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Association genetics of sucrose, glucose, and fructose contents with SNP markers in *Solanum tuberosum* Group Phureja

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Abstract

High contents of sucrose (non-reducing sugar), glucose and fructose (reducing sugars) in potato tubers represent an undesirable trait for fry processing because reducing sugars lead to potato darkening and the production of toxic compounds such as acrylamide that reduce consumer's acceptance and generate risks for human health. Association genetics analysis is a strategy to study the molecular basis of complex traits as tuber sugar contents. Colombia leads the production of diploid genotypes named "Creole potato", which belong to the cultivated Group Phureja and present outstanding organoleptic and nutritional properties. Currently, there are not Phureja cultivars suitable for chip processing because of high levels of reducing sugars in the tubers. The main purpose of this research is to determine the candidate gene regions influencing sucrose, glucose and fructose contents in *Solanum tuberosum* Group Phureja. Tubers from 108 accessions of the Colombian Core Collection and four commercial cultivars were sown in pots in Soacha (Cundinamarca, 2850 m above the sea level) for sugar content analyses. The harvest of three plants of each genotype constituted three biological replicates. The sugar contents of Phureja genotypes were quantified through a liquid chromatographic method developed and validated using an AMINEX87H column with sulfuric acid 10 mM as eluent. Sucrose, glucose and fructose genotypic mean values varied from 6.39-29.48 mg/g tuber dried weight (DW), 0.46-28.04 mg/g tuber DW, and 0.29-27.23 mg/g tuber DW, respectively. Association analysis was carried out with 111 SNP markers identified in candidate genes with key function in carbohydrate metabolism. This analysis revealed four SNP markers in the locus *InvGE* from an apoplastic invertase and one SNP marker in the locus *SssI* from a soluble starch synthase with significant effect in sugar content variation. These enzymes have not been found expressed in mature tubers, therefore these SNP-trait associations might be indirect resulting from the linkage disequilibrium with causal variants, or direct through a potential novel role of these candidate genes controlling sugar contents in tubers.

Keywords: Reducing sugars, frying quality, association mapping, HPLC

Resumen

El alto contenido de sacarosa (azúcar no reductor), glucosa y fructosa (azúcares reductores) en los tubérculos de papa representa un rasgo indeseable en la industria del procesamiento en frito, pues lo azúcares reductores conducen al ennegrecimiento de la papa frita y a la producción de compuestos tóxicos como la acrilamida que reducen la aceptación por los consumidores y ocasionan riesgos para la salud humana. El análisis de asociación genética es una estrategia para estudiar las bases moleculares de rasgos complejos como la acumulación de estos azúcares en tubérculos. Colombia lidera la producción de genotipos diploides conocidos como "papa criolla", que pertenecen al grupo cultivado Phureja y presentan propiedades organolépticas y nutricionales sobresalientes. Actualmente, no existen cultivares de Grupo Phureja aptos para el procesamiento en frito debido a la alta acumulación de azúcares en los tubérculos. El objetivo de esta investigación fue establecer las regiones de genes candidatos con influencia en los contenidos de sacarosa, glucosa, y fructosa en *Solanum tuberosum* Grupo Phureja. Tubérculos de 108 accesiones de la Colección Central Colombiana y cuatro cultivares comerciales fueron sembrados en macetas en Soacha (Cundinamarca, 2850 m sobre el nivel del mar). La cosecha de tres plantas de cada genotipo constituyeron tres replicas biológicas. Los azúcares se cuantificaron con cromatografía líquida utilizando una metodología que fue desarrollada y validada con una columna AMINEX HPX 87H utilizando ácido sulfúrico 10 mM como eluyente. Se encontraron valores medios genotípicos de sacarosa, glucosa, y fructosa variando entre 6.39-29.48 mg/g peso seco (PS) de tubérculo, 0.46-28.04 mg/g PS de tubérculo, y 0.29-27.23 mg/g PS de tubérculo, respectivamente. El análisis de asociación genética se efectuó con 111 marcadores SNP identificados en genes candidatos que codifican para enzimas en el metabolismo de carbohidratos. Este análisis reveló cuatro marcadores SNP en el locus *InvGE* de una invertasa apoplástica y un marcador en el locus *SssI* de un almidón sintasa soluble con efecto significativo en la variación del contenido de azúcares. Estas enzimas no han sido encontradas expresadas en tubérculos maduros, en consecuencia estas asociaciones rasgo-marcador pueden ser indirectas resultado del desequilibrio de ligamiento con variantes causales, o directa a través de un potencial rol novedoso de estos genes candidatos en el control de la acumulación de azúcares en tubérculos.

Palabras claves: Azúcares reductores, calidad de fritura, mapeo por asociación, HPLC

Content

Introduction

Potato (*Solanum tuberosum*) is the third crop of global relevance after wheat and rice thus playing an important role in worldwide food security and nutrition as it is a supplement or a substitute in cereal-based diets (Camire *et al*., 2009; Mosquera and Cuéllar, 2013). *S. tuberosum* Group Phureja is distributed from central Bolivia to western Venezuela and represents an important genetic resource for Colombia as in the south region of Nariño is located a center of diversity (Estrada, 1996; Spooner *et al*., 2007). This cultivated group consists primarily on diploid potatoes with short day adaptation and tubers sprouting at harvest (Huamán and Spooner, 2002; Spooner *et al*., 2007).

Colombia leads the production, consumption and export of diploid Phureja genotypes commonly known as "Creole potato", which are characterized for their round tubers with yellow flesh and skin (Bonilla *et al*., 2009; Rodríguez *et al*., 2009). These genotypes present an interesting possibility for their widespread use because of their outstanding nutritional and organoleptic properties and their processing potential (Rivera *et al*. 2006; Rodríguez *et al*., 2009; Peña *et al*., 2015). In Colombia this crop is an important source of income for small farmers (Rodríguez *et al*., 2009). The generation of basic information for Group Phureja is therefore relevant to promote its processing alternatives.

Currently, the demand for potato chips and French fries continues to grow because of changes in consumption habits (Faulkner, 2015; Kirkman, 2007). High contents of sucrose (non-reducing sugar), glucose, and fructose (reducing sugars) in potato tubers represent an undesirable trait for fry processing because reducing sugars are precursors of the Maillard Reaction and sucrose is the main source of glucose and fructose during its enzyme-catalyzed hydrolysis in cold storage below 10 ºC (Eck, 2007; Isla *et al*., 1998). The Maillard Reaction refers to a series of non-enzymatic reactions between non-reducing sugars and principally amino groups from amino acids during the thermal processing of potatoes that lead to the production of dark pigments, the development of off-flavours, and the production of toxic compounds such as acrylamide that reduces consumer's acceptance and also generates risks for human health (Eck, 2007; Halford *et al*., 2012).

There are not Phureja cultivars suitable for fried processing, thus crops for frying purposes must be grown less than 2,600 meters above the sea level to decrease the probabilities of high or accelerated accumulation of reducing sugars in tubers due to lower temperatures (Ñústez-López, 2011). Because of the lack of dormancy in tubers, the development of Phureja cultivars with tolerance to cold-induced sweetening will have the additional advantage of allowing the storage at low temperatures of tubers for processing to avoid sprouting (Sowokinos, 2008). The aforementioned considerations show the importance of guiding breeding programs towards the development of cultivars specific for processing.

The phenotypic assessment of potato frying quality has been performed frequently by means of visual scales of frying color (Werij *et al*., 2012; Li *et al*., 2013). Notwithstanding, it is relevant for potato breeders the evaluation of genotypes for their sugars profiles through the measurement of sucrose, glucose, and fructose contents. This type of evaluation also constitutes a quantitative approach for the understanding of the frying quality trait as it is possible to identify the potential of different genotypes for the synthesis of dark pigments and acrylamide (Muttucumaru *et al*., 2014).

Chromatographic methods are the most powerful analytical techniques for the identification and quantification of monosaccharides and oligosaccharides in foods. High Performance Liquid Chromatography (HPLC) is currently the most common chromatographic method for analyzing these compounds as it is capable of rapid, specific, sensitive, and precise measurements (McClements, 2003). However, there are neither details on method development nor in validation steps for sugar extraction and chromatographic analysis in potato. Therefore, it is necessary the development, validation and implementation of an extraction method and a HPLC method for the identification and reliably quantification of sucrose, glucose, and fructose in Phureja raw tubers.

The generation of such quantitative data of the frying quality phenotype in Group Phureja is also important to implement association mapping strategies where precise quantification of the trait phenotype in the population is imperative for the genetic association analyses (Ersoz *et al*., 2007). These analyses are relevant to understand the polygenic control of sugar accumulation in diploid genotypes of interest for Colombia as this trait is regulated by several pathways that influence starch-sugar equilibrium. The knowledge of genomic regions with influence on sugar contents can provide advantages in the increase of the speed of developing new cultivars through the use of marker assisted selection. This strategy can contribute in the decrease of the number and the extent of field trials by the identification of genotypes with desirable frying quality before measuring frying phenotype or sugar contents in field trials (Ramakrishnan *et al*., 2015).

Association mapping uses populations of diverse individuals to assess alleles of each locus for association, assuming that one or more of the considered loci are in linkage disequilibrium with the genomic region that is causal of the variation of the trait of interest (Rafalski, 2010). Using this approach there is a major record of ancient recombination events than in a bi-parental population, that lead to the reorganization of the chromosomes in small regions. Thus, there is a greater possibility that the marker associated significantly with the phenotype will be located near the causal region, therefore allowing a higher mapping resolution (Ersoz *et al*., 2007; Hamblin *et al*., 2011). This strategy has shown to be a less time-consuming approach for the discovery of marker-trait associations compared with linkage mapping (Álvarez *et al*., 2015). The use of natural populations or sets of breeding materials in association mapping analysis is useful in the generation of diagnostic molecular markers that can be readily implemented in breeding schemes for the selection of outstanding genotypes (Gebhardt *et al*., 2007).

There is a significant knowledge on the metabolic pathways and enzymes involved in starch synthesis, degradation and transport in potato. Sixty nine functional genes in carbohydrate metabolism have been mapped in potato (Chen *et al*., 2001). From these candidate genes, a set have been tested for association of natural DNA variation with frying quality or reducing sugar contents in tetraploid potato germplasm (Baldwin *et al*., 2011; Li *et al*., 2005, 2008, 2013; Schreiber *et al*., 2014). These studies have revealed the multiloci genetic architecture of sugar contents and frying color related to genes coding enzymes functional in carbohydrate metabolism in tetraploid potato populations. Therefore, it is relevant to identify the candidate gene loci that underlie these traits in Group Phureja that represent a potato gene pool with a particular metabolic behavior regarding sugar accumulation in tubers.

The main purpose of this research is to establish the genetic association of sucrose, glucose, and fructose contents, measured with a HPLC method, with a set of SNP markers identified in candidate genes with key function in carbohydrate metabolism and influence in potato frying color and sugar contents in tetraploid potatoes (Baldwin *et al*., 2011; Fisher *et al*., 2013; Li *et al*., 2005, 2008, 2013; Schreiber *et al*., 2014). This document presents in Chapter 1 the development and validation of a HPLC method to quantify sucrose, glucose, and fructose in tubers of Group Phureja. In Chapter 2 this method is implemented to reveal the natural variation of sucrose, glucose, and fructose contents in a Colombian germplasm collection. Chapter 3 analyzed the association between these accurate sugar measurements and a set of SNP markers identified in ten candidate gene loci. This research is the first attempt to understand the molecular basis of sugar accumulation in Group Phureja tubers through an association mapping strategy with phenotypic data obtained from the application of analytical chemistry methodologies.

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Chapter 1

Development and validation of a liquid chromatographic method to quantify sucrose, glucose, and fructose in tubers of *Solanum tuberosum* Group Phureja

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ABSTRACT

A High Performance Liquid Cromatography (HPLC) method was developed and validated to quantify sucrose (non-reducing sugar), glucose, and fructose (reducing sugars) in raw tubers of *Solanum tuberosum* Group Phureja. Chromatographic analysis was performed using an AMINEX HPX 87H column, at 18 ºC, linked to a refraction index detector, at 35 ºC. The eluent was 10 mM sulfuric acid. The conditions established for the method provided an optimum separation of sugars, citric acid, and malic acid, with resolution values higher or equal to one. Among the four sugar extraction methods tested, the double 50 % (*v/v*) aqueous methanol extraction gave the highest level of analytes. Recovery of this extraction method ranged between 94.14 to 99.77 %. The HPLC method was validated for repeatability, reproducibility, linearity, and limits of detection, and quantification. Relative standard deviation was found to be lower than five, when testing repeatability and reproducibility, which is suitable considering a range of acceptability from 5.3 to 7.3. Additionally, the regression analyses supported the method linearity in a range of quantification from 3 to 100 mg/L with regression coefficients values greater than 0.998 for the three analytes. Limits of detection were 3.0 mg/L for the three sugars and limits of quantification were 2.0 mg/L for sucrose and 3.0 mg/L for glucose and fructose. Four Colombian commercial cultivars (Criolla Guaneña, Criolla Paisa, Criolla Galeras, and Criolla Colombia) and five landrace accessions from the Colombian Core Collection of Group Phureja were grown in the district of Usme (Bogotá) fields to analyze their sugar contents. Sucrose, glucose, and fructose contents were found ranging from 0.93 to 3.11 g/100 g tuber dried weight (DW), from 0.25 to 4.53 g/100 g tuber DW, and from 0.10 to 1.49 g/100 g tuber DW, respectively. Therefore, a high range in the variability of sugar contents was found among genotypes. However, the variability was low among technical replicates of the same genotype, revealing an accurate quantification of sugars in Group Phureja. This method can be used to assess the amount of reducing and non-reducing sugars accumulation in potato germplasm.

Keywords: HPLC, reducing sugars, non-reducing sugars, Group Phureja.

1. Introduction

Potato (*Solanum tuberosum*) plays an important role in worldwide food security and nutrition as it is the third crop of global relevance [1]. Tetraploid potatoes are the most cultivated worldwide, however, there is an interesting potential for the widespread use of diploid genotypes, 'Creole potato', that are known for their round tubers with yellow flesh and skin, and outstanding organoleptic properties [2]. Creole potato genotypes belong to the cultivated *Solanum tuberosum* Group Phureja, consisting primarily on diploid potatoes, that present a center of diversity in the south Andean region of Colombia and the north of Ecuador [3]. This cultivated group is characterized by lack of tuber dormancy, short day growing period, and short vegetative season (120 days) [4]. Colombia is positioned as the greatest producer, consumer, and exporter of these genotypes [5]. As owners of a high diversity of Phureja potatoes, it is crucial to generate knowledge on the processing quality, especially the sugar type and contents.

Glucose and fructose (reducing sugars) accumulation is a phenomenon that increases at low temperatures, as these compounds contribute to freezing tolerance [6]. The plant uses sucrose (non-reducing sugar) as the main source for the accumulation of reducing sugars in the tuber. Non-enzymatic browning is the result of the reaction between free amino acids and reducing sugars at high temperatures during frying process, generating the production of dark pigments and rancid flavors in chips or French fries, due to the Maillard reaction [7]. Reducing sugars contents are conditioned by the levels of starch synthesis, degradation, and transport, which depend on the genotype and the temperatures at which the tubers are stored [7,8].

A creole potato cultivar for frying has yet not been developed and the current cultivars show several undesirable properties for processing, mainly because of the accumulation of high levels of reducing sugars in tubers leading to non-enzymatic browning [9]. Due to a worldwide increase in the demand of processed food, the potato processing industry is a fast growing sector [5]. Hence, Colombia has a big challenge in the development of Phureja cultivars for frying. The phenotypic assessment of potato frying quality has been performed frequently by means of visual scales of frying color [9-11]. There is no report on the concentration of sucrose, glucose, and fructose of Phureja germplasm collections. Such quantitative data are important for the breeding programs, to implement strategies such as association mapping where precise quantification of the phenotype is imperative for the genetic association analyses [12].

Chromatographic methods are the most powerful analytical techniques for the identification and quantification of monosaccharides and oligosaccharides in foods. High Performance Liquid Chromatography (HPLC) is currently the most common chromatographic method for analyzing these compounds as it is capable of rapid, specific, sensitive, and precise measurements [13]. For method validation it is necessary to demonstrate that a particular protocol applied to a sample is suitable for obtaining analytical results with an acceptable uncertainty level [14], including the protocol for the extraction of the analytes of interest.

Sugar extraction methods for potato tubers include the use of e.g. 80 % (*v/v*) aqueous ethanol either at 80 ºC [15] or at 60 ºC [16], 50 % (*v/v*) aqueous methanol [17], 100% (*v/v*) methanol with activated charcoal at room temperature [18], or water [19]. Ion exchange resin columns are of widespread use in carbohydrate HPLC analysis since they do not require complex eluents for effective separations [20,21]. Extracts of sugars from potato tubers have been analyzed using these columns, e.g. with a Carbopac PA1 column using gradient elution with sodium hydroxide [17,19], an Inertsil NH_2 column

with 80% (*v/v*) aqueous acetonitrile as eluent [22], and with an AMINEX HPX 87H column using sulfuric acid 8 mM for elution [23]. In the mentioned studies, nevertheless, there are neither details on method development nor in validation steps for sugar extraction and chromatographic analysis. This study, therefore, reports the development and validation of an extraction method and a HPLC method for the identification and quantification of sucrose, glucose, and fructose in *S. tuberosum* group Phureja raw tubers, involving the method implementation using an AMINEX HPX 87H ion exchange column in the analysis of nine genotypes.

2. Materials and methods

2.1. Chemicals

Sulfuric acid (98% analytical grade) and methanol (99.96% analytical grade) were from J.T. Baker (Center Valley, Pennsylvania, USA). Sucrose, glucose, and fructose HPLC standards were provided by Sigma-Aldrich (St. Louis, Missouri, USA). Water was obtained from a Water ProPs purification system (Labconco, Kansas, Missouri, USA).

2.2. Plant material and field design

Potato tubers were produced in the field in a loam soil (pH 5.02), in the district of Usme (Bogotá, Colombia; with an altitude of 3,400 meters above sea level, latitude of 4°20′23″N and longitude of 74°10′55″W). Tubers were sown on the beginning of April 2013 and plants were harvested on the mid of August 2013. Tuber maturity was assessed at the end of the growth season (140-150 days in these altitudes), when foliage was senescent and the skin was set to the tubers [24,25]. A randomized block design with three replicates was used to study the sugar content of four Colombian commercial cultivars (Criolla Guaneña, Criolla Paisa, Criolla Galeras, and Criolla Colombia) and five landraces from the Colombian Core Collection of Group Phureja (CCC) (CCC 8, CCC 52, CCC 80, CCC 108, and CCC 123). Each experimental unit consisted of tubers from one to three plants from each genotype in each block. Tubers from the three blocks were bulked to generate a composite sample of each genotype. Then, sugar content analyses were done with three technical replicates.

2.3. Tuber sample preparation

After harvest, mature and healthy tubers without mechanical damages were washed with distilled water and stored at 18°C. Two days later, the tubers were cut into slices (0.3-0.5 cm) and stored at -20°C. The frozen slices were freeze-dried for 72 h using a freeze drier model Free Zone 7806020 (Labconco, Kansas, Missouri, USA), homogenized using a domestic blender, and fine ground using a pestle and a mortar. Homogenized tissue was sieved with a mesh, obtaining a particle size of maximum 0.8 mm.

2.4. Optimization of sugar extraction and chromatographic analysis

2.4.1. Optimization of sugar extraction

Four extraction methods were tested using 0.5 g of freeze-dried tubers of Criolla Galeras: i) extraction with 4 mL HPLC grade water at 92 ºC [26]; ii) extraction with 4 mL 50% (*v/v*) aqueous methanol using reflux [27]; iii) extraction with 4 mL aqueous methanol in activated charcoal [18]; iv) double extraction with 4 mL aqueous methanol [28]. Each of these four extraction methods were repeated three times, thus three technical replicates were performed. Methanol was removed from the extracts by roto-evaporation and diluted to 10 mL in a volumetric flask with 10 mM sulfuric acid. Extracts were purified using 500 mg of C-18 cartridges (Agilent Technologies, Santa

Clara, California, USA), to remove less polar compounds and avoid possible co-elution with sugars during HPLC analysis [29]. Cartridges were activated with 2 mL of methanol and washed with 3 mL of 10 mM sulfuric acid. Then, 1 mL of extract was loaded into the cartridge and washed with 3 mL of 10 mM sulfuric acid. The aqueous eluate was taken to a 10 mL volumetric flask and topped up with 10 mM sulfuric acid.

Recovery of the selected method was calculated in extractions of raw tissue from Criolla Guaneña, by analyzing triplicate samples spiked with a standard mixture of 10 mg/L and three un-spiked samples, according to Eq. (1).

$$\%\ Recovery = \frac{C_{spk} - C_s}{C_{ad}} * 100 \quad (1)$$

Where C_{spk} is the average concentration of spiked samples, C_s is the average concentration of sugars from the original sample, and C_{ad} is the concentration of standards added to spiked samples.

2.4.2. Optimization of chromatographic analysis

The samples were analyzed in an UHPLC Ultimate 3000 (Dionex, Sunnyvale, California, USA) equipped with a pump, an autosampler and a refraction index (RI) detector (Shodex, New York, New York, USA), at 35 ºC, using an AMINEX HPX 87H column (300 mm length x 7.8 mm particle size) (Biorad, Hercules, California, USA), with a pre-column (30mm x 4.6 mm). Column temperature and concentration of sulfuric acid were tested in an interval from 18 to 35 °C and from 5 to 12 mM sulfuric acid. Those conditions that yielded the best resolution in compound separation were selected for sugar content analysis. The elution was isocratic during 35 min. A volume of 20 µL was injected per sample. The compound identification was based on comparison of retention time and co-elution after spiking with authentic standards. Quantification was performed by the external standard method. Calibration curves were established using a mixture of sucrose, glucose, and fructose standards, at concentrations ranging from 5 to 100 mg/L. Operation of the instrument and data processing were implemented using Chromeleon v. 7.1.2. (Dionex, Sunnyvale, California, USA).

Area of the peaks was integrated according to the maximum peak width from the baseline. Compound separation was determined based on the resolution for each pair of peaks, in injections of three mixtures of standards, at a concentration of 100 mg/L, using the European Pharmacopeia formula shown in Eq. (2).

$$Resolution = \left| \frac{t_{RefPeak} - t_R}{W_{50\%,RefPeak} + W_{50\%,R}} \right| \quad (2)$$

Where t_R is the retention time of the current peak; $t_{RefPeak}$ is the retention time of the reference peak which by default is the peak after the current peak; $W_{50\%,R}$ and $W_{50\%,RefPeak}$ are the width of these two peaks at 50% of the peak height.

2.5. Chromatographic method validation and analysis of sugar content in a set of group Phureja genotypes

The method developed above was validated for repeatability, reproducibility, linearity, and limits of detection (LOD), and quantification (LOQ). Inter-day repeatability was assessed over three days using a different 40 mg/L mixture of standards. Similarly, the reproducibility was evaluated based on five injections in the same day of a 40 mg/L mixture of standards. The Relative Standard Deviation (RSD) was used to evaluate the repeatability and reproducibility. AOAC's Peer Verified Method Program (PVMP) levels of acceptability of RSD values for given concentrations of the analyte, were used as a reference to analyze the precision of the method [30].

Linearity was evaluated based on linear regression analyses of three calibration curves run in different days, at concentrations of 5, 10, 20, 40, 60, 80, and 100 mg/L. The peak areas for the same concentration were averaged. Using this data a linear regression model was developed, and a regression coefficient greater than 0.998 was considered to have a linear relationship between the peak area and the concentration of the analyte [31,32]. LOD and LOQ were established using a mixture of standards with concentrations of 0.5, 1, 2, 3, 4, and 5 mg/L, prepared and injected in triplicates. LOD was visually assessed by identifying the minimum concentration at which each analyte was reliably detected in the three injections. Likewise, LOQ was determined based on the minimum concentration of analyte to which the peak area response and concentration showed linearity [32]. Thus, a regression model was settled using the average areas of the injections with lower concentrations. Using this validated method, three technical replicates, for each freeze-dried tuber samples, of nine genotypes were analyzed.

2.6. Statistical analyses

First, an analysis of variance of a completely randomized design was carried out to determine if at least one sugar extraction method was different from the others. Then, the Tukey's test was employed to identify differences among mean values, to select the extraction method that gave the highest level of extraction. Regression analyses for linearity and LOQ included testing the hypothesis for the model significance using the *F* statistic and testing the significance of the model's slope using a two-tailed *t* test. Hypotheses were tested with a level of significance of $p < 0.05$. All the analyses were carried out using R software v. 3.1. [33].

3. Results and discussion

3.1. Optimization of sugar extraction and chromatographic analysis

3.1.1. Optimization of sugar extraction

The analysis of variance revealed that for each sugar content at least one sugar extraction methodology was significantly different from the others. Table 1 shows the average contents of sucrose, glucose, and fructose for each method. Double extraction with 50% (*v/v*) aqueous methanol yielded the highest extraction levels for each analyte as well as it was determined that the mean value for this extraction method was significantly different from the others in glucose and fructose contents according to the Tukey's test. Despite sucrose mean value in this method was not significantly different from reflux with 50% (*v/v*) aqueous methanol, it is important to underline that the mean value for the other two sugars was significantly different, and that double extraction method allows carrying out an easier parallel processing of more samples than reflux.

Table 1. Average concentrations of sugars for each extraction method. Averages with different letters in each column indicate significant differences according to Tukey's test (p <0.05). Bold values show the highest contents of each analyte.

Method	Average sucrose concentration (mg/L)	Average glucose concentration (mg/L)	Average fructose concentration (mg/L)
Water	2,566.22 ± 81.29 a	308.2 ± 3.14 a	747.44 ± 3.36 a
Methanol double treatment	**4,925.64** ± 240.2 **b**	**1,035.36** ± 50.74 **b**	**1,487.26** ± 81.24 **b**
Methanol with activated charcoal	3,479.68 ± 189.74 c	672.42 ± 60.28 c	939.16 ± 82.79 ac
Methanol with reflux	4,322.76 ± 417.87 b	817.85 ± 91.58 c	1,169.59 ± 135.31 c

Karkacier *et al*. [28] compared the sugar contents of apple samples extracted using water and the methanol extraction method adapted herein. Contrary to our results, these authors concluded that water was a more effective solvent than methanol for sugar extraction, since in the latter not all the sugars were soluble, because of solvent vaporization. In contrast, Johansen *et al*. [34] concluded that water and 50% alcohol (methanol or ethanol) produced similar sugar extraction levels and that during water extraction there was the risk of starch and oligosaccharides degradation due to enzymatic action.

Whether enzymatic degradation of starch occurred during water extraction, this phenomenon was overcome by the higher extraction power of 50% (*v/v*) aqueous methanol, since in all cases methanol extractions yielded higher sugar contents than those with water extraction. This result can be explained because the method used with water was with a temperature (92 ºC) that caused tuber protein denaturation, which later might have trapped soluble sugars and diminished their extractions [35]. From the methods that use methanol, the one using activated charcoal yielded less sugar contents suggesting that charcoal could have adsorbed sugars into its carbon surface due to its preference towards organic molecules [36].

Thus, recovery analysis was performed with the double extraction method, revealing the highest percentages for sucrose (99.77 ± 1.98) and glucose (99.69 ± 1.55). The lowest recovery was found for fructose (94.14 ± 1.00), indicating that this compound was more prone to be lost during sample processing. This analysis demonstrates that extraction and sample purification interfere somehow in the loss of sugar contents during those procedures. Fructose data presented were not adjusted by its recovery value. Recovery values presented were within the acceptability range (90 – 107%, *w/v*) for analytes at a concentration of 100 mg/L [14].

3.1.2. Optimization of chromatographic analysis

From the different concentration values of sulfuric acid and temperatures of the column that were tested, a concentration of 10 mM sulfuric acid, at a flow rate of 0.3 mL/min, and a column temperature of 18 ºC were the conditions that resulted in the best resolution for the compounds analyzed. The conditions established for chromatographic analysis allowed not only the separation of sucrose, glucose, and fructose, but also the separation and identification of two additional peaks corresponding to citric acid and malic acid (Figure 1A). Selectivity of the method was assessed by checking the UV-vis

answer of each chromatographic peak. The absence of UV-vis response in the peaks corresponding to the sugars studied was an indicative of the absence of compounds such as organic acids or phenolic compounds co-eluting with the sugars, compounds that are expected to be present in potato tuber extracts [37].

Resolution for each pair of the identified peaks is shown in Figure 1A, using a mixture of sugars and organic acid standards in a concentration of 100 mg/L. Less resolved peaks were those from citric acid/glucose and fructose/malic acid with resolution values close to one. Authors as Kupiec [38] have reported that resolution values equal or greater than one indicate appropriate quality in compound separation. Figure 1B shows a chromatogram of a potato extract from accession CCC 52 with sucrose, glucose, and fructose quantifications revealing as well an adequate separation of the compounds analyzed. Thus, the chromatographic method established provides good separation as well as proper quantification of sugars, supported by a satisfactory measurement of glucose and fructose containing citric acid and malic acid in the mixture of standards and in the sample.

Citric acid and malic acid play important roles in the Krebs cycle and are the most abundant organic acids in potato tubers [39,40]. It was expected, therefore to detect these compounds in Phureja tubers eluting close to sugars using an AMINEX 87H column [21]. Authors do not agree in specific malic acid and citric acid roles and accumulation trends in the physiological processes during storage [39,41,42]. Even though, it is reported that both acids increases their amounts when the tubers are stored at low temperatures [41]. Consequently, for the better understanding of the dynamics of sugars and major organic acids during storage in diploid potatoes, it might be appropriate to use this chromatographic method, as it is possible to simultaneously quantify sugars and major potato organic acids using the RI detector. It is important to underline, however, that UV detection is more sensitive than RI detection for the quantification of organic acids. In addition, the simultaneous quantification of sugars and organic acids are useful in the characterization of food products as wine because these contents analyses are required for quality evaluation [43].

Using the same type of column to quantify sugars in the Colombian tetraploid cultivar R-12 (Diacol Capiro), Fonseca & Urueña [23] did not report the presence of additional peaks corresponding to organic acids. This fact can be explained because of lower concentration of sulfuric acid (8 mM) and higher column temperature (35 ºC) used by them, did not allow resolving acids from sugars or due to the existence of non-detectable amounts of citric acid and malic acid in the cultivar studied. In contrast, Eyéghé-Bikong *et al*. [44] reported the co-elution of fructose and malic acid, using AMINEX 87H column, operated at 55 ºC with 5 mM sulfuric acid in wine and grapevine samples. The chromatographic method used in our research shows the possibility to resolve fructose from malic acid without using organic modifiers in sulfuric acid solutions as proposed by Castellari *et al*. [43], but by increasing acid concentration and diminishing column temperature. In this scenario, it is expected an accurate quantification of fructose with RI detection as it separates from malic acid in the RI detection. This approach is more suitable than those that comprise the quantification by RI detection of sugars and UV detection of organic acids, without resolving organic acids from sugars in RI detection [45,46].

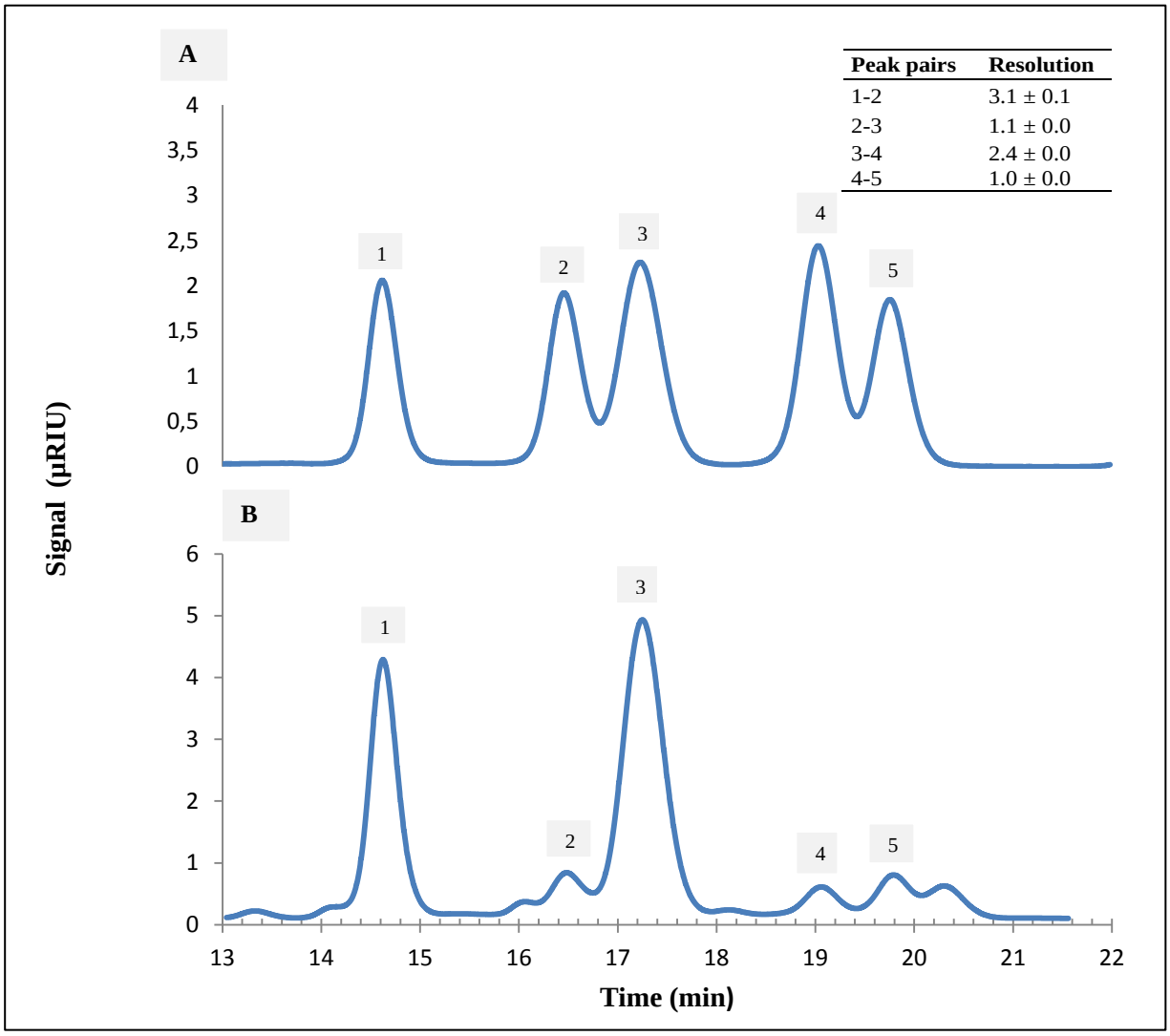

Figure 1. HPLC-Chromatograms illustrating: 1) Sucrose; 2) Citric acid; 3) Glucose; 4) Fructose; 5) Malic acid. A) Mixture of sugars and organic acid standards in a concentration of 100 mg/L. Insert shows resolution for each pair of peaks from chromatogram (e.g. resolution for peaks 1-2 means resolution for sucrose and citric acid). B) Sample of *Solanum tuberosum* group Phureja accession CCC 52. Concentrations found in accession were as follows: 1) 153.90 mg/L; 3) 225.32 mg/L); 4) 21.72 mg/L. µRIU: micro refractive index units.

3.2. Chromatographic method validation and analysis of sugar content in a set of group Phureja genotypes

3.2.1. Method validation

Reproducibility and repeatability were determined for each analyte according to the RSD values shown in Table 2. As injections were performed on three different days, the repeatability assay revealed a higher RSD values than reproducibility. Taverniers *et al*. [14] indicated that the AOAC's PVMP proposes levels of acceptability of RSD values with greater accuracy than the Horwitz function [47]. Hence, for an analyte in a concentration ranging from 10 to 100 mg/L, a RSD between 5.3 and 7.3 is acceptable. Mixtures of 40 mg/L standards were used for both assays, yielding RSD values even lower than the range previously mentioned. The RSD values obtained when testing

reproducibility and repeatability, assures the precision of the method developed for the quantification of sucrose, glucose, and fructose.

Table 2. Reproducibility and repeatability analysis of each analyte using a 40 mg/L mixture of standards.

	Reproducibility		Repeatability	
Sugar	**Average (mg/L)**	**RSD**	**Average (mg/L)**	**RSD**
Sucrose	40.08 ± 0.49	1.21	42.06 ± 2.40	5.71
Glucose	40.42 ± 0.34	0.83	41.33 ± 1.52	3.67
Fructose	40.41 ± 0.29	0.72	40.49 ± 1.80	4.44

RSD**,** relative standard deviation.

The averaged regression coefficients for the independent calibration curves analyzed revealed values greater than 0.998 (Table 1 of Supplementary Data), indicating an acceptable fit of the data to the regression curves which supports a proportional relationship between the response of the analyte and its concentration [31]. Using the averaged values of areas in different concentrations, a regression model was fitted for each sugar. The regression coefficients obtained revealed an acceptable fit of the averaged data for all sugars; as well, the *F* tests for the regression showed significance ($p < 0.05$). The two-tailed *t* test for the slope ($p < 0.05$) demonstrated that the values were different from zero which indicated that the sugar concentration had a significant effect on peak area (Table 1 of Supplementary Data). These analyses supports the significance of the adjusted models, which reinforces the linearity of the analyte response in the range studied and the accuracy in sample quantification performed at different times with different calibration curves.

LOD and LOQ showed the same values for both parameters in glucose and fructose (3 mg/L) and a lower value for sucrose in LOD (2 mg/L). The adjusted models for LOQ analysis and their slopes were significant according to the *t* and *F* tests respectively, indicating that the method quantification was linear at concentrations ranging from 3 to 100 mg/L for the three sugars (Table 2 of Supplementary Data). To summarize, the validation analyses performed demonstrated the method precision in a range of linearity from 3 mg/L to 100 mg/L.

3.2.2. Sugar content in group Phureja genotypes based on validated method

The sugar contents of nine Phureja genotypes were calculated and shown in Table 3. According to the AOAC's levels of acceptability of RSD (5.3 - 7.3), two values were out of range, but considering the Horwitz function these values are acceptable [14,47]. Besides, these RSD values belong to glucose and fructose, and the quantification of these compounds showed greater variability which was reinforced by higher RSD means and higher RSD deviations with respect to sucrose values. Thus, the reliability found in the quantification of these genotypes supported the method validation presented here.

Glucose and fructose contents found in Phureja potatoes were not equimolar as fructose amounts were lower. This result agrees with a potential high activity of fructokinase which is the responsible for fructose metabolism into hexose-phosphate cycle, thus

diminishing the fructose content in tubers [48]. There was a high variability in the sugar content among the four commercial and five CCC landraces studied (e.g. sucrose content ranged from 0.93 to 3.11 g/100 g tuber DW). McCann *et al*. [16] analyzed, based on HPLC, the sugar content of two group Phureja accessions stored at 2 ºC during three months. As these authors studied tubers from plants grown from botanical seeds of these accessions subjected to cold storage, wider ranges for sugar contents were found, especially for sucrose (ranging from 1.6 to 16.9 g/100 g tuber DW). A detailed multi-environmental study of sugar contents of all genotypes from CCC using this HPLC method is necessary to conclude about the current extent of the natural variation of sugar contents in Colombian Phureja germplasm.

4. Conclusions

The chromatographic method developed and validated allows a simple and appropriate quantification of sucrose, glucose, and fructose in *Solanum tuberosum* Group Phureja, also offering the possibility of their simultaneous quantification with the most abundant organic acids (citric acid and malic acid) in potato using a RI detector. In addition, in comparison with water extraction and 50% (*v/v*) aqueous methanol extractions with reflux and activated charcoal, the double extraction method with 50% (*v/v*) aqueous methanol provides higher sugar contents, an appropriate recovery, and it is easy to implement when having a large number of samples as required for the assessment of germplasm collections. With the purpose of a wider knowledge of the natural variation of sugar contents in Group Phureja, it is necessary to include all genotypes from CCC. Therefore, the chromatographic method established will contribute to an accurate phenotypic characterization of this collection that will impact in the understanding of the process of reducing and non-reducing sugars accumulation in tubers of Phureja potatoes from Colombia.

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Table 3. Average content of sugars in tubers of commercial cultivars and landraces from CCC. Bold numbers indicate those RSD values out of the levels of acceptability established by the AOAC [14,30].

Genotype	Sucrose Average (g/100 g tuber DW)	RSD	Glucose Average (g/100 g tuber DW)	RSD	Fructose Average (g/100 g tuber DW)	RSD	Reducing sugars (g/100 g tuber DW)	Total sugars (g/100 g tuber DW)
Guaneña	1.47 ± 0.04	3.02	0.27 ± 0.02	**8.13**	0.10 ± 0.01	4.88	0.36 ± 0.04	1.83 ± 0.08
Paisa	2.10 ± 0.05	2.24	0.26 ± 0.01	4.89	0.13 ± 0.01	4.61	0.39 ± 0.01	2.49 ± 0.04
Galeras	0.93 ± 0.05	5.74	0.29 ± 0.02	5.98	0.12 ± 0.01	**8.50**	0.40 ± 0.03	1.33 ± 0.08
Colombia	1.31 ± 0.04	2.76	0.25 ± 0.01	2.53	0.15 ± 0.01	5.05	0.40 ± 0.01	1.71 ± 0.05
CCC 8	1.05 ± 0.05	4.65	0.70 ± 0.04	5.60	0.32 ± 0.01	3.33	1.00 ± 0.07	2.05 ± 0.11
CCC 52	3.11 ± 0.04	1.16	4.53 ± 0.03	1.06	0.44 ± 0.01	2.17	4.97 ± 0.04	8.09 ± 0.07
CCC 80	1.36 ± 0.05	3.52	0.44 ± 0.02	4.36	0.14 ± 0.01	5.38	0.59 ± 0.02	1.94 ± 0.07
CCC 108	1.92 ± 0.04	1.98	2.79 ± 0.08	2.74	1.49 ± 0.02	1.10	4.28 ± 0.09	6.19 ± 0.13
CCC 123	1.99 ± 0.05	2.73	1.50 ± 0.06	4.17	0.35 ± 0.01	2.62	1.89 ± 0.02	3.85 ± 0.01
Average	1.46 ± 0.44	3.09 ± 1.40	1.23 ± 1.50	4.38 ± 2.11	0.36 ± 0.44	4.18 ± 2.18	1.59 ± 1.80	3.28 ± 2.35
Maximun	3.11	5.74	4.53	8.13	1.48	8.50	4.97	8.09
Minimum	0.93	1.16	0.25	1.06	0.10	1.10	0.36	1.33

CCC, Colombian Core Collection of *Solanum tuberosum* group Phureja; DW, dried weight; RSD, relative standard deviation.

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Supplementary Data

Supplementary Table 1 indicates linearity analyses performed for the method and Supplementary Table 2 shows limit of detection and limit of quantification analyses for each sugar.

Supplementary Table 1. Linearity analyses.

Sugar	[a] Average R^2	[b] R^2	[c] Slope
Sucrose	0.9997 ± 0.0002	0.9994**	0.0084**
Glucose	0.9998 ± 0.0002	0.9996**	0.0105**
Fructose	0.9997 ± 0.0002	0.9994**	0.0098**

[a] Average regression coefficients of three models fitted for three calibration curves of each sugar.
[b] Regression coefficients of the regression models fitted with the average areas of the sugars in each concentration.
[c] Slopes of the regression models fitted with the average areas of the sugars in each concentration.
** Significant *p*-values ($p < 0.05$) of the *F* test for the regression model and the two-tailed *t* test for the slope hypothesis.

Supplementary Table 2. Limit of detection (LOD) and limit of quantification (LOQ) analyses.

Sugar	LOD (mg/L)	LOQ (mg/L)	[a] R^2	[b] Slope
Sucrose	2.00	3.00	0.9984**	0.0089**
Glucose	3.00	3.00	0.9980**	0.0102**
Fructose	3.00	3.00	0.9992**	0.0105**

[a] Regression coefficients for the models fitted with the average areas of the sugars in the concentrations considered.
[b] Slopes of the regression models fitted.
** Significant *p*-values ($p < 0.05$) of the *F* test for the regression model and the two-tailed *t* test for the slope hypothesis.

Chapter 2

Natural variation of sugar contents in landraces of *Solanum tuberosum* Group Phureja

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Abstract

Potato light frying color is dependent on the reduced accumulation of sugars in the tubers. Contents of sucrose, glucose, and fructose were studied in 108 landraces and four commercial cultivars of *Solanum tuberosum* Group Phureja in a single environment. Sucrose, glucose and fructose genotypic mean values, analyzed based on liquid chromatography, ranged from 6.39 to 29.48 mg/g tuber dried weight (DW), from 0.46 to 28.04 mg/g tuber DW, and from 0.29 to 27.23 mg/g tuber DW, respectively. Sugar content analysis in Group Phureja revealed an extensive variability and it is consistent with previous frying color data from the landraces. Glucose content was higher than fructose in genotypes, which indicates that this reducing sugar might be the most relevant in defining frying color of Phureja genotypes at harvest. Five clusters of genotypes with biological significance were recognized and the results showed that high accumulation of sucrose and low accumulation of reducing sugars is the common feature of Group Phureja.

Keywords: Sucrose**,** reducing sugars, glucose, fructose, sugar ratios, frying quality.

1. Introduction

Potato (*Solanum tuberosum* L.) is the third crop of global relevance after wheat and rice thus it plays an important role in worldwide food security and nutrition (Camire, Kubow, & Donnelly, 2009; Mosquera & Cuéllar, 2013). The demand for potato chips and French fries continues to grow because of changes in consumption habits (Faulkner, 2015; Kirkman, 2007). High contents of sucrose (non-reducing sugar), glucose, and fructose (reducing sugars) in potato tubers represent an undesirable trait for fried processing because they tend to accumulate during cold storage at temperatures below

10 ºC. Reducing sugars are precursors of the Maillard reaction and sucrose is the main source of glucose and fructose during its enzyme-catalyzed hydrolysis (Eck, 2007; Isla, Vattuone, & Sampietro, 1998).

The Maillard reaction refers to a series of non-enzymatic reactions between non-reducing sugars and principally amino groups from amino acids during the thermal processing of potatoes. This reaction leads to the production of dark pigments, the development of off-flavours, and the production of toxic compounds such as acrylamide that reduce consumer's acceptance and generate risks for human health (Eck, 2007; Halford *et al*., 2012). The fact that there is a wide range of sugar concentrations among different potato genotypes indicates that these contents are under polygenic control, therefore highly influenced by environmental conditions such as tuber storage temperature (Halford *et al*., 2012).

The phenotypic assessment of potato frying quality has been performed frequently by means of visual scales of frying color (Li *et al*., 2013; Ñústez-López, 2011a; Werij, Furrer, Eck, Visser, & Bachem, 2012). Nevertheless, it is relevant for potato breeders the evaluation of genotypes for their contents of precursors of the Maillard reaction, such as sugars. This type of evaluation also constitutes a quantitative approach for the understanding of the frying quality trait as it is possible to identify genotypes prone to the synthesis of dark pigments and acrylamide (Muttucumaru, Powers, Elmore, Briddon, Mottram, & Halford, 2014). Such improved quantitative phenotypic data are also important to implement strategies as association mapping, where accurate quantification of the phenotypic trait in the population is crucial to find highly reliable associated variants (Ersoz, Yu, & Buckler, 2007; Manolio *et al*., 2009).

S. tuberosum Group Phureja consists primarily of diploid genotypes with short day adaptation and tuber sprouting at harvest that represent an important genetic resource for Colombia as in the south region of Nariño is located a center of diversity (Estrada, 1996; Huamán & Spooner, 2002; Spooner, Núñez, Trujillo, Herrera, Guzmán, & Ghislain, 2007). Colombia leads the production, consumption and export of diploid Phureja genotypes commonly named "Creole potato", which are known for their round tubers with yellow flesh and skin (Bonilla, Cardozo, & Morales, 2009; Rodríguez, Ñústez, & Estrada, 2009). These genotypes present an interesting possibility for their widespread use because of their outstanding nutritional and organoleptic properties and their processing potential (Bonilla *et al*., 2009; Peña, Restrepo-Sánchez, Kushalappa, Rodríguez-Molano, Mosquera, Narváez-Cuenca, 2015; Rivera, Herrera, & Rodríguez, 2006). The crossability of Group Phureja with tetraploid potatoes, the most cultivated worldwide, is notable, therefore this cultivated group constitutes an important gene pool with prospective use in tetraploid breeding programs (Ñústez-López, 2011a).

Currently, there are not Phureja cultivars suitable for fried processing, thus crops for frying purposes must be grown less than 2,600 meters above the sea level (masl) to decrease the probabilities of high or accelerated accumulation of reducing sugars in tubers due to lower temperatures (Ñústez-López, 2011a). Because of the lack of dormancy in tubers, the development of Phureja cultivars with tolerance to cold-induced sweetening would have the additional advantage of allowing the storage at low temperatures of tubers for processing to avoid sprouting (Sowokinos, 2008). Therefore, it is relevant to guide breeding programs towards the development of specific cultivars for processing.

Accessions from the Colombian Core Collection of Group Phureja (CCC) were characterized previously for their frying quality in a multi-environmental trial using a visual scale with equivalence of darkening percentage, identifying a wide genetic base for the trait (Ñústez-López, 2011a). Recently, a high performance liquid chromatographic (HPLC) method for the analysis of sugars in Phureja tubers was validated (Duarte-Delgado, Narváez-Cuenca, Restrepo-Sánchez, Kushalappa, & Mosquera-Vásquez, 2015). The aim of this study was to introduce the natural variation of sucrose, glucose, and fructose contents measured with a chromatographic technique in Phureja landraces, with the future perspective of elucidating the molecular basis of sugar accumulation in Phureja tubers through an association mapping strategy.

2. Materials and methods

2.1. Germplasm

A set of 108 diploid landrace accessions from CCC (previously studied by Ñústez-López, 2011a) and four commercial diploid cultivars (Criolla Colombia, Criolla Latina, Criolla Galeras, and Criolla Guaneña) (Ñústez-López, 2011b; Rodríguez *et al*., 2009) were studied for their sugar contents. Landrace accessions have been maintained through vegetative propagation.

2.2. Field trial

The field trial was located in a plot in the municipality of Soacha (Cundinamarca, Colombia; with an altitude of 2,850 masl, latitude of 4°28′40″N and longitude of 74°11′48″W). Tubers from the landrace accessions and the commercial cultivars were sown in pots with a substrate of three parts of soil by one of sand. To prepare the substrate, organic moor soil with loam texture was brought from a close area of the plot. A tuber per pot was sown at the end of September 2013 and plants were harvested after 137 days at tuber physical maturity, when foliage was senescent and tubers had developed a degree of skin set (Kumar, Singh, & Kumar, 2004). Plants were irrigated when required. Potato tubers from three pots of each genotype constituted three biological replicates in a completely randomized design.

2.3. Tuber sample preparation and HPLC analysis

Sugar content analysis was carried out after harvest. Mature and healthy raw tubers without mechanical damages were prepared for freeze-drying as described previously (Duarte-Delgado *et al*., 2015). Sucrose, glucose, and fructose were extracted with 50% (*v/v*) aqueous methanol from freeze-dried tissue and measured using an HPLC method. This method was developed and validated for Phureja potatoes using an AMINEX 87H column, 10 mM sulfuric acid as eluent, and refraction index detection (Duarte-Delgado *et al*., 2015).

Calibration curves were evaluated every 48 samples. The lack of carryover in the long-term HPLC analysis was verified by injecting blanks every eight samples. The stability of the HPLC system during independent experiments was assessed by the injection of a 40 mg/L mixture of standards every nine samples; the mean relative standard deviation (RSD) values were 10.09 ± 0.84, 3.94 ± 1.24, and 3.97 ± 0.85 for sucrose, glucose, and fructose, respectively. These RSD values (below 11.30) assured the data reliability through long-term HPLC runs, according to levels of acceptability of RSD values

proposed in the Horwitz function for analytes in concentrations ranging from 10 to 100 mg/L (Codex Alimentarius Commission, 2001; Taverniers, De Loose, & Van Bockstaele, 2004).

2.4. Statistical analyses

Mean values of each sugar content were established for each genotype and expressed as mg/g of dried weight (DW) tuber. Reducing sugar contents values were obtained from the sum of glucose and fructose, and total sugar contents were calculated from the sum of glucose, fructose, and sucrose. Average and standard deviation values of reducing and total sugars were also calculated for each genotype. Mean values of glucose/fructose and sucrose/reducing sugars ratios were calculated for each genotype with the purpose of analyzing the differential genotype metabolism regarding the proportion of sugars accumulated.

Data of CCC accessions from a multi-environmental Bayesian estimate of mean chip darkening % calculated using the International Potato Center scale of frying color (Ñústez-López, 2011a), were included as an additional variable in the correlation analysis. Spearman`s correlation among sugar contents, sugar ratios, and chip darkening % was carried out considering a level of significance of $\alpha = 0.05$. Correlation analysis was performed using SAS v.9.2 software (SAS Institute Inc., Cary, NC, USA).

A normed Principal Component Analysis (PCA) was employed with mean genotypic data of sugar contents and sugar ratios, to illustrate a biplot representing the amount of inertia accumulated in the first two principal components together with a correlation circle showing the direction of the increase of the variables represented by vectors and their degree of correlation. Norm of the vectors was also calculated to conclude about the degree of representation of the variables in the factorial space, thus values greater than 0.6 indicated an appropriate representation of the variable in the biplot (Supplementary Data 2). A factor-based hierarchical cluster analysis was implemented using the nearest neighbor method, to incorporate in the PCA biplot the information of clusters. Number of clusters was determined from the analysis of a dendrogram that was partitioned at the level of five groups with biological significance. These multivariate analyses were performed using SPAD v. 5.6 (Coheris, Suresnes, France).

3. Results and discussion

3.1. Sugar contents

Sugar content analysis showed an extensive range of variation among the three sugars, and among reducing sugar and total sugar contents studied in all accessions (Table 1; Supplementary Data 1), supporting that these contents at harvest are genetically influenced (Kumar *et al*., 2004). The most abundant sugar found in tubers was sucrose, followed by glucose and fructose, as sucrose is the predominant sugar in potato tubers during growth and development.

Sucrose contents of commercial cultivars are close to the average value of CCC (Table 1). Potato genotypes capable of storing low sucrose levels before harvest have high potential for processing as these genotypes have greater ability to control sucrose accumulation as growth stage is completed (Kumar *et al*., 2004). Criolla Guaneña is the

commercial cultivar with highest sucrose content, indicating that this cultivar might be the one with greatest potential of reducing sugars accumulation during low temperature storage. It is possible that the Phureja clones with lower sucrose accumulation at harvest would also accumulate less reducing sugars during low temperature storage, thus should have better frying qualities than the current cultivars.

Commercial cultivars show values closer to minimum values of reducing sugars from CCC, with the exception of Criolla Colombia that tends to accumulate higher amounts of glucose and fructose (Table 1). A previous analysis of commercial cultivars sown in a higher plot at 3,400 masl, showed an increased accumulation of reducing sugars at harvest (from 3.6 to 4.0 mg/g tuber DW; Duarte-Delgado *et al*. 2015). This reveals that commercial cultivars have a similar trend in reducing sugar accumulation under a high pressure of the environment at high altitudes. The outstanding accumulation of reducing sugars found in Criolla Colombia in the current assessment in a low altitude (2,850 masl) supports that this cultivar that corresponds to an earlier clonal selection from landraces of CCC is not suitable for frying (Rodríguez *et al*., 2009; Ñústez-López, 2011a).

The five Phureja landraces with the lowest sucrose contents are: CCC 80, CCC 61, CCC 2, CCC 20, and CCC 4 (from 6.39 to 8.65 mg/g tuber DW), while the five landraces with the lowest reducing sugar contents are: CCC 27, CCC 4, CCC 91, CCC 35, and CCC 41 (from 0.87 to 0.98 mg/g tuber DW). The nine landraces aforementioned are of interest for further analysis in breeding programs that consider frying quality, as it is relevant to include genetic diversity that shows not only low reducing sugars levels but also a diminished potential for the generation of reducing sugars through the low accumulation of sucrose. Noteworthy, CCC 27 and CCC 35 are the landraces with the lowest multi-environmental estimates of chip darkening of 3.33% and 4.45%, respectively (Ñústez-López, 2011a). The genotype CCC 4 is also of great importance because it accumulates both low amounts of sucrose and reducing sugars, thus this genotype might present specific genetic mechanisms that contribute to the reduced accumulation of sugars in tubers.

3.2. Sugar ratios

The glucose/fructose ratio range found revealed that landrace genotypes and commercial cultivars presented values greater than 1.00, indicating that fructose tends to accumulate in lesser amounts than glucose (Table 1). Glucose concentration can be found up to six times higher than fructose as in the case of CCC 2 (Figure 1; Supplementary Data 1). Accessions CCC 42 and CCC 108 were the exception, as glucose and fructose contents were nearly equimolar (Supplementary Data 1). The variability of glucose and fructose contents in cold-stored tubers at 2 ºC have also been shown across wild *Solanum* species, revealing similar amounts of glucose and fructose or higher contents of fructose than of glucose (McCann, Bethke, & Simon, 2010).

The range of glucose/fructose ratio found in Group Phureja at harvest suggests differential activities and affinities of fructokinases and hexokinases, which are enzymes responsible of fructose and glucose irreversible phosphorylation before their use in metabolic processes (Davies & Oparka, 1985; Granot, David-Schwartz, & Kelly, 2013; Kumar *et al*., 2004; Sowokinos, 2001). The previous finding suggests that Phureja landraces possess a heterogeneous metabolism regarding the initiation of respiratory process because of the lack of a tuber dormancy period in these genotypes

(Rodríguez & Moreno, 2010; Spooner *et al*., 2007). Therefore, the greater contents of glucose than those of fructose observed in landraces, reflected in the mean ratio of 1.79 ± 0.71, supports that fructose 6-phosphate might be preferentially used in glycolysis (Junker *et al*., 2006).

McCann *et al*. (2010) in the study of the sugar content of 22 genotypes from a botanical seed accession of Group Phureja found a mean glucose/fructose ratio of 0.52. These contrasting results suggest an important influence of the low temperatures of storage (2 ºC) in the tubers analyzed (McCann *et al*., 2010). The storage temperature might contribute to the potential preferential enzymatic action of sucrose synthase, which is related to cold stress response and converts sucrose into UDP-glucose and fructose, thus generating higher concentrations of fructose in tubers and with different effect from that of invertase that produces equimolar amounts of both sugars (McCann *et al*., 2010; Sturm & Tang, 1999).

The range observed for the sucrose/reducing sugars ratio (Table 1) indicated the presence of genotypes with values lower than 1.00, representing individuals with greater accumulation of reducing sugars than that of sucrose. Besides, there were genotypes accumulating up to 14 times more sucrose than reducing sugars as was the case of CCC 41 (Table 1; Supplementary Data 1), which is caused by the low reducing sugar content present in this accession. The variability in this ratio has been correlated with the invertase activity in cold-stored tubers (Zrenner, Schüler, & Sonnewald, 1996), hence there might be a differential enzymatic activity in Phureja accessions.

3.3. Correlation among variables

Table 2 shows the correlation coefficients that were found among the eight variables considered in the current study. A strong and significant correlation was found between glucose and fructose contents. Sucrose is significantly correlated with reducing sugar contents revealing that the disaccharide content has a moderate relationship with the reducing sugar contents in Phureja genotypes at harvest, which is contrary to results of Zhu, Cai, Ke, & Corke (2010) that also performed tuber analysis at harvest ($r < 0.14$, $p > 0.05$, n=16). Notwithstanding, Halford, Muttucumaru, *et al*. (2012) in the analysis of ten tetraploid potato cultivars stored at temperatures ranging from 8.5 to 9.5 ºC, found a significant correlation between sucrose and reducing sugars ($r = 0.921$, $p \leq 0.001$) which reveals that strong correlations between these variables are also possible in nature. Sucrose/reducing sugar ratio presents a strong negative correlation with reducing sugar contents and a non-significant correlation with sucrose contents. These results indicate that ratio variability is more related to reducing sugars variation.

Chip darkening % presents between weak to moderate significant correlations with each sugar contents and with sucrose/reducing sugar ratio. The positive correlations with sugar contents suggest that each sugar is related to frying color, showing sucrose the lowest correlation value, and glucose the greater correlation coefficient. Therefore, it is proposed that non-reducing sugars and reducing sugars measured in a single environment weakly influence the variation of the multi-environmental estimate of chip darkening % as sucrose represents the potential for the production of glucose and fructose in cold environments. These correlation results show that despite of the specific interactions of the genotypes and the environment, there is a defined relationship between sugar contents and frying color in Group Phureja.

Furthermore, these weak to moderate correlations also suggests the influence in chip color variation of other precursors of the Maillard reaction as the free amino acids contents in tubers or other unidentified compounds that also can participate in these non-enzymatic reactions (Halford *et al*., 2012; McCann *et al*., 2010; Muttucumaru *et al*., 2014). This observation is relevant because genotypes with low amino acid contents will present light frying color even if accumulate high contents of sugars.

3.4. Principal component analysis

Data revealed the extensive variation present in Phureja genotypes regarding sugar contents and sugar ratios, which is of potential use in breeding programs. This variation is depicted in the PCA biplot (Figure 1), where the two principal components explain 77.1% of the total inertia thus displaying a high amount of the total variability. The norm calculated for the vectors that denote the variables in the biplot (with values higher than 0.6, Supplementary Data 2), supports that all the variables are well represented in the factorial space as well. Less represented variables correspond to glucose/fructose and sucrose/reducing sugars ratios and variables with greater representation are glucose and reducing sugar contents.

The disposition of the vectors in the biplot is representative of the correlation coefficients found among the variables (Table 2, Section 3.3). The proximity of the vectors representing glucose, fructose, and reducing sugar contents supports the high positive correlation found among them. The opposite direction of the sucrose/reducing vector reflects the negative correlation with reducing sugar and total sugar contents. Finally, the independent variation that shows the orientation of the vector of glucose/fructose ratio reveals the non-significant or poor correlation between this ratio and the other variables. Therefore, the PCA biplot (Figure 1) is an appropriated tool to demonstrate the relationships among all the analyzed variables and to show their variability.

3.5. Cluster analysis

The factor-based cluster analysis incorporated in PCA analysis, shows five groups in the factorial space (Figure 1). These clusters are influenced by the direction of the variables represented by the vectors in the factorial space. Supplementary Data 1 presents the group corresponding to each genotype. Group Five (2.7% of genotypes) is the scarcest and relates to genotypes with an outstanding accumulation of reducing sugars and sucrose. Accessions CCC 108 and CCC 120 that present the highest reducing sugars contents, also had an outstanding multi-environmental estimate of chip darkening of 79.46 % and 68.26 %, respectively (Ñústez-López, 2011a), indicating that extreme phenotypes with undesirable estimate of chip darkening also coincide with present higher contents of glucose and fructose.

Group One (3.6% of genotypes) represents the clones with outstanding glucose/fructose ratios, revealing that this superior accumulation of glucose at harvest is a rare phenomenon in the natural variation of Phureja landraces. This cluster includes four genotypes with a glucose/fructose ratio higher than 3.5 (Supplementary Data 1), suggesting that these landraces might have undergone through a respiratory metabolism that favored fructose phosphorylation as discussed in Section 3.2. Group Two (9.8% of genotypes) covers clones with high contents of sucrose and intermediate values of reducing sugars.

Table 1. Average content of sugars and average glucose/fructose and sucrose/reducing sugars ratios from landraces of CCC (108 genotypes) and four commercial cultivars from *Solanum tuberosum* Group Phureja.

Genotypes	Sucrose[a]	Glucose[a]	Fructose[a]	Reducing sugars[a]	Total sugars[a]	Glucose/Fructose[b]	Sucrose/ Reducing sugars[b]
CCC	14.32 ± 4.70	2.68 ± 4.27	1.77 ± 3.76	4.45 ± 7.98	18.77 ± 10.25	1.79 ± 0.71	5.65 ± 2.91
Criolla Guaneña[c]	14.87 ± 3.49	0.80 ± 0.14	0.41 ± 0.10	1.21 ± 0.22	16.07 ± 2.24	1.95 ± 0.36	12.32 ± 2.24
Criolla Latina[c]	10.44 ± 1.35	0.69 ± 0.14	0.37 ± 0.07	1.05 ± 0.20	11.49 ± 2.53	1.88 ± 0.14	9.94 ± 2.53
Criolla Galeras[c]	11.15 ± 0.32	0.79 ± 0.11	0.38 ± 0.14	1.17 ± 0.25	12.32 ± 2.01	2.07 ± 0.58	9.51 ± 2.01
Criolla Colombia[c]	12.00 ± 1.26	1.67 ± 0.14	1.31 ± 0.33	2.99 ± 0.47	14.99 ± 1.07	1.28 ± 0.22	4.02 ± 1.07
Minimum	6.39	0.46	0.29	0.87	7.53	1.01	0.27
Maximum	29.42	28.04	27.23	55.25	74.05	6.67	14.48

CCC, Colombian Core Collection of *Solanum tuberosum* Group Phureja.
[a] Average sugar contents are expressed in mg/g of dried weight tuber.
[b] Ratios are given in *w/w.*
[c] Commercial cultivars.

Group Four (30.3% of genotypes) comprises individuals with high values of sucrose/reducing sugar ratios, which includes the Phureja genotypes with lowest contents of reducing sugars. Group Three (53.6% of genotypes) is the most frequent and defines genotypes with lower sucrose/reducing sugar ratios than Group Four, thus these genotypes tend to present greater contents of reducing sugars. The biplot shows that Groups Three and Four are closely related clusters, mainly conditioned by glucose contents, which is the variable that influences greatly the variation of sucrose/reducing sugar ratio as proposed in Section 3.3.

Cluster analysis indicates that in fact the most common feature of Phureja landraces in the current environment assessed, is a high accumulation of sucrose and a low accumulation of reducing sugars (represented in Groups Three and Four), which is also reflected in the PCA biplot by the dense cumulus of genotypes towards the minimum values of this variable in the factorial space. These results support the use of accessions of Group Phureja in tetraploid potato breeding programs, to produce offspring with improved chip color scores (Hamernik, Hanneman, & Jansky, 2009; Jakuczun & Zimnoch-Guzowska, 2004). The rare Phureja genotypes with extreme phenotypic values are of great interest for deeper studies because they are often enriched with uncommon causal variants that can contribute to explain complex trait variability and present a large effect on the trait (Korte & Farlow, 2013; Lee, Abecasis, Boehnke, & Lin, 2014).

Conclusions

Sugar content analysis in Group Phureja revealed an extensive variability and is consistent with previous data of frying color from the collection. The correlation analysis supports that each sugar individually is related to frying color, showing sucrose the lowest correlation value, and glucose the greater correlation coefficient. Analysis of glucose/fructose and sucrose/reducing sugar ratios disclosed also a wide range that is related to differential metabolism in sugar accumulation in Group Phureja. Glucose was the sugar predominantly found in tubers, which indicates that this reducing sugar might be the most relevant in defining frying color of Phureja genotypes at harvest. The lack of tuber dormancy and the heterogeneous respiratory metabolism in Phureja germplasm represent the main factors that might influence their sugar contents and frying quality at harvest.

This single-environment analysis revealed five clusters of genotypes with biological significance and shows that the most common feature of Group Phureja is a high accumulation of sucrose and a low accumulation of reducing sugars. Nevertheless, Phureja landraces with extreme phenotypes are scarce and are of great interest to understand complex trait variability. The landraces that represent the genotypes for further analysis in breeding programs that consider frying quality were identified. For breeding schemes it would be relevant to study the accumulation of sugars in these genotypes under cold-storage conditions to contribute in the understanding of the cold-induced sweetening trait in Group Phureja, as commercial cultivars are inclined to accumulate high contents of sucrose that can be hydrolyzed during storage.

Table 2. Spearman`s correlation coefficients among average sugar contents, average sugar ratios, and average chip darkening % studied in landraces of CCC (108 genotypes) and four commercial cultivars from *Solanum tuberosum* Group Phureja.

	Sucrose[a]	**Glucose[a]**	**Fructose[a]**	**Reducing sugars[a]**	**Total sugars[a]**	**Glucose/Fructose[b]**	**Sucrose/ Reducing sugars[b]**	**Chip darkening %[c]**
Sucrose[a]		0.48***	0.51***	0.49***	0.94***	-0.10^{ns}	-0.02^{ns}	0.34**
Glucose[a]	0.48***		0.90***	0.99***	0.72***	-0.05^{ns}	-0.85***	0.44***
Fructose[a]	0.51***	0.90***		0.94***	0.72***	-0.40***	-0.78***	0.38***
Reducing sugars[a]	0.49***	0.99***	0.94***		0.73***	-0.14^{ns}	-0.85***	0.43***
Total sugars[a]	0.94***	0.72***	0.72***	0.73***		-0.13^{ns}	-0.31**	0.43***
Glucose/Fructose[b]	-0.10^{ns}	-0.05^{ns}	-0.40***	-0.14^{ns}	-0.13^{ns}		-0.11^{ns}	0.04^{ns}
Sucrose/Reducing sugars[b]	-0.02^{ns}	-0.85***	-0.78***	-0.85***	-0.31**	-0.11^{ns}		-0.32**
Chip darkening %[c]	0.34**	0.44***	0.38***	0.43***	0.43***	0.04^{ns}	-0.32**	

CCC, Colombian Core Collection of *Solanum tuberosum* Group Phureja.
[a] Average sugar contents were expressed in mg/g of dried weight tuber.
[b] Average ratios were given in *w/w*.
[c] The mean chip darkening % from 108 genotypes from CCC are data that come from a Bayesian estimation from the assessment of multi-environmental trials as described by Ñústez-López (2011a).
p-values: ***, $p \leq 0.0001$; **, $p \leq 0.001$; *, $p \leq 0.05$; ns, $p > 0.05$ the coefficient is not significant.

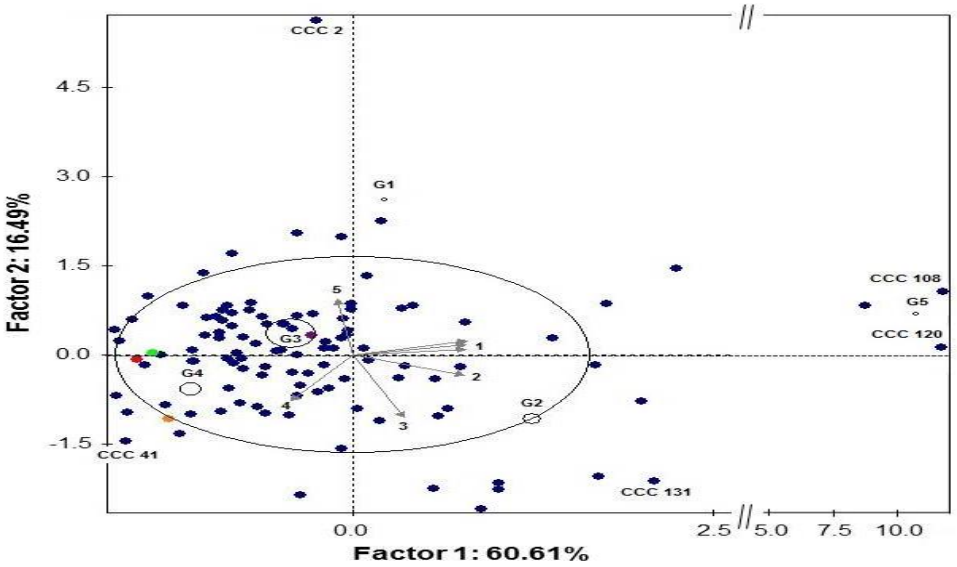

Figure 1. Principal component analysis biplot with cluster analysis incorporated of sugar contents and sugar ratios in *Solanum tuberosum* Group Phureja genotypes. The axes present the percentage of variance accounted in the first two principal components. Two scales were generated in the X axis to provide detail in the high-populated X axis up to 2.5 units. The blue spots represent 108 genotypes from CCC (Colombian Core Collection of Group Phureja), and the other colors in parenthesis correspond to commercial cultivars as follows: Criolla Latina (red), Criolla Galeras (green), Criolla Colombia (purple), and Criolla Guaneña (orange).The grey arrows represent the vectors indicating the direction of the increase of the variables and the magnitude in which each one is represented in the factorial space. The extreme genotypes in variables are labeled in the biplot and are shown in parenthesis as follows with the number of the grey vectors: 1) Glucose, reducing sugars, and fructose (CCC 108), 2) total sugars (CCC 120), 3) sucrose (CCC 131), 4) sucrose/reducing sugars (CCC 41), and 5) glucose/fructose (CCC 2). The white spots indicate the clusters of genotypes, being their size proportional to the abundance of genotypes in each cluster. Each cluster is noted with G, thus G1, G2, G3, G4, and G5 group together the 3.6%, 9.8%, 53.6%, 30.3%, and 2.7% of the genotypes respectively.

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Supplementary Data

Supplementary Table 1. Average content of sugars and average glucose/fructose and sucrose/reducing sugars ratios from landraces of CCC (108 genotypes) and four commercial cultivars from *Solanum tuberosum* Group Phureja. The genotypic group from cluster analysis of Section 3.4 is also shown for each genotype.

Genotype	Sucrose[a]	Glucose[a]	Fructose[a]	Reducing sugars[a]	Total sugars[a]	Glucose/fructose[b]	Sucrose/ reducing sugars[b]	Group
CCC 2	8.05 ± 1.96	5.40 ± 2.28	0.81 ± 0.34	6.21 ± 2.16	14.26 ± 3.68	6.67 ± 2.92	1.29 ± 0.15	1
CCC 3	16.96 ± 2.97	2.46 ± 0.28	1.62 ± 0.36	4.07 ± 0.64	21.03 ± 3.61	1.52 ± 0.17	4.16 ± 0.08	3
CCC 4	8.65 ± 0.60	0.62 ± 0.16	0.30 ± 0.00	0.92 ± 0.07	9.57 ± 0.77	2.05 ± 0.22	9.43 ± 1.86	4
CCC 5	12.69 ± 3.63	1.50 ± 0.06	1.01 ± 0.35	2.51 ± 0.40	15.21 ± 5.45	1.49 ± 0.97	5.05 ± 1.23	3
CCC 6	16.64 ± 3.79	3.88 ± 1.35	0.85 ± 0.39	4.73 ± 1.25	21.37 ± 4.86	4.58 ± 2.87	3.52 ± 0.58	1
CCC 7	13.53 ± 5.28	2.19 ± 0.98	0.98 ± 0.06	3.17 ± 1.01	16.70 ± 6.27	2.24 ± 0.99	4.28 ± 0.39	3
CCC 8	16.79 ± 1.55	1.65 ± 0.63	1.50 ± 0.34	3.16 ± 0.98	19.94 ± 2.77	1.10 ± 0.17	5.31 ± 1.08	3
CCC 9	16.54 ± 2.50	1.57 ± 0.07	0.84 ± 0.04	2.41 ± 0.08	18.95 ± 2.48	1.86 ± 0.13	6.87 ± 1.11	4
CCC 11	14.55 ± 2.13	0.92 ± 0.03	0.64 ± 0.05	1.56 ± 0.03	16.11 ± 2.14	1.44 ± 0.16	9.35 ± 1.32	4
CCC 13	17.60 ± 2.28	1.35 ± 0.33	0.79 ± 0.06	2.14 ± 0.38	19.74 ± 2.65	1.71 ± 0.34	8.23 ± 0.56	4
CCC 14	12.83 ± 1.20	1.56 ± 0.13	1.16 ± 0.25	2.72 ± 0.38	15.55 ± 0.83	1.35 ± 0.19	4.71 ± 1.18	3
CCC 15	25.55 ± 5.14	2.45 ± 0.37	1.88 ± 0.26	4.33 ± 0.63	29.88 ± 5.77	1.31 ± 0.02	5.90 ± 0.36	2
CCC 16	11.66 ± 1.02	0.91 ± 0.12	0.55 ± 0.08	1.46 ± 0.20	13.12 ± 1.64	1.66 ± 0.02	7.98 ± 0.12	4
CCC 17	14.58 ± 3.27	1.04 ± 0.24	0.86 ± 0.19	1.90 ± 0.43	16.49 ± 3.70	1.20 ± 0.03	7.66 ± 0.10	4
CCC 19	14.02 ± 3.99	1.75 ± 0.01	1.30 ± 0.28	3.05 ± 0.29	17.07 ± 3.76	1.34 ± 0.29	4.60 ± 0.65	3
CCC 20	8.23 ± 1.42	1.03 ± 0.25	0.85 ± 0.22	1.89 ± 0.46	10.11 ± 1.85	1.22 ± 0.04	4.36 ± 0.51	3
CCC 21	19.00 ± 2.59	1.89 ± 0.44	1.39 ± 0.15	3.27 ± 0.59	22.28 ± 3.17	1.36 ± 0.17	5.80 ± 0.25	3
CCC 23	15.35 ± 4.81	1.61 ± 0.16	1.28 ± 0.24	2.89 ± 0.39	18.24 ± 4.64	1.25 ± 0.13	5.31 ± 2.28	3

CCC 24	9.61 ± 1.10	1.48 ± 0.49	1.09 ± 0.50	2.57 ± 0.98	12.18 ± 2.01	1.35 ± 0.23	3.74 ± 0.95	3
CCC 27	9.45 ± 0.85	0.46 ± 0.01	0.41 ± 0.05	0.87 ± 0.06	10.32 ± 0.54	1.13 ± 0.09	10.89 ± 0.18	4
CCC 30	11.34 ± 2.24	1.93 ± 0.33	1.15 ± 0.07	3.08 ± 0.40	14.42 ± 2.64	1.67 ± 0.20	3.68 ± 0.28	3
CCC 31	13.10 ± 1.13	1.60 ± 0.62	1.12 ± 0.45	2.72 ± 1.07	15.82 ± 1.68	1.43 ± 0.04	4.81 ± 2.77	3
CCC 32	13.23 ± 0.92	1.91 ± 0.15	1.32 ± 0.06	3.23 ± 0.20	16.45 ± 0.86	1.44 ± 0.05	4.10 ± 0.46	3
CCC 33	10.04 ± 0.31	1.04 ± 0.33	0.76 ± 0.31	1.81 ± 0.63	11.85 ± 0.82	1.37 ± 0.13	5.55 ± 1.74	3
CCC 34	11.73 ± 0.62	1.15 ± 0.22	0.77 ± 0.16	1.92 ± 0.38	13.64 ± 0.96	1.49 ± 0.05	6.12 ± 0.99	4
CCC 35	11.87 ± 1.99	0.58 ± 0.15	0.38 ± 0.04	0.96 ± 0.19	12.83 ± 2.44	1.52 ± 0.26	12.36 ± 0.10	4
CCC 36	14.37 ± 1.96	1.00 ± 0.02	0.74 ± 0.17	1.74 ± 0.19	16.11 ± 0.04	1.35 ± 0.29	8.24 ± 0.92	4
CCC 37	22.78 ± 2.85	1.22 ± 0.16	0.68 ± 0.20	1.90 ± 0.36	24.68 ± 3.03	1.78 ± 0.31	11.99 ± 2.34	4
CCC 38	11.44 ± 2.34	1.02 ± 0.02	0.84 ± 0.14	1.86 ± 0.16	13.30 ± 2.46	1.21 ± 0.17	6.17 ± 0.96	4
CCC 40	15.14 ± 1.83	2.30 ± 0.24	1.52 ± 0.23	3.82 ± 0.47	18.96 ± 1.74	1.51 ± 0.10	3.96 ± 0.83	3
CCC 41	14.14 ± 0.70	0.62 ± 0.06	0.35 ± 0.04	0.98 ± 0.10	15.12 ± 0.79	1.77 ± 0.15	14.48 ± 0.77	4
CCC 42	11.35 ± 2.69	0.98 ± 0.38	0.97 ± 0.22	1.95 ± 0.49	13.30 ± 3.16	1.01 ± 0.01	5.81 ± 0.16	3
CCC 43	14.18 ± 2.60	2.26 ± 0.40	1.25 ± 0.13	3.51 ± 0.51	17.68 ± 2.94	1.81 ± 0.21	4.04 ± 0.57	3
CCC 44	12.37 ± 2.65	2.69 ± 0.35	1.46 ± 0.27	4.14 ± 0.60	16.52 ± 3.08	1.84 ± 0.16	2.99 ± 0.47	3
CCC 45	12.54 ± 1.36	1.81 ± 0.06	1.01 ± 0.09	2.82 ± 0.13	15.36 ± 1.37	1.79 ± 0.14	4.45 ± 0.54	3
CCC 47	9.40 ± 0.45	1.23 ± 0.25	0.74 ± 0.13	1.97 ± 0.38	11.37 ± 0.81	1.67 ± 0.05	4.77 ± 0.70	3
CCC 51	13.53 ± 0.29	2.01 ± 0.09	1.37 ± 0.14	3.38 ± 0.22	16.92 ± 0.47	1.47 ± 0.09	4.00 ± 0.23	3
CCC 52	9.36 ± 0.57	1.53 ± 0.11	0.95 ± 0.07	2.48 ± 0.04	11.83 ± 0.59	1.61 ± 0.25	3.78 ± 0.19	3
CCC 53	9.63 ± 2.77	1.31 ± 0.36	0.74 ± 0.29	2.05 ± 0.65	11.68 ± 3.26	1.78 ± 0.20	4.69 ± 1.35	3
CCC 56	13.17 ± 3.62	2.37 ± 0.46	1.44 ± 0.35	3.81 ± 0.81	16.98 ± 3.25	1.65 ± 0.10	3.45 ± 1.36	3
CCC 57	12.23 ± 3.25	2.43 ± 0.36	1.48 ± 0.32	3.91 ± 0.67	16.14 ± 3.60	1.65 ± 0.18	3.13 ± 0.85	3
CCC 59	17.79 ± 1.34	3.41 ± 1.26	2.00 ± 0.17	5.41 ± 0.72	23.20 ± 1.84	1.71 ± 0.11	3.29 ± 0.15	3
CCC 61	7.43 ± 0.44	1.25 ± 0.50	0.66 ± 0.21	1.92 ± 0.71	9.34 ± 1.15	1.89 ± 0.18	3.88 ± 1.28	3
CCC 62	13.77 ± 3.11	2.31 ± 0.10	1.66 ± 0.07	3.96 ± 0.17	17.73 ± 2.71	1.39 ± 0.00	3.47 ± 0.50	3
CCC 63	11.73 ± 0.71	0.91 ± 0.06	0.53 ± 0.05	1.44 ± 0.10	13.17 ± 0.81	1.71 ± 0.08	8.12 ± 0.18	4
CCC 65	12.96 ± 2.17	2.77 ± 0.91	1.31 ± 0.38	4.08 ± 1.27	17.05 ± 3.39	2.11 ± 0.29	3.18 ± 0.70	3

CCC 66	10.69 ± 0.90	1.50 ± 0.17	0.80 ± 0.05	2.31 ± 0.22	13.00 ± 0.95	1.87 ± 0.09	4.63 ± 0.58	3
CCC 67	11.06 ± 1.18	0.80 ± 0.10	0.42 ± 0.06	1.22 ± 0.15	12.27 ± 1.73	1.92 ± 0.02	9.10 ± 0.13	4
CCC 69	10.65 ± 2.41	0.79 ± 0.10	0.29 ± 0.05	1.09 ± 0.14	11.74 ± 2.54	2.72 ± 0.20	9.82 ± 1.24	4
CCC 70	20.02 ± 2.67	2.71 ± 0.88	1.80 ± 0.66	4.51 ± 1.54	24.53 ± 1.13	1.51 ± 0.09	4.44 ± 2.90	3
CCC 71	21.80 ± 4.15	5.41 ± 1.12	3.61 ± 1.09	9.02 ± 2.21	30.81 ± 2.80	1.50 ± 0.15	2.42 ± 1.15	2
CCC 72	22.60 ± 4.25	2.68 ± 0.15	1.27 ± 0.39	3.95 ± 0.54	26.55 ± 0.80	2.10 ± 0.55	5.72 ± 1.05	3
CCC 73	25.71 ± 3.99	2.00 ± 0.81	1.21 ± 0.50	3.21 ± 1.30	28.92 ± 5.11	1.65 ± 0.23	8.01 ± 2.33	2
CCC 74	15.72 ± 0.58	1.14 ± 0.11	0.53 ± 0.02	1.67 ± 0.12	17.38 ± 0.63	2.16 ± 0.20	9.43 ± 0.71	4
CCC 76	12.01 ± 1.12	1.20 ± 0.21	0.58 ± 0.23	1.78 ± 0.44	13.80 ± 0.85	2.08 ± 0.47	6.74 ± 1.96	4
CCC 79	13.14 ± 4.99	0.74 ± 0.19	0.45 ± 0.05	1.19 ± 0.25	14.32 ± 1.68	1.66 ± 0.24	11.07 ± 0.63	4
CCC 80	6.39 ± 1.52	0.70 ± 0.13	0.44 ± 0.21	1.14 ± 0.34	7.53 ± 1.33	1.57 ± 0.40	5.61 ± 2.73	3
CCC 81	16.33 ± 1.09	1.39 ± 0.40	0.61 ± 0.12	2.00 ± 0.52	18.33 ± 1.61	2.29 ± 0.22	8.16 ± 1.49	4
CCC 83	15.72 ± 0.44	0.91 ± 0.01	0.44 ± 0.01	1.35 ± 0.00	17.07 ± 0.45	2.06 ± 0.07	11.63 ± 0.30	4
CCC 86	12.18 ± 1.27	1.10 ± 0.26	0.69 ± 0.24	1.79 ± 0.50	13.98 ± 1.75	1.60 ± 0.26	6.80 ± 1.46	4
CCC 87	19.87 ± 1.64	1.46 ± 0.18	1.08 ± 0.25	2.54 ± 0.43	22.41 ± 2.07	1.35 ± 0.17	7.81 ± 0.77	3
CCC 88	12.42 ± 1.92	0.79 ± 0.08	0.34 ± 0.05	1.13 ± 0.13	13.54 ± 2.05	2.35 ± 0.14	11.02 ± 0.51	4
CCC 89	25.85 ± 1.93	2.36 ± 0.21	1.88 ± 0.12	4.24 ± 1.41	30.09 ± 19.21	1.26 ± 0.90	6.10 ± 3.90	2
CCC 91	9.57 ± 1.94	0.65 ± 0.14	0.31 ± 0.03	0.96 ± 0.17	10.54 ± 2.11	2.11 ± 0.30	9.94 ± 0.40	4
CCC 92	17.10 ± 0.87	1.23 ± 0.24	0.68 ± 0.26	1.91 ± 0.50	19.01 ± 0.37	1.81 ± 0.32	8.94 ± 2.58	4
CCC 93	16.58 ± 4.55	0.86 ± 0.04	0.41 ± 0.09	1.27 ± 0.02	17.85 ± 5.49	2.12 ± 0.26	13.11 ± 4.29	4
CCC 94	13.91 ± 4.93	1.35 ± 0.41	0.81 ± 0.38	2.16 ± 0.79	16.07 ± 5.66	1.66 ± 0.26	6.44 ± 1.17	4
CCC 96	16.16 ± 2.89	22.83 ± 9.55	17.81 ± 7.87	40.64 ± 17.35	56.80 ± 20.14	1.28 ± 0.08	0.40 ± 0.16	5
CCC 98	13.08 ± 3.47	1.71 ± 0.84	0.45 ± 0.12	2.17 ± 0.28	15.24 ± 4.31	3.79 ± 0.37	6.04 ± 1.02	1
CCC 99	18.63 ± 2.25	5.41 ± 1.85	3.40 ± 0.78	8.81 ± 2.57	27.44 ± 4.19	1.59 ± 0.27	2.12 ± 0.61	2
CCC 100	15.46 ± 0.30	1.52 ± 0.20	1.18 ± 0.24	2.70 ± 0.43	18.16 ± 0.71	1.29 ± 0.12	5.72 ± 0.92	3
CCC 101	10.18 ± 0.51	1.12 ± 0.36	0.59 ± 0.25	1.71 ± 0.61	11.89 ± 1.12	1.88 ± 0.21	5.95 ± 1.72	4
CCC 102	18.08 ± 3.51	5.38 ± 0.97	2.57 ± 0.49	7.96 ± 1.46	26.04 ± 4.11	2.09 ± 0.06	2.27 ± 0.50	2
CCC 103	17.60 ± 5.51	2.86 ± 1.01	1.90 ± 0.74	4.76 ± 1.74	22.36 ± 7.25	1.51 ± 0.08	3.69 ± 0.20	3

CCC 104	10.36 ± 4.34	1.49 ± 0.27	1.07 ± 0.27	2.55 ± 0.54	12.91 ± 4.88	1.40 ± 0.13	4.06 ± 1.00	3
CCC 106	12.91 ± 0.83	1.96 ± 0.83	1.35 ± 0.42	3.31 ± 1.25	16.22 ± 1.92	1.46 ± 0.15	3.90 ± 1.17	3
CCC 108	14.98 ± 1.58	28.03 ± 2.77	27.23 ± 2.55	55.25 ± 5.31	70.23 ± 3.78	1.03 ± 0.01	0.27 ± 0.05	5
CCC 109	13.01 ± 0.76	4.01 ± 0.87	2.82 ± 0.60	6.83 ± 1.45	19.83 ± 2.20	1.42 ± 0.09	1.91 ± 0.32	3
CCC 110	14.07 ± 4.91	9.30 ± 3.29	3.88 ± 1.54	13.18 ± 4.83	27.26 ± 9.22	2.39 ± 0.13	1.07 ± 0.06	2
CCC 112	14.15 ± 2.43	1.47 ± 0.44	1.03 ± 0.25	2.50 ± 0.68	16.65 ± 3.10	1.43 ± 0.08	5.66 ± 0.59	3
CCC 113	14.60 ± 4.44	1.59 ± 0.63	1.11 ± 0.52	2.70 ± 1.15	17.30 ± 5.56	1.43 ± 0.18	5.40 ± 1.24	3
CCC 114	10.63 ± 1.01	3.43 ± 0.92	2.61 ± 0.38	6.04 ± 1.26	16.67 ± 1.83	1.31 ± 0.23	1.76 ± 0.11	3
CCC 115	26.55 ± 6.04	2.04 ± 0.03	1.74 ± 0.08	3.78 ± 0.10	30.34 ± 5.96	1.17 ± 0.05	7.02 ± 1.72	2
CCC 116	15.32 ± 7.11	3.44 ± 1.46	1.41 ± 0.56	4.85 ± 2.01	20.16 ± 7.59	2.44 ± 0.25	3.16 ± 1.59	3
CCC 117	15.38 ± 2.48	1.37 ± 0.1	1.03 ± 0.14	2.40 ± 0.23	17.78 ± 2.70	1.32 ± 0.08	6.41 ± 0.45	3
CCC 118	18.56 ± 5.93	2.79 ± 1.29	1.30 ± 0.53	4.09 ± 1.82	22.65 ± 7.23	2.15 ± 0.15	4.54 ± 2.33	3
CCC 119	13.82 ± 3.05	1.45 ± 0.57	0.67 ± 0.19	2.12 ± 0.76	15.94 ± 3.81	2.15 ± 0.25	6.51 ± 0.95	4
CCC 120	21.31 ± 3.27	28.04 ± 11.44	24.69 ± 11.18	52.73 ± 22.62	74.05 ± 21.94	1.14 ± 0.06	0.40 ± 0.22	5
CCC 121	10.2 ± 4.00	2.80 ± 0.57	0.94 ± 0.23	3.74 ± 0.78	13.94 ± 3.24	2.97 ± 0.45	2.73 ± 1.57	3
CCC 122	22.28 ± 4.72	6.86 ± 2.83	1.89 ± 0.52	8.75 ± 3.27	31.03 ± 7.19	3.63 ± 0.84	2.54 ± 0.80	1
CCC 123	9.22 ± 0.90	1.27 ± 0.23	0.85 ± 0.18	2.12 ± 0.41	11.34 ± 1.09	1.49 ± 0.03	4.35 ± 0.75	3
CCC 124	11.66 ± 2.45	3.43 ± 1.44	1.10 ± 0.32	4.52 ± 1.75	16.18 ± 4.18	3.12 ± 0.47	2.58 ± 0.61	3
CCC 125	11.97 ± 3.65	1.74 ± 0.68	0.98 ± 0.36	2.72 ± 1.04	14.69 ± 4.53	1.77 ± 0.07	4.41 ± 0.86	3
CCC 126	11.26 ± 4.96	0.94 ± 0.10	0.53 ± 0.17	1.47 ± 0.27	12.73 ± 4.10	1.77 ± 0.41	7.66 ± 1.52	4
CCC 127	10.66 ± 1.63	1.25 ± 0.18	0.75 ± 0.13	2.00 ± 0.31	12.66 ± 1.93	1.68 ± 0.10	5.34 ± 0.14	3
CCC 128	9.64 ± 0.54	1.03 ± 0.01	0.48 ± 0.08	1.50 ± 0.09	11.15 ± 0.66	2.14 ± 0.32	6.41 ± 0.86	4
CCC 129	28.98 ± 6.55	3.84 ± 1.54	2.08 ± 0.49	5.93 ± 1.91	34.90 ± 8.17	1.85 ± 0.52	4.89 ± 0.84	2
CCC 131	29.42 ± 4.74	4.42 ± 0.13	2.66 ± 0.15	7.08 ± 0.27	36.50 ± 4.47	1.66 ± 0.06	4.15 ± 0.85	2
CCC 132	26.90 ± 1.05	10.76 ± 1.56	7.96 ± 1.63	18.72 ± 3.19	45.62 ± 4.57	1.35 ± 0.08	1.44 ± 0.17	2
CCC 133	9.08 ± 1.13	3.33 ± 1.32	2.05 ± 1.25	5.38 ± 3.02	14.46 ± 4.60	1.63 ± 0.07	1.69 ± 0.90	3
CCC 135	12.79 ± 2.62	1.47 ± 0.46	0.96 ± 0.36	2.43 ± 0.81	15.22 ± 3.42	1.54 ± 0.11	5.27 ± 0.75	3
CCC 136	12.63 ± 3.48	1.19 ± 0.29	0.74 ± 0.29	1.93 ± 0.58	14.55 ± 4.05	1.61 ± 0.22	6.55 ± 0.24	4

CCC 137	11.75 ± 0.68	1.73 ± 0.13	1.02 ± 0.12	2.75 ± 0.21	14.50 ± 0.64	1.70 ± 0.19	4.27 ± 0.45	3
CCC 138	9.92 ± 1.07	0.99 ± 0.10	0.66 ± 0.11	1.65 ± 0.22	11.58 ± 1.26	1.50 ± 0.10	6.01 ± 0.45	3
CCC 140	11.46 ± 0.94	1.28 ± 0.10	0.79 ± 0.13	2.07 ± 0.23	13.53 ± 1.06	1.63 ± 0.16	5.53 ± 1.25	3
CCC 141	9.37 ± 1.46	1.15 ± 0.24	0.78 ± 0.14	1.93 ± 0.37	11.30 ± 1.82	1.46 ± 0.08	4.85 ± 0.23	3
CCC 142	12.93 ± 1.29	2.28 ± 0.22	1.52 ± 0.06	3.80 ± 0.27	16.73 ± 1.56	1.50 ± 0.09	3.41 ± 0.09	3
CCC 145	15.65 ± 0.66	1.87 ± 0.51	1.32 ± 0.42	3.19 ± 0.93	18.84 ± 0.28	1.42 ± 0.06	4.91 ± 1.48	3
Criolla Guaneña[c]	14.87 ± 3.48	0.80 ± 0.14	0.41 ± 0.10	1.21 ± 0.22	16.07 ± 3.63	1.95 ± 0.36	12.32 ± 2.24	4
Criolla Latina[c]	10.44 ± 1.35	0.68 ± 0.14	0.37 ± 0.07	1.05 ± 0.20	11.49 ± 1.35	1.87 ± 0.14	9.94 ± 2.53	4
Criolla Galeras[c]	11.15 ± 0.32	0.79 ± 0.11	0.38 ± 0.14	1.17 ± 0.25	12.32 ± 0.41	2.07 ± 0.58	9.51 ± 2.01	4
Criolla Colombia[c]	12.00 ± 1.26	1.67 ± 0.14	1.31 ± 0.33	2.99 ± 0.47	14.99 ± 0.79	1.41 ± 0.22	3.76 ± 1.07	3

CCC, Colombian Core Collection of *Solanum tuberosum* Group Phureja
[a] Average sugar contents are expressed in mg/g of dried weight tuber
[b] Ratios are given in *w/w*
[c] Commercial cultivars

Supplementary Table 2. Norm values of vectors representing variables in the principal component analysis. Values greater than 0.6 indicate an appropriate representation of the variable in the factorial space.

Variable	Norm value
Sucrose[a]	0.80
Glucose[a]	0.99
Fructose[a]	0.96
Reducing sugars[a]	0.99
Total sugars[a]	0.98
Glucose/Fructose[b]	0.62
Sucrose/Reducing sugars[b]	0.74

[a] Average sugar contents were expressed in mg/g of dried weight tuber
[b] Average ratios were given in *w/w*

Chapter 3

Genetic analysis of the natural variation of sugar contents and frying color using SNP markers in *Solanum tuberosum* Group Phureja

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Abstract

Potato frying color is an agronomic trait dependent on sugar accumulation in tubers. A candidate gene approach was implemented to elucidate the molecular basis of sugar contents and frying color variation in Group Phureja, through the study of candidate gene regions coding functional enzymes in carbohydrate metabolism using an association mapping strategy to find associated variants. Association analysis was carried out with 111 SNP markers identified in ten candidate genes with key function in carbohydrate metabolism. This analysis revealed four SNP markers in the locus *InvGE* from an apoplastic invertase and one SNP marker in the locus *SssI* from a soluble starch synthase with significant effect in sugar content and frying color. These enzymes have not been found expressed in mature tubers, therefore these SNP-trait associations might be indirect resulting from the linkage disequilibrium with causal variants, or direct through a potential novel role of these candidate genes controlling sugar contents in tubers. Three markers from *InvGE* were found in strong linkage disequilibrium; the presence of a larger haplotype block with associations needs to be proven through the study of the variation in closer genomic regions. Most of the associated SNPs were low-frequency variants, thus revealing that these types of variants present important effects on sugar contents and frying color in Group Phureja. These results suggest that despite the differences in carbohydrate metabolism in both diploid and tetraploid germplasm, there are conserved genes related to sugar content and frying color variation.

1. Introduction

Potato frying quality depends on sugar contents in the tuber, because the hydrolysis of sucrose (non-reducing sugar) is the main source of glucose and fructose (reducing sugars), which are precursors of the Maillard reaction. This reaction produces dark pigments and toxic products as acrylamide during the non-enzymatic reaction of reducing sugars and free amino acids at high temperatures (Halford *et al*., 2012; Isla *et al*., 1998). Sucrose, glucose, and fructose are important metabolic signals in transduction pathways that affect the expression of several classes of genes involved in all stages of plant development and related to stress response, therefore sugar contents of storage organs are complex traits controlled by multiple genetic and environmental factors (Roitsch and González, 2004; Ruan, 2014; Schreiber *et al*., 2014; Sturm and Tang, 1999).

Reducing sugars tends to accumulate in tubers in response to cold tolerance when there are low respiration rates at temperatures below 8ºC (Hertog *et al*., 1997; Kumar *et al*., 2004; Malone *et al*., 2006); consequently, of particular interest for frying purposes, it is the development of potato cultivars with little accumulation of reducing sugars after harvest and during cold storage. Association mapping is a strategy for the study of the molecular basis of complex traits that has shown to be a less time-consuming approach for the discovery of marker-trait associations compared to linkage mapping (Álvarez *et al*., 2014). Using this approach there is major record of ancient recombination events than in a bi-parental linkage population, that lead to the reorganization of the chromosomes in smaller regions consequently increasing mapping resolution (Ersoz *et al*., 2007; Hamblin *et al*., 2011). The use of natural populations or sets of breeding materials in association mapping analysis is useful in the generation of diagnostic molecular markers that can be readily implemented in breeding schemes for the selection of outstanding genotypes (Gebhardt *et al*., 2007).

There is a significant knowledge on the metabolic pathways and enzymes involved in starch synthesis, degradation and transport in potato. Sixty nine functional genes in carbohydrate metabolism have been mapped (Chen *et al*., 2001). From these candidate genes, a set has been found to be associated with natural variation for frying quality or reducing sugar contents (Baldwin *et al*., 2011; Li *et al*., 2005, 2008, 2013; Schreiber *et al*., 2014). These studies have revealed the multiloci genetic architecture for sugar contents and frying color related to genes coding for functional enzymes in carbohydrate metabolism in tetraploid potato populations ($2n = 4x = 48$). Therefore, it is relevant to identify the loci that underlie these traits and are located in genes with key function in carbohydrate metabolism in other potato populations of current use in breeding programs and with different metabolic behaviors.

S. tuberosum Group Phureja is mainly constituted by diploid genotypes ($2n = 2x = 24$) with short day adaptation and tuber sprouting at harvest (Huamán and Spooner, 2002). Genotypes with round tubers, yellow flesh and skin present an interesting potential for their extensive use due to their desirable organoleptic properties and processing quality (Bonilla *et al*., 2009; Rivera *et al*., 2006; Rodríguez *et al*., 2009). This cultivated group is also a valuable genetic resource for the introgression of genes of agronomic importance to tetraploid potatoes that are the most consumed worldwide (Straadt and Rasmussen, 2003; Ghislain *et al*., 2006; Faostat, 2012). In Andean countries, Group Phureja constitutes an important crop, especially for small farmers; also this potato

group has shown important properties for its nutritional content (Peña *et al*., 2015). The sequencing of the doubled-monoploid genotype DM1-3516 R44 from Group Phureja represents the potato reference genome and its analyses has contributed to the understanding of the genetic basis of various agronomic traits in tetraploid potato (Jupe *et al*., 2013; Potato Genome Sequencing Consortium *et al*., 2011; Schreiber *et al*., 2014).

Accessions from the Colombian Core Collection of Group Phureja (CCC) were characterized for their frying quality, using a color scale, through an evaluation in a multi-environmental trial (Ñústez-López, 2011a). Recently, a liquid chromatographic method was applied to measure the sucrose, glucose, and fructose contents in these accessions in a single-environment trial (see Chapter II). These phenotypic assessments were used in the current study to implement an association mapping strategy in order to elucidate the molecular basis of sugar accumulation and frying color in Phureja tubers using a candidate gene approach. Association analysis was performed with SNP markers identified in candidate genes with key function in carbohydrate metabolism and influence in potato frying color and sugar contents in tetraploid potatoes (Baldwin *et al*., 2011; Fischer *et al*., 2013; Li *et al*., 2005, 2008, 2013; Schreiber *et al*., 2014).

2. Materials and methods

2.1. Plant material and phenotypes

A set of 108 diploid landrace accessions from CCC and four commercial diploid cultivars (Criolla Colombia, Criolla Latina, Criolla Galeras, and Criolla Guaneña) (Ñústez-López, 2011b; Rodríguez *et al*., 2009) were used in this study for the association analysis. These genotypes were characterized previously for their frying quality using a multi-environmental Bayesian estimate of mean chip darkening % (Ñústez-López, 2011a). Recently, these genotypes were also assessed for their sucrose, glucose, fructose, reducing and total sugar contents, and for their glucose/fructose and sucrose/reducing sugars ratios in a single environment (see Chapter II). Mean genotypic values of these eight variables were used for the independent association analysis for each trait.

2.2.Candidate gene amplicon sequencing and SNP calling

Genomic DNA was isolated with DNeasy Plant Mini Kit™ (Qiagen) from plant young leaf tissue. DNA was quantified with a Thermo Scientific NanoDrop 2000c and adjusted to a concentration of 10 ng μL^{-1} for the Polymerase Chain Reaction (PCR) amplification. A set of candidate gene loci were selected to manually design primers for amplicon sequencing using the potato reference genome (Potato Genome Sequencing Consortium, 2011) in the SPUD data base (Hirsch *et al*., 2014). In addition, primers in other candidate gene regions analyzed by Fischer *et al*. 2013 and Schreiber *et al*. (2014) were also tested in Group Phureja. Amplicons with appropriated sequence quality in eight genotypes and with four or more SNP markers identified were selected for amplicon sequencing in the whole population (Table 1). A total of 50 ng of genomic DNA template in 25 µL of 1X PCR buffer (Invitrogen), 2.5 mM MgCl2, 0.2 mM each dNTP, and 0.5 mM each forward and reverse primers were amplified with 1 unit Taq polymerase (Incitrogen). Amplification cycling conditions used were the same

described by Schreiber *et al*. (2014). Annealing temperatures of each primer are specified in Table 1.

Amplicons were visualized in 1.5% (w/v) agarose gels in 1X TAE buffer and stained with ethidium bromide (0.4 μg mL^{-1}). PCR products were cleaned using the ExoSAP mix of enzymes (Affymetrix) and sequenced at the Max-Planck-Genome-Center Cologne using the dideoxy chain-termination sequencing method, an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, and an ABI PRISM 3730 automated DNA Sequencer (Applied Biosystems, Weiterstadt, Germany). SNPs were detected by visual examination of the sequence trace files for overlapping base calling peaks using Geneious Software (Biomatters, Auckland, New Zealand). The SNP allele dosage was estimated first manually and after with Data Acquisition and Analysis Software (DAx) (Van Mierlo Software Consultancy, Eindhoven, The Netherlands); in the case of heterozygous individuals, overlapping base calling peaks were identified. These analyses allowed the identification of 111 SNP markers in ten candidate gene regions shown in Table 1.

2.3. Association analysis

Association analysis was carried out assuming that Phureja accessions studied do not present a marked population structure as described previously (Juyó *et al*., 2015). Markers included in the analysis were those with a minimum allele frequency higher than 0.05. A compressed mixed linear model (Zhang *et al*., 2010) and an enriched compressed mixed linear model (Li *et al*., 2014) were implemented with GAPIT R Package (Lipka *et al*., 2012) for the analyses. These models included population structure and kinship estimates from molecular markers analyzed. Quantile-quantile (QQ) plots of the expected and observed *F*-test probabilities for the SNP markers were assessed to identify the appropriate model in controlling type I errors caused by population structure and familial relatedness (Sukumaran *et al*., 2012) (Supplementary Figure 1).

The threshold for significant marker-trait association is expressed on a $-\log_{10}$ scale, being the SNP markers with a *p*-value higher than two considered with significant effect on the variable studied. To support the associations found for the SNP markers with the mixed model approach, an analysis of variance (ANOVA) was performed using Genstat software (VSN International, Hemel Hempstead, United Kingdom) to assess the effect of the allele dosage in the trait, considering a level of significance of $\alpha = 0.05$. Linkage disequilibrium (LD) was estimated between pairs of SNP markers that were found with significant associations in the same chromosome, using the r^2 statistic (Hill and Robertson, 1968).

3. Results

The analysis of amplicon sequences with appropriate quality, allowed the identification of 111 SNP markers in ten candidate gene regions. The mixed model analysis generated seven significant marker-trait associations that were supported by single-marker ANOVAs. Thus, five SNP markers in two chromosomes were associated with sugar contents and chip darkening in Group Phureja (Table 2). From ten candidate gene regions studied, two loci were found with significant associations. The QQ-plots showed that the models adjusted for each phenotype were effective in controlling type I

error (Supplementary Figure 1). The box plots in Figure 1 supported the consistency of the associations by showing the effect of the SNP allele dosage in the trait variation.

Four markers were found in the locus *InvGE* from an apoplastic invertase in the chromosome IX. The average distance between adjacent markers was of 88 bases (Supplementary Data 1). The minor frequency alleles of these loci were not common enough to be found in homozygous state in the population (Figure 1A-F), which indicated that these alleles correspond to low-frequency variants (Mitchell *et al*., 2004). Finally, one SNP marker trait-association was found in the locus *SssI* from a soluble starch synthase in the chromosome III (Supplementary Data 1). In this case, the three genotypic classes observed reflect the highest minor allele frequency found for this associated SNP marker (Figure 1G).

The SNP *InvGE*-$C_{2476550}$T had a negative effect in chip darkening variation (Figure 1A). This marker presented the minor allele with the lowest frequency and with the highest effect in the trait variation. The markers *InvGE*-$G_{2476660}$A and *InvGE*-$C_{2476709}$A were associated with a positive and similar effect in fructose contents (Figure 1B-C). Both markers also shared a similar minor allele frequency. The SNP *InvGE*-$G_{2476817}$A showed significant associations with positive effect in fructose, glucose, and total sugar contents (Figure 1D-F). *InvGE*-$G_{2476660}$A, *InvGE*-$C_{2476709}$A, and *InvGE*-$G_{2476817}$A were found in strong LD, which is reflected in the similar effects in the trait and the similar distributions of the markers in the population. The marker *SssI*-$C_{45603698}$T is associated with a negative effect in sucrose variation and it is also outstanding for the amount of variance that explains of the trait (Figure 1G).

4. Discussion

The study of ten candidate genes with key function in carbohydrate metabolism allowed the identification of SNP markers in two genes associated with sugar contents and frying color in Group Phureja**.** This result demonstrates the effectiveness of the candidate gene approach to find associated variants in Group Phureja through the exploration of regions with known function in carbohydrate metabolic pathway and associated in previous studies to sugar contents and chip color variation in tetraploid populations. The association analysis validated the relevance of the *InvGE* and *SssI* loci in the variation of frying color and sugar contents in diverse potato genetic backgrounds including different ploidy levels. The association of SNP markers from *InvGE* locus with chip darkening, fructose, glucose, and total sugar contents supports the relationships among these variables that were discussed in Chapter II.

InvGE and *InvGF* in chromosome IX are apoplastic invertase genes tandem duplicated and separated by 1.8 kb of DNA that co-localize with the cold-sweetening QTL *Sug9a*, which is highly reproducible among environments (Maddison *et al*., 1999; Menéndez *et al*., 2002). Experimental evidence reveals that these genes conform a haplotype block associated to chip quality described previously by Li *et al*., 2008 and Draffehn *et al*., 2010. The presence of SSR alleles located in both genes, with positive or negative effect in reducing sugar contents under cold-storage, have been observed in studies in tetraploid potato landraces from Argentina and in a collection of cultivars and breeding lines from New Zealand (Baldwin *et al*., 2011; Colman *et al*., 2009). The current results in Phureja support the assumption that the genomic region that includes *InvGE* has an important effect in potato tuber sugar accumulation that is consistent in this cultivated group.

Table 1. Candidate gene PGSC0003DMG loci analyzed from the potato reference genome (Potato Genome Sequencing Consortium, 2011; Hirsch *et al*., 2014), chromosome (Chr.), primer sequences, annealing temperature (Ta), amplicon sizes, and number of SNP markers scored in Group Phureja.

Gene Acronym (GenBank Accesion No.)	Locus PGSC0003DMG	Chr.	Primer Sequences 5'-3'	Ta (°C)	Amplicon size (bp)	No. SNPs scored
Stp23 (D00520)	400007782	III	*f-cagatatgttacatactctacc r-tcattagtcacaactttatcgg	59	998	4
StpL (X73684)	400028382	V	*f-ttacattgcacaagcacaagc r-gtgtacatacaatactctatcc	57	984	14
SssI (Y10416)	402018552	III	*f-aacaataggaatttaccataacc r-atattcccaaacaaaacagagc	57	970	12
InvGE (AJ133765)	400008943	IX	f-caattcttcgattcttcatagg *r-aattgaagcagatcatgtagg	57	797	9
Pain1 (X70368)	400013856	III	f-catacattacactatagatcc *r-aattgaagcagatcatgtagg	56	926	5
			f-caaaatgaatacatattaagagg[a] *r-cttaagcagttgcttagagc	56	711	4
UGPase (D00667)	401013333	XI	f-atgatgttctccacttaaaagc *r-tttcagattttcagaagagagg	56	807	11
			*f-tgattaacgatactatacgtcc r-ttaaaacttccttatactatatgg	56	933	12
GWD (Y09533)	400007677	V	f-ttctgttatctacttagttacg *r-gttttatatcttgcttctttgg	56	994	7
BMY-8/2 (AF393847)	400001855	VIII	f-gctactggacatggtgacaga[b] *r-ttacatagaggtctgtcctgcttgag	57	560	9
PWD (AY747068)	400016613	IX	*f-ggtctgatgatctatctgattgc[b] r-gacatcttgaggagaaccaaactt	57	871	16
LapN (X77015)	400007831	XII	f-gcttcctggtcttggctc[c] *r-gataggcatacgcagccaggtcagaaatcaa	60	985	8

* Primer strand used for amplicon sequencing
[a] From the promoter region of the Pain1 candidate gene
[b] From Schreiber *et al*., 2014
[c] From Fischer *et al*., 2013

Studies in tetraploid potatoes have shown *InvGE* and *InvGF* alleles associated with better chip quality, explaining less amount of the trait variation than in the current work (Li *et al*., 2005, 2008). The higher percent of variance explained on the trait variation found for Group Phureja might reflect a large phenotypic effect of the low-frequency variants in *InvGE* locus, thus adding evidence that these variants can present important effects on complex trait architecture (Panoutsopoulou *et al*., 2013). These alleles are likely to be rare because of purifying selection; consequently these alleles are removed from the population because they might not be advantageous for the carrier individuals (Lee *et al*., 2014). The magnitude of the effect is relevant considering that genotypes with these minor frequency alleles in homozygous state were not found. The current study shows the opposite effects in the trait variation of the SNP marker *InvGE*-$C_{2476550}$T and the haplotype block constituted by the markers *InvGE*-$G_{2476660}$A, *InvGE*-$C_{2476709}$A, and *InvGE*-$G_{2476817}$A. The results reveal that both regions are inherited independently; additional research is required to validate the opposite effect for the low-frequency variants present in these regions given the outstanding positive effect of the lowest allele frequency of *InvGE*-$C_{2476550}$T in frying color.

Apoplastic invertases are considered key enzymes in plant source/sink balance, thus regulating the import of glucose and fructose for tuber initiation (Fotopoulos, 2005; Minhas *et al*., 2004). It is remarkable that vacuolar acid invertases rather than apoplastic invertases have been found to control sucrose/reducing sugar ratio in cold-stored tubers (Li *et al*., 2005; Zrenner *et al*., 1996). Transcripts of *InvGE* and *InvGF* have been found expressed in leaves and flowers but not in mature tubers. Furthermore, functional analyses of potato alleles are not consistent with their statistical association with chip quality (Draffehn, 2010; Maddison *et al*., 1999). The aforementioned observations suggest a novel regulatory role for these genes in sugar accumulation of mature tubers or the existence of a large haplotype block associated to chip quality including both *InvGE* and *InvGF* (Draffehn, 2010; Li *et al*., 2005).

The potential functional effect of apoplastic invertases in sugar contents of mature tubers is yet to be determined but it might be an effect earlier during tuber initiation. Transcription analyses have shown that invertase genes are expressed in a genotype specific manner (Draffehn, 2010) therefore it is relevant the design of appropriate experiments to reveal the functional influence of apoplastic invertases in sugar accumulation of tubers from Group Phureja. The association of the SNP *InvGE*-$G_{2476817}$A with total sugar contents and reducing sugar contents bring up the potential effect of the gene in a process upstream the glucose and fructose accumulation in tubers. This potential effect is supported by the effect of the QTL *Sug9a* in sucrose contents as well as reducing sugar contents (Menéndez *et al*., 2002).

The presence of a haplotype block in the distal region of chromosome IX is supported by the co-segregation of two microsatellite loci in landraces from Argentina. One locus is located in a region of the gene *InvGF* while the other is in the gene *SbeII* which encodes a starch branching enzyme (Colman *et al*., 2009; Feingold *et al*., 2005). Even though *SbeII* is not the predominant starch branching enzyme expressed in tubers, antisense inhibition experiments have shown that it has a major effect on starch structure (Jobling *et al*., 1999; Larsson *et al*., 1998); recently, an association study revealed the effect of the polymorphisms in this gene with the degree of starch phosphorylation, which is a process related to starch degradation (Carpenter *et al*., 2015).

Table 2. Candidate gene SNP markers associated with sugar contents and chip darkening in *Solanum tuberosum* Group Phureja. Associations presented are significant with a mixed model approach and with an analysis of variance (ANOVA).

Gene-SNP [a]	Trait	Minor Allele Frequency, %	$-\log_{10}(p\text{-value})$ [b]	Percent variance explained [c]
InvGE-$C_{2476550}$T	Chip darkening	9.0 (T)	2.4**	15.6 ↓
***InvGE*-$G_{2476660}$A** [d]	Fructose	19.6 (A)	2.2**	7.2 ↑
***InvGE*-$C_{2476709}$A**	Fructose	19.2 (C)	2.2***	7.0 ↑
***InvGE*-$G_{2476817}$A**	Fructose	18.2 (A)	2.7***	8.5 ↑
InvGE-$G_{2476817}$A	Glucose	18.2 (A)	2.0*	6.6 ↑
InvGE-$G_{2476817}$A	Total sugars	20.0 (A)	2.0***	8.8 ↑
SssI-$C_{45603698}$T	Sucrose	35.9 (C)	2.0*	13.5 ↓

[a] SNP reference allele is followed by its position in the chromosome and the nucleotide from the allelic variant.
[b] SNP markers with a $-\log_{10}(p\text{-value})$ higher than two were considered with significant effect on the variables studied with the mixed model approach. These markers were tested with an ANOVA considering a level of significance of $\alpha = 0.05$. *p*-values: ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$.
[c] The percent variance explained expresses the effect of the minor frequency allele in the trait. Arrows indicate the direction of the effect on the trait, upwards for a positive effect (high sugar contents or dark chip color) and downwards for a negative effect (low sugar contents or light chip color).
[d] Bold SNP markers were in strong linkage disequilibrium, showed similar effects in the trait, and presented similar distributions in the population.

These previous remarks stand for a conserved linkage disequilibrium region in chromosome IX containing *SbeII*, whose potential function in sugar accumulation and chip color variation in potato has to be proven. Therefore it is relevant to explore the presence in Group Phureja of a larger haplotype block than the one reported here in *InvGE*, through the evaluation of markers in *InvGF*, *SbeII*, and other close genomic regions. The identification of associated regions in large haplotype blocks is favorable for breeding purposes because it might allow the design of diagnostic markers with high predictive value due to the low recombination rates in the region (Li *et al*., 2008).

The association of the SNP *SssI*-$C_{45603698}$T reflects the effect of a marker with three genotypic classes and consequently with a minor allele with a higher frequency than the minor alleles frequencies from markers in *InvGE*. The association of *SssI* locus with sucrose is novel since this gene has been reported previously with effects in tuber starch content and chip quality after harvest and after storage (Li *et al*., 2008; Schreiber *et al*., 2014). Starch synthases catalyze the glycosyl transfer from ADP-glucose to glucan. The expression of *SssI* in tubers is low, therefore suggesting a minor role of this particular enzyme in starch synthesis in storage organs (Kossmann *et al*., 1999). As in the previous case, this SNP-trait association might be indirect resulting from the LD with causal variants, or direct through a potential novel role of *SssI* in the control of starch degradation in tubers.

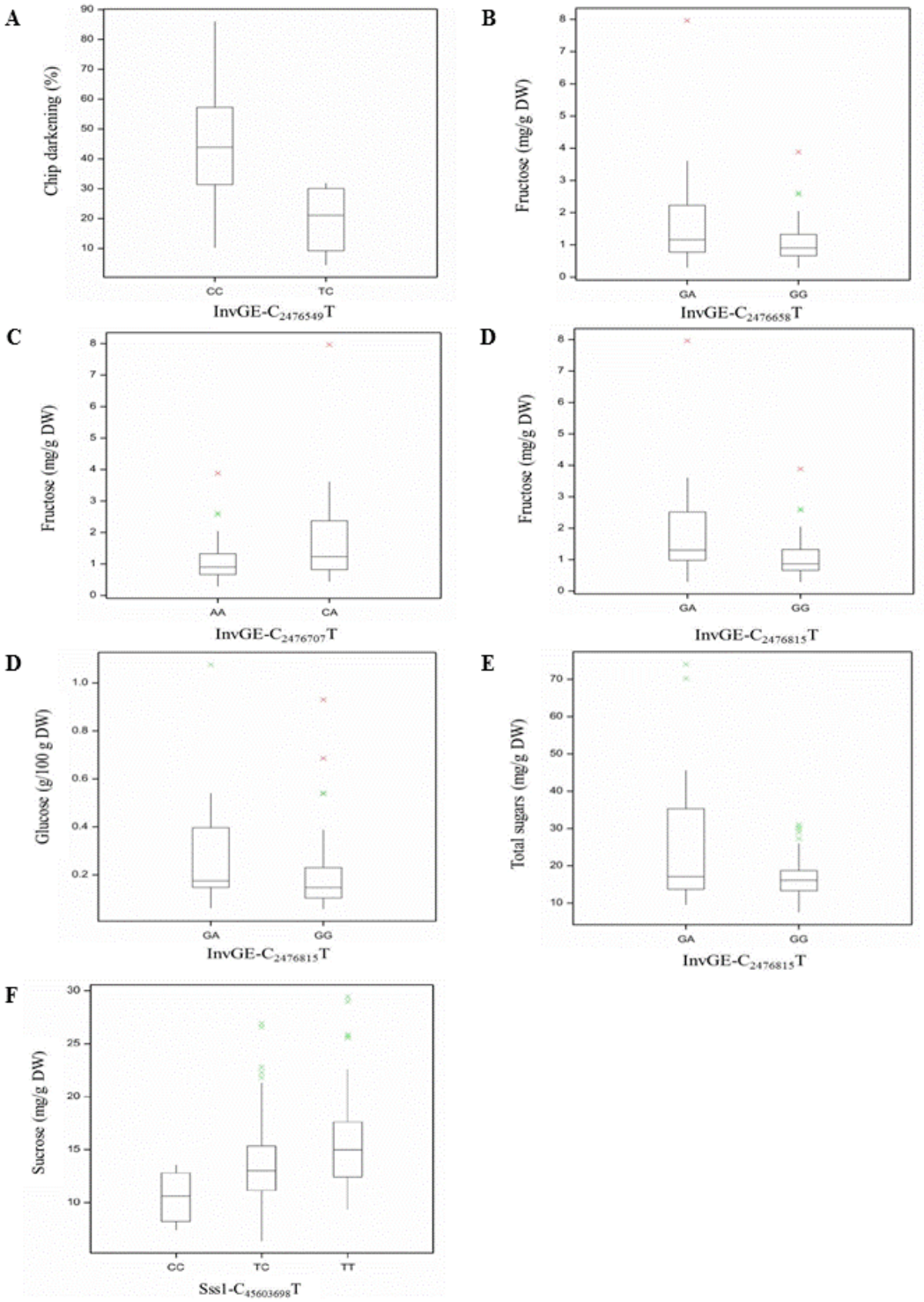

Figure 1. Box plots representing the effect of the allele dosage in seven significant SNP marker-trait associations. Each plot is labeled with the SNP identification indicated in Table 2. Y-axis: Values for chip darkening (%) and sugar contents (mg/g DW or g/100g DW). X-axis: Allelic dosage in the locus analyzed. Box plots from A to F show the effect of SNP markers in locus *InvGE* in chromosome IX. Box plot G indicates the effect of the SNP marker located in locus *SssI* in chromosome III.

5. Conclusions

The candidate gene approach allowed the identification of SNP markers in two genes associated with sugar contents and frying color in Group Phureja that were previously reported with associations in tetraploid germplasm. This might suggest that despite the differences in carbohydrate metabolism in both genetic backgrounds, there are conserved genes related to sugar content and frying color variation. The enzymes coded by these genes have not been found expressed in mature tubers, therefore these SNP-trait associations might be indirect resulting from the linkage disequilibrium with causal variants, or direct through a novel role of these candidate genes controlling sugar contents in tubers. Most of the associated SNPs were low-frequency variants, thus revealing that these types of variants present important effects on sugar contents and frying color in Group Phureja.

The loci found with associations explain a limited amount of the overall trait variability in Group Phureja. Therefore, selecting the individuals combining the superior alleles for these loci will not guarantee *per se* better chip quality because there are other regions influencing the trait and the interaction with other loci might affect its expression. The study of additional candidate gene regions with key function in carbohydrate metabolism that were not tested here is relevant to support the presence of a haplotype block in chromosome IX and to evaluate the variation in other relevant enzymes that could not be included in the current work. The implementation of a genome wide approach is necessary to include genomic regions in other pathways that control starch-sugar equilibrium as well. The genome wide study is also required to assess the distribution of LD regions in all the chromosomes to establish the mapping resolution. These strategies will contribute to the understanding of the genetic architecture of sugar accumulation and frying color of Group Phureja from Colombia.

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Supplementary Data

Supplementary Data 1. Amplicon sequences of candidate genes with associations, SNP alleles and SNP positions from the potato reference genome (Potato Genome Sequencing Consortium, 2011). The sequences were retrieved from pseudomolecules v4.03 (http://potato.plantbiology.msu.edu/cgi-bin/gbrowse/potato/) in the SPUD data base (Hirsch et al., 2014). Primer positions are underlined and SNP markers with associations are highlighted blue.

1. *InvGE*, Apoplastic invertase (PGSC0003DMG400008943)

chr09:2476346...2477143

acaattcttcgattcttcatagggtcatagaatgacttagatgcatagaaattcccatagtcaagcctcaatcccttcgaaccatcg
atagaattgttatcaggaatgtacctatctttttttggtgtcatacataccaatagtgtaatactcaaacctattaacatcaaggctattc
2476534 .6550 .6574 .6595
ttaaggacat[g/a]tttgacatttttttcc[g/a]cgatacgatgcatctaagccatt[a/t]gtatttttttaatgatacagg[g/a]aaaa
.6660
aatcaggacattcccaatttccagtatgaggagatgaatgaagtggatgttgggctttgg[c/t]ccatttaatgaagtctctacttc
.6709
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.6817
ggatcacgaaatttggttttgttgatgctattgtcaggtacaatcaa[c/t]gggttgttattaggtttgatccatttacgaagaaatg
.6934
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.6937
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ggatccaatttatcaagtcttttgagactgaatgagcccaaacaatattgccccatactgatccttttggattgtattgatagaataa
atgatatactccattataatacataggtgctgt

2. *Sss1*, Soluble starch synthase (PGSC0003DMG402018552)

chr03:45603327…45604297

atattcccaaacaaaacagagcttcccaaatactaaaacgatgtcgttctgagtatctgtccacctctttctaggtacttgtgttctt
catcagatcctatactagtactgtagtagtagaatatagaatgagtgtttcattttcacttttttttcacttttcagattttctatattgaaa
45603548
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.3662
aagtcatgtttatgtgtgtcagggagagttgtgaggggtttgagggtagaaagacaagtggggttggg[a/g]ttttcttggttgtt
.3698 .3744
gaagggacgaagaaacagaaa[g/a]gttcaatctttgtgtgttacaagtagtgtttcagatggttcatca[a/g]ttgctgaaaat
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.3936 .3968 .3991 .4002
[t/c]tagtgaaaaatctgttttttaagatatcata[a/g]ttcatagtttcctatttgatta[a/t]acaatgcccc[a/g]tgctgccaat

.4050
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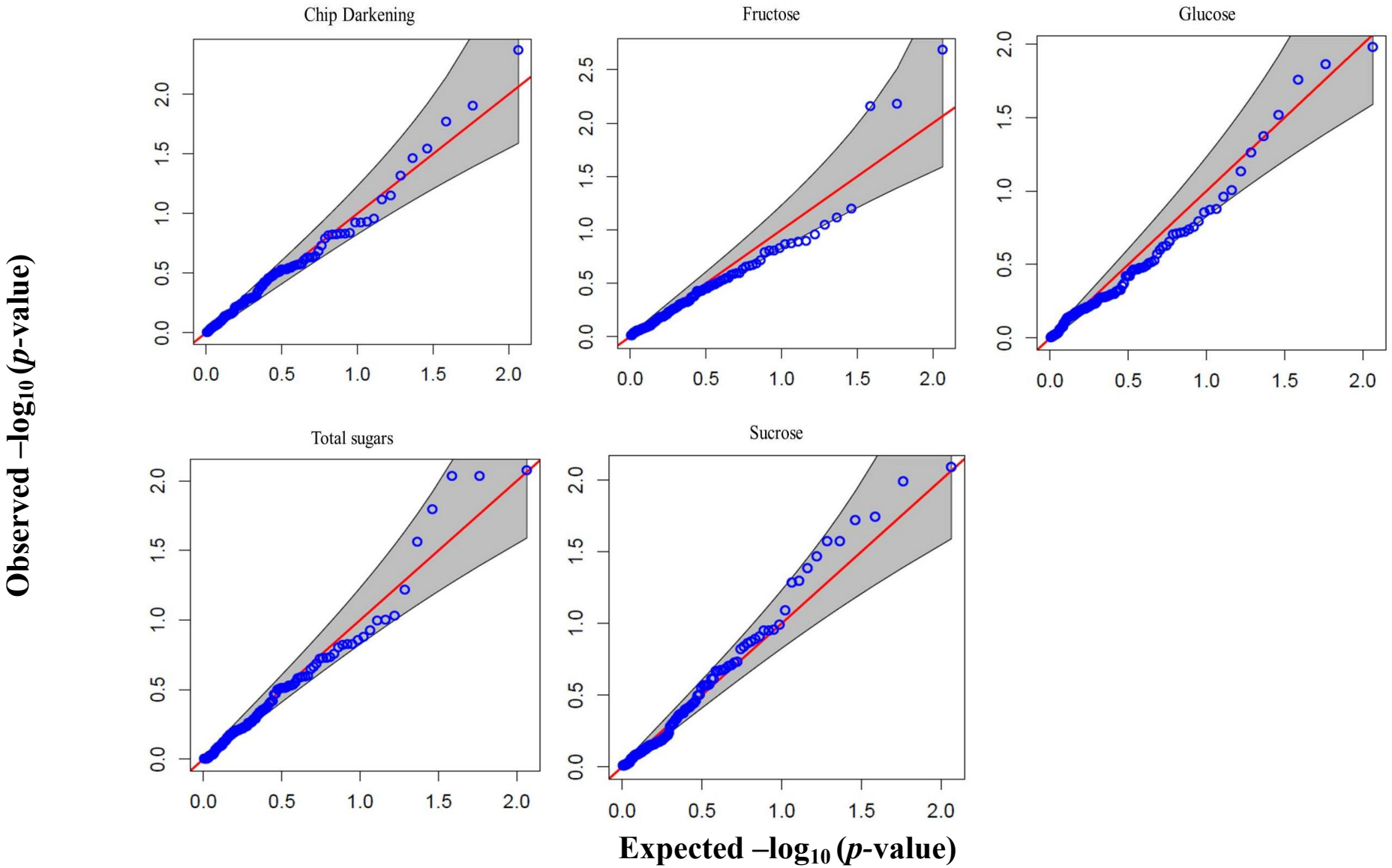

Supplementary Figure 1. Quantile-quantile (QQ) plots representing the observed (X-axis) and expected (Y-axis) *F*-test probabilities for the SNP markers tested for association in the five traits that showed significant markers. The grey area shows the 95% confidence interval for the QQ-plot under the null hypothesis of no association between the SNP and the trait.

General discussion

The chromatographic method developed and validated allowed to perform an accurate and exact phenotypic characterization of sugar contents of tubers from accessions of Group Phureja. This analysis revealed an extensive variability regarding sugar contents and sugar ratios that reflected the existence of variation regarding sugar accumulation metabolism in Phureja germplasm. This variability of sugar contents is consistent with multi-environmental data of frying color from the collection (Ñústez-López, 2011), thus showing the association of both experimental approaches.

Glucose was the predominant sugar found in tubers, which indicates that this reducing sugar might be the most relevant in defining frying quality at harvest and supports that fructose 6-phosphate might be preferentially used in glycolysis (Junker *et al*., 2006). The unequal amount of both reducing sugars suggests the effect of the lack of tuber dormancy in this cultivated group (Huamán and Spooner, 2002) and shows a potential variation in the activities and affinities of fructokinases and hexokinases, which are enzymes responsible of fructose and glucose irreversible phosphorylation before their use in metabolic processes (Davies & Oparka, 1985; Granot *et al*., 2013; Kumar *et al*., 2004; Sowokinos, 2001). Further association and expression studies are necessary to establish the specific enzymes that influence the glucose/fructose ratio in Group Phureja.

The multivariate analysis allowed the identification of two clusters with few individuals. Group One represents four genotypes with outstanding values of glucose/fructose ratio and Group Five includes three genotypes with the highest contents of reducing sugars. This rare Phureja genotypes with extreme phenotypic values are of great interest for deeper studies because they are often enriched with uncommon causal variants that can contribute to explain complex trait variability and present a large effect on the trait (Lee *et al.*, 2014). Accordingly, it is important to highlight that most of the associated SNPs were low-frequency variants, thus revealing that these types of variants present important effects on sugar contents and frying color in Group Phureja.

The HPLC assessment and the implementation of a candidate gene approach enabled the identification of two genes with key function in carbohydrate metabolism with association to sugar contents that were previously reported with associations in tetraploid germplasm. This might suggest that despite of the differences in carbohydrate metabolism in both genetic backgrounds influenced by divergences in the dormancy period (Huamán & Spooner, 2002), there are conserved genes related to sugar contents and frying color variation. Although a relationship was found between the variation of sugar contents and frying color, the haplotype block in *InvGE* with association was obtained through the mixed model analysis with the HPLC measurements. This result is relevant because the identification of associated regions in haplotype blocks is favorable for breeding purposes because it might allow the design of diagnostic markers with high predictive value due to the low recombination rates in the region (Li *et al*., 2008).

The loci found with associations explain a limited amount of the overall trait variability in Group Phureja and the validation of the effect of the associated SNP markers is

necessary in a different population. Selecting the individuals combining the superior alleles for these loci will not guarantee *per se* better chip quality because there are other regions influencing the trait and the interaction with other loci might affect its expression (Li *et al*., 2005). Therefore, for a comprehensive understanding of the genetic architecture of sugar accumulation and frying color of Group Phureja, the implementation of a genome wide approach is necessary to include genomic regions in other pathways that control starch-sugar equilibrium.

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Conclusions and perspectives

A HPLC-RI method for sugar content analysis has been developed and validated for accurate measurements in *S. tuberosum* Group Phureja tubers. This method is relevant because it offers the opportunity to obtain an accurate phenotypic assessment of sugar contents that impacted in the reliability of association analysis. Sugar content results revealed variability in Group Phureja and are consistent with previous data of frying color. Similarly, analysis of glucose/fructose and sucrose/reducing sugar ratios disclosed also an extensive variability regarding metabolisms in sugar accumulation. The generation of this basic information of sugar profiles of Phureja germplasm is important for breeding programs to identify genotypes with little accumulation of the three sugars.

Sugar accumulation profiles are particular for the specific environment of assessment; therefore, it is of great interest the understanding of the changes in sugar accumulation in the genotypes caused by differential environmental conditions and to study their stability. Likewise, it is important to evaluate the potential of the different genotypes for sucrose hydrolysis under cold storage as in the current study most of the genotypes tend to accumulate higher contents of sucrose than of reducing sugars. This information is also necessary for the breeding programs, as Phureja commercial cultivars tend to accumulate high contents of sucrose, which can be hydrolyzed during storage consequently diminishing frying quality.

According to the knowledge of the author, this is the first study that uses a biochemical approach and analytical chemistry methodologies to perform accurate sugar quantifications to find associated regions to frying quality using a candidate gene approach. These results should contribute to advance in the design of molecular markers easy-to-hand for the implementation of marker assisted selection.