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Evidence that genes from the male parent may influence the morphology of potato dihaploids

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Abstract A number of recent studies have provided evidence that potato dihaploids (*S. tuberosum*) contain and express DNA from the male (dihaploid inducer) parent, *S. phureja*. The importance of this for breeding programmes that use dihaploid potatoes is to some extent dependent upon whether the *S. phureja* DNA influences dihaploid morphology. In the present study, 21 characters were used to compare the morphology of six dihaploids with those of their parents: *S. tuberosum* (cvs 'Pentland Dell' and 'Pentland Crown') and *S. phureja* (IVP48). Characteristics of *S. phureja* were found in all of the dihaploids examined. In principal component analyses, dihaploids formed intermediate groupings positioned between those of the parents, although much closer to *S. tuberosum*. It is concluded there is evidence that DNA originating from the dihaploid inducer can affect the morphology of potato dihaploids. Implications of the findings are discussed.

Key words Potato dihaploids · Principal component analysis · Dihaploid induction · Microsatellite · Isozyme analysis

Introduction

The genetics of the cultivated potato (*Solanum tuberosum* L., 2n=4x=48) is complex and many research workers have used dihaploid potatoes (2n=2x=24) to simplify cytological and genetical studies of the crop (Suska 1985; De Maine and Jervis 1989). Dihaploids have also been used for tissue culture (Fish and Jones 1988; Visser et al. 1989), in the construction of linkage maps (Bonierbale et al. 1988; Gebhardt et al. 1993) and for breeding purposes (Wenzel et al. 1979; Peloquin et al. 1990).

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Most dihaploids are created by pollinating tetraploid potatoes using pollen from selected clones of *S. phureja* known as 'dihaploid inducers'. The majority of dihaploid inducers are homozygous for the dominant gene, embryo seed spot marker (Hermsen and Verdenius 1973). This gene is expressed as a dark-purple spot on the embryo and is visible in the mature seed. The offspring from a dihaploid induction cross usually includes a proportion of seeds which lack the marker. Diploid individuals grown from these seeds (dihaploids) closely resemble the maternal parent and were believed to have arisen by female parthenogenesis (Hermsen and Verdenius 1973; Rowe 1974; Van Breukelen et al. 1977). In consequence, they were thought also to be genetically pure *S. tuberosum*. The discovery that many dihaploids contain additional chromosomes in some cells (Clulow et al. 1991) and also possess DNA markers specific to the dihaploid inducer demonstrated this is not entirely the case (Clulow et al. 1991; Waugh et al. 1992; Wilkinson et al. 1995). Clulow et al. (1993) reported that isoforms specific to the dihaploid inducer were also present in some dihaploids and inferred from this that genetic material from the inducer can be expressed in potato dihaploids. It is still unclear, however, whether sufficient genes are transmitted from the inducer to affect overall morphology of the dihaploids and so be worthy of practical consideration for breeding purposes. In the study described here, we use principal component analysis as an aid to determining whether the genetical contribution of the dihaploid inducer has influenced the morphology of six dihaploids derived from the cultivars 'Pentland Crown' and 'Pentland Dell'.

Materials and methods

Plant material

Tubers of cv 'Pentland Dell' and cv 'Pentland Crown' were obtained from the breeders germplasm collection at the Scottish Crop Research Institute (SCRI). Tubers of the *S. phureja* 'dihaploid inducer' clone IVP48 and of the *S. tuberosum* dihaploids PDH 52, 55, 348,

831, 834 and 849 were taken from the SCRI potato dihaploid collection. Two triploid hybrids were grown from spotted seed produced in dihaploid induction crosses between IVP48 and cvs 'Pentland Crown' and 'Pentland Dell' made in 1992 by M. J. De Maine (SCRI, UK). Fifteen tubers of cv 'Pentland Dell', cv 'Pentland Crown', IVP48 and dihaploids PDH52 and PDH55 were used in the following experiment. Three suitable tubers were available for the triploid hybrids (H1, H2) and dihaploids PDH348, 831, 834 and 849.

Cytological analysis

Roots were pre-treated in iced water (24 h), fixed in 3:1 ethanol:glacial acetic acid (3 days) and stored in ethanol. The roots were washed (in distilled water, 3–5 min), hydrolysed in 5N HCl (45 min), washed again and stained in Schiff's reagent for 2 h. Stained roots were then washed and softened enzymically (20% w/v pectinase, 2% w/v cellulase, pH 8) for 1 h at 37°C. Softened roots were rinsed in distilled water and transferred to 70% ethanol. Root tips were removed, pipetted onto a clean glass slide and spread under a drop of 45% acetic acid. Preparations were viewed by fluorescence microscopy on a Nikon Axiophot compound microscope using a 580 nm excitation filter.

Polymerase chain reaction (PCR) analysis

DNA was extracted from 150 mg of fresh leaf material using the procedure of Hu and Quiros (1991).

PCR analysis was conducted according to the method described by Charters et al. (1996) using 5′ anchored primers for two dinucleotide repeats and one trinucleotide repeat: BDB-[CA]7, HVH-[TG]7, BDB[CAC]5 (University of British Columbia, set 9) where B=C, G or T (i.e. not A); D=not C; H=not G and V=not T. Genomic DNA samples of the tetraploid cultivars, IVP48 and the dihaploids were diluted to a concentration of 10 mg/l and then 2 µl (containing 20 ng of DNA) was added to the reaction mixture containing: 1 unit *Taq* DNA polymerase, 1×5 m*M* $MgCl_2$ buffer, 0.2 m*M* of each dNTP (all Boehringer Mannheim) and 0.2 µ*M* of primer. DNA was amplified on a Hybaid Omingene Thermocycler using one of the manufacturers' standard programmes: 1 min at 94°C, 30 cycles of (2 min at 55°C, 30 s at 72°C, 1 min at 94°C) with a final 5-min extension at 72°C.

Amplification products were loaded onto precast polyacrylamide gels (0.5 mm thickness) comprising a 5% w/v acrylamide stacking gel and a 10% w/v acrylamide separating gel (Pharmacia, Cleangel 48S). They were separated and stained according to the method described by Charters et al (1996).

Leaf isozymes (α-esterase)

Leaf material (0.1 g) was ground in 100 ml of extraction buffer (25 m*M* Na_2HPO_4, 10 m*M* DTT, 10% w/v sucrose, pH 7.4), centrifuged for 4 min at 12,000 *g* and the supernatant stored on ice. Isoelectric focusing gels (4.5% w/v acrylamide, 14% glycerol, 5% ampholyte) were prefocused at 0.5 kV, 22 mA, 40 W for 4.5 (kV)h. The electrode strips were soaked in 1 *M* H_3PO_4 (anode) and 1 *M* NaOH (cathode). Gels (pH 4–6.5) were stained for α-esterase activity according to the method described by Clulow et al. (1993).

Experimental design

Sprouted tubers of approximately equal size (3–2 cm diameter) and age (1 year) were given a specific code number and planted into 12.5 cm square pots. The pots were randomly arranged in a heated glasshouse (temperature range 23°C–28°C) and grown under sodium lighting (12 h photoperiod). The first shoots emerged from the soil after 10 days. Pots in which shoots failed to emerge after 20 d were discarded in order to minimize variation in plant maturity. The plants remaining consisted of 12 individuals of 'Pentland Dell', 7 representatives of 'Pentland Crown' and IVP48, 10 plants of PDH52, 10 plants of PDH55, 3 of PDH348, 3 of PDH834 and 1 each of PDH831, PDH849 and the triploid hybrids between IVP48 and cvs 'Pentland Dell' and 'Pentland Crown'. Twenty-one morphological characters were recorded from all plants 10 weeks after planting, as shown in Table 1. Quantitative characters were measured directly but the 11 qualitative traits were transformed into a three-state numerical form (i.e. 0, 1 or 2) before use in Principal component analysis on the Minitab statistical program package (Minitab Inc). Separate analyses were performed on two sets of plants. The first set ('Pentland Dell') comprised: four dihaploids of cv 'Pentland Dell' (8 plants in total), 12 plants of cv 'Pentland Dell', 7 plants of IVP48 and 1 plant of the hybrid H2. The second set ('Pentland Crown') con-

Table 1 Characters used in Principal component analyses (PCA)

A) PCA of cvs Pentland Crown, P. Dell, IVP48, H1, H2, and dihaploids PDH52, 55, 348, 831, 834 and 849	B) PCA of cvs Pentland Crown, IVP48 and diploid and tetraploid regenerants of dihaploid PDH52
1. Terminal leaflet length	1. Terminal leaflet length
2. Terminal leaflet width	2. Terminal leaflet width
3. Mean internode length (2nd–5th nodes)	3. Mean internode length (2nd–5th nodes)
4. Total leaf length	4. Total leaf length
5. Ratio of 1st lateral leaflet length/terminal leaflet length	5. Ratio of 1st lateral leaflet length/terminal leaflet length
6. Ratio of 2nd leaflet length/1st lateral leaflet length	6. Ratio of 2nd leaflet length/1st lateral leaflet length
7. Ratio of distance from leaf apex to basal leaflet/leaf length	7. Ratio of distance from leaf apex to basal leaflet/leaf length
8. Mean distance between leaflet pairs	8. Mean distance between leaflet pairs
9. No. of lateral leaflets	9. Mean no. of prominent secondary veins on terminal leaflet
10. Mean no. of prominent secondary veins on terminal leaflet	
11. Terminal leaflet apex shape[a]	
12. Terminal leaflet infolded/flat[a]	
13. Anthocyanin disposition on leaflet veins, (abaxial surface)[a]	
14. Anthocyanin disposition on rachis[a]	
15. Anthocyanin disposition on stems[a]	
16. Anthocyanin disposition on stem nodes[a]	
17. Anthocyanin disposition on the margins of terminal leaflet[a]	
18. Presence of interjective leaflets[a]	
19. Petiolule wholly or partly laminate/not[a]	
20. Presence of B-type trichomes on stems[a]	
21. Shape of terminal leaflet base caudate/lanceolate	

[a] Qualitative characters

tained two dihaploids of cv 'Pentland Crown' (20 plants in total), 7 plants of cv 'Pentland Crown', 7 plants of IVP48 and 1 individual of the hybrid H1. Tuber characteristics are used for the early selection of desirable dihaploids at SCRI. Dihaploids exhibiting unwanted tuber traits such as deep eyes and irregular shape (including many features found in IVP48 tubers) are routinely discarded. For this reason, tuber characters were not included in the analysis.

It is possible that differences in ploidy level could influence the expression of quantitative characters. To test for this, diploid and tetraploid clones of dihaploid PDH52 were regenerated from leaf disks according to the method described by Coleman et al. (1991). Plants were transferred to the glasshouse and allowed to produce tubers. The chromosome number of these plants was determined from actively growing root tips according to the method of Wilkinson et al. (1995). In 1994, tubers of 14 diploid and seven tetraploid regenerants were planted along with the other plants used in the study (listed above). Nine quantitative characters were recorded from all individuals 10 weeks after planting as shown in Table 1. Principal component analysis was applied to all representatives of IVP48, cv 'Pentland Crown' and the diploid and tetraploid regenerants of PDH52 using the Minitab statistical program package (Minitab Inc).

Results

Cytology

All cells examined from the *S. tuberosum* parents were tetraploid (2n=4x=48) and those from *S. phureja* dihaploid inducer IVP48 were diploid (2n=2x=24). The 2 plants grown from spotted seeds were triploids (2n=3x=36) and were therefore considered to be F_1 hybrids between *S. tuberosum* and IVP48. Root squash preparations of the dihaploids contained a predominance of cells with 24 chromosomes, although all dihaploids, except PDH849, also contained a small proportion of cells with 25 chromosomes (Table 2). No triploid or tetraploid cells were observed in any dihaploid.

Table 2 Cytological variation in potato dihaploids. Frequency of diploid, hyperdiploid and polyploid cells in root tips of six potato dihaploids, cv 'Pentland Crown', cv 'Pentland Dell', IVP48 and the hybrids H1, H2

	Female parent	Dihaploid inducer	Number of diploid, aneuploid and polyploid cells identified					Total no. of cells karyotyped
			Number of chromosomes per cell					
			24	25	26	36	48	
P. Crown							11	11
P. Dell							52	52
IVP48			52					52
H1	P. Crown	IVP48				12		12
H2	P. Dell	IVP48				15		15
PDH52	P. Crown	IVP48	322	4				326
PDH55	P. Crown	IVP48	496	11	1			508
PDH348	P. Dell	IVP48	30	2				32
PDH831	P. Dell	IVP48	70	2				72
PDH834	P. Dell	IVP48	53	1				54
PDH849	P. Dell	IVP48	27					27

Table 3 The distribution of IVP48-specific PCR and isozyme markers in: (A) the 'P. Dell' dihaploids PDH348, 831, 834 and 849 using α-esterase and primers 891, 1424; (B) the 'Pentland Crown' dihaploids PDH52 and 55 using α-esterase and primer 888

A	PCR markers							Isozyme marker α-esterase
	Primer 891				Primer 1424			
	2260 bp	1290 bp	1060 bp	780 bp	920 bp	860 bp	560 bp	
P. Dell	–	–	–	–	–	–	–	–
IVP48	+	+	+	+	+	+	+	+
PDH348	–	+	+	–	–	–	+	–
PDH831	+	–	–	+	+	+	+	+
PDH834	–	–	–	–	+	–	–	–
PDH849	+	–	–	–	+	–	–	–

B	PCR marker Primer 888 750 bp	Isozyme marker α-esterase
P. Crown	–	–
IVP48	+	+
PDH52	+	–
PDH55	–	+

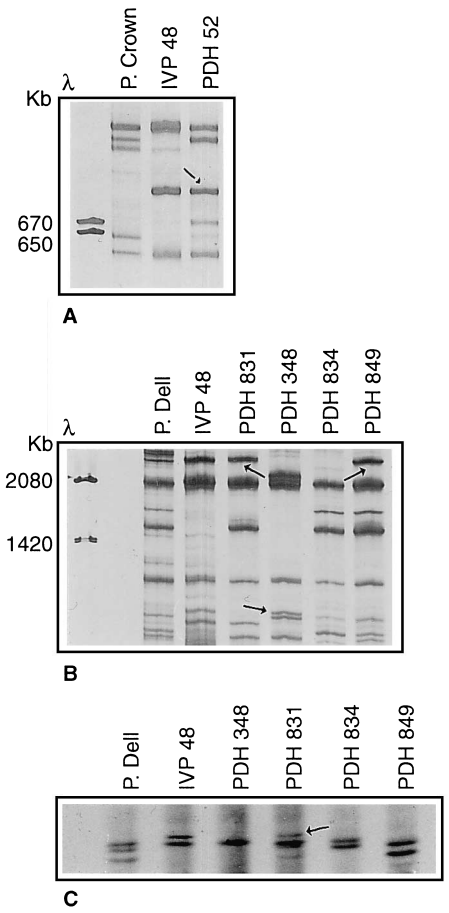

Fig. 1A–C The presence of IVP48-specific markers in: **A** the 'Pentland Crown' dihaploid PDH52 using primer 888; **B** the 'Pentland Dell' dihaploids PDH348, 831 and 849 using primer 891; **C** the 'Pentland Dell' dihaploid 831 using α-esterase

PCR and leaf isozyme analysis

Several markers were found to be present in the dihaploid inducer (IVP48) but absent from the *S. tuberosum* parents. At least one such marker was detected in all dihaploids used in the study (Fig. 1A,B, Table 3). This suggests that DNA originating from IVP48 is present in the dihaploids used in the study.

Leaf extracts of IVP48 contained one α-esterase band that did not appear in either of the tetraploid *S. tuberosum* clones (Table 3). The IVP48-specific α-esterase band was present in leaf extracts of PDH831 but was absent in the other 'Pentland Dell' dihaploids (Fig. 1C). PDH55 also expressed an α-esterase band characteristic of IVP48.

Plant morphology

None of the plants had an abnormal phenotype and, for all characters examined, the majority of dihaploids resembled the *S. tuberosum* parent. However, all dihaploids possessed at least one characteristic of IVP48.

For example, only two dihaploids flowered during the course of the experiment (PDH348 and PDH831) and for this reason it was not possible to use flowering characteristics in the principal component analyses. It was noted, however, that PDH831 possessed red-purple flowers although the cultivar from which it is derived (cv 'Pentland Dell') had only white flowers. The inducer parent (IVP48) produced deep-purple flowers.

The terminal leaflets of all IVP48 plants had a caudate base, whereas those of cv 'Pentland Dell' tapered gradually to the point of attachment with the petiolule. The triploid hybrid between these plants (H2) and dihaploid PDH849 both possessed the caudate bases of IVP48. The terminal leaflet bases of dihaploids PDH348 and PDH834 were intermediate between the character states of the parents.

A significant difference was noted between the mean distance between interjective leaflets on the leaves of cv 'Pentland Dell' and those of IVP48 (*t*-test, $t=9.93$, $P<0.001$). Individuals of the former had a mean separation of between 16 and 24.5 cm whereas those of the latter ranged from 6.2 cm to 12 cm. Mean distance between interjectives in most dihaploids fell into the range observed in cv 'Pentland Dell' (17–20 cm), but dihaploid PDH831 bore the phenotype of IVP48, with a mean distance between interjectives of 9 cm. It should be pointed out, however, that 1 of the 3 individuals of PDH348 also fell into the range of IVP48 (10 cm), whereas the other 2 representatives of the clone resembled cv 'Pentland Dell' (17 and 19 cm).

Multivariate analysis of morphological data

In the analysis of the dihaploids of cv 'Pentland Dell', the first four principal components accounted for 60.8%, 13.4%, 9.2% and 5.8% of the variation. This represents 89.2% of the total variation observed. The first principal component was determined largely by two sets of contrasting characters of approximately equal value. The first set consisted of: shape of terminal leaflet apex (acute/obtuse), leaflet margins reflexed/not, presence/absence of anthocyanin on leaf veins, rachis, nodes and margin of apical leaflet and petiolules laminated/not. These characters contrasted strongly with the second set, comprising: terminal leaflet length and width, leaf length and mean distance between interjective leaflets. The second principal component was influenced primarily by the ratio between first lateral leaflet length/terminal leaflet length, the ratio of second lateral leaflet length/first lateral leaflet length and by the ratio of first lateral leaflet length/terminal leaflet length. The main determining characters of the third and fourth principal components were the number of primary lateral veins on the terminal leaflet contrasted by the frequency of interjective leaflets (third principal component), and the presence of B-type trichomes on the stem contrasted by stem length (2nd-5th node) and total leaf length (fourth principal component).

The parents were well-separated along the axis of the first principal component and formed distinct groupings in

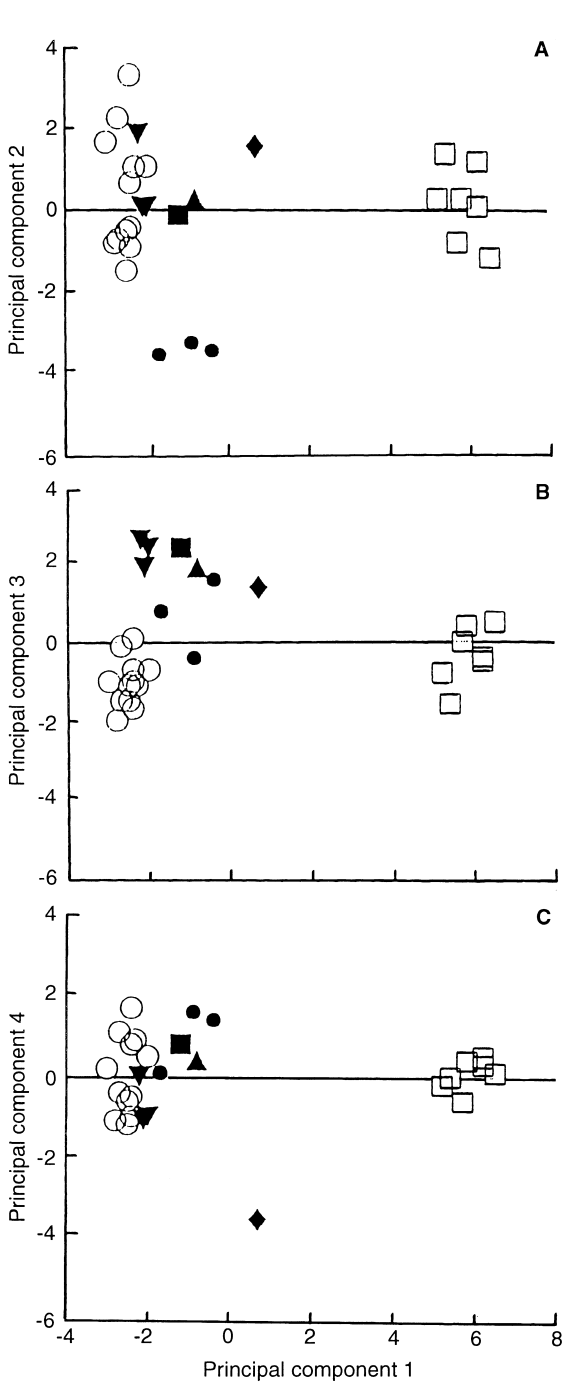

Fig. 2A–C Principal component analyses of 'Pentland Dell' (○), IVP48 (□), H2 (◆) and the dihaploids PDH348 (●), PDH831 (▲), PDH834 (▼) and PDH849 (■) on the basis of 21 morphological characters. **A** First and second principal components, **B** first and third principal components, **C** first and fourth principal components

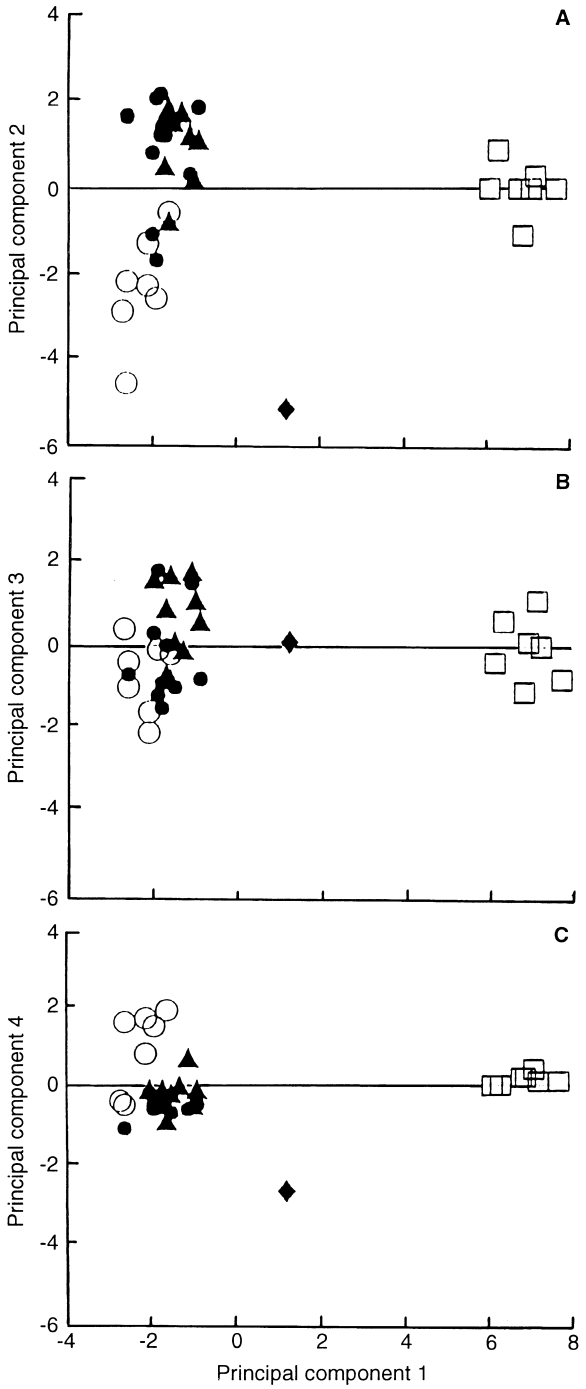

Fig. 3A–C Principal component analyses of 'Pentland Crown' (○), IVP48 (□), H1 (◆) and the dihaploids PDH52 (●) and PDH55 (▲) on the basis of 21 morphological characters. **A** first and second principal components, **B** first and third principal components, **C** first and fourth principal components

plots of the first principal component against either the second, third or fourth principal components (Fig. 2A–C). The triploid hybrid was intermediate between the parents along the axis of the first component (score=0.7) and was positioned 38% of the distance between the mean value for cv 'Pentland Dell' (–2.5) and that of IVP48 (5.9). All dihaploids formed a discrete cluster between the parents but were much closer to the *S. tuberosum* parent. The mean score for the first principal component of the dihaploids was –1.4 compared with –2.5 for cv 'Pentland Dell' and 5.9 for IVP48.

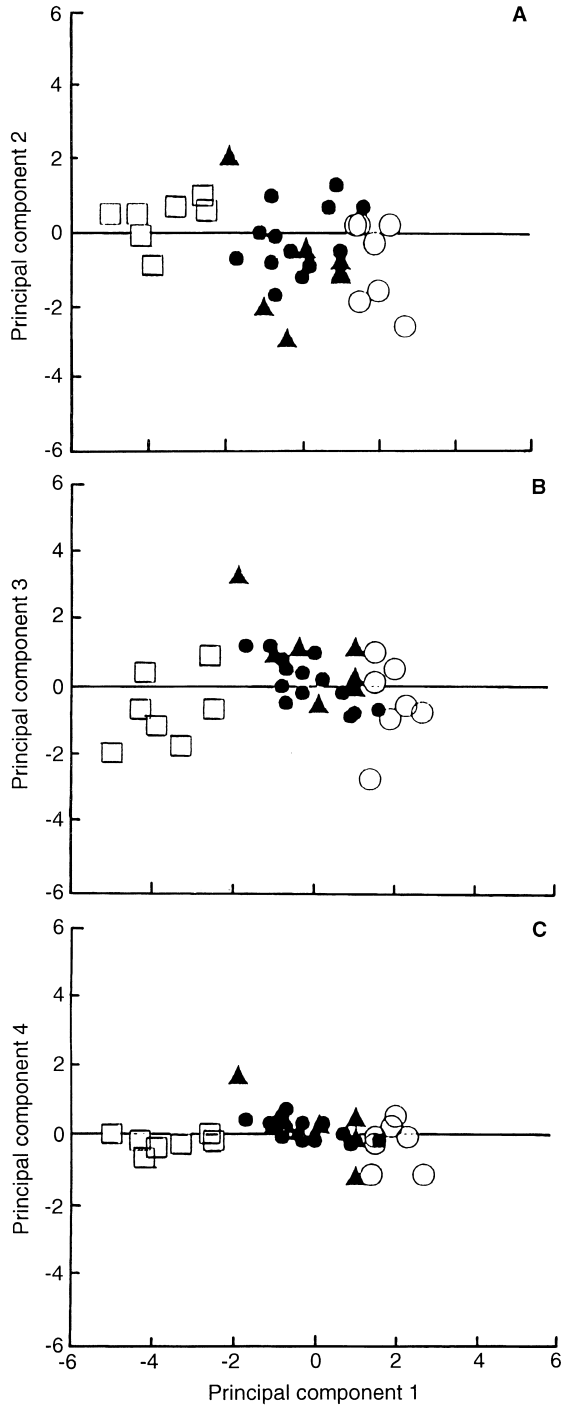

Fig. 4 Principal component analysis of cv 'Pentland Crown' (○), IVP48 (□), diploid (●) and tetraploid (▲) regenerants from PDH52 on the basis of 9 quantitative characters. **A** First and second principal components, **B** first and third principal components, **C** first and fourth principal components

For the comparison of cv 'Pentland Crown' dihaploids, the first four components accounted for 58.6%, 17%, 5.6% and 4.5% of the total variability. This represents 85.7% of the variance observed. The first principal component was determined principally by two contrasting sets of characters of approximately equal value. The first group consisted of terminal leaflet base caudate/lanceolate, presence/absence of anthocyanin on leaf veins, rachis, nodes and margin of apical leaflet, leaflet margins reflexed/not and the shape of the terminal leaflet apex (acute/obtuse). These characters contrasted with the length and width of the terminal leaflet, leaf length and mean internode length (2nd–3rd nodes). The main determinant characters of second principal component were the presence of interjective leaflets and the ratio of the lengths of the terminal leaflets and the first lateral leaflets, contrasted with the mean number of prominent lateral veins on the terminal leaflets. The ratios between the lengths of the first and second lateral leaflets and between the distance from leaf apex to basal leaflet and total leaf length were the dominant characters influencing the third principal component. The fourth principal component was influenced largely by petiolule length contrasted by the ratio between first lateral leaflet length and terminal leaflet length.

The parents were well-separated along the first principal component. Distinct groupings were observed when the first principal component was plotted with the second, third or fourth components (Fig. 3A–C). The triploid hybrid was placed in an intermediate position along the first principal component but positioned closer to the *S. tuberosum* parent. The ratio of the distance from the hybrid to *S. tuberosum* represented 38% of the separation between the parents. The first principal component scores of the dihaploids were not distinct from those of cv 'Pentland Crown', although reasonable separation of the two was achieved when first principal component scores were plotted against the second or fourth principal component scores (Figs 3A, 4C). Mean first principal component scores of the two dihaploids used were between those of the parents although much closer to those of *S. tuberosum* (*S. tuberosum*, –2.2; PDH55, –1.7, PDH52, –1.4, *S. phureja*, 6.9).

The effect of ploidy level on dihaploid morphology

In the analysis of the PDH52 regenerants, the first four components accounted for 42%, 22%, 14% and 9% of the variation, respectively. The parents of PDH52 were well-separated along the axis of the first principal component and formed distinct groupings in plots of the first component against the second, third or fourth principal components (Fig. 4A–C). The diploid and tetraploid regenerants of PDH52 occupied an intermediate space between the parents in all three plots and did not separate into distinct clusters.

Discussion

Most of the dihaploids examined possessed additional chromosomes in some cells, and all contained PCR markers specific to the inducer parent. The latter was taken as strong evidence for the presence of DNA from IVP48 in

the dihaploids being studied but provides no evidence on the nature of the genetic transfer. It is possible, for instance, that the occasional additional chromosomes originate from the inducer parent and that these account for at least some of the DNA markers. Alternatively, Wilkinson et al. (1995) reported that genomic DNA from IVP48 hybridized to three segments on PDH55 chromosomes, suggesting that direct integration into the *S. tuberosum* genome may also be possible. In the present study, insufficient cytological variation was observed between dihaploids to make meaningful comparisons between the frequency of additional chromosomes and morphological similarity to the inducer parent. Thus, the importance of additional chromosomes in carrying DNA from the male parent requires further investigation. One possibility would be to make combined use of linkage analysis and genomic *in situ* hybridization.

The appearance of cells containing extra chromosomes in PDH55 contradicts earlier reports that this dihaploid contains only 24 chromosomes (Clulow et al. 1991; Wilkinson et al. 995). This discrepancy may have several causes including intraclonal variation, chromosome breakage or instability of additional chromosomes during root growth.

All dihaploids possessed at least 1 trait of IVP48, although the character present differed between dihaploids. The most likely explanation of this variability is that the genetic contribution of IVP48 also differs between dihaploids. The variation between dihaploids in the possession of IVP48-specific molecular markers reported here and in previous works (Clulow et al. 1991, 1992; Waugh et al. 1992; Wilkinson et al. 1995) provides further evidence that variation exists between dihaploids in the genetic contribution originating from the inducer parent.

Principal component analysis resulted in the spacial separation of potato dihaploids and their parents. The dihaploids of 'Pentland Dell' formed a distinct grouping positioned close to *S. tuberosum* and, whilst those of cv 'Pentland Crown' formed a slightly less well defined cluster, they also were positioned near to the tetraploid parent. Tetraploid and diploid regenerants of dihaploid PDH52 were not distinguished by PCA, even though the analysis used only quantitative characters which were considered the most likely to be affected by changes to chromosome number. This result suggests the observed morphological separation of dihaploids and their tetraploid (cultivar) parents should not be attributed simply to the change in ploidy level. It follows, therefore, that the intermediate positioning of dihaploids provides further evidence of a morphological influence by the inducer parent.

The triploid hybrids contain two genomes from the *S. tuberosum* parent and one from the inducer (IVP48). Both triploids occupied an intermediate point along the axis of the first principal component, positioned 38% of the distance from the *S. tuberosum* parent to IVP48. Should the degree of intermediacy be proportional to the genetic contribution made by each parent, then these data would suggest a genome composition of 1:1.6 IVP48:*S. tuberosum* rather than the expected 1:2 IVP48:*S. tuberosum*. Thus, whilst the morphological analysis identified the triploids as intermediate, it provided only a modest guide to the genetic contributions made by the two parents. It would be unwise, therefore, to use the degree of intermediacy exhibited by the dihaploids to estimate the genetic contribution made by the male (inducer) parent, although it is likely to be small in comparison with that made by *S. tuberosum*.

The observation that DNA from the inducer IVP48 can influence the gross morphology of dihaploids may have wider implications for the use of dihaploids for breeding purposes. A small number of *S. phureja* clones are routinely used in the production of potato dihaploids and none of these have been reported to contain agronomically desirable traits. However, *S. phureja* and several other species reported to be capable of dihaploid induction are known to possess resistance traits to important pests and diseases (Hawkes 1990; Wilkinson et al. 1995). This raises the possibility of using dihaploid induction for the direct introgression of agronomically desirable traits into the cultivated potato. The success of such an approach would depend on a number of factors but particularly upon the efficiency of dihaploid production and on whether DNA can be transferred by dihaploid induction from species other than *S. phureja*.

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