Theor Appl Genet (2003) 107:1384–1390
DOI 10.1007/s00122-003-1420-z

ORIGINAL PAPER

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A fluorogenic 5′ nuclease (TaqMan) assay to assess dosage of a marker tightly linked to red skin color in autotetraploid potato

Received: 30 December 2002 / Accepted: 31 May 2003 / Published online: 12 September 2003

Abstract We have recently identified an allele of dihydroflavonol 4-reductase (*dfr*) that cosegregates with the ability of potato (*Solanum tuberosum* L) to produce red pelargonidin-based anthocyanin pigments. A rapid assay to assess dosage of this allele in cultivated potato, an autotetraploid, would be useful for breeding programs that develop red-skinned cultivars. To identify regions of *dfr* that are conserved between alleles, as well as regions that are variable, a portion of the gene was sequenced from several cultivated and wild potato clones. In one region the sequence of the 'red' *dfr* allele differed at two nucleotide positions from the three other sequence classes observed. A fluorogenic oligonucleotide probe labeled with 6-FAM was designed to anneal specifically to the red allele in this region, while a second probe labeled with VIC was designed to anneal to the 'not-red' *dfr* alleles. PCR primers that annealed to conserved sequences flanking the variable region were also developed. When subjected to a fluorogenic 5′ nuclease (TaqMan) allelic discrimination assay all diploid clones tested clustered into three distinct groups based on the relative amounts of FAM and VIC released. These three groups represented clones homozygous for the red allele, heterozygous for the red allele, and homozygous for the not-red allele(s). When tetraploid clones were tested they separated into five distinct clusters, three of which were shared with diploid clones. The five clusters were interpreted to represent clones quadruplex, triplex, duplex, simplex and nulliplex for the red *dfr* allele. This interpretation was supported by monitoring the segregation of red allele dosage in several tetraploid crosses. To the best of our knowledge this is the first report of a fluorogenic 5′ nuclease assay being used for allelic discrimination in an autopolyploid.

Communicated by J.W. Snape

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Introduction

Issues related to allele dosage complicate genetic analyses and breeding in autotetraploid crops like cultivated potato (*Solanum tuberosum* L.). Unlike diploid individuals, which can harbor just one or two alleles at any locus, autotetraploid plants can carry up to four alleles, in a wide range of combinations. In the simplest case, where just two alleles are considered, an autotetraploid may be nulliplex (*aaaa*), simplex (*Aaaa*), duplex (*AAaa*), triplex (*AAAa*) or quadruplex (*AAAA*). For breeding it is often useful to know the dosage of desirable alleles in parental clones because transmission frequencies are determined primarily by allele copy number. When a locus is tightly linked to the centromere, so that double reduction does not occur, simplex clones will transmit an allele to half of their progeny while duplex parents will transmit it to five-sixths. Clones that are triplex or quadruplex transmit alleles of interest to all progeny, or most progeny, in the case of a triplex parent subject to double reduction. In some cases dosage also influences trait expression, e.g., the enzyme activity of potato granule-bound starch synthase is linearly correlated with dosage of the wild-type allele (Flipse et al. 1996).

At present allele dosage in potato is normally determined through the time-consuming process of making multiple crosses and analyzing resulting segregation ratios (e.g., Mendoza et al. 1996). One recent study described a more rapid alternative, pyrosequencing, for quantitating allele dosage in tetraploid potato (Rickert et al. 2002). Although powerful this method requires extensive sample processing as well as highly specialized and costly equipment. In contrast fluorogenic 5′ nuclease (TaqMan; Applied Biosystems, Foster City, Calif.) assays require no post-PCR sample processing and are employed by many investigators for real-time PCR, as well as for allelic discrimination assays (Meksem et al. 2001; Salvi et

al. 2001). While not inexpensive the necessary equipment is much more widely available. Fluorogenic nuclease assays exploit the 5′ to 3′ nucleolytic activity of *Taq* DNA polymerase to cleave fluorescent dyes from the 5′ end of fluorescence-quenching oligonucleotide probes annealed to target DNA (Livak et al. 1995). The probes are designed to complement internal regions of PCR products so that when levels of targets increase during amplification, increasing amounts of fluorescent dye are released into solution. Allelic discrimination assays utilize multiple probes, each labeled with a different dye, in a single tube (Livak 1999). After PCR the relative dosage of each allele is determined by comparing the amount of fluorescence released from each probe (Livak 1999). A significant recent advance in fluorogenic nuclease methodology was the development of minor groove binders (MGBs) that can be conjugated to the 3′ end of oligonucleotide probes. MGBs dramatically increase the affinity of short oligonucleotide probes for their target sequence (Kutyavin et al. 2000). MGBs also serve to increase the difference in melting temperature between a perfectly annealed probe and one with a sequence mismatch, thereby enhancing specificity in *Taq*Man assays (Kutyavin et al. 2000).

We have recently identified an allele of dihydroflavonol 4-reductase (*dfr*) that cosegregates (De Jong et al. 2003) with the dominant allele at *R*, a potato locus required for the production of red pelargonidin-based anthocyanin pigments (Dodds and Long 1955). Because anthocyanin synthesis requires *dfr*, this gene constitutes a good candidate for the *R* locus. High dosage at *R* is desirable in parental clones since it increases the frequency of red-skinned progeny. By employing a cleaved amplified polymorphic sequence assay (Konieczny and Ausubel 1993) we have been able to determine whether clones are nulliplex or quadruplex for the 'red' *dfr* allele, but have not been able to reliably quantify intermediate states of dosage.

To the best of our knowledge fluorogenic 5′ nuclease assays have not previously been employed to discriminate between alleles in any autopolyploid. To determine if this methodology can be used to distinguish between nulliplex, simplex, duplex, triplex and quadruplex dosage states in general, and at *R* in particular, two fluorogenic oligonucleotide probes were constructed, one specific for the red *dfr* allele and the other diagnostic for all other potato *dfr* alleles. A MGB was included at the 3′ end of each probe to enhance binding specificity. We report here that the five genotypic classes could clearly be differentiated in tetraploid potato, demonstrating that it is possible to use a fluorogenic 5′ nuclease assay for allelic discrimination in an autopolyploid. We report further that dosage of the red-linked *dfr* allele is positively correlated with intensity of red skin color, although the contribution of dosage to skin color is not large.

Materials and methods

DNA isolation

Template DNA for PCR amplification from named cultivars, breeding clones and other *Solanum* species was isolated from young leaf material with a DNeasy plant mini kit (Qiagen). For analysis of segregating populations DNA was isolated either with the DNeasy kit or with the rapid miniprep method of Edwards et al. (1991).

Plant material

Leaf material was sampled from potato cultivars and advanced clones being maintained or evaluated by the Cornell University potato variety development program. *Solanum andigena* PI 160215, *Solanum stenotomum* PI 234008 and *Solanum phureja* PI 225676 were obtained from the US Potato Genebank (NRSP-6) (Sturgeon Bay, Wis.). Leaves were pooled from three clones of each accession before DNA isolation. DNA from *Solanum microdontum* (CPC No. 2551) was isolated from a clone maintained by the Commonwealth Potato Collection (Scottish Crop Research Institute, Invergowrie, UK). Diploid clones W5281.2, 07506-01, 79-48, 320-02, AC Candy Cane, BPH32-03, CH072-03, CIP590001.7, DW84-1457, W5295.7. 07248-02, 09429-01, 09433-09, 09459-07, 09465-03, 09479-05, 09901-01, 09904-04, 09970-02, 10602-02, 10607-01, 10610-03, 10618-01, 10875-04, 10908-05, 10909-18, 11364-22, 11426-08, 11448-02, 11827-09, 11910-25, 12060-14, 12066-05, 12334-07, 12613-02, 12586-05, 12901-01, 12901-02, 12936-04 and 12937-03 were kindly provided by H. De Jong (Potato Research Centre, Agriculture and Agri-Food Canada). W5281.2 is homozygous dominant *RR* (De Jong and Burns 1993), 07506-01 is homozygous recessive *rr* (De Jong 1987; De Jong and Burns 1993), 79-48 is heterozygous RR^{pw} (De Jong 1987) and 320-02 is heterozygous *Rr* (unpublished data). Crosses were made between Eva and NY97, and between NY118 and Redsen, to develop F_1 populations segregating for the *dfr* alleles of each parent. A cross between Eva and Redsen was made to evaluate the influence of red allele dosage on the intensity of red skin color. Seedlings from the latter cross were started indoors in the spring, transplanted to six-inch pots containing an artificial soil mix (1:2 peat:vermiculite, plus lime and fertilizer), and then grown outdoors during the summer. Tubers were harvested in the fall. All progeny with white-skinned tubers were discarded. The tubers from red-skinned progeny were visually evaluated and the clones sorted in order of skin color, from darkest to lightest.

Sample sequencing of *dfr* from multiple sources

Primers potDFR1 (5′*GGCTCTTGGCTTGTCATGAG*) and potDFR2 (5′*AGCATTCCCCTGACTGTTGG*) were used to amplify an approximately 530 base-pair fragment of the *dfr* gene from PI 160215, PI 234008, PI 225676, CPC No. 2551, W5281.2, the cultivar Eva and breeding clone NY97. The approximately 530-bp PCR amplification products were cloned into vector pGEM-T (Promega). We have previously shown that a polymorphic *Bam*HI restriction site differentiates some alleles of *dfr* (De Jong et al. 2003). Prior to sequencing, cloned *dfr* gene fragments were screened with *Bam*HI. If *dfr* clones possessing the *Bam*HI site and clones lacking the site were both observed from a potato clone or accession, then at least one *dfr* clone of each type was sequenced. Up to four independent clones were sequenced in one direction from each genotype or accession. A total of 21 sequences were aligned and compared with each other, as well as to previously sequenced alleles from 320-02 and 07506-01, using LaserGene sequence-analysis software (DNASTAR, Madison, Wis.). Single nucleotide differences in one sequence relative to all other sequences were occasionally observed and were assumed to result from *Taq* DNA polymerase-mediated copying errors

(Bracho et al. 1998). Such singletons were ignored for the purpose of classifying sequences into distinct haplotypes.

Assay to assess dosage of red-linked *dfr* in diploid clones

Genomic DNA from all diploid clones was amplified by PCR with primers potDFR1 and potDFR2. The resulting PCR products were digested with *Bam*HI. Clones that revealed just two fragments of 370 and 162 bp after digestion were judged to be homozygous for the red allele of *dfr*. Clones that did not display the 370- and 162-bp fragments were deemed to be homozygous for not-red allele(s) of *dfr*. Clones that exhibited 370- and 162-bp fragments together with other fragments (approximately 230-, 160- and 145-bp in size) were considered to be heterozygous for the red allele of *dfr*. This assay and its rationale is described more fully elsewhere (De Jong et al. 2003).

Fluorogenic 5′ nuclease allelic discrimination assay

Primer Express Version 1.5 software (Applied Biosystems) was used to design probes and amplification primers (Fig. 1). One probe (*5′AATACTAATGGACTTCTTAC*, labeled at the 5′ end with the fluorophore 6-FAM), was used to quantify levels of the red allele of *dfr*. A second probe (*5′CAATACTTATGGACTTGTTAC*, labeled at the 5′ end with the fluorescent reporter VIC) was used to quantify the combined dosage of all other known alleles of *dfr*. Both probes were conjugated, at their 3′ ends, to a minor groove binder and a non-fluorescent quencher, and were purchased from Applied Biosystems. DFRFOR4 (*5′CCGATGGATTTCGAGTCCAA* and DFRREV3 (*5′CTTCGTTCTGTCACGTATATCAATGTC*) were used as amplification primers. Amplifications were performed in a total volume of 50 μl or 25 μl (both worked acceptably well) using TaqMan Universal PCR Master Mix (Applied Biosystems, catalog 4324018) with each primer at a concentration of 900 nM and each probe at a concentration 200 nM. Thermal cycling parameters for the assay were 40 cycles of (95°C, 15 s; 62°C, 1 min). After amplification the fluorescence of samples was measured with an ABI PRISM 7900HT Sequence Detection System and analyzed using SDS software version 2.0 (Applied Biosystems). This software automatically normalizes (R_n) fluorescence signals relative to a passive reference dye, a component of the Universal PCR Master Mix.

Results

We have recently found that red-colored potatoes appear to possess an identical allele of *dfr*. This allele differs from all other alleles in that it lacks a specific *Bam*HI restriction site (De Jong et al. 2003). To further test the hypothesis that red-colored potatoes carry an identical allele of *dfr*, as well as to obtain comparative sequence information from other alleles necessary for developing a fluorogenic 5′ nuclease assay, a *dfr* fragment approximately 530-bp long was amplified, cloned and sequenced from a wide range of *Solanum* germplasm. Clones and accessions sequenced included the potato cultivar Eva, the Cornell breeding clone NY97, one cultivated tetraploid species (*S. andigena*), two cultivated diploid species (*S. phureja* and *S. stenotomum*) and a non-cultivated diploid (*S. microdontum*). The accessions of the cultivated species selected, as well as NY97, are known to produce red anthocyanin pigments, while Eva and *S. microdontum* do not.

```
          -- DFRFOR4 --------->     *
haplotype 1   CCGATGGATT TCGAGTCCAA GGACCCAGAG gtactataaa gtaacagagt  50
haplotype 2   CCGATGGATT TCGAGTCCAA GGATCCAGAG gtactataaa gtaacagagt
haplotype 3   CCGATGGATT TCGAGTCCAA GGATCCAGAG gtactataaa gtaacagagt

                        *         *                                 *
haplotype 1   aactcatcaa tactaatgga cttcttacat ttctggatga ttaatactaa 100
haplotype 2   aactcatcaa tacttatgga cttgttacat ttctggatga ttaatactaa
haplotype 3   aactcatcaa tacttatgga cttgttacat ttctggatga ttaatactag

              6-FAM-AA TACTAATGGA CTTCTTAC-NFQMGB   red probe
               VIC-CAA TACTTATGGA CTTGTTAC-NFQMGB   not-red probe

                   *
haplotype 1   cataactctt atgacattga tatacgtgac agAACGAAG 139
haplotype 2   cataactctc atgacattga tatacgtgac agAACGAAG
haplotype 3   cataactctc atgacattga tatacgtgac agAACGAAG
                        <-------- DFRREV3 -- ---------
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Fig. 1 Alignment of three *dfr* haplotypes and position of primers and probes used in the 5′ fluorogenic nuclease assay. Fragments of *dfr* were sequenced from several *Solanum* clones and aligned. Haplotype 1 was detected in breeding clone NY97, diploid *S. phureja*, tetraploid *S. andigena* and diploid *S. stenotomum*, and appears to be present in all red-skinned potatoes (De Jong et al. 2003). Haplotype 2 was detected in the cultivar Eva, diploid wild species *S. microdontum* and diploid clone 320-02. The sequence of 320-02 differs in sequence from Eva and *S. microdontum* at positions not shown in this alignment, thus haplotype 2 represents at least two alleles. Haplotype 3 was detected in *S. andigena* and *S. phureja*. The positions where amplification primers DFRFOR4 and DFRREV3 anneal are shown. The sequences of the 'red probe' and the 'not-red' probes are also shown. The red probe was labeled with fluorophore 6-FAM, the not-red probe with VIC. Both probes were conjugated to a nonfluorescent quencher (NFQ) and minor groove binder (MGB). *Asterisks* denote positions where the sequence varies between the three haplotypes. A polymorphic *Bam*HI restriction site, absent in the red-linked haplotype, is *underlined*. The location of an intron in the alignment is indicated with *lower case type*

A total of 14 cloned *dfr* fragments lacking the *Bam*HI site were sequenced from NY97, *S. phureja*, *S. andigena* and *S. stenotomum*. All 14 shared an identical sequence. The same *dfr* sequence has previously been described for the allele of diploid clone 320-02 that is present in all red-skinned progeny of 320-02 (De Jong et al. 2003). The *dfr* allele of 320-02 that is not associated with red skin color has also been (partially) sequenced previously (De Jong et al. 2003). Seven cloned *dfr* fragments possessing the *Bam*HI restriction site were sequenced from Eva, *S. phureja*, *S. andigena* and *S. microdontum.* None of the seven were identical in sequence to either allele of 320-02. The four sequenced *dfr* clones from Eva and one sequenced *dfr* clone from *S. microdontum* were identical. The sequence of *S. phureja* and *S. andigena dfr* clones were identical to each other, as well as to the sequence previously obtained from diploid clone 07506-01, but were distinct from Eva and *S. microdontum*. Thus, a total of four *dfr* sequence classes were observed, one of which has previously been found to be associated with the ability to produce red anthocyanin pigments.

Figure 1 shows an alignment of 139 nucleotides from the four sequence classes of *dfr*, and summarizes both the sequence conservation and the sequence variation utilized in developing a fluorogenic 5′ nuclease assay. Note that because two of the four sequence classes were identical in this region, the alignment shows only three haplotypes. Amplification primers DFRFOR4 and DFRREV3 were

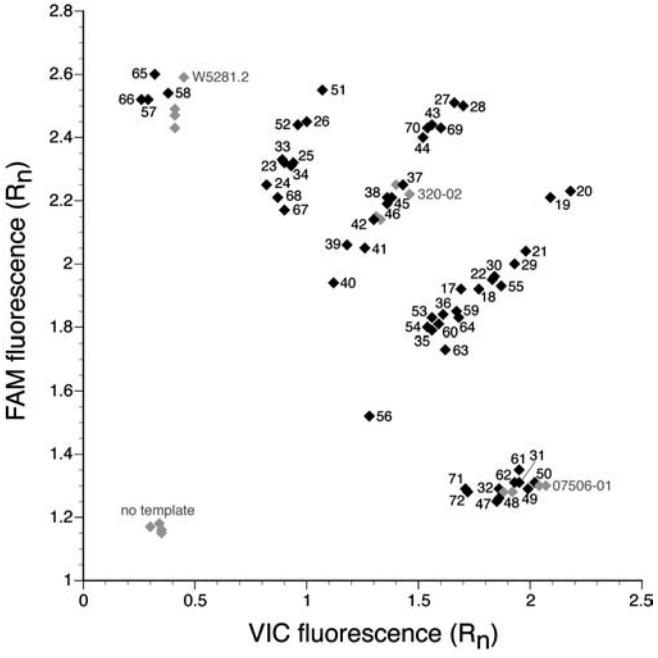

Fig. 2 Scatter plot illustrating normalized (R_n) 6-FAM and VIC fluorescence signals from a representative fluorogenic 5′ nuclease allelic discrimination assay with diploid and tetraploid potatoes. Signals from standards and no template controls are shown with *grey diamonds*: diploid W5281.2 is homozygous *RR*, diploid 320-02 is heterozygous *Rr* and diploid 07506-01 is homozygous *rr*. Signals from other genotypes, all replicated twice, are shown with *black diamonds*. Identities of numbered data points: Atlantic (*17*, *18*), Chieftain (*19*, *20*), Chippewa (*21*,*22*), Dakota Rose (*23*, *24*), Idarose (*25*, *26*), Kanona (*27*, *28*), Katahdin (*29*, *30*), Kennebec (*31*, *32*), Nordonna (*33*, *34*), Dark Red Norland (*35*, *36*), Pike (*37*, *38*), Red Companion (*39*, *40*), Red Pearl (*41*, *42*), Redsen (*43*, *44*), Rosa (*45*, *46*), Snowden (*47*, *48*), Stirling (*49*, *50*), Superior (*51*, *52*), Yagana (*53*, *54*), Yukon Gold (*55*, *56*), NY97 (*57*, *58*), NY99 (*59*, *60*), Marcy (*61*, *62*), NY126 (*63*, *64*), T15-1 (*65*, *66*), T15-3 (*67*, *68*), diploid 79-48 (*69*, *70*), diploid 07248-02 (*71*, *72*)

designed to anneal to regions conserved in all four *dfr* sequence classes. An oligonucleotide coupled to the fluorophore 6-FAM (the 'red' probe) was designed to anneal specifically to the *dfr* sequence known to be associated with red color, while a second oligonucleotide coupled to the fluorescent dye VIC (the 'not-red' probe) was constructed to anneal to all remaining *dfr* sequence classes. To help ensure binding-specificity the red and not-red probes were deliberately designed to anneal to a region where the red *dfr* sequence differed by two nucleotide positions from all other *dfr* sequences.

Before testing these primers and probes in a fluorogenic 5′ nuclease assay we first assessed the allelic configuration at *dfr* in 40 diploid clones with an independent PCR + restriction digestion assay (see Materials and methods). Briefly, genomic DNA was amplified using primers potDFR1 and potDFR2, the PCR products were digested with *Bam*HI, and then the digestion products were visualized on an ethidium bromide-stained agarose gel. Based on the resulting fragment patterns six of these diploid clones (typified by clone W5281.2) were found to be homozygous for the red allele of *dfr*, four (typified by clone 07506-01) were found to be homozygous for not-red allele(s) of *dfr*, and the remaining 30 (typified by clone 320-02) were found to be heterozygous, possessing both red and not-red alleles (data not shown). When these same diploid clones were subjected to a fluorogenic 5′ nuclease allelic discrimination assay they were observed to separate into three distinct clusters, based on the ratio of FAM to VIC fluorescence for each sample (Fig. 2 and data not shown). As expected, the six diploid clones known to be homozygous red exhibited high FAM:VIC fluorescence signal ratios, while the four homozygous not-red clones exhibited low FAM:VIC ratios. The remaining 30 heterozygous clones clustered together and exhibited intermediate FAM:VIC signal ratios.

In contrast, when the same allelic discrimination assay was applied to a large number of tetraploid clones, five distinct clusters were observed (Fig. 2). Three of these groups corresponded to the diploid groups defined by W5281.2 (*RR*), 320-02 (*Rr*) and 07506-01 (*rr*). The tetraploids that clustered with these three diploids were interpreted to be quadruplex, duplex and nulliplex for the red allele, respectively. Other tetraploid clones were observed in two additional clusters, halfway between W5281.2 and 320-02 or halfway between 320-02 and 07506-01. Given their relative positions these two clusters were interpreted to correspond to triplex and simplex dosages, respectively. A summary of the inferred genotype at the *R* locus in selected tetraploid potato cultivars and breeding clones, based on analyses of *dfr* allele dosage like that shown in Fig. 2, is provided in Table 1.

Table 1 Inferred genotype at *R* in several potato cultivars and breeding clones

Clone name	Dosage	Clone name	Dosage
Allegany	*RRrr*	Redsen	*RRrr*
Andover	*rrrr*	Rideau	*Rrrr*
Atlantic	*Rrrr*	Rosa	*RRrr*
Chieftain	*Rrrr*	Russett Bake King	*Rrrr*
Chippewa	*Rrrr*	Salem	*RRrr*
Dakota Rose	*RRRr*	Sebago	*Rrrr*
Elba	*Rrrr*	Serrana	*rrrr*
Eva	*rrrr*	Snowden	*rrrr*
Genesee	*rrrr*	Stirling	*rrrr*
Green Mountain	*rrrr*	Superior	*RRRr*
Hampton	*Rrrr*	Yagana	*Rrrr*
Idarose	*RRRr*	Yukon Gold	*Rrrr*
Kanona	*RRrr*	NY97	*RRRR*
Katahdin	*Rrrr*	NY99	*Rrrr*
Kennebec	*rrrr*	NY102	*rrrr*
Keuka Gold	*rrrr*	NY115	*RRrr*
Lenape	*rrrr*	NY118	*RRrr*
Marcy	*rrrr*	NY120	*RRrr*
Nordonna	*RRRr*	NY126	*Rrrr*
Dark Red Norland	*Rrrr*	NY129	*RRrr*
Pike	*RRrr*	L235–4	*rrrr*
Purple Peruvian	*rrrr*	S45–5	*Rrrr*
Reba	*rrrr*	S48–6	*RRrr*
Red Companion	*RRrr*	T15–1	*RRRR*
Red Pearl	*RRrr*		

Table 2 Segregation of red-linked *dfr* allele dosage in two crosses

Cross	Nulliplex	Simplex	Duplex	Triplex	Quadruplex	Other	Total
Eva × NY97 (nulliplex × quadruplex)	0	0	45	0	0	1[a]	46
NY118 × Redsen (duplex × duplex)	1	24	34	28	4	0	91

[a] This clone displayed a FAM:VIC fluorescence signal ratio intermediate to *RRRr* and *RRrr*

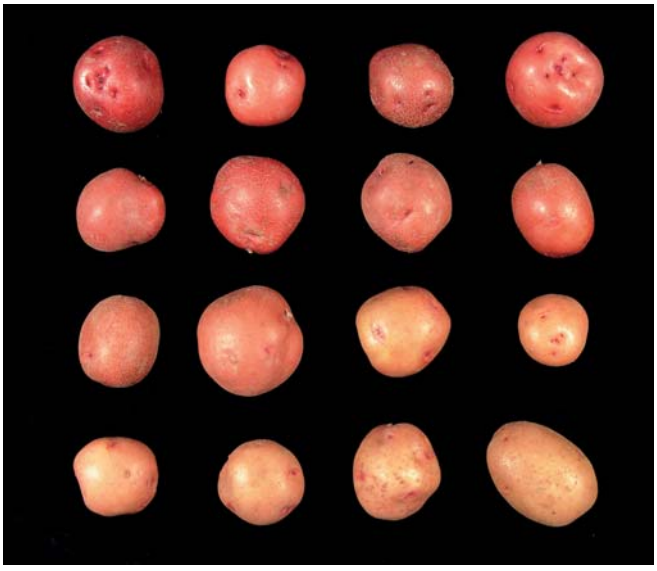

Fig. 3 Selected progeny tubers illustrating segregation of skin color intensity in a cross between the cultivars Eva and Redsen. A total of 266 red-skinned progeny were sorted visually based on the intensity of red skin color—from darkest red to lightest red—after being grown in an artificial soil mix. In the following year tubers were grown in the field and photographed 6 weeks after harvest. The *top left corner* shows a tuber from the potato clone deemed to have the darkest red skin; the *bottom right corner* shows a tuber from the clone judged to have the lightest red skin. From left to right, the remaining tubers shown are from clones that ranked (*top row*) 11th, 21st, 31st, (*second row*) 41st, 51st, 61st, 71st, (*third row*) 196th, 206th, 216th, 226th, (*fourth row*) 236th, 246th and 256th in intensity of red-skin color. Of these clones, the following rankings were duplex for the red-linked *dfr* allele: 1, 11, 31, 41, 61, 226. These clones were simplex: 21, 51, 71, 196, 206, 216, 236, 246, 256. Allele dosage of 266 was not determined

Table 3 Frequency of simplex and duplex clones in the 85 darkest and 86 lightest red progeny of a cross between Eva (nulliplex) and Redsen (duplex)[a]

Type	Duplex	Simplex	No data
85 darkest red progeny	40	44	1
86 lightest red progeny	23	58	5

[a] The cross yielded 266 red-skinned progeny. White segregants were not evaluated for dosage

To test whether dosage values assigned to tetraploids were accurate, several clones were crossed and their progeny evaluated with the same allelic discrimination assay. A cross between Eva (nulliplex) and NY97 (quadruplex) is predicted to give entirely duplex progeny. When 46 progeny of this cross were evaluated, 45 gave FAM:VIC ratios similar to diploid 320-02 and were thus inferred to have a duplex genotype (Table 2). The remaining clone consistently yielded FAM:VIC ratios that were higher than the other 45. When eight replicated samples of this clone were carefully compared to eight replicates each of Nordonna (triplex) and 320-02, the FAM:VIC ratios of this clone were observed to consistently fall between the two, but closer to Nordonna than to 320-02 (data not shown). Although chromosome number in this individual was not determined, the FAM:VIC signal ratio was consistent with a genotype of *RRr* rather than *RRRr* or *rrrr*, such as might occur if this clone is aneuploid for the chromosome that encodes *dfr*. A cross between two duplex clones, NY118 and Redsen, was also evaluated. As summarized in Table 2 this cross yielded a full spectrum of progeny genotypes from nulliplex through quadruplex, as would be expected from a duplex by duplex cross. A chi-square test revealed that the observed frequency distribution is consistent (P=0.08) with that expected from such a cross if there was no double reduction (expected segregation ratio of 1:8:18:8:1). Moreover, for any value of double reduction (α) less than or equal to the theoretical maximum of 1/6, the observed frequency distribution is still consistent with that expected from a duplex × duplex cross, e.g. P=0.34 at α=1/6.

Two of the most intensely red-colored potato clones in the Cornell breeding program, T15-1 and NY97, are both quadruplex for the red-linked *dfr* allele (Table 1 and De Jong et al. 2003). This raised the question of whether dosage of this allele might be positively correlated with the intensity of red skin color. To test this, a cross between Eva (nulliplex) and Redsen (duplex) was evaluated both for tuber skin color and for dosage at *dfr*. Approximately 400 progeny from this cross were grown outdoors in six inch pots in an artificial soil mix. At harvest all white-skinned progeny were discarded, leaving 266 red-skinned progeny. The white-skinned progeny were assumed to represent segregants that either lacked *R* and/or a second locus, *I*, also required for red skin color (Dodds and Long 1956). The red-skinned progeny were visually sorted from darkest to lightest red in color; selected segregants are shown in Fig. 3. Dosage of the red allele in 84 of the darkest 85 progeny and 81 of the lightest 86 progeny was then assessed (Table 3). As expected all red-skinned progeny were either simplex or duplex for the red allele. While duplex clones were observed among both the darkest and lightest red progeny, the frequency of duplex clones was significantly higher among the darkest clones (chi-square test; P=0.01).

Nevertheless, many light-red clones were duplex for red-linked *dfr*, and many dark-red clones were simplex (Table 3 and Fig. 3). Thus although dosage of *R* appears to contribute to intensity of skin color, it does not appear to be the primary genetic factor to influence this trait.

Discussion

This paper has presented evidence that a 5′ fluorogenic nuclease allelic discrimination assay can be used to assess dosage in an autotetraploid, cultivated potato. When diploid potato clones were evaluated only three genotypic classes were observed, corresponding to clones homozygous for the red *dfr* allele, homozygous for not-red allele(s), or heterozygous. In contrast, tetraploid clones were separated into five distinct classes, three of which were shared with the diploid clones. The simplest interpretation, supported by dosage analyses of progeny from several crosses, is that the five tetraploid clone clusters correspond to nulliplex, simplex, duplex, triplex and quadruplex genotypic classes. To the best of our knowledge this is the first report of a TaqMan assay being used to assess dosage in an autopolyploid. Indeed, the allelic discrimination software currently provided by Applied Biosystems (SDS version 2.0) has not been programmed to anticipate the possibility of more than three genotypic classes. During inspection of scatter plot data the program only provides three options to label individual data points—allele X, allele Y, or allele X and Y. In general, the approach described here with *dfr* in potato may prove useful for investigators who wish to develop dosage-sensitive marker assays at other loci in potato, or in any other autopolyploid species (e.g., alfalfa, peanut or salmon).

Prior to conducting these experiments it was not obvious to us whether a 5′ fluorogenic assay would generate meaningful data in potato. Two issues were of particular concern. The first was whether there would be sufficient separation on scatter plots between the five genotypic classes. This is not a serious problem for diploids as any individual that exhibits above-threshold fluorescence from both probes must be heterozygous. However, for autotetraploids with just two alleles there are three possible heterozygous genotypes (simplex, duplex, triplex) that need to be differentiated. In an effort to maximize specificity we deliberately utilized MGB probes (Kutyavin et al. 2000) and designed them so that they would anneal to regions that differed at two nucleotide positions. Whether either or both of these factors are necessary for effective discrimination in potato is not clear since these probes were not compared to any others. A second issue was whether novel undetectable alleles of *dfr* would be encountered that could not be amplified by primers DFRFOR4 and DFRREV3 or would not anneal to the red or not-red probes. Such alleles would complicate genotypic assignments, e.g., a tetraploid with genotype *Rrrr* would be erroneously scored as quadruplex if none of the *r* alleles amplified during the assay. To reduce the chances of encountering such alleles a portion of *dfr* from a wide range of germplasm was sequenced before designing primers and probes. None of the 30 diploid clones known to be heterozygous on the basis of a PCR amplification + *Bam*HI digestion assay was judged to be homozygous red or homozygous not-red with the fluorogenic 5′ nuclease assay. Thus, if alleles not recognized by the 5′ nuclease assay exist, they do not appear to be very common in cultivated potato germplasm.

Two key assumptions were made in generating Table 1, which reports the inferred genotype at *R* in several potato cultivars and breeding clones. The first assumption is that the red allele of *dfr* is always associated with *R*, while the second is that the red allele is absent in potatoes of genotype *rrrr*. We have not yet observed a clear violation of either assumption. All 154 red-skinned diploid and tetraploid clones tested with the *Bam*HI assay to-date have been found to possess the red allele, while none of the 209 clones found to lack the red allele have displayed red skin (De Jong et al. 2003 and unpublished data). In the present study 165 red-skinned progeny from a cross between Eva and Redsen were successfully genotyped at *dfr*. All were found to carry the red allele (Table 3). It should nevertheless be noted that the fluorogenic and *Bam*HI assays described here are almost certainly not based on the sequence polymorphism(s) that functionally differentiate *R* from *r*. The two single-nucleotide polymorphisms evaluated by the fluorogenic 5′ nuclease assay both reside in an intron and are not expected to affect DFR function. Similarly, although the polymorphic *Bam*HI restriction site resides in an exon, it is translationally silent. Thus the crucial functional differences between *R* and *r* presumably reside elsewhere in *dfr* or in some other tightly linked gene.

The specific assay described in this paper should be of most use to potato breeding programs that develop red-skinned potato cultivars. By choosing to use parents with higher dosage at *R*, fewer white segregants will result from crosses. The test should be of particular value in developing true potato seed (TPS) ‘varieties’, where developing parental clones homozygous for *R* and/or *I* is required if skin color is not to segregate. The gel-free assay described here is easy to perform, requiring only DNA isolation, PCR, and software-aided data analysis. Best results are obtained with pure DNA, e.g., isolated with a Qiagen DNeasy mini-kit, but acceptable results were also obtained with crude DNA, i.e., isolated from young leaf tissue with the method of Edwards et al. (1991). The current cost of reagents for the fluorogenic assay described here is approximately $5 per sample, quite reasonable compared to the alternative of assessing dosage by making crosses and evaluating progeny. Necessary equipment includes a thermal cycler and an ABI PRISM sequence detection system or a fluorescence-capable microtiter plate reader, all relatively common in large research institutions.

The intensity of red skin color is an economically important trait—other things being equal; potatoes with

deeper-red color sell for a higher price. In a large population of red-skinned potatoes that were either simplex or duplex for the red-linked *dfr* allele, a positive correlation was observed between the dosage of red-linked *dfr* and skin color. The effect was not large as many dark-red potatoes were simplex and many light-red potatoes were duplex. One or more additional loci thus appear to control most variation for skin color. Dosage or allelic configuration at *I*, a regulatory locus that is also required for tissue-specific expression of anthocyanin pigments in tuber skin (Dodds and Long 1956), may be the primary determinant of color intensity. In maize it is known that the expression level of transcriptional regulators of anthocyanin biosynthesis are directly correlated with the amount of anthocyanin produced (Patterson et al. 1993; Hollick et al. 2000). Once *I* is isolated or markers tightly linked to it are developed, testing whether dosage at *I* contributes to the intensity of skin color would be warranted.

Acknowledgements We thank S. Kresovich for suggesting the 5′ nuclease assay, H. De Jong for providing diploid clones and for reviewing the manuscript, K. Paddock for assistance in maintaining potato clones, and the US Potato Genebank (NRSP-6) for providing accessions of cultivated *Solanum* spp. This work was supported in part by Federal Hatch Funds and a grant from the Empire State Potato Growers, Incorporated.

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