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Overexpression of *IbPAL1* promotes chlorogenic acid biosynthesis in sweetpotato

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ABSTRACT

Sweetpotato [*Ipomoea batatas* (L.) Lam.], a food crop with both nutritional and medicinal uses, plays essential roles in food security and health-promoting. Chlorogenic acid (CGA), a polyphenol displaying several bioactivities, is distributed in all edible parts of sweetpotato. However, little is known about the specific metabolism of CGA in sweetpotato. In this study, *IbPAL1*, which encodes an endoplasmic reticulum-localized phenylalanine ammonia lyase (PAL), was isolated and characterized in sweetpotato. CGA accumulation was positively associated with the expression pattern of *IbPAL1* in a tissue-specific manner, as further demonstrated by overexpression of *IbPAL1*. Overexpression of *IbPAL1* promoted CGA accumulation and biosynthetic pathway genes expression in leaves, stimulated secondary xylem cell expansion in stems, and inhibited storage root formation. Our results support a potential role for *IbPAL1* in sweetpotato CGA biosynthesis and establish a theoretical foundation for detailed mechanism research and nutrient improvement in sweetpotato breeding programs.

1. Introduction

Specific nutrients and health-promoting values in food crops are of high current interest. Sweetpotato [*Ipomoea batatas* (L.) Lam.] is an important food, feed, industrial, and energy crop yielding about 200 million metric tons per year on 9 million ha. Both leaves and storage roots of sweetpotato are used for human consumption and provide carbohydrates, fibers, carotenes, thiamine, riboflavin, niacin, minerals, vitamins A and C, and high-quality protein [1]. Several functional ingredients in sweetpotato give it medicinal value, in particular chlorogenic acid (CGA), whose health-promoting effects include antioxidant, antimicrobial, anti-inflammatory, and antitumor activities [2–4].

Classically, CGA refers to 5-*O*-caffeoylquinic acid. A wider definition of CGAs includes a large group of compounds formed between hydroxycinnamic acids (caffeic, ferulic, and *p*-coumaric acids) and quinic acid that include mono-, di-, tri-, and tetra-esters and mixed esters [5]. Some of these derivatives are specific to plant species or families, but CGA can be found in several herbs and foods. While herbal medicinal sources always come from honeysuckle [6–8], mikania [9], tea [10,11], and gardenia [12], most

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dietary sources of CGA are coffee beans [13], apples [14], potatoes [15], and sweetpotatoes [16]. CGA's wide distribution in the leaves, stems, and roots of sweetpotato suggests the potential of increasing the crop's resistance to biotic stresses and nutritional benefits [17–21]. It is desirable to increase CGA content in sweetpotato in order to achieving the goals of guaranteeing stable production for food security and providing available nutrients to a large population of the world.

Plant biosynthesis of CGAs occurs downstream of the phenylpropanoid pathway, a plant secondary metabolism pathway mediated by phenylalanine ammonia lyase (PAL). There is little clarity about the pathways forming other isomers of the extended CGA family, but the enzymes catalyzing the conversions that produce CGA are well established. Three routes have been proposed (Fig. S1): route 1 with the conversion of caffeoyl CoA and quinic acid to CGA through hydroxycinnamoyl CoA: quinate hydroxycinnamoyl transferase (HQT), route 2 through the conversion of *p*-coumaroyl quinic acid to CGA catalyzed by *p*-coumarate 3′ hydroxylase (C3H), and route 3 through the conversion of caffeoyl-D-glucose to CGA through hydroxyl cinnamoyl D-glucose: quinate hydroxycinnamoyl transferase (HCGQT) [22]. Numerous CGA biosynthesis pathway genes have been reported to affect CGA accumulation [23], suggesting that it is a direct and efficient way to explore gene engineering and synthetic biology applications of CGA by identifying the molecular mechanism. However, information about the functional genes and specific pathway of CGA synthesis in sweetpotato remains limited.

As a crucial rate-limiting enzyme that mediates the conversion of L-phenylalanine to trans-cinnamic acid in the first step of the phenylpropanoid pathway, PAL links primary and secondary metabolism [24–26]. PALs are helix-containing proteins that contain a common domain, 4-methyldiene-imidazol-5-one (MIO), in their active site for catalysis [27,28]. In plants, the PAL proteins are encoded mostly by a multigene family and specific *PAL* isogenes often show a unique but overlapping expression pattern, suggesting that PAL isoforms have distinct but redundant functions [29]. In *Coffea canephora*, three *PAL* genes have been characterized and appear to fall into two different groups. *CcPAL1* and *CcPAL3* are associated with the accumulation of CGA, whereas *CcPAL2* may contribute more to flavonoid accumulation [30]. Sweetpotato *PALs* are known [31,32], and two proteins (IPBPAL, M29232; IPBPALA, D78640) were isolated from sweetpotato root following wounding [33]. However, little is known about their function in CGA biosynthesis.

In this study, we cloned a novel PAL gene, *IbPAL1*, from a Sushu 16 transcriptome database and validated the positive correlation between the expression level of *IbPAL1* and CGA content in sweetpotato. Overexpression of *IbPAL1* in transgenic sweetpotato greatly increased the CGAs contents and altered the expression level of CGA biosynthetic pathway genes in leaves. Enlarged secondary xylem cells in stems and reduced storage roots were observed in overexpressing transgenic plants. Collectively, this study provides the basic insights for nutritional quality improvement and synthetic biology application of functional nutrients in sweetpotato.

2. Materials and methods

2.1. Plant materials

A transcriptome database from Sushu 16 (a variety developed by Jiangsu Academy of Agricultural Sciences) was constructed in our laboratory and used for gene cloning. The cloned gene was further introduced into sweetpotato cv. Xushu 29 (a variety suitable for transformation) for function characterization. Cuttings (sweetpotato tips, top 10 cm) obtained from the field were planted in sterilized soil in 25 L plastic (35 cm height × 25 cm top diameter × 28 cm bottom diameter) pots at 25 °C under 16 h light/8 h dark in growth chamber. For biological replicates, at least five pots were included in each treatment.

For *IbPAL1* expression pattern analysis in Sushu 16, the 3rd fully expanded leaves and stems from shoot apical meristems, fibrous roots, pigment roots, and storage roots were collected. For expression level and PAL content analysis in overexpressing transgenic plants and Xushu 29, the 3rd fully expanded leaves, stems, and roots from two-month-old plants were collected. Samples were immediately frozen in liquid nitrogen and stored at –80 °C for RNA extraction or freeze-dried for high performance liquid chromatography (HPLC) analysis.

2.2. RNA isolation and first-strand cDNA synthesis

Total RNA was extracted from sweetpotato plants using RNAprep Pure Plant kit (Polysaccharides & Polyphenolics-rich) (Tiangen Biotech Co., Beijing, China) according to the manufacturer's protocol. First-strand cDNA was synthesized using a Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase kit (Promega Biotech Co., Beijing, China) with 1 μg of total RNA.

2.3. Isolation and cloning of IbPAL1

According to the Sushu 16 transcriptome database, a unigene (C41375.Contig_All) predicted to encode a phenylalanine ammonia lyase was used to design primers to amplify a full-length fragment of the candidate gene (primer pair IbPAL1-F/R, Table S1). The PCR product was amplified by KOD FX (TOYOBO Co., Osaka, Japan) and then cloned into a pEASY-Blunt Cloning Vector (TransGen Biotech Co., Beijing, China) for sequencing. Five independent clones were sequenced to confirm the sequence.

2.4. Protein sequence analysis

Theoretical molecular weight and isoelectronic point (pI) were calculated by ProtParam tool (http://web.expasy.org/protparam/). The active site of the IbPAL1 protein was identified with InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/). Prediction of the 3-D structure was performed using phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/). Sequences similar to IbPAL1 were identified using the BLASTP search program of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). A phylogenetic tree

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was constructed by the UPGMA method using MEGA 6 [34]. Multiple sequence alignments were performed with BioEdit software. Prediction of IbPAL1 subcellular localization was performed with Localizer [35] and CELLO2GO [36].

2.5. Subcellular localization

Subcellular localization analysis of IbPAL1 in 3-week-old tobacco (*Nicotiana benthamiana*) leaves was performed as described previously [37]. The coding sequence of *IbPAL1* was amplified and fused to the N-terminus of GFP under control of the *CaMV35S* promoter in the transient expression vector pCAMBIA1305-GFP (primer pair PALG-F/R, Table S1). A transient expression construct was introduced into *Agrobacterium tumefaciens* strain GV1301, which was then infiltrated into *N. benthamiana* leaves. After 2 days, the infiltrated parts of tobacco leaves were sampled and used to isolate *N. benthamiana* protoplasts. Confocal imaging analysis was performed using a Leica TCS SP5 laser scanning confocal microscope.

2.6. Primer design and expression analysis

Specific primer pairs for quantitative real-time PCR (qRT-PCR) amplification were designed with the GenScript online tool (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool) to amplify products of 80–150 bp, with an optimal primer melting temperature (T_m) of 60 °C and GC contents between 40% and 60%. qRT-PCR was performed using a SYBR Premix Ex Taq kit (TaKaRa Biomedical Technology Co., Beijing, China) on an ABI prism 7900 Real-Time PCR System. The $2^{-\Delta\Delta CT}$ method [38] was used to analyze relative changes in gene expression. The sweetpotato *Tubulin* gene was used as a reference. For qRT-PCR of genes in the CGA pathway, the primers were initially selected by separation of their PCR products on a temperature gradient from 60 to 95 °C to confirm the presence of a single PCR product and specificity of the qRT-PCR. Primers are listed in Table S1.

2.7. Vector construction and plant transformation

The coding region of *IbPAL1* was introduced into the plant expression vector pCAMBIA1305 under the *CaMV35S* promoter (primer pair OPAL-F/R, Table S1). The resulting *CaMV35S*::IbPAL1 plasmid was transferred into *A. tumefaciens* EHA105 and introduced into Xushu 29 for overexpression analysis [39]. After transgenic sweetpotato plants were generated, vine cuttings from them were propagated. Regenerated plants were transplanted into pots and grown in a greenhouse for further analysis.

2.8. PAL content assay

The protein content of PAL was assayed with a Plant PAL ELISA Kit (Shanghai Jianglai Industrial Limited By Share Ltd., Shanghai, China). Homogenized samples were prepared in PBS buffer (pH 7.4). After centrifugation at 2500 ×*g* for 20 min, the supernatant was collected as enzyme source. After addition of stop solution, the concentration was measured at 450 nm with a spectrophotometer. The Plant PAL ELISA Kit provided a set of calibration standards ranging from 0.2 U L^{-1} to 4.0 U L^{-1}, which were assayed at the same time to create a standard curve. The concentration of PAL in the samples was determined by comparison of the A_{450} absorbance to the standard curve.

2.9. Paraffin sectioning

The stems between the 6th and 7th fully expanded leaves from the top harvested from the field were excised and fixed in FAA solution (5 mL formalin, 5 mL glacial acetic acid, 90 mL 70% ethanol) for 48 h. After dehydration in an ethanol gradient series from 50% to 100%, the samples were cleared with xylene and embedded in paraffin. Sections of 10 μm were prepared with a rotary microtome (LEICA RM2245, Wetzlar, Germany), dewaxed in xylene, and stained with safranin solution (1% safranin dissolved in 50% ethanol) and Fast Green solution (5 g of Fast Green in 100 mL of 95% ethanol). The sections were viewed and photographed under an Olympus BX51 microscope (Olympus, Tokyo, Japan).

2.10. Histochemical staining

Histochemical staining was performed by the Wiesner method [40], using stems between the 3rd and 4th fully expanded leaves from the top harvested from 1.5-month-old same-sized plants in the greenhouse. More than five stems of the *IbPAL1*-overexpressing and control plants were cut into sections by hand and dipped in phloroglucinol-HCl solution (one volume of concentrated HCl mixed with two volumes of 3% phloroglucinol in ethanol) for 3–5 min. The stained sections were observed and photographed with an Olympus BX51 microscope.

2.11. HPLC quantification of CGA and isochlorogenic acids (ICGAs)

The same samples used for real-time PCR experiments were assayed for CGA content, following Kobayashi et al. [41], with some modifications. Fifty milligrams of freeze-dried powder were added to 5 mL of 70% ethanol (v/v) and incubated at 85 °C for 40 min. After centrifugation at 3000 r min^{-1} for 15 min, the supernatant was collected, filtered through a 0.22 μm membrane filter, and stored at 4 °C in the dark.

After fivefold dilution of all test solutions, 10 μL was injected for HPLC analysis using an Agilent HP 1100 series HPLC system (Agilent Technologies, Santa Clara, California, USA) with an InertSustain C18 250 mm × 4.6 mm column (Shimadzu Global Laboratory Consumables Co., Shanghai, China). The mobile phase included acetonitrile (solvent A), 40% methanol (solvent B) and aqueous formic acid (100:0.1, v/v, solvent C) in the following gradient system: initial, 80% A/10% B/10% C; 0–3 min, 65% A/10% B/25% C; 3–15 min, 55% A/10% B/35% C; 15–15.1 min, 75% A/10% B/ 15% C; 15.1–20 min, back to the initial condition for re-equilibration. The flow rate was 0.5 mL min^{-1} and the detection wavelength was set at 318 nm.

Standards for CGA, isochlorogenic acid A (ICAA), isochlorogenic acid B (ICAB), and isochlorogenic acid C (ICAC) were from Sigma. For CGA content measurement in Sushu 16, CGA standard solutions ranging from 1 to 50 μg mL^{-1} were prepared in 70% ethanol. For content measurement of CGA and

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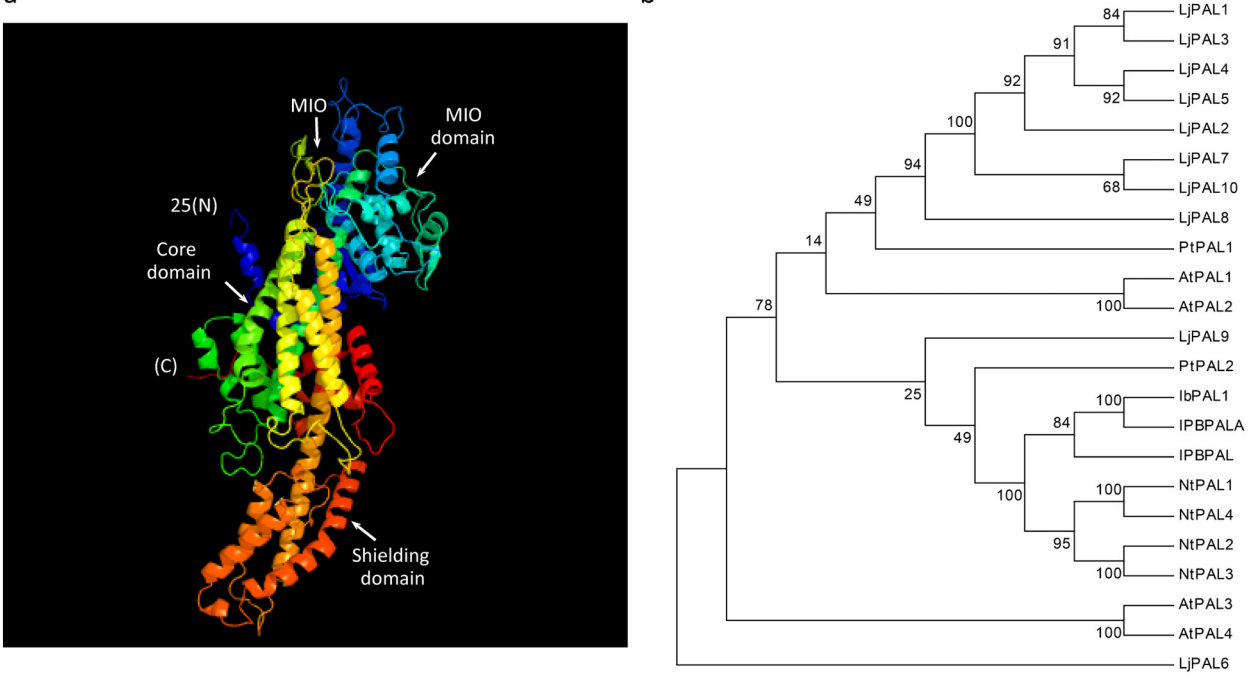

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three isochlorogenic acids (ICGAs) in transgenic plants, standard solutions in a concentration range of 1 to 50 μg mL^{-1} containing CGA, ICAA, ICAB, and ICAC were prepared in 70% ethanol.

2.12. Statistical analysis

All values are presented as mean ± SD from at least three independent experiments with three replicates each. Statistical analysis was performed with the GLM procedure of SAS (SAS Institute Inc., Cary, North Carolina, USA) to reveal significant differences (*, $P < 0.05$; **, $P < 0.01$).

3. Results

3.1. Isolation and characterization of IbPAL1

The PAL cDNA clone (C41375.Contig1_All) was obtained from the transcriptome sequencing database of Sushu 16. PCR was performed to amplify the gene from transcripts of Sushu 16 tender leaves. Sequencing of the fragment showed a complete open reading frame of 2130 bp, which encodes a protein of 710 amino acids (Fig. S2, GenBank: MN823653). The estimated molecular weight of the protein was 77.1 kDa and the *pI* was 6.02. Structure analysis revealed that the helix-containing protein harbored a phenylalanine ammonia-lyase active site (amino acids 191–207) which formed a MIO (4-methyldiene-imidazol-5-one) domain on top of three polar helices. Two additional structural elements, a specific shielding domain sites over the active center and a mobile N-terminal extension (Fig. 1a), indicated that the isolated gene encoded a typical PAL protein.

Sequence alignment showed that the protein had high similarity with the other two known PAL enzymes from sweetpotato, in particular IPBPALA (96.4%) (Fig. S3). As this protein showed the highest sequence similarity to AtPAL1 (83.6%) from *Arabidopsis*, we named it IbPAL1. A phylogenetic tree constructed with the full-length amino acid sequences showed that IbPAL1 clustered with PALs from sweetpotato and tobacco (Fig. 1b). Whereas PAL proteins in tobacco can be divided into two distinct groups, subfamily I with NtPAL1 and NtPAL4, and subfamily II with NtPAL2 and NtPAL3, the amino acid sequence of IbPAL1 shared higher similarity with NtPAL1 (87.2%) and NtPAL2 (87.6%) (Fig. 1b, Fig. S3).

It has been reported that PALs may be located in cytoplasm, mitochondria, Golgi apparatus, chloroplast, peroxisome, and other membrane organelles at subcellular level [25]. To determine the subcellular localization of IbPAL1 protein, we transiently expressed the fusion protein IbPAL1-GFP in tobacco (*N. benthamiana*) leaves. As shown in Fig. 1c, the control vector 35S::GFP showed expression in both nuclei and cytosol, while IbPAL1::GFP showed a typical endoplasmic reticulum (ER) localization, indicating that IbPAL1 was targeted to the ER.

3.2. Expression profiles of IbPAL1 and CGA content in sweetpotato organs

Transcripts of *IbPAL1* were detected in all tested tissues. The lowest relative expression was in stems, with higher expression in leaves and pigment roots (Fig. 2a). Maximum CGA content was found in leaves, containing 8.55 ± 1.41 mg g^{-1} (DW), followed by pigment roots (7.14 ± 0.86 mg g^{-1} DW) and fibrous roots (3.50 ± 0.24 mg g^{-1} DW). In accordance with the expression level, stems and storage roots contained less CGA (Fig. 2b). These results showed that the expression pattern of *IbPAL1* corresponded with CGA distribution in tissues of sweetpotato.

3.3. Identification of overexpressed IbPAL1 transgenic sweetpotato

To confirm that *IbPAL1* is responsible for the biosynthesis of CGA in sweetpotato, we constructed and transformed an overexpression vector of *IbPAL1* into Xushu 29. By PCR identification of genomic DNA with both hygromycin (*Hyg*-F/*Hyg*-R, Table S1) and 35S::IbPAL1-specific fragment which includes a portion of the 35S promoter and *IbPAL1* (dePAL-F/dePAL-R, Table S1), 17 positive plants were identified, suggesting that the recombined *IbPAL1* gene had been integrated into the genome. The 17 independent positive lines and Xushu 29 were propagated and used for qRT-PCR analysis and PAL content assay after 2 months. Three independent transgenic lines showing about 2- to 4-fold higher expression of *IbPAL1* than Xushu 29 were selected as representative lines and referred to as OVs (Fig. 3a). The PAL content in overexpressing transgenic plants was also increased 2- to 2.5-fold (Fig. 3b)

3.4. Phenotypic characterization of IbPAL1-overexpressing transgenic plants

The OV lines showed no marked difference in morphology of aboveground parts but showed harder stems than Xushu 29. Anatomical examination of stems showed enlarged expanding secondary xylem cell in OV lines (Fig. 4a). In younger stems, phloroglucinol-HCl staining also revealed a thicker secondary xylem with more lignified cells in OV lines (Fig. 4b). These results suggested that overexpression of

Fig. 1 - Characterization of IbPAL1. (a) 3-D structure of IbPAL1. IbPAL1 contains three domains including MIO domain, core domain, and shielding domain. Image coloured by rainbow N to C terminus. Model dimensions (Å): X:49.781 Y:135.025 Z:59.937 (b) Evolutionary relationships of PAL proteins. Other PALs used for analysis were from the following plant species: Arabidopsis (*Arabidopsis thaliana*, At), tobacco (*Nicotiana tabacum*, Nt), honeysuckle (*Lonicera japonica*, Lj), aspen (*Populus tremuloides*, Pt), and sweetpotato. LjPAL1 (Locus ID: Lj1g3v4590760), LjPAL2 (Locus ID: Lj1g3v4590770.1), LjPAL3 (Locus ID: Lj1g3v4590840.1), LjPAL4 (Locus ID: Lj1g3v4590850.1), LjPAL5 (Locus ID: Lj1g3v4590850.2), LjPAL6 (Locus ID: Lj2g3v3339740.1), LjPAL7 (Locus ID: Lj3g3v0602630.2), LjPAL8 (Locus ID: Lj5g3v0659760.1), LjPAL9 (Locus ID: Lj5g3v1811400.1), LjPAL10 (Locus ID: Lj0g3v0350889.1), AtPAL1 (Locus ID: At2g37040), AtPAL2 (Locus ID: At3g53260), AtPAL3 (Locus ID: At5g04230), AtPAL4 (Locus ID: At3g10340), NtPAL1 (P25872), NtPAL2 (P35513), NtPAL3 (P45733), NtPAL4 (ACJ66297), PtPAL1 (AF480619), PtPAL2 (AF480620). (c) Subcellular localization of IbPAL1, Scale bars, 10 μm.

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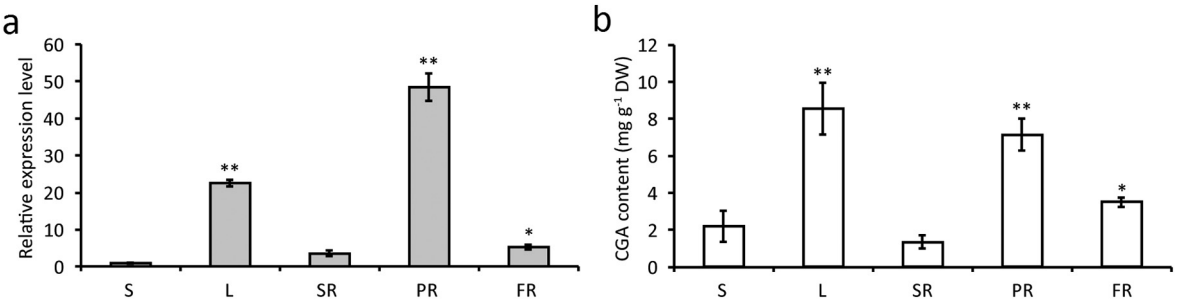

Fig. 2 – *IbPAL1* expression and CGA content in organs of sweetpotato. (a) Expression profiles of *IbPAL1* in tissues of sweetpotato cv. Sushu 16. (b) Measurement of CGA content in corresponding tissues of Sushu 16. S, stem; L, leaf; SR, storage root; PR, pigment root; FR, fibrous root; CGA, chlorogenic acid. Values represents means ± SD. * and ** indicate significant differences compared to the stem at $P < 0.05$ and $P < 0.01$ (t-test), respectively.

IbPAL1 promoted lignification in the early stage of secondary growth and thus stimulated secondary xylem cell expansion in sweetpotato stem.

Compared with Xushu 29, marked phenotypic changes in storage roots were observed in OV lines after the plants were harvested from the field (Fig. 5a). Compared with Xushu 29 (2–4 storage roots per plant), fewer storage roots but more pigment roots were produced by *IbPAL1* OV lines (Fig. 5b, c). The smaller size of storage roots in OV lines was accompanied by severe lignification. Thus, overexpressed *IbPAL1* inhibited storage root formation.

3.5. *Overexpression of IbPAL1 promoted CGAs accumulation and altered the CGA biosynthetic pathway genes expression in leaves*

In comparison with Xushu 29 (5.80 ± 0.17 mg g^{-1} DW), the leaf CGA content was significantly increased in all the three transgenic plants, which contained 17.76 ± 0.47, 10.94 ± 0.15, and 7.79 ± 0.39 mg g^{-1} DW) (Fig. 6a). ICAA, ICAB and ICAC were also detected in Xushu 29 and transgenic plants (Fig. S4). Fig. 6a shows that ICAA was the main form of three ICGAs in leaves and accumulated to the highest level in both Xushu 29 and OV lines. The abundances of ICGAs were higher in transgenic plants, and the most striking increase was in OV1 with an approximately 2-fold increase over Xushu 29. The increasing trend of the contents of these three ICGAs in OV3 was less marked. A significant increase of ICAB content (4.25 mg g^{-1}) was observed in OV2, whereas that in Xushu 29 was 1.89 mg g^{-1}. In the other 14 transgenic lines, the CGA and ICGAs concentrations varied with *IbPAL1* expression level, from unchanged to a greater than 2-fold increase (Fig. S5).

The effect of overexpressed *IbPAL1* on downstream CGA pathway genes expression was analyzed to further study the mechanism of sweetpotato CGA biosynthesis. From the Sushu 16 transcriptome sequencing library, we finally isolated six CGA biosynthesis pathway genes including *IbC4H*, *Ib4CL*, *IbHCT*, *IbC3H*, *IbHQT*, and *IbUGCT*. As shown in Fig. 6b, the transcript levels of both *IbC4H* and *IbUGCT* were significantly increased in OV lines. In comparison with Xushu 29, a slight increase was observed in expression of *Ib4CL* and *IbHCT* in three transgenics. Expression of *IbC3H* was not significantly different between Xushu 29 and OV lines, whereas expression of *IbHQT* was decreased by 26%–35% relative to Xushu 29. These results suggested that *IbPAL1* promotes the biosynthesis of CGA by influencing the transcript levels of downstream pathway genes.

4. Discussion

4.1. *IbPAL1 plays a key role in sweetpotato CGA biosynthesis*

We isolated a novel PAL gene, *IbPAL1*, from sweetpotato cv. Sushu 16. *IbPAL1* encodes a 710-amino-acid protein with conserved

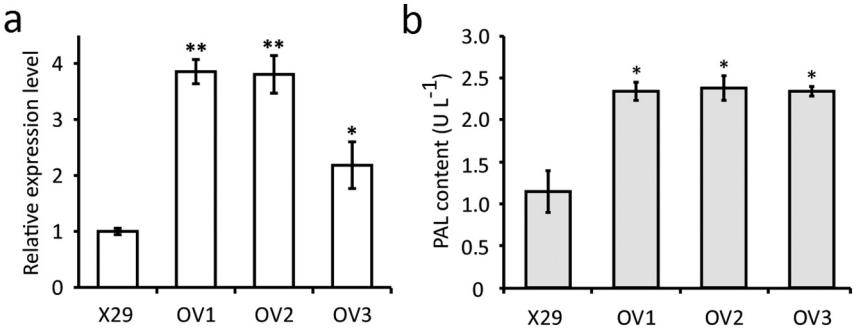

Fig. 3 – Transcript levels of *IbPAL1* (a) and PAL protein content (b) in Xushu 29 (X29) and overexpressing transgenic plants. Values represent means ± SD. Asterisks indicate a significant difference compared to X29 at *, $P < 0.05$; **, $P < 0.01$ (t-test). PAL, phenylalanine ammonia-lyase.

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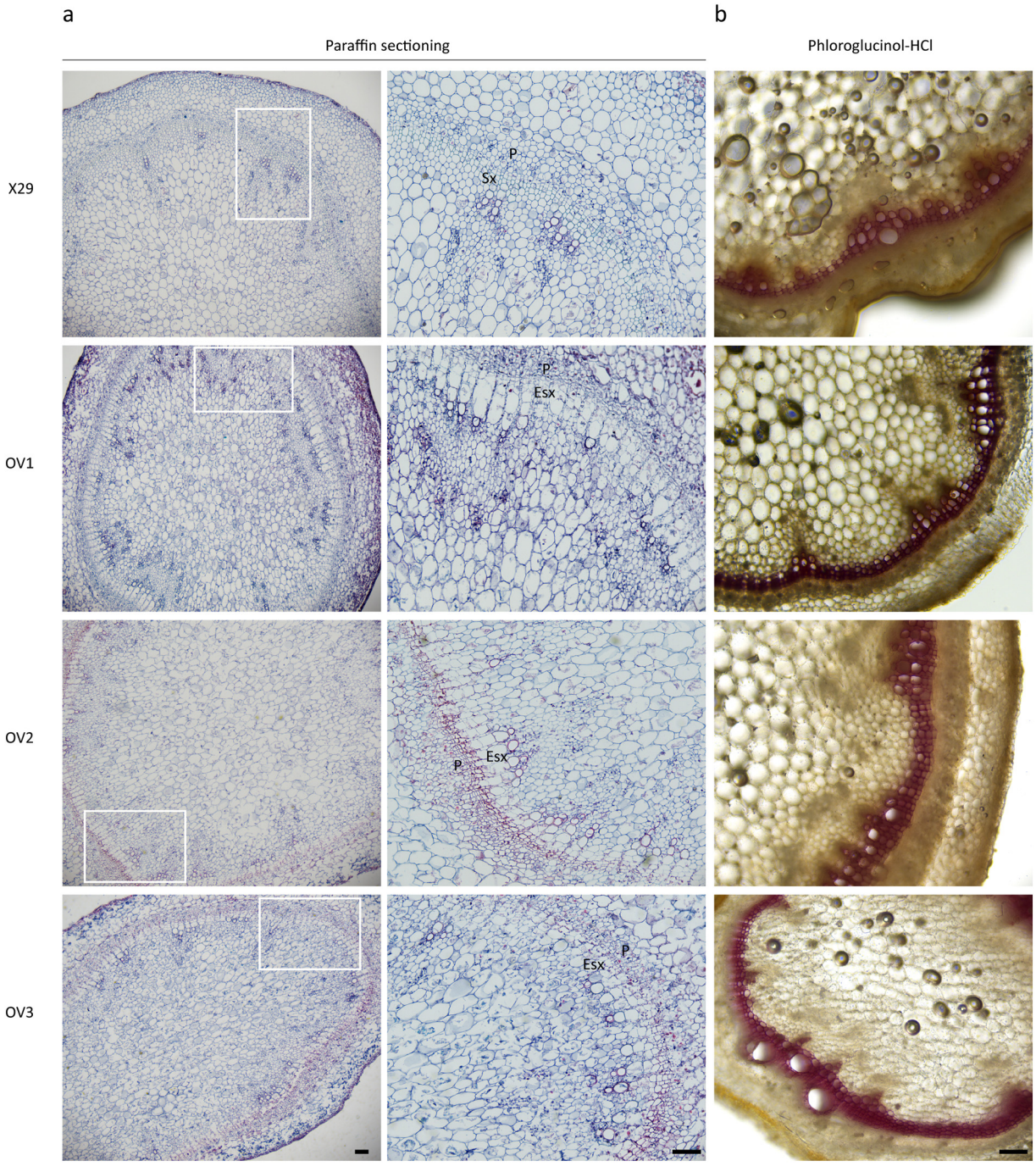

Fig. 4 – Phenotypic characterization of stems. (a) Paraffin sectioning of the 7th stem showed enlarged secondary xylem cell in OVs. P, phloem; Esx, expanding secondary xylem. (b) Phloroglucinol-HCl staining of the 4th stem showed thickened and more lignified secondary xylem cells in OVs. Scale bars, 100 μm.

domains and active site, especially the Ala-Ser-Gly triad (195–197) which can be converted into a MIO domain (Fig. 1a, Fig. S2). The formation of a MIO domain in the active site reveals an active PAL enzyme able to catalyze the synthesis of trans-cinnamic acid from L-phenylalanine [27,28]. Phylogenetic analysis and amino acid sequence alignment also demonstrated that IbPAL1 shares high identity with two previously isolated PAL proteins from sweetpotato (Fig. 1b, Fig. S3). However, there have been few studies describing functional analysis of these two proteins, and whether they are involved in CGA biosynthesis is unclear. PALs are soluble proteins that are localized mostly in the cytosol, whereas some more active ones are associated with the ER [42–44]. Subcellular localization of IbPAL1 showed that IbPAL1 is an ER-localized protein (Fig. 1c). These results demonstrate that IbPAL1 is a member of the PAL family and may possess high enzyme activity.

A positive association between *IbPAL1* expression and CGA content was confirmed. *IbPAL1* was preferentially expressed

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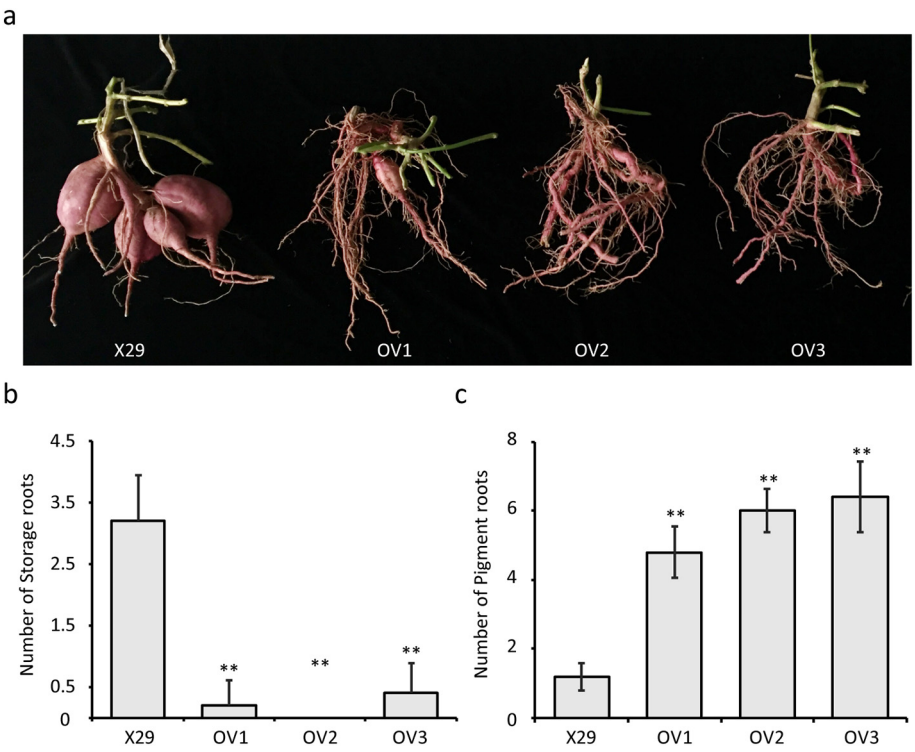

Fig. 5 – Phenotypic characterization of roots. (a) Inhibited storage root formation with more pigment roots in *IbPAL1*-overexpressing plants. (b) Storage root numbers of Xushu 29 (X29) and OVs. (c) Pigment root numbers of Xushu 29 and OVs. Values represents means ± SD. Asterisks indicate a significant difference compared to X29 at *, $P < 0.05$; **, $P < 0.01$ (*t*-test).

in leaves and pigment roots accumulating higher content of CGA, and weakly expressed in tissues containing much lower CGA including stems, storage roots, and fibrous roots (Fig. 2). In comparison with Xushu 29, overexpression of *IbPAL1* promoted CGA accumulation in leaves (Figs. 3, 6a, Fig. S5). Three ICGAs were also detected in leaves of Xushu 29 and OVs, and HPLC results confirmed that ICAA, which accounts for a high proportion of ICGAs, is the main isomer of ICGAs in sweetpotato leaves. The contents of three ICGAs showed various degrees of increase in OVs leaves (Fig. 6a, Fig. S5). Little is known about the biosynthesis of dicaffeoylquinic acid, but caffeoyl moiety can be combined with quinic acid to form either monocaffeoylquinic or dicaffeoylquinic acid [45]. CGA can be converted to ICAA by CGA pathway genes or enzymes in plants [46–48]. It can thus be speculated that promoted CGA accumulation provides the necessary precursor or substrate for ICGAs synthesis. The changes in the expression levels of *IbPAL1* and CGAs were not completely consistent in OVs (Figs. 3, 6a, Fig. S5), for unknown reasons. We speculate that *IbPAL1* is metabolically feedback-regulated at both transcriptional and post-transcriptional level.

4.2. *IbPAL1 promotes CGA biosynthesis by affecting expression of downstream pathway genes*

Flux through the phenylpropanoid pathway depends mainly on the expression levels of pathway genes [22,49]. Although the plant biosynthetic route of CGA remains unknown, major genes encoding key enzymes have been reported [23,50,51] to control the biosynthesis of CGA. As we obtained six downstream pathway genes from the Sushu 16 transcriptome database, the effect of overexpressed *IbPAL1* on these downstream genes was investigated. The effect seems to be related to their position in the biosynthesis pathway. Overexpressed *IbPAL1* may stimulate the accumulation of cinnamic acid, which activates the subsequent catalytic reaction controlled by *IbC4H* and *IbUGCT*. The transcripts of both the two genes were significantly accumulated in *IbPAL1*-overexpressing transgenic plants (Fig. 6b). However, the effectiveness of *IbPAL1* may gradually decreased as specialized branches of phenylpropanoid metabolism shunt common metabolic intermediates in different directions. *p*-Coumaroyl-CoA is a key branch point in phenylpropanoid biosynthesis and 4CL plays a central role in redirecting the biosynthetic pathway towards either the CGA or other branches in the phenylpropanoid pathway [5,52]. Thus, while there was a slight increase in expression of *Ib4CL* and *IbHCT*, the effect on *IbC3H* of overexpressing *IbPAL1* was smaller (Fig. 6b). As for *IbHQT*, the transcript level was even decreased in OVs compared with Xushu 29 (Fig. 6b). The correspondence between HQT and CGA was less clear than that between the other pathway genes and CGA. The role of *HQT* in CGA accumulation has been reported in many plants [22,53–56]. However, some studies in potato [57–60] showed no association between *HQT* expression and CGA concentration. The activity of HQT is fully reversible: HQT can catalyze a reverse reaction by which caffeoyl-CoA is

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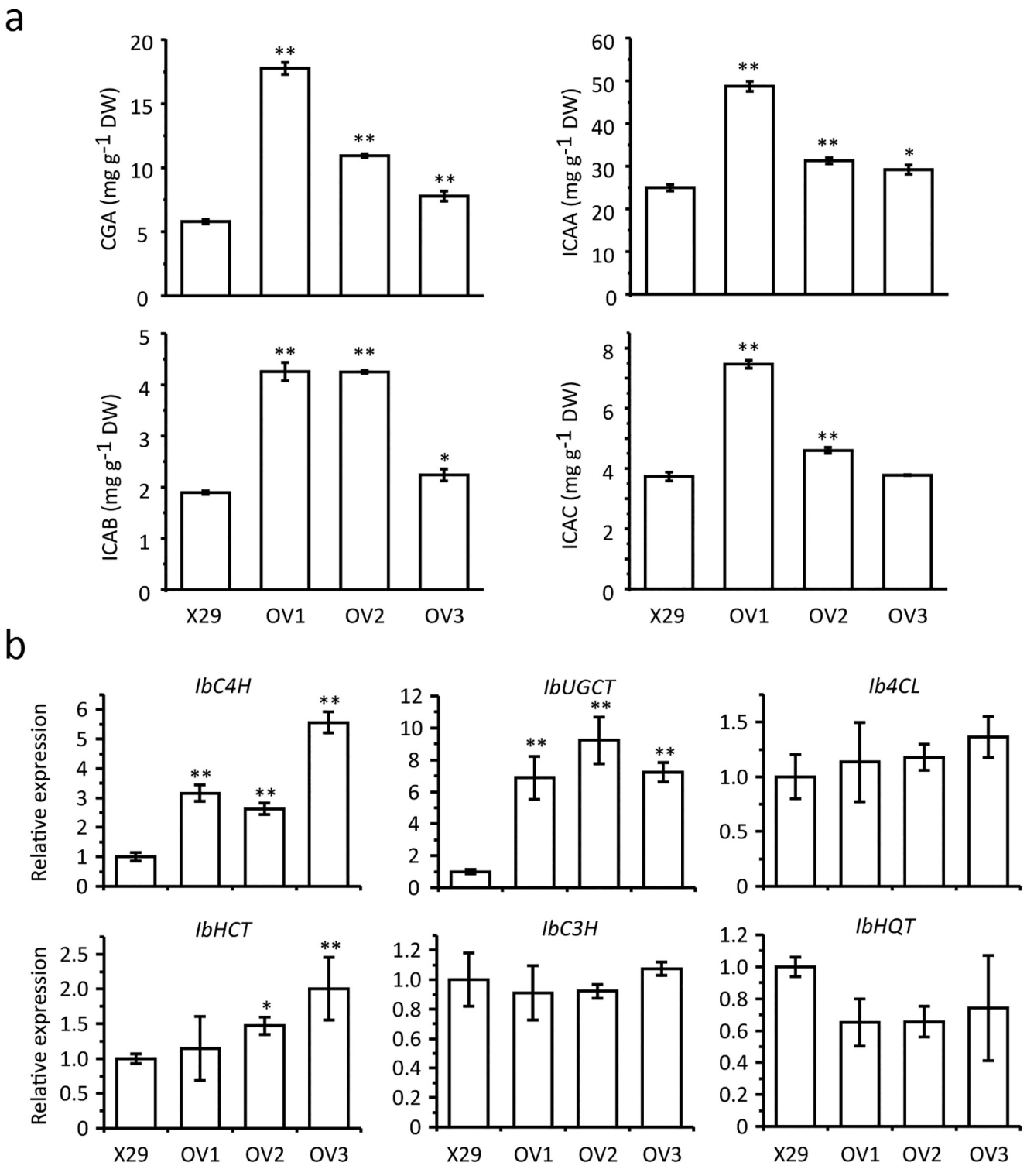

Fig. 6 – Overexpression of *IbPAL1* increased CGAs accumulation and altered CGA biosynthetic pathway genes expression in leaves. (a) CGA and ICGA contents in leaves of Xushu 29 and overexpression lines. CGA, chlorogenic acid; ICAA, isochlorogenic acid A; ICAB, isochlorogenic acid B; ICAC, isochlorogenic acid C. (b) Expression levels of CGA pathway genes in Xushu 29 and overexpressing transgenic plants. Values represent means ± SD. * indicates statistically significant based on t-test (*, $P < 0.05$; **, $P < 0.01$).

made from CGA, consistent with a complex dynamic regulation in CGA biosynthesis by metabolic feedback. We propose that overexpressed *IbPAL1* increased carbon flux into the CGA biosynthetic pathway, increasing CGA accumulation in leaves by increasing the transcription of downstream pathway genes.

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4.3. *IbPAL1 may possess a tissue-specific function in sweetpotato secondary metabolism*

As the entry-point enzyme controlling general phenylpropanoid metabolism, PAL affects the production of various bioactive aromatic compounds other than CGAs, such as flavonoids, anthocyanins, lignin, and condensed tannins. Overexpressed *IbPAL1* caused thickened lignified secondary xylem cell in stems (Fig. 4). As for the effect of overexpressed *IbPAL1* on storage root development, inhibited storage root formation with more pigment roots production was observed in OVs (Fig. 5). The production of sweetpotato storage roots is a complex process: initially formed adventitious roots transform to fibrous roots, with some of these subsequently acquiring pigmentation and undergoing thickening growth to form pigment roots that ultimately develop into storage roots [61]. Recent studies [31,32] have shown that storage root formation involves the regulation of lignin and starch biosynthesis, and increased lignification can suppress starch accumulation in developing storage roots, causing the reduction of storage root yield. We accordingly propose that an initial large increase in lignified pigment roots affected the formation of storage roots in our *IbPAL1*-overexpressing plants. Taking into account the expression pattern of *IbPAL1* (Fig. 2a), we speculate that *IbPAL1* is involved mainly in guiding the flux into the lignin biosynthesis pathway in stems and developing storage roots, suggesting a tissue-triggered functional specialization of *IbPAL1* in sweetpotato secondary metabolism.

5. Conclusions

We report the function of *IbPAL1* in sweetpotato CGA biosynthesis. *IbPAL1* is involved in the regulation of CGA accumulation. Overexpressing *IbPAL1* altered phenotype and affected phenylpropanoid flux, depending on the organ. More detailed studies would shed light on the molecular regulation of *IbPAL1* and specific metabolism of CGA biosynthesis in sweetpotato. Suppression of *IbPAL1* is one promising approach. An interesting question concerns the extent to which specific CGA biosynthesis can be promoted in sweetpotato without concomitant increases in other phenylpropanoid branches.

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Declaration of competing interest

Authors declare that there are no conflicts of interest.

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Author contributions

Yang Yu and Xiaofeng Bian conceived the research. Yang Yu, Yingjie Wang, Yue Yu, Peiyong Ma, and Zhaodong Jia performed the experiments. Xiaoding Guo and Yizhi Xie provided technical assistance. Yang Yu analyzed the data and wrote the manuscript. Xiaofeng Bian revised the manuscript. All authors read and approved the manuscript.

REFERENCES

[1] A.F.E.I. Sheikha, R.C. Ray, Potential impacts of bioprocessing of sweet potato: review, Crit. Rev. Food Sci. Nutr. 57 (2017) 455–471.
[2] N. Liang, D.D. Kitts, Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions, Nutrients 8 (2016) 16.
[3] Y.J. Liu, C.Y. Zhou, C.H. Qiu, X.M. Lu, Y.T. Wang, Chlorogenic acid induced apoptosis and inhibition of proliferation in human acute promyelocytic leukemia HL-60 cells, Mol. Med. Rep. 8 (2013) 1106–1110.
[4] J.G. Xu, Q.P. Hu, Y. Liu, Antioxidant and DNA-protective activities of chlorogenic acid isomers, J. Agric. Food Chem. 60 (2012) 11625–11630.
[5] M.N. Clifford, I.B. Jaganath, I.A. Ludwig, A. Crozier, Chlorogenic acids and the acyl-quinic acids: discovery, biosynthesis, bioavailability and bioactivity, Nat. Prod. Rep. 34 (2017) 1391–1421.
[6] T. Wang, X. Jiang, L. Yang, S. Wu, pH-gradient counter-current chromatography isolation of natural antioxidant chlorogenic acid from *Lonicera japonica* Thumb. using an upright coil planet centrifuge with three multi-layer coils connected in series, J. Chromatogr. A 1180 (2008) 53–58.
[7] Z. Xiang, Z. Ning, Scavenging and antioxidant properties of compound derived from chlorogenic acid in South-China honeysuckle, LWT-Food Sci. Technol. 41 (2008) 1189–1203.
[8] B. Zhang, R. Yang, C.Z. Liu, Microwave-assisted extraction of chlorogenic acid from flower buds of *Lonicera japonica* Thumb, Separ. Purif. Tech. 62 (2008) 480–483.
[9] L.V. de Melo, A.C. Sawaya, UHPLC-MS quantification of coumarin and chlorogenic acid in extracts of the medicinal plants known as guaco (*Mikania glomerata* and *Mikania laevigata*), Rev. Bras. Farmacogn. 25 (2015) 105–110.
[10] S.W. Zhang, C. Yang, E. Idehen, L. Shi, L.S. Lyu, S.M. Sang, Novel theaflavin-type chlorogenic acid derivatives identified in black tea, J. Agric. Food Chem. 66 (2018) 3402–3407.
[11] Z. Wang, M.N. Clifford, P. Sharp, Analysis of chlorogenic acids in beverages prepared from Chinese health foods and investigation, *in vitro*, of effects on glucose absorption in cultured Caco-2 cells, Food Chem. 108 (2008) 369–373.
[12] M.N. Clifford, W. Wu, J. Kirkpatrick, R. Jaiswal, N. Kuhnert, Profiling and characterisation by liquid chromatography/multistage mass spectrometry of the chlorogenic acids in *Gardeniae fructus*, Rapid Commun. Mass Spectrom. 24 (2010) 3109–3120.
[13] V.R. Preedy, Coffee in Health and Disease Prevention, Academic Press, London, UK, 2014.

[14] M.A. Awad, A. de Jager, L.M. van Westing, Flavonoid and chlorogenic acid levels in apple fruit: characterisation of variation, Sci. Hortic. 83 (2000) 249–263.
[15] L. Dao, M. Friedman, Chlorogenic acid content of fresh and processed potatoes determined by ultraviolet spectrophotometry, J. Agric. Food Chem. 40 (1992) 2152–2156.
[16] W. Zheng, M.N. Clifford, Profiling the chlorogenic acids of sweet potato (*Ipomoea batatas*) from China, Food Chem. 106 (2008) 147–152.
[17] M.Y. Liu, H.J. Sun, J.J. Wang, K.Y. Wang, J. Shen, D. Liu, J. Zhang, Y.P. Xie, The study on phenol metabolism in sweetpotato infected by *Ceratocystis fimbriata* Ellis et Halsted, Chin. Agric. Sci. Bull. 28 (2012) 226–230 (in Chinese with English abstract).
[18] H.C. Liu, L.Q. Duan, H.P. Li, S.J. Feng, B.B. Zhang, Chlorogenic acid enhances the virulence of *Lymantria dispar* necleopolyhydrovirus (LdNPV), Acta Entomol. Sin. 59 (2016) 568–572 (in Chinese with English abstract).
[19] M. Aidilla, P.B. Catherine, H.L. Alex, J.C. Michael, R. Lisa, M. Emilie, D.C. Kevin, M.H. Jonathan, Acute effects of chlorogenic acid on nitric oxide status, endothelial function, and blood pressure in healthy volunteers: a randomized trial, J. Agric. Food Chem. 60 (2012) 9130–9136.
[20] H. Jang, H.R. Ahn, H. Jo, K.A. Kim, E.H. Lee, K.W. Lee, S.H. Jung, C.Y. Lee, Chlorogenic acid and coffee prevent hypoxia-induced retinal degeneration, J. Agric. Food Chem. 62 (2014) 182–191.
[21] J. Santana-Gálvez, L. Cisneros-Zevallos, D.A. Jacobo-Velázquez, Chlorogenic acid: recent advances on its dual role as a food additive and a nutraceutical against metabolic syndrome, Molecules 22 (2017) 358.
[22] R.S. Payyavula, R. Shakya, V.G. Sengoda, J.E. Munyaneza, P. Swamy, D.A. Navarre, Synthesis and regulation of chlorogenic acid in potato: rerouting phenylpropanoid flux in HQT-silenced lines, Plant Biotechnol. J. 13 (2015) 551–564.
[23] Y. Yuan, Z.Y. Wang, C. Jiang, X.M. Wang, L.Q. Huang, Exploiting genes and functional diversity of chlorogenic acid and luteolin biosyntheses in *Lonicera japonica* and their substitutes, Gene 534 (2014) 408–416.
[24] M. Kamalipourazad, M. Sharifi, H.Z. Maivan, M. Behmanesh, N.A. Chashmi, Induction of aromatic amino acids and phenylpropanoid compounds in *Scrophularia striata* Boiss. cell culture in response to chitosan-induced oxidative stress, Plant Physiol. Biochem. 107 (2016) 374–384.
[25] R.F. Ma, Q.Z. Liu, Y. Xiao, L. Zhang, Q. Li, J. Yin, W.S. Chen, The phenylalanine ammonia-lyase gene family in *Isatis indigotica* Fort.: molecular cloning, characterization, and expression analysis, Chin. J. Nat. Med. 14 (2016) 801–812.
[26] S. Masuo, Y. Kobayashi, K. Oinuma, N. Takaya, Alternative fermentation pathway of cinnamic acid production via phenyllactic acid, Appl. Microbiol. Biotechnol. 100 (2016) 8701–8709.
[27] L. Poppe, Methylidene-imidazolone: a novel electrophile for substrate activation, Curr. Opin. Chem. Biol. 5 (2001) 512–524.
[28] S. Strom, U. Wanninayake, N.D. Ratnayake, K.D. Walker, J.H. Geiger, Insights into the mechanistic pathway of the *Pantoea agglomerans* phenylalanine aminomutase, Angew. Chem. Int. Ed. Engl. 51 (2012) 2898–2902.
[29] X.B. Zhang, C.J. Liu, Multifaceted regulation of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids, Mol. Plant 8 (2015) 17–27.
[30] M. Lepelley, V. Mahesh, J. McCarthy, M. Rigoreau, D. Crouzillat, N. Chabrillange, A. de Kochko, C. Campa, Characterization, high-resolution mapping and differential expression of three homologous *PAL* genes in *Coffea canephora* Pierre (Rubiaceae), Planta 236 (2012) 313–326.
[31] N. Firon, D. LaBonte, A. Villordon, Y. Kfir, J. Solis, E. Lapis, T.S. Perlman, A. Doron-Faigenboim, A. Hetzroni, L. Althan, L.A. Nadir, Transcriptional profiling of sweetpotato (*Ipomoea batatas*) roots indicates down-regulation of lignin biosynthesis and up-regulation of starch biosynthesis at an early stage of storage root formation, BMC Genomics 14 (2013) 460.
[32] H. Wang, J. Yang, M. Zhang, W. Fan, N. Firon, S. Pattanaik, L. Yuan, P. Zhang, Altered phenylpropanoid metabolism in the maize *Lc*-expressed sweet potato (*Ipomoea batatas*) affects storage root development, Sci. Rep. 6 (2016) 18645.
[33] Y. Tanaka, M. Matsuoka, N. Yamanoto, Y. Ohashi, Y. Kano-Murakami, Y. Ozeki, Structure and characterization of a cDNA clone for phenylalanine ammonia-lyase from cut-injured roots of sweet potato, Plant Physiol. 90 (1989) 1403–1407.
[34] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[35] J. Sperschneider, A.M. Catanzariti, K. DeBoer, B. Petre, D.M. Gardiner, K.B. Singh, P.N. Dodds, J.M. Taylor, Localizer: subcellular localization prediction of both plant and effector proteins in the plant cell, Sci. Rep. 7 (2017) 44598.
[36] C.S. Yu, C.W. Cheng, W.C. Su, K.C. Chang, S.W. Huang, J.K. Hwang, C.H. Lu, CELLO2GO: a web server for protein subcellular localization prediction with functional gene ontology annotation, PLoS One 9 (2014), e99368.
[37] R. Waadt, J. Kudla, In planta visualization of protein interactions using bimolecular fluorescence complementation (BiFC), CSH Protoc. 5 (2008) t4995.
[38] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real–time quantitative PCR and the 2(–Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[39] S.H. Kim, Y.O. Ahn, M.J. Ahn, H.S. Lee, S.S. Kwak, Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweetpotato, Phytochemistry 74 (2012) 69–78.
[40] P.P. Mitra, D. Loqué, Histochemical staining of *Arabidopsis thaliana* secondary cell wall elements, J. Vis. Exp. 87 (2014), e51381.
[41] T. Kobayashi, R. Kurata, Y. Kai, Seasonal variation in the yield and polyphenol content of sweet potato (*Ipomoea batatas* L.) foliage, Hort. J. 88 (2019) 270–275.
[42] J.E. Bassard, J. Mutterer, F. Duval, D. Werck-Reichhart, A novel method for monitoring the localization of cytochromes P450 and other endoplasmic reticulum membrane associated proteins: a tool for investigating the formation of metabolons, FEBS J. 279 (2012) 1576–1583.
[43] G. Hrazdina, G.J. Wagner, Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes, Arch. Biochem. Biophys. 237 (1985) 88–100.
[44] G.J. Wagner, G. Hrazdina, Endoplasmic reticulum as a site of phenylpropanoid and flavonoid metabolism in hippeastrum, Plant Physiol. 74 (1984) 901–906.
[45] G. Legrand, M. Delporte, C. Khelifi, A. Harant, C. Vuylsteker, M. Mörchen, P. Hance, J.L. Hilbert, D. Gagneul, Identification and characterization of five BAHD acyltransferases involved in hydroxycinnamoyl ester metabolism in chicory, Front. Plant Sci. 7 (2016) 741.
[46] M. Kojima, T. Kondo, An enzyme in sweet potato root which catalyzes the conversion of chlorogenic acid, 3-caffeoylquinic acid, to isochlorogenic acid, 3,5-dicaffeoylquinic acid, Agric. Biol. Chem. 49 (1985) 2467–2469.
[47] L.A. Lallemand, C. Zubieta, S.G. Lee, Y. Wang, S. Acajjaoui, J. Timmins, S. McSweeney, J.M. Jez, J.G. McCarthy, A.A. McCarthy, A structural basis for the biosynthesis of the major chlorogenic acids found in coffee, Plant Physiol. 160 (2012) 249–260.
[48] A. Moglia, S. Lanteri, C. Comino, L. Hill, D. Knevitt, C. Cagliero, P. Rubiolo, S. Bornemann, C. Martin, Dual catalytic activity of hydroxycinnamoyl-CoA quinate transferase from tomato

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allows it to moonlight in the synthesis of both mono- and dicaffeoylquinic acids, Plant Physiol. 166 (2014) 1777–1787.
[49] G. Sonnante, R. D'Amore, E. Blanco, C.L. Pierri, M. De Palma, J. Luo, M. Tucci, C. Martin, Novel hydroxycinnamoyl-coenzyme A quinate transferase genes from artichoke are involved in the synthesis of chlorogenic acid, Plant Physiol. 153 (2010) 1224.
[50] G.B. Bai, X.X. Peng, W.D. Li, W.Q. Wang, Cloning and characterization of a cDNA coding a hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase involved in chlorogenic acid biosynthesis in *Lonicera japonica*, Planta Med. 76 (2010) 1921–1926.
[51] Z.X. Chen, G.H. Liu, Y.Q. Liu, Z.Q. Xian, N. Tang, Overexpression of the *LmHQT1* gene increases chlorogenic acid production in *Lonicera macranthoides* Hand-Mazz, Acta Physiol. Plant. 39 (2017) 27.
[52] M.M. Rigano, A. Raiola, T. Docimo, V. Ruggieri, R. Calafiore, P. Vitaglione, R. Ferracane, L. Frusciante, A. Barone, Metabolic and molecular changes of the phenylpropanoid pathway in tomato (*Solanum lycopersicum*) lines carrying different *Solanum pennellii* wild chromosomal regions, Front. Plant Sci. 7 (2016) 1484.
[53] K. Cheevarungnapakul, G. Khaksar, P. Panpetch, P. Boonjing, S. Sirikantaramas, Identification and functional characterization of genes involved in the biosynthesis of caffeoylquinic acids in sunflower (*Helianthus annuus* L.), Front. Plant Sci. 10 (2019) 968.
[54] A. Moglia, A. Acquadro, K. Eljounaidi, A.M. Milani, C. Cagliero, P. Rubiolo, A. Genre, K. Cankar, J. Beekwilder, C. Comino, Genome-wide identification of BAHD acyltransferases and in vivo characterization of HQT-like enzymes involved in caffeoylquinic acid synthesis in globe artichoke, Front. Plant Sci. 7 (2016) 1424.
[55] R. Niggeweg, A.J. Michael, C. Martin, Engineering plants with increased levels of the antioxidant chlorogenic acid, Nat. Biotechnol. 22 (2004) 746–754.
[56] J. Zhang, M. Wu, W. Li, G. Bai, Regulation of chlorogenic acid biosynthesis by hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase in *Lonicera japonica*, Plant Physiol. Biochem. 121 (2017) 74–79.
[57] C.M. André, R. Schafleitner, S. Legay, I. Lefèvre, C.A.A. Aliaga, G. Nomberto, L. Hoffmann, J.F. Hausman, Y. Larondelle, D. Evers, Gene expression changes related to the production of phenolic compounds in potato tubers grown under drought stress, Phytochemistry 70 (2009) 1107–1116.
[58] D.A. Navarre, R.S. Payyavula, R. Shakya, R.N. Knowles, S.S. Pillai, Changes in potato phenylpropanoid metabolism during tuber development, Plant Physiol. Biochem. 65 (2013) 89–101.
[59] R.S. Payyavula, D.A. Navarre, J.C. Kuhl, A. Pantoja, S.S. Pillai, Differential effects of environment on potato phenylpropanoid and carotenoid expression, BMC Plant Biol. 12 (2012) 39.
[60] R.S. Payyavula, R.K. Singh, D.A. Navarre, Transcription factors, sucrose, and sucrose metabolic genes interact to regulate potatophenylpropanoid metabolism, J. Exp. Bot. 64 (2013) 5115–5131.
[61] S.A. Noh, H.S. Lee, Y.S. Kim, K.H. Paek, J.S. Shin, J.M. Bae, Down-regulation of the *IbEXP1* gene enhanced storage root development in sweetpotato, J. Exp. Bot. 64 (2013) 129–142.

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