Transcription factors, sucrose, and sucrose metabolic genes interact to regulate potato phenylpropanoid metabolism

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Abstract

Much remains unknown about how transcription factors and sugars regulate phenylpropanoid metabolism in tuber crops like potato (Solanum tuberosum). Based on phylogeny and protein similarity to known regulators of phenylpropanoid metabolism, 15 transcription factors were selected and their expression was compared in white, yellow, red, and purple genotypes with contrasting phenolic and anthocyanin profiles. Red and purple genotypes had increased phenylalanine ammonia lyase (PAL) enzyme activity, markedly higher levels of phenylpropanoids, and elevated expression of most phenylpropanoid structural genes, including a novel anthocyanin O-methyltransferase. The transcription factors Anthocyanin1 (StAN1), basic Helix Loop Helix1 (StbHLH1), and StWD40 were more strongly expressed in red and purple potatoes. Expression of 12 other transcription factors was not associated with phenylpropanoid content, except for StMYB12B, which showed a negative relationship. Increased expression of AN1, bHLH1, and WD40 was also associated with environmentally mediated increases in tuber phenylpropanoids. Treatment of potato plantlets with sucrose induced hydroxycinnamic acids, flavonols, anthocyanins, structural genes, AN1, bHLH1, WD40, and genes encoding the sucrose-hydrolysing enzymes SUSY1, SUSY4, and INV2. Transient expression of StAN1 in tobacco leaves induced bHLH1, structural genes, SUSY1, SUSY4, and INV1, and increased phenylpropanoid amounts. StAN1 infiltration into tobacco leaves decreased sucrose and glucose concentrations. In silico promoter analysis revealed the presence of MYB and bHLH regulatory elements on sucrolytic gene promoters and sucrose-responsive elements on the AN1 promoter. These findings reveal an interesting dynamic between AN1, sucrose, and sucrose metabolic genes in modulating potato phenylpropanoids.

Key words: Anthocyanins, bHLH, chlorogenic acid, nutrition, MYB, phenolics, phenylpropanoids, phytonutrients, potato, sucrose, tobacco.

Introduction

Plants synthesize an array of phenylpropanoids with diverse roles including in plant growth and development, flowering, pigmentation, signal transduction, and structural integrity (Fig. 1; Koes et al., 2005; Vogt, 2010). Phenylpropanoids are also important plant dietary constituents that possess various health-promoting properties, including against cardiovascular disease and cancers (Parr and Bolwell, 2000). White potatoes (Solanum tuberosum L.) contain modest amounts of phenylpropanoids and are the third largest source of dietary phenylpropanoids because of high consumption (Chun et al.,...
Red- and purple-flesh potatoes contain decidedly higher amounts of phenylpropanoids (André et al., 2007a) and the increase is largely, but not solely, due to anthocyanin biosynthesis (Navarre et al., 2011).

Numerous factors mediate expression of phenylpropanoid genes, including sugars and various transcription factors including MYBs (Dubos et al., 2010). MYBs have single or multiple imperfect repeats (R) of structurally conserved DNA-binding domains (Fig. 2A). R2R3 genes comprise the largest of the four classes of plant MYBs and have a conserved N-terminal DNA-binding domain that is in direct contact with the DNA and a variable C-terminal domain that activates or represses its targets (Dubos et al., 2010).

R2R3 MYBs are divided into 22 subgroups in Arabidopsis thaliana based on conserved motifs (Stracke et al., 2001). Various R2R3 MYBs regulate phenylpropanoid biosynthesis, some of which interact with basic helix–loop–helix (bHLH) proteins (Grotewold, 2005). A mutant maize (Zea mays) P1 MYB activates some flavonoid genes but not anthocyanins in the absence of a bHLH, whereas AtMYB12 regulates flavonol biosynthesis without a bHLH interaction (Grotewold et al., 2000; Mehrtens et al., 2005).

Unlike the flavonol branch of the pathway, anthocyanin biosynthesis is typically regulated by a complex in which MYB, bHLH, and WD40 transcription factors interact. When this complex is formed, MYB and bHLH bind to promoters with consensus nucleotide sequences like MACCW and CANNNG (Sablowski et al., 1994; Zimmermann et al., 2004). The first 200 aa of bHLH proteins are required to interact with MYB transcription factors, and aa 200–400 interact with WD40 proteins (Pattanaik et al., 2010). The C-terminal ACT-like domain facilitates binding of MYB to the promoter (Grant, 2006). WD proteins have four to eight imperfect tandem repeats and interact with other proteins through the WD repeat region (Neer et al., 1994).

Sucrose modulates transcriptional and post-translational regulation of many pigment-related genes (Koch, 1996). Sucrose induces anthocyanins in Arabidopsis through induction of PAP1/MYB75 (Production of Anthocyanin Pigment 1) and fails to induce anthocyanins in the papi1 mutant (Teng et al., 2005; Solfanelli et al., 2006). Anthocyanin induction in Arabidopsis seems specific to sucrose, but in grapes (Vitis vinifera), other sugars also stimulate anthocyanin synthesis (Gollop et al., 2002).
As a staple food, potatoes are an attractive target for phytonutrient enhancement. Tuber-specific overexpression of the MYB transcription factor StMtf1 resulted in elevated amounts of phenylpropanoids (Rommens et al., 2008). Tuber anthocyanin synthesis in the periderm is controlled in part by three loci, D, P, and R. P and R were found to be structural genes, whereas D encodes an R2R3 MYB (Jung et al., 2005, 2009; Zhang et al., 2009a). The D locus maps to a region of chromosome 10 that harbours StAN2, which was later renamed StANI (Jung et al., 2009). StANI expression correlated with anthocyanin levels in drought-stressed potatoes (André et al., 2009).
The goal of this study was to determine how sugars and transcription factors modulate biosynthesis of potato phenylpropanoids, including hydroxycinnamic acids, flavonols, and anthocyanins. The involvement of sugars and 15 transcription factors was characterized and provided evidence that AN1 along with bHLH1, sucrose, and sucrose metabolic genes interact to regulate the pathway.

Materials and methods

Plant materials
Small tubers (~25–50 g) from NY144, Challenger, ORO4198-1, AmaRosa, and Magic Molly were harvested from field-grown plants in Moses Lake, WA, USA, in August 2011. Tubers were peeled and frozen in liquid nitrogen within minutes of harvest. Analysis of environmental influences on transcription factor expression was performed on potatoes grown in Texas, Florida, and four locations in Alaska (Payyavula et al., 2012). For sucrose feeding studies, internodes from 1-month-old potato (cv. Purple Majesty) plants were propagated on MS medium supplemented with 0 or 120 mM sucrose at 25 °C with 16:8 light. Plantlets were collected after 5 d.

Phenylpropanoid analysis
Phenolics were extracted from 50 mg freeze-dried homogenized sample with 1.5 ml of 50% methanol, 1 mM EDTA, and 2.5% metaphosphoric acid using a validated method (Shakya and Navarre, 2006). Total phenolics were estimated by the Folin–Ciocalteu method (Singleton and Rossi, 1965). Individual phenolics were analysed on a 100 × 4.6 mm Onyx monolithic C-18 (Phenomenex) column with an Agilent 1100 HPLC system equipped with a quaternary pump, refrigerated autosampler, and column heater. Detectors were a DAD and SL ion trap with an electrospray ionization (ESI) source operated in both positive and negative ion mode, as described previously (Navarre et al., 2011). Anthocyanins were extracted from 50 mg of dry powder using a total of 2 ml of 50% methanol containing 2.5% formic acid. Total anthocyanins were estimated by a pH differential method (Wrolstad et al., 2005). Individual anthocyanins were determined by liquid chromatography/mass spectrometry (LC/MS) as described previously (Payyavula et al., 2012). Retention times and MS data for the quantitated compounds are shown in Supplementary Tables S1 and S2 at JXB online.

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)
RNA was extracted using hot cetyl trimethylammonium bromide (Chang et al., 1993), with slight modification (Payyavula et al., 2012). Samples were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and quality was assayed on a 1% agarose gel. cDNA synthesis used 2 μg of total RNA, Moloney murine leukemia virus reverse transcriptase (New England BioLabs), and oligo(dT) 20VN primers at 42 °C, RNA, Moloney murine leukemia virus reverse transcriptase (New England BioLabs), and oligo(dT) 20VN primers at 42 °C. Analysis of environmental influences on transcription factor expression was performed on potatoes grown in Texas, Florida, and four locations in Alaska (Payyavula et al., 2012). For sucrose feeding studies, internodes from 1-month-old potato (cv. Purple Majesty) plants were propagated on MS medium supplemented with 0 or 120 mM sucrose at 25 °C with 16:8 light. Plantlets were collected after 5 d.

Phylogenetic tree and protein similarity
Protein sequences collected by the best BLAST match from the Potato Genome Sequencing Consortium (PGSC) database (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml) or NCBI were aligned and a phylogenetic tree developed using MEGA4 (Tamura et al., 2007). The evolutionary history was inferred using the neighbour-joining method with 500 bootstrap replicates. The evolutionary distances were computed using the Poisson correction method with units of the number of amino acid substitutions per site. Percentage similarity was calculated with GeneDoc (Nicholas et al., 1997).

Antioxidant and enzyme assays
A ferric reducing antioxidant power (FRAP) assay was performed as reported elsewhere (Benzie and Strain, 1996). To an aliquot of 15–30 μl of phenolic extract, 1 ml of the pre-warmed (37 °C) FRAP reagent was added, incubated 5 min at 37 °C, and then centrifuged for 1 min. Absorbance at 593 nm (A593) was read and antioxidant capacity was estimated as trolox equivalents. Phenylalanine ammonia lyase (PAL) catalyses the first reaction of the phenylpropanoid pathway (Fig. 1) and activity was measured as described previously (Zucker, 1965). In a 1.5 ml tube, 700 μl of 50 mM sodium borate buffer (pH 8.8) and 200 μl of 50 mM phenylalanine were pre-warmed at 37 °C prior to adding 100 μl of extract. The reaction was continued for 1 h at 37 °C and then stopped with 200 μl of 12% trichloroacetic acid and measured at A590. Sugars were extracted twice at 80°C for 15 min from 25 mg of freeze-dried sample with a total of 2 ml of 80% ethanol. Pigments were removed by re-extracting with 50 mg of activated charcoal. Sucrose and glucose were estimated using Sigma kits (SCA20 and GAHK20).

Cloning and leaf infiltrations
Full-length coding sequences of the potato transcription factors AN1, bHLH1, and WD40 and the structural gene AOMT3 (anthocyanin O-methyltransferase 3) were amplified from Magic Molly or Purple Majesty tuber cDNA using Phusion® DNA polymerase (New England Biolabs). Full-length fragments were ligated into the pTOPO-TOPO5 vector (Invitrogen). Sequences were confirmed by sequencing (Retrogen). Sequences for StAOM3 (JX848659), StbHLH1 (JX848660), StWD40 (JX848661) and StAOMT3 (JX848662) were submitted to GenBank. A single positive colony was cultured in 5 ml of LB medium overnight and used to inoculate 50 ml of medium. Cells were harvested and redissolved in 10 mM MgCl2, containing 100 μM acetylsyringone and adjusted to an optical density of 0.5. Cultures were then diluted (1:1) with the gene silencing suppression vector p19TBSV of tomato bushy stunt virus (Voinnet et al., 2003) to avoid co-suppression. Samples were infiltrated into leaves of 3-week-old tobacco plants (Nicotiana tabacum cv. Samsun and Nicotiana benthamiana) maintained in a growth chamber under conditions of 15 h light. Leaves were harvested at 4 d post-infiltration.

Results
Phylogenetic analysis and protein similarity
A total of 12 MYBs were selected for characterization based on BLAST searches conducted against the potato genome database (The Potato Genome Sequencing Consortium, 2011) using nucleotide sequences of transcription factors described previously in the literature. Arabidopsis has 198 MYB genes (Yanhui et al., 2006) of which AtPAPI (GenBank accession no. AF325123) and AtPAP2 (MYB90, AF325124) are known to be involved in anthocyanin synthesis (Dubos et al., 2003).
Quattrocchio et al., 2010). Three putative potato MYB genes, StMYB75A, StMYB7B, and StMYB75C, were identified based on homology to AtMYB1 and AtMYB2. StMYB12A and StMYB12B are homologous to SIMYB12 (EU419748) and AtMYB12 (NM_130314), which regulate flavonols in tomato (Solanum lycopersicum) and Arabidopsis (Mehrtens et al., 2005). StAN1 and StAN2 (AY841217 and AY841131) are MYBs implicated in regulating anthocyanin biosynthesis in tuber skin and drought-stressed potatoes (André et al., 2009; Jung et al., 2009).

Numerous sequences were found in the PGSC database with homology to StAN1 and StAN2, but only the two with highest similarity were chosen. StMTF1 (EU310399) and StMTF2 (CV506186) are MYBs shown to regulate potato phenylpropanoids with varying efficacy (Rommens et al., 2008). StMYb73A and StMYb73B were collected by blasting a MYB73 sequence that was upregulated 44-fold in the purple portions of tubers compared with the white portions (Stushnoff et al., 2010). StMYB10 was homologous to NiAn2 (FJ472647) and MdMYB10 (EU518249), which regulate anthocyanin biosynthesis in tobacco floral tissue and apple (Malus domestica), respectively (Espley et al., 2007; Pattanaik et al., 2010).

AtTT8 (AJ277509), AtGL3 (NM_148067.3), JAFL3 (AF020545.1), and NiAN1 (HQ589209) are bHLH genes involved in anthocyanin synthesis (Nesi et al., 2000; Quattrocchio et al., 1998; Feyissa et al., 2009; Bai et al., 2011). After BLASTing these sequences, the two best matches in potato were bHLH1 and bHLH2. AtTTG1 (AJ133743), MtWD40-I (EU040206), and PhAN11 (U94748) are WD40 members that regulate anthocyanin synthesis (Vetten et al., 1997; Walker et al., 1999; Pang et al., 2009) and their sequences were used to identify StWD40.

The protein sequences of these 12 MYBs, two bHLHs, and one WD40 from potato were used to develop a phylogenetic tree (Fig. 2B–D). StAN1 and StAN2 were closely associated with the MYBs of other solanaceous species, SIAN1, NtAN2, and CaMYB, known to regulate anthocyanins (Mathews et al., 2003; Borovsky et al., 2004; Pattanaik et al., 2010). StAN1 was 66 and 58% similar to StAN2 and NtAN2, respectively, and StMTF1 was 89% similar to SIAN1 (Supplementary Table S4A at JXB online). StMYB12A and StMYB12B clustered with AtMYB12 and SIMYB12 (Luo et al., 2008; Ballester et al., 2010). MYB73A and MYB73B formed a unique clade. All the MYBs from solanaceous species had highly conserved R2 and R3 MYB domains (Supplementary Fig. S1A at JXB online). StbHLH1 clustered and shared around 80% similarity with NtAN1a and PhAN1, which regulate anthocyanins in tobacco and petunia (Petunia hybrida) and was only 43% similar to StbHLH2. However, StbHLH2 was 86% similar to PhJAF13 (Supplementary Table S4B). These proteins are conserved in MYB and bHLH domains but are diversified in other regions (Supplementary Fig. S1B).

StWD40 is clustered with NiTTG2, PhAN11, and VvWDR1, and is 97% similar to NiTTG2 and 94 and 88% similar to PhAN11 and VvWDR1, respectively (Supplementary Table S4C), which regulate anthocyanin synthesis in petunia and grapes (Vetten et al., 1997; Matus et al., 2010). These proteins are slightly distinct at the N-terminal but are highly conserved in the middle and C-terminal end (Supplementary Fig. S1C).

Basal phenylpropanoid metabolism

Five genotypes, NY144, Challenger, ORO4198-1, AmaRosa and Magic Molly with white, light yellow, dark yellow, red, and purple flesh, respectively, were selected for analysis (Fig. 3A). These genotypes were chosen because they were expected to have markedly different phenylpropanoid profiles; consequently, a comparative analysis of transcription factor expression would be informative. Prior to transcription factor analysis and to provide a context to interpret the results, phenylpropanoid profiles and structural gene expression were evaluated in each genotype. Tubers were collected from field-grown plants, peeled, and processed within minutes of harvest to avoid potential post-harvest effects. Total phenolic levels ranged from 2.9 to 12 mg g⁻¹ (Fig. 3B) and were 3–4-fold higher in red and purple potatoes than in white. Anthocyanins were detectable only in red and purple cultivars, with higher amounts in the purple (10.4 mg g⁻¹) than

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**Fig. 3.** Characterization of the five genotypes used in this study. (A) Flesh colour of NY144 (a), Challenger (b), ORO4198-1 (c), AmaRosa (d), and Magic Molly (e). (B) Levels of total phenolics, anthocyanins, and carotenoids. Carotenoids are shown on the y-axis on the right. The data represents the means±SEM of three biological replicates. Values with the same letter are not significantly different (P<0.05).
in the red (6.5 mg g⁻¹) genotype. Carotenoids were highest (60.7 μg g⁻¹) in the dark yellow genotype.

Individual phenylpropanoids were measured by HPLC/ESI-MS/DAD (Supplementary Table S1). 5-Chlorogenic acid (5CGA) was the predominant phenylpropanoid in all genotypes, varying from 0.12 to 6.3 mg g⁻¹ (Fig. 4A). Caffeoyl putrescine (CP) amounts were higher in dark yellow, red, and purple genotypes, while that of feruloyl quinic acid (FQA2) was higher in red and purple genotypes (Fig. 4B). Relative amounts of hydroxycinnamic acid amides were measured, and only bis-dihydrocaffeoyl spermine (BDCS), and bis-dihydrocaffeoyl spermidine (BDCSD) were detected in all genotypes (Fig. 4C) and levels of both were least in white potatoes.

Concentrations of shikimate-derived aromatic amino acids were lowest in the light yellow genotype (Fig. 4D). Concentrations of phenylalanine (Phe), the precursor for phenylpropanoid biosynthesis, did not associate with total phenolic or 5CGA concentrations. However, the enzyme activity of PAL, which catalyses the first committed step of phenylpropanoid pathway where cinnamic acid is formed by deamination of Phe, was about 35–45-fold higher in potatoes.

Fig. 4. Individual metabolites measured by LC/MS in the five genotypes. 5CGA and CGA isomers, with 3CGA and C4CGA in the inset (A); caffeoyl and feruloyl derivatives and flavonols (B); polyamines (C); ascorbic acid, aromatic amino acids, and glycoalkaloids (D). (E, F) Individual anthocyanins in red (E) and purple (F) potatoes. The data represents the means±SEM of three biological replicates. Values with the same letter are not significantly different (P<0.05). The key for all panels is shown in (A). Abbreviations are listed in Supplementary Tables S1 and S2.
with higher amounts of phenylpropanoids (Supplementary Fig. S2 at JXB online). Another major source of tuber antioxidant capacity is ascorbic acid, which varied from 1.2 to 2.0 mg g\(^{-1}\) among genotypes (Fig. 4D). Antioxidant capacity was measured by FRAP and was greater in red and purple potatoes (Supplementary Fig. S2).

**Flavonols and anthocyanins**

Among these genotypes, flavonols were observed only in red and purple potatoes (Fig. 4B). Purple potatoes accumulated myricetin-3-O-rutinoside (Myr), quercetin-3-O-rutinoside (Rut), and kaempferol-3-O-rutinoside (Kmp), and the red genotype only accumulated Kmp but in greater amounts than the total flavonols in purple potatoes. Numerous anthocyanins were present in red and purple genotypes, and the more abundant were analysed (Supplementary Table S2). Pelargonidin-3-(coumaroyl)-rutinoside-5 glucoside (PlCRG) was the predominant anthocyanin in the red genotype, contributing to >90% of total anthocyanins (Fig. 4E). In purple potatoes, petunidin-3-(coumaroyl)-rutinoside-5 glucoside (PtCRG) and peonidin-3-(coumaroyl)-rutinoside-5 glucoside (PeCRG) comprised >80% of total anthocyanins (Fig. 4F).

**Phenylpropanoid structural gene expression**

Relative expression of PAL was about 40-fold higher in red and purple potatoes compared with white (Fig. 5A), which is consistent with the amount of phenylpropanoids and PAL enzyme activity in these genotypes (Fig. 4 and Supplementary Fig. S2). The primary pathway to CGA biosynthesis is thought to be through hydroxycinnamoyl-CoA quinate transferase (HQT). Although 5CGA was the most abundant phenylpropanoid, HQT transcript expression did not track with the 5CGA levels (Fig. 5A). Expression of \(\beta\)-coumarate 3-hydroxylase (C3H) and hydroxycinnamoyl transferase (HCT) expression was 2–5-fold higher in red than in other genotypes. The expression of late genes like chalcone synthase (CHS), dihydroflavonol reductase (DFR), and UDP-glucose:flavonoid 3-O-glucosyltransferase (UGFT) were strongly expressed in red and purple genotypes and correlated with anthocyanin levels (Fig. 5B). To our knowledge, no anthocyanin O-methyltransferase (AOMT) gene has been reported in potato or any Solanaceae. By similarity search with grape AOMT (Hugueney et al., 2009), three isoforms were identified and aligned (Supplementary Fig. S1D). AOMT1 and AOMT2 shared high similarity (90%), so only one set of primers was used for both. The expression of AOMT1/2 showed no specific trend, while that of

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**Fig. 5.** Gene expression in the five genotypes. Expression of structural genes from the early (A) and late (B) phenylpropanoid pathway, or genes encoding transcription factors (C). In (C), only AN1 uses the left y-axis, and all others use the right axis. In the inset are expression values of MYB75B, MYB75C, and MYB7512B. The data represent the means±SEM of three biological replicates. Values with the same letter are not significantly different (\(P<0.05\)).
Expression of the 15 potato transcription factors listed in the phylogenetic analysis was studied by qRT-PCR in the flesh of the five genotypes. Expression of *MTF1*, *MTF2*, *MYB10*, *MYB12A*, and *MYB75A* were below quantifiable levels. Primers amplified an appropriately sized fragment from genomic DNA eliminating sequence compatibility as an issue. The expression data for the remaining ten transcription factors is shown in Fig. 5C. *ANI* was by far the most abundantly expressed of those analysed, and was more than 100-fold higher in the flesh of red and purple potatoes. To evaluate whether differences in the AN1 protein might account for the differential phenylpropanoid profiles observed among the five genotypes, full-length coding regions were cloned and sequenced. The sequence was identical among Challenger, AmaRosa, and Purple Majesty, whereas a white and yellow cultivar each had two nucleotide changes resulting in leucine-to-phenylalanine and leucine-to-valine substitutions (Supplementary Fig. S3a at JXB online).

Sequencing of ~1500 bp of the *StANI* promoter region revealed substantial differences. The promoter region was 97% similar between the low-phenylpropanoid white and yellow genotypes, and 93% similar between the purple and red potatoes. However, only 37% similarity occurred between the white/yellow and red/purple genotypes. Interestingly, the white/yellow genotypes had only one sucrose-responsive (SURE) element, whereas the red and purple potatoes had six (Supplementary Fig. S3b). Similarly, no methyl jasmonate-responsive elements were present in the promoter region examined in the white and yellow potatoes, but five were present in red and four in purple potatoes. Despite using various primers, the promoter sequence of Challenger could not be amplified.

The expression of *AN2*, *MYB75B*, and *MYB75C* was much lower and did not associate with total phenylpropanoid concentrations. Excluding *ANI*, the expression levels of *MYB73A* and *MYB73B* were higher than other transcription factors but did not associate with phenylpropanoid concentrations. *MYB12B* was poorly expressed in all genotypes, but was the only gene that showed a clear inverse association with phenylpropanoid amounts. *MYB12B* expression was 2–4-fold higher in white and yellow genotypes compared with that in red and purple potatoes.

The expression of *bHLH1* in tuber flesh was ~10–20-fold lower than that of *ANI* and was detected only in red and purple potatoes (Fig. 5C), whereas *bHLH2* was expressed in all potatoes but did not show any association with phenylpropanoid concentrations. *WD40* expression was 3–5-fold higher in the red and purple potatoes.

*Environmental effects on transcription factor expression*

These results suggested that the differences in the phenylpropanoid concentrations among different cultivars were probably partly determined by *ANI*, *bHLH1*, and *WD40*, i.e. they have a role in determining the inter-genotypical variation. However, phenylpropanoid concentrations can also vary significantly within potatoes of a single genotype. Potatoes from the same cultivar grown under the same management regime can have different concentrations in different years or locations. To determine whether *ANI*, *bHLH1*, and *WD40* were also associated with intra-genotypical variation, purple potatoes grown in four locations in Alaska, plus in Texas and Florida, were examined. The Alaskan potatoes had significantly higher amounts of phenylpropanoids (*Payyavula et al.*, 2012) that correlated with higher expression of *ANI*, *bHLH1*, and *WD40* (Supplementary Fig. S4 at JXB online). *ANI* expression was strongest in tuber samples from Wiseman, the northern-most site located in the Arctic Circle, and least in the southern-most locations. Similar patterns were observed for *bHLH1* and *WD40.*

*Evaluation of the role of sugars in phenylpropanoid metabolism*

Sugars were measured in the five genotypes (Fig. 6). The red and purple genotypes accumulated up to 30 and 60% higher sucrose and glucose levels, respectively, which is consistent with a role for sugars in modulating tuber phenylpropanoid content (*Zucker and Levy, 1959; Leggewie et al., 2003*). On the other hand, there were differences among phenylpropanoids in white and yellow potatoes (Fig. 4A), which did not correlate with sugar content. For example, the dark yellow genotype had higher phenylpropanoid concentrations than the white genotype but equivalent amounts of sucrose and lower glucose.

Preliminary sugar feeding studies showed increases in phenylpropanoids in both white and purple potatoes.
Subsequently, the cultivar Purple Majesty was selected for in-depth analysis, and the effect of sucrose on metabolites and gene expression was studied in plantlets cultured on 0 and 120 mM sucrose (Fig. 7). Total phenolics increased by 40% reaching 14 mg g⁻¹ in 120 mM sucrose-treated plantlets (Fig. 7A). Sucrose induced an almost 5-fold increase in total chlorogenic acid content. Numerous other phenylpropanoids including multiple flavonols showed a strong induction by sucrose, with the most abundant flavanol, Kmp, induced almost 15-fold (Fig. 7A–C). Likewise, the anthocyanin branch of the pathway was strongly induced, with total anthocyanins increasing by ~85% (Fig. 7A). PtCRG was the predominant anthocyanin and increased by 2.5-fold with sucrose feeding (Fig. 7A). Among the less-abundant anthocyanins, some decreased in response to sucrose, such as petunidin 3-rutinoside-5-glucoside (PtRG), which may reflect competition with the more abundant anthocyanins for common precursors. Unlike a majority of the phenylpropanoids examined, the three shikimate-derived aromatic amino acids decreased in sucrose-fed plantlets (Fig. 7D).

Expression of the structural genes PAL, CHS, F3H, DFR, UFGT, AOMT3, and transcription factors AN1 and bHLH1 was more than 3-fold higher in plantlets fed 120 mM sucrose, but WD40 and AN2 showed only a slight increase (Fig. 7F, G). Genes encoding isoforms of the sucrose-hydrolysing enzymes sucrose synthase (SUSY) and invertase (INV) were also studied. The expression of SUSY1 was several fold higher than SUSY4. Both were induced by sucrose, and SUSY4 underwent a >100-fold increase in expression after sucrose feeding (Fig. 7F). Expression of INV1 was greater than INV2, but did not increase in response to sucrose, whereas INV2 expression increased.

Fig. 7. Metabolite and gene expression changes associated with sucrose feeding. (A–E) Amounts of total phenolics, total chlorogenic acids (5CGA plus isomers), and total anthocyanins (A), caffeoyl and feruloyl derivatives (B), individual flavonols (C), aromatic amino acids (D), and anthocyanins (E). PtCRG is shown on the right y-axis, and all others on the left. (F, G) Expression of phenylpropanoid and sugar-related genes, with expression of INV2 shown in the inset (F), and transcription factors in potato plantlets (G) cultured on 0 mM (open bars) and 120 mM (dotted bars) sucrose. Data represents the means±SEM of three biological replicates. Asterisks indicate treatments that are significantly different (P<0.05).
Transient leaf infiltration assays

The functionality of the selected potato transcription factors was tested using tobacco leaf infiltration. Leaves infiltrated with empty vector, bHLH, WD40, or combined bHLH plus WD40 did not show pigmentation (Fig. 8A). Anthocyanin accumulation was time dependent and all leaves infiltrated with any combination of AN1 showed pigmentation by 96 h post-infiltration (Fig. 8A). However, at 48 h, visible purple pigmentation was only observed on leaves co-infiltrated with AN1+bHLH (Supplementary Fig. S5 at JXB online). This accelerated anthocyanin induction was observed across multiple independent experiments. After 72 h, leaves infiltrated with AN1 alone or AN1+WD40 constructs also accumulated purple pigmentation, but were less intense than leaves infiltrated with AN1+bHLH or AN1+bHLH+WD40.

Phenylpropanoid profiles in leaves infiltrated with bHLH1, WD40, or bHLH1+WD40 were similar to those infiltrated with empty vector, but AN1 or any of its combinations induced 2–4-fold higher levels of total phenolics and CGA (Fig. 8B). Interestingly, concentrations of the three aromatic amino acids increased with AN1 infiltration or its combinations (Fig. 8C). Rhamnetin rutinoside (RR) accumulated in infiltrated leaves, but with empty vector, but the major flavonol, increased ~30-fold (Fig. 8D).

Cyanidin 3-rutinoside (CR) was by far the most abundant anthocyanin induced in N. tabacum (Fig. 8E and Supplementary Fig. S6A, E at JXB online). In contrast, when N. benthamiana leaves were infiltrated with S1AN1, the major anthocyanin was delphinidin 3-rutinoside (DR; Supplementary Fig. S6B, F). Purple and blue potatoes contain petunidin and malvidin derivatives, which are methylated products of delphinidin (Fig. 4F; Hillebrand et al., 2009). A possible reason for not forming downstream products of delphinidin in tobacco could be due to a lack of AOMT activity. To address this question and address the functionality of the potato gene, N. benthamiana leaves were infiltrated with potato AOMT3 alone or in combination with AN1. Anthocyanins were not observed in leaves infiltrated with AOMT3 alone (Supplementary Fig. S6C), but when infiltrated with AOMT3 and AN1, the amounts of DR were greatly reduced, and two major peaks appeared of m/z 625 and 639 (M+H)+ that were identified as the methylated anthocyanins petunidin 3-rutinoside (PR) and malvidin 3-rutinoside (MR) (Supplementary Fig. S6D, G, H).

From the above data, it was clear that the metabolite changes were predominantly due to AN1 infiltration, so subsequent experiments did not use combinatorial infiltrations. Changes in sucrose and glucose levels, PAL activity, and the expression of several key phenylpropanoid genes, transcription factors, and sugar metabolism genes were estimated in leaves infiltrated with empty vector, bHLH1, WD40, or AN1 (Fig. 8F–H and Supplementary Fig. S7 at JXB online). In tobacco, four PAL isoforms have been reported, of which PAL1 and PAL4, and PAL2 and PAL3 have high similarity (Reichert et al., 2009). Therefore, PAL1 and PAL4 were amplified together with one only primer set, as were PAL2 and PAL3. Expression of both PAL1/4 and PAL2/3 increased, along with the late pathway genes CHS, DFR, and DFR in AN1-infiltrated samples (Fig. 8G). PAL enzyme activity increased more than 25-fold in AN1-infiltrated samples (Supplementary Fig. S7). Notably, the expression of NbHLH1 was increased in AN1-infiltrated samples. In leaves infiltrated with StbHLH1, the expression of bHLH1 increased, probably because NbHLH primers amplified StbHLH1, which has a high sequence similarity.

Strikingly, infiltration with AN1 induced a marked change in sugar metabolism, as seen by the stimulation of SUSY1, SUSY4, and INVI expression (Fig. 8H) and the sizeable decrease in sucrose and glucose concentrations (Fig. 8F).

Promoter elements

The regulatory elements in the promoters of selected potato genes were predicted in the 1500 bp sequence upstream of ATG using the PLACE database (Higo et al., 1999). We were not able to retrieve promoter sequences for UFGT and AOMT3. The interaction of transcription factors and sugars was of particular interest; therefore, MYB, bHLH, and SURE elements were examined. The occurrence of the three M/YB recognition sites, MYBCORE (CNGTTR; Solanol et al., 1995), MYBPLANT (MACCWAMC; Sablowski et al., 1994), and MYBPZM (CCWACC; Grotewold et al., 1994), one bHLH recognition site (G-box) MYCONSENSUS (CANNTG; Blackwell and Weintraub, 1990; Hartmann et al., 2005); and one SURE (AATAGAAAA; Grierson et al., 1994) for different gene promoters is shown in Supplementary Table S5 at JXB online. MYBCORE was present in all the promoters except INVI and was over-represented in WD40 and SUSY1. MYCONSENSUS was the most abundantly represented element and was present multiple times (two to nine) in many of the promoters except HQT. SURE was present only in AN1, WD40, SUSY1, HCT; and HQT (Supplementary Table S5).

Discussion

Relatively few studies have focused on the regulation of phenylpropanoid metabolism in a tuber crop. To identify candidate transcription factors that regulate the phenylpropanoid pathway, metabolite and gene expression profiles were determined in tubers of five genotypes with decidedly different phenylpropanoid profiles (Fig. 3B). Field-grown tubers were used so results would relate to the crop and avoid the potentially non-representative results noted previously in studies using greenhouse-grown potatoes (Chawla et al., 2012; Navarre et al., 2013). Peeled samples were used to more clearly differentiate the samples by ensuring that all samples represented distinct flesh colours and not a mix such as red skin and white flesh. Moreover, phenylpropanoid metabolism differs between tuber skin and flesh, and less is known about the flesh (Jung et al., 2005) than periderm, in which genetic analysis has revealed three major loci controlling pigmentation, D, R, and P (De Jong et al., 2004).
AN1, bHLH1, and WD40 regulate tuber anthocyanins and other phenylpropanoids

Red and purple potatoes had higher expression of phenylpropanoid structural genes and contained higher amounts of phenylpropanoids, not just anthocyanins but colourless compounds such as 5CGA (Figs 3B and 4A, B). Compared with concentrations reported previously in transgenic tubers overexpressing the R2R3 MYB StMTFI (Rommens et al., 2008), the wild-type red and purple potatoes in this study contained...
higher levels of phenylpropanoids, including almost 4-fold and 2-fold higher amounts of 5CGA and Kmp, respectively. Analysis of the potential involvement of 15 transcription factors was measured by qRT-PCR. ANI was the most abundantly expressed transcription factor and was >100-fold higher in red and purple potatoes than in white. Besides ANI, only two of the other examined transcription factors, bHLH1 and WD40, showed a positive correlation with phenylpropanoid concentrations (Supplementary Fig. S8 at JXB online). Over 30 phenylpropanoid-related compounds were measured, of which most were only minor components of total soluble tuber phenylpropanoids in red and purple potatoes (Fig. 4). Notably 5CGA and a single anthocyanin accounted for 80-90% of the total. Amounts of CGA and total phenylpropanoids strongly correlated with ANI expression in genotypes with different phenylpropanoid profiles, and in response to environmental signals, sucrose feeding, and ANI infiltration. These results suggested that ANI can mediate marked changes in phenylpropanoids outside the anthocyanin pathway, as chlorogenic acid is synthesized by a different branch of the pathway from anthocyanins. Consistent with this finding is the fact that 5CGA is found in much higher amounts in red and purple potatoes than in white or yellow (André et al., 2007b; Navarre et al., 2011).

To our knowledge, this is the first report of a potato WD40 whose expression correlates with phenolics and anthocyanins (Supplementary Fig. S8). WD40 was not able to induce phenylpropanoid expression on its own in infiltration studies (Fig. 8A), consistent with previous results where overexpression of WD40 from Medicago truncatula (MtWD40) in hairy roots failed to induce flavonols, proanthocyanidins, or anthocyanins (Pang et al., 2009). In contrast, overexpression of MtWD40 was able to complement the lack of red-pigmented phenotype of a mutant line NF0977, which suggests that WD40 is required but not sufficient for anthocyanin pathway activation (Pang et al., 2009). However, ectopic expression of a grape WD40 induced anthocyanins in Arabidopsis leaves (Matus et al., 2010). These results illustrate the complex interactions among the transcription factors.

bHLH1 but not bHLH2 showed a strong association with phenylpropanoid expression. A quantitative trait locus study linked a potato bHLH to anthocyanin synthesis and reported that it was expressed in all the coloured genotypes and in 21 of 53 white or yellow genotypes, suggesting that it is required but not sufficient for anthocyanin synthesis (Zhang et al., 2009b). While we were unable to determine if these bHLHs are the same because sequence information was not available for the previous bHLH, both are localized on chromosome 9. The expression patterns of ANI, bHLH1, and WD40 suggest that they are determinants of the amounts of phenylpropanoids that a given genotype will contain. ANI, bHLH1, and WD40 were also implicated in the intra-genotypical variation that occurs in potatoes in response to environmental variations, such as when the same purple genotype was grown in Alaska, Texas, and Florida (Supplementary Fig. S4). The higher expression in the Alaskan-grown potatoes might be due to the lower temperatures or longer day length, and potentially reflects greater stress.

Unlike a previous microarray study that reported an ~40-fold increase in MYB73 in the purple flesh of a sectored potato that had both white and purple regions (Stushnoff et al., 2010), elevated expression of MYB73 was not observed in the red or purple genotypes relative to the white or yellow potatoes (Fig. 5C). MYB12, which regulates the flavonol branch of the pathway in Arabidopsis (Mehrtens et al., 2005), appeared to be negatively associated with tuber phenylpropanoid content. The minimal repression domain (TLLLFR) present at the C terminus of AtMYB12, a negative regulator of anthocyanin biosynthesis in Arabidopsis (Matsui et al., 2008), was not found in StMYB12B or in other MYB transcription factors in this study. Nor was the ERF-associated amphipathic repression (EAR) motif, associated with repression of anthocyanin gene transcription, present in any of these potato MYBs (Ohta et al., 2001; Lin-Wang et al., 2011). Expression of other transcription factors varied among the genotypes but did not seem to be associated with phenylpropanoid content. Among the potato MYBs examined, StMTF1 has the highest percentage similarity with AtPAP1 (Supplementary Table S4); however, the StMTF1 transcript was not present at detectable levels in any of the genotypes examined, suggesting that the native gene does not have a major role in tuber phenylpropanoid metabolism. A previous study showing a stimulatory effect of StMTF1 (Rommens et al., 2008) may be due to its being expressed under a heterologous promoter. Potentially StMTF1 under its own promoter is active in other tissues or conditions.

Importantly, the stimulatory effects of ANI on phenylpropanoid metabolism were not limited to anthocyanins because other compounds were also upregulated, notably 5CGA, the predominant soluble phenylpropanoid in potatoes (Fig. 8B). The increase in expression of CHS and F3H (Fig. 8G) along with a 30-fold increase in Rut (Fig. 8D) suggested that StANI upregulates 3'-hydroxylated flavonones. Collectively, the relationship between StANI expression and high pelargonidin and petunidin concentrations in coloured potatoes and the induction of cyanidin or delphinidin derivatives in StANI-infiltrated N. tabacum or N. benthamiana leaves suggest that StANI regulates flavanone 3-hydroxylase (F3H), flavonoid 3-hydroxylase (F3'-H), and flavonoid 3',5'-hydroxylase (F3'5'-H).

Differences were seen in aromatic amino acid pools among the samples. No obvious relationship was observed between these amino acids and phenylpropanoids in the field-grown tubers, whereas sucrose feeding lowered aromatic amino acid concentrations but increased phenylpropanoids, ANI, bHLH1, and WD40 expression. However, ANI infiltration decreased sucrose and glucose but increased the amounts of aromatic amino acids. The increase was particularly clear with tyrosine and not quite so dramatic with phenylalanine and tryptophan. Thus, sucrose feeding and ANI infiltration both increased phenylpropanoids, whereas sucrose treatments decreased aromatic amino acid amounts but ANI increased them. Understanding the basis for these differences will require further study.
To our knowledge, a gene for AOMT has not been reported previously in potatoes or other solanaceous plants. AOMT3 but not AOMT1/2 showed a strong correlation with anthocyanins (Supplementary Fig. S8) and altered anthocyanin profiles when infiltrated into leaves. AOMT3 probably plays a key role in determining the type of anthocyanins that accumulate in potatoes, and its identification offers another potential target for efforts to manipulate tuber anthocyanin composition. The failure of *N. benthamiana* leaves to synthesize methylated anthocyanins when infiltrated with *St*AN suggests that either AN1 does not regulate AOMT or that tobacco leaves lack a functional AOMT. BLAST searches for an *AOMT3* homologue in tobacco did not identify any candidates.

**Sucrose induces AN1 and phenylpropanoid biosynthesis**

In addition to providing carbon for phenylpropanoid metabolism, sugars also regulate anthocyanin biosynthesis (Teng et al., 2005). During grape berry development, the increase in sugars modulates expression of anthocyanin biosynthetic genes (Boss et al., 1996). High-phenylpropanoid red and purple potatoes had substantially higher amounts of sucrose and glucose than white or yellow tubers (Fig. 6). Similar relationships were seen in a tuber developmental study in which sugar and phenylpropanoid concentrations were correlated (Navarre et al., 2013). To gain direct evidence, potato plantlets were cultured on medium supplemented with 0 or 120 mM sucrose, resulting in large increases in phenylpropanoids (Fig. 7A). Sucrose stimulated significant increases in the expression of *AN1*, *bHLH1*, and *WD40*, demonstrating that the stimulatory effect of sucrose on potato phenylpropanoid metabolism is at least partly modulated through these transcription factors. Supporting this finding is the higher expression of *AN1*, *bHLH1*, and *WD40* in the field-grown potatoes that had highest sucrose and phenylpropanoid concentrations.

The presence of SURE elements in the promoter of *AN1* (Supplementary Fig. S3b) is consistent with its regulation by sucrose. In addition to greater amounts of sucrose, purple and red potatoes contained six SURE elements in the *AN1* promoter, whereas white and yellow potatoes had a single SURE element. A mechanism for the 5-fold increase in CGA (Fig. 7A) in response to sucrose is suggested by the *MYB*, *bHLH*, and SURE elements present in the promoter of *HQT*. SURE elements were not observed in the *bHLH1* promoter, but *bHLH1* expression was upregulated by sucrose treatment. A possible explanation is that the potato *AN1* induced expression of tobacco *bHLH* in transient assays (Fig. 8G), which is consistent with a previous report of tobacco transcription factors (Bai et al., 2011). *AN1*-infiltrated leaves showed a 24 h delay in anthocyanin formation compared with *AN1* + *bHLH1* co-infiltrated leaves (Supplementary Fig. S5), and the delay may reflect the time needed for *AN1* to recruit *bHLH*. The promoters of potato *PAL*, *CHS*, *F3H*, and *DFR* did not have SURE elements, but had MYB and bHLH regulatory elements (Supplementary Table S5), suggesting that the increased expression after sucrose feeding was due to *AN1* and *bHLH1*. Anthocyanin levels in potato correlated with the expression of *AN5* and *UGFT* (Hu et al., 2011; Keifenheim et al., 2006) and higher expression of these genes was observed in this study in the high-phenylpropanoid potatoes and sucrose-treated potato plantlets (Figs 5B and 7F).

Interestingly, sucrose treatments of potato plantlets induced large increases in *AN1* and *bHLH1* expression, and to a lesser extent that of *WD40* (Fig. 7G), whereas infiltration of potato *AN1* into tobacco leaves increased expression of *SUSY* and *INV* genes (Fig. 8H). This suggests the possibility of a regulatory loop in which sucrose increases *AN1* expression but *AN1* decreases sucrose concentrations by inducing sucrolytic enzymes that liberate hexoses that are channelled to the phenylpropanoid pathway (Fig. 9). In contrast, *bHLH1* and *WD40* infiltration alone did not increase expression of *SUSY* or *INV* or mobilize sucrose. The extent that sucrose–AN1 interactions modulate tuber phenylpropanoid metabolism is an interesting question that awaits future research.

In addition to their important *in planta* roles, phenylpropanoids are desirable in the diet because of their health-promoting properties, which include antioxidant, anti-inflammatory, hypotensive, and chemopreventative effects (Manach et al., 2004; Prior, 2003; Kaspar et al., 2011; Vinson et al., 2012). High-phenylpropanoid potatoes would be especially valuable because of the high consumption of this staple food. The potential of potatoes to provide dietary phenylpropanoids, including anthocyanins, is significant. For example, the purple potatoes in this study contained anthocyanin amounts that approach those of high-anthocyanin transgenic tomatoes (Butelli et al., 2008), and exceed amounts in many fruits.
and vegetables (see Table 2 in Wu et al., 2006). Collectively, these data suggest that interactions among sucrose, sucrolytic enzymes, and AN1 modulate the pathway, and that AN1 is a key regulator of the most abundant tuber phenylpropanoids. Increased understanding of tuber phenylpropanoid metabolism will facilitate efforts to develop potatoes with optimal types and concentrations of phenylpropanoids.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Protein sequence alignment of representative (A) MYBs, (B) bHLH, (C) WD40, and (D) AOMT from potato and from other species.

Supplementary Fig. S2. PAL enzyme activity and FRAP antioxidant activity in the five genotypes.

Supplementary Fig. S3. (A) StAN1 protein sequence of the five potato genotypes used in this study. (B) StAN1 promoter region from four genotypes used in this study.

Supplementary Fig. S4. Expression of selected transcription factors in purple potatoes grown in Alaska (Wiseman, Fairbanks, Palmer and Juneau), Texas and Florida.

Supplementary Fig. S5. Tobacco leaves 48 and 72h after infiltrating with a binary construct harbouring AN1 (A) alone or AN1 + bHLH1 (AB).

Supplementary Fig. S6. MS extracted ion data of the most abundant anthocyanins formed in (A) N. tabacum (Samsun) infiltrated with AN1. (B) N. benthamiana leaves infiltrated with AN1, (C) AOMT3, and (D) AN1 + AOMT3.

Supplementary Fig. S7. PAL activity in tobacco leaves infiltrated with (E) empty vector, (B) bHLH1, (W) WD40, or (A) AN1.

Supplementary Fig. S8. Correlation analysis of transcript and metabolite expression in tubers from five potato genotypes.

Supplementary Table S1. Retention times and MS data of select compounds present in potato phenolic extracts separated by HPLC.

Supplementary Table S2. Retention times and MS data of anthocyanins in potato extracts separated by HPLC.

Supplementary Table S3. List of primers used in this study.

Supplementary Table S4. Protein similarity matrix of transcription factors from different species.

Supplementary Table S5. List of regulatory elements in promoters of different genes from S. tuberosum group Phureja.

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