effect of proline on PPO activity, although it would be interesting in the different effects of proline on PPO activity between in vivo and in vitro.

Sun et al. (2011) reported that the MDA contents in litchi pericarp were increased during postharvest preservation along with enzymatic browning. It was reported that exogenous proline enhanced the activity of enzymes involved in the ascorbate-glutathione cycle (Hoque et al., 2007), suggesting its involvement in the improvement of the antioxidant ability of cells. In our study, exogenous proline didn't affect the MDA contents in rice cultured cells (Fig. S1C). Similarly, no difference in polyphenol contents that were substrate of PPO was observed between proline treated and untreated rice cultured cells (Fig. S1B). These results suggest that the lower browning of rice cultured cells after proline application is due to the inhibition of PPO activities rather than suppression of intracellular oxidation and polyphenol compound accumulation.

In conclusion, proline has different effects on plant growth among species. Favorable or unfavorable effects might result from different proline uptake abilities. Proline has an inhibitory effect on PPO activity by affecting the enzyme-substrate complex and suppressed the browning in rice cultured cells. Being a compound with a slightly sweet but not unpleasant taste (Schiffman and Dackis, 1975), proline could be an useful and effective food browning preventing agent on food system and food preservation.

Disclosure statement

No potential conflict of interest was reported by the authors.

Author contribution

M. Suekawa performed the experiments and wrote the paper. Y. Fujikawa and M. Esaka designed research and gave the advice of paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.032.

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