Theor Appl Genet (2003) 107:1375–1383
DOI 10.1007/s00122-003-1395-9

ORIGINAL PAPER

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An allele of dihydroflavonol 4-reductase associated with the ability to produce red anthocyanin pigments in potato (*Solanum tuberosum* L.)

Received: 12 November 2002 / Accepted: 11 June 2003 / Published online: 3 September 2003

Abstract The potato *R* locus is necessary for the production of red pelargonidin-based anthocyanin pigments in any tissue of the plant, including tuber skin and flower petals. The production of pelargonidins in plants requires the activity of dihydroflavonol 4-reductase (DFR) to catalyze the reduction of dihydrokaempferol into leucopelargonidin. To test the hypothesis that potato *R* encodes DFR, portions of both *dfr* alleles were sequenced from a diploid potato clone known to be heterozygous *Rr*. Sequence comparison revealed a polymorphic *Bam*HI restriction site. The presence or absence of this site was monitored in three diploid populations that segregated for *R*, as well as in a wide range of tetraploid breeding clones and cultivars, by amplifying a fragment of *dfr* and digesting the products with *Bam*HI. An identically sized *dfr* restriction fragment lacking the *Bam*HI site was present in all potato clones that produced red anthocyanin pigments, while the same fragment was absent in many potato clones with white tuber skin and flowers. An independent RFLP test using *Dra*I to detect sequence polymorphism was performed on a subset of the potato clones. This test also revealed *dfr*-derived bands that were present in all red-colored potatoes and absent in several white clones. The presence of shared restriction fragments in all red-colored potatoes provides strong evidence that *R* does indeed code for DFR. The data are also consistent with a 48 year-old hypothesis by Dodds and Long, that *R* was selected just once during the domestication of potato. A cDNA clone corresponding to the red allele of *dfr* was sequenced and compared to two other alleles. The red allele is predicted to encode a 382 amino acid protein that differs at ten amino acid positions from the gene products of the two alternative alleles. Several of these differences map in a region known to influence DFR substrate specificity in *Gerbera*.

Keywords Crop domestication · Secondary metabolism · Marker-assisted selection · Allele dosage

Communicated by J. Dvorak

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Introduction

The skin of cultivated diploid and tetraploid potato (*Solanum* spp.) tubers can be colored either red, purple or white. This natural variation in color is controlled by three independent loci—*R*, *P* and *I* (reviewed in De Jong 1991). *R* and *P* are required for the production of red and purple anthocyanin pigments, respectively, anywhere in the potato plant, e.g. in tubers, flowers, sprouts or stems, while *I* is required for the tissue-specific expression of anthocyanins in tuber skin (Dodds and Long 1955, 1956). The tubers of wild potato species are only purple or white, which has led to the suggestion that the ability to produce red anthocyanin pigments was selected just once, and at the diploid level, during domestication (Dodds and Long 1955). *R*, *P* and *I* have been genetically mapped to chromosomes 2, 11 and 10, respectively (Van Eck et al. 1993, 1994).

Biochemical analyses have shown that the red anthocyanins in tuber skin and flesh are primarily derived from the anthocyanidin pelargonidin, while the purple pigments are derived from the related compound petunidin (Lewis et al. 1998; Naito et al. 1998; Rodriguez-Saona et al. 1998). Some pink-colored clones of the diploid species *Solanum phureja* and *Solanum stenotomum* have been shown to contain peonidin-derived pigments instead of

pelargonidin derivatives (Dodds and Long 1955). The enzyme dihydroflavonol 4-reductase (DFR) plays a necessary role in the synthesis of pelargonidins, peonidins and petunidins, although it catalyzes the reduction of slightly different substrates, dihydrokaempferol (DHK), dihydroquercitin (DHQ) and dihydromyricetin (DHM), respectively, in each case (Holton and Cornish 1995). The structure of DHK differs from DHQ and DHM in having one and two fewer hydroxyl groups, respectively, on the B phenyl ring. In some genera, including *Petunia* (Forkmann and Ruhnau 1987) and *Cymbidium* (Johnson et al. 1999), DFR is not able to efficiently reduce DHK although it is still able to reduce DHQ and DHM. In other genera, e.g. *Gerbera*, DFR can utilize DHK, DHQ and DHM as substrates (Johnson et al. 2001). The variation in DFR substrate specificity explains why red pelargonidin-pigmented flowers are observed naturally in *Gerbera* but not in *Petunia* (Johnson et al. 2001).

The *dfr* gene has previously been mapped in tomato to chromosome 2 (Bongue-Bartelsman et al. 1994). This chromosome shares extensive conservation of marker order with potato chromosome 2 (Tanksley et al. 1992). Because *R* also maps to this chromosome, and natural variation at the *R* locus is suggestive of variation in the substrate specificity of DFR, we wondered if *R* might correspond to *dfr*. To test this we followed the segregation of *dfr* alleles in several diploid populations that segregated for color, as well as in a wide range of tetraploid potato breeding clones and cultivars. We report here that all red-colored potato clones tested, but not all white potatoes, carry an allele of *dfr* that lacks a specific *Bam*HI restriction site. An independent RFLP test revealed that red-colored potatoes also share a common *dfr*-derived *Dra*I restriction fragment, which is absent in some white clones. These results suggest that *R* does indeed code for DFR, and are consistent with the hypothesis (Dodds and Long 1955) that the dominant *R* allele in modern potatoes results from the identification and subsequent selection of a single plant, most likely with red tuber skin, during crop domestication.

Materials and methods

Plant material

Ploidy and color characteristics of potato clones used in this study are summarized in Table 1. Several of the diploid clones have previously been assigned genotypes at two loci—*I* and *R*—that are together required for the synthesis of red anthocyanin pigments in tuber skin (De Jong 1991). These clones are 79-48 (genotype *II* RR^{pw}) (De Jong 1987), 320-02 (*Ii Rr*; unpublished), 07506-01 (*ii rr*) (De Jong 1987; De Jong and Burns 1983) and W5281.2 (*Ii RR*) (De Jong and Burns 1983). CIP590001.7 was described by Watanabe et al. (1994). Diploids W5281.2, W5337.3 and W5295.7 were developed at the University of Wisconsin. The NY clones listed in Table 1 were developed by the Cornell University potato breeding program. 320-02 was selfed to generate an S_1 population that segregates for tuber skin color. 79-48 was crossed as a female with 07506-01 to develop an F_1 population that segregates for flower color. 07248-02 was crossed as a female with 320-02 to generate an F_1 population that segregates for tuber skin color.

Allelic discrimination

In early experiments, template DNA for PCR amplification was isolated with a DNeasy plant mini kit (Qiagen). For some large-scale screening experiments potato DNA was isolated with the rapid miniprep method of Edwards et al. (1991). Both methods yielded acceptable results. PCR primers potDFR1 (*5′GGCTCTTG-GCTTGTCATGAG*) and potDFR2 (*5′AGCATTCCCCTGACT-GTTGG*) were designed to anneal to regions conserved between a tomato *dfr* cDNA sequence (GenBank accession Z18277) and a potato *dfr* EST sequence (accession BE923282). Based on comparison to the genomic DNA sequence of *Petunia dfrA* (accession X79723) the primers were anticipated to flank a region near the 5′ end of *dfr* that would include two small introns. The two primers were used to amplify genomic DNA of diploid 320-02 using the following thermal profile: 94°C for 2 min, followed by 35 cycles of 94°C, 15 s; 55°C, 15 s; and 72°C, 60 s. This and other amplifications below were carried out in a volume of 50 μl and contained 200 nM of each primer, 200 μM of dNTPs, 10 mM of Tris-Cl pH 8.3, 50 mM of KCl, 2.5 mM of $MgCl_2$, 0.05% of Nonidet-P40, 50–200 ng of template DNA and 2.5 units of *Taq* DNA polymerase. The resulting PCR products were ligated into vector pGEM-T following the manufacturer's (Promega) instructions. Eight independent clones were sequenced and aligned for comparison; this revealed a polymorphic *Bam*HI restriction site (see Results). To evaluate potato clones for this polymorphic site, PCR was performed with primers potDFR1 and potDFR2, and the products were then digested with *Bam*HI. Digested products were electrophoretically separated on a 2% agarose gel and visualized by staining with ethidium bromide.

RFLP analysis

DNA isolation and gel-blot analysis were essentially as described (Bonierbale et al. 1988). Genomic DNA was digested separately with *Dra*I or *Sca*I prior to blotting. An approximately 1.5 kb fragment of the tomato *dfr* gene, obtained by amplification with primers *5′CACTCTCCTCCGAAGACGAC* and *5′TCCATTGTCT-GCAGTGCTTC*, was used as a hybridization probe. Random hexamer labeling of the probe, hybridization and washing methods were as described previously (Feinberg and Vogelstein 1983). Filters were washed to a final stringency of 0.5×SSC at 65°C (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate).

cDNA library screening and cDNA sequencing

A cDNA library was constructed using a ZAP-cDNA synthesis kit (Stratagene) with mRNA isolated from pigmented flowers and stems of W5281.2. The tomato *dfr* probe described above was used for library screening. Positively hybridizing phage were plaque-purified and the inserts then excised to permit propagation in a pBlueScript SK(-) phagemid vector, following the manufacturer's instructions. The largest cDNA insert was completely sequenced on both strands. Partially sequenced (accession BG595686) potato cDNA clone cSTS11C14 was provided by S.D. Tanksley (Cornell University). This clone is derived from the potato cultivar Kennebec and was also completely sequenced on both strands.

Amplification and sequencing of dfr from genomic DNA

Primers to amplify the entire open reading frame of *dfr* alleles from W5281.2 and 07506-01 were designed based on the cDNA sequences obtained from W5281.2 and Kennebec, respectively. Primers DFRORF-F (*5′ CTTGAGGTTAAATTTTCTGACTC*) and DFRORF-R3 (*5′GCCTTTTTATTTTGTTAGGTTGTG*) were used to amplify *dfr* from W5281.2, while primers DFRORF-F and DFRORF-R (*5′ CCTTTTTATTTCATATGGTTGCG*) were used to amplify *dfr* from 07506–01 and the *dfr* allele linked to R^{pw} in 79-48. Thermal cycling parameters were 94°C for 2 min, followed by 35

Table 1 Characteristics of potato clones utilized in this study

Clone	Ploidy	Tuber skin color[a]	Flower color[a]	Sprout color[a]	*dfr* allele without *Bam*HI[b]	Genotype (where known)
07248-02	2*x*	White	White	ND[c]	No	
07506-01	2*x*	White	White	ND	No	*ii rr*
09479-05	2*x*	Red	Red	ND	Yes	
75-10	2*x*	White	Blue	ND	No	
79-48	2*x*	Red	Red	ND	Yes	*Ii* RR^{pw}
320-02	2*x*	Red	Red	ND	Yes	*Ii Rr*
11364-09	2*x*	White	Blue	ND	No	
11827-09	2*x*	Red	Red	ND	Yes	
AC Candy Cane	2*x*	Red	Red	ND	Yes	
CIP590001.7	2*x*	Red	Red	ND	Yes	
W5281.2	2*x*	Purple	Purple	Purple	Yes	*Ii RR*
W5295.7	2*x*	Red eyes	ND	Red	Yes	
W5337.3	2*x*	Red eyes	Red	ND	Yes	
Andover	4*x*	White	White	Purple	No	
Allegany	4*x*	White	Red	ND	Yes	
Atlantic	4*x*	White	Red	ND	Yes	
Banana	4*x*	White	Red	ND	Yes	
Chieftain	4*x*	Red	Red	ND	Yes	
Chippewa	4*x*	White	Red	Red	Yes	
Dakota Rose	4*x*	Red	Red	ND	Yes	
Dark Red Norland	4*x*	Red	Red	ND	Yes	
Elba	4*x*	White	Red	ND	Yes	
Eva	4*x*	White	White	White	No	
Genesee	4*x*	White	White	Purple	No	
Hampton	4*x*	White	White	Red	Yes	
Idarose	4*x*	Red	Red	ND	Yes	
Jacqueline Lee	4*x*	White	Red	Red	Yes	
Kanona	4*x*	White	Red	Red	Yes	
Katahdin	4*x*	White	Red	Red	Yes	
Kennebec	4*x*	White	White	White	No	
Keuka Gold	4*x*	White	White	Purple	No	
Lenape	4*x*	White	Blue	Purple	No	
Marcy	4*x*	White	White	Purple	No	
Monona	4*x*	White	White	Red	Yes	
Nordonna	4*x*	Red	Red	ND	Yes	
Pike	4*x*	White	White	Purple	Yes	
Reba	4*x*	White	White	Purple	No	
Red Companion	4*x*	Red	ND	ND	Yes	
Red Pearl	4*x*	Red	ND	ND	Yes	
Redsen	4*x*	Red	Red	Red	Yes	
Rideau	4*x*	Red	ND	ND	Yes	
Rosa	4*x*	Red eyes	Red	ND	Yes	
Salem	4*x*	White	White	Purple	Yes	
Serrana Inta	4*x*	White	Blue	Purple	No	
Snowden	4*x*	White	White	Purple	No	
Stirling	4*x*	White	White	Purple	No	
Superior	4*x*	White	Red	Red	Yes	
Yagana	4*x*	White	Red	Red	Yes	
Yukon Gold	4*x*	Red eyes	Red	Red	Yes	
NY97	4*x*	Red	Red	Red	Yes	
NY99	4*x*	White	Red	ND	Yes	
NY102	4*x*	White	White	Purple	No	
NY115	4*x*	White	White	Red	Yes	
NY118	4*x*	Red	Red	Red	Yes	
NY120	4*x*	White	White	Purple	Yes	
NY121	4*x*	White	White	White	No	
NY123	4*x*	White	Blue	Purple	No	
NY124	4*x*	White	White	Red	Yes	
NY125	4*x*	White	White	Purple	No	
NY126	4*x*	White	White	Purple	Yes	
NY129	4*x*	Red	Red	ND	Yes	
NYE11-45	4*x*	White	Red	Red	Yes	
NYL235-4	4*x*	White	Blue	Purple	No	
NYR6-4	4*x*	White	White	Purple	No	
NYR17-7	4*x*	White	Red	Red	Yes	
NYS48-6	4*x*	Red	White	ND	Yes	
NYT3-9	4*x*	White	White	Purple	No	
NYT15-1	4*x*	Red	Red	ND	Yes	

Table 1 (continued)

Clone	Ploidy	Tuber skin color[a]	Flower color[a]	Sprout color[a]	*dfr* allele without *Bam*HI[b]	Genotype (where known)
NYT15-3	4*x*	Red	Red	ND	Yes	
NYT17-2	4*x*	Red	Red	ND	Yes	
NYT28-1	4*x*	White	White	Purple	Yes	
NYU47-2	4*x*	White	White	Purple	No	
NYU75-1	4*x*	White	White	Purple	No	
NYU85-12	4*x*	White	White	Purple	No	
NYU100-87	4*x*	White	White	Purple	Yes	
NYU106-26	4*x*	White	White	Purple	Yes	
NYW2-8	4*x*	Red	ND	ND	Yes	
NYW2-19	4*x*	Red	ND	ND	Yes	
NYW2-24	4*x*	Red	ND	ND	Yes	
NYW2-37	4*x*	Red	ND	ND	Yes	
NYW2-42	4*x*	Red	ND	ND	Yes	
NYW2-112	4*x*	Red	ND	ND	Yes	
NYW2-123	4*x*	Red	ND	ND	Yes	
NYW2-127	4*x*	Red	ND	ND	Yes	
NYW3-10	4*x*	Red	ND	ND	Yes	
NYW3-31	4*x*	Red	ND	ND	Yes	
NYY4-1	4*x*	Red	ND	ND	Yes	

[a] Color was scored visually. "Red" denotes the presence of red pigment. For flowers and sprouts this included colors that ranged from light pink to deep red
[b] Presence or absence of the *dfr* allele lacking the polymorphic *Bam*HI restriction site (see text for details)
[c] Not determined

cycles of 94°C, 15 s; 50°C, 15 s; and 72°C, 150 s. Amplification products were ligated into pGEM-T. One full-length insert from each amplification was chosen at random and sequenced in its entirety on both strands.

Results

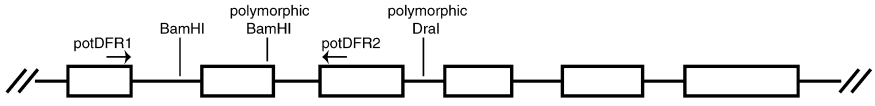
The red-skinned diploid potato clone 320-02 is heterozygous at the *R* locus (genotype *Rr*). To determine if 320-02 possesses two alleles of *dfr* a fragment of the gene was amplified with primers potDFR1 and potDFR2 (see Materials and methods). The resulting PCR products, approximately 530 base pairs (bp) in length, were cloned into a plasmid vector. Sequencing eight independent clones revealed the presence of two alleles, the sequences of which are shown in Fig. 1. Three of the clones matched allele 1 while the other five matched allele 2. The sequence data suggested that the two alleles would differ with regard to the presence or absence of a *Bam*HI restriction site (Fig. 1). The single C/T nucleotide polymorphism underlying the polymorphic *Bam*HI restriction site is in the wobble position of the 106th codon and is not predicted to change the amino acid sequence of DFR. To confirm the presence of this polymorphic site, 320-02 was again amplified with potDFR1 and potDFR2, and then uncloned products were digested with *Bam*HI (referred to hereafter as "the *Bam*HI assay"). The resulting banding pattern after electrophoretic separation on a 2% agarose gel was consistent with the presence of two alleles, with one allele yielding fragments of 370 and 162 bp and the other allele yielding fragments of 231, 158 and 144 bp (Fig. 2).

To determine if either allele could be related to red skin color 320-02 was selfed to yield an S_1 population of 56 individuals. Of these 39 had red skin and 17 had white skin. The *Bam*HI assay revealed that the 370-bp fragment was present in all 39 clones with red skin and absent in eight of the progeny with white skin (data not shown). The *R* locus in potato is necessary but not sufficient for the production of red anthocyanin pigments in tuber skin (Dodds and Long 1955, 1956). Such production also requires the action of a dominant allele at the *I* locus (Dodds and Long 1955, 1956). Because the 370-bp fragment was present in all red clones, *dfr* must be tightly linked to *R* or *I*. It is known that both *R* and *dfr* map to chromosome 2, while *I* maps to chromosome 10, so the linkage must be between *dfr* and *R*. The presence of the 370-bp fragment in nine of the white clones could be explained by assuming that some or all of these nine clones are homozygous recessive *ii*.

To further assess if *dfr* could be related to red skin color white-skinned diploid 07248-02, which does not exhibit the 370-bp fragment (Fig. 2), was crossed with 320-02 to yield an F_1 population of 199 individuals. Forty three of the progeny had red skin while the remaining 156 were white. The observed segregation ratio is consistent with 07248-02 having genotype *ii rr* and 320-02 having genotype *Ii Rr*. The 370-bp fragment was observed in all 43 red-skinned progeny as well as in 51 of the white-skinned clones (data not shown). The presence of the 370-bp fragment in all red-skinned progeny further established a tight genetic linkage between *dfr* and *R*.

A third population resulting from a cross between 79-48 (genotype *II* RR^{pw} *FF*) and 07506-*01 (ii rr ff*) was evaluated next. The *F* locus is required for the production

A



B

```
          ---- potDFR1 ------->
allele 1 GGCTCTTGGC TTGTCATGAG ACTCCTTGAA CGCGGTTATA ATGTCCATGC TACTGTTCGT  60
allele 2 GGCTCTTGGC TTGTCATGAG ACTCCTTGAA CGCGGTTATA ATGTCCATGC TACTGTTCGT  60

allele 1 GATCCTGgta cgttttcatt ttatttaaga gtttaacttc tatagat--- -tgtcaaata 116
allele 2 GATCCTGgta tgttttcgtt ttatttaa-- ------cttc tatagactgt ctgtcaaata 112

allele 1 atttataaac ccattaatca atctgtacaa agctgcattt tgaggatcca gagagatgtg 176
allele 2 atttataaac ccattaatca atccgtacaa agctgcattt tgaggatcca gagaagtgta 172

allele 1 tctcgttgt- ----atgcaa tgacccttat ataaagatgc atcaactttt gtagAGAACC 231
allele 2 tgacggtgct agaagttcaa tgacccttac ataaagatgc atgaactttt gtagAGAACC 232

allele 1 AGAAGAAGGT GAAACATCTG TTGGAACTGC CAAAAGCTGA TACAAACTTA ACGCTGTGGA 291
allele 1 AGAAGAAGGT GAAACATCTG TTGGAATTGC CAAAAGCTGA TACAAACTTA ACGCTGTGGA 292

allele 1 AAGCAGACTT GGCAGTGGAA GGAAGCTTTG ATGAAGCCAT TCAAGGCTGT CAAGGAGTAT 351
allele 2 AAGCAGACTT GGCAGTGGAA GGAAGCTTTG ATGAAGCCAT TCAAGGTTGT CAAGGAGTAT 352

allele 1 TTCATGTGGC TACACCGATG GATTTCGAGT CCAAGGACCC AGAGgtacta taaagtaaca 411
allele 2 TTCATGTGGC TACACCGATG GATTTCGAGT CCAAGGATCC AGAGgtacta taaagtaaca 412

allele 1 gagtaactca tcaatactaa tggacttctt acatttctgg atgattaata ctaacataac 471
allele 2 gagtaactca tcaatactta tggacttgtt acatttctgg atgattaata ctaacataac 472

allele 1 tcttatgaca ttgatatacg tgacagAACG AAGTAATTAA ACCAACAGTC AGGGGAATGC T 532
allele 2 tctcatgaca ttgatatacg tgacagAACG AAGTAATTAA ACCAACAGTC AGGGGAATGC T 533
                                                  <----- potDFR2 ----- -
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Fig. 1A,B Diagram of *dfr* gene organization and alignment of partial sequences of the two alleles of *dfr* present in diploid potato clone 320-02. **A** Schematic diagram of *dfr* gene organization. The *open boxes* represent coding sequence, which is divided among six exons. The locations where primers potDFR1 and potDFR2 anneal are shown, as are the locations of polymorphic *Bam*HI and *Dra*I restriction sites. The position of a conserved *Bam*HI restriction site is also shown. **B** Alignment of the sequence of two alleles of *dfr* present in diploid potato clone 320-02 between the annealing sites of primers potDFR1 and potDFR2. The regions where primers potDFR1 and potDFR2 anneal are highlighted with *hyphens and arrows*. Exon sequence is shown in *upper case type* while introns are denoted in *lower case*. Insertions or deletions of several nucleotides in one allele relative to the other are shown with *short dashes*. A *Bam*HI restriction site present in both alleles is underlined with a *thin line*. A polymorphic *Bam*HI site, present in allele 2 but absent in allele 1, is underlined with a *thick line*. The sequence of W5281.2 *dfr* in this region is identical to that shown for allele 1, except that the sequence of base 528 is G instead of A and base 531 is T instead of C

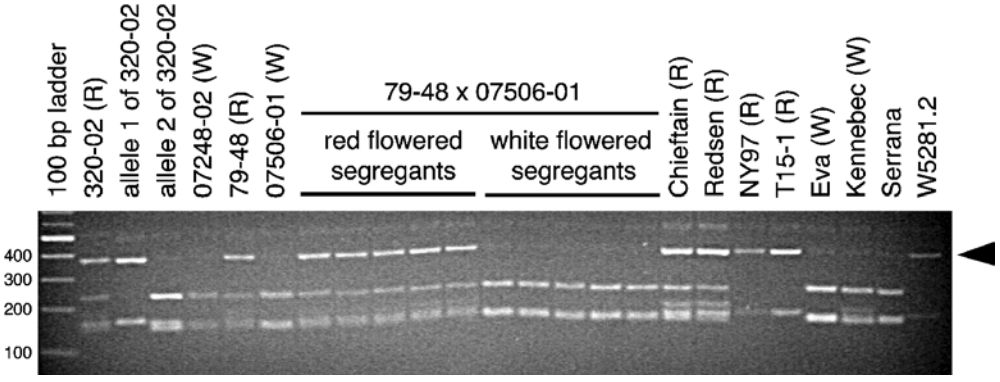

Fig. 2 Presence of a *dfr Bam*HI restriction fragment in red-colored potato clones and absence of this fragment in some white potato clones. Genomic DNA was amplified with primers potDFR1 and potDFR2, and then the products were digested with the restriction enzyme *Bam*HI. Digested products were separated on a 2% agarose gel, visualized by staining with ethidium bromide, and then photographed. DNA size markers, with the lengths of selected bands shown, were loaded in the *leftmost lane*. The allele 1 and allele 2 *lanes* show digestion products after amplification of cloned *dfr* fragments from 320-02 and correspond to allele 1 and allele 2 of Fig. 1. The following potato clones or cultivars have red tuber skin: 320-02, 79-48, Chieftain, Redsen, T15-1, NY97. Their names are followed by an R to indicate this. The following clones have white flowers and white tuber skin: 07248-02, 07506-01, Eva, Kennebec. Their names are followed by a W. Serrana has white tubers and pale-blue flowers. W5281.2 has purple-skinned tubers and is known to be homozygous *RR*. Digestion patterns for five red-flowered and five white-flowered progeny of 79-48×07506-01 are also shown

of anthocyanin pigments in potato flowers and is known to be tightly linked to *I* (Dodds and Long 1956). The phenotype of the R^{pw} allele has only been described to-date in several diploid species and is associated with the production of *p*ink-skinned tubers and *w*hite flowers (Dodds and Long 1955). The pink pigment in such tubers is derived from peonidin (a methylated derivative of cyanidin), not pelargonidin (Dodds and Long 1955). Of the 70 progeny evaluated, 28 had red flowers and red tubers while 42 had white flowers and pink tubers. 79-48 displayed a *Bam*HI fragment that migrated at the same position as the 370-bp fragment of 320-02, while 07506-01 did not exhibit a similar band (Fig. 2). Examination of their progeny revealed that this large fragment was present in all red-skinned/red-flowered clones and absent in all pink-skinned/white-flowered clones (some progeny are shown in Fig. 2). Thus, in the three segregating populations examined, the *Bam*HI assay revealed an apparently identical *dfr* restriction fragment in all progeny with red tuber skin.

Since 79-48 and 320-02 do not share any immediate ancestors yet nevertheless yield an indistinguishable 370-bp restriction fragment with the *Bam*HI assay, we wondered how widely distributed this fragment might be in other potato clones and whether the fragment would always be associated with the ability to produce red anthocyanin pigments. Accordingly a wide range of potato cultivars, breeding clones and diploid clones were evaluated with the *Bam*HI assay. All 46 cultivars and breeding clones tested with red skin, red flowers, and/or red sprouts (see Table 1) exhibited a *Bam*HI fragment indistinguishable in size from the 370-bp band of 320-02 (some clones are shown in Fig. 2). Two red-skinned breeding clones, NY97 and T15-1, appeared to be quadruplex for this allele since both clones yielded only two *Bam*HI fragments that co-migrated with the 370- and 162-bp fragments of 320-02 (Fig. 2). All six diploid clones tested with red skin or red tuber eyes also revealed fragments indistinguishable in size from the 370-bp fragments of 320-02 and 79-48 (data not shown).

Potato clones that do not produce any anthocyanin pigments in tubers, flowers or sprouts are relatively rare. The three such clones evaluated (Eva, Kennebec and NY121) did not display the 370-bp fragment (Fig. 2 and data not shown). Several potato clones (Lenape, Serrana, NY123, L235-4, 75-10 and 11364-09) appear, on the basis of their light blue flowers, to produce only blue petunidin-based pigments. If red pelargonidin-based pigments were also present the flowers would be expected to be purple. None of these six clones exhibited the large *Bam*HI fragment either (Fig. 2 and data not shown). Thus, similar to what was observed in the segregating diploid populations, an indistinguishable *dfr*-derived *Bam*HI restriction fragment was found in all potato cultivars, breeding clones and assorted diploid clones known to produce red pigments in their tuber skin, flowers and/or sprouts, while this fragment was absent in all potato clones tested that do not produce any red anthocyanins in the same tissues.

We also evaluated 21 white-skinned and white-flowered cultivars and breeding clones that produce purple pigments in tuber sprouts. The purple color suggests that all these plants possess a regulatory locus that directs expression of anthocyanin biosynthetic genes in sprouts, and that these plants can synthesize petunidin-based pigments; but because we have never observed blue-colored sprouts, even on plants with blue flowers, it was not possible to visually discern whether any of these plants also produced red pigment that might be masked by the production of purple pigment. Of the 21 clones, 14 did not display the 370-bp *Bam*HI fragment. The remaining seven (Pike, Salem, NY120, NY126, NYT28-1, NYU100-87 and NYU106-26) did reveal a large *Bam*HI fragment (data not shown).

To provide an independent test of whether unique or closely related alleles of *dfr* are associated with the ability to produce red pigments, we also subjected 17 potato clones to RFLP analysis, using a portion of the tomato *dfr* gene as a hybridization probe. As shown in Fig. 3 all tested clones known to produce red pigments displayed an identical RFLP marker allele approximately 3.6 kb in size after digestion with *Dra*I. This RFLP marker allele was absent in clones that do not produce red anthocyanins (Eva, Kennebec, Serrana, NY121, 07506-01) (Fig. 3). Probing *Sca*I-digested DNA blots yielded similar results; all clones known to produce red pigments shared a common RFLP band and this band was absent in clones free of red pigment (data not shown). Similar to the *Bam*HI data described above, conventional RFLP data also indicates that clones NY97 and T15-1 are quadruplex for the allele linked to red color as both clones displayed only the red-linked *Dra*I RFLP marker band (Fig. 3). Because the *Dra*I and *Sca*I tests assessed polymorphism distinct from the *Bam*HI assay, more than one sequence difference distinguishes the *dfr* allele(s) associated with red pigment production from all other *dfr* alleles.

The diploid clone W5281.2 is known to be homozygous *RR* (De Jong and Burns 1993). To provide a foundation for future comparison of *dfr* alleles a cDNA clone prepared from W5281.2 and a second cDNA clone obtained from the white-skinned cultivar Kennebec were completely sequenced (deposited in GenBank with accession numbers AY289921 and AY289922, respectively). These two cDNA clones as well as a third cDNA sequence from the purple-skinned cultivar Jashim (Jeon et al., unpublished; GenBank accession AF449422) are predicted to encode proteins of 382 amino acids (Fig. 4). The predicted W5281.2 gene product differs at ten amino acid positions from both Kennebec and Jashim (Fig. 4). The W5281.2 protein also differs at three additional positions from Kennebec, and at five additional positions from Jashim (Fig. 4). Of the three cDNA sequences only W5281.2 lacked the *Bam*HI restriction site highlighted in Figure 1. Curiously the W5281.2 cDNA sequence revealed two mismatches with the potDFR2 primer, albeit near the 5′ end of the primer, so that this primer is nevertheless able to participate in the amplification of this allele (Fig. 2). The sequence of

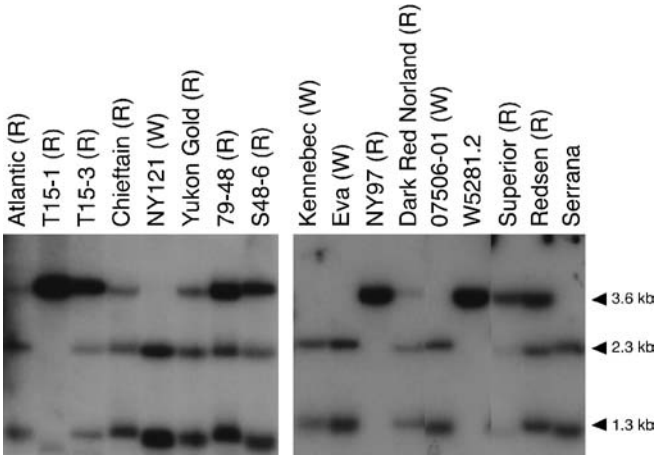

Fig. 3 RFLP analysis of potato *dfr*. Genomic DNA was digested with the restriction enzyme *Dra*I, separated by agarose-gel electrophoresis, blotted onto a nylon membrane, hybridized to a radioactively labeled tomato *dfr* gene fragment, and then the membrane was used to expose X-ray film. The samples loaded are shown above each lane. *The left and right hand panels* represent data from different experiments. Approximate sizes of restriction fragments are shown to the right. Clones denoted with an R have either red tubers or red flowers, or both. Clones denoted with a W have white tubers, flowers, and sprouts. Serrana has white tubers and blue flowers. W5281.2 has purple-skinned tubers and flowers and is known to be homozygous *RR*

"allele 1" shown in Fig. 1 was obtained from a PCR product where potDFR2 was one of the amplification primers. Thus if allele 1 shares these two nucleotide differences with W5281.2 they would not have been apparent when PCR products of allele 1 were sequenced.

Primers based on the 5′ and 3′ non-coding regions of W5281.2 and the Kennebec cDNA sequences were used to amplify genomic DNA between the translational start and stop codons of W5281.2 and 07506-01. Primers based on the Kennebec cDNA sequence were also used to amplify the *dfr* allele linked to R^{pw} in 79-48. The PCR products were cloned into a plasmid vector and then one randomly selected full-length clone from each genotype was completely sequenced (sequences not shown; genomic DNA sequence data for W5281.2 and 07506-01 have been deposited in GenBank with accession numbers AY289923 and AY289924, respectively). The sequence of W5281.2 in the region between the potDFR1 and potDFR2 annealing sites was found to be identical to the sequence of allele 1 shown in Fig. 1. The corresponding sequence of 07506-01 differs at several nucleotide positions from both allele 1 and allele 2 of Fig. 1, as well as from the Jashim cDNA sequence, and thus may represent a fourth allele of *dfr*. Interestingly, the genomic sequence of the *dfr* allele linked to R^{pw} in 79-48 was identical to the genomic sequence from 07506-01. Regions corresponding to the predicted exons of 07506-01 and 79-48 were identical to the cDNA sequence of Kennebec. A basis for the different *Dra*I RFLP patterns of Fig. 3 was found by comparing the genomic DNA sequence from W5281.2, 07506-01 and 79-48. W5281.2 contains no *Dra*I restriction sites within the gene, while 07506-01 and 79-48 carry a *Dra*I site approximately 350 bases downstream of the polymorphic *Bam*HI site, in the third intron (Fig. 1).

Discussion

The potato *R* locus encodes a basic factor required for the production of red pelargonidin-based anthocyanin pigments. To test the hypothesis that *R* encodes dihydroflavonol 4-reductase we monitored the segregation of

Fig. 4 Comparison of predicted potato DFR protein sequences. cDNA clones isolated from homozygous *RR* diploid W5281.2 (GenBank accession AY289921) and white-skinned tetraploid cultivar Kennebec (accession AY289922) were completely sequenced. Their predicted gene products and that from a *dfr* cDNA isolated from the potato cultivar Jashim (accession AF449422) are shown aligned. Each of the ten amino acid positions where W5281.2 DFR differs from both Kennebec and Jashim is highlighted in *black*. Other amino acid positions that are not conserved in these three alleles are highlighted in *gray*

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W5281.2     MASEVHAVVD AHSPPKTPTV CVTGAAGFIG SWLVMRLLER GYNVHATVRD PENQKKVKHL  60
Kennebec    MASEVHSVVD AHSPPKTPTV CVTGAAGFIG SWLVMRLLER GYNVHATVRD PENQKKVKHL
Jashim      MASEVHAVVD AHSPPKTPTV CVTGAAGFIG SWLVMRLLER GYNVHATVRD PENQKKVKHL

W5281.2     LELPKADTNL TLWKADLAVE GSFDEAIQGC QGVFHVATPM DFESKDPENE VIKPTVRGVL  120
Kennebec    LELPKADTNL TLWKADLAVE GSFDEAIQGC QGVFHVATPM DFESKDPENE VIKPTVRGML
Jashim      LELPKADTNL TLWKADLAVE GSFDEAIKGC QGVFHVATPM DFESKDPENE VIQPTVRGML

W5281.2     SIIESCAKAN TVKRLVFTSS AGALDVQEDQ KLPCDETSWS DLDFIYAKKM TGWMYFVSKI  180
Kennebec    SIIESCAKAN TVKRLVFTSS AGTLDVQEDQ KLFYDETSWS DLDFIYAKKM TGWMYFVSKI
Jashim      SIIESCAKAK TVKRLVFTSS AGTLDVQEDQ KLFYDETSWS DLDFIYAKKM TGWMYFVSKI

W5281.2     LAEKAAMEEA KKNNIDLISI IPPLVVGPFI TPTFPPSLIT ALSLITGNEA HYGIIKQGQY  240
Kennebec    LAEKAAMKEA KKNNINFISI IPPLVVGPFI TPTFPPSLIT ALSLITGNEA HYGIIKQGQY
Jashim      LAEKAAMEEA KKNNINFISI IPPLVVGPFI TPTFPPSLIT ALSLITGNEA HYGIIKQGQY

W5281.2     VHLDDLCEAH IFLYEHPKAE GRFICSSHHA IIYDVAKMVR QKWPEYYVPT EFKGIDKDLP  300
Kennebec    VHLDDLCEAH IFLYEHPKAE GRFICSSHHA IIYDVAKMVR QKWPEYYVPT EFKGIDKDLP
Jashim      VHLDDLCEAH IFLYEHPKAE GRFICSSHHA IIYDVAKMVR QKWPEYYVPT EFKGIDKDLP

W5281.2     IVSFSSKKLM DMGFLFKYTL EDMYKGAIET CRQKQLLPFS TQSTADNGRD KEAIPIPTEN  360
Kennebec    IVSFSSKKLM DMGFQFKYTL EDMYKGAIET CRQKQLLPFS TRSSADNGKD KEAIPISTEN
Jashim      IVSFSSKKLM DMGFQFKYTL EDMYKGAIET CRQKQLLPFH TRSTAANGKD KEAIPITTEN

W5281.2     YSSGKENAPV ANCTGKLTNG EI  382
Kennebec    YSSGKENAPV ANCTGKFTNG EI
Jashim      YSSGKENAPV ANCTGKFTNG EI
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dfr alleles in three diploid populations, as well as in a diverse set of diploid and tetraploid germplasm. In every case examined, potato clones known to produce red anthocyanins were found to contain an allele of *dfr* that lacks a *Bam*HI restriction site just upstream of the gene's second intron. The corresponding 370-bp *Bam*HI restriction fragment was absent in many white-skinned and white-flowered segregants. The notion that *R* might encode DFR was motivated by the precedent of DFR substrate specificity in *Petunia* (Forkmann and Ruhnau 1987) and *Cymbidium* (Johnson et al. 1999). In these two genera it is known that DFR can efficiently reduce dihydroquercitin (DHQ) en route to producing cyanidin-based pigments, and dihydromyricetin (DHM) en route to producing delphinidin-derived pigments, but not dihydrokaempferol (DHK), a step required to produce red pelargonidin-based pigments. DHK, DHQ and DHM are similar in structure, differing only in the presence of one or two hydroxyl groups on a phenyl ring that is not the site of enzymatic action. The *dfr* gene is known to be expressed in red tuber skin (Hung et al. 1997). Although the present study does not provide formal proof, given the combination of the biochemical precedent and the absolute co-segregation reported here, it appears very likely that *R* does code for DFR. It should be noted that because the polymorphic *Bam*HI and *Dra*I restriction sites described in this study are not expected to change the amino acid sequence of DFR, the functional polymorphism(s) responsible for red color almost certainly reside elsewhere within *dfr* or, if *R* does not encode DFR, in some other tightly linked gene.

Several alleles have previously been described at the *R* locus including the dominant *R*, associated with the ability to produce red pelargonidin pigments in tubers; R^{pw}, recessive to *R* but dominant to *r*, and associated with the production of peonidin-based pigments in tubers; and recessive *r*, associated with the absence of red tuber anthocyanins (Dodds and Long 1955; De Jong 1991). In the current study we found that the DNA sequences of *dfr* associated with *r* in 07506-01 and with R^{pw} in 79-48 were indistinguishable. This result was not a complete surprise. At the biochemical level the key difference between pelargonidin and peonidin synthesis is that peonidin requires flavonoid 3′-hydroxylase (F3′H) activity (Holton and Cornish 1995). F3′H catalyzes the hydroxylation of DHK, creating DHQ. The differential requirement for F3′H activity could be interpreted to indicate that the *R* locus codes for F3′H, although it is difficult to postulate natural variation in F3′H that would account for the spectrum of phenotypes and dominance relationships observed among *R*, R^{pw} and *r*. A plausible explanation can be devised, however, by postulating that the primary basis for the observed variation in tuber color results from the substrate specificity of DFR, and that superimposed on this is natural variation for tuber expression of F3′H. In this model the *dfr* allele present in all red-skinned potatoes encodes an isozyme that is able to utilize DHK as a substrate; this permits the synthesis of pelargonidins (the *R* phenotype). In genetic backgrounds that lack the red allele of *dfr* but do express F3′H, e.g. some clones of *S. phureja* and *S. stenotomum*, DHQ will be synthesized. Because DHQ can be utilized by all alleles of *dfr*, this permits the synthesis of pink peonidin-based anthocyanins (the R^{pw} phenotype). When neither DHK-competent DFR nor F3′H activities are present, no pink or red anthocyanins are produced (the *r* phenotype). Testing of this model will require extensive additional analyses at the biochemical level.

Domain swapping experiments between *Gerbera* and *Petunia* have shown that the ability of DFR to metabolize DHK resides in the first 170 amino acids (Johnson et al. 2001). Moreover, altering a single amino acid at position 134 has been shown to affect substrate specificity. The resulting *Gerbera* point mutant preferentially utilized DHK over DHQ and was no longer able to utilize DHM (Johnson et al. 2001). *Gerbera* amino acid position 134 corresponds to potato position 145. There are two differences in W5281.2 relative to Kennebec and Jashim near 145, at positions 143 and 154; both of these changes are in the third exon. If the red-linked *dfr* can metabolize DHK, these changes may be responsible.

Independent of whether or not *R* actually codes for DFR, from the perspective of potato domestication and current breeding, it is nevertheless intriguing that all red-colored potato cultivars appear to share an identical allele of this gene. Both the PCR/*Bam*HI test and conventional RFLP evaluation with *Dra*I and *Sca*I revealed indistinguishable bands in all red-colored clones tested. In the course of developing a TaqMan assay to measure dosage of the red-linked *dfr* allele we have also found that an oligonucleotide probe identical to bases 424 through 443 of allele 1 (numbering as in Fig. 1) specifically anneals to DNA from genotypes that generate the 370-bp PCR/*Bam*HI fragment (De Jong et al. 2003). Dodds and Long (1955) have previously noted that although pelargonidin-based pigments are present in many cultivated diploid and tetraploid potatoes they have never been reported in wild potato species. From this observation they hypothesized that the ability to produce red pigments in cultivated potatoes only arose once. They further postulated that a mutation conferring this ability was selected at the diploid level during domestication. Thus the gene controlling red color in modern cultivated tetraploids would be directly descended from the gene conferring color in the first domesticated red diploid. The data presented in the current study is consistent with the Dodds and Long scenario. One of the diploid clones evaluated, 79-48, has only two cultivated diploids, *S. phureja* and *S. stenotomum,* in its ancestry. As evident in Figs. 2 and 3, PCR/*Bam*HI and RFLP analyses revealed no differences between the red-linked allele of 79-48 and the allele shared by all modern red (tetraploid) cultivars.

The existence of a shared *dfr* allele also raises the question of whether alleles of any genes tightly linked to *dfr*, and present in the putative diploid ancestor, are also present in most or all modern red-colored potato cultivars. Because potato is clonally propagated it has undergone many fewer meioses during domestication than seed-

propagated crops like maize. Infrequent sexual reproduction could lead to multiple gene-containing linkage blocks that have never been separated by recombination. Further work to investigate whether there are indeed such linkage blocks in modern cultivars and, if so, determining how large such blocks are, would be of practical value to breeders in defining the genomic architecture of modern potato.

Potato breeding clones that are quadruplex for a desirable allele are valuable because they transmit the corresponding trait to all progeny. Two tetraploid potato clones evaluated in this study, NY97 and T15-1, are quadruplex for the red-linked allele of *dfr*, and thus may be of value in developing new red-skinned cultivars. The need for quadruplex *RRRR* and/or *IIII* clones is particularly acute when developing true potato seed (TPS) "varieties" that do not segregate for skin color. Several years ago while evaluating a breeding cross between NY97 and a North Dakota clone, ND2225-IR, we observed that all 184 progeny had red skin. When crossed with other clones both NY97 and ND2225-IR yield some white-skinned progeny. Taken together these observations suggest that one of these parents (NY97) is *RRRR* and the other (ND2225-IR) is *IIII*. The *Bam*HI assay described in this study provides a relatively simple means for identifying quadruplex *RRRR* clones. Should the absence of red color be desirable, the assay could also be used to identify *rrrr* individuals.

Acknowledgements We thank S.D. Tanksley for providing the Kennebec cDNA clone and K. Paddock for maintaining the plants in the field. This work was supported in part with Federal Hatch funds provided to W.D.

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