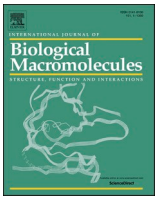
International Journal of Biological Macromolecules 253 (2023) 126702

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



TgLCYB1 regulated by TgWRKY22 enhances the tolerance of *Torreya grandis* to waterlogging stress

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ARTICLE INFO

Keywords:
Torreya grandis
β-Carotene
Waterlogging
TgLCYB1
TgWRKY22

ABSTRACT

β-Carotene functions in plant growth and development and plays an important role in resisting abiotic stress, such as drought and salt stress. The specific function and mechanism by which β-carotene responds to waterlogging stress, however, remain elusive. In this study, we found that β-carotene content and lycopene cyclase (*TgLCYB1*) expression, both in leaves and roots of *Torreya grandis*, were increased under waterlogging treatment. Subcellular localization assays indicated that TgLCYB1 was localized in the chloroplasts. Phenotypic, physiological, and metabolome analysis showed that overexpression of *TgLCYB1* enhanced the tolerance of tomato plants to waterlogging stress. Furthermore, application of a LCYB enzyme inhibitor, 2-(4-chlorophenylthio)-triethylamine hydrochloride, markedly enhanced the sensitivity of *T. grandis* to waterlogging stress. In addition, yeast one-hybrid assay, the dual luciferase assay system, and real-time quantitative PCR indicated that waterlogging stress induced *TgWRKY22* to increase *TgLCYB1* expression by binding to the *TgLCYB1* promoter. Collectively, our results indicated that TgWRKY22 positively regulated *TgLCYB1* expression to improve the activities of antioxidant enzyme and increase the levels of some key metabolites, thereby relieving waterlogging-induced oxidative damage, and consequently modulating the waterlogging stress response. This study contributes to a more comprehensive understanding of carotenoid functions and the role *LCYB* genes play in plant stress response.

1. Introduction

Carotenoids, ubiquitous in bacteria, fungi, algae, and higher plants, represent one of the most abundant pigments that produce yellow-red hues [1,2]. They primarily encompass α-carotene, β-carotene, lutein, zeaxanthin, lycopene, and cycloflavin. Carotenoids contribute significantly to diverse biological processes within the plant system, including development and photosynthesis [3]. Moreover, they serve as precursors for plant hormones, such as abscisic acid (ABA) and strigolactones (SLs), that function as stress hormones. The highly unsaturated structure of carotenoids, which is attributed to their main chain containing up to 15 conjugated double bonds, confers a robust antioxidant activity [4]. Thus, carotenoids are implicated in adaptive stress responses to various environmental stimuli. The accumulation of α-carotene, β-carotene, and lutein, for instance, notably enhances the tolerance of sweet potato to salt and drought stresses [5]. Tobacco plants with higher lutein content exhibit increased chilling stress resistance [6]. Despite the comprehensive study of carotenoids' roles in responding to salt and drought stress, however, understanding their function and mechanism in addressing waterlogging stress remains limited.

In plants, the synthesis of carotenoids occurs on the plastid membrane [3]. The process commences with isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), catalyzed by IPP isomerase (IPI) and GGPP synthase (GGPS) to yield geranylgeranyl pyrophosphate (GGPP), which is the precursor of methylerythritol (MEP). Subsequently, phytoene synthase (PSY) induces the condensation of two GGPP molecules to form phytoene, which is transformed into lycopene through the action of multiple enzymes, including phytoene desaturase (PDS) and 15-cis-ζ-carotene isomerase (*Z*-ISO). Lycopene cyclases (LCYB and LCYE) further catalyze lycopene to synthesize α- and β-carotene [7,8]. Numerous studies have demonstrated the pivotal role of LCYB in mediating abiotic stress responses [5,9–11]. For instance,

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https://doi.org/10.1016/j.ijbiomac.2023.126702
Received 7 July 2023; Received in revised form 31 August 2023; Accepted 2 September 2023
Available online 4 September 2023

overexpression of *IbLCYB2* in sweet potato notably enhances its tolerance to salt and drought stress [5]. Similarly, overexpression of *LcLCYB* in tobacco bolsters its tolerance to salt stress [10]. Research concerning the role and potential mechanisms of the *LCYB* gene in plant responses to waterlogging stress, however, remains scarce and unclear.

Torreya grandis (*T. grandis*), a member of the Torreya family, is an evergreen tree species predominantly found in China [12]. As a unique economic tree species, the fruit of *T. grandis* is not only highly nutritious but also possesses significant medicinal value [13,14]. Notably, *T. grandis* primarily thrives in the southern provinces of China, including Zhejiang and Anhui. The region's distinct geographical locations, climates, and geological environments are characterized by frequent flooding, leading to long-term waterlogging [15,16]. This persistent waterlogging adversely affects the growth and development of *T. grandis*, resulting in severe yield reductions. Despite this, the response and regulatory mechanisms of *T. grandis* in response to waterlogging stress remain unclear.

In this study, we initially explored alterations in the main carotenoids present in *T. grandis* subjected to waterlogging stress. We identified that TgLCYB1 could enhance the waterlogging resistance of tomato plants. Furthermore, we delineated the transcriptional regulation mechanism of *TgLCYB1* in reaction to waterlogging stress. Collectively, these findings offer novel insights into carotenoid metabolism, stress resistance, and transcriptional regulation mechanisms in economic tree species in response to waterlogging stress conditions.

2. Materials and methods

2.1. Plant materials and growth condition

We cultivated one-year-old seedlings of *Torreya grandis* cv. Merrillii (*T. grandis*) in pots in a growth chamber. The conditions in the chamber were as follows: 14 h at 25 °C, with 60 % humidity and a photosynthetically active radiation of 500 μmol m^{-2} s^{-1} during the day; and 10 h at 23 °C with 60 % humidity during the night. For the waterlogging treatments, the water level in the pots containing *T. grandis* was kept 2–3 cm above the soil surface for varying durations (0, 3, and 6 d). Subsequently, leaves and roots were harvested for carotenoid content determination.

2.2. Quantitative analysis of carotenoid content

We quantified the carotenoid content following the method outlined in a recent study [2]. In brief, *T. grandis* samples were freeze-dried and then extracted with an n-hexane: acetone solution (1:1, *V*/V). Subsequently, this extract was vortexed for 20 min and subjected to ultrasonication for 30 min at room temperature. The supernatants were gathered and evaporated under a nitrogen gas stream until dry, and then were reconstituted in a mixture of methanol and ethyl acetate. After filtration through a 0.22 μm filter, we analyzed the samples using the liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry (LC-APCI-MS/MS) system (UPLC ExionLC™ AD; MS Sciex Triple Quadrupole 4500, Toronto, Canada). The UPLC parameters were as follows: column, YMC C30 (3 μm, 2 mm × 100 mm); solvent A consisted of methanol: acetonitrile (3:1, *V*/V) with 0.01 % BHT, and solvent B was composed of ethyl acetate with 0.01 % BHT. We set the gradient program to 100 % solvent A from 0 to 1 min, 20 % solvent A from 6 to 15 min, and back to 100 % solvent A from 15.1 to 18 min. Other conditions included a flow rate of 0.4 mL/min, temperature of 50 °C, and an injection volume of 3 μL. The Triple Quadrupole 4500 LC/MS/MS System, equipped with an APCI Turbo Ion-Spray interface, was operated in positive ion mode and managed by Analyst 1.6.3 software (Sciex). Carotenoid levels were quantified based on a standard curve, which were calculated as follows: [concentration (μg/mL) × volume (mL)] / [weight (g)].

2.3. Reverse transcription and real-time PCR analysis

We extracted total RNA from both *T. grandis* and tomato samples using the FastPure Plant Total RNA Isolation Kit (polysaccharide and polyphenolics–rich) (Vazyme, Nanjing, China). We assessed the RNA's quality and purity using a NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Only high-quality RNA (A260/A280 ratios >1.8 and A260/A230 ratios above 2.0) was utilized for cDNA synthesis, facilitated by the PrimeScript RT Reagent Kit with genomic DNA (gDNA) Eraser (TaKaRa, Dalian, China). For each reverse transcription reaction, 1 μg of total RNA was utilized in a 20 μL system. We conducted real-time polymerase chain reaction (RT-PCR) using a CFX96 RT-PCR system (BioRad, Hercules, CA, USA) in alignment with the manufacturer's protocol using ChamQ SYBR® qPCR Master Mix (Vazyme). Each 20 μL reaction incorporated 200 ng of cDNA, 0.2 μM of each primer, and 2 × qPCR MasterMix. The PCR cycling protocol consisted of an initial 94 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 58 °C for 15 s, and 72 °C for 10 s. We determined the target genes' relative expression according to the $2^{-\Delta\Delta CT}$ method, referencing *TgActin* as an internal standard [17].

2.4. Subcellular localization

We amplified the encoding region of *TgLCYB1* devoid of the stop codon using specific primers to generate a TgLCYB1-GFP fusion protein integrated into the expression vector pCAMBIA1300-GFP. Following this, the recombinant vectors were transferred into *Agrobacterium tumefaciens* strain GV3103-pSoup. The collected bacteria were resuspended with resuspension solution (10 mM 2-morpholinoethanesulphonic acid, 10 mM $MgCl_2$, 150 μM acetosyringone, pH 5.7), and transiently expressed in the leaf epidermal cells of *Nicotiana benthamiana* using Agrobacterium infiltration. After a 72-h incubation, we analyzed the infiltrated tobacco leaves using a confocal fluorescence microscope (LSM510, Karl Zeiss, Oberkochen, Germany; excitation: 488 nm, emission: 527 nm).

2.5. Generation of TgLCYB1 transgenic tomato plants

We integrated the coding sequence (CDS) of *TgLCYB1*, including the stop codon, into the pCAMBIA1300 vector to produce the overexpression vector, pC1300-*TgLCYB1*. Subsequently, we introduced this vector into the *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method. We used genetically modified Agrobacterium to transform callus derived from the cotyledons of tomato (*Solanum lycopersicum* L.) cv. Micro-Tom, following the method outlined in [18]. Putative transgenic plants were identified using RT-PCR analysis with specific primers (Table S1).

2.6. Phenotypic analysis

We exposed one-month-old tomato seedlings, including the *OE-TgLCYB1#1* and *OE-TgLCYB1#2* lines, and wild-type seedlings, to waterlogging stress for 10 d, or maintained under normal conditions. Following these treatments, we captured images and recorded the fresh weight of shoots, the fresh weight of roots, and the root length. For the 2-(4-chlorophenylthio)-triethylamine hydrochloride (CPTA) treatments, we subjected one-year-old *T. grandis* plants to waterlogging stress and sprayed them with 100 μM CPTA at three-day intervals. We subsequently captured new images and recorded both the fresh weights. The morphological characteristics of the nine cultivars (e.g., Zaolv, Chang, Qiefei1), including leaf chlorosis, were documented through direct observation. We scored and computed the waterlogging injury index according to established methods [19].

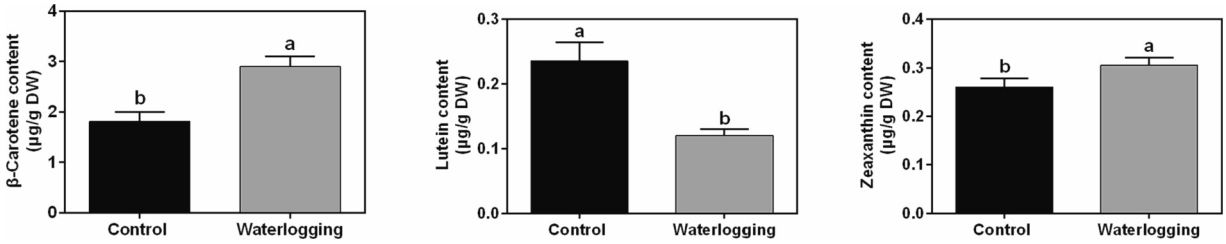

Fig. 1. The contents of β-carotene, lutein, and zeaxanthin in *T. grandis* leaves exposed to waterlogging stress. Error bars indicate SD ($n = 3$). Different letters indicate a significant difference compared with the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

2.7. Physiological index determination

After waterlogging stress, we harvested the samples, prepared them in 10 % trichloroacetic acid containing 0.65 % 2-thiobarbituric acid, and incubated them at 95 °C for 25 min. We calculated the content of malondialdehyde (MDA) according to a previously described method [12]. For measurement of the electrolyte leakage (%), fresh samples (0.1 g) were placed in 50 mL tubes with 40 mL distilled deionized water. These tubes were reacted at 30 °C for 2 h, and then we measured the initial electrical conductivity of the medium (EC1). After reaction at 100 °C for 15 min, we measured the second electrical conductivity of the medium (EC2). We calculated electrolyte leakage (%) using the following equation: electrolyte leakage = EC1/EC2 × 100 [12].

Tomato leaves were subjected to waterlogging stress or were maintained under normal conditions for 7 d. Then they were homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 1 mM EDTA, 1 % polyvinylpyrrolidone, and 1 mM sodium ascorbate. After centrifugation at 12,000*g* for 30 min at 4 °C, we used the supernatant to assess the activity of antioxidant protective enzymes. We determined ascorbate peroxidase (APX) activity by adding 1 mM sodium ascorbate and measuring the decrease in ascorbic acid absorbance postoxidation at 290 nm. The catalase (CAT) activity was gauged by noting the reduction in absorbance following the decomposition of hydrogen peroxide (H_2O_2) at 240 nm. Superoxide dismutase (SOD) activity was quantified by monitoring the inhibition of nitroblue tetrazolium at 560 nm [20]. We evaluated the root vigor using the enzymatic reduction assay of triphenyl tetrazolium chloride at 485 nm as previously detailed [21].

2.8. Determination of H_2O_2 level

To stain for H_2O_2, tomato leaves either subjected to waterlogging stress or maintained under normal conditions for 10 d were treated with 3,3′-Diaminobenzidine (DAB), following the method detailed in [22]. Briefly, detached leaves were immediately immersed in 1 mg mL^{-1} DAB solution (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 8 h at 28 °C. Subsequently, the leaves were dipped in 95 % (*v*/v) and 75 % (v/v) ethanol to decolorize, and then photographed. We performed quantification of H_2O_2 in the leaves using a Hydrogen Peroxide Assay Kit (Beyotime, China, Beijing, China), according to the manufacturer's instructions.

2.9. Determination of ABA content

We quantified ABA content using an enzyme-linked immunosorbent assays (ELISA) kit (Shanghai Meilian Biotechnology Co, Ltd. Shanghai, China) as outlined in [23] with minor modifications. Briefly, seedling leaves were ground in a mortar and homogenized in a PBS extraction buffer (pH 7.3). The resulting extracts were centrifuged at 4 °C, 10,000 rpm for 20 min, after which we collected the supernatant, which was stored at 4 °C in preparation for ELISAs.

2.10. Metabolites extraction and analysis

Tomato samples were pulverized after being frozen with liquid nitrogen and subsequently resuspended in 2 mL of cold 80 % methanol. They were then incubated on ice for 5 min, followed by centrifugation at 15,000*g* and 4 °C for 20 min. We transferred the supernatant after dilution with 53 % methanol to a fresh Eppendorf tube and centrifuged it again under the same conditions. We then analyzed the supernatant using the LC-MS/MS system. We performed this analysis using the Waters ACQUITY UPLC I-Class plus/Thermo QE plus at Oebiotech Co., Ltd. (Shanghai, China) in both positive and negative polarity modes. The supernatant was loaded onto an ACQUITY UPLC HSS T3 (100 mm × 2.1 mm, 1.8 μm) with a 16-min linear gradient at a flow rate of 0.35 mL/min. Eluent A consisted of 0.1 % formic acid in water, and eluent B consisted of 0.1 % formic acid in an acetonitrile solution.

2.11. Y1H assays

We integrated the coding sequences (CDS) of transcription factors devoid of stop codons into the pB42AD vector. Simultaneously, the promoter region of *TgLCYB1*, spanning 2000 base pairs, was incorporated into the pLacZi vector. These plasmids were cotransformed into the yeast strain EGY48 and cultivated on a synthetic dropout/-Trp-Ura medium at 28 °C for 3 d. To verify the interaction between TF and *TgLCYB1* promoter, we selected and cultivated the positive clones on a synthetic dropout/-Trp-Ura/Gal/Raf/X-Gal (80 μg/mL) plate (Coolaber, Beijing, China) at 28 °C for 3 d.

2.12. Luciferase assays

We cloned the coding sequence (CDS) of *TgWRKY22* into the pGreenII 62-SK vector and incorporated the promoter of *TgLCYB1*, encompassing 2000 base pairs, into the pGreenII 0800-LUC vector. We subsequently introduced the vectors into the *Agrobacterium tumefaciens* strain GV3101, which was then transformed into the leaves of four-week-old *Nicotiana benthamiana* plants, following the protocol outlined in [24]. Three days postinfiltration, we took samples from the infected site and then determined the LUC (firefly luciferase)/REN (renilla luciferase) ratio in accordance with the instructions provided by the Dual-LumiTM II Luciferase Reporter Gene Assay Kit (Beyotime).

3. Results

3.1. The changes in carotenoid content in T. grandis exposed to waterlogging stress

We measured the changes in carotenoid content in *T. grandis* leaves in response to waterlogging conditions to investigate the effect of carotenoids in the response of *T. grandis* to waterlogging stress. Notably, the β-carotene content in *T. grandis* significantly increased upon waterlogging treatment, almost doubling compared with the control group (Fig. 1). Likewise, zeaxanthin content in waterlogged *T. grandis* leaves was substantially higher than in the control group. Lutein content in *T.*

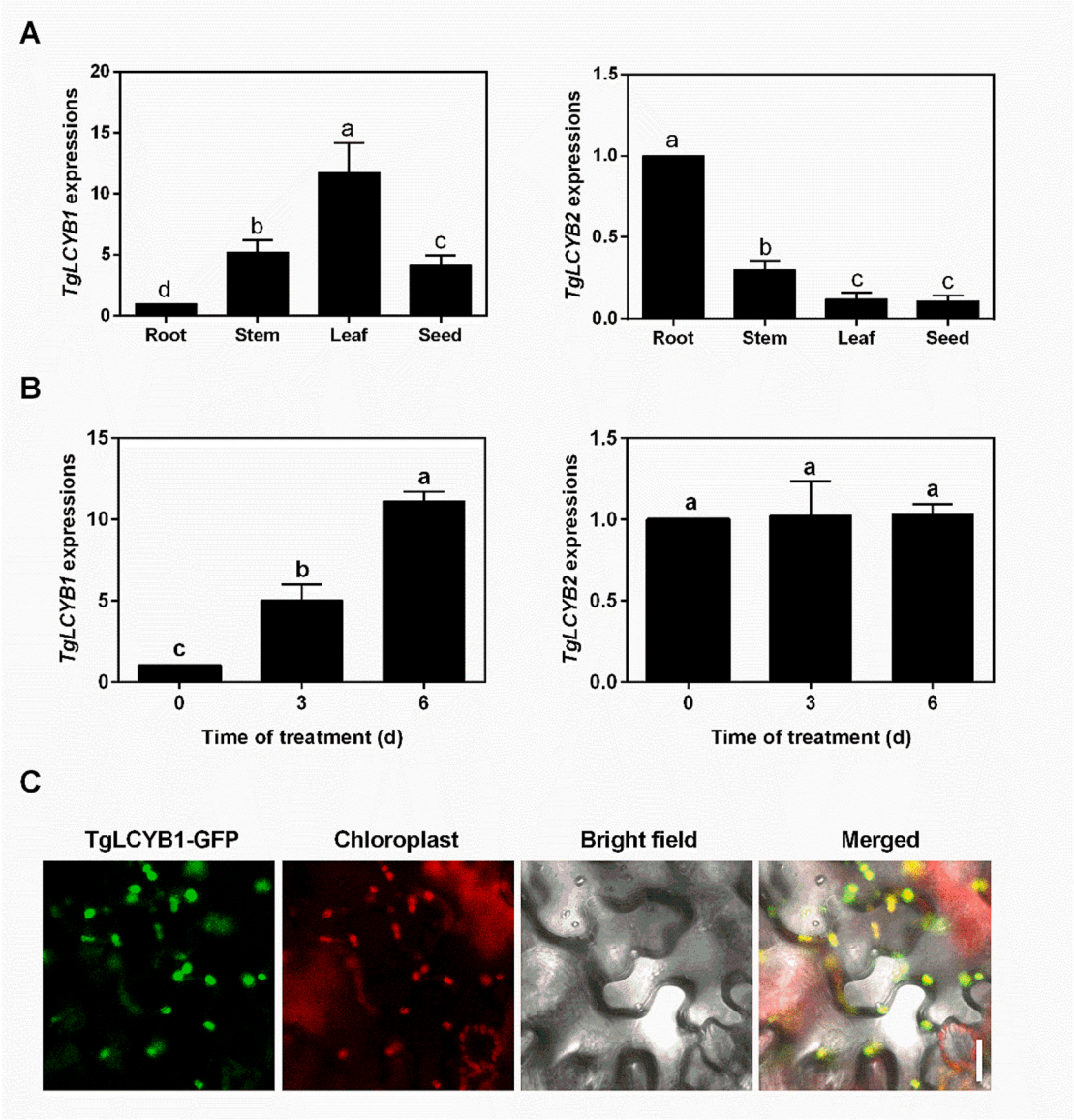

Fig. 2. The expression patterns of *TgLCYB1* and *TgLCYB2*. (A) The expression levels of *TgLCYB1* and *TgLCYB1* in various tissues (root, stem, leaf, and seed) of *T. grandis*. (B) Expression analysis of *TgLCYB1* and *TgLCYB2* in *T. grandis* leaves exposed to waterlogging stress for 0 d, 3 d, and 6 d. Error bars in (A) and (B) indicate SD ($n = 3$). Different letters in (A) and (B) indicate a significant difference compared with the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$. (C) The subcellular localization of TgLCYB1 protein. The scale bar in (C) indicates 20 μm. We performed the experiment in (C) at least three times with similar results.

grandis, however, significantly decreased following waterlogging stress exposure (Fig. 1). Meanwhile, the β-carotene content in *T. grandis* roots was significantly increased upon waterlogging treatment (Fig. S1A).

3.2. The expression level of TgLCYB1 and TgLCYB2

From observing the accumulation of β-carotene in response to waterlogging stress, we hypothesized that this metabolic pathway might play a crucial role in the waterlogging stress response. Consequently, we identified two *LCYB* genes responsible for direct β-carotene synthesis in the *T. grandis* genome [25]. Initial qRT-PCR analysis revealed that *TgLCYB1* expression in *T. grandis* leaves was significantly higher than in roots, stems, or seeds, whereas *TgLCYB2* expression was highest in roots (Fig. 2A). Waterlogging stress treatment significantly increased the expression level of *TgLCYB1* in *T. grandis* leaves. In contrast, the expression level of *TgLCYB2* remained unaffected for the duration of the treatment (Fig. 2B). Furthermore, we also observed this inducement of *TgLCYB1* by waterlogging stress in *T. grandis* roots (Fig. S1B). These observations suggest that TgLCYB1 may participate in the waterlogging stress response.

We further examined the subcellular localization of TgLCYB1 in the epidermal cells of *N. benthamiana* leaves. As shown in Fig. 2C, the green fluorescence emitted by TgLCYB1-GFP aligned with the red auto-fluorescence of chloroplasts, which suggested the localization of the TgLCYB1 protein in chloroplasts.

3.3. Overexpression of TgLCYB1 in tomato confers tolerance to waterlogging

We transformed tomato plants with *TgLCYB1*, driven by the *35S* promoter, to elucidate the role of TgLCYB1 in the waterlogging response. RT-PCR analysis confirmed the successful expression of *TgLCYB1* in the tomato plants, and we selected two lines demonstrating equivalent expression levels of *TgLCYB1* for waterlogging

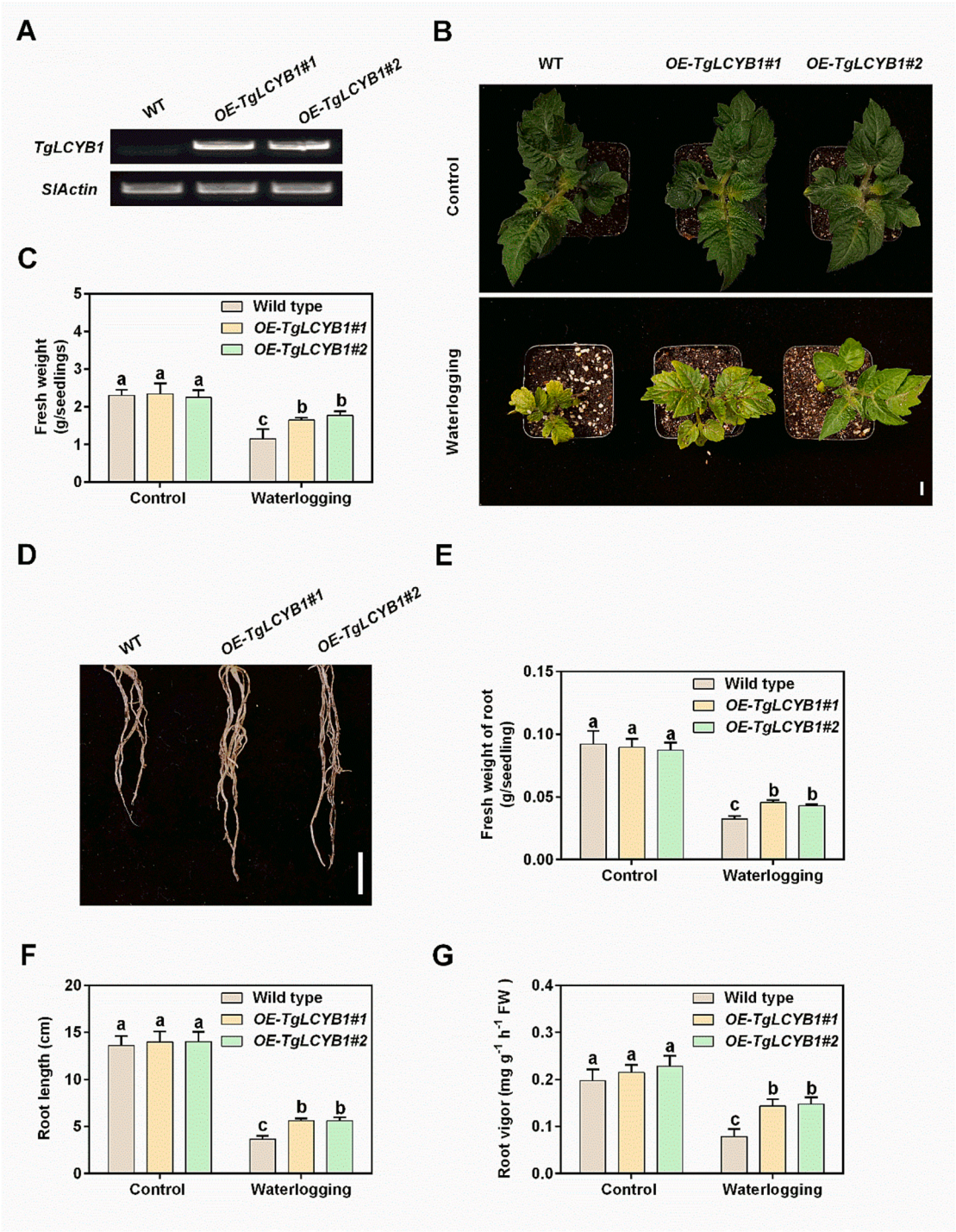

Fig. 3. Analysis of waterlogging tolerance of tomato seedlings. (A) RT-PCR analysis of *TgLCYB1* in *TgLCYB1* transgenic lines (*OE-TgLCYB1#1* and *OE-TgLCYB1#2*) and wild type. (B) Phenotype analysis of tomato seedlings under waterlogging treatment. The scale bar in (B) indicates 1 cm. We performed the experiment in (A) at least three times with similar results. (C) Analysis of shoot fresh weight. Shoot fresh weight of tomato seedling after treatment with waterlogging stress. (D) Phenotype analysis of tomato roots under waterlogging treatment. The scale bar in (D) indicates 1 cm. (E) Analysis of root fresh weight. (F) The root length. (G) Analysis of root activity. Error bars in (C), (E), (F), and (G) indicate SD ($n = 3$). Different letters in (C), (E), (F), and (G) indicate a significant difference compared with the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

hypersensitivity tests (Fig. 3A). In the absence of waterlogging treatment, we did not observe any discernible differences between wild-type and transgenic plants (*OE-TgLCYB1#1* and *OE-TgLCYB1#2*). Following waterlogging stress treatment, however, *OE-TgLCYB1#1* and *OE-TgLCYB1#2* plants exhibited less chlorosis and growth inhibition compared with the wild-type plants (Fig. 3B). Additionally, the fresh weight of the transgenic lines significantly exceeded that of wild-type plants in response to waterlogging treatment (Fig. 3C).

The root system is the direct organ stressed by waterlogging. Thus, to explore the effect of *TgLCYB1* on roots under waterlogging stress, we analyzed the fresh weight of root, root length, and root vitality of tomato roots in response to waterlogging stress. Under waterlogging stress treatment, the root length, the fresh weight, and root vitality of roots of *OE-TgLCYB1#1* and *OE-TgLCYB1#2* plants were greater than those of

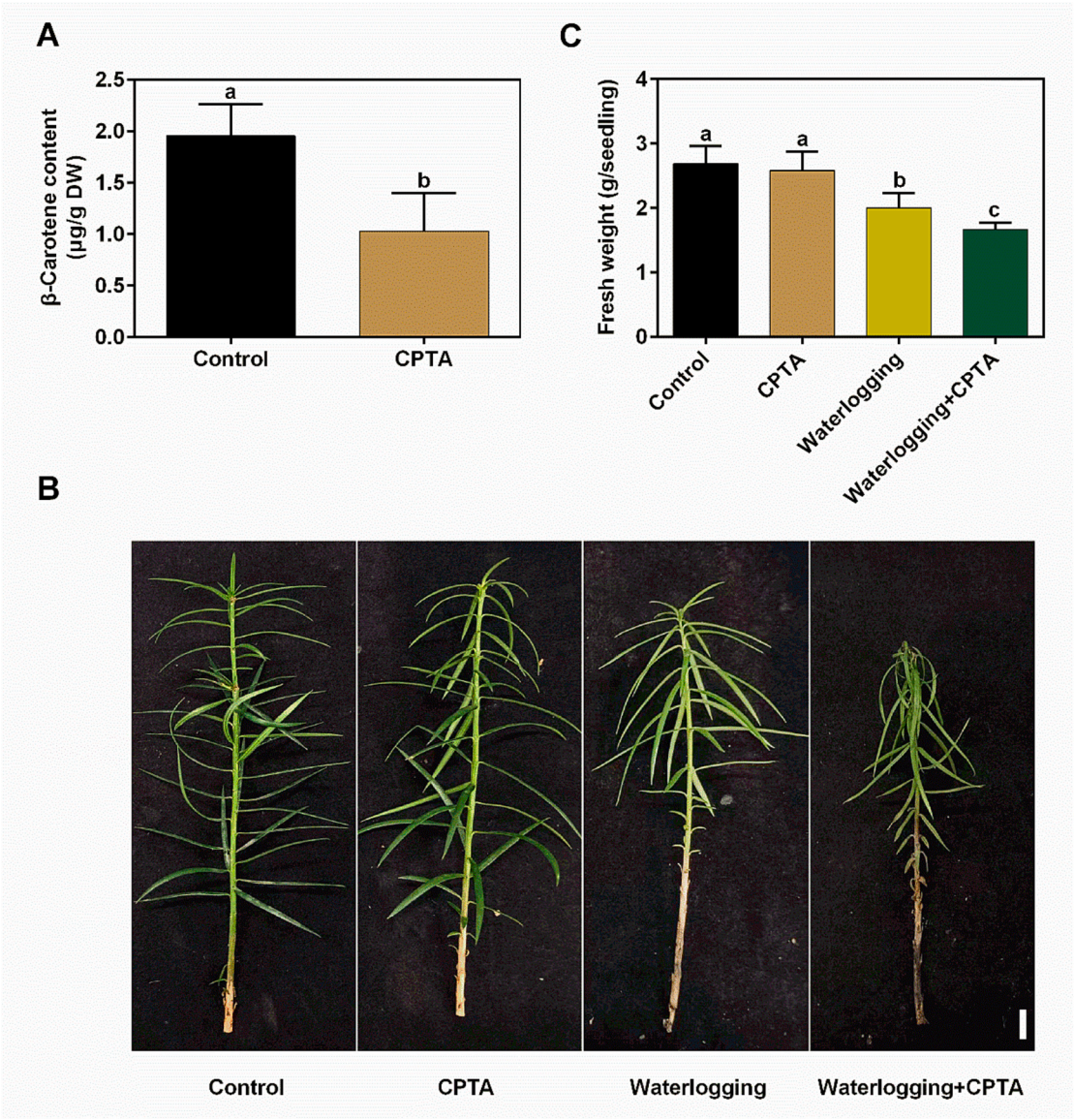

Fig. 4. Effect of CPTA on the β-carotene content and waterlogging tolerance of *T. grandis*. (A) β-carotene content in *T. grandis* exposed to waterlogging stress and exogenous CPTA. (B) Phenotype analysis of *T. grandis* exposed to waterlogging stress and exogenous CPTA. The scale bar in (B) indicates 1 cm. (C) Analysis of shoot fresh weight. Shoot fresh weight of *T. grandis* after treatment with waterlogging stress and exogenous CPTA. Error bars in (A) and (C) indicate SD ($n = 3$). Different letters in (A) and (C) indicate a significant difference compared to the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

the wild-type plants (Fig. 3D–G). These findings suggested that *TgLCYB1* overexpression enhanced tomato plant tolerance to waterlogging stress.

3.4. Application of CPTA markedly enhances the sensitivity of T. grandis to waterlogging stress

Because of the challenges in the genetic transformation process of *T. grandis*, in particular, bacterial contamination, we did not obtain any *TgLCYB1* transgenic lines. Thus, to explore the role of TgLCYB1 in *T. grandis*, we used an LCYB enzyme inhibitor, CPTA, which has been frequently utilized in prior research [26–28]. As shown in Fig. 4A, CPTA treatment indeed led to the reduction of β-carotene. Also as expected, CPTA treatment markedly enhanced the sensitivity of *T. grandis* to waterlogging stress (Fig. 4B and C). Furthermore, we detected the expression levels of *TgLCYB1* in nine *T. grandis* cultivars (e.g., Zaolv, Chang, Qiefei1) with varying waterlogging index. The results demonstrated a significant correlation between the expression of *TgLCYB1* and waterlogging index (Fig. S3). This result underscored the crucial role of TgLCYB1 in modulating *T. grandis*'s response to waterlogging stress.

3.5. Overexpression of TgLCYB1 alleviates the oxidative damage caused by waterlogging stress in tomato

Waterlogging stress commonly incites oxidative damage in plants. To understand the impact of *TgLCYB1* overexpression on the damage induced by waterlogging stress, we initially measured electrolyte leakage and MDA content in waterlogged tomato leaves. As shown in Fig. 5A and B, the electrolyte leakage and MDA content in unstressed tomato leaves were substantially lower than in leaves subjected to waterlogging stress. In response to waterlogging stress treatment, wild-type plants and transgenic lines showed a notable increase in electrolyte leakage and MDA content, and this increase was more modest in the transgenic lines compared with the wild-type plants.

Subsequently, we assessed the accumulation of H_2O_2, a contributor to oxidative damage, using DAB staining. Fig. 5C illustrated that the wild-type leaves, indicated by darker brown precipitation, had a higher

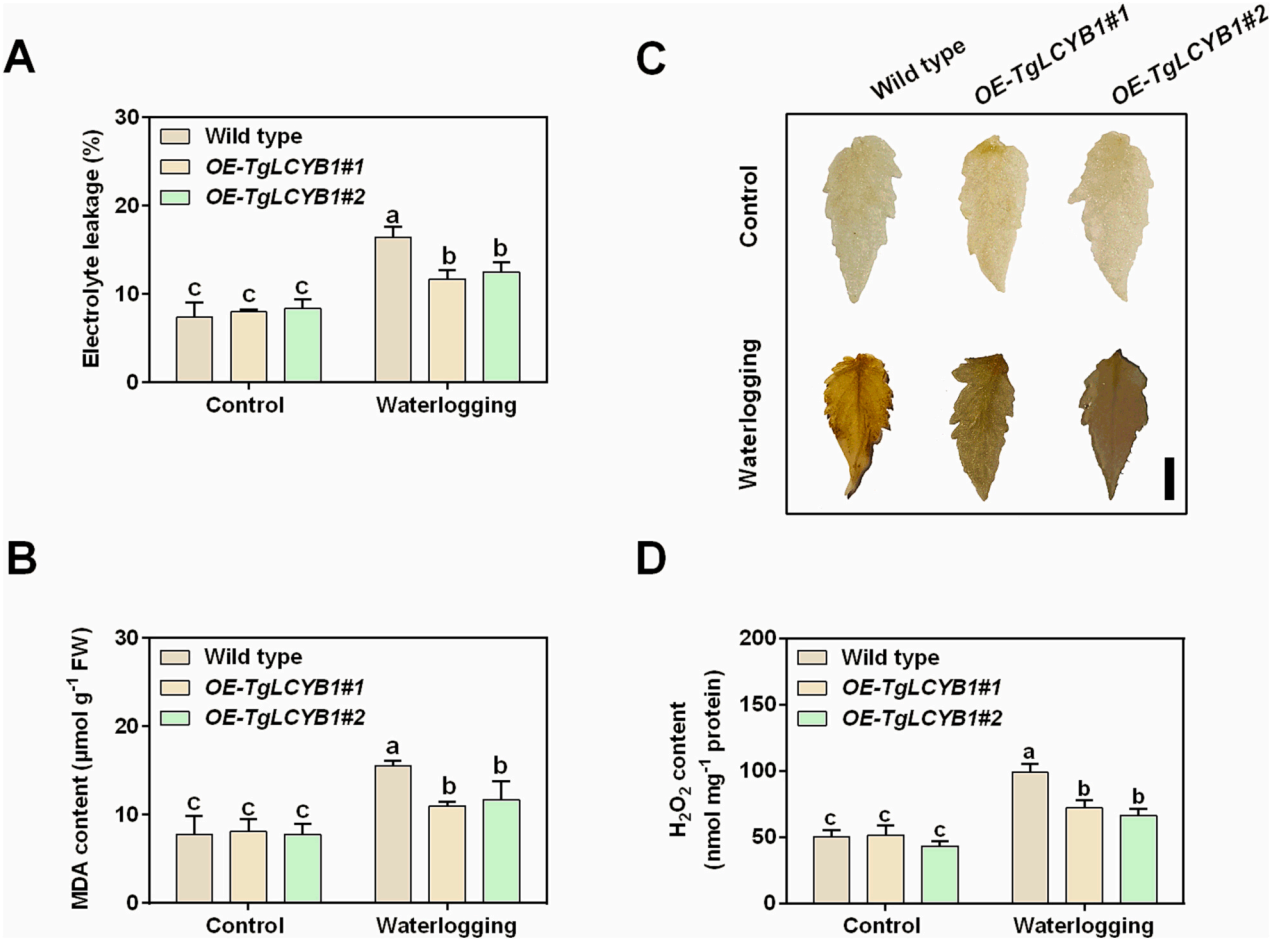

Fig. 5. Effect of TgLCYB1 on the oxidative damage in tomato leaves in response to waterlogging stress. (A) Electrolyte leakage rates in tomato leaves of transgenic lines and wild type under waterlogging treatment. (B) MDA contents in tomato leaves of transgenic lines and wild type under waterlogging treatment. (C) H_2O_2 accumulations in tomato leaves of transgenic lines and wild type under waterlogging treatment detected by NBT staining. The bars indicate 1 cm. We performed the experiment in (C) at least three times with similar results. (D) The H_2O_2 contents in tomato leaves of transgenic lines and wild type under NaCl treatment. Error bars in (A), (B), and (D) indicate SD (n = 3). Different letters in (A), (B), and (D) indicate a significant difference compared with the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

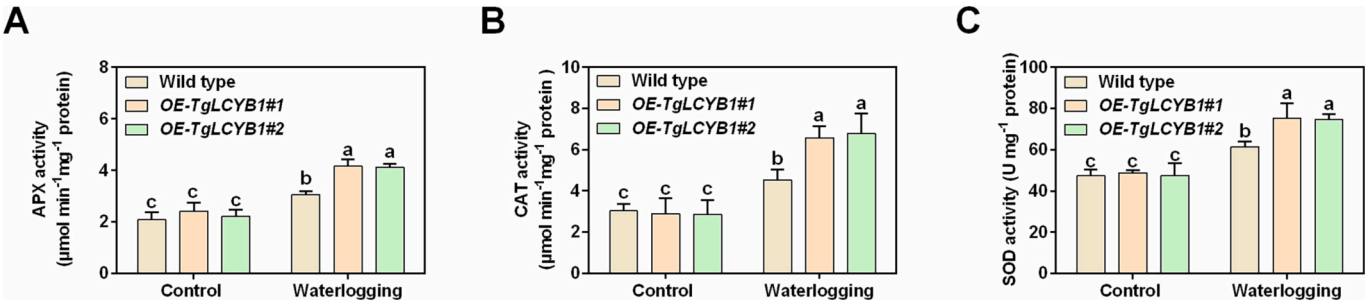

Fig. 6. Effect of *TgLCYB1* on the activities of antioxidant enzyme (APX, CAT, and SOD) in tomato leaves in response to waterlogging stress. The activities of APX (A), CAT (B), and SOD (C) in tomato leaves of transgenic lines and wild type under waterlogging treatment. Error bars in (A), (B), and (C) indicate SD (n = 3). Different letters in (A), (B), and (C) indicate a significant difference compared with the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

level of H_2O_2 accumulation compared with the transgenic lines in response to waterlogging stress. Supporting the results of DAB staining, the H_2O_2 content in wild-type plants was significantly higher than that in the transgenic lines subjected to waterlogging stress (Fig. 5D). Collectively, these results suggested that overexpressing *TgLCYB1* mitigated H_2O_2 accumulation, thereby alleviating oxidative damage caused by waterlogging stress.

3.6. Overexpression of TgLCYB1 in tomato leaves increases antioxidant activity

Reactive oxygen species (ROS)-scavenging enzymes, such as APX, CAT, and SOD play vital roles in maintaining ROS homeostasis [29]. We measured APX, CAT, and SOD activities in tomato leaves subjected to waterlogging stress to investigate the contribution of *TgLCYB1* to antioxidant activity. Fig. 6 illustrates the activities of APX, CAT, and SOD in

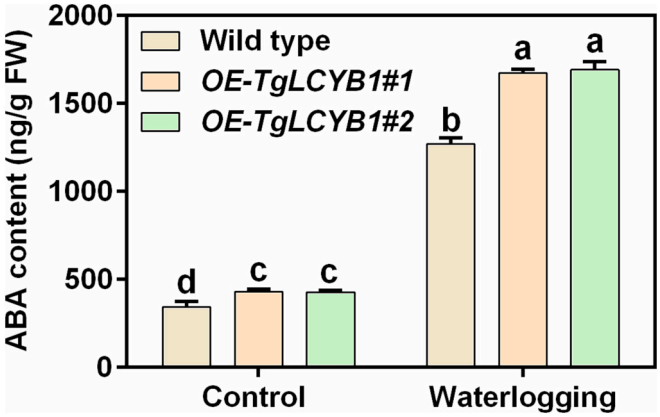

Fig. 7. Effect of *TgLCYB1* on the ABA content in tomato leaves in response to waterlogging stress. Error bars indicate SD (n = 3). Different letters indicate a significant difference compared with the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

the transgenic line leaves, which were significantly higher compared with those in wild-type leaves exposed to waterlogging stress. Furthermore, we also analyzed the ABA content derived from β-carotene in tomato leaves after exposure to waterlogging stress. Under normal conditions, the ABA content in transgenic lines was slightly higher than that in wild-type plants. After treatment with waterlogging stress, the ABA content both in wild-type and transgenic lines was increased, and in the transgenic lines, this inducement was much higher than in the wild-type plants (Fig. 7). These results suggested that the overexpression of *TgLCYB1* could improve the activities of antioxidant enzymes by increasing the ABA content in tomato.

3.7. Overexpression of TgLCYB1 in tomato alters metabolic dynamic changes in response to waterlogging stress

We performed a nontargeted metabolome of *OE-TgLCYB1#1* and wild-type plants treated with waterlogging stress to further explore the effect of TgLCYB1 on the changes of metabolites in tomato under waterlogging stress. The principal component analysis (PCA) showed that the samples were closely gathered in the electrospray ionization (ESI) modes, indicating that the repeatability of the experiment was good (Fig. 8A). The statistical analysis identified 431 differentially accumulated metabolites (DAMs), including 251 up-regulated and 180 down-regulated DAMs based on the following criteria: variable importance in the projection (VIP) > 1.0 and *P*-value < 0.05 (Fig. 8B and Table S2). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that DEMs with different comparative combinations were enriched in multiple pathways, including "autophagy," "aminoacyl-tRNA biosynthesis," "valine, leucine and isoleucine biosynthesis," "monobactam biosynthesis," and "citrate

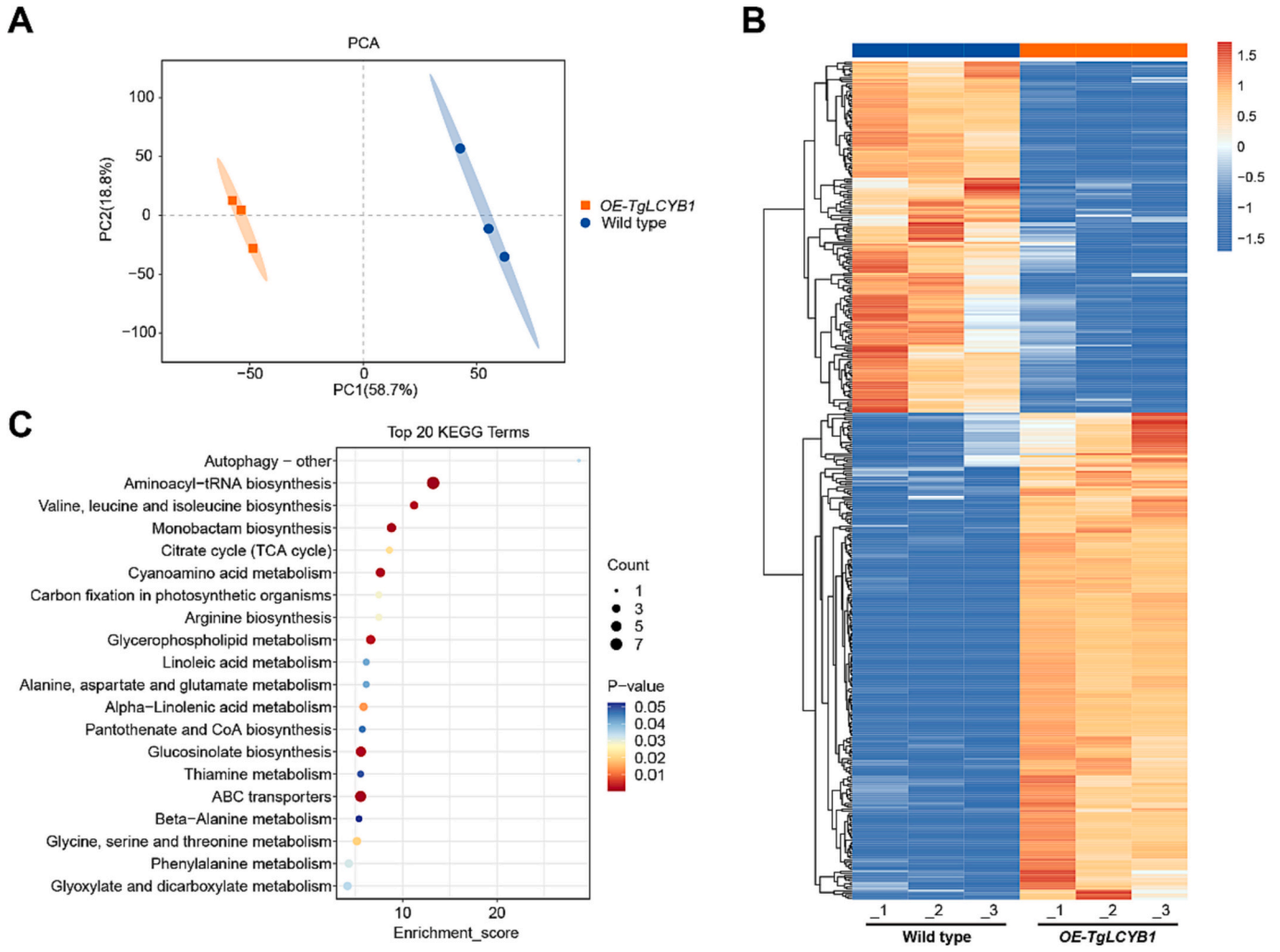

Fig. 8. Effect of TgLCYB1 on the changes of metabolites in tomato under waterlogging stress. (A) Principal component analysis (PCA) of monitored metabolites. (B) Heat map of differential expressed metabolites (DEMs). (C) KEGG analysis of differential expressed metabolites.

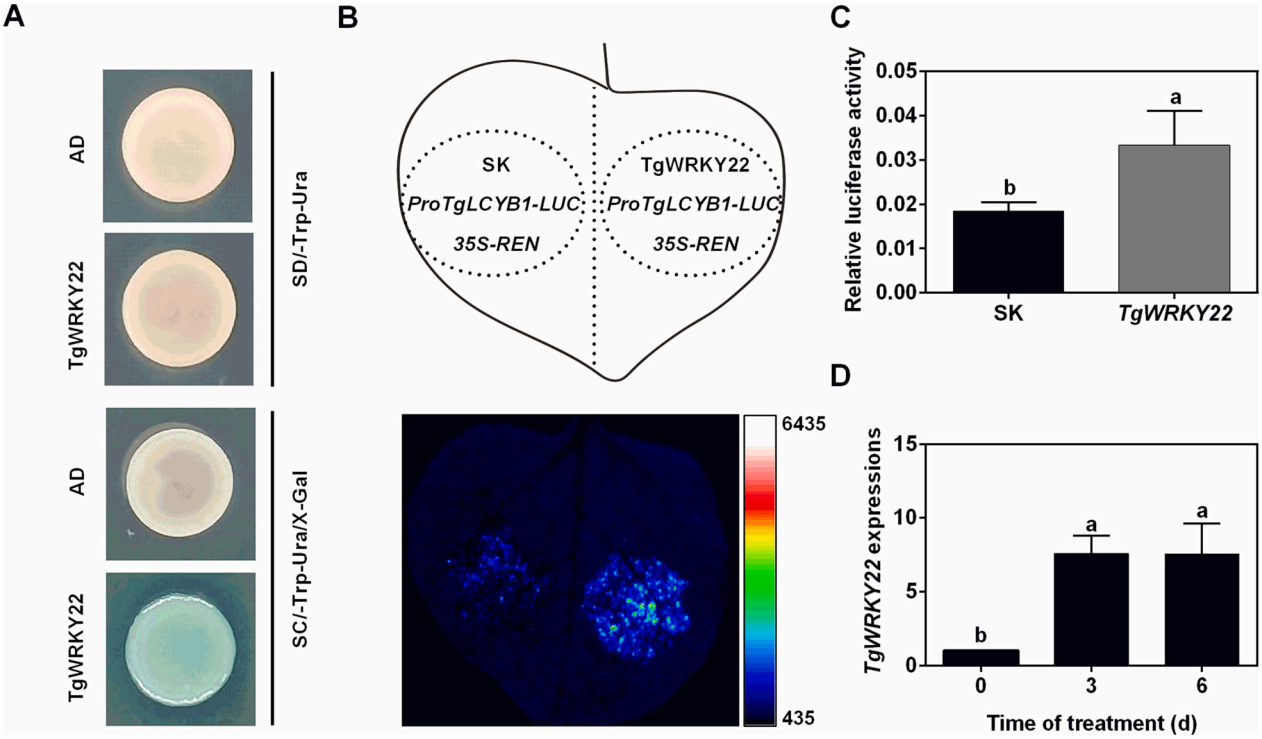

Fig. 9. TgWRKY22 directly binds to the *TgLCYB1* promoter to positively regulate its expression. (A) TgWRKY22 directly binds to the *TgLCYB1* promoter in yeast. (B) and (C) The effect of TgWRKY22 on luciferase activity driven by *TgLCYB1* promoter in tobacco leaves through dual-luciferase reporter assay. (D) Expression analysis of *TgWRKY22* in *T. grandis* leaves exposed to waterlogging stress for 0 d, 3 d, and 6 d. We performed the experiment in (A) and (B) at least three times with similar results. Error bars in (C) and (D) indicate SD (n = 3). Different letters in (C) and (D) indicate a significant difference compared to the control as determined by one-way analysis of variance (ANOVA) at $P < 0.05$.

cycle" (Fig. 8C and Table S2). Notably, the levels of some key metabolites, such as D-glucose, D-fructose, sucrose, malic acid, and citric acid, in the *TgLCYB1* transgenic line were much higher than those in the wild type (Table S2). These results indicated that overexpression of *TgLCYB1* in tomato increased the levels of some key metabolites (e.g., D-glucose, D-fructose) to enhance the waterlogging tolerance.

3.8. *TgWRKY22 positively regulates TgLCYB1 expression by directly binding to the TgLCYB1 promoter in response to waterlogging stress*

Given the clear induction of *TgLCYB1* transcription level in response to waterlogging stress, we hypothesized that the transcriptional regulation of *TgLCYB1* should exist. Therefore, we performed a yeast one-hybrid assay to investigate potential transcription factors regulating *TgLCYB1* expression assay as the first step. Fig. 9A shows that yeast EGY48 transformed with TgWRKY22 and the *TgLCYB1* promoter (2000 bp) exhibited a blue color, which indicated the direct binding of TgWRKY22 to the *TgLCYB1* promoter in yeast.

To ascertain the regulatory role of TgWRKY22 on *TgLCYB1* expression, we conducted a dual-luciferase analysis. We cloned the *TgLCYB1* promoter and fused to the coding region of firefly luciferase protein (Fluc), along with a renilla luciferase (Rluc). We introduced *TgWRKY22* into the pGreen II -62SK vector. The Fluc/Rluc ratio indicated the transcriptional activation ability of this transcription factor on the *TgLCYB1* promoter. As shown in Fig. 9B and C, TgWRKY22 augmented the activity of the luciferase (LUC) gene while under the control of the *TgLCYB1* promoter. Additionally, we examined the expression of *TgWRKY22* in *T. grandis* at various time points during waterlogging treatment. As shown in Fig. 9D, the expression of *TgWRKY22* was up-regulated when exposed to waterlogging stress. These results indicated that TgWRKY22 was induced by waterlogging stress and increased *TgLCYB1* expression by directly binding the *TgLCYB1* promoter.

4. Discussion

The nuts of *T. grandis*, a valuable economic tree species indigenous to the subtropical mountainous regions in China, possess high nutritional value and exhibit health-promoting and medicinal effects on human health [30–32]. Currently, research efforts primarily focus on exploring the economic and nutritional value of *T. grandis*. Although *T. grandis* exhibits relative drought tolerance, it is highly susceptible to waterlogging. Importantly, seasonal waterlogging frequently occurs in *T. grandis* cultivation areas, significantly affecting its growth, yield, and quality [33–35]. Nevertheless, research is limited on the waterlogging tolerance of *T. grandis*. In this study, we investigated an association between carotenoids and waterlogging tolerance of *T. grandis* for the first time, revealing a significant increase in β-carotene content in *T. grandis* in response to waterlogging conditions (Fig. 1). This association implied that the accumulation of β-carotene might positively regulate the response of *T. grandis* to waterlogging stress.

Lycopene β-cyclase (LCYB) is a pivotal enzyme in the biosynthesis pathway of β-carotene. As anticipated, the β-carotene level in the transgenic line was significantly elevated compared with the wild type (Fig. S2). This result affirmed TgLCYB1's pivotal role in carotene biosynthesis. Previous studies have demonstrated that alterations in *LCYB* can influence carotenoid accumulation and have a positive impact on plant responses to abiotic stressors [5,36]. In sweet potato, the overexpression of *IbLCYB2* led to elevated levels of α-carotene, β-carotene, lutein, β-cryptoxanthin, and zeaxanthin, consequently enhancing its tolerance to salt and drought stresses [5]. The overexpression of *SeLCYB* from *Salicornia europaea* in *Arabidopsis* enhanced salt tolerance by up-regulating carotenoid synthesis [36]. Notably, some studies have indicated that LCYB exerted a negative regulatory role in abiotic stress responses. For instance, down-regulation of the lycopene β-cyclase gene in sweet potato enhanced abiotic stress tolerance in transgenic calli [9]. In this study, we discovered that TgLCYB1 plays a positive regulatory

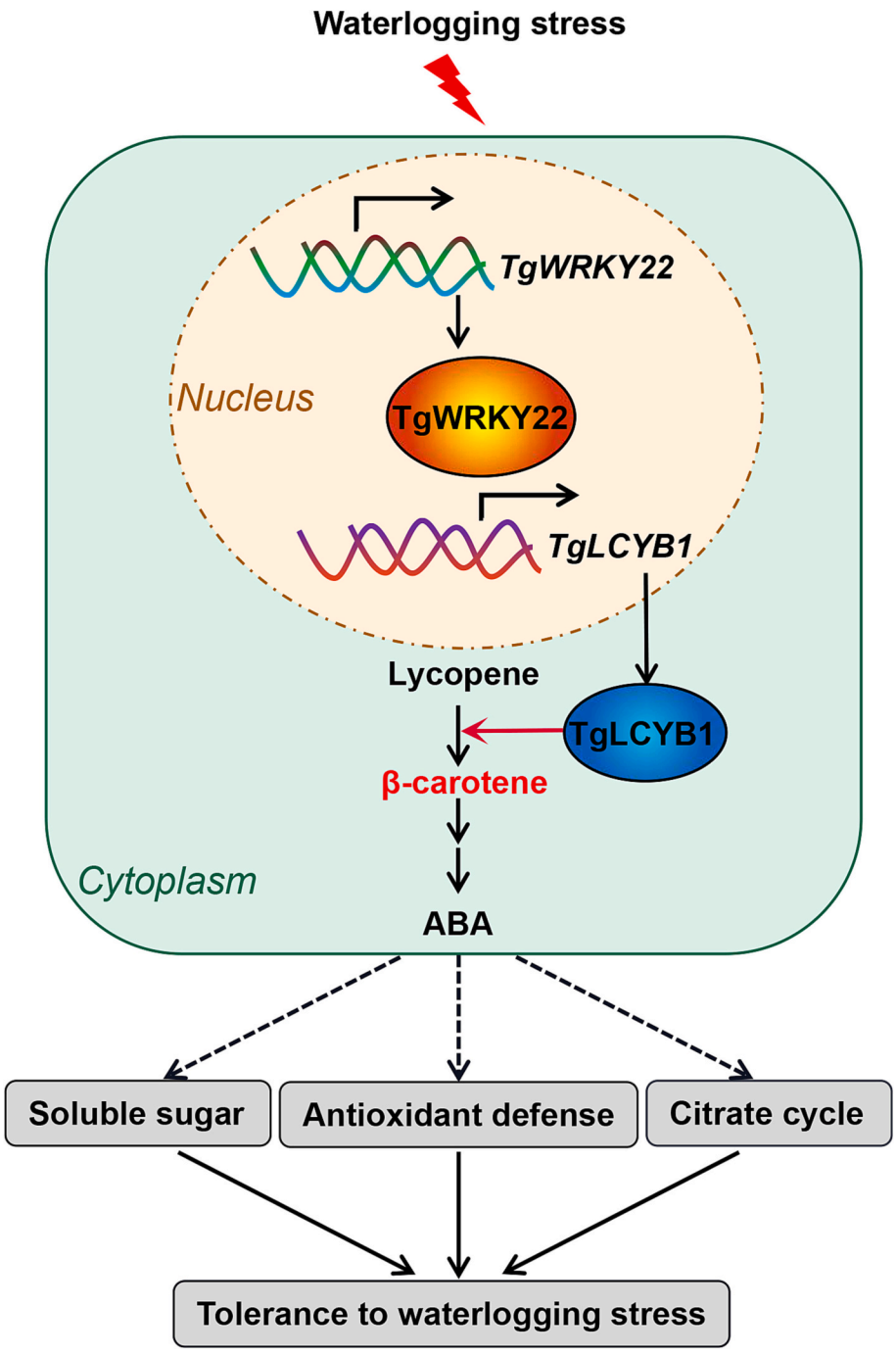

Fig. 10. A working model of TgWRKY22-TgLCYB1 involvement in response to waterlogging stress in *Torreya grandis*.

role in the waterlogging stress response based on the following evidence: first, waterlogging significantly up-regulated the expression of *TgLCYB1* (Fig. 2B); second, overexpression of *TgLCYB1* improved the activity of antioxidant enzymes to mitigate waterlogging-induced oxidative damage, thereby enhancing the tolerance of tomato plants to waterlogging stress (Figs. 3, 5 and 6); third, application of CPTA markedly enhanced the sensitivity of *T. grandis* to waterlogging stress (Fig. 4); and, fourth, a significant correlation between *TgLCYB1* expression and the waterlogging index existed according to an evaluation of *TgLCYB1* expression across nine *T. grandis* cultivars with varying waterlogging index (Fig. S3). This result suggested that the role of the *LCYB* gene in stress responses varied across different plant species. The carotenoid biosynthesis pathway is highly intricate, consisting of multiple branches [1]. Consequently, the accumulation of specific metabolites resulting from the *LCYB* gene could potentially affect other metabolites within this pathway, particularly upon exposure to adverse environmental conditions.

Several studies have demonstrated that the rapid accumulation of ABA can enhance stress tolerance by inducing the antioxidant defense system [37,38]. β-carotene serves as a precursor in ABA biosynthesis. Therefore, we hypothesized that overexpression of *TgLCYB1* in tomato could enhance the activity of antioxidant enzymes by increasing ABA content. Consistently, our findings revealed significantly higher ABA content in *TgLCYB1* transgenic lines compared with wild-type plants (Fig. 7).

Transcriptional regulation plays a crucial role in the biosynthesis of

carotenoids in plants [39]. To date, various transcription factors have been discovered as regulators of carotenoid metabolism, including ERFs, NACs, WRKYs, MYBs, and MADSs, among others [3]. A study by [40] demonstrated that 79 transcription factors directly influenced the expression of *LCYB* in banana fruit. Specifically, these factors were associated with the degradation of lycopene to carotenoids by up-regulating the expression of *CpLCYB* and *CpCHYB* during papaya fruit ripening [41]. In *Citrus sinensis*, CsMADS6 enhanced carotenoid biosynthesis by promoting the expression of *CsLCYB1*, *CsPSY*, *CsPDS*, and *CsCCD1* through direct binding to their respective promoters [42]. Limited reports, however, have been published regarding the transcription factors that are directly involved in the regulation of carotenoid synthesis during exposure to stress conditions. In this study, we identified TgWRKY22 as a transcription factor that directly binds to the *TgLCYB1* promoter (Fig. 9A). Dual-luciferase analysis revealed that TgWRKY22 was induced by waterlogging stress and functioned as a positive regulator in controlling the expression of *TgLCYB1* (Fig. 9B–D). A recent study showed that several transcription factors, such as, TgMYB48, enhanced β-carotene biosynthesis by promoting the expression of *TgLCYB1* during the seed development of *T. grandis* [2]. It remains unclear, however, whether these transcription factors also regulate *TgLCYB1* expression in response to waterlogging stress.

Based on our findings, we proposed a model wherein TgWRKY22 acted as a positive regulator of *TgLCYB1* expression, leading to enhanced antioxidant enzyme activities. This regulation, in turn, mitigated the accumulation of H_2O_2, reduced MDA content, and decreased the percentage of electrolyte leakage induced by waterlogging stress. Consequently, these effects significantly influenced the response to waterlogging stress (Fig. 10). The insight gained from this study contribute to a deeper understanding of the biological aspects of carotenoid metabolic regulation in the context of waterlogging stress. Moreover, these results provide a scientific foundation and practical implications for future research endeavors in this field.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.126702.

CRediT authorship contribution statement

Zhihui Liu: Methodology, Validation, Investigation, Writing – original draft. **Jiawen Yan:** Methodology, Validation, Investigation. **Tongtong Wang:** Methodology. **Weijie Chen:** Methodology, Validation. **Jinwei Suo:** Writing – review & editing, Supervision, Funding acquisition. **Jingwei Yan:** Writing – review & editing, Supervision, Funding acquisition. **Jiasheng Wu:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (32001349 and 32371921); the Scientific Research Startup Fund Project of Zhejiang A&F University (2022LFR033). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

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