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Selection of independent *Ds* transposon insertions in somatic tissue of potato by protoplast regeneration

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Abstract Potato is an autotetraploid crop plant that is not very amenable to the deployment of transposon tagging for gene cloning and gene identification. After diploidisation it is possible to get potato genotypes that grow well, but they are self-incompatible. This prevents the production of selfed progeny that are normally used in gene tagging approaches to select for parental lines with the target gene to be tagged in a homozygous stage. We describe here an alternative selection method for directed transposon tagging for a gene of interest in a heterozygous background. Diploid potato plants with a *Ds* transposon linked to the desired gene of interest (the *Phytophthora infestans R1* resistance locus) in a heterozygous stage were used for the development of this directed transposon tagging strategy. After crossing to a diploid *Ac* transposon-containing genotype, 22 'interesting' seedlings (R1Ds/r–; Ac/–) were selected that showed active *Ds* transposition as displayed by DNA blot hybridisation, empty donor site PCR and sequencing. Protoplast isolation and the use of the hygromycin gene as a cell-specific selection marker of *Ds* excision enabled the direct selection of *Ds* excision sectors in these highly chimaeric seedlings. This somatic selection of *Ds* transpositions and the regeneration through protoplasts resulted in the development of a large population of almost 2000 hygromycin-resistant plants. Southern blot analysis confirmed the insertion of *Ds* at independent positions in the genome. Every selected plant displayed independent *Ds* excisions and re-insertions due to the expression of the *Ac* transposase throughout development. This population, which is developed from seedlings with the desired *R1* gene in a heterozygous stage, is directly useful for searching for transposon-tagged *R1* mutants. In general, this approach for selecting for somatic transpositions is particularly suitable for the molecular isolation of genes in a heterozygous crop like potato.

Key words *Ac/Ds* transposon tagging · Somatic selection · Hygromycin · *Phytophthora infestans* · *R1* resistance gene

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Introduction

McClintock's genetic studies in maize resulted in the characterisation of *Dissociation* (*Ds*) and *Activator* (*Ac*) elements that cause chromosome breakage and mutations (McClintock 1948, 1950). The molecular isolation and characterisation of this two-component maize *Ac-Ds* transposable element (Fedoroff et al. 1983) directly lead to the isolation of genes that were 'tagged' by these elements (Fedoroff et al. 1984). *Ac-Ds* and other maize transposable elements (*En*/*Spm*) are also functional tools for isolating genes in several heterologous plant species like Arabidopsis (Aarts et al. 1993), petunia (Chuck et al. 1993), tobacco (Whitham et al. 1994), tomato (Jones et al. 1994) and flax (Lawrence et al. 1995). These are all self-fertilising plant species. Either random tagging strategies (Arabidopsis, petunia) by screening large populations for mutants or the targeted tagging of specific genes (tobacco, tomato, flax) were applied. In addition, positive selection strategies (tobacco and tomato) facilitated the direct selection of a mutant phenotype. By self- or testcrossing, large populations were produced for the direct screening of possible transposon-tagged mutants. The number of the germinal transmissions of transposon insertions determines the number of useful mutation events in such large selfed progeny populations.

Phytophthora infestans causes potato late blight and is one of the most economically important diseases that occurs in the autotetraploid potato crop. A large number of *R* (resistance) genes are present in the hexaploid wild species *Solanum demissum* and its introgressions in tetraploid potato cultivars. These race-specific *R* genes do not provide durable field resistance because of the rapid evolution of new virulent races of the fungus. To improve understanding of the molecular basis of *R* gene-mediated disease resistance we initiated a transposon tagging strategy in diploidised potato aimed at the molecular isolation of the *S. demissum R1* gene. Knapp et al. (1988) and Pereira et al. (1991) had previously shown that the maize *Ac* transposable elements are functional in potato. To tag the *R1* gene, diploid potato genotypes were induced and, after crossing, selected for a high *Agrobacterium tumefaciens* transformation frequency and segregation for the *R1* gene (El-Kharbotly et al. 1995), earlier mapped on chromosome 5 (Leonards-Schippers et al. 1992). Self-incompatibility at the diploid level hindered the production of vigorous homozygous plants, and the selection of *R1* homozygous diploid potato turned out to be a difficult and time-consuming process (unpublished). Therefore, an alternative method was developed to employ directed transposon tagging in diploid potato that was heterozygous for *R1*.

A common property of transposable elements is somatic instability within a plant, displayed as variegation or chimerism for wild-type and mutant sectors. Visual transposon phenotypic excision assays with marker genes encoding streptomycin resistance (Jones et al. 1989), β-glucuronidase (GUS) (Masson and Fedoroff 1989), rolC (Spena et al. 1989) or GBBS (Pereira et al. 1991) made somatic excision events screenable. In this way the occurrence and frequency of transposition could be determined and used for the selection of excision events. When kanamycin (Baker et al. 1987) or hygromycin (Rommens et al. 1992) marker genes are used, the selection of excision events at the cellular level is feasible and, in combination with effective *in vitro* selection and somatic propagation procedures, the production of large numbers of transposon mutants can be facilitated.

Diploid potato plants heterozygous for the *P. infestans R1* resistance gene were transformed with an *Agrobacterium* strain containing a *Ds*-transposon T-DNA construct. After mapping of the *Ds* T-DNA insertions (Jacobs et al. 1995), two plants harbouring the *Ds* T-DNA on chromosome 5 were further propagated. Sexual progeny of one of these *Ds* plants was obtained, and plants with the *R1* gene and the *Ds* T-DNA in coupling phase (18 cM) were selected (El-Kharbotly et al. 1996). By further crossing, an active *Ac* was introduced for the induction of *Ds* transposition.

This paper describes the selection of *R1* resistant diploid potato plants with an *R1*-linked *Ds* showing transposition late in development producing a population of highly chimaeric plants. Instead of searching for germinal transmission, we selected directly for somatic excision events by protoplast isolation and the use of hygromycin as a cell-specific selection marker for *Ds* excision. In this way a population of hygromycin-resistant regenerants was produced, and Southern blot analysis confirmed the selection of independent *Ds* mutations. This approach shows that somatic selection for excision and probably independent transposition events can facilitate the production of large tagging populations. This is particularly suitable for the isolation of genes in heterozygous crops like potato.

Materials and methods

Plant material

The diploid potato clone J92–6400-A16, *R1* resistant against *P. infestans* (R1r), was transformed with the *Ds* transposon-containing T-DNA construct pHPT::*Ds*-Kan shown in Fig. 1 (Pereira et al. 1992; El-Kharbotly et al. 1995). Transformants BET92-Ds-A16–259 (Ds259) and BET92-Ds-A16–416 (Ds416) were selected for a *Ds* T-DNA insertion on chromosome 5 (El-Kharbotly et al. 1996). Both were linked in repulsion phase to the previously mapped *P. infestans R1* resistance gene (Leonards-Schippers et al. 1992). Crossing Ds259 to diploid genotypes susceptible for *P. infestans* resulted in very few progeny. Crossing Ds416 to the same susceptible diploid genotypes resulted in offspring and enabled the selection of four recombinant plants (Ds53–3, –15, –22 and –34) having the *R1* gene and the *Ds* T-DNA in coupling phase (18 cM) (El-Kharbotly et al. 1996). In Ds416 two pHPT::*Ds*-Kan copies were present at one locus (unpublished).

Seeds were generally germinated on MS medium (Murashige and Skoog 1962) supplemented with 30 mg/l sucrose and 8 g/l agar (MS30). Shoot tips or axillary buds from *in vitro*-grown seedlings were used for the *in vitro* selection of kanamycin- and hygromycin-resistant seedlings. After transfer to the greenhouse all seedlings were tested for *P. infestans R1* resistance as described previously (El-Kharbotly et al. 1994), using *P. infestans* 89148–09 (race 0, kindly supplied by Dr. F. Govers, Laboratory of Phytopathology, Wageningen University and Research Centre, The Netherlands).

Molecular analysis

Plant genomic DNA was isolated from greenhouse-grown plants according to Pereira and Aarts (1998). Primers were designed on pHPT::*Ds*-Kan, and the polymerase chain reaction (PCR) was used to show excision of *Ds* (Fig. 1). Southern analysis was performed using *Pst* I-restricted genomic DNA and the blot probed with a NOS promoter DNA fragment. With this probe both the *Ac* as well as the *Ds* T-DNA loci revealed hybridising fragments. PCR products were separated on a 0.8% TBE agarose gel, and the specific empty donor site PCR fragments (EDS-PCR) were cut out of the gel. DNA was isolated from the agarose using Qiaex II (Qiagen), and the EDS-PCR fragments were cloned in a pGEM -T

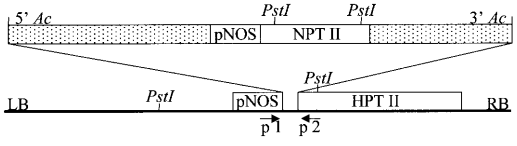

Fig. 1 Schematic drawing of pHPT::*Ds*-Kan showing the positions of primer 1 (p1, GCG CGT TCA AAA GTC GCC TA), primer 2 (p2, GTC AAG CAC TTC CGG AAT CG) and *Pst* I restriction sites. *LB*=Left border, *RB*=right border, *pNOS*=nopaline synthase promoter, *NPT II*=neomycin phosphotransferase gene, *HPT II*=hygromycin phosphotransferase gene

easy vector (Promega Corp) and sequenced using an automated ABI 373 DNA sequencer.

Protoplast isolation, culture and regeneration

Protoplasts were isolated from 4-week-old *in vitro*-grown shoots according to Uijtewaal et al. (1987). Before cutting, the plants were placed in the dark at 4°C for 6 h. After the second centrifugation step the protoplast pellet was not further purified but immediately re-suspended in culture medium TM2G (Wolters et al. 1991) to a final concentration of 500,000 pp/ml. In the first week the protoplast cultures were diluted 1:1 several times with fresh TM2G medium, and in the second week they were diluted 1:1 with TMD medium (Wolters et al. 1991). In the third week the calli were transferred to callus growth medium, and after 2 weeks of growth the largest calli were collected on shoot induction medium (Mattheij et al. 1992). Finally the calli were maintained on shoot elongation medium (Mattheij et al. 1992) until regenerated plants could be harvested. To select specifically for protoplast regenerants with excision events, we added 10 mg/l hygromycin to the callus growth medium 14 days after protoplast isolation. The hygromycin concentration was increased to 20 mg/l on day 21 and maintained during the whole protocol. In additional selection experiments the hygromycin concentration in the culture media was increased to 30 mg/l and 40 mg/l, and selection was started on day 14, 7 and 0 after protoplast isolation.

Hygromycin resistance testing

Hygromycin resistance of seedlings selected by PCR for *Ds* excision was tested by scoring for the rooting ability of *in vitro*-grown plants on MS30 supplemented with different concentrations of hygromycin (0, 10, 20, 30, 40, 60, 100 mg/l). Root formation was scored on day 10.

Rooting on MS30 supplemented with 40 mg/l hygromycin was used to test all regenerated shoots from the protoplast selection experiments as well as the control experiments. Regenerants with at least two growing roots were considered to be resistant; regenerants with no roots or with only one small, slow-growing root were considered to be sensitive.

Results

Development of transposon genotypes by crossing

To activate *Ds* linked to *P. infestans R1* resistance we crossed two of the four selected recombinants, Ds53–22 and -34 (Materials and methods), with TM17–2, a diploid potato clone susceptible to *P. infestans* and transformed with the *Ac* transposon-containing T-DNA construct pMK1GBSS*Ac* (Pereira et al. 1991). TM17–2 contains one functional *Ac* that is transferred to progeny lines and was selected for *Ac* excisions early in development (unpublished). The progeny of these crosses, populations EE96–4311 (Ds53–22 X TM17–2; 18 seedlings) and EE96–4312 (Ds53–34 X TM17–2; 96 seedlings), were tested for segregation of kanamycin (100 mg/l), hygromycin (30 mg/l) and *P. infestans R1* resistances. Since both parental lines contained a kanamycin resistance gene, used as a selection marker during T-DNA transformation, the expected segregation for kanamycin resistance was 3:1; while *P. infestans R1* resistance, only present in the *Ds* parent, was expected to segregate 1:1. The observed segregation data for kanamycin resistance and *P. infestans R1* resistance are displayed in Table 1. Population EE96–4312 showed a distorted segregation for *R1* resistance ($\chi 2$ 1:1=4.26, $0.05>P>0.025$), while in both populations segregation for kanamycin resistance was as expected. Finally, 47 (8 and 39) kanamycin-resistant *R1* seedlings (KanR *R1*) were selected from the two populations (Table 1). None of the seedlings showed clear hygromycin resistance in the first rooting test on 30 mg/l hygromycin, indicating that *Ds* was not excised in the majority of the cells in these plants.

The 47 KanR *R1* seedlings were further investigated by PCR to select *R1* resistant seedlings showing active *Ds* excision due to the presence of the introduced *Ac*. Using specific primers (Fig. 1), we determined the presence of empty donor sites (EDS-PCR) in 22 of the KanR *R1* seedlings as a 450-bp PCR product (data not shown).

The EDS-PCR data for a subset of plants were confirmed by Southern blot analyses (Fig. 2). Hybridisation with a NOS promoter probe revealed a *Pst* I restriction fragment of 4.0 kb corresponding to two copies of the original *Ds* T-DNA construct in the Ds53–22 parent and in 7 of the selected KanR *R1* seedlings from population

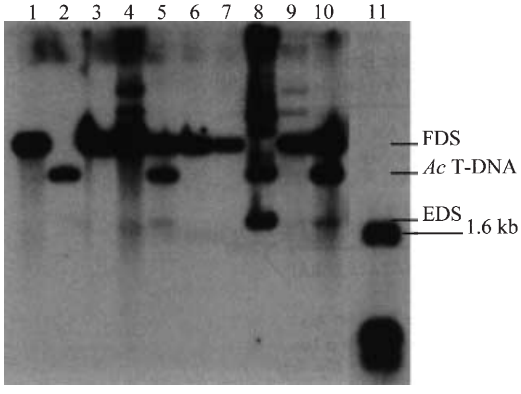

Fig. 2 *Pst* I restriction of genomic DNA hybridised to NOS promoter probe to select for presence of the full donor site (*FDS*=4.0 kb), empty donor site (*EDS*=2.3, kb) and *Ac* T-DNA construct (3.5 kb) in Ds53–22 (*lane 1*), TM17–2 (*lane 2*) and 8 KanR *R1* seedlings EE96–4311–3 (*lane 3*), 6 (*lane 4*), 8 (*lane 5*), 9 (*lane 6*), 10 (*lane 7*), 12 (*lane 8*), 14 (*lane 9*) and 15 (*lane 10*). *Lane 11* 1-kb DNA size marker

Table 1 Segregation data for kanamycin resistance versus susceptibility (KanR-KanS) and *P. infestans R1* resistance versus susceptibility (*R1*-*r*) for the populations EE96–4311 and EE96–4312

	KanR *R*	KanS *R1*	KanR *r*	KanS *r*	Unknown	Total
EE96–4311	8	1	3	2	4	18
EE96–4312	39	8	14	15	20	96

original site pHPT::*Ds*-Kan:	GT TGC GTG ACC	Ds	GCG TGA CCC GG
EE96-4311-12:	GT TGC GTG ...	-	... .. A CCC GG
EE96-4312-05:	GT TG.	-	... .. A CCC GG
	.. ..	-	... .. A CCC GG
EE96-4312-60:	GT TGC GTG AC.	-	... ... GG
EE96-4312-63:	GT TGC GTG ...	-	... .. A CCC GG

Fig. 3 Sequences flanking the *Ds* transposon in pHPT::*Ds*-Kan and the EDS sequences isolated from 4 R1Ds/r–; Ac/– seedlings displayed also in Table 2. The *underlined* sequence represents the target site duplication flanking the *Ds* insertion site

EE96–4311. Complete excision of *Ds* will result in a bandshift from 4.0 kb to 2.3 kb. In the KanR *R1* seedling EE96–4311–12, a 2.3-kb *Pst* I fragment is clearly visible. This corresponds to an empty donor site, indicating complete *Ds* excision early in development. Two other KanR *R1* seedlings (EE96–4311–8 and -15) showed a faint 2.3-kb fragment, the intensity corresponding to about 10% EDS-containing cells. The *Pst* I restriction fragment of ±3.5 kb in the TM17–2 parent reflects the presence of the pMK1GBSS*Ac* T-DNA construct in the *Ac* transposon-containing parent, which was inherited by the 3 plants showing an EDS fragment. The other 5 plants did not inherit this *Ac* T-DNA and did not show active *Ds* excision. This same analysis was also performed for 16 of the 39 KanR *R1* seedlings selected from the EE96–4312 population. Nine seedlings were selected by EDS-PCR for *Ds* excision, which was confirmed by the presence of a faint 2.3-kb EDS fragment in Southern blot analyses (data not shown). The other 10 KanR *R1* seedlings with active *Ds* excision were selected from this population solely on the basis of the EDS-PCR. In total 22 (3+19) out of 47 KanR *R1* seedlings showed clear empty donor sites, indicating active *Ds* transposition in the presence of the *Ac*.

To confirm that the EDS-PCR fragments were the result of *Ds* excision from the original T-DNA construct, we isolated the EDS-PCR fragments of 4 plants from an agarose gel and sequenced them. The DNA sequence of these four EDS-PCR fragments confirmed excision of *Ds* from the original T-DNA construct (Fig. 3).

Following selection of the 22 *R1* resistant seedlings showing active *Ds* excision (R1Ds/r–; Ac/–), as confirmed by PCR analyses, Southern blot analyses and EDS sequencing, the expression of hygromycin resistance was tested by rooting on MS30 supplemented with 10, 20, 30, 40, 60 and 100 mg/l hygromycin. Parental plant Ds53–22 and 2 of its R1Ds/r–; Ac/– progeny plants (EE96–4311–8 and -15) were only able to form roots on 10 mg/l hygromycin. EE96–4311–12 showed good rooting on 20, 30 and 40 mg/l hygromycin. The parental plant Ds53–34 and 18 of its R1Ds/r–; Ac/– progeny plants (EE96–4312–3, -5, -6, -14, -23, -27, -28, -30, -31, -37, -40, -43, -46, -52, -60, -63, -76, -89) showed good rooting on 10 and 20 mg/l hygromycin. EE96–4312–49 showed also some rooting on 30 mg/l hygromycin but not on 40 mg/l hygromycin. Despite the presence of empty donor sites shown by PCR and Southern blot analyses only 2 plants, EE96–4311–12 and EE96–4312–49, displayed an improved level of hygromycin resistance. In the case of plant EE96–4311–12 the complete EDS (absence of a full donor site; FDS), as shown on Southern blot (Fig. 2), explained the high level of hygromycin resistance. EE96–4312–49 did not show complete *Ds* excision on a Southern blot. These results suggest that *in vitro* rooting of shoots on 40 mg/l hygromycin can be used as a stringent criterion for complete *Ds* excision.

Protoplast isolation and selection of hygromycin-resistant regenerants

The 22 selected R1Ds/r–; Ac/- plants did display *Ds* excision at low levels, as shown by EDS-PCR and Southern blot analyses. Since only 1 seedling showed early and complete excision of *Ds*, resulting in high levels of hygromycin resistance (HygR), it seems that most excision events occurred late in shoot development. It is expected that in these cases different excision events would result in different somatic sectors of *Ds* transposition. These sectors of *Ds* excision should contain HygR cells. The individual cells were separated using protoplast isolation to investigate whether this is the case. After regeneration of the protoplasts, all regenerated plants were individually tested for their hygromycin resistance. As negative controls the parental plants Ds53–22 and Ds53–34 and a progeny plant EE96–4312–21 (R1Ds/r–; –/–) were used.

Table 2 gives an overview of the protoplast regeneration data. The parental genotype Ds53–22 performed poorly in the protoplast experiments, and no regenerants were obtained at all. The other parental plant, Ds53–34, and the control EE96–4312–21 showed a better protoplast regeneration capacity. From the 22 selected R1Ds/r–; Ac/– plants 16 genotypes formed sufficient amounts of calli and 15 of them also showed shoot regeneration from these calli.

From parental clone Ds53–34, control EE96–4312–21 and selected R1Ds/r–; Ac/– seedlings a maximum of 100 calli were collected and at least 50 regenerating shoots were harvested when possible. All of the regenerated shoots were tested for their rooting ability on MS30 supplemented with 40 mg/l hygromycin (Table 2). The percentages of recovered hygromycin-resistant regenerants from the different genotypes are shown in Fig. 4. As expected, the parent Ds53–34 and the control EE96–4312–21 produced no HygR protoplast regenerants. EE96–4311–12 gave 45% HygR protoplast regenerants, suggesting early excisions. The other 14 R1Ds/r–; Ac/– plants that performed well showed a regeneration of 4–33% of HygR shoots (Table 2, Fig. 4). These data confirmed the presence of HygR cells, indicating excision of *Ds* from its original T-DNA location.

To select for cells with excision events represented by HygR protoplast regenerants at an earlier stage of the regeneration process, we added hygromycin during the

Table 2 Number of calli, shoots and selected hygromycin-resistant (HygR) regenerants for parental genotypes Ds53–22 and Ds53–34, control genotype EE96–4312–21 (R1Ds/r–; –/–) and 22-selected R1Ds/r–; Ac/– genotypes from the seedling populations EE96–4311 and EE96–4312 after protoplast isolation and regeneration experiments with and without hygromycin selection

Genotype	No selection during protoplast regeneration			Hygromycin selection during protoplast regeneration		
	Calli	Shoots	HygR	Calli	Shoots	HygR
Ds53–22	10	0		47	0	
Ds53–34	100	45	0	134	1	0
EE96–4312–21	100	21	0	900	10	0
EE96–4311–08	0[a]			0[a]		
EE96–4311–12	100	49	22	1000	198	98
EE96–4311–15	300	82	11	800	160	101
EE96–4312–03	100	23	3	1000	166	83
EE96–4312–05	100	29	8	1000	198	121
EE96–4312–06	100	6[a]	2	1000	205	139
EE96–4312–14	100	70	15	1000	208	118
EE96–4312–23	100	51	2	1000	211	91
EE96–4312–27	10	0		10	0	
EE96–4312–28	100	47	7	1000	143	82
EE96–4312–30	100	0		419	0	
EE96–4312–31	100	30	2	570	21	4
EE96–4312–37	100	52	2	1000	248	92
EE96–4312–40	33	2	0	67	0	
EE96–4312–43	100	45	8	650	207	101
EE96–4312–46	14	9	0	103	0	
EE96–4312–49	100	48	3	1000	206	109
EE96–4312–52	3	3	0	1	1	1
EE96–4312–60	100	52	7	1000	203	93
EE96–4312–63	100	50	7	1000	203	130
EE96–4312–76	0			24	0	
EE96–4312–89	100	49	4	274	41	19
Total		691	103		2619	1382

[a] Low due to infection

protoplast culture starting at day 14 after isolation (see Materials and methods). From parent Ds53–34 and control EE96–4312–21 calli were obtained on hygromycin-containing medium but these rarely regenerated to shoots and they did not root on 40 mg/l hygromycin, confirming thereby the lack of *Ds* excision as expected. All 16 R1Ds/r–; Ac/– seedlings that performed well in protoplast culture were able to form relatively high numbers of actively growing calli on hygromycin-containing callus growth medium. Regenerated shoots were collected and tested for hygromycin (40 mg/l) resistance. Genotype EE96–4311–12 showed rooting for 50% of the regenerants (Table 2, Fig. 4). This percentage was comparable to the 45% HygR regenerants collected after protoplast regeneration without hygromycin selection and suggests that there is most likely an upper limit of real HygR regenerants that can be selected from this *Ds* excision locus. For all the other seedlings the use of hygromycin selection during callus culture and regeneration of shoots greatly increased the recovery of HygR regenerants to levels varying from 19% to 68% (Table 2, Fig. 4). These results indicate that *Ds* excision resulted in HygR cells in all of the selected R1Ds/r–; Ac/– plants. On average, 53% of these protoplast regenerants were HygR in a rooting test on MS30 supplemented with

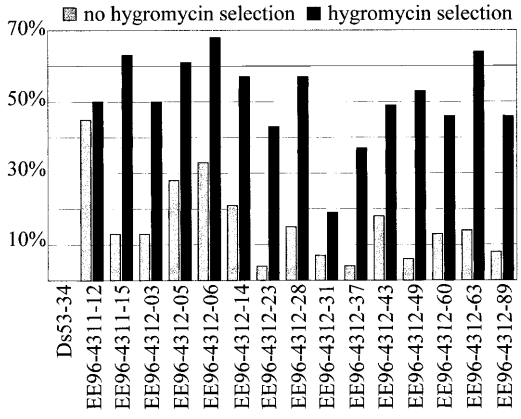

Fig. 4 Percentage hygromycin (40 mg/l)-resistant protoplast regenerants obtained with and without hygromycin selection during protoplast culture

40 mg/l hygromycin. This is a 3.8-fold increase in HygR regenerants when compared to the average of 14% HygR regenerants obtained without using hygromycin selection in the tissue culture phase.

To further improve the frequency of HygR regenerant selection, we made the selection more stringent. Hygro-

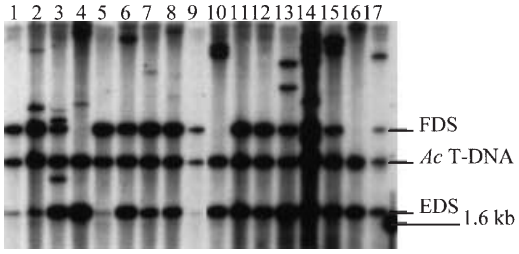

Fig. 5 *PstI* restriction of genomic DNA hybridised to NOS promoter probe to select for presence of the full donor site (FDS=4.0 kb), empty donor site (EDS=2.3 kb), *Ac* T-DNA construct (3.5 kb) and *Ds* re-insertion sites in the R1Ds/r–; Ac/– selected seedlings EE96–4311–37 (*lane 1*), EE96–4312–43 (*lane 5*) EE96–4312–49 (*lane 9*) and HygR protoplast regenerants from EE96–4311–37 (*lanes 2, 3 and 4*), EE96–4312–43 (*lanes 6, 7 and 8*), EE96–4312–49 (*lanes 10, 11, 12 and 13*), EE96–4311–15 (*lanes 13, 15*), EE96–4312–05 (*lane 14*), EE96–4312–76 (*lane 16*) and EE96–4312–06 (*lane 17*). *Lane 18* the 1-kb DNA size marker

mycin was added starting on day 0, 7 or 14 after protoplast isolation, and the concentration of hygromycin in the culture media was increased to 30 or 40 mg/l. These more stringent conditions in the earlier stages of the protoplast culture process had a negative effect on the formation of calli and on the regeneration capacity of these calli into shoots. For only a few genotypes was it possible to collect reasonable amounts of calli and regenerating shoots (data not shown). After re-testing these shoots, the percentages of regenerants showing good rooting on 40 mg/l hygromycin had not increased, indicating that the percentages shown in Fig. 4 are the maximum numbers of HygR regenerants that can be obtained for the R1Ds/r–; Ac/– seedlings. In total 1,485 (103+1,382) HygR regenerants were selected from all R1Ds/r–; Ac/– seedlings used in the first protoplast regeneration experiment (Table 2). From later experiments with more stringent hygromycin selection 480 additional HygR regenerants were selected, making a total of 1,973 HygR protoplast regenerants available for further investigation.

Molecular analysis of *Ds* excision and re-insertion

We performed Southern analysis on a subset of selected R1Ds/r1–; Ac/– seedlings and some of their HygR protoplast regenerants in order to analyse *Ds* excision in the HygR protoplast regenerants; some of these analyses are shown in Fig. 5. The R1Ds/r1–; Ac/– seedlings used for protoplast isolation all displayed two *Pst* I fragments, 4.0- and 3.5-kb, corresponding to the *Ds* T-DNA construct and the *Ac* T-DNA construct respectively. Faintly visible fragments of 2.3 kb were also detected that correspond to a low amount of EDS fragments being present in these seedlings. All HygR protoplast regenerants showed a strong hybridising EDS fragment, indicating the early or repeated excision of *Ds*, which in turn corresponds to the high level of hygromycin resistance for which these plants were selected. The original *Ds* parent accommodated two copies of *Ds*. Full donor site fragments were detected in most of the HygR protoplast regenerants, which indicates that one of the two *Ds*'s was not excised. Four plants (3 are shown in Fig. 5) showed complete EDS, indicating that in the starting protoplast of these plants both *Ds*'s were excised.

Most HygR protoplast regenerants showed clear *Ds* re-insertion fragments varying from one to eight new positions per individual HygR regenerant. Different regenerants from one single seedling showed different re-insertion patterns, indicating that they originated from independent transposition events. These data confirm the expectation that all selected HygR protoplast regenerants originated from independent excision sectors in the original R1Ds/r1–; Ac/– seedling.

Discussion

The efficient transposon tagging of specific genes can only be achieved when a large population of different insertional mutants can be produced easily. In plant species that produce large amounts of seeds, self- or testcross populations with a high germinal transmission frequency of transposon mutations can be used. However, attempts to develop efficient transposon tagging strategies in potato encountered the problem that at the tetraploid level this approach is rather difficult. After diploidisation the development of vigorous homozygous *Phytophthora infestans* resistant R1R1 plants turned out to be very time-consuming (unpublished). Therefore, an alternative method was developed to use the maize *Ac-Ds* transposable elements in a directed transposon tagging strategy for tagging the *R1* resistance gene. This strategy started with the selection of diploid potato plants heterozygous for the *R1* gene and having a *Ds* linked in coupling phase. By crossing these plants with a diploid potato containing an active *Ac* we were able to select R1Ds/r–; Ac/– seedlings showing *Ds* transposition in the somatic cells. These chimaeric seedlings were used in a protoplast regeneration approach to select cells with a *Ds* transposition event.

The transposase source used was from TM17–2, a diploid potato plant that contained an active autonomous *Ac* (unpublished). This plant was selected from a population of transformants showing various patterns of *Ac* transposition (Pereira et al. 1991). These differences were probably due to a combination of position and dosage copy-number effects. In the original transformant, TM17–2, early *Ac* excision occurred from one of the two copies of the pMK1GBSS*Ac* construct, resulting in two *Ac* homologous fragments. One of these appeared to be a complete *Ac*, while the other one (linked) had a deletion in the 3′ site and was presumably not functional (unpublished). Crossing of TM17–2 with other diploid potato genotypes was expected to result in progeny having only one segregating active *Ac* transposase source. The heterogeneity in the sizes of the excision sectors seen in the one- and two-copy transformants from the

pMK1GBSS*Ac* population as described in Pereira et al. (1991) can, therefore, also be expected for progeny of TM17–2. Presumably these plants show a continuous expression of transposase activity throughout shoot development (Scofield et al. 1992). The level of transposase enzyme activity is probably low because significant somatic variegation has already resulted from an extremely low level of transcription from the wild-type *Ac* promoter (Fusswinkel et al. 1991).

In this study R1Ds/r–; Ac/– seedlings were selected by searching for EDS by PCR analyses. Southern blot analyses (Fig. 2) confirmed the presence of these EDS in somatic tissue. The DNA sequence of a few EDS-PCR fragments offered supplementary evidence for the excision of *Ds* from its original T-DNA construct (Fig. 3). Testing of these seedlings for rooting on hygromycin-containing media showed that most of these plants did not display any hygromycin resistance, indicating that only a small number of the somatic cells contained excised *Ds* elements. This observation shows that *Ds* excision induced by the one-copy autonomous *Ac* transposase source seems to occur relatively late in development of the seedling, resulting in chimaeric plants with small sectors of independent excision events. Only 1 progeny seedling, EE96–4311–12 showed complete *Ds* excision early in development. Instead of looking further at the germinal transmission of these excision events, the chimaeric plants with an EDS site were considered as being an interesting pool of independent *Ds* transpositions.

Protoplast isolation and regeneration has proven to be an efficient method in potato to create a large progeny from an individual genotype. It was possible to produce a large population of protoplast regenerants from our selected R1Ds/r–; Ac/– seedlings. The regenerated plants were tested for hygromycin resistance, which confirmed the excision of *Ds*. All plants that displayed an EDS-PCR fragment were able to produce HygR protoplast regenerants, indicating that this selection criterion was accurate and that *Ds* excision from the T-DNA resulted in the expression of the hygromycin resistance gene. In experiments without hygromycin selection during the callus phase on average 14% of the regenerated plants were hygromycin-resistant, indicating that they were regenerated from somatic cells expressing *Ds* excision events. This percentage confirms the 10% EDS-containing cells estimated from the intensity levels of the EDS fragments on Southern blots. Hygromycin selection during protoplast regeneration made it possible to directly select for *Ds* excisions in protoplasts of the chimaeric R1Ds/r-; Ac/- plants. This resulted in a significant increase in the number of HygR protoplast regenerants selected – 53%, the upper limit of HygR regenerants that can be obtained from this *Ds* excision locus.

The somatic selection of *Ds* transpositions from individual cells facilitated the production of a large population of shoots with *Ds* excision events. Southern blot analysis confirmed the selection of independent *Ds* insertions as all investigated regenerants derived from one progeny plant showed different re-insertion sites of *Ds*, indicating independent excision and re-insertion. The occurrence of different re-insertion sites for *Ds* is an indication for the presence of small independent sectors with *Ds* transposition in the selected R1Ds/r–; Ac/– seedlings. Within the seedlings several independent *Ds* excisions and re-insertions probably occur due to the continuous expression of the *Ac* transposase throughout development.

In total, almost 2000 HygR protoplast regenerants were selected that potentially represent 2000 independent *Ds* insertions. This number of *Ds* transposon-induced mutations should theoretically be enough for the isolation of tagged mutants of the linked *R1* gene. Previously it was shown that in tomato the *Cf9* gene was tagged at a frequency of 1 in 1000 *Ds* transposition events from a position located at a distance of 3 cM (Jones et al. 1994). In Arabidopsis tagging of the *FAE1* genes by an *Ac* at a 22-cM distance resulted in 1 insertion from about 500–1000 transpositions (James et al. 1995). The somatic selection of *Ds* transposition and the rapid production of independent plants containing these transpositions as described here facilitate the production of large tagging populations needed for the transposon mutagenesis of selected genes. This is particularly suitable for the mutagenesis of genes in heterozygous crops like potato.

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References

Aarts MGM, Dirkse WG, Stiekema WJ, Pereira A (1993) Transposon tagging of a male sterility gene in *Arabidopsis*. Nature 363:715–717

Baker B, Coupland G, Fedoroff N, Starlinger P, Schell J (1987) Phenotypic assay for excision of the maize controlling element *Ac* in tobacco. EMBO J 6:1547–1554

Chuck G, Robbins T, Nijjar C, Ralston E, Courtney-Gutterson N, Dooner HK (1993) Tagging and cloning of a petunia flower color gene with the maize transposable element Activator. Plant Cell 5:371–378

El-Kharbotly A, Leonards-Schippers C, Huigen DJ, Jacobsen E, Pereira A, Stiekema WJ, Salamini F, Gebhardt C (1994) Segregation analysis and RFLP mapping of the *R1* and *R3* alleles conferring race-specific resistance to *Phytophthora infestans* in progeny of dihaploid potato parents. Mol Gen Genet 242: 749–754

El-Kharbotly A, Jacobsen E, Stiekema WJ, Pereira A (1995) Genetic localisation of transformation competence in diploid potato. Theor Appl Genet 91:557–562

El-Kharbotly A, Jacobs JME, Te Lintel Hekkert B, Jacobsen E, Ramanna MS, Stiekema WJ, Pereira A (1996) Localization of *Ds*-transposon containing T-DNA inserts in the diploid transgenic potato: linkage to the *R1* resistance gene against *Phytophthora infestans* (Mont.) de Bary. Genome 39:249–257

Fedoroff N, Wessler S, Shure M (1983) Isolation of the transposable maize controlling elements *Ac* and *Ds*. Cell 35:235–242

Fedoroff NV, Furtek DB, Nelson OE (1984) Cloning of the *bronze* locus in maize by a simple and generalizable procedure using the transposable controlling element *Activator* (*Ac*). Proc Natl Acad Sci USA 81:3825–3829

Fusswinkel H, Schein S, Courage U, Starlinger P, Kunze R (1991) Detection and abundance of mRNA and protein encoded by transposable element *Activator* (*Ac*) in maize. Mol Gen Genet 225:186–192

Jacobs JME, Van Eck HJ, Arens P, Verkerk-Bakker B, Te Lintel Hekkert B, Bastiaanssen HJM, El-Kharbotly A, Pereira A, Jacobsen E, Stiekema WJ (1995) A genetic map of potato (*Solanum tuberosum*) integrating molecular markers, including transposons, and classical markers. Theor Appl Genet 91:289–300

James DW, Lim E, Keller J, Plooy I, Ralston E, Dooner HK (1995) Directed tagging of the Arabidopsis *FATTY ACID ELONGATION* (*FAE1*) gene with the maize transposon *Activator*. Plant Cell 7:309–319

Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789–793

Jones JDG, Carland FM, Maliga P, Dooner HK (1989) Visual detection of transposition of the maize element *Activator* (*Ac*) in tobacco seedlings. Science 244:204–207

Knapp S, Coupland G, Uhrig H, Starlinger P, Salamini F (1988) Transposition of the maize transposable element *Ac* in *Solanum tuberosum*. Mol Gen Genet 213:285–290

Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG (1995) The *L6* gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell 7:1195–1206

Leonards-Schippers C, Gieffers W, Salamini F, Gebhardt C (1992) The *R1* gene conferring race-specific resistance to *Phytophthora infestans* in potato is located on potato chromosome V. Mol Gen Genet 233:278–283

Masson P, Fedoroff NV (1989) Mobility of the maize Suppressor-mutator element in transgenic tobacco cells. Proc Natl Acad Sci USA 86:2219–2223

Mattheij WM, Eijlander R, De Koning JRA, Louwes KM (1992) Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *Solanum circaeifolium* subsp *circaeifolium* Bitter exhibiting resistance to *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens: 1. somatic hybrids. Theor Appl Genet 83:459–466

McClintock B (1948) Mutable loci in maize. Carnegie Inst Washington Yearb 47:155–169

McClintock B (1950) The origin and behavior of mutable loci in maize. Proc Natl Acad Sci USA 36:344–355

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

Pereira A, Aarts MGM (1998) Transposon tagging with the *En-I* System. In: Martinez-Zapater J, Salinas J (eds) Methods in molecular biology, vol. 82: Arabidopsis protocols. Humana Press, Totowa, N.J., pp 329–338

Pereira A, Aarts M, Van Agtmaal S, Stiekema WJ, Jacobsen E (1991) Waxy variegation in transgenic potato. Maydica 36: 323–327

Pereira A, Jacobs JME, Te Lintel Hekkert W, Rutgers E, Jacobsen E, Stiekema WJ (1992) Towards the isolation of resistance genes by transposon targeting in potato. Neth J Plant Pathol 98:215–221

Rommens CMT, Van Haaren MJJ, Buchel AS, Mol JNM, Van Tunen AJ, Nijkamp HJJ, Hille J (1992) Transactivation of *Ds* by *Ac*-transposase gene fusions in tobacco. Mol Gen Genet 231:433–441

Scofield SR, Harrison K, Nurrish SJ, Jones JDG (1992) Promoter fusions to the *Activator* transposase gene cause distinct patterns of *Dissociation* excision in tobacco cotyledons. Plant Cell 4:573–582

Spena A, Aalen RB, Schulze SC (1989) Cell-autonomous behavior of the *rolC* gene of *Agrobacterium rhizogenes* during leaf development: A visual assay for transposon excision in transgenic plants. Plant Cell 1:1157–1164

Uijtewaal BA, Suurs LCJM, Jacobsen E (1987) Protoplast fusion of monohaploid (2n=×=12) potato clones: identification of somatic hybrids using malate dehydrogenase as a biochemical marker. Plant Sci 51:277–284

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene *N*: similarity to Toll and the Interleukin-1 receptor. Cell 78:1101–1115

Wolters AMA, Schoenmakers HCH, Van der Meulen-Muisers JJM, Van der Knaap E, Derks FHM, Koornneef M, Zelcer A (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet 83:225–232