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RESEARCH ARTICLE

Enzymological mechanism for the regulation of lanthanum chloride on flavonoid synthesis of soybean seedlings under enhanced ultraviolet-B radiation

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Abstract In order to probe into the enzymological mechanism for the regulation of lanthanum chloride ($LaCl_3$) on flavonoid synthesis in plants under enhanced ultraviolet-B (UV-B) radiation, the effects of $LaCl_3$ (20 and 60 mg l^{-1}) on the content of flavonoids as well as the activities of phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate:coenzyme A ligase (4CL), and chalcone synthase (CHS) in soybean seedlings under enhanced UV-B radiation (2.6 and 6.2 kJ m^{-2} day^{-1}) were investigated. Enhanced UV-B radiation (2.6 and 6.2 kJ m^{-2} day^{-1}) caused the increase in the content of flavonoids as well as the activities of PAL, C4H, 4CL, and CHS in soybean seedlings. The treatment of 20 mg l^{-1} $LaCl_3$ also efficiently increased these indices, which promoted the flavonoid synthesis and provided protective effects for resisting enhanced UV-B radiation. On the contrary, the treatment of 60 mg l^{-1} $LaCl_3$ decreased the content of flavonoids as well as the activities of C4H, 4CL, and CHS in soybean seedlings except increasing the activity of PAL, which were not beneficial to the flavonoid synthesis and provided negative effects for resisting enhanced UV-B radiation. In conclusion, enhanced UV-B radiation caused the increase in the flavonoid synthesis by promoting the activities of PAL, C4H, 4CL, and CHS in soybean seedlings. The treatment of $LaCl_3$ could change flavonoid synthesis in soybean seedlings under enhanced UV-B radiation by regulating the activities of PAL, C4H, 4CL, and CHS, which is an enzymological mechanism for the regulation of $LaCl_3$ on flavonoid synthesis in plants under enhanced UV-B radiation.

Keywords UV-B radiation · Lanthanum · Soybean seedlings · Flavonoid synthesis · Enzymatic activity · Phenylalanine ammonia-lyase

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Introduction

Due to human activities, large amounts of chlorofluorocarbon compounds have been released into the atmosphere. The long-term retention of such chlorofluorocarbon in the atmosphere has catalytically destroyed the stratospheric ozone layer and thus resulted in higher levels of ultraviolet-B (UV-B) radiation reaching the earth's surface (Butchart and Scaife 2001; McKenzie et al. 2011). Statistically, the concentration of mid-latitude stratospheric ozone in winter and spring decreases by 6 % every decade, and in summer and autumn, it decreases by 3 % every decade (Tang et al. 2002). It has reported that 1 % depletion of ozone is expected to cause ~2 % increase in UV-B intensity (Guarnieri et al. 2004; Kane 2002; McKenzie et al. 2011). Therefore, the depletion of stratospheric ozone has led to the 12 % increase in the level of UV-B radiation in winter and spring, as well as 6 % increase in the level of UV-B radiation in summer and autumn. It has been reported that enhanced UV-B radiation are responsible for multiple biologically harmful effects in plants, such as DNA damage, membrane changes, protein denaturation (Zlatev et al. 2012), because some macromolecules, such as DNA and proteins in plants, can absorb ultraviolet light

(Caldwell et al. 1998), which commonly causes the decreases in chlorophyll content, stomatal conductance, carbon assimilation, transpiration, and many other processes (Bornman 1989). Enhanced UV-B radiation finally affects the growth, root:shoot ratio (Ziska et al. 1992), leaf anatomical features (Li et al. 2001), leaf thicknesses, leaf epidermis thicknesses, and stomatal density of plants (Kostina et al. 2001).

Fortunately, plants contain many protective substances for shielding from enhanced UV-B radiation including flavonoid (Das et al. 2013; Eichholz et al. 2012; Takahama and Oniki 1997; Yao et al. 2003; Zhang et al. 2011). In general, flavonoid induces two distinct categories of mechanisms for resisting enhanced UV-B radiation. One mechanism is that flavonoid strongly absorbs UV-B radiation and effectively reduces the damage effects of enhanced UV-B radiation on nucleic acids, proteins, and other macromolecules. The other mechanism is that flavonoid can be often used to quench free radicals, so as to resist the oxidative stress induced by enhanced UV-B induction (Takahama and Oniki 1997; Yao et al. 2003). Some studies reported that enhanced UV-B radiation can increase the content of total flavonoids in different crops (Das et al. 2013; Eichholz et al. 2012; Zhang et al. 2011). These studies focus on the accumulation of flavonoid in plants induced by enhanced UV-B radiation, and little information is available regarding the accumulative mechanism of flavonoid in plants under enhanced UV-B radiation especially the changes in the activities of key enzymes [phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate:coenzyme A ligase (4CL), and chalcone synthase (CHS)] of flavonoids synthesis in plants under enhanced UV-B radiation.

Previous studies showed that rare earth elements (REEs) can alleviate the harmful effects of UV-B radiation on plants (Liang et al. 2006b, c; Peng and Zhou 2009; Wang et al. 2009) because of their special properties including enhancing the protective ability, regulating the contents of endogenous hormones, improving the several metabolic activities (such as photosynthesis and nitrogen metabolism), and promoting the growth of plants (Hu et al. 2004; Redling 2006; von Tucher and Schmidhalter 2005). Therefore, it is important to elucidate the underlying mechanism where REEs efficiently alleviate the damage in plants exposed to UV-B radiation. Our previous studies found that the treatment by lanthanum chloride ($LaCl_3$), one type of REE salt, could increase the content of total flavonoids in soybean seedlings under enhanced UV-B radiation, which was one of the mechanisms where REEs efficiently alleviate the damage in plants exposed to UV-B radiation (Liang et al. 2006a; Peng and Zhou 2008). However, the mechanism for the regulation of $LaCl_3$ on flavonoid synthesis in plants under enhanced UV-B radiation has not been reported. In the present work, the effects of $LaCl_3$ on the content of total flavonoid as well as the activities of PAL, C4H, 4CL, and CHS in soybean seedlings under enhanced UV-B radiation were investigated. The aims to probe into the regulation mechanism of $LaCl_3$ on flavonoid synthesis in plants under enhanced UV-B radiation.

Materials and methods

Plant materials and growth conditions

The soybean seeds of "Kennong18" were surface-sterilized for 10 min with $HgCl_2$ (0.1 %) and washed five times with deionized water. After soaking for 4 h, the soybean seeds were placed in dishes that were underlaid with three pieces of filter paper and germinated in an incubator at 25±1 °C. When the lengths of the soybean hypocotyls had grown to approximately 2 cm, the seedlings were transplanted into plastic pots (20-cm diameter, 5 plants per pot) filled with deionized water; the water was replaced each day. When two true leaves had developed (after approximately 10 days from germination), the seedlings were cultured in one half strength Hoagland's solution (pH 7.0) in a greenhouse at 25±5 °C. Deionized water was added to the solution to maintain its volume (Pan 2001). The nutrient solution was replaced every 3 days to stabilize the pH (pH 7.0) of the culture. The nutrient solution was aired twice each day with an electronic air pump. The photosynthetic photon flux density provided by the incandescent lamps at the greenhouse was 300 μmol m^{-2} s^{-1}, as measured by a photometer (Fluke 941, US). UV-A bulbs (ZOOMMED SL-25E R63 E27 25W, USA) were suspended perpendicularly over the plants to supply an ambient amount of UV-A radiation for the soybean seedlings. An ultraviolet radiation meter (Lanhe Electronic Instrument, Guangzhou, China) was used to determine the UV-A level. Using the summer solstice weighted against the generalized plant response action spectrum described by Caldwell (1971), the ambient UV-B radiation (7.6 kJ m^{-2} day^{-1}) was artificially provided by UV-B 40 W fluorescent lamps (Nanjing Lamp Factory, Nanjing, China). Three lamps (120-cm-long) per bank spaced 30 cm apart on a steel frame were suspended perpendicularly over the plants. The lamps were covered by 0.13 mm cellulose diacetate film (transmission down to 280 nm) to provide the UV-B radiation at the ambient level by regulating the height of the lamps everyday, respectively. The UV-B radiation level was measured by ultraviolet radiac (Photo-Electricity Instrument Factory of Beijing Normal University, Beijing, China). The cellulose diacetate film was replaced every 2 days to avoid the photodegradation of the film.

$LaCl_3$ and enhanced UV-B radiation treatment

In a previous experiment (results not shown here), we treated soybean leaves with 10, 15, 20, 25, 30, 60, and 120 mg l^{-1}

$LaCl_3$ solution. The soybean seedlings that were treated with 20 mg l^{-1} $LaCl_3$ showed the highest photosynthetic rate and growth. When the concentration of $LaCl_3$ was increased to 60 mg l^{-1}, the photosynthetic rate and growth of soybean seedlings were decreased. Thus, 20 or 60 mg l^{-1} $LaCl_3$ solution was selected for further investigation. The $LaCl_3$ solution (20 or 60 mg l^{-1}) was sprayed evenly on the leaves until drops began to fall. The same amount of deionized water was applied to another set of seedlings as the control. After 48 h, during which the amount of $LaCl_3$ absorbed by the leaves reached a maximum (Hu et al. 2004), the seedlings that were pretreated with $LaCl_3$ or deionized water were placed under fluorescent lamps. The control plants received only ambient levels of UV-B radiation. The lamps in the frames were adjusted each day to provide a mean enhanced UV-B radiation of either 2.6 or 6.2 kJ m^{-2} day^{-1} to plant apices for 5 h daily from 10:00 to 15:00. The experimental plants beneath the cellulose diacetate film received UV-B levels (ambient light+2.6 m^{-2} day^{-1} or ambient light+6.2 kJ m^{-2} day^{-1}) that mimicked a 15 or 30 % reduction in the stratospheric ozone at Wuxi, China, during clear sky conditions (Green et al. 1980; Guarnieri et al. 2004; Kane 2002; McKenzie et al. 2011) normalized at 300 nm. The ozone column thickness was assumed to be 3.0 mm, the albedo was set to 0, and the scatter was set to 1.0. The seedlings were irradiated by UV-B lamps for 5 days. Enhanced UV-B radiations ceased from the sixth to 11th days to allow the soybean seedlings to self-recover. Six experimental sets were prepared: the control group (sprayed with deionized water), the $LaCl_3$ group (sprayed with 20 or 60 mg l^{-1} $LaCl_3$ solution), the T_1 group (irradiated with 2.6 kJ m^{-2} day^{-1} enhanced UV-B radiation), the T_2 group (irradiated with 6.2 kJ m^{-2} day^{-1} enhanced UV-B), the La20/60+T_1 group (sprayed with 20 or 60 mg l^{-1} $LaCl_3$ solution and then exposed to 2.6 kJ m^{-2} day^{-1} enhanced UV-B radiation), and the La20/60+T_2 group (sprayed with 20 or 60 mg l^{-1} $LaCl_3$ solution and then exposed to 6.2 kJ m^{-2} day^{-1} enhanced UV-B radiation). The treatments were performed in triplicate. The leaves were sampled for the determination of the test indices on the first, third, fifth, seventh, ninth, and 11th days.

Measurement of the content of total flavonoids

Fresh samples of 0.5 g were extracted in 10 ml of acidified methanol (methanol:water:hydrochloric acid, 79:20:1, v/v) for UV-B absorbing compounds, according to the procedure of Caldwell (1977). The hydrochloric acid used was 36 % HCl. Extract absorbance at 415 nm was measured using a spectrophotometer (UV-3000; Hitachi, Japan). The content of total flavonoids was determined using a standard curve with quercetin (Sigma-Aldrich Chemie, Steinheim, Germany).

Measurement of PAL activity

The phenylalanine ammonia-lyase (PAL) activity was determined according to the previous report (Nazi et al. 2012). PAL was extracted from fresh leaves (1 g) with 6.5 ml of 50 mM pH 8.8 Tris–HCl buffer containing 15 mM of β-mercaptoehanol in an ice-cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged for 30 min, and the supernatant was collected for enzyme assay. PAL activity was determined based on the rate cinnamic acid production. Briefly, 1 ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine, 0.4 ml of deionized water, and 0.1 ml of enzymatic extract were incubated at 37 °C for 1 h. The reaction was terminated by the addition of 0.5 ml ethyl acetate followed by evaporation to remove the extract solvent. The solid residue was suspended in 3 ml of 0.05 M NaOH, and the cinnamic acid concentration was measured spectrophotometrically by the absorbance at 290 nm. PAL activity (1 unit) was expressed as 0.1 $A_{290}/g_{FW}/h$.

Measurement of C4H activity

The cinnamate-4-hydroxylase (C4H) activity was measured with reference to the methods of Kusukawa et al. (1994). Soybean leaves (0.5 g) were homogenized in four volumes of an extraction buffer (50 mM Tris–HCl at pH 8.0) containing 0.3 M D-sorbitol, 1 mM EDTA-2Na, 1 % sodium isoascorbate, and Polyclar AT (0.05 g/g) at 2–4 °C. The homogenate was forced through four layers of cotton gauze, and the filtrate was centrifuged at 500×g for 10 min to remove the debris and Polyclar AT. The supernatant was centrifuged at 20,000×g for 15 min, and the precipitate was suspended in 3 ml of the extraction buffer. The resulting supernatant was centrifuged again at 100,000×g for 60 min, the precipitate being suspended in 7 ml of the extraction buffer. This supernatant (0.4 ml) was mixed with 3 μl of dimethyl sulfoxide containing a test compound and 0.4 ml of a 50 mM Tris–HCl buffer (pH 8.0) containing 3 mM NADP (sodium salt), 10 mM glucose-6-phosphate dehydrogenase (4 units/ml), and then incubated first at 30 °C for 10 min. To the mixture was added 0.4 ml of a 2 mM *t*-cinnamic acid solution in the buffer, bringing the final concentration to 0.66 mM, before the mixture was incubated at 30 °C for 1 h while vigorously shaking. The reaction was stopped by adding 24 μl of acetic acid and boiling the mixture for 1 min. The mixture was then cooled and centrifuged at 1,000×g for 10 min. The concentration of *p*-coumaric acid in the supernatant was assayed by an HPLC system equipped with an M&S ODS column (4.6 mm i.d.× 15 cm), which was eluted with MeOH:*n*-BuOH:AcOH:H_2O=5:1:2:92 (v/v) while monitoring at 280 nm. The concentration of *p*-coumaric acid after the reaction was substracted from that before the reaction, and the amount of

p-coumaric acid produced was calculated from the remainder in each experiment.

Measurement of 4CL activity

Extracts were prepared as described by previously reported method (Lee et al. 1997). Briefly, plant tissues were ground into a fine powder in liquid nitrogen, resuspended in 200 mM Tris, pH 7.8, and 15 mM β-mercaptpethanol, and rotated in the presence of 10 % Dowex AG 1-X2 Resin (Bio-Rad) at 4 °C for 15 min. The mixture was centrifuged twice to remove debris, and the supernatant was made to 30 % glycerol, frozen in liquid nitrogen, and stored at −80 °C. 4-Coumarate:coenzyme A ligase (4CL) activity was measured according to the methods reported by Lee et al. (1997). 4CL reaction mixtures contained protein extracts, 5 mM ATP, 5 mM $MgCl_2$, 0.33 mM CoA, and 0.2 mM cinnamic acid derivatives. The blank (reference) mixtures contained the same components but without CoA. Enzyme activity was measured as the increase in absorbance at the absorption maximum of the 4-coumaroyl-CoA ester. The extinction coefficients of 4-coumaroyl-CoA ester (21,000 $l \cdot mol^{-1} \cdot cm^{-1}$) were used to calculate enzyme activities.

Measurement of CHS activity

Chalcone synthase (CHS) was prepared according to the previously reported method (Claudot et al. 1997). Lyophilized leaves powder mixed with polyvinylpolypyrrolidone (PVPP), and Dowex (Sigma, St. Louis, MO, USA) 1X2-200 (1:1:1, by weight; Dowex pre-equilibrated with the extraction liquid) was extracted under nitrogen for 20 min at 4 °C. The extraction liquid contained 0.7 M potassium phosphate buffer (pH 8.0), 0.1 % (w/v) BSA, 1.5 % (w/v) polyethyleneglycol (PEG) 20,000, 0.4 M sucrose, 1 M $CaCl_2$, 0.2 M ascorbic acid, 50 mM EDTA, 50 mM cysteine, and 5 mM sodium diethyldithiocarbamate. After filtration on nylon cloth, the filtrate was centrifuged for 20 min at 37,000×g. The supernatant was used as crude enzyme extract.

The assay mixture contained 50 μl of crude extract to 8 ml of reaction solution, and the reference was made with the extraction solution. Aliquots of the crude enzyme extract were incubated with 0.1 M potassium phosphate buffer (pH 8.0) containing 2 % (w/v) BSA, 25 μl of 4-coumaroyl-CoA and 16 μM [2-^{14}C]malonyl-CoA (8.34 MBq) for 15 min at 35 °C. The reaction was stopped by adding 350 μl of ethyl acetate containing naringenin. This resulted in the extraction of the labeled reaction products which were separated from the substrates. After mixing for 3 min, samples were centrifuged and radioactivity as labeled naringenin 50 μl aliquots of the ethyl acetate phase was assessed in a scintillation counter Beckman (LS 6000).

Statistical analysis

Differences among treatments were analyzed by one-way analysis of variance using SPSS 11.5 and Origin 8.0. Student's *t* test was applied to determine the significance among the different treatments ($p<0.05$; Ke et al. 2003).

Results

Effects of $LaCl_3$ on the content of total flavonoids in soybean seedlings under enhanced UV-B radiation

As shown in Fig. 1, during the stress period (from the first to fifth day), the content of total flavonoids in the 20 mg l^{-1} $LaCl_3$ group was always higher than that of the control, which did not change with prolonging the treating time. On the contrary, the content of total flavonoids in the 60 mg l^{-1} $LaCl_3$ group was less than that of the control, and the effect was aggravated as the treating time went on. The contents of total flavonoids in the T_1 and T_2 groups were gradually increased from the first to third day and then gradually decreased from the third to fifth day, which were higher than those of the control group from first to fifth day. The increase in the content of total flavonoids in the T_1 group was higher than that in the T_2 group. For the La20+T_1 group, the content of total flavonoids was increased compared with that of the control and T_1 group, and it was gradually increased from the first to third day and then decreased from the third to fifth day. The similar effect was observed in the La20+T_2 group, and the increase in the La20+T_2 group was higher than that in the La20+T_1 group and T_2 group. When the concentration of $LaCl_3$ increased to 60 mg l^{-1}, the content of total flavonoids in the $LaCl_3$+T1 group turned to decrease compared with that of the control, and the effect was gradually aggravated as the

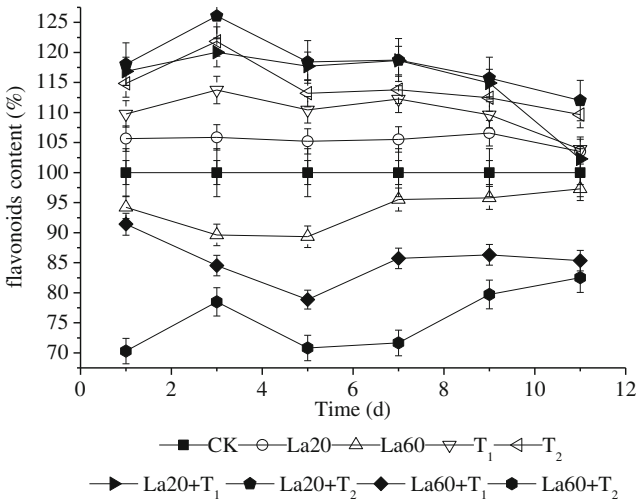

Fig. 1 Dynamics of relative value of the content of total flavonoids in the $LaCl_3$-treated soybean seedlings under enhanced UV-B radiation

treating time went on. For the $LaCl_3+T_2$ group, the decrease in the content of total flavonoids was still observed, and the effect was firstly aggravated and then gradually alleviated as the treating time went on.

During the recovery period (from the 6th to 11th day), the content of total flavonoids in the 20 mg l^{-1} $LaCl_3$ group was still higher than that of the control, but the increased flavonoids during the stress period began to decrease until the 9th day. The content of total flavonoids in the 60 mg l^{-1} $LaCl_3$ group gradually returned to the control level. The contents of total flavonoids in the T_1, T_2, or La20+T_1/T_2 group were still higher than that of the control, but they gradually recovered to the control level. The degree and rate of recovery in the La20+T_1 group was higher than those in the T_1 and T_2 group. Similarly, in the 60 mg l^{-1} $LaCl_3$ and La60+T_1/T_2 group, the contents of total flavonoids were also close to the control level. And the recovery efficiency of the content of total flavonoids in the La60+T_1 group was higher than that in the La60+T_2 group.

Effects of $LaCl_3$ on the PAL activity of soybean seedlings under enhanced UV-B radiation

As shown in Fig. 2, during the stress period (from the first to fifth day), the 20 mg l^{-1} $LaCl_3$ treatment made the PAL activity always higher than that of the control, and the increase extent was largest on the third day, up to 79 %. In comparison with the control group, the PAL activity in the 60 mg l^{-1} $LaCl_3$ group was continuously increased as the treating time went on. The PAL activity in the T_1 or T_2 group was higher than that of the control, which rose with the increase in the treating time and the level of enhanced UV-B radiation. The similar results were also observed in the La20+T_1/T_2 and La60+T_1/T_2 groups. Moreover, the PAL activity in different groups followed this order: La20+T_2 group>T_2 group>La20+T_1 group>T_1 group>La60+T_2 group>La60+T_1 group.

During the recovery period (from the 6th to the 11th day), the PAL activity in any of groups was still higher than that of the control. But in comparison with the stress period, the PAL activity gradually recovered to the control level as the treating time went on.

Effects of $LaCl_3$ on the C4H activity of soybean seedlings under enhanced UV-B radiation

As shown in Fig. 3, during the stress period (from the first to the fifth day), the C4H activity in the 20 mg l^{-1} $LaCl_3$ group was increased compared with the control group, which gradually increased with prolonging the treating time. The opposite result was observed in the 60 mg l^{-1} $LaCl_3$ group. The C4H activities in the T_1 and T_2 groups were also increased compared with that of the control group, which became larger with increasing the level and time of enhanced UV-B radiation. The change order of the C4H activities in the La20+T_1/T_2 groups was similar with that of the T_1/T_2 groups, but the change degree in the La20+T_1/T_2 groups was higher than that of the T_1/T_2 groups. For the La60+T_1/T_2 groups, the C4H activities were firstly higher and then lower than those of the control, and the C4H activities were gradually decreased with prolonging the treating time.

During the recovery period (from the 6th to the 11th day), the C4H activity in the 20 mg l^{-1} $LaCl_3$ group was still higher than that of the control group, and the increase degree did not changed with prolonging the treating time. The C4H activities in the T_1, T_2, La20+T_1, and La20+T_2 groups began to decrease compared with those during the stress period, but still higher than that of the control group. Moreover, the C4H activities in 60 mg l^{-1} $LaCl_3$ group and La60+T_1 and La60+T_2 groups continued to decrease compared with that

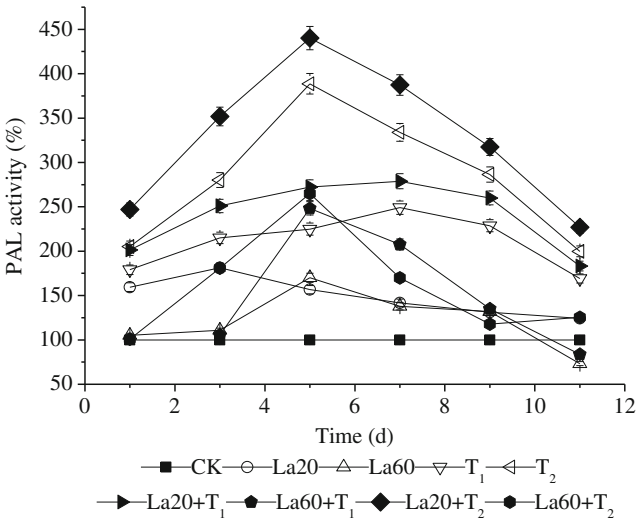

Fig. 2 Dynamics of relative value of PAL activity in the $LaCl_3$-treated soybean seedlings under enhanced UV-B radiation

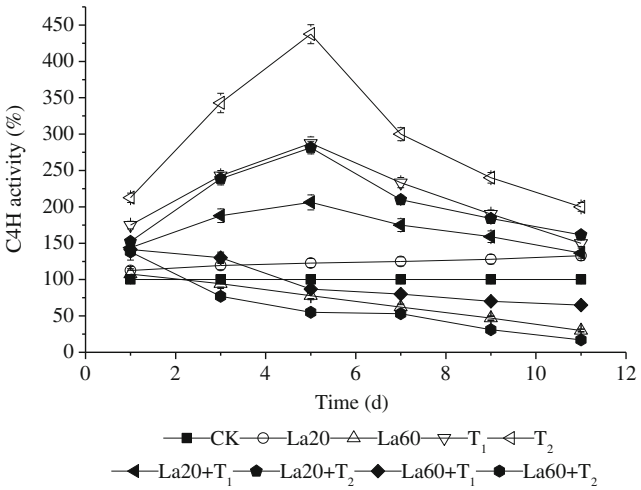

Fig. 3 Dynamics of relative value of C4H activity in the $LaCl_3$-treated soybean seedlings under enhanced UV-B radiation

during the stress period, and the decrease degree became larger as the treating time increased.

Effects of $LaCl_3$ on the 4CL activity of soybean seedlings under enhanced UV-B radiation

As shown in Fig. 4, during the stress period (from the first to the fifth day), the treatment of 20 mg l^{-1} $LaCl_3$ did not affect the 4CL activity. The treatment of 60 mg l^{-1} $LaCl_3$ caused the decrease in the 4CL activity, and the effect was aggravated with prolonging the treating time. The 4CL activities in the T_1 and T_2 groups were always higher than that in the control group, and they gradually rose as the treating time went on. The same change order of the 4CL activity was observed in the La20+T_1 and La20+T_2 groups. The change degree of the 4CL activity in the La20+T_1/T_2 group was lower than that of the T_1/T_2 group. For the La60+T_1/T_2 group, the 4CL activity was gradually close to the control level with prolonging the treating time.

During the recovery period (from the 6th to the 11th day), the 4CL activity in the 20 mg l^{-1} $LaCl_3$ group gradually increased compared with that during the stress period. On the contrary, the 4CL activities in other groups were decreased to below the control level, and the effects in the different groups followed the order: T_2 group>T_1 group, La20+T_2 group>La20+T_1 group, La60+T_2 group>La60 group>La60+T_1 group.

Effects of $LaCl_3$ on the CHS activity of soybean seedlings under enhanced UV-B radiation

As shown in Fig. 5, during the stress period (from the first to the fifth day), the CHS activity in the 20 mg l^{-1} $LaCl_3$ group was always higher than that of the control group. On the contrary, the CHS activity in the 60 mg l^{-1} $LaCl_3$ group was lower than that of the control group, and the discrepancy between the CHS activities in the 60 mg l^{-1} $LaCl_3$ group and the control group became larger with prolonging the treating time. The CHS activities in the T_1, T_2, La20+T_1, and La20+T_2 groups were continually increased with prolonging the treating time. The discrepancies between the CHS activities in these groups and the control followed the order: La20+T_1 group>T_2 group>T_1 group>La20+T_2 group. Compared with the control group, the CHS activity in the La60+T_1/T_2 group was gradually far away the control group as the treating time went on.

During the recovery period (from the 6th to the 11th day), the CHS activities in the 20 and 60 mg l^{-1} $LaCl_3$ groups was still higher and lower than that of the control, respectively. The change of the CHS activities in the T_1, T_2, and La20+T_1/T_2 groups were opposite to those during the stress period. The decreases in the CHS activities of La60+T_1/T_2 groups turned to be stable, and the decrease degree in the La60+T_2 group was larger than that of the La60+T_1 group.

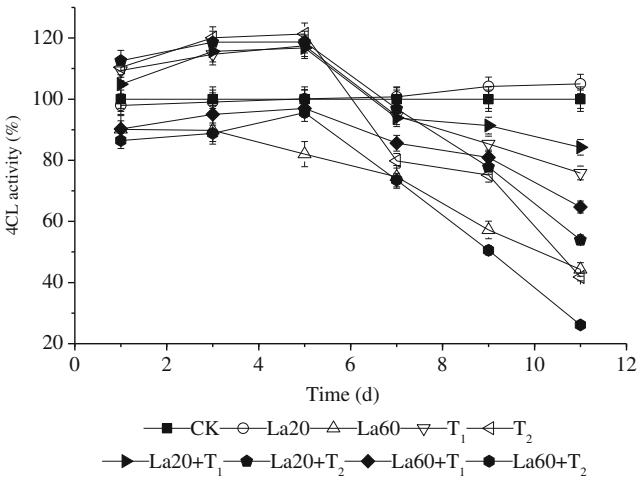

Fig. 4 Dynamics of relative value of 4CL activity in the $LaCl_3$-treated soybean seedlings under enhanced UV-B radiation

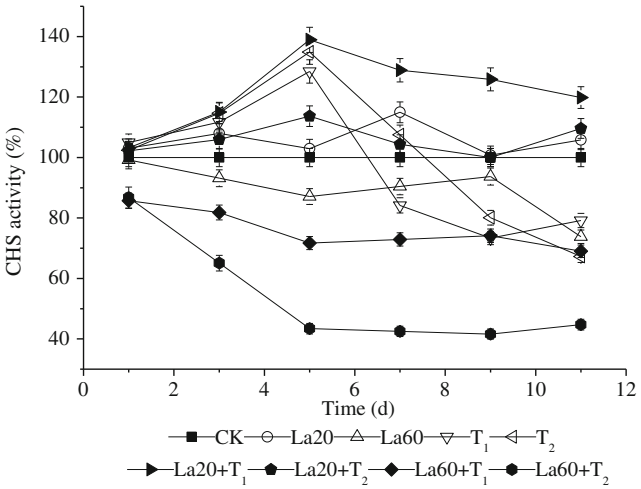

Fig. 5 Dynamics of relative value of CHS activity in the $LaCl_3$-treated soybean seedlings under enhanced UV-B radiation

Discussion

Many studies reported that flavonoid in plants plays an positive role in the response to enhanced UV-B radiation (Das et al. 2013; Eichholz et al. 2012; Takahama and Oniki 1997; Yao et al. 2003; Zhang et al. 2011). In the present work, we found that enhanced UV-B radiation induced the increase in the content of total flavonoids in soybean seedlings and the effect depended on the level of enhanced UV-B radiation (Fig. 1), which was consistent with the work of Xiang et al. (2009). The combined treatment of 20 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation caused a greater increase in the content of total flavonoids of soybean seedlings than that caused by the single treatment of enhanced UV-B radiation

(Fig. 1), indicating that the pretreatment of 20 mg l^{-1} $LaCl_3$ exhibited the promotion effects on the content of total flavonoids in soybean seedlings under enhanced UV-B radiation. This effect was different from some reports on the alleviation effect of Se supply on plants exposed to UV-B radiation (Breznik et al. 2005; Pennanen et al. 2002; Shanker 2006). Most reports found that Se supply alleviate the damage effect of UV-B radiation via enhancing antioxidative capacity not the content of total flavonoids in plants (Breznik et al. 2005; Pennanen et al. 2002; Shanker 2006). On the contrary, the combined treatment of 60 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation led to a greater decrease in the content of total flavonoids in soybean seedlings than that caused by the single treatment of enhanced UV-B radiation, indicating that the pretreatment of 60 mg l^{-1} $LaCl_3$ has negative effects on the content of total flavonoids in soybean seedlings under enhanced UV-B radiation. Tian et al. (2007) reported that enhanced UV-B radiation causes the increase in the content of total flavonoids in wheat, while the content of total flavonoids approaches to the control level when enhanced UV-B radiation ceased. In our present work, the content of total flavonoids increased in soybean seedlings under enhanced UV-B radiation also recovered to the control level (Fig. 1). The recoveries at different degrees were observed in the other treatments (Fig. 1). In summary, in this work, we understood the effects of enhanced UV-B radiation on the content of total flavonoids in soybean seedlings and the regulation of $LaCl_3$ on the content of total flavonoids in soybean seedlings under enhanced UV-B radiation.

However, some key scientific questions are not clear and as follows: what was the reason for enhanced UV-B radiation increased the content of total flavonoids in soybean seedlings; how did $LaCl_3$ regulate the change of content of total flavonoids in soybean seedlings under enhanced UV-B radiation. We speculated the change in the content of total flavonoids of soybean seedlings was related to the flavonoid synthesis in the plant. Flavonoid is synthesized via the phenylpropanoid pathway (Liu 2001). It has been reported that PAL, C4H, 4CL, and CHS are the rate-limiting enzymes of the flavonoids synthesis (Shirley 1996). PAL catalyzes the conversion of phenylalanine to cinnamate, C4H catalyzes the synthesis of *p*-hydroxycinnamate from cinnamate, 4CL converts *p*-coumarate to its coenzyme-A ester, activating it for reaction with malonyl CoA, and CHS catalyzes the reaction of 4-counmaroyl-CoA and malonyl-CoA to yield naringenin chalcone (Shirley 1996). In summary, the changes in the activities of PAL, C4H, 4CL, and CHS in soybean seedlings might be the direct reason for that enhanced UV-B radiation increased the content of total flavonoids. The regulation of $LaCl_3$ on the content of total flavonoids was, in fact, the regulation on the activities of PAL, C4H, 4CL, and CHS.

During the stress period (from the first to the fifth day), enhanced UV-B radiation caused the increases in the activities of PAL, C4H, 4CL, and CHS in soybean seedlings (Figs. 2–5). Therefore, the increase in the content of total flavonoids induced by enhanced UV-B radiation was relative to the promotion of the conversion of phenylalanine to cinnamate, the synthesis of *p*-hydroxycinnamate from cinnamate, conversion of *p*-coumarate to its coenzyme-A ester, and the reaction of 4-counmaroyl-CoA and malonyl-CoA to yield naringenin chalcone, all of which were catalyzed by PAL, C4H, 4CL, and CHS. In the combined treatment of 20 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation, the changes in the activities of PAL, C4H, 4CL, and CHS were similar to those in the treatment of enhanced UV-B radiation. However, in comparison with the treatment of enhanced UV-B radiation, after the combined treatment of 20 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation, the activities of PAL and CHS increased, the activity of C4H decreased, and the activity of 4CL did not changed, which indicated that under enhanced UV-B radiation, 20 mg l^{-1} $LaCl_3$ regulated the content of total flavonoids by interfering the activities of PAL, C4H, and CHS. Enhanced UV-B radiation activated the signal produced by various types of light receptors in the cytoplasm, which resulted in the increase in the Ca^{2+} concentration in the cytoplasm as well as the promotion of the genetic transcription and expression of PAL, C4H, and CHS (Liang and Zhou 2007). The charge and ionic potential of La(III) was higher than those of Ca^{2+} (Hu et al. 2004). Therefore, La(III) could displace calcium ion in the enzyme and interfere the normal physiological function of calcium ion by involving in the enzyme reaction (Hu et al. 2004). The combined treatment of 60 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation decreased the activities of C4H, 4CL, and CHS, but increased the activity of PAL, and the change degrees were larger than those of the single treatment of enhanced UV-B radiation, which was similar to the change of the content of total flavonoids (Figs. 2–5). The results indicated 60 mg l^{-1} $LaCl_3$ decreased the flavonoid synthesis in soybean seedlings under enhanced UV-B radiation by decreasing the activities of C4H, 4CL, and CHS and increasing the PAL activity.

After enhanced UV-B radiation was removed, the activities of PAL, C4H, 4CL, and CHS in soybean seedlings under enhanced UV-B radiation could be recovered (Figs. 2–5). The same phenomena were observed in the combined treatment of 20 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation (Figs. 2–5). The results indicated that the recovery of the flavonoid synthesis was one of the reasons for the recovery of the content of total flavonoids in soybean seedlings in the treatment of enhanced UV-B radiation and the combined treatment of 20 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation. However, in the combined treatment of 60 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation, only the activity of PAL in soybean seedlings could be recovered (Figs. 2–5). The results indicated that although the activities of C4H, 4CL, and CHS continuously decreased, the content of total flavonoids could be

recovered due to the recovery of the activity of PAL in the combined treatment of 60 mg l^{-1} $LaCl_3$ and enhanced UV-B radiation.

Conclusions

In summary, enhanced UV-B radiation led to the increases in the activities of PAL, C4H, 4CL, and CHS, which promoted the flavonoid synthesis, and increased the content of total flavonoids in soybean seedlings. $LaCl_3$ could affect the flavonoid synthesis by regulating the activities of PAL, C4H, 4CL, and CHS in soybean seedlings under enhanced UV-B radiation. However, the present work has been performed in the laboratory conditions excluding soil and the processes occurring to xenobiotics in soil (e.g., microbial degradation and sorption). Therefore, the results obtained need to be confirmed by extending the investigation to real and natural soil–plant systems.

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