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Induced biosynthesis of chlorogenic acid in sweetpotato leaves confers the resistance against sweetpotato weevil attack

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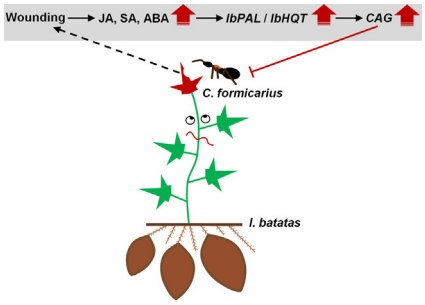
GRAPHICAL ABSTRACT



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ABSTRACT

Sweetpotato weevil is among the most harmful pests in some major sweetpotato growing areas with warm climates. To enable the future establishment of safe weevil-resistance strategies, anti-weevil metabolites from sweetpotato should be investigated. In the present study, we pretreated sweetpotato leaves with exogenous chlorogenic acid and then exposed them to sweetpotato weevils to evaluate this compound's anti-insect activity. We found that chlorogenic acid applied to sweetpotato conferred significant resistance against sweetpotato-weevil feeding. We also observed enhanced levels of chlorogenic acid in response to weevil attack in sweetpotato leaves. To clarify how sweetpotato weevils regulate the generation of chlorogenic acid, we examined key elements of plant-herbivore interaction: continuous wounding and phytohormones participating in chlorogenic acid formation. According to our results, sweetpotato weevil-derived continuous wounding induces increases in phytohormones, including jasmonic acid, salicylic acid, and abscisic acid. These phytohormones can upregulate expression levels of genes involved in chlorogenic acid formation, such as *IbPAL*, *IbC4H* and *IbHQT*, thereby leading to enhanced chlorogenic acid generation. This information should contribute to understanding of the

Abbreviations: ABA, abscisic acid; CAF, caffeic acid; CGA, chlorogenic acid; *C3H*, *p*-coumarate 3-hydroxylase; *C4H*, cinnamate 4-hydroxylase; *4CL*, 4-coumarate: CoA ligase; *HCGQT*, hydroxycinnamoyl glucose: quinate hydroxycinnamoyl transferase; *HCT*, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; *HQT*, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; *Ib*, *Ipomoea batatas*; JA, jasmonic acid; *PAL*, phenylalanine ammonia lyase; SA, salicylic acid; *UGCT*, UDP glucose: cinnamate glucosyl transferase; UPLC-QTOF-MS, Ultra-performance liquid chromatography/ quadrupole time-of-flight mass spectrometry.

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occurrence and formation of natural anti-weevil metabolites in sweetpotato in response to insect attack and provides critical targets for the future breeding of anti-weevil sweetpotato cultivars.
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Introduction

Sweetpotato, *Ipomoea batatas* (L.) Lam., an herbaceous, perennial plant belonging to the *Convolvulaceae* family, is cultivated in more than 100 countries, mainly in tropic and subtropical regions between latitudes of 20 °N and 40 °N [1]. Sweetpotato roots are used as food, feed, and fuel, while the foliage is used as a vegetable in some regions. The storage root of sweetpotato is rich in active ingredients beneficial for human nutrition, such as soluble sugar, vitamin A, mucins, dehydroepiandrosterone, and fiber [2]. The vine system of sweetpotato expands easily and rapidly, thereby guaranteeing a stable, high yield and making sweetpotato a national strategic food. However, sweetpotato production is seriously affected by insect pests worldwide, causing up to 80% reduction in production [3]. Sweetpotato weevil (*Cylas formicarius* Fab.), a major devastating pest present in almost all sweetpotato cultivars worldwide, is primarily hazard to the veins and roots of sweetpotato, rendering a bitter taste for human to consume [4]. Sweetpotato weevil is considered to be the most critical preharvest limiting factor in sweetpotato production, causing crop losses as high as 98% [4,5]. Because sweetpotato is the fifth most important crop in developing countries, maintaining a steady yield is critical. Given that sweetpotato weevil can occur year-round and has a high natural reproduction capacity and hunger tolerance, this insect constitutes a major challenge to field planting and postharvest storage [5]. The low diversity of insect-resistant sweetpotato resources has impeded research progress on insect-resistance mechanisms. Present pest management of this insect mainly relies on agronomic measures and chemical pesticides, which are either inefficient or eventually lead to food safety issues and environmental pollution [3].

Because the use of natural phytochemicals produced by plants as anti-herbivore ingredients is targeted and safe, this approach been widely accepted over the last decade. Plant defensive allelochemicals can be mainly divided into five categories, namely, volatile hydrocarbons, phenolic acids, alkaloids, terpenoids, and plant proteins [6]. Among these compounds, phenolic acids have roles as antifeedants or toxins that prevent further damage from pests [6]. Chlorogenic acid (CGA) has been reported to show broad-spectrum activity against noctuids, thrips, and beetles [7]. Apart from protecting plant from herbivorea and pathogens, CGA also participate in the growth and development of plant, such as shoot organogenesis and fruit ripening [7]. Compared with synthetic pesticides, CGA therefore appears to be more environmentally friendly and possibly safer for biological use. Evidence is lacking, however, for whether CGA can inhibit sweetpotato weevil attack. Much effort has previously been focused on studying specific genes or enzymes in the CGA biosynthetic pathway, whereas the CGA regulatory mechanism is relatively poorly understood. Sweetpotato contains abundant amounts of CGA, which suggests a huge potential exists for the exploitation of this compound [8]. The present study aimed to elucidate the impact of CGA on sweetpotato weevil and to explore the regulation of CGA in response to sweetpotato weevil. We also investigated how insect-derived wounding, phytohormones, and CGA-related genes participate in this process. The information uncovered in this study should contribute to understanding of the occurrence and formation of natural anti-weevil metabolites in sweetpotato in response to insect attack and provide critical targets for the future breeding of anti-weevil sweetpotato cultivars.

Materials and methods

Chemicals and regents

Caffeic acid (CAF), CGA were purchased from Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China. Jasmonic acid (JA) was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Salicylic acid (SA) and abscisic acid (ABA) were purchased from Aladdin Industrial Co., Ltd, Shanghai, China. [$^2H_6$]ABA was purchased from Toronto Research Chemicals, Inc., North York, Canada. [$^2H_5$]JA and [$^2H_4$]SA were purchased from CDN Isotopes, Vaudreuil, Quebec, Canada.

Methanol and acetonitrile were purchased from Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA. Formic acid was purchased from Sigma-Aldrich Co., Ltd., St. Louis, MO, USA. Ethanol was purchased from Shanghai Macklin Biochemical Technology Co., Ltd., Shanghai, China. De-ionized water was obtained using Milipore Water Purification System (Synergy UV; Merck, Darmstadt, Germany). PrimeScript RT Reagent Kit with gDNA Eraser was purchased from Takara Bio Inc., Kyoto, Japan. Quick Plant Total RNA Kit was purchased from Huayueyang Biotechnology Co., Ltd., Beijing, China.

Plant materials and treatments

Sweetpotato cultivar Guangshu No. 87, a main crop variety in Guangdong Province, China, was obtained from Professor Hongbo Zhu of Guangdong Ocean University (Guangdong, China) in May 2019. For use in analysis of the distribution of CGA in different tissues, Guangshu No. 87 plants cultivated for 4 months in a sweetpotato field were harvested and quickly preserved in liquid nitrogen. For remaining experiments, plant material were the new branches (approximately 40 days) grown from cuttings transplanted from the sweetpotato field in May 2019. The transplanted cuttings were planted in soil and cultivated in an incubator (12-h light/12-h dark; 25 °C; 80% humidity; 6,720 Lux, twice-weekly irrigation). Sweetpotato weevils (*Cylas formicarius*) were captured from the sweetpotato field and identified by Professor Hongbo Zhu at the Guangdong Ocean University in May 2019.

CGA treatments: After approximately 40 days, new vines grown from the sweetpotato cuttings (approximately five leaves) were cut, and the incision was soak in a solution containing 100 μM CGA (including 1% ethanol for solubilization) in an incubator for 1 day (12-h light/12-h dark; 25 °C; 80% humidity; 6,720 Lux). After the solution was completely absorbed, more water was added. Sweetpotato vines (approximately five leaves) subjected to the same conditions but fed with water containing 1% ethanol served as the control group. One day later, third or fourth leaf of the vines from both groups was placed inside a petri dish, and 10 sweetpotato weevils were added and allowed to feed on the leaves (shown in Figure S1 in Supplementary Materials). Each leaf served as one repetition, with five replicates performed per group. After exposed to sweetpotato weevils for an additional day, the leaves were subjected to damage quantification assay as described in the following.

Distribution of CGA among different tissue: Fresh leaves, stems, root flesh and root skin from the same strain of sweetpotato were harvested from the sweetpotato field in May 2019 and quickly preserved in liquid nitrogen. The different samples were ground with a ball mill in liquid nitrogen and stored at −80 °C until used for phenolic acid extraction and analysis and transcript expression analysis as described below.

Insect attack treatments: Forty-day-old, newly grown sweetpotato vines (approximately five leaves) were cut and soaked in water to ensure survival in an incubator for 12 and 24 h (12-h light/12-h dark; 27 °C; 80% humidity; 6,720 Lux). Each piece of sweetpotato leaf was sealed in a net bag along with ten sweetpotato weevils (shown in Figure S2 in Supplementary Materials). Each replicate consisted of six leaves from three vine, with three biological replicates per group. Sweetpotato vines under the same conditions but without insects in the bag served as the control group. The samples were then ground with a ball mill in liquid nitrogen and stored at −80 °C until subjected to extraction and analysis of phenolic acid and phytohormones and transcript expression analysis as described below.

Phytohormone treatments: Forty-day-old, newly grown sweetpotato vines (approximately five leaves) were cut and soaked in a solution containing either a low concentration of phytohormones (0.2 μM JA, 2 μM SA and 20 μM ABA respectively, with 1% ethanol for solubilization) or a high concentration (1 μM JA, 10 μM SA and 100 μM ABA, with 1% ethanol for solubilization) in an incubator for 1 or 2 days (12-h light/12-h dark; 27 °C; 80% humidity; 6,720 Lux). Sweetpotato vines under the same conditions but fed with water containing 1% ethanol served as the control group. Each replicate consisted of five leaves from a vine, with three biological replicates per group. The samples were then ground with a ball mill in liquid nitrogen and stored at −80 °C until subjected to phenolic acid and phytohormone extraction and analysis and transcript expression analysis as described below.

Mechanical wounding treatments: Forty-day-old, newly grown sweetpotato leaves were isolated from a vine, tiled on thick cardboard, and covered with plastic wrap. The leaves were then continuously pricked for 3 or 6 h at a rate of one prick every 6 s using a continuous damage meter (CNC Engraving Machines, Shenzhen, China). Each replicate consisted of five intact leaves, with three replicates per group. Isolated intact sweetpotato leaves tiled on thick cardboard and covered with plastic wrap without mechanical wounding treatment served as the control group. The leaves were then ground with a ball mill in liquid nitrogen and stored at −80 °C until subjected to phenolic acid and phytohormone extraction and analysis and transcript expression analysis as described below.

Within-leaf mechanical wounding treatment: Six entire sweetpotato plants were placed in an incubator (27 °C; 80% humidity; 6,720 Lux). Five newly grown sweetpotato leaves (approximately 40 days old) were used from each plant. Half of each sweetpotato leaf was manually jabbed with a needle for 3 or 6 h at a rate of five pricks every 15 min, while the other side was left unharmed. Each replicate consisted of five half leaves, with three replicates per group. After treatment for 3 or 6 h, the leaves were separated along the vein with a knife, quickly frozen in liquid nitrogen, and stored at −80 °C until subjected to phenolic acid extraction and analysis and transcript expression analysis as described below.

Extraction and analysis of phenolic acids in sweetpotato leaf

The method was referred to a previous report with modification [9]. 200 mg fresh weight of sweetpotato leaves powder was extracted with 2 mL cold methanol. The mixture was vortexed, ultrasonic extracted in ice water for 30 min, and then centrifuged at 5000 *g* for 10 min at 4 °C. The resultant supernatant was filtered through a 0.22 μm membrane and analyzed using UPLC-QTOF-MS (Waters Corp., Milford, MA, USA) with a UPLC AQUICTY HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters) at 35 °C with a flow rate of 0.3 mL/min. Gradient elution was performed using two mobile phases: A, water containing 0.1% (v/v) formic acid; and B, acetonitrile containing 0.1% (v/v) formic acid. The gradient profile was described as follow: 0 to 15 min, 10 to 30% B; 15 to 15.1 min, 30% B to 90% B; 15.1 to 18 min, 90% B, 18 to 18.1 min, 90% to 10% B, 18.1 to 21 min, 10% B. The electrospray ionization was operated in negative mode. The MS conditions were capillary voltage: 1.5 kV; source temperature: 100 °C; desolvation temperature: 250 °C; cone gas flow: 50 L/h; and desolvation gas flow: 550 L/h. Ultraviolet absorption wavelength was 280 nm. Standards of CAF and CGA were used to qualify and quantify the metabolites in the sweetpotato samples.

Extraction and analysis of phytohormones in sweetpotato leaf

The method was referred to a previous report with modification [10]. Three hundred mg finely powdered tea leaves were collected in 10 mL tubes. Three mL cold ethyl acetate was added to resolve it. After vortexing for 1 min, 30 μL of 1 μM [$^2H_5$]-JA, 2 μM [$^2H_4$]-SA and 1 μM [$^2H_6$]-ABA were added to the solution as internal standards. The solution was ultrasonic extracted in cold ice water for 20 min. 2.4 mL of supernatant solution of each samples was dried under nitrogen and re-dissolved in 200 μL methanol. Then the supernatants were filtered through a 0.22 μm membrane, and subjected to an UPLC-QTOF-MS (Acquity UPLC I-Class/ Xevo G2-XS QTOF, Waters Corporation, MA, USA). Each sample (5 μL) was injected onto a Waters ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters). Solvent A was water with 0.1% (v/v) formic acid. Solvent B was acetonitrile with 0.1% (v/v) formic acid. The solvent gradient was started at 20% B, then linearly increased to 35% within 10 min, later increased to 95% B in 0.1 min and kept for 3 min. In that moment, it suddenly dropped to 20% B within 0.1 min and maintain for 3 min. The flow rate was 0.4 mL/min. The column temperature was 30 °C. The electrospray ionization was operated in negative mode. The MS conditions were capillary voltage: 1.5 kV; source temperature: 100 °C; desolvation temperature: 300 °C; cone gas flow: 50 L/h; and desolvation gas flow: 600 L/h. Standards of JA, SA and ABA were used to qualify and quantify the phytohormones in the sweetpotato samples.

Damage quantification assay of sweetpotato leaf assaulted by sweetpotato weevil

Each single sweetpotato leaf was cut off from the stem, placed on a lightening plain and photographed. The area of injury was calculated from the resulting photo using Image J 1.52a.

Transcript expression analysis of CGA synthesis genes in sweetpotato leaf

The method was referred to a previous report [11]. Total RNA was isolated immediately after dissection using a Quick RNA isolation Kit (Huayueyang Biotechnology (Beijing) Co., Ltd., Beijing, China). The reactions were performed using iTaqTM Universal SYBR® Green Supermix (BioRad, Hercules, CA, USA) in a 20 μL volume containing 10 μL of iTaqTM Universal SYBR® Green Supermix (2 ×), 0.4 μM each specific forward and reverse primers listed in Table S1. Templates were diluted 20-fold, and 4 μL of each one was used for 20 μL PCR reaction. The quantitative real time PCR (qRT-PCR) was carried out by Roche LightCycle 480 (Roche Applied Science, Mannheim, Germany) with one cycle of 95 °C 30 s, 40 cycles of 95 °C 5 s, 60 °C 30 s. A melt curve was performed at the end of each reaction to verify PCR product specificity. The $2^{-\triangle\triangle CT}$ method was used to calculate the relative expression level. *IbARF*

was used as an internal reference. Changes in mRNA level of the test gene for each treatment were normalized to that of *IbARF*.

Statistical analysis

All the data were under normality and homogeneity of variance test by means of Kolmogorov-Smirnov test and One-way ANOVA test respectively, using IBM SPSS Statistics 24. Independent samples *t*-test was used to determine the differences between the two groups (95% CI). One-way ANOVA followed by Duncan's multiple range test was used to rank the differences among more than two groups (alpha = 0.05). Independent samples *t*-test and one-way ANOVA were processed by using IBM SPSS Statistics 24.

Results and discussion

Effect of exogenous CGA treatment on sweetpotato weevil resistance in sweetpotato leaves

To understand the anti-herbivore effects of CGA in sweetpotato leaves, a damage quantification assay was conducted on leaves attacked by sweetpotato weevils. Compared with the control group, the injury area of sweetpotato leaves in the CGA pretreatment group was significantly decreased (Fig. 1A), thus indicating that exogenously applied CGA significantly restricted the feeding behavior of sweetpotato weevils.

CGA is widely present in plants, ranging from ferns to higher dicotyledons. Except for CGA-rich species such as *Eucommia ulmoides*, honeysuckle, sunflower, coffee, and sweetpotato, most plants possess only small amounts of CGA. CGA has well-known biological activities, including antibacterial properties, viral resistance, tumor resistance, and cardiovascular-protective effects [12]. Furthermore, CGA has been reported to effectively defend against multiple herbivores (primarily noctuids, thrips, and beetles) in species such as tomato, *Salix pentandra*, *Arachis hypogaea*, *Dendranthema, Solanum tuberosum*, and *Nicotiana attenuata* [7]. No direct evidence has been obtained, however, that shows CGA can inhibit attack of sweetpotato weevils on sweetpotato. In this study, we accordingly investigated whether anti-weevil activity was enhanced when excessive exogenous CGA was applied to sweetpotato vines. We found that CGA directly and significantly reduced feeding by sweetpotato weevils on sweetpotato leaves. As an anti-nutritive ingredient, CGA is oxidized by polyphenol oxidase in insects to form chloroquinone, which can easily combine with amino acids, such as cysteine, histidine, methionine, and lysine, to significantly inhibit the growth rate of insects [13]. Notably, CGA was inhaled by the plants in our study, which might result in transformations into other metabolites through enzyme reactions *in vivo*, potentially allowing CGA to be directly or indirectly involved in resistance to sweetpotato weevils. Further evidence is needed to confirm its direct efficacy-for example, by adding CGA to an artificial diet to evaluate its effect on injury area, herbivore growth, and survival rate.

Distribution of CGA in different parts of sweetpotato

To explore the distribution of CGA in sweetpotato, we measured the CGA contents of leaves, stems, root skin, and root flesh of the same strain of sweetpotato. The CGA contents of different sweetpotato parts were as follows: leaf (380.20 μg/g) > stem (206.39 μg/g) > root skin (121.30 μg/g) > root flesh (53.41 μg/g) (Fig. 1B). This result is consistent with values in the reported literature (leaf > stem > root), which suggests a tissue-dependent pattern [14]. Adult sweetpotato weevils mainly feed on the leaves and veins. Female sweetpotato weevils will then oviposit on the surface of tubers in the same plant [15]. The developing larvae subsequently exist on the storage roots until maturity and then drill tunnels [16]. In response to the invasion, the tuber produces terpene phytoalexins, which makes it inedible and leads to economic loss [15]. Although storage root damage is the predominant cause of loss, host-plant selection occurs prior to tuber infection [17]. Investigating the distribution of CGA as a candidate substance for resistance to sweetpotato weevil in different parts of sweetpotato plants is therefore of interest. Among the different parts of the sweetpotato plant, we found that leaves had the highest CGA content, followed by stems, root skin, and root flesh (Fig. 1B), which was in agreement with the chronological order in which sweetpotato organs were exposed to sweetpotato weevil. While CGA content has been found to vary among varieties, this order of distribution is consistent with previous findings [14]. Although current research on pest resistance in sweetpotato has mainly focused on tubers, our study findings suggest that the role of leaves in protection against weevils deserves more attention.

Monitoring transcript levels of candidate genes involved in the CGA biosynthesis is an obvious step toward elucidation of its anti-insect properties. The CGA biosynthetic pathway in potato plants, which is better understood, includes three proposed routes via the phenylpropanoid pathway (Fig. 1C) [18]. Among these routes, evidence for route B, which involves *PAL*, *C4H*, *C3H*, *4CL*, *HCT*, and *HQT,* is the strongest (Fig. 1D) [18]. To further explore the intensity of CGA synthesis in different sweetpotato parts, we assessed expression levels of five key genes, none of which, other than *IbC3H*, have been reported thus far in sweetpotato. In general, our analysis found that transcript levels were not in agreement with CGA levels in different sweetpotato organs (Fig. 1D). Leaves had the highest CGA content but the lowest levels of *IbPAL*, *Ib4CL*, and *IbHQT* gene expression, while root skin showed the highest expression levels of *IbPAL*, *IbC4H*, *Ib4CL*, and *IbHQT* but lower CGA levels. The explanation for this discrepancy is unclear, but it might reflect the fact that CGA is mainly synthesized in root skin and transported upwards to leaves and stems for protection, or it could be related to inter-organ differences in substrates and enzyme activities [19]. To sufficiently explain the CGA biosynthesis mechanism in sweetpotato, these factors should also be screened. In regard to distribution within roots, CGA and gene expression levels were found to be higher in the skin than in flesh (Fig. 1D); this result is similar to the situation in potato, hinting that rhizome crops may have evolved this mechanism to counteract biotic stress [20].

Effect of sweetpotato weevil treatment on the contents of CGA and phytohormones in sweetpotato leaves

Given the above results, we investigated the influence of sweetpotato weevils on the CGA content of sweetpotato leaves. We detected a marked increase in the levels of CAF and CGA in sweetpotato leaf exposed to weevils for 24 h (Fig. 2A). Weevils attack also induces *IbPAL, IbHCT and IbHQT* up-regulation (Fig. 2B). Phytohormones, which are the most important upstream regulators responding to insect feeding, were also monitored, with salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) found to be significantly elevated after treatment for 24 h (Fig. 2C).

Plants possess a complex defense system to protect themselves against herbivores. Upon insect attack, plants mobilize the formation of defensive secondary metabolites. The steps taken by plants, from suffering attack to counterattack, are recognition of mechanical damage or the insect elicitor, enzyme phosphorylation/dephosphorylation cascade reaction, unraveling of plant hormone signaling, and, finally, resistance-activated substance production [21]. Phytohormones are critical regulatory signals that modulate plant immune responses against herbivores. JA is the chief regulator of

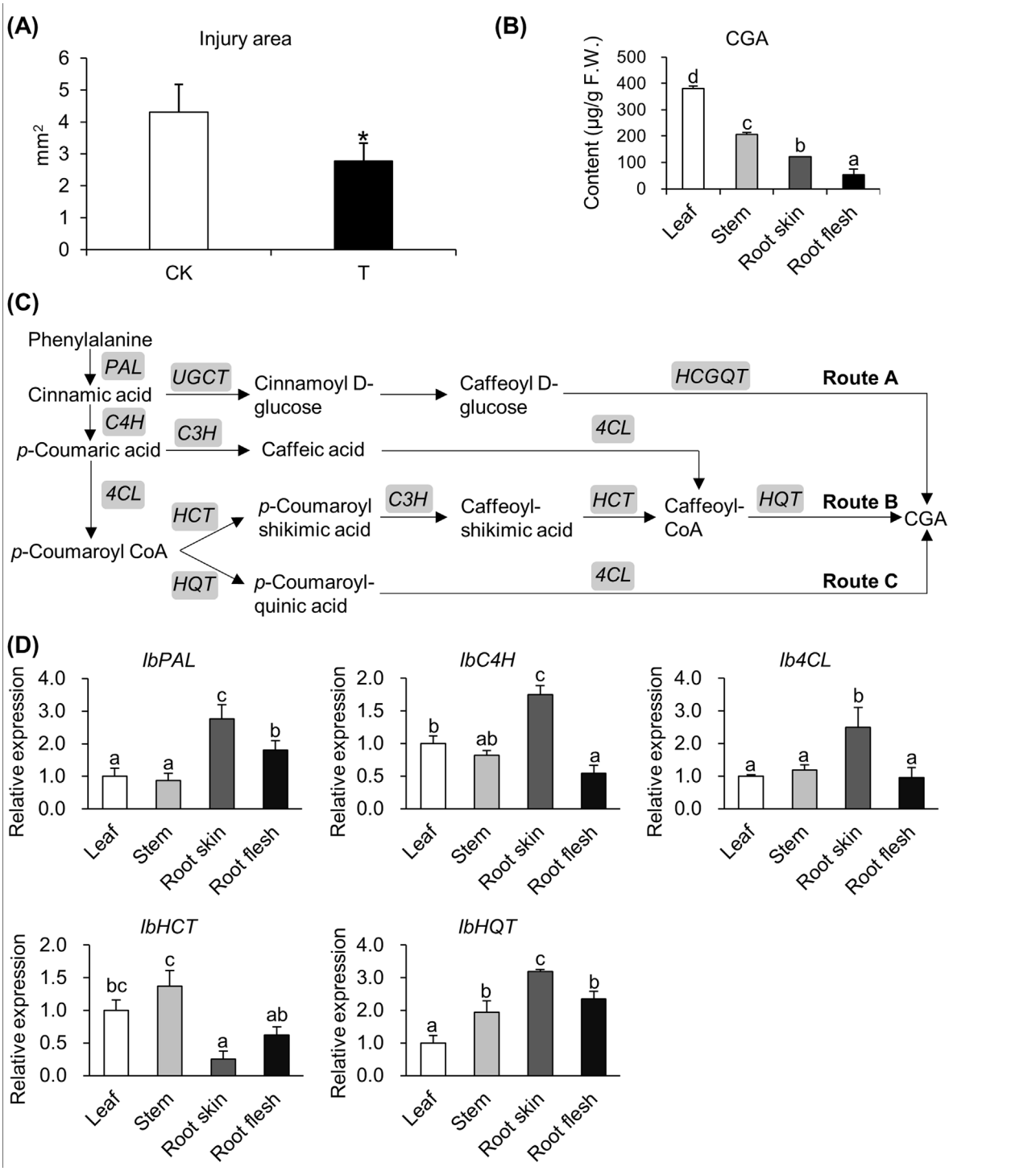

Fig. 1. Insect resistance evaluation of chlorogenic acid application in sweetpotato leaves against sweetpotato weevils (A), distribution of chlorogenic acid in different parts of sweetpotato plants (B), the schematic overview of the chlorogenic acid synthesis pathway (C), as well as expression profiles of biosynthetic genes of chlorogenic acid in different parts of sweetpotato plants (D). (A) Damage quantification assay of sweetpotato leaf assaulted by sweetpotato weevil using Image J (1.52a). CK, non-chlorogenic acid treated group. T, chlorogenic acid pretreated group. All data are expressed as mean ± S.D. (n = 5). Significant differences between CK and T group are indicated as (* $p \leq 0.05$, and ** $p \leq 0.01$), determined by independent samples *t*-test. (B and D) *Ib*, *Ipomoea batatas*. All data are expressed as mean ± S.D. (n = 3). Significant differences among the different part of sweetpotato plants are indicated as different letter (Means accompanied by same letters are not significantly different from each other ($p \leq 0.05$, Duncan's test).

stress response and is responsible for a wide range of plant defenses, both physical (trichomes) and chemical (toxins and volatiles) [22]. Other phytohormones, such as SA and ABA, also collaboratively participate in the defense process [23]. To understand plant defense, comprehensive analysis of multiple phytohormones is therefore necessary in most cases [22]. JA, SA, and ABA were accordingly measured in this study. From the results, we concluded that sweetpotato weevils elicit an increase in phytohormones (JA, SA, and ABA) in sweetpotato leaves (Fig. 2C). Phytohormones subsequently mediate the regulation of defensive gene expression.

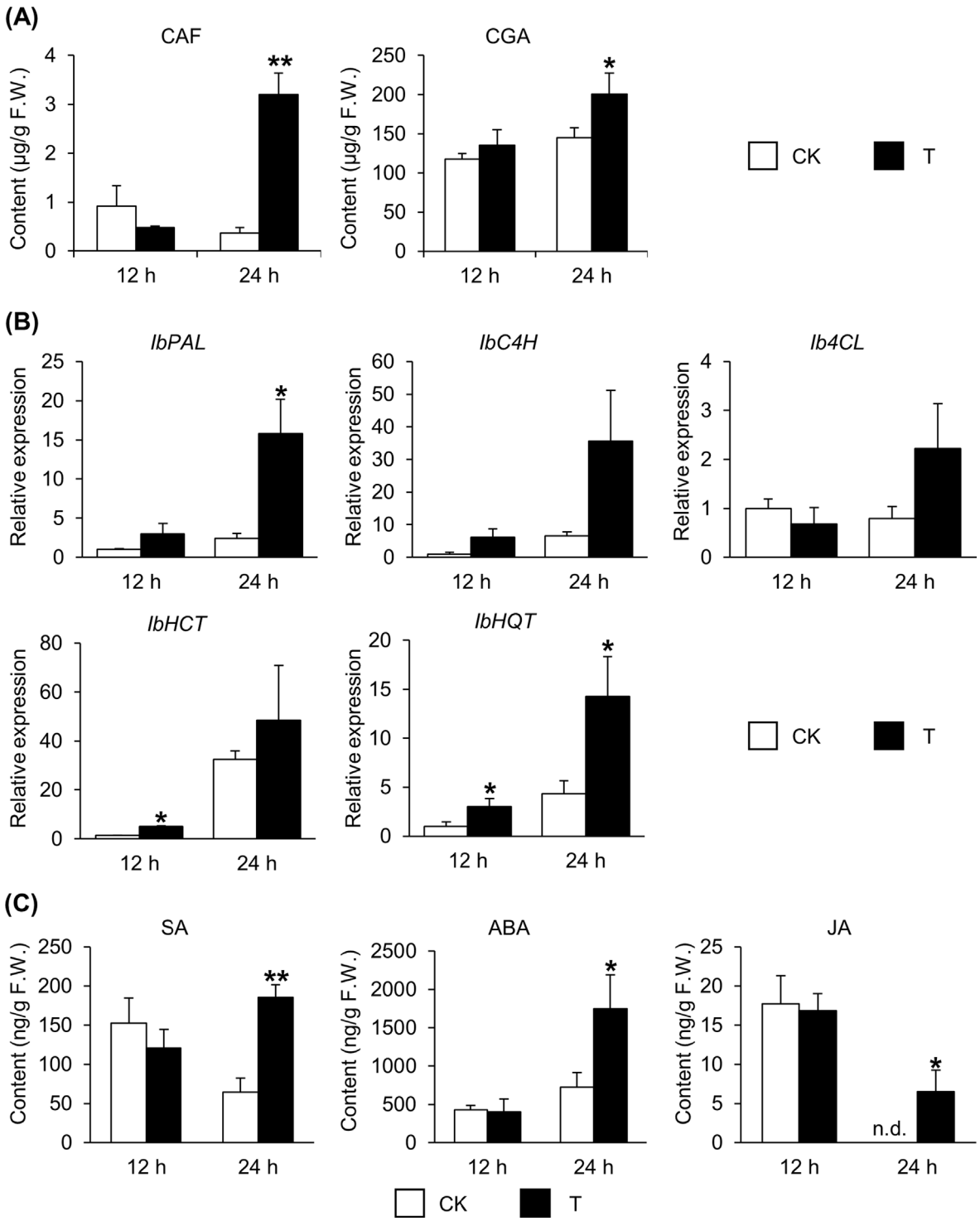

Fig. 2. Effect of sweetpotato weevils attack (for 12 h and 24 h) on the contents of phenolic acids (A), the transcript levels of chlorogenic acid biosynthesis corresponding genes (B) and the contents of phytohormones (C) in sweetpotato leaves. n.d., Not detected. The × axis indicates treatment time. CK, non-insect treated group. T, sweetpotato weevils treated group. All data are expressed as mean ± S.D. (n = 3). Significant differences between CK and T group are indicated as (* $p \leq 0.05$, and ** $p \leq 0.01$), determined by independent samples *t*-test.

Among the genes mentioned above, *HQT* is considered to be responsible for CGA biosynthesis [19,21]. Furthermore, *PAL* plays a critical role in the regulation of CGA synthesis, and also manipulates the synthesis of many other defensive phenolic compounds and phenylpropanoid volatiles [22,24]. According to our results, transcript levels of *HQT* and *PAL* also responded to the weevil signal (Fig. 2B). Upregulation of gene expression helps produce resistance components. Because of their universal existence among plants and pro-oxidant effects on insects, phenolic acids (such as SA, CAF, and CGA) are considered important components of both constitutive and induced defenses against herbivores [25]. Furthermore, CAF and CGA production induced by insect pests has been widely reported in many species [26–28]. CAF also serves as an important precursor in the CGA synthesis pathway (Fig. 1C).

Therefore, the contents of CAF and CGA were detected simultaneously in the present study. Although healthy sweetpotato leaves contain a certain amount of CAF and CGA, these compounds were induced in response to weevil attack, a reaction attributed to both constitutive and induced defenses (Fig. 2A). These results confirm that sweetpotato weevils can cause an increase in the content of phytohormones (JA, SA, and ABA), the expression of related genes (*IbPAL*, *IbHCT*, and *IbHQT*), and the production of phenolic acids (CAF and CGA) in sweetpotato leaves. We next aimed to explore whether phytohormones (JA, SA, and ABA) are directly involved in the CGA biosynthesis.

Effect of phytohormone treatment on CGA content in sweetpotato leaves

Exogenous JA, SA, and ABA were separately absorbed by sweetpotato leaves at the applied concentration, referred to as the response concentration (shown in Fig. 2C, two-fold or ten-fold

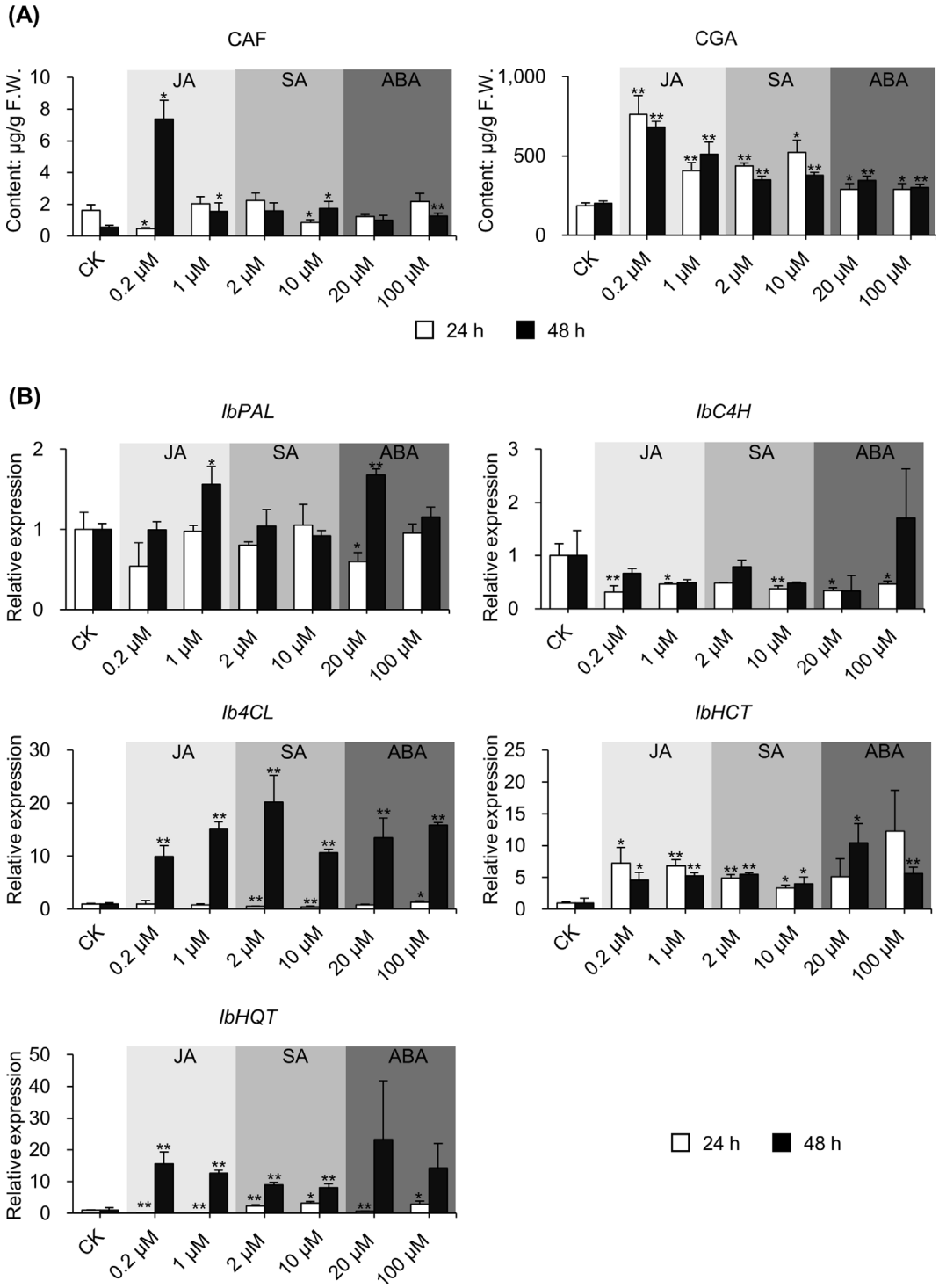

Fig. 3. Effect of phytohormone treatment (for 24 h and 48 h) on the contents of phenolic acids (A), the transcript levels of chlorogenic acid biosynthesis corresponding genes (B) in sweetpotato leaves. *Ib*, *Ipomoea batatas*. The × axis indicates solution concentration. CK, non-phytohormone treated group. All data are expressed as mean ± S.D. (n = 3). Significant differences between CK and phytohormone treated group are indicated as (* $p \leq 0.05$, and ** $p \leq 0.01$), determined by independent samples *t*-test.

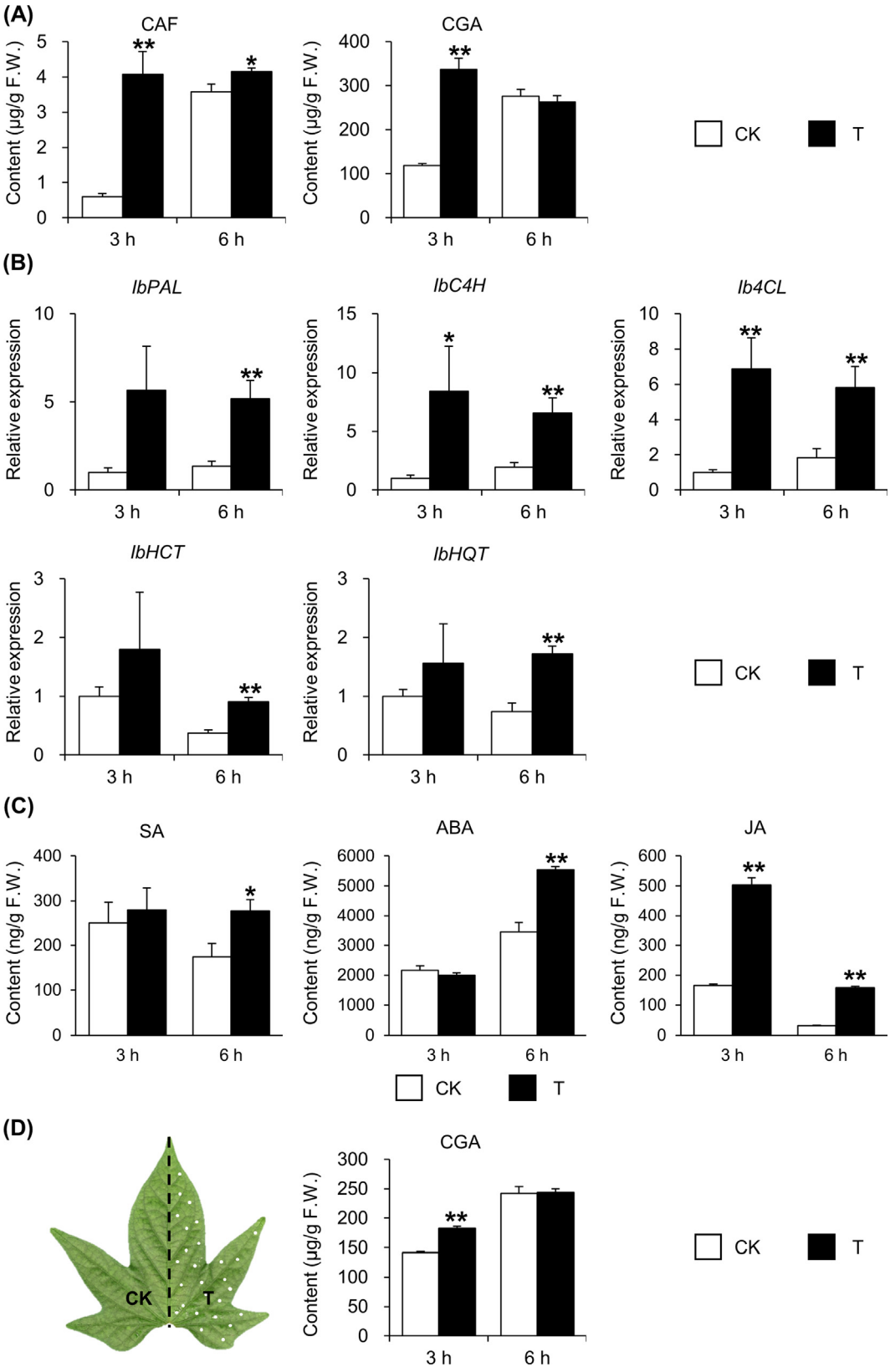

Fig. 4. Effect of mechanical wounding treatment (for 3 h and 6 h) on the contents of phenolic acids (A), the transcript levels of chlorogenic acid biosynthesis corresponding genes (B) and the contents of phytohormones in sweetpotato leaves (C). Analysis of CGA alterations in the wounding stress (for 3 h and 6 h) within the sweetpotato leaf (D). (A-C) *Ib*, *Ipomoea batatas*. The × axis indicates treatment time. CK, non-wounding treated group. T, wounding treated group. All data are expressed as mean ± S.D. (n = 3). Significant differences between CK and T group are indicated as (* $p \leq 0.05$, and ** $p \leq 0.01$), determined by independent samples *t*-test. (D) CK, non-wounded regions. T, wounded regions. All data are expressed as mean ± S.D. (n = 3). Significant differences between CK and T group are indicated as (* $p \leq 0.05$, and ** $p \leq 0.01$), determined by independent samples *t*-test.

respectively). In general, all three hormones were found to promote CGA synthesis, whereas CAF content first decreased and then increased upon application of JA or SA. The results showed that, in general, all three hormones promoted CGA synthesis, whereas the CAF content first decreased and then increased with the application of JA and SA (Fig. 3A). This result may be due to a sacrifice of CAF flux to compensate for CGA formation [29]. In comparison, no such clear pattern was observed in transcript level variation among the five genes (Fig. 3B). First, JA and ABA induced the accumulation of *IbPAL*, consistent with previous reports in other species (Fig. 3B) [30,31]. Second, we observed a steady decrease in the expression level of *IbC4H* and an increase in the expressions of *Ib4CL*, *IbHCT*, and *IbHQT* in response to all tested phytohormones after treatment, which indicates that *IbC4H* is marginally associated with CGA (Fig. 3B). Collectively, these results suggest that JA, SA, and ABA cofacilitate CGA generation to varying degrees by modulating the expressions of *Ib4CL*, *IbHCT*, and *IbHQT* (Fig. 3B). Furthermore, JA and ABA can both increase the expression of *IbPAL* (Fig. 3B).

In plant-pest interactions, phytohormones manipulate defensive genes by activating transcription factors (TF). Well-known JA-dependent TFs include JAZ and JIN1/MYC2, while SA-dependent TFs include NPR [32]. These TFs are transported to the nucleus and activate responsive genes by binding to their promoters, leading to gene transcription. Furthermore, complex cross-talk also occurs among these phytohormones in response to various biotic stresses. According to our experimental results, sweetpotato weevil infestation induces the release of phytohormone JA, SA, and ABA, which subsequently co-regulate the transcription of CGA synthesis-related genes and finally lead to CGA accumulation. Available evidence exists that CGA is subject to regulation by JA, SA and ABA in other species [33,34]. CGA production in response to *Bemisia tabaci* is inhibited in salicylate-deficient tobacco plants [35]. Furthermore, may other studies have confirm that JA, SA, and ABA are involved in modulating the expression of *HQT* [26,30,31,33,34]. However, Ghasemzadeh announced that only exogenous ABA mediates CGA synthesis in sweetpotato plants [36]. After comparison of the experimental parameters between their previous study and our present investigation, we conclude that the different inferences regarding JA and SA may be due to the applied phytohormone concentration, as the previous study sprayed 100 μM of MeJA, SA, and ABA on plants. These conflicting results suggest that sweetpotato plants react differently to different hormone concentrations. In the present study, the applied concentration was based on the self-response of sweetpotato under insect infestation to simulate herbivore attack conditions (Fig. 2C).

Effect of continuous wounding treatment on CGA content in sweetpotato leaves

Once a wound is recognized, the host-plant cell rapidly produces signal molecules, namely phytohormones, which are able to coordinate the intricate defense network system to achieve self-protection [21]. The regulation mechanism underlying CGA production by hormones has been explained previously. To improve our understanding of the role of wounding in this process, we measured CAF and CGA contents, phytohormone accumulations, and expression levels of CGA synthesis genes under wounding treatment. As sweetpotato weevil feeding follows a sucking-piercing pattern, continuous wounding was adopted to simulate this eating behavior. According to our results, the above indicators all displayed an overall significant upward trend (Fig. 4A-4C), this suggests that the damage induced the release of JA, SA, and ABA, which in turn mediated gene activation and resulted in CGA accumulation. No significant difference in CGA content was observed between the control group and the wounding group treated for 6 h. CGA could be converted into lignin, a major contributor to plants biotic and abiotic stress responses, but the signal driving transformation from CGA to lignin is not yet clear [37]. We further explored the effect of continuous wounding on the CGA content within sweetpotato leaf. Interestingly, CGA content initially differed between treatment and control groups and then converged at 6 h, probably as a result of injury signal transduction or CGA transportation (Fig. 4D). More evidence is needed to explain this result.

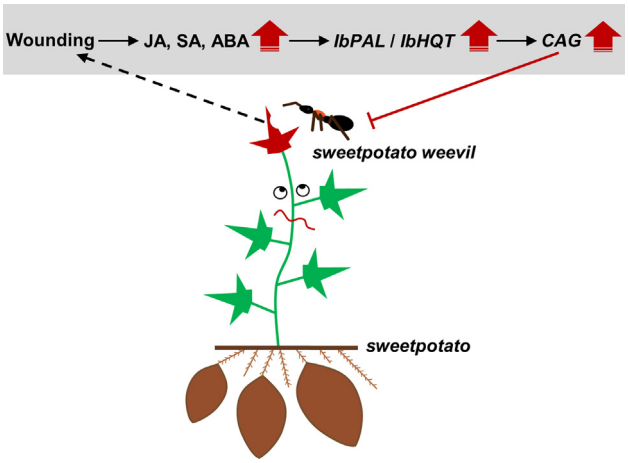

Fig. 5. Summary on formation of chlorogenic acid in sweetpotato in response to sweetpotato weevil attack.

Recognition of herbivore arrival depends both on wounding and oral secretions from the invader [21]. Plant-insect interaction response patterns can accordingly be divided into herbivore-associated molecular patterns (HAMPs) and damage-associated molecular patterns (DAMPs). HAMP elicitors are herbivore-specific compounds originating from the herbivore; they include inceptins, caeliferins, *N*-acyl glutamines, and bruchin A. DAMP elicitors, such as saponins, pectins, cardenolides, cicutoxin, and systemin, are endogenous molecules produced by plants through mechanical wounding [23]. Specific receptors on a cell membrane bind with their elicitor and trigger early signal transduction in the following order: (1) changes in membrane potentials, (2) intracellular [Ca^{2+}] transients, (c) protein phosphorylation/dephosphorylation cascades, (d) hydrogen peroxide generation, and (e) phytohormone generation [38]. Unfortunately, little is known about HAMPs in plants. Wounding regulates phenylpropanoid metabolism through the induction of gateway *PAL* [39]. Torres-Contreras et al. have also demonstrated that wounding is the prime inducer of increased CGA content in potato tubers [40]. Our results are in agreement with this conclusion (Fig. 4A and 4B). These phenotypic results were indeed achieved through hormone mediation. Wounding-induced production of JA, SA, and ABA is also well documented [21]. Altogether, wounding is an important upstream signal for directing CGA-mediated defense. Wounding caused by pest would induce phytohormones (JA, SA, and ABA) releasing, resulted in CGA accumulation by activating its biosynthesis genes, which in turn inhibit the pest activity (Fig. 5). Further study is needed to determine whether oral secretions of sweetpotato weevils participate in this process.

Conclusions

The distribution of CGA among different sweetpotato plant parts (leaf > stem > root skin > root flesh) was roughly in agreement with the chronological order in which sweetpotato organs were exposed to sweetpotato weevils. This result indicates that

more attention should be paid to breeding sweetpotato-weevil resistance into sweetpotato leaves. In conclusion, sweetpotato weevil attack caused a marked upregulation of CGA in sweetpotato leaves. More specifically, the continuous wounding produced by herbivore invasion significantly elevates levels of SA, ABA, and JA, which cofacilitate CGA generation by modulating the expression of the key genes *IbPAL* and *IbHQT*. Our study provided the first *in vivo* evidence that CGA confers remarkable resistance against sweetpotato weevils. These results aid understanding of the regulatory mechanism associated with CGA formation and are a promising foundation for future development of CGA-mediated pest control in the field.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.06.011.

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