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Genetic localisation of transformation competence in diploid potato

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Abstract In the course of improving diploid potato genotypes for transformation ability, selection for specific components affecting regeneration and transformation was carried out. From a segregating population between two good regenerating clones a selection was made to yield an optimal well-transforming and fertile genotype J92-6400-A16. This plant yielded predominantly diploid transformants and was heterozygous for the gene *R1*, conferring resistance to *Phytophthora infestans*. The speed of, and competence for, regeneration and transformation on both sides of the stem explant were improved. A competence factor for tranformation was found to be linked with the *R1* locus and a molecular marker on chromosome 5. The male fertility of transformants was frequently decreased to a great extent, whereas female fertility was not so markedly affected.

Key words *Solanum tuberosum* · *Phytophthora infestans* · Transposable element · Transformation competence · Linkage analysis

Introduction

Transformation of plants with *Agrobacterium tumefaciens* T-DNA is genotype- and protocol-dependent. Efforts have been made to improve transformation efficiency with modifications in culture technique, by interaction with specific genotypes (McCormick et al. 1986; Valvekens et al. 1988), or by the transfer of genetic factors from wild species (Koornneef et al. 1986). Transformation of heterologous plants with maize transposable elements is an extension of transposon-tagging technology to isolate genes in other species. While some plants like *Arabidopsis* are naturally well suited for the use of this technique (Aarts et al. 1993; Bancroft et al. 1993) other crop plants like potato are not. The tetraploid nature of the crop necessitates the use of diploid potato genotypes for genetic analysis and transposon tagging. For successful isolation of genes in heterologous plants it is recognised that a relatively high tagging frequency of a specific locus is expected with linked transposons, as the maize elements transpose preferentially to linked locations (reviewed in Haring et al. 1991). This can be achieved by selecting for transposon-containing, T-DNA inserts located near to the target gene but requires the production of large numbers of transformants.

The maize elements transpose effectively in potato (Knapp et al. 1988; Frey et al. 1989; Pereira et al. 1991) but appropriate genotypes for employing tagging strategies have yet to be developed for the potato. This means that well-transforming diploid genotypes, preferably bearing the gene of interest, need to be selected. In potato, it is well known that a large variation for competence for regeneration (Taylor and Veillieux 1992) and transformation exist and that both factors are highly protocol- and genotype-dependent (Visser 1991). The effect of genotypes on regeneration has been analyzed in a number of plant species. The genetic factors involved can be dominant, as suggested in diploid alfalfa (Reisch and Bingham 1980) and described and localised in tomato (Koornneef et al. 1987, 1993), or recessive, as found in the diploid cultivated potato *Solanum phureja* (Taylor and Veilleux 1992).

Another problem related to the regeneration and transformation of diploid potato is tetraploidisation. For transposon-tagging research diploid transformants are required. For potato, it is generally known that

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tetraploidisation is genotype dependent. Competence for fast regeneration on stem explants is a key factor in avoiding this phenomenon (Visser 1991).

In the present paper the selection of a genotype with good transformation ability and the discovery of linkage between transformation competence and the resistance locus *R1*, from the wild species *S. demissum*, is described. This plant provides the basis for tagging of the resistance locus *R1* to *P. infestans* by a *Ds*-element (Pereira et al. 1992).

Materials and methods

Plant material

R1r1 parent plants included the diploid (2n = 2x = 24) clone J91-6167-2, derived from a cv Hertha dihaploid cross "Her 64 × 87-1029-31", and clones J91-6146-(3, 4, 8, 9, 12, 15, 17, 19, 22 and 24), derived from a cv Saturna dihaploid cross "J90-6026-4 × 87-1029-31" (El-Kharbotly et al. 1994). The fertile *r1r1* diploid clone 87.1024-2 (Jacobsen et al. 1989) was used as the crossing parent because of its known competence for regeneration and transformation (Pereira et al. 1992). These plants were grown in vitro as shoot cultures in 400-ml jars (four plants/jar) under 16-h light (3200 Lux) at 22–24 °C on MS medium (Murashige and Skoog 1962), supplemented with 30 g/l sucrose. They were also grown in a glasshouse and used as the female parent in a crossing program.

Regeneration from stem explants

Stem segments without the axillary buds from in vitro-grown shoots were used for the regeneration experiments. Forty or 80–120 explants per genotype were used in estimating the regeneration competence. They were placed on shoot-induction medium (MS with 8 g/l agar, supplemented with 30 g/l sucrose, 1 mg/l zeatin), 200 mg/l cefotaxime and 200 mg/l vancomycin. The plates were closed with cellophane tape and maintained under a 16-h photoperiod (3200 Lux) at 24 °C for 56 days.

Transformation procedure

Transformation efficiency (TE) was determined with 37–43 and 19–40 explants per genotype from the 11 diploids and from the family J92-6400-A respectively. TE is the frequency of explants regenerating shoots after 10 weeks of growth on selective medium following treatment with *A. tumefaciens*.

The transformation was carried out according to Visser (1991) but with different media. The explants were pre-cultured for 1 day on M300 (Visser 1991) covered with a piece of sterile Whatman no. 1 filter paper saturated with 2 ml of MS supplemented with 30 g/l sucrose, 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 0.5 mg/l kinetin and 2 g/l casein hydrolysate without antibiotics. One-day later the explants were inoculated in a 2-day culture of *A. tumefaciens* for 15 min, blotted dry and placed back on the same medium. Two-days later the explants were transferred to a selection medium (shoot-induction medium; see regeneration), supplemented with 200 mg/l cefotaxime, 200 mg/l vancomycin and 100 mg/l kanamycin as a selectable antibiotic. The explants were subcultured on fresh medium 2 and 5 weeks after transformation and the Petri dishes were maintained under conditions for normal regeneration.

Bacterial strains

The *A. tumefaciens* strain AM8706 (Visser 1989a) was used. This strain contains the binary vector pBI121 (Jefferson et al. 1987) which has both the reporter gene β-glucuronidase (GUS) and the NPT II gene for kanamycin resistance.

In addition, the *A. tumefaciens* strain GV3101 (pMP90PK) (Koncz and Schell 1986), containing the recombinant binary vector pHPT: :*Ds*-Kan (Pereira et al. 1992), was used for the production of *Ds*-containing transformants.

Determination of fertility

Female fertility of the selected clones was determined, after pollination with the fertile diploids J89-5040-2 and BE93-4002-3, in terms of berry set and seeds per berry. Male fertility was estimated after staining pollen with lactophenol acid fuchsin.

Screening for resistance

Preparation of the *P. infestans* inoculum and the inoculation procedure were carried out according to El-Kharbotly et al. (1994). Races 0 and 1 of *P. infestans* were kindly supplied by L. J. Turkesteen (Research Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands) and F. Govers (Department of Phytopathology, Wageningen Agricultural University, The Netherlands).

Ploidy level determination

The ploidy level of the transformants was estimated by counting the number of chloroplasts in the stomatal guard cells (Frandsen 1967).

RFLP analysis

Parental clones (87-1024-2, J91-6167-2) and their F_1 progeny were used for RFLP analysis. DNA extraction (Dellaporta et al. 1983), restriction digests, electrophoresis, blotting and hybridization procedures were all done as recommended by the suppliers. The RFLP probe GP21 (kindly supplied by Dr. C. Gebhardt, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany), linked to the *R1* locus (Leonards-Schippers et al. 1992) was used for hybridization of the Southern blots.

Results

Selection and testing of well-transforming *R1r1* genotypes

The competence for regeneration and transformation was investigated in 11 diploid *R1r1* clones originating from the tetraploids cv Saturna and cv Hertha. The results, shown in Table 1, indicate that in this material there is a large variation for regeneration ability, which is the first step in the selection process for well-transforming genotypes. The percentage of regenerating explants varied between 55% and 95%. Screening for transformation showed only one genotype, J91-6167-2, derived from cv Hertha, with a high transformation efficiency (TE; 24%). J91-6167-2 appeared to be male sterile and berry and seed set were both relatively low, namely 0.2 berries/pollination and 21.2 seeds/berry. To overcome these fertility problems J91-6167-2 was crossed with clone 87.1024-2. The offspring plants of this cross (family J92-6400-A) were tested for regeneration, transformation efficiency, resistance to *P. infestans* and fertility traits.

Table 1 Regeneration and transformation competence of 11 diploid clones

Genotypes	Percentage of explants with shoots	
	Regeneration (31 days)	Transformation (70 days)
Originated from Saturna cv		
J91-6146-3	55 ± 7.9 a*	0 ± 0.0
J91-6146-4	88 ± 5.2 b	0 ± 0.0
J91-6146-8	75 ± 6.8 ab	0 ± 0.0
J91-6146-9	90 ± 4.7 b	0 ± 0.0
J91-6146-12	83 ± 6.0 ab	0 ± 0.0
J91-6146-15	75 ± 6.8 ab	0 ± 0.0
J91-6146-17	93 ± 4.2 b	3 ± 2.5
J91-6146-19	95 ± 3.4 b	0 ± 0.0
J91-6146-22	72 ± 7.1 ab	0 ± 0.0
J91-6146-24	78 ± 6.6 ab	0 ± 0.0
Originated from Hertha cv		
J91-6167-2	93 ± 4.2 b	24 ± 7.1

* There is no significant difference between percentages with the same letter at $P = 0.05$

Analysis of competence for stem-explant regeneration

Rapid regeneration is an important way of avoiding tetraploidisation. Therefore, regeneration of explants was scored after 18, 31 and 56 days (Table 2). Compared with the parental clones, after 18 days, three groups of plants were distinguished in respect of the frequency of shooting explants: lower (four genotypes), no difference (ten) or higher (six). Thus, in six genotypes more stem explants were regenerating shoots than in the parental clones. After 31 days the frequency of regeneration was significantly increased for all genotypes but differences were still observed. Three genotypes were lower, 13 had no significant differences and four were higher than the parents. These differences disappeared after 56 days, except in the case of genotype J92-6400-A2 which invariably showed a relatively low frequency of regeneration.

In the Table 2 the phenomenon of regeneration at both cut surfaces of the stem explants is also described. After 18 days, except for J92-6400-A10 and J92-6400-A16, the parental clones and the sexual offspring had a low frequency of, or else no regeneration at both cut surfaces. Compared to the parental clones, scoring of this phenomenon after 56 days showed three groups, namely "low" frequency (four genotypes), no difference (seven) and "high" frequency (nine) regeneration. The two best-performing genotypes were the same as those observed after 18 days (J92-6400-A10 and J92-6400-A16). Early regeneration was positively correlated with both early and late regeneration from both sides ($r = 0.65$ and $r = 0.54$, respectively). The fact that in most genotypes regeneration started at the original basal side of the explant, followed by regeneration at the apical side, suggests that regeneration on one side stimulates that on the other side. This was not the case in J92-6400-A10 and J92-6400-A16, with simultaneous regeneration

Table 2 Regeneration competence of the progeny of 20 plants and their parents

Genotypes	Percentage of explants with shoots				
	Within			From two sides within	
	18 days	31 days	56 days	18 days	56 days
Parents					
87-1024-2	17 ± 3.8 a*	86 ± 3.5 a	99 ± 1.0 a	2 ± 1.4 a	18 ± 3.8 ab
J91-6167-2	15 ± 4.0 ab	88 ± 3.7 a	94 ± 2.7 ab	0 ± 0.0 a	34 ± 5.3 b
J92-6400-					
A1	4 ± 2.0 b	97 ± 1.7 a	98 ± 1.4 a	0 ± 0.0 a	12 ± 3.2 a
A2	2 ± 1.2	14 ± 3.2	32 ± 4.2	0 ± 0.0 a	0 ± 0.0
A3	18 ± 3.7 a	92 ± 2.7 a	99 ± 0.9 a	2 ± 1.3 a	11 ± 3.1 a
A4	5 ± 2.1 b	94 ± 2.3 a	99 ± 1.0 a	0 ± 0.0 a	63 ± 4.7
A5	18 ± 3.8 a	98 ± 1.4	96 ± 2.0 a	0 ± 0.0 a	22 ± 4.1 ab
A6	18 ± 3.8 a	91 ± 2.9 a	98 ± 1.4 a	3 ± 1.7 a	79 ± 4.1
A7	37 ± 4.8	95 ± 2.2 a	99 ± 1.0 a	4 ± 2.0 a	28 ± 4.5 ab
A8	1 ± 1.0	53 ± 5.0	81 ± 3.9 b	0 ± 0.0 a	3 ± 1.7
A9	4 ± 1.9 b	58 ± 4.9	86 ± 3.4 b	0 ± 0.0 a	0 ± 0.0
A10	60 ± 4.8	100 ± 0.0	100 ± 0.0 a	35 ± 4.6	92 ± 2.7
A11	3 ± 1.7	73 ± 5.0 a	99 ± 1.2 a	0 ± 0.0 a	5 ± 2.4 a
A12	19 ± 4.0 a	100 ± 0.0	100 ± 0.0 a	2 ± 1.4 a	57 ± 5.0
A13	14 ± 3.5 a	97 ± 1.7 a	99 ± 1.0 a	3 ± 1.7 a	59 ± 4.9
A14	25 ± 4.3 a	96 ± 2.0 a	97 ± 1.7 a	3 ± 1.7 a	82 ± 3.8
A15	30 ± 5.0 a	92 ± 3.1 a	98 ± 1.7 a	2 ± 1.7 a	63 ± 5.3
A16	41 ± 4.7	98 ± 1.3	100 ± 0.0 a	14 ± 3.3	93 ± 2.5
A17	42 ± 4.9	96 ± 1.9 a	98 ± 1.4 a	3 ± 1.7 a	67 ± 4.7
A18	0 ± 0.0	90 ± 3.0 a	91 ± 2.9 a	0 ± 0.0 a	13 ± 3.4 a
A19	39 ± 4.8	88 ± 3.2 a	94 ± 2.3 a	0 ± 0.0 a	0 ± 0.0
A20	51 ± 4.9	91 ± 2.8 a	100 ± 0.0 a	3 ± 1.6 a	33 ± 4.6 ab

* Singnificant difference from the parental clones at $P = 0.05$ is indicated by letters. The use of the same letter indicates no significant difference

at both sides, or in J92-6400-A2, J92-6400-A9 and J92-6400-A19, with regeneration only at one side.

Analysis of competence for plant transformation

The parents 87.1024-2 and J91-6167-2 and 26 sexual offspring plants (J92-6400-A) were investigated for TE. Besides resistance to race 0 of *P. infestans* and susceptibility to race 1, the segregation pattern of RFLP probe GP21, as well as the ability of regeneration under selective conditions after 18 days and from the two cut surfaces, were investigated (Table 3). In comparison with the TE of the parental clones differently performing progeny genotypes were found. After 18 days, no parental and four offspring plants with transformed shoots were observed. After 70 days, four genotypes (J92-6400-A8, J92-6400-A10, J92-6400-A11 and J92-6400-A13) had a lower frequency of explants with transformants than the best-performing parental genotype. The differences between several other genotypes and the parental clones were clear but not significant, due to the relatively low number of explants per genotype used. The phenomenon of transformation at two cut surfaces was not observed in the parental clones whereas it occurred in eight offspring genotypes. This is clearly different to what has been observed in the regeneration assay without transformation and selective growth (Table 2).

Table 3 Competence for transformation, segregation for resistance to *Phytophthora infestans* race 0, and restriction polymorphisms with probe GP21 of a progeny of 26 plants and their parents

Genotypes	Transformation efficiency (%) within	Shoot induction within		RFLP[a] allele
	70 days	18 days	70 days from two sides	
Parents				
87-1024-2	15 ± 5.0 ab*	–	–	–
J91-6167-2	24 ± 6.6 a	–	–	+
Resistant J92-6400-				
A4	13 ± 6.8 a	–	–	+
A5	22 ± 8.0 a	–	+	+
A6	8 ± 5.2 a	–	–	+
A7	13 ± 6.2 a	+	+	+
A12	25 ± 7.7 a	+	+	+
A15	12 ± 6.5 a	–	+	+
A16	23 ± 8.3 a	+	+	+
A20	4 ± 3.9 a	–	–	+
A59	4 ± 3.8 a	–	–	+
A63	42 ± 9.7 a	–	–	+
A65	30 ± 8.0 a	–	+	+
A71	38 ± 9.9 a	–	+	+
A89	20 ± 7.3 a	–	–	+
A99	21 ± 8.3 a	–	–	+
Susceptible J92-6400-				
A1	12 ± 6.3 a	–	–	+
A2	5 ± 5.1 a	–	–	–
A8	0 ± 0.0 b	–	–	–
A10	0 ± 0.0 b	–	–	–
A11	0 ± 0.0 b	–	–	–
A13	3 ± 2.5 b	–	–	–
A17	0 ± 0.0 a	–	–	–
A18	0 ± 0.0 a	–	–	–
A19	9 ± 4.9 a	+	–	–
A50	30 ± 10.2 a	–	+	+
A51	8 ± 5.4 a	–	–	–
A64	0 ± 0.0 a	–	–	–

+ = the phenomenon is present
– = the phenomenon is absent
* The significant difference from the parental clones at $P = 0.05$ is indicated by different letters
[a] Marker GP21 linked to the *R1* locus on chromosome 5

The offspring in Table 3 have been divided into resistant (*R1r1*) and susceptible (*r1r1*) genotypes. A significant positive correlation was found between TE and resistance ($r = 0.57$). This is a clear indication that, as a result of genetic linkage, an important part of the genetic variation connected with competence for transformation is associated with variation at the *R1* locus. To substantiate this observation all clones were investigated for polymorphisms with the probe GP21 which is closely linked to *R1* (Leonards-Schippers et al. 1992; El-Kharbotly et al. 1994). As expected, a positive correlation between GP21 and both *R1* and TE ($r = 0.86$ and $r = 0.67$ respectively) was observed. The magnitude of the correlations indicate that the order of the loci involved is *R1*–GP21–TE. The recombination percentage between *R1* and GP21 is 7.7%. The transformation competence of some susceptible clones (J92-6400-A2 and J92-6400-A51) are probably influenced by other genetic factors present in parental clone 87.1024-2.

For transposon-tagging purposes the resistant clones J92-6400-A16 and J92-6400-A12 appeared to combine most of the desired factors needed for the rapid production of a large number of transformants. J92-6400-A12 had the disadvantage of producing too many tetraploid regenerants (data not shown). Therefore J92-6400-A16 was selected for further research.

Fertility of J92-6400-A16 and its diploid transformants

The fertility of the parent J92-6400-A16 has been compared with that of the J91-6167-2 parent (Table 4). The pollen stainability, berry set per pollination, and seed set per berry of J92-6400-A16 were all much higher. The only disadvantage of J92-6400-A16 appeared to be late flowering.

Genotype J92-6400-A16 has been transformed with the *Ds*-element-containing *A. tumefaciens* strain. As expected, a high percentage of transformants (83%) proved to be diploid. A sample of diploid transformants with one or more T-DNA copies has been investigated for fertility traits. It turned out that pollen stainability was decreased to a level between 33.0% and 0.6%. Berry set per pollination from all transformants (except BET92-Ds-A16-18) was lower than that of the untransformed control J92-6400-A16 (Table 4), varying from

Table 4 Male and female fertility of J91-6167-2, the selected clone J92-6400-A16, and 19 *Ds* transformants

Genotype		Male fertility (pollen stainability %)	Female fertility			
			Total no. of			
			Crosses	Berries	Seeds	Seeds/berry
J91-6167-2		0	41	10	212	21.2
J92-6400-A16		40	10	9	723	80.3
Single T-DNA copy						
BET92-*Ds*-A16	-5	8.9	30	25	1453	58.1
	-7	22.9	9	3	88	29.3
	-8	6.7	15	13	656	50.5
	-100	25.0	27	18	736	40.9
	-112	13.7	13	7	132	18.9
	-234	5.9	19	8	424	53.0
	-317	7.5	32	25	681	27.2
	-379	33.0	11	2	128	64.0
	-397	15.6	10	4	148	74.0
	-409	8.0	23	18	636	35.3
	-410	9.6	11	8	450	56.3
	-416	7.0	11	8	269	33.6
	-428	28.4	10	6	127	21.2
	-438	14.5	57	38	1364	35.9
	-465	0.6	50	37	1566	42.3
	-473	6.7	33	20	1412	70.6
More than one T-DNA copy						
BET92-*Ds*-A16	-18	4.9	40	36	1197	33.3
	-340	1.9	13	5	387	77.4
	-453	16.5	40	24	869	36.2

18.2 to 86.7%. The seed set per berry in all transformants was lower than in the control clone, varying from 18.9 to 77.4 seeds. These observations clearly indicate that both the male and female fertility of transformants are frequently affected to a certain degree.

Discussion

The correlations between the *R1* locus, the competence for transformation and the RFLP allele GP21 confirm the localization of the transformation-competence factor on chromosome 5. The number of genetic studies on transformation efficiency described in the literature is low. The early study by Koornneef et al. (1986) showed the possibility of transferring the competence for transformation from *Lycopersicon peruvianum* to the cultivated tomato *L. esculentum*. Other studies have been mainly restricted to the detection of genotypic effects within a species, as recently described by Du et. al. (1994) in the cultivated alfalfa. In potato, as in alfalfa (Du et al. 1994), competence for regeneration and transformation can be due to different factors. Genotypes like J92-6400-A10 and J92-6400-A17 (Tables 2 and 3) are early and well-regenerating but their competence for transformation is low, whereas genotypes such as J92-6400-A16 bear both competence factors. Variation in competence for transformation has earlier been described between tetraploid potato varieties (Hoekema et al. 1989).

Despite the good regeneration competence of cv Saturna (M. Goveia, personal communication) and its dihaploid progeny (unpublished data) a very low TE was found. This cultivar could only be transformed by an altered transformation protocol which has not been applied here. Our experiments showed that diploids, originating from this variety, were also recalcitrant for transformation. However a positive competence factor enabling transformation seemed to be linked to *R1* in the diploid *R1r1* clone J91-6167-2. This clone has been derived from the *R1*-bearing cultivar Hertha.

In the present study, genetic factors influencing shoot regeneration on stem explants appeared to be present but they were not investigated in detail. The outcome was that, in the sexual offspring, plants could be found with early and two-sided regeneration in a relatively high frequency of explants. In addition, the speed of regeneration differed between individual genotypes of the same progeny. The six genotypes which were faster than the parental clones in regeneration may be the result of the accumulation of genetic factors controlling this character. Indications of genetic variability in the speed of regeneration have been found earlier among diploids and monoploids derived from different diploid potato clones (M' Ribu and Veilleux 1990).

The phenomenon of regeneration from two cut surfaces of the explant in diploid potato has earlier been described by Visser (1989b). In the present study it is shown that this can be combined with high frequency competence for early regeneration and transformation. The advantage of this phenomenon is the induction of more independent transformants from the same number of explants, which save both labour and plant material.

Another important observation is that male fertility is decreased drastically in a relatively high frequency of transformants with one or more T-DNA inserts (Table 4). Female fertility is much less affected. This phenomenon was also reported earlier in diploid potato (Visser et al. 1989c). For transposon-tagging research, female fertility must be relatively high when high numbers of seeds are needed in screens for the "insertion mutant" among sexual offspring.

It can be concluded that variation is available for both regeneration and transformation competence. These competence factors could easily be selected in combination with the well-segregating resistance factor *R1* and with sufficient male and female fertility in the diploid clone J92-6400-A16.

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