Features and genetic basis of chlorogenic acid formation in diploid potatoes

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ABSTRACT

The concentration of chlorogenic acids (CGAs), is tightly associated with the appearance, taste, and nutrient content of potato tubers. Manipulation of tuber CGA concentrations allows for the breeding of quality traits in potatoes. Currently, a hybrid potato breeding system that aims to convert tetraploid potato into a diploid seed crop represents a new development in potato breeding. Unfortunately, however, a systematic study of CGA formation is very limited in diploid potatoes. Here, using a diverse panel of diploid potatoes, including 40 ancestors and 374 landraces, we analyzed the influence of location, environment, genetic basis, as well as expression of enzymes, in affecting the CGA concentrations in diploid lines. We revealed a selection of the decreased CGA level of tuber flesh in the domestication of diploid potatoes. Moreover, we identified 18 SNPs associated with tuber CGA levels using re-sequenced genome data. This study provides a basis for the breeding of high-quality potato by taking into consideration customer preferences.

1. Introduction

Potatoes (Solanum tuberosum L.) are grown in ~ 125 countries and are consumed as the third most important staple food by over 1 billion people (Devaux, Goffart, Petsakos, Kromann, & Hareau, 2020). Potato tubers provide not only large amounts of carbohydrates as an energy source, but also nutrient-rich elements including minerals, vitamins, and fiber to human diets. Potatoes represent the largest supply of vegetable phenolics and antioxidants in the American diet (Song et al., 2010). Unfortunately, quality traits such as micronutrients and flavors are commonly neglected and are often lost in breeding processes that prioritize high yield (Sands, Morris, Dratz, & Pilgeram, 2009; Tieman et al., 2017). This can result in consumer complaints and unmet market demands. Thus, potato breeders need to develop elite cultivars with high-quality traits and more desirable flavors.

The flavor, nutrient content, and disease resistance of crops is commonly associated with a vast array of specialized metabolites, such as phenolics, terpenoids, and alkaloids. These compounds are biosynthesized by plants in order to mediate the interactions with the constantly changing environment. In potato tubers, chlorogenic acids (CGAs), ester of caffeeic acid and quinic acid, represent over 80% of all phenolic compounds (Friedman, 1997a; Im et al., 2008; Riciputi et al., 2018; Valinas, Lanteri, Have, & Andreu, 2017). They exist in three major isomers, CGA (5-O-cafeoylquinic acid, the predominant isomer), neo-chlorogenic acid (3-O-cafeoylquinic acid), and cryptochlorogenic acid (4-O-cafeoylquinic acid), respectively (Maldonado, Mudge, Gänzle, & Schieber, 2014). Given their high antioxidant activities, CGAs exhibit potential health benefits including anti-inflammatory, anti-diabetic, anti-carcinogenic, and anti-obesity effects, which can prevent cardiovascular and other degenerative diseases (Onakpoya, Spencer, * Corresponding authors.
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were planted in Xundian (102°E, 23°N), Dehong (97°E, 25°N), and 2019; Valinas et al., 2017), higher contents of CGAs are usually observed in colored potatoes which can negatively influence a customer’s preference. Moreover, over accumulation of CGAs in tubers (˃120 mg/100 g) may induce unwanted sourness and bitterness, severely affecting the flavors of the potato tuber (Jansky, 2010). Since the concentration of chlorogenic acids (CGAs), is tightly associated with the appearance, taste, and nutrient content of potato tubers, CGA levels should be controlled to provide bioactive nutrients, but not to detrimentally influence tuber flavors.

Currently, the breeding process to introduce the desired traits to the potato is labor-intensive and time-consuming, as most potato cultivars should be controlled to provide bioactive nutrients, but not to detrimentally influence tuber flavors.

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Currently, the breeding process to introduce the desired traits to the potato is labor-intensive and time-consuming, as most potato cultivars should be controlled to provide bioactive nutrients, but not to detrimentally influence tuber flavors.

Here, we analyzed the influence of location, environment, genetic basis, as well as the expression of key enzymes, in affecting the abundance of the major isoflavonoids (5-O-cafeoylquinic acid, CGA), using a natural variation diploid population including 40 wild species and 374 landraces. These results provide a basis to understand the mechanisms underlying the CGA formation in diploid potatoes, and also serve as guidance for the future breeding of high-quality potato by taking into consideration customer preferences.

2. Material and methods

2.1. Plant material

414 diploid potatoes, including *Solanum candolleanum* (40), *Solanum stenotomum* (113), *Solanum goniocalyx* (38), and *Solanum phureja* (223) were planted in Xundian (102°41’-103°33’E, 25°20’-26°01’N, in March 2018) and Dehong (97°31’-98°43’E, 23°50’-25°20’N, in November 2018 and 2019) in the Yunnan Province. Each line was cultivated in fields containing ten replications, and tubers from each line were simultaneously collected at end of the growing season. For each line, skin or flesh from five tubers were pooled together to generate a sample, while young leaves from five plants were also pooled together to generate a sample. The samples were immediately frozen in liquid nitrogen, then freeze-dried and stored at 4 °C until analysis. Three samples were used as three biological repeats in the following assays.

2.2. Sample preparation

200 mg powdered freeze-dried sample was homogenized with 2 mL of 75% ethanol (v/v) and incubated for 30 min in an ultrasonic bath, before being centrifuged at 13,945g for 2 min at 4 °C. The supernatant was then filtered (0.22 µm microporous organic filtration membrane) and transferred into a clean tube for analysis.

2.3. Quantification of chlorogenic acid, caffeic acid, ferulic acid, and antioxidant activity

Chlorogenic acid (5-O-cafeoylquinic acid), caffeic acid, and ferulic acid were determined by High-Performance Liquid Chromatography (HPLC) as described by Valinas et al. (2017) with minor modifications, which was carried out using an ACQUITY UPLC system (Waters, Milford, USA). A flow rate of 0.5 mL/min was used and 10 µL samples were injected onto a C-18 Phenomenex Luna column (250 × 3 mm i.d.; 5 µm particle size) at 25 °C. The mobile phases were (A) HPLC-grade water acidified with 4% H3PO4 (Damao, Tianjin, China), and (B) acetonitrile (Damao, Tianjin, China). The solvent program was as follows: 0 to 25 mins, gradient from 10% to 25% B, then linear 30% B until 36.5 mins, return to 10% B between 36.5 and 37 mins and linear at 10% B until 40 mins. Simultaneous monitoring was set at 320 nm. 5-O-cafeoylquinic acid (Pusi, Chengdu, China), caffeic acid (Pusi, Chengdu, China), and ferulic acid (Pusi, Chengdu, China) were used as standards for the calibration curve. Chlorogenic acid, caffeic acid, and ferulic acid concentrations are expressed in µg/g DW. In addition to HPLC analysis, a UV–Vis spectrophotometer (Younike, Shanghai, China) was used to determine the content of chlorogenic acid at 327 nm for population samples. Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Valinas, Lanteri, Have, & Andreu, 2015) with minor modification. Briefly, 150 µL of extracts were added to 2850 µL DPPH (Yuanye, Shanghai, China) solution (100 µM) freshly prepared in methanol. The reaction was kept in the dark at room temperature for 24 h, and the absorbance at 515 nm was measured using a UV–Vis spectrophotometer (Younike, Shanghai, China). Trolox (Yuanye, Shanghai, China) was used as the standard for the calibration curve and the results are expressed as µg of Trolox equivalents g−1 DW. For all determinations, three independent extractions from a pool of five tubers were conducted.

2.4. RNA extraction, cDNA synthesis, and qRT-PCR experiments

Total RNA from tubers was extracted using RNAprep Pure polysaccharide polyphenol plant total RNA extraction kit (Tiangen, Beijing, China) following the manufacturer’s instruction. First-strand cDNA synthesis was prepared using 1 µg total RNA with PrimerScript RT reagent kit (TaKaRa, Kusatsu, Japan). qRT-PCRs were performed using SYBR Premix (Takara, Kusatsu, Japan) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster, USA). Three independent biological experiments were performed in all cases. ACTIN was used as an internal control gene for normalization. The relative gene expression was calculated using 2−ΔΔCt. The primers used for qRT-PCRs are listed in Supplementary Table 1.

2.5. Statistical analysis of phenotypic data

The variation assessments were compared before and after filter the data from the Goniocalyx group. The boxplot of the CGA content trait was plotted using Boxplot in R (https://cran.r-project.org). The best linear unbiased predictions (BLUPs) can eliminate the environmental deviation and estimate the real individual value. Therefore, the BLUPs were determined using the ‘lme4’ package of the R3.5.3 software (www.r-project.org), with year and location selected as random effects in the model $\Delta \Delta CT = lmer(X + (1|Line) + (1|Year) + (1|Line:Year). The descriptive statistics of the CGA content, correlations between BLUP and the measured values among different years, and those among BLUPs were analyzed using correlation in R3.5.3 and corrplot0.8.4 (https://cran.r-project.org/web/packages/corrplot).

2.6. Genome-wide association studies (GWAS) for the CGA content of tuber flesh, skin, and leaf

PCA was conducted to evaluate the genetic structure of 271 diploid
lines using Genome-wide Complex Trait Analysis (GCTA) software. The top five principal components were used to construct the covariate matrix, with which we performed the association analysis. GWAS were performed using the Emmax method (Valinas et al., 2015). Single nucleotide polymorphisms (SNPs) collected from the Resequencing project (Kang et al., 2010) were filtered with a missing rate ≤ 10%, and a Minor Allele Frequency (MAF) ≥ 5%. The BLUPs of tuber CGA content from plants grown in 2018 and 2019, and CGA content of the leaf and the tuber skin from plants grown in 2019 were utilized for the association study. GWAS threshold was set using a Bonferroni correction in the analysis of the tuber CGA content, and GWAS threshold was set as $1 \times 10^{-6}$ in the analysis of the CGA content of leaf and tuber skin. Manhattan plots were generated using the qqman package in R (https://github.com/stephenturner/qqman). The effectiveness of the significant SNPs was calculated using gcta-1.93.2 (http://cnsgenomics.com/software/gcta). To fully cover the candidate region, we extended the region to calculate the pairwise linkage disequilibrium (LD) using the SNPs revealed by the GWAS analysis (p value < 0.001 for chromosome 4 and 8, and p value < 0.0001 for chromosome 10). Only the LD blocks containing at least one significant SNP were regarded as significant loci.

3. Results and discussion

3.1. Selection of decreased CGA level of tuber flesh in the domestication of diploid potatoes

To systematically investigate the characteristics of CGA formation in diploid potatoes, a diversity panel consisting of a domestication progenitor Solanum candolleanum (40 lines) and the Andean landraces Solanum tuberosum groups Stenotomum (113 lines), Goniocalyx (38 lines), and Phureja (223 lines) were planted at Xundian (102°41'-103°33' E, 25°20'-26°01’N, in March 2018) and Dehong (97°31'-98°43'E, 23°50'-25°20’N, in November 2018 and 2019) in Yunnan Province (Supplementary Table 2). After the growth season, tubers of 158 species including
S. stenotomum (109), S. goniocalyx (14), and S. phureja (35) were successfully harvested from Xundian. In contrast, tubers of 190 species including S. stenotomum (83), S. goniocalyx (27), and S. phureja (80) were harvested from Dehong in 2019, while 226 lines including S. stenotomum (99), S. goniocalyx (33), and S. phureja (94) were harvested from Dehong in 2020 because both field management and weather conditions were much better at Dehong, a well-known winter farming area in China, than that of Xundian. This study found that a large number of our chosen diploid species, especially those from the progenitor S. candollei group, failed to produce any tubers in each growing season, likely due to slow plant growth and pest infection. Thus, we had to use flowerpots and greenhouses to re-culture these S. candollei lines in Kunming (102°10′-103°40′E, 24°23′-26°22′N, July 2019), and finally, tubers from 16 progenitor species were successfully collected (Supplementary Fig. 1).

Tuber CGA level was determined using the Ultraviolet-visible spectroscopy method as well as HPLC, as both methods have been shown as efficient and accurate ways to determine CGA content in plants (Friedman, 1997b). The CGA concentrations in S. phureja tubers grown in the Xundian area, ranged from 0.60 to 4020.73 μg/g DW, while those in S. goniocalyx and S. stenotomum tubers ranged from 5.80 to 435.27 μg/g DW and 0.24 to 987.16 μg/g DW, respectively (Fig. 1A). A similar result was also observed for the tubers collected from both growing seasons at Dehong (Fig. 1B,C). More intriguingly, the average CGA concentrations of tuber flesh (1848.11 μg/g DW) are much higher in the immediate ancestor S. candollei group, when compared to that of the three Andean landraces (Fig. 2), although it should be noted that these ancestral lines were not grown in the same field as the other diploid lines.

A clear decrease in the abundance of CGAs present in Andean landrace tubers suggests a tuber CGA level was selected during the domestication of potato (Meyer et al., 2015; Nzaramba, Bamberg, Scheuring, & Miller, 2006). While other studies have also found comparable or even higher phenolic content in potato cultivars than in wild lines (Navarre, Pillai, Shakya, & Holden, 2011; Soltys-Kalina, Murawksa, Strzelecki-Zyta, Waslelewicz-Fis, & Marczewski, 2019), this discrepancy may due to a limited number of wild and cultivated lines used in this research. Given that domestication selects for certain metabolic processes, including the shikimate pathway which generates a tryptophan sink, and tryptophan catabolism which produces kynurenic acid (Hardigan et al., 2017), the metabolic direction of tryptophan to kynurenic acid may decrease the abundance of tryptophan and phenylalanine in tubers (Yao & Brisson, 1995). This would limit the metabolic flux forward phenolic compounds such as CGAs and lignin.

Another possible explanation for the decreased CGA content is customer preference. Similar to the result in tetraploid potatoes (Orsák, Hamouz, Lachman, & Kasal, 2019; Ru et al., 2019; Valinas et al., 2017), higher CGA levels of tuber flesh and peel were observed in the colored diploid lines than that of ordinary potatoes (Supplementary Table 3-5). Our results (Supplementary Fig. 2) in accordance with previous studies (Albishi, John, Al-Khalifa, & Shahidi, 2013; Glosek-Sobieraj, Cwalina-Ambrozia, Waszkiewicz, Hamouz, & Perczak, 2019; Orsák et al., 2019; Valinas et al., 2017), found that purple and red-pigmented tubers have a greater concentration of total phenolics, anthocyanins, and antioxidant activities. Generally, US consumers prefer white-flesh varieties of potato rather than colored flesh (Navarre et al., 2011).
Consumer preference likely encourages potato breeders to cultivate varieties with a low tuber CGA content to avoid pigmented tubers.

3.2. Major factors in affecting the CGA level of tuber flesh in diploid potatoes

Consistent with a previous study using tetraploid potatoes (Reddivari, Hale, & Miller, 2007), the most significant determinant of CGA levels in diploid potatoes was the effect of genotype (Fig. 2). A 2.80, 9.19, 1.87, and 7.23 fold change of the CGA level of tuber flesh were observed for the ancestor group *S. candollei*, the landrace groups *S. stenotomum*, *S. goniocalyx*, and *S. phureja*, respectively. This diversity in tuber CGA concentrations could only be explained by the significant phylogenetic distance between these diploid lines.

Following genetic variation, the second major determinant of the CGA content of tuber flesh is the geographic location. For all lines grown at Xundian, the distribution patterns of tuber CGA contents were different from those of the plants grown at Dehong, especially for the 11 species of *S. goniocalyx* (Fig. 3A). The most contrasting examples were C107 and C22, whose tuber flesh had the highest CGA level in *S. stenotomum* and *S. goniocalyx* group, respectively, when they were grown at Xundian. However, once these two lines were planted at Dehong, their tuber flesh accumulated the lowest CGA in their respective group (Fig. 3A). This result further confirmed the role of location in determining the CGA level in potatoes (Reddivari et al., 2007). However, we also documented lines from *S. stenotomum* and *S. phureja* group, whose CGA levels were relatively stable and were only weakly influenced by the location. For instance, tube flesh of C206 from *S. phureja* accumulated the highest CGA content at both locations (Fig. 3A), indicating the genotype of this line is the decisive factor in controlling its CGA level.

Moreover, planting time was the third most influential factor in controlling the CGA content in diploid potatoes. CGA levels were relatively stable when 144 diploid potatoes were collected after two growing seasons at Dehong, although inconsistent results were also observed for a few of the lines (Fig. 3B).

Science a year is the least influential factor in controlling CGA content in diploid potatoes, the CGA contents of tuber flesh collected at between 2018 and 2019 were...
leaves and tuber skins were much higher than that of tuber flesh in the contribution of genotype to the CGA content trait, the genetic architecture of the trait was inspected further. The heritability of the SNPs involved in producing the by-product CA, this result is consistent with our expectations. However, for 4CL, this result indicates this upstream enzyme catalyzes the committed steps for CGA biosynthesis, although their roles were not consistent in different studies (Valinas et al., 2015; Niggeweg et al., 2004; Valinas et al., 2015) while CSE is an enzyme responsible for the biosynthesis of p-Coumaro-CoA, a common precursor for CGA production and lignin biosynthesis (Howles et al., 1996; Niggeweg et al., 2004; Valinas et al., 2015), while CSE is an enzyme competing with HCT for the common substrate, Caffeoyl shikimic acid, to produce CA during lignin pathway (Valinas et al., 2015). In contrast to PAL, 4CL, and CSE, the two enzymes, HQT, and HCT are supposed to catalyze the committed steps for CGA biosynthesis, although their roles were not consistent in different studies (Valinas et al., 2017; Niggeweg et al., 2004; Payyavula et al., 2015). Finally, we also analyzed levels of CA and FA in tuber peel and flesh Valinas et al., 2015). Interestingly, the CGA content of tuber peel was co-related with the expression levels of HQT and HCT, but not with PAL (Fig. 6). While the CGA content of tuber flesh was co-related with the expression level of all the three enzymes. In addition, expression of CSE and 4CL were negatively co-related with the CGA contents of tuber peel and flesh (Fig. 6). Since CSE is potentially involved in producing the by-product CA, this result is consistent with our expectations. However, for 4CL, this result indicates this upstream enzyme may also participate in the downstream reaction to direct metabolic flux toward by-products.

FA was not detected in either tuber peel or flesh, while the CA content of the tuber flesh was very low, even in the line that has the highest CGA level (Fig. 6). Although this result is inconsistent with previous findings that CA and FA are co-related with the CGA level in tuber peel and flesh (Valinas et al., 2015), a similar result was also observed in purple potato flesh (Albishi et al., 2013; Ru et al., 2019; Valinas et al., 2017), and color potato could be explained by the fact that both CA and FA were in bound fraction, not in a free status, as in the colored potato (Ru et al., 2019).

To locate loci associated with the CGA content in diploid potatoes, the best linear unbiased prediction (BLUP) value was obtained from the CGA content of each accession grown at Dehong (2018 and 2019). The populations we selected were likely suitable for GWAS analysis. Thus, the populations we selected were likely suitable for GWAS analysis.

3.3. Potential genetic basis of the CGA formation in diploid potato

3.4. Expression patterns of the key enzymes involved in the CGA metabolism

In addition to utilizing GWAS analysis to investigate the potential genetic basis in controlling the CGA levels in diploid potatoes, three lines with a low, medium, or high CGA content of tuber peel and flesh were selected from S. stenotomum group to study the expression of the CGA biosynthetic enzymes. We focused on the enzymes, such as phenylalanine ammonia-lyase (PAL), 4coumarate-CoA ligase (4CL), caffeoyl shikimate esterase (CSE), hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT), and hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), that are involved in driving the metabolic flux toward CGA or byproducts, such as caffeic acid (CA) and ferulic acid (FA). Among these enzymes, PAL and 4CL are responsible for the biosynthesis of p-Coumaro-CoA, a common precursor for CA production and lignin biosynthesis (Howles et al., 1996; Niggeweg et al., 2004; Valinas et al., 2015), while CSE is an enzyme competing with HCT for the common substrate, Caffeoyl shikimic acid, to produce CA during lignin pathway (Valinas et al., 2015). In contrast to PAL, 4CL, and CSE, the two enzymes, HQT, and HCT are supposed to catalyze the committed steps for CGA biosynthesis, although their roles were not consistent in different studies (Valinas et al., 2017; Niggeweg et al., 2004; Payyavula et al., 2015). Finally, we also analyzed levels of CA and FA in tuber peel and flesh Valinas et al., 2015). Interestingly, the CGA content of tuber peel was co-related with the expression levels of HQT and HCT, but not with PAL (Fig. 6). While the CGA content of tuber flesh was co-related with the expression level of all the three enzymes. In addition, expression of CSE and 4CL were negatively co-related with the CGA contents of tuber peel and flesh (Fig. 6). Since CSE is potentially involved in producing the by-product CA, this result is consistent with our expectations. However, for 4CL, this result indicates this upstream enzyme may also participate in the downstream reaction to direct metabolic flux toward by-products.

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4. Conclusion

CGA levels of tuber flesh in diploid potatoes are associated with flesh color and antioxidant activity and are mainly influenced by genetic variation of the plants and the environment. During the domestication of diploid potatoes, a decreased tuber CGA level was potentially selected for, likely due to the customer preference of white flesh potatoes and the selected metabolic flux toward side products, such as kynurenic acid. We also provide the SNPs and candidate genes associated with the CGA level of tuber flesh using re-sequencing genome data of diploid lines. This study provides a basis for the future breeding of high-quality potatoes by manipulation of the tuber CGA level.

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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