WHAT MAKES A RED POTATO RED: IDENTIFYING AMINO ACIDS THAT INFLUENCE SUBSTRATE SPECIFICITY OF POTATO DFR

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfilment of the Requirements for the Degree of

Master of Science

by

TEDDY SAMUEL YESUDASAN

MAY 2019

ABSTRACT

What Makes a Red Potato Red: Identifying Amino Acids that Influence Substrate Specificity of Potato DFR

Teddy Samuel Yesudasan, Cornell University, School of Integrative Plant Science, Section of Plant Breeding and Genetics

Pigmented potatoes (*Solanum tuberosum* L.) are a rich source of anthocyanin pigments. Dihydroflavonol 4-reductase (DFR) is a rate limiting enzyme in the flavonoid pathway that gives rise to the anthocyanin pigments pelargonidin (red), cyanidin (pink) and delphinidin (purple) by reducing dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. It has previously been shown that *dfr* corresponds to the *R* locus in potato, where one specific allele is able to direct synthesis of red pigments and other alleles are unable to do so. To test whether either of two amino acids (alanine at position 143, cysteine at position 154) in a putative substrate binding region determines ability to produce red anthocyanins, six chimeric constructs of *dfr* were made by reciprocal exchanges between red and non-red alleles. These constructs, as well as wild type red and non-red alleles, were introduced into the potato cultivar Prince Hairy (genotype *dddd rrrr P-*), which has pale blue flowers. The flowers of engineered lines were monitored for shifts to purple coloration and anthocyanins were analyzed using UHPLC/MS. Although individual amino acid changes altered both flower color and anthocyanin profiles, possessing alanine at position 143 and cysteine at 154 gave the largest effect. Editing both of these positions may be sufficient to convert white potatoes to red potatoes, and vice-versa.

BIOGRAPHICAL SKETCH

Teddy Yesudasan was born and raised in Coimbatore, India. Being an ardent fan of any outdoor activity, he initially envisioned a career in cricket. Due to certain circumstances and thanks to his high school science teacher at Lisieux, Prathiba, life took a turn for the good. He graduated from Tamil Nadu Agricultural University (TNAU) with a Bachelor in Bioinformatics (an Indian who's not an engineer or a doctor, imagine that!). His inspiration to further pursue graduate work was Dr. Raveendran, his mentor at TNAU. He was a recipient of the prestigious Ratan Tata scholarship under a TNAU-Cornell partnership for a Master of Science degree in Plant Breeding & Genetics. Thanks to his mentor at Cornell, Dr. De Jong, he was able to continue to pursue his studies and complete his Master of Science in Plant Breeding & Genetics.

என் அன்புள்ள அம்மா மற்றும் அப்பாவுக்காக

The Yesudasans & The Millers

ACKNOWLEDGEMENTS

Honest confession: the following is a result of blood, sweat and tears; literally and figuratively.

"Let us now praise men of renown… (and women, might I add)." ~ Sirach 44:1

First and foremost, I would like to thank God for this opportunity for it was here in Ithaca that I was re-introduced to Christ.

I would like to thank my advisor Walter Sytze De Jong for putting up with my shenanigans and for giving me the opportunity at a second shot with regards to getting my Master of Science degree. I'm incredibly grateful to him for mentoring me and for giving me many a life lesson. From our common distaste for bureaucracy to lessons from The Gospels to potato dihydroflavonol, I have enjoyed each and every conversation with him. It has been an interesting journey. Thank you, Walter!

I would like to thank Shuping; whose guidance, patience and help with experiments helped me immensely. 非常非常感谢你!

I would like to thank my committee members, Dr. Margaret Smith and Dr. Lailiang Cheng for their timely encouragement and support

I would like to thank The Millers, "my-home-away-from-home-peeps". Their Christ-like love, constant encouragement, support and help throughout the different stages of my life in the past six years is the reason I still breathe.

I would like to thank my parents, The Yesudasans, for being the best non-traditional outlier-ish Indian parents I could ever have.

I'm incredibly grateful to Emily, my fiancée, for putting first things first and helping me pursue this degree as first priority through her constant encouragement and support.

I'd like to thank my friends the Gendrues, the Yus, the Kuhns, the Holdas, Mr. Wright, Ko-Ying, Cindy & Bo, Ellie, Melanie, Joe, Caroline, and Rosalie, for their support.

I'd like to thank the Cornell undergraduate biology department for instilling in me a love for teaching through their teaching assistantship.

Last but not the least, a special mention goes out to the Cornell-TNAU-TATA fellowship without which I wouldn't have been able to cross the ocean in the first place.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Role of Potato

Within the grand scheme of history, the potato is a relatively new crop in the Old World as it was brought from South America to Europe in the 16th century (Salaman, 1949). Potatoes were first cultivated in the Andes several thousand years ago, near Lake Titicaca, which is located on the present-day border of Bolivia and Peru (Morales Garzon, 2007). Followed by rice and wheat, potato ranks third in world food crop consumption and fourth in food production (FAOSTAT, 2013). Potato yields many calories per acre of land and contains important vitamins and nutrients so that, from a nutritional standpoint, potatoes are superior to cereal crops when consumed as the sole component of a diet (Davidson, 1975). A recent comprehensive study on the impact of agricultural productivity on population growth and economic development concluded that the introduction and adoption of potatoes in the Old World was responsible for approximately 25% of the growth in population and urbanization between the 18th and 19th century (Nunn & Qian, 2011).

Cultivated varieties of potatoes that give rise to red or purple flesh are a rarity beyond the center of origin in the Andes (Brown et al., 2005). In recent years, potato varieties with pigmented flesh have garnered much interest (Manach et al., 2004; Tajner-Czopek et al., 2012; Perla et al., 2012), mainly because of the high anthocyanin content of the tubers. As a result, potato breeders have started developing pigmented varieties. The antioxidant activity of red or purple fleshed potatoes is akin to spinach or brussels sprouts (Brown 2005). Brown (2005) reported that, on average, purple and red cultivars contain 0.009 to 0.038 g of total anthocyanins per 100 g fresh

weight. Pigments from colored potatoes can potentially be used as natural dyes (Wrolstad et al. 2001). Since potatoes are a low-cost crop, they may be a better source for natural dyes than other conventional sources. There is also potential for the use of colored potato flour for innovative snacks (Nems et al., 2015; Nayak et al., 2011).

Anthocyanins

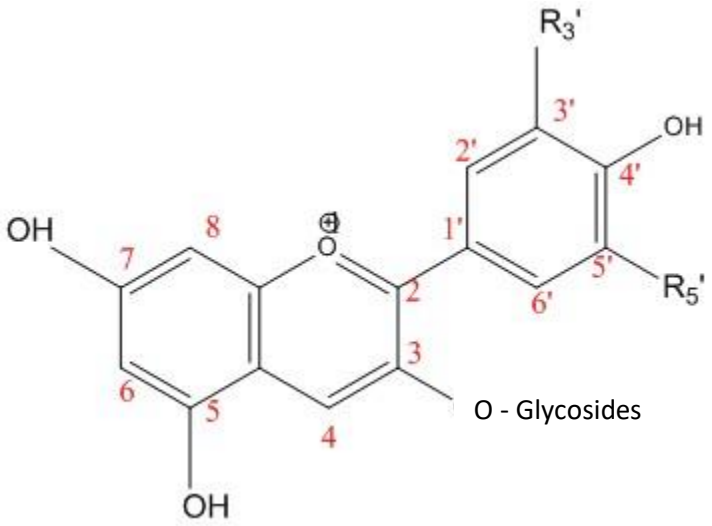
Anthocyanins (from Greek, *Anthos* meaning flower and *kyanos* meaning blue) are a group of water-soluble phenolic compounds, collectively called flavonoids, responsible for the attractive colors of many flowers, fruits and vegetables (Kong et al., 2003). Anthocyanins act as an electron sink aiding in antioxidant activity, protect plant tissues from adverse effects of excess light and ultraviolet radiation, act as pollinator attractants, and help signal fruit ripening (Chalker-Scott, 1999). Anthocyanins also play an indirect role in plant growth and development encompassing response to stress and gene expression affected by modulating reactive oxygen signaling (Lev-Yadun & Gould, 2008). In addition to being beneficial to plants themselves, anthocyanins may be advantageous to animals and humans (Sandoval-Ramírez et al., 2018). The benefits of the consumption of anthocyanin-rich foods are still poorly understood (Daneshzad et al., 2018; Khoo et al., 2017; Pojer et al., 2013).

Anthocyanidins are aglycones of anthocyanins. The six commonly occurring anthocyanidins are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Fig. 1). In addition to the aglycone, the basic anthocyanin structure is that of a flavylium ion which has methoxy and hydroxyl attachments based on the type of the pigment.

There are two groups of genes involved in the synthesis of anthocyanins: structural genes that encode anthocyanin biosynthetic enzymes and regulatory genes responsible for the expression

of structural genes (Jaakola et al., 2002). At least nine structural genes have been elucidated (Fig. 2) (Holton and Cornish 1995). This dissertation focuses on the structural gene, *dfr*, that codes for dihydroflavonol reductase in potato.

a



b

Anthocyanidin	R3'	R5'
Pelargonidin	H	H
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H

Figure 1. Anthocyanin structure

a. Basic anthocyanin structure, b. Table showing chemical groups at 3' and 5' positions of various anthocyanidins

Dihydroflavonol 4-reductase (DFR) and substrate specificity

DFR is a rate-limiting biosynthetic enzyme in the anthocyanin pathway and catalyzes an essential reaction that reduces colorless dihydroflavonols dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyricetin (DHM) to leucoanthocyanidins, eventually leading to the production of anthocyanidin pigments pelargonidin (red), cyanidin (pink) and delphinidin (purple), respectively (Holton and Cornish 1995). The three substrates (DHK, DHQ, and DHM) are similar in structure: the only difference between them is the number of hydroxyl groups on the B phenyl ring (Fig. 2). Two other important enzymes in anthocyanidin synthesis are flavonoid 3'hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) that convert DHK to DHQ and DHM, respectively (Brugliera et al., 1999; Holton et al., 1993). The ability of DFR to reduce the substrates DHK, DHQ and DHM varies across species; in some species, DFR can efficiently metabolize all three, in others, only a subset (Heller et al., 1985; Meyer et al., 1987; Stich et al., 1992; Tanaka et al., 1995; Fischer et al., 2003; Obsuwan et al., 2007). In *Matthiola incana,* DFR is capable of reducing DHK (Heller et al., 1985). DFR reduces DHQ and DHK to cyanidin and pelargonidin, respectively, in both *Zea mays L.* and *Antirrhinum majus*. Obsuwan et al. (2007) studied orchid DFR and concluded that it does not show substrate specificity: it can efficiently reduce DHK, DHQ and DHM.

In *Petunia* (Forkmann & Ruhnau, 1987) and *Cymbidium* (Johnson et al., 1999), DFR can efficiently reduce DHQ and DHM, but not DHK, while in *Gerbera,* DFR is able to efficiently reduce all three substrates (Johnson et al., 2001). Johnson et al. (2001) demonstrated that substrate specificity of DFR could be altered by changing a single amino acid in *Gerbera* DFR. When this

mutant was introduced into a *Petunia* line that lacked DFR activity, the mutant DFR preferentially used DHK as a substrate *en route* to the production of pelargonidin.

Competition between enzymes may also influence color. Forkmann et al. (1987) demonstrated in *Petunia hybrida* that enhanced expression of flavonol synthase (FNS) resulted in DHK being converted into kaempferol, instead of leucopelargonidin, suggesting that FNS can compete with DFR to mask DFR activity. Similarly, Obsuwan et al. (2007) hypothesized that mutations in F3'H, which converts DHK to DHQ, can increase the amount of DHK available to DFR, leading to the production of more pelargonidin and less cyanidin.

The red (*R*) locus in potato is required for the production of red anthocyanins in any tissue of the potato plant (Salaman, 1910). It has been shown that *R* codes for DFR (*dfr)*, where a specific dominant allele enables the production of red pelargonidin pigment (Zhang et al., 2009). While this allele of *dfr* is capable of reducing DHK, other potato *dfr* alleles cannot do so (De Jong et al., 2003).

The goal of this study was to examine the basis of *dfr* substrate specificity in potato by identifying the amino acids responsible for the ability to utilize DHK. De Jong et al. (2003) compared *dfr* alleles from three different cultivars, namely W5281.2, Kennebec, and Jashim and found that the protein sequences differ at a relatively small number of positions. The W5281.2 protein, which is capable of utilizing DHK, differed at ten amino acid positions when compared to both Kennebec and Jashim. Two of the ten polymorphic amino acids in W5281.2 map to a 26 amino acid region known to affect substrate specificity in *Gerbera* (Johnson et al. 2001). Prior transgenic experiments in *Petunia* (De Jong and Cheng, unpublished) demonstrated that the 'non-red' allele of *dfr* in Kennebec is functional, able to convert white petunia flowers to pink. In this

dissertation, chimeric versions of the 'red' allele of *dfr* from W5281.2 and the 'non-red' allele from Kennebec were created to identify the nucleotide/amino acid positions that influence substrate specificity of potato DFR.

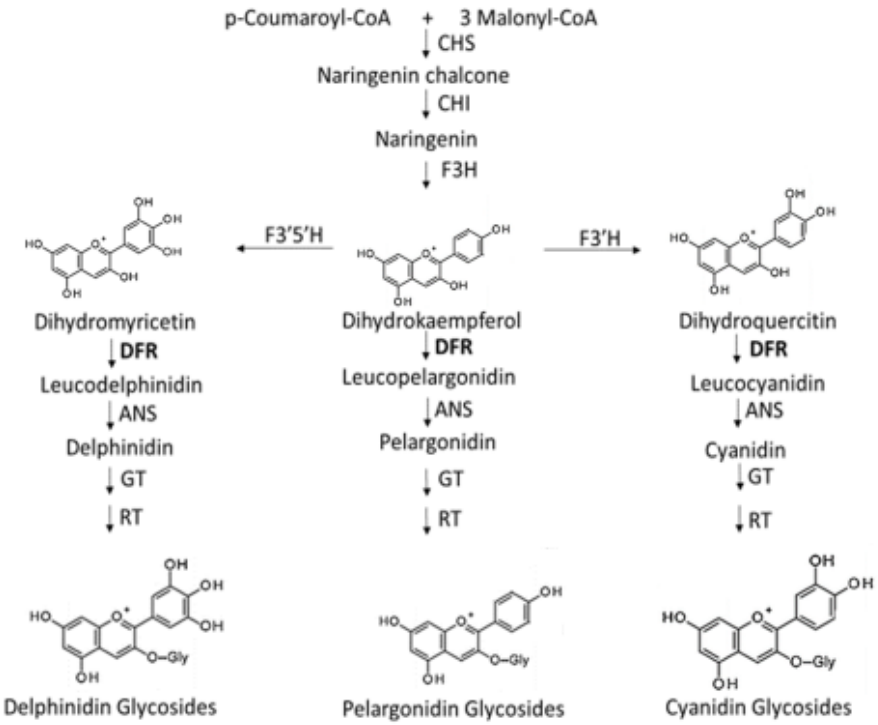


Figure 2. Anthocyanin biosynthesis pathway adapted from Holton and Cornish 1995
Enzyme abbreviations: CHS chalcone synthase, CHI chalcone isomerase, F3H flavanone 3-hydroxylase, F3'H flavonoid 3'-hydroxylase, F3'5'H flavonoid 3',5'-hydroxylase, DFR dihydroflavonol 4-reductase, ANS anthocyanidin synthase, GT UDP- glucose anthocyanidin 3-O-glucosyltransferase, RT UDP-rhamnose anthocyanidin-3-glucoside rhamnosyltransferase

CHAPTER II

Materials and Methods

Plant material

The potato used in this study was the tetraploid cultivar *Solanum tuberosum* L., cv. 'Prince Hairy', also known as NYL235-4 (Plaisted et al., 1992). Prince Hairy is homozygous recessive (*rrrr*) at the red locus (De Jong et al., 2003).

Constructs

To determine which region(s) of potato *dfr* are responsible for the production of red pelargonidin-based pigments, we created chimeric alleles, combining portions of an allele from W5281.2 (red allele; R), which can produce red pigment, with portions of an allele from 'Kennebec' (non-red allele; W), which cannot. The native R allele has previously been cloned into vectors pIBT210.1 (Haq et al. 1995) and pPS1 (Zhang et al., 2009). The W allele was cloned into the same vectors using the same procedure described by Zhang el al. (2009). Because there were no convenient restriction sites to make the desired exchanges, synthetic fragments of *dfr* with the desired sequence were purchased from Integrated DNA Technologies, Inc (Coralville, Iowa). Each synthetic fragment contained one or two nucleotide changes (see Figure 3 and Table 1) as well as *PsiI* and *BstXI* restriction sites, which were used to replace *PsiI-BstX1* fragments of wild type *dfr* alleles harbored in pIBT210.1. Chimeric *dfr* alleles with desired sequence were then transferred from pIBT210.1 as *XhoI-KpnI* fragments into the *XhoI* and *KpnI* sites of the binary vector pPS1 (Huang and Mason 2004). The pPS1 vector (12544 bp) contains an *nptII* selectable marker gene that confers resistance to kanamycin and drives transcription with a doubled CaMV

35S promoter (Odell et al.1985); this vector also contains a tobacco etch virus translational enhancer to further increase gene expression.

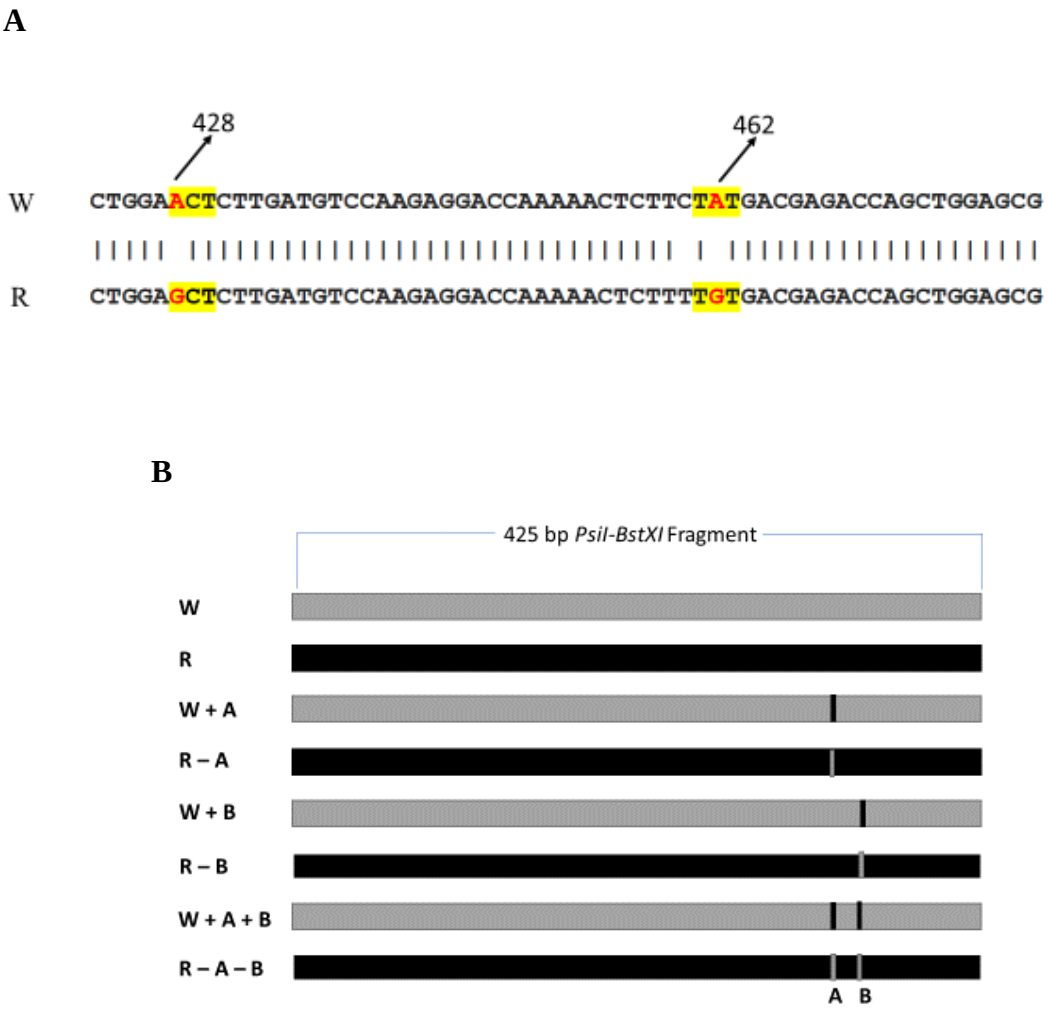


Figure 3. The non-synonymous nucleotide differences between the W and R alleles that were evaluated for impact on substrate specificity.

A. Partial sequence of two *dfr* alleles. W – white (i.e., non-red) allele from Kennebec, R – red allele from W5281.2. The polymorphic nucleotides at positions 428 and 462 that alter predicted amino acid sequence are in red text, the codons they are part of are highlighted in yellow. **B.** Chimeric *dfr* alleles. Position A represents nucleotide 428, position B represents nucleotide 462. W + A denotes a white allele with R allele sequence at position 428, while R - B denotes the red allele with white allele sequence at position 462, etc.

Table 1. The nucleotides and corresponding amino acids of chimeric *dfr* alleles.

Construct	Nucleotide at position 428	Nucleotide at position 462	Amino acid at position 143	Amino acid at position 154
W	A	A	Threonine	Tyrosine
R	G	G	Alanine	Cysteine
W + A	G	A	Alanine	Tyrosine
W + B	A	G	Threonine	Cysteine
W + A + B	G	G	Alanine	Cysteine
R – A	A	G	Threonine	Cysteine
R – B	G	A	Alanine	Tyrosine
R – A – B	A	A	Threonine	Tyrosine

Wild type and chimeric constructs were introduced into Prince Hairy via *Agrobacterium tumefaciens* strain LBA4404 as described by Jung et al. (2005). Transformed plants were propagated *in vitro* in magenta boxes containing Murashige and Skoog (MS) salts (Murashige and Skoog 1962) in a growth chamber maintained under fluorescent lights with a photoperiod of 16 h at 19 ˚C.

Transformation

LBA4404 cultures containing individual constructs were inoculated into liquid YM medium [1 g/L yeast extract, 53 mM mannitol, 2.8 mM KH_2PO_4, 0.81 mM $MgSO_4$ 1.7 mM NaCl] and grown to OD_{600} = 0.6–0.7 at 28 ˚C. The culture was then pelleted at 6000 rpm for 10 min and resuspended in 10 mL of YM medium. Internodes and leaves from 6-week-old *in vitro* grown plants were cut into approximately 0.5 – 1 cm sections, inoculated for 10 mins with *A. tumefaciens*, blotted on sterile paper, and placed on callus induction medium (CIM) [4.3 g/l Murashige and Skoog salts, 26.64 µM glycine, 4.06 µM nicotinic acid, 2.43 µM pyridoxine HCl, 1.19 µM

thiamine HCl, 0.57 µM folic acid, 0.20 µM biotin, 0.555 mM myo-inositol, 87.64 mM sucrose, 4.44 µM 6-benzylamino purine (BAP), 10.74 µM 1-naphthaleneacetic acid (NAA), and 2.5 g/l Phytagel] (Sigma) plates. Explants on CIM (without antibiotics) were incubated for 48 hrs at 19^{o}C in the dark. After the 48 hr cocultivation period, explants were then transferred to shoot induction medium (SIM) [4.3 g/l Murashige and Skoog salts, 2.96 µM thiamine HCl, 4.06 µM nicotinic acid, 2.43 µM pyridoxine HCl, 0.555 mM myo-inositol, 87.6 mM sucrose, 2.85 µM 3-indoleacetic acid (IAA), 8.54 µM trans-zeatin-riboside, and 2.5 g/l Phytagel (Sigma), with pH adjusted to 5.9] containing 50 mg/1 kanamycin monosulfate and 300 mg/l timentin (ticarcillin disodium and clavulanic acid) (GlaxoSmithKline, Research Triangle Park, N.C., USA). Explants were then transferred weekly for one month and every 10 – 14 days after one month to SIM. After approximately 8 weeks, regenerated shoots were excised (1.5 cm long) and transferred into rooting medium (CM) [(4.3 g/l Murashige and Skoog salts, 1.19 µM thiamine HCl, 0.555 mM myo-inositol, 58.43 mM sucrose, and 2.5 g/l Phytagel, with pH adjusted to 5.7)], with kanamycin and timentin, for selective rooting. After four weeks these were then transferred to fresh medium containing 300 mg/l timentin without kanamycin. After another four weeks, plantlets were transferred to CM without timentin and kanamycin, to ensure that the plants were free of bacteria. Only plantlets with well-developed roots were excised and grown in magenta boxes. To test for the presence of the construct and to rogue out untransformed lines, plant genomic DNA was extracted as described in Edwards et al. (1991) and amplified by PCR with primers pPS13162F (5’ CGA ATC TCA AGC AAT CAA GCA) and pPS13443R (5’ CGT AGG TAC GTG GAG TGT CTT); these flank the cloning sites of pPS1 and amplify a 1414-bp product from *dfr* constructs. The amplification conditions were as follows: 94 ˚C for 3 min for one cycle, followed by 35 cycles of 94 ˚C for 20 sec, 50 ˚C for 20 sec, and 72 ˚C for 1 min. The transformed lines were then

transferred to the green house and maintained under a 14 hr photoperiod with temperatures of 24-28 ˚C during the day and 16-18 ˚C at night.

RT-PCR Analysis of Gene Expression

Total RNA was isolated from leaf samples with an EZ-10 Spin Column Plant RNA Miniprep Kit (Bio Basic Amherst, NY). Total RNA was treated with RQ1 RNase-free DNase (Promega) to eliminate genomic DNA prior to performing reverse transcription. DNase treated total RNA was then converted into cDNA using random primers and a reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). The resulting cDNA was then used as a template for PCR using the following thermal profile: 94 ˚C for 2 min for one cycle, followed by 35 cycles of 94 ˚C for 15 sec, 55 ˚C for 15 sec, and 72 ˚C for 1 min. The amplification primers used were potDFR1 (5' GGCTCTTGGCTTGTCATGAG) and potDFR2 (5' AGCATTCCCCTGACTGTTGG). These primers flank a region near the 5' end of *dfr* that includes two introns; this enables amplification products generated from genomic and cDNA templates to be readily distinguished. The PCR products were visualized on a 2% agarose gel. The preceding PCR test does not, however, differentiate between the expression of endogenous and introduced *dfr* alleles. To test whether the introduced alleles were expressed, cDNA was also amplified with primers DFREx3F (5' CCA ACA GTC AGG GGA ATG CT) and DFREx3R (5' GAC CTT GTT TAA TGA TGC CG), which flank the third exon, where nucleotide changes had been made. This cDNA was Sanger-sequenced to confirm that the introduced alleles, as well as Prince Hairy's endogenous alleles, were expressed. This sequencing also confirmed that no other mutations were present than those deliberately introduced.

Flower Collection and Anthocyanin Extraction

Flowers from four or more independent lines of each construct were collected and photographed to record color. The petals were then stored at -80 ˚C prior to being freeze-dried for long-term storage.

Anthocyanins were extracted from 50 mg of freeze-dried pulverized flower petals by adding 1000 µL methanol/water/formic acid (70:30:1, v/v). Samples were mixed for 30 s and centrifuged at 9.1 × 1000 g for 10 minutes. Anthocyanidins were prepared from anthocyanins by adding 360 µL of 37% concentrated HCl to 800 µL aliquots of supernatant; samples were then mixed for 10 seconds and incubated in a water bath at 95°C. After 15 minutes, the samples were taken out of the water bath and placed in cold water to cool down. Samples were diluted by adding 20 µL of hydrolysate to 980 µL of methanol. The diluted samples were mixed, filtered through 0.22 µm PTFE filters and transferred into HPLC vials for analysis.

Liquid Chromatography and Mass Spectrometry

Hydrolyzed samples were characterized with a Vanquish Flex UHPLC system (Dionex Softron GmbH, Germering, Germany) coupled with a TSQ Quantis mass spectrometer (Thermo Scientific, San Jose, CA). The UHPLC was equipped with a Kinetex 2.6 µm EVO C18 100 Å column (150 mm × 2.1 mm). The mobile phase consisted of (A) 1% formic acid in water and (B) 1% formic acid in acetonitrile. The temperature of the column was maintained at 40°C throughout the run and the flow rate was 500 µL/min. The elution program was the following: 0-1 min (5% B, isocratic), 1-6 min (5%-45% B, linear gradient), 6-6.1 min (45%-100% B, linear gradient), 6.1-7.5 min (100% B, column wash), 7.5-8 min (100%-5% B, linear gradient), 8-8.5 min (5% B, re-

equilibration). The flow from the LC was directed to the mass spectrometer through a Heated Electrospray probe (H-ESI). The settings of the H-ESI were: spray voltage 1500 V, Sheath gas 45 (arbitrary unit), Auxiliary gas 24 (arbitrary unit), Sweep gas 0.5 (arbitrary unit), Ion transfer tube temperature 325°C, Vaporizer temperature 350°C.

The mass spectrometer was operated in positive mode and the detection of the anthocyanidins was carried out using Selected Reaction Monitoring (SRM). The SRM parameters for each anthocyanidin are summarized in Table 2. The resolution of both Q1 and Q3 was set at 0.7 FWHM, the dwell time for each transition was 20 ms and the pressure of the collision gas (argon) was set at 1.5 mTorr.

Table 2. SRM parameters for the detection of the six anthocyanidins.

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Delphinidin	3.57	0.6	Positive	303	153	30	143
Delphinidin	3.57	0.6	Positive	303	229	32	143
Cyanidin	3.86	0.6	Positive	287	187	38	156
Cyanidin	3.86	0.6	Positive	287	213	32	156
Petunidin	3.97	0.6	Positive	317	245	36	156
Petunidin	3.97	0.6	Positive	317	302	25	156
Pelargonidin	4.17	0.6	Positive	271	121	33	176
Pelargonidin	4.17	0.6	Positive	271	197	31	176
Peonidin	4.28	0.6	Positive	301	201	39	152
Peonidin	4.28	0.6	Positive	301	286	25	152
Malvidin	4.34	0.6	Positive	331	287	30	173
Malvidin	4.34	0.6	Positive	331	315	30	173

Results

The potato cultivar ‘Prince Hairy’ lacks the allele of *dfr* needed for synthesis of red pigments (De Jong et al., 2003) and produces white tubers and pale blue flowers. The pale blue color results from the accumulation of delphinidin and methylated delphinidin (malvidin, petunidin) pigments. To test whether either of two amino acid positions that differ between alleles of *dfr* that can (R) and cannot (W) direct the synthesis of red pelargonidins, Prince Hairy was transformed with a series of wild type and chimeric alleles (Figure 3, Table 1). Our expectation was that alleles capable of reducing DHK would change Prince Hairy flower color from pale blue to purple (Zhang et al., 2009) – by virtue of containing a mixture of blue delphinidins and red pelargonidins – while flowers of Prince Hairy transformed with alleles that cannot utilize DHK would remain pale blue.

The success of each transformation was first assessed by using amplification primers that flanked the transformation construct (see Methods). Subsequent Sanger-sequencing of *dfr* cDNA amplified from each transgenic line was used to confirm that alleles with the desired nucleotide changes – in addition to Prince Hairy’s own *dfr* alleles – were expressed (see Methods; data not shown). In the process of engineering Prince Hairy, we noted that a few transgenic lines had white flowers. Figure 4 shows that the white-flowered lines no longer expressed any *dfr* alleles, presumably a result of transgene-mediated gene silencing (Elomaa et al., 1995), while Prince Hairy and engineered lines with pale blue or purple colored flowers all expressed *dfr*.

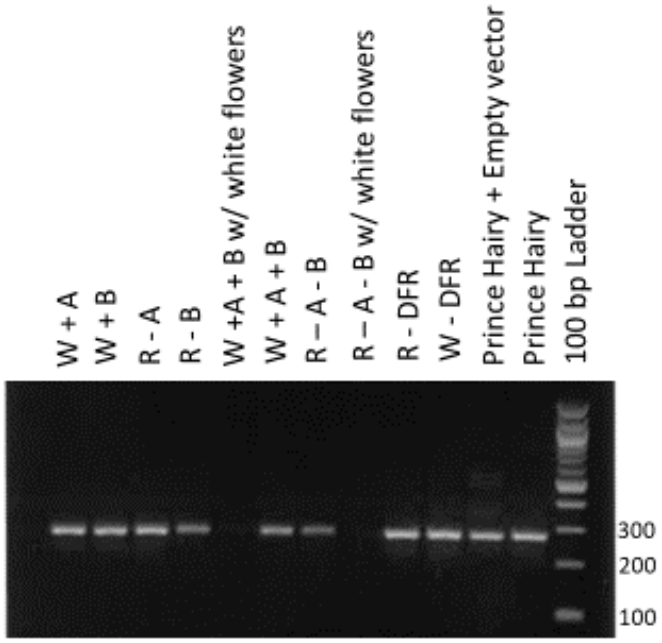


Figure 4. Evaluation of *dfr* gene expression in wild-type Prince Hairy and engineered lines using potDFR primers (see methods), which amplify both endogenous and added *dfr* alleles.

Flower Phenotype

Four or more independent lines of Prince Hairy transformed with each of the two wild type (R, W) and six chimeric (R-A, R-B, R-A-B, W+A, W+B, W+A+B) constructs, as well as Prince Hairy transformed with an empty vector, were grown in the greenhouse. Flowers were photographed to record color and subsequently processed to analyse anthocyanidin content. A representative flower from each construct, as well as untransformed and empty vector controls, is shown in Figure 5. As expected, we observed pale blue flowers in Prince Hairy, Prince Hairy transformed with an empty vector, and Prince Hairy transformed with the W allele of *dfr* (Figure 5). Similarly, as previously reported (Zhang et al., 2009), flowers of Prince Hairy transformed with the R allele were purple (Figure 5). The flower color of constructs R-A, R-B, and W+B, each

of which harbor individual nucleotide substitutions, were also changed, and in all cases, to a lighter purple color than that mediated by the wild type R allele, suggesting that amino acid positions 143 and 154 both influence DFR substrate specificity, but are not individually sufficient to direct high levels of red pigment production. The remaining construct with a single nucleotide substitution, W+A, yielded pale blue flowers visually indistinguishable from Prince Hairy. Construct W+A+B produced flowers considerably more purple in color than those transformed with W+A or W+B, while flowers from R-A-B constructs yielded flowers paler in color than R-A or R-B (Figure 5), i.e., constructs with two nucleotide changes had more striking changes in flower color than those with individual nucleotide changes.

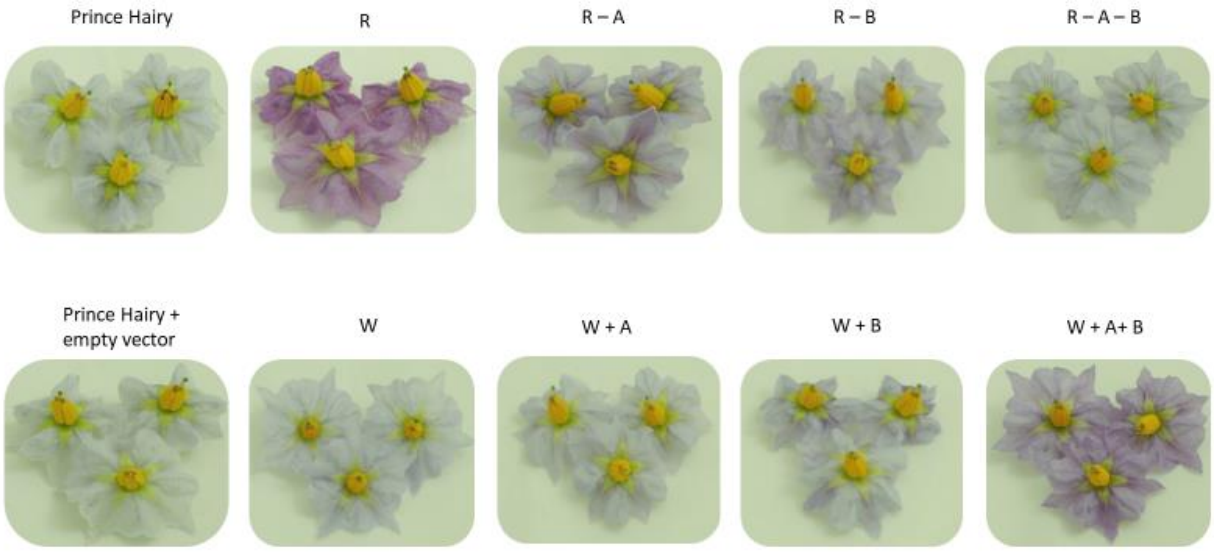


Figure 5. Flowers of Prince Hairy transformed with an empty vector, wild type and chimeric *dfr* alleles.

Flower Anthocyanidin Content

To evaluate the effects of each transgene at a biochemical level, we assessed the anthocyanidins present in petals of each engineered line (Table 3, Figure 6). Pelargonidin was detected by LC-MS in all flowers that exhibited a change in flower color from pale blue to various intensities of purple (Table 3, Figure 6). Pelargonidin was not detected in Prince Hairy, Prince Hairy transformed with an empty vector, or Prince Hairy transformed with either the W or W+A alleles. Trace amounts of pelargonidin were found in W+B. W+A+B and R both displayed a substantially greater amount of pelargonidin compared to other treatments, with respective difference in mean content of 7.3-10.6 (t-test, $p < 0.01$) and 17.9-21.2 (t-test, $p < 0.001$), while pairwise comparisons among all other treatments were close to zero (mean difference 0-3.3 (t-test, p range: 0.26 – 1.00)). Mean pelargonidin content in R was about twice as much as in W+A+B, with a mean difference of 10.6 (t-test, $p = 0.016$) (Table 3, Figure 6).

Table 3. Pelargonidin content (µg/g) of wild type and engineered lines

Construct	n	pelargonidin	% of total anthocyanidins
Prince Hairy	2	ND[1]	0
empty vector	3	ND	0
R	7	21.2 ± 8.3[2]	0.4
R - A	4	3.3 ± 1.0	0.3
R - B	5	1.3 ± 0.9	0.07
R - A - B	7	0.7 ± 0.7	0.02
W	7	ND	0
W + A	7	ND	0
W + B	5	1.0 ± 0.6	0.1
W+A+B	7	10.6 ± 10	0.2

[1] ND - not detectable

[2] mean ± standard deviation

A one-way analysis of variance (ANOVA) between treatments was conducted to compare the effect of the different constructs on pelargonidin production. There was statistically significant variation among constructs for the amount of pelargonidin production at $p < 0.05$ [$F(9, 44) = 14.30$, $p = 0.0001$].

For all plants, the amount of pelargonidin production was small compared to total anthocyanidin production (Table 3). Pelargonidin produced by the red allele (R) was about 0.39% of the total, while alleles W+B and W+A+B comprised 0.1% and 0.2% respectively. Figure 6 reports levels of all anthocyanidins measured, i.e., pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. These latter five pigments, other than pelargonidin, were present in wild-type Prince Hairy flowers and all engineered flowers. A correlation analysis revealed that pelargonidin levels were positively correlated with cyanidin and its methylated derivative peonidin, and negatively correlated to delphinidin and its methylated derivatives, petunidin and malvidin.

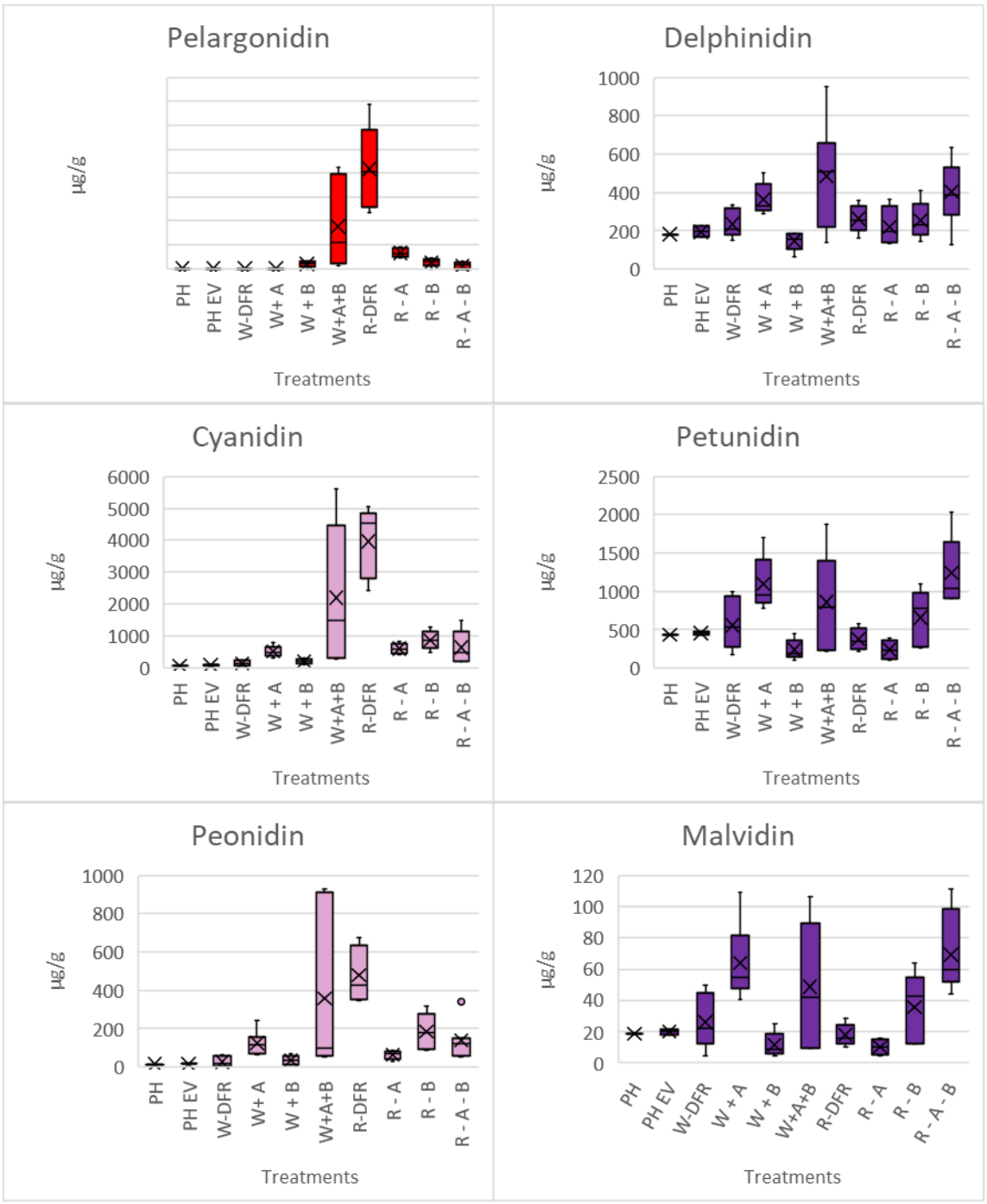


Figure 6. Box and whiskers plot for anthocyanidin content across various constructs. PH is Prince Hairy, PH EV is Prince Hairy transformed with an empty vector, other constructs are as given in Table 1, 'X' is the mean, the horizontal bar within each box shows the mean, and the upper and lower box bounds show 25th and 75th percentiles, respectively.

Discussion

The objective of this study was to identify the amino acids that determine substrate specificity of DFR in potato. Johnson et al. (2001) demonstrated that altering a single amino acid in *Gerbera* DFR and introducing it into *Petunia* led to preferential catalysis of DHK over DHQ and DHM, i.e., modifying the substrate specificity of DFR. While comparing the sequence of different *dfr* alleles in potato, De Jong et al. (2003) noted that amino acid positions 143 and 154, which are in a putative 26 amino acid substrate binding pocket (Johnson et al., 2001), are polymorphic between 'red' and 'non-red' alleles. By making reciprocal changes between 'red' and 'non-red' alleles we demonstrated here that these two amino acid positions together confer upon potato DFR the ability to reduce DHK, an essential step in the production of red pelargonidin pigment.

Even though the 'non-red' allele with a change at position 154 (tyrosine to cysteine) can make trace amounts of pelargonidin, the non-red allele with changes at both positions 143 (threonine to alanine) and 154 (tyrosine to cysteine) yielded the most pelargonidin of any chimeric construct (Table 1). The yield of pelargonidin in W+A+B was, nevertheless, only about half that of the wild type red allele, suggesting that additional amino acid changes are needed for maximal activity. Even so, it may possible to convert some white potatoes to red and vice-versa, just by changing the amino acids at positions 143 and 154.

We observed that increased pelargonidin levels were associated with substantially increased accumulation of cyanidin and peonidin (Figure 6). Thus, the amino acid changes introduced appear not only to have conferred the ability to utilize DHK, but to have improved the ability to utilize DHQ as well. Heterologous expression in *Escherichia coli* of DFR genes from

Lotus japonicus (Shimada et al., 2005) and *Medicago trunculata* (Xie et al., 2004) has shown that an aspartic acid (D) residue in the substrate binding region - at the position that corresponds to position 145 in potato (Figure 7) - leads to the preferential catalysis of DHQ over DHK. Potato DFR has an aspartic acid at position 145, which may explain why more cyanidin and peonidin than pelargonidin were produced by our chimeric constructs. An alternative explanation for the increase in cyanidin and peonidin is that F3'H and/or F3'5'H, which compete with DFR for DHK, may also hydroxylate downstream products in the pelargonidin biosynthetic pathway at the 3' hydroxyl position, driving them into the cyanidin/peonidin biosynthetic pathway. Further experiments will need to be conducted to differentiate between these alternative explanations.

```
Zea       112 RILRACKEAGTVRRIVFTSSAGTVNLEERQRPVYDEESWTDVDFCRRVKMTGWMYFVSKS 171
Gerbera   110 SIIRSCVKAKTVKKLVFTSSAGTVNGQEKQLHVYDESHWSDLDFIYSKKMTAWMYFVSKT 169
Petunia   119 SIIESCAKANTVKRLVFTSSAGTLDVQEQQKLFYDQTSWSDLDFIYAKKMTGWMYFASKI 178
Kennebec  121 SIIESCAKANTVKRLVFTSSAGTLDVQEDQKLFYDETSWSDLDFIYAKKMTGWMYFVSKI 180
W5281.2   121 SIIESCAKANTVKRLVFTSSAGALDVQEDQKLFCDETSWSDLDFIYAKKMTGWMYFVSKI 180
```

Figure 7 DFR protein sequence comparison

The black box shows the 26 amino acid substrate binding region in *Gerbera* as defined by Johnson et al. (2001). The amino acid (asparagine (N)) highlighted in red has been shown to affect substrate specificity in *Gerbera*. Polymorphic potato amino acids are highlighted in yellow. Potato amino acid position 145 (aspartic acid (D)) is highlighted in green.

The effect of individual and double amino acid exchanges on pigment levels varied for the three major groups of anthocyanidins (pelargonidin, cyanidin/peonidin, delphinidin/petunidin/malvidin). For pelargonidin levels, position 154 had more impact than position 143 (Figure 6). For levels of cyanidin, peonidin, petunidin, malvidin and possibly also delphinidin, position 143 had more impact than position 154 (Figure 6). The highest levels of

pelargonidin, cyanidin and peonidin were observed with R and W+A+B (Figure 6), i.e., the two constructs which had 'red' amino acids at positions 143 and 154. Surprising was that W+A+B and R-A-B, which are mirror-image constructs with double amino acid changes, both gave rise to increased levels of delphinidin, petunidin, and malvidin. Ultimately crystallographic approaches will be needed to fully understand how potato DFR substrate specificity is determined.

Conclusion and Future Work

We have identified two amino acids that affect substrate specificity in potato. Further studies involving either 3D modeling and/or crystallography will help to establish how these amino acid positions affect substrate specificity at a mechanistic level. That is beyond our expertise. Nevertheless, we do plan on crossing our transgenic lines with a cultivar that is simplex at R (genotype D-Rrrr pppp), to test if any of the amino acid changes are able to change tuber skin from white to red, as the current study only looked at color in flowers.

BIBLIOGRAPHY

Brown, C. R., Culley, D., Yang, C. P., Durst, R., & Wrolstad, R. (2005). Variation of anthocyanin and carotenoid contents and associated antioxidant values in potato breeding lines. *Journal of the American Society for Horticultural Science*, *130*(2), 174-180.

Brown, C.R. 2005. Antioxidants in potato. *American Journal of Potato Research*. 82:163-172.

Brugliera, F., Barri-Rewell, G., Holton, T. A., & Mason, J. G. (1999). Isolation and characterization of a flavonoid 3'-hydroxylase cDNA clone corresponding to the Ht1 locus of Petunia hybrida. *Plant Journal*, *19*(4), 441–451.

Chalker-Scott, L. (1999). Environmental Significance of Anthocyanins in Plant Stress Responses. *Photochemistry and Photobiology*, 70: 1-9.

Daneshzad, E., Shab-Bidar, S., Mohammadpour, Z., Djafarian, K. (2018). Effect of anthocyanin supplementation on cardio-metabolic biomarkers: A systematic review and meta-analysis of randomized controlled trials. *Clinical Nutrition*. ISSN 0261-5614.

Davidson, S. (1975). Human nutrition and dietetics. 6th ed. Edinburgh: Churchill Livingstone: distributed in the U.S.A. by Longman.

De Jong, W. S., De Jong, D. M., De Jong, H., Kalazich, J., & Bodis, M. (2003). An allele of dihydroflavonol 4-reductase associated with the ability to produce red anthocyanin pigments in potato (Solanum tuberosum L.). *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *107*(8), 1375–1383.

Edwards, K., Johnstone, C., Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res* 19:1349.

FAOSTAT 2013

Fischer, T. C., Halbwirth, H., Meisel, B., Stich, K., & Forkmann, G. (2003). Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductases from Malus domestica and Pyrus communis cultivars and the consequences for flavonoid metabolism. *Archives of Biochemistry and Biophysics*, *412*(2), 223–230.

Forkmann, G., & Ruhnau, B. (1987). Distinct substrate specificity of dihydroflavonol 4-reductase from flowers of Petunia hybrida. *Z. Naturforsch., 42c*, 1146–1148.

Gerats, A. G., de Vlaming, P., Doodeman, M., Al, B., & Schram, A. W. (1982). Genetic control of the conversion of dihydroflavonols into flavonols and anthocyanins in flowers of Petunia hybrida. *Planta*, *155*.

Giusti M, Wrolstad RE. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochem Eng J.*, 14(3):217–225.

Haq, T. A., Mason, H. S., Clements, J. D., & Arntzen, C. J. (1995). Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science (New York, N.Y.)*, *268*(5211), 714–716.

Heller, W., Forkmann, G., Britsch, L., & Grisebach, H. (1985). Enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-cis-diols with flower extracts from Matthiola incana and its role in anthocyanin biosynthesis. *Planta*, *165*(2), 284–287.

Holton, T. A., & Cornish, E. C. (1995). Genetics and Biochemistry of Anthocyanin Biosynthesis. *The Plant Cell*, *7*(7), 1071–1083.

Holton, T. A., Brugliera, F., Lester, D. R., Tanaka, Y., Hyland, C. D., Menting, J. G., … Cornish, E. C. (1993). Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature*, *366*(6452), 276–279.

Huang, Z., Mason H.S. (2004). Conformational analysis of hepatitis B surface antigen fusions in an Agrobacterium-mediated transient expression system. *Plant Biotechnol J* 2:241–249

Jaakola, L., Määttä, K., Pirttilä, A. M., Törrönen, R., Kärenlampi, S., & Hohtola, A. (2002). Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. *Plant Physiology*, *130*(2), 729-739.

Johnson, E. T., Ryu, S., Yi, H. K., Shin, B., Cheong, H., & Choi, G. (2001). Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. *Plant J, 25*.

Johnson, E. T., Yi, H., Shin, B., BJ, O., Cheong, H., & Choi, G. (1999). Cymbidium hybrida dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonidin-type anthocyanins. *Plant J, 19*.

Jung, C. S., Griffiths, H. M., De Jong, D. M., Cheng, S., Bodis, M., & De Jong, W. S. (2005). The potato P locus codes for flavonoid 3',5'-hydroxylase. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *110*(2), 269–275.

Katsu, K., Suzuki, R., Tsuchiya, W., Inagaki, N., Yamazaki, T., Hisano, T., Matsui, K. (2017). A new buckwheat dihydroflavonol 4-reductase (DFR), with a unique substrate binding structure, has altered substrate specificity. *BMC Plant Biology*, *17*(1), 239.

Khoo, H. E., Azlan, A., Tang, S. T., & Lim, S. M. (2017). Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food & Nutrition Research, 61*.

Kong, J., Chia, L., Goh, N., Chia, T., & Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 64*(5), 923-933.

Lev-Yadun S., Gould K.S. (2008). Role of Anthocyanins in Plant Defence. *In: Winefield C., Davies K., Gould K. (eds) Anthocyanins.* Springer, New York, NY.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition, 79*(5), 727-747.

Mazza, G., & Brouillard, R. (1987). Recent developments in the stabilization of anthocyanins in food products. *Food Chemistry, 25*(3), 207-225.

Meyer, P., Heidmann, I., & Forkmannr, G. (1987). A new petunia flower colour generated. *Nature*.

Morales Garzón, F.J. (2007). Pre-Columbian societies associated with the domestication and cultivation of potato (Solanum tuberosum) in South America. *Revista Latinoamericana de la Papa* 14:1-9.

Murashige, T. and F. Skoog. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497

Nayak, B., Berrios, J. D. J., Powers, J. R., Tang, J., & Ji, Y. (2011). Colored Potatoes (Solanum Tuberosum L.) Dried for Antioxidant-Rich Value-Added Foods. *Journal of Food Processing and Preservation, 35*(5), 571–580.

Nemš, A., Pęksa, A., Kucharska, A. Z., Sokół-Łetowska, A., Kita, A., Drozdz, W., & Hamouz, K. (2015). Anthocyanin and antioxidant activity of snacks with coloured potato. *Food Chemistry, 172*, 175-182.

Nunn, N., & Qian, N. (2011). The potato's contribution to population and urbanization: Evidence from a historical experiment. *Quarterly Journal of Economics*, 126(2), 593–650.

Obsuwan, K., Hieber, D. A., Mudalige-jayawickrama, R. G., & Kuehnle, A. R. (2007). Functional Characterization of Dendrobium and Oncidium Dfr in Petunia hybrida Model, 137–144.

Odell, J. T., Nagy, F., & Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature, 313*(6005), 810–812.

Perla, V., Holm, D. G., & Jayanty, S. S. (2012). Effects of cooking methods on polyphenols, pigments and antioxidant activity in potato tubers. *LWT - Food Science and Technology, 45*(2), 161-171.

Petit, P., Granier, T., d'Estaintot, B. L., Manigand, C., Bathany, K., Schmitter, J.-M., Gallois, B. (2007). Crystal structure of grape dihydroflavonol 4-reductase, a key enzyme in flavonoid biosynthesis. *Journal of Molecular Biology, 368*(5), 1345–1357.

Plaisted, R.L., Tingey, W.M., Stevens, J.C. (1992) The germplasm release of NYL 235–4, a clone with resistance to the Colorado potato beetle. *Am Pot J* 69:843–846

Pojer, E., Mattivi, F., Johnson, D., & Stockley, C. S. (2013). The case for anthocyanin consumption to promote human health: A review. *Comprehensive Reviews in Food Science and Food Safety, 12*(5), 483-508.

Salaman, R. (1949). The History and Social Influence of the Potato. Cambridge, MA: Cambridge University Press.

Salaman, R.N. (1910). The inheritance of colour and other characters in the potato. *J Genet* 1:7–46

Sandoval-Ramírez, B. A., Catalán, U., Fernández-Castillejo, S., Rubió, L., Macià, A., Solà. R. (2018). Anthocyanin Tissue Bioavailability in Animals: Possible Implications for Human Health. A Systematic Review. *Journal of Agricultural and Food Chemistry* 2018 66 (44), 11531-11543

Shimada, N., Sasaki, R., Sato, S., Kaneko, T., & Tabata, S. (2005). A comprehensive analysis of six dihydroflavonol 4-reductases encoded by a gene cluster of the Lotus japonicus genome, *56*(419), 2573–2585.

Stich, K., Eidenberger, T., Wurst, F., & Forkmann, G. (1992). Enzymatic conversion of dihydroflavonols to flavan-3,4-diols using flower extracts of Dianthus caryophyllus L. (carnation). *Planta*, *187*(1), 103–108.

Tajner-Czopek, A., Rytel, E., Kita, A., Pęksa, A., & Hamouz, K. (2012). The influence of thermal process of coloured potatoes on the content of glycoalkaloids in the potato products. *Food Chemistry, 133*(4), 1117-1122.

Tanaka, Y., Fukui, Y., Fukuchi-Mizutani, M., Holton, T. A., Higgins, E., & Kusumi, T. (1995). Molecular cloning and characterization of Rosa hybrida dihydroflavonol 4-reductase gene. *Plant & Cell Physiology*, *36*(6), 1023–1031.

Xie, D. Y., Jackson, L. A., Cooper, J. D., Ferreira, D., & Paiva, N. L. (2004). Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol-4-reductase from Medicago truncatula. *Plant Physiol, 134*.

Zhang, Y., Cheng, S., De Jong, D., Griffiths, H., Halitschke, R., & De Jong, W. (2009). The potato R locus codes for dihydroflavonol 4-reductase. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *119*(5), 931–937.