Theor Appl Genet (1988) 75:452–459

Regeneration and characterization of plants from potato root lines transformed by *Agrobacterium rhizogenes*

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Received June 25, 1987; Accepted August 31, 1987
Communicated by G. Wenzel

Summary. Transformed root lines were obtained after infection of leaf segments and tuber discs of tetraploid potato cvs Bintje and Desirée with *Agrobacterium rhizogenes*. In response to shoot induction, about 10% of the root lines produced shoots through callus formation. The tests for opine suggest that all 26 shoot lines of cv Bintje (Ri-Bintje) and 13 of Desirée (Ri-Desirée) were transformed. All shoot lines were tetraploid except for one octoploid subshoot line of cv Desirée; no aneuploids were observed. With the exception of two shoot lines derived from the same root line, all other Ri-Bintje plants showed a pattern of phenotypic variation, generally observed among transformed plants. In contrast, the phenotype of Ri-Desirée plants was uniform and normal; variation was observed in tuber form and size. Phenotypic variation observed among Ri-plants appeared to be mainly root line-dependent, particularly for height of plants and tuber size and form. Variation was also observed within root and shoot lines and was more pronounced among the Ri-Bintje plants. Segregation of phenotypic characteristics was observed among transformed plants, resulting in the occurrence of phenotypes resembling the control. Chromosomal stability and the frequent reversion to normal phenotype of Ri-plants make *A. rhizogenes* particularly suitable as a virulence vector in the binary transformation system for the transfer of desirable genes.

Key words: *Solanum tuberosum* L. – *Agrobacterium rhizogenes* – Plant regeneration – Genetic stability – Phenotypic characterization

Introduction

Tetraploidy and heterozygosity are requirements for the vigour and productivity of potato cvs (*Solanum tuberosum* L.). However, these properties are a barrier as well for breeders interested in further improvement of the crop (Howard 1970). It is difficult to distinguish morphological characteristics among the progeny from crosses; until now only two genes have been mapped (Hermsen et al. 1973; Wagenvoort 1982). Therefore, genetic manipulation of potato for the introduction of desirable genes has received much attention. One requirement for successful genetic manipulation is genetically stable plant regeneration from transformed protoplasts or cells. Results from several research groups have shown that plant regeneration from protoplasts of various potato cvs is, genetically, a very unstable process (reviewed in Sree Ramulu 1986). The occurrence at high frequency of polyploidy, aneuploidy and point mutations is the result of early events during protoplast culture (Sree Ramulu et al. 1984).

Potato callus from other origins also appeared to be genetically unstable. These callus tissues were induced by hormone treatment of explants and by infection of tuber discs of cv Bintje with different *Agrobacterium tumefaciens* strains and mutants. All the tissues showed rapid polyploidization, irrespective of hormonal regime and explant source (Hänisch ten Cate and Sree Ramulu 1987). In contrast, *A. rhizogenes* infection produced hairy roots without visible callus formation on the tuber discs of cv Bintje, and the transformed roots appeared genetically stable upon continuous culture in liquid and on solid media (Hänisch ten Cate et al. 1987).

The present article reports on plant regeneration from various Ri-transformed root lines of cvs Bintje

and Desirée. Distinguishing between general effects and those of individual transformation events was achieved by comparison of the morphogenesis of the regenerated plants. Confirmation of the transformed condition of regenerants and genetic stability was obtained by opine analysis and by measurement of nuclear DNA content and chromosome counts, respectively. In cv Bintje, considerable differences were observed between plants from different root clones.

Materials and methods

Induction and culture of transformed root lines

Sterile tuber discs and leaf segments of the tetraploid potato cvs Bintje and Desirée (*Solanum tuberosum* L., 2n=4x=48) were infected with the agropine strains of *Agrobacterium rhizogenes* LBA 9402 (a derivative of AR 1855) and AR 15834, according to the method of Anand and Heberlein (1977). The infected tuber discs were plated on H_2O-agar (1%) and leaf segments were plated on MS medium (Murashige and Skoog

Table 1. Origin of transformed roots and regeneration of shoots in potato cvs Bintje and Desirée

Potato cv	Plant organ	*A. rhizogenes* strain	No. of root lines tested showing shoot development		No. of primary shoots obtained	Code of root lines as in Tables 2, 3
Bintje	leaf	AR 15834	10	4	16	*a, b, c, d (d1, d2)*[a]
		LBA 9402	12	1	8	*g*
	tuber	AR 15834	10	1	1	*f*
		LBA 9402	40	1	1	*e*
Desirée	tuber	AR 15834	10	1	5	*i*[b]
		LBA 9402	10	2	8	*h, j*[b]

[a] Root line *d* generated two types of primary shoots: *d1* and *d2*
[b] Root lines *i* and *j* generated several shoot lines among which one (*i*) and two (*j*) shoot lines segregated in two types of shoots (a and b) after subculture (*see text*)

Table 2. Characteristics of Ri-Bintje plants regenerated from root lines

Root line	*a*	*b*	*c*	*d1*	*d2*	*e*	*f*	*g*	Control
No. of shoot lines	2[a]	6	5	1	2	1	1	8	7
Opines in vitro shoots:[b]									
agropine	++	+	±	–	±	±	±	±	–
mannopine	++	++	±	±	±	+	±	+	–
Height of plants:[c]									
long	–	–	–	–	2	–	–	–	7
intermediate	–	6	5	1	–	1	1	–	–
small	–	–	–	–	–	–	–	8	–
Leaves:									
normal	–	3	–	–	2	2	–	3	–
deviant	–	1[d]	5	1	–	–	1	3	7
segregating	–	2[e]	–	–	–	–	–	2	–
Tuber form:									
oblong	–	5	5	1	–	1	1	8	–
round	–	–	–	–	2	–	–	–	7
Tuber weight: (g/plant)	–	5.0±3.4	41±16	27±8	89±5	26±7	18±4	2.1±2.0	88±10
Decreased apical dominance	–	2	5	–	–	–	–	7	–
Rooting (excessive)	–	3	1	–	–	–	–	–	–
Flowering	–	3[f]	–	–	–	–	–	–	–

[a] Both these shoot lines from rootline *a* did not produce plants after potting
[b] –: not detectable; ±: low level; +: average; ++: high level
[c] The three classes of height were discontinuous: small: 20–30 cm; intermediate: 40–60 cm; long: 80–150 cm
[d] One shoot line that showed deviant leaves formed no tubers.
[e] Two lines gave rise to excessive rooting and normal rooting plants, which showed normal and deviant leaves, respectively
[f] Three of the lines with normal leaf development showed each one flowering plant

1962) supplemented with cefotaxime (200 mg/l) to suppress bacterial growth. Root lines were obtained after excision of single roots, and were propagated on MS medium supplemented with cefotaxime during 6 subcultures as described by Hänisch ten Cate et al. (1986).

Plant regeneration and culture

Shoot regeneration from transformed roots was induced according to Ooms et al. (1985), with shorter periods of root dedifferentiation and shoot induction: 4 and 2 weeks, respectively. Shoot lines were obtained from primary shoots via micropropagation on MS medium with 2% sucrose. The root lines producing shoots were designated "*a–g*" for Bintje and "*h, i, j*" for Desirée (Tables 1 and 2). All plants regenerated from transformed roots of cvs. Bintje and Desirée (hereafter called Ri-Bintje and Ri-Desirée), and in vitro cultured control plants were transferred to soil and grown under controlled conditions: 17 °C during the day with a photoperiod of 14 h at 15,000 lux, and 15 °C during the night; relative humidity was approximately 80%. After 3 weeks the plants were grown to maturity in the greenhouse.

Phenotypic characterization of plants

Three plants per Ri shoot line were analysed to determine the following phenotypic characteristics: growth and height of plants; branching; axillary rooting; leaf colour, shape, texture and size; flowering and tuber characteristics.

In vitro tuber induction

Axillary shoot segments were cultured on MS medium supplemented with 8% sucrose and 33 μM benzylaminopurine (BAP), and incubated in the dark at 24 °C for a minimum of 6 weeks.

Flow cytometric (FCM) measurements

Nuclear DNA contents of cells from regenerated shoots were measured with a Fluorescent Activated Cell Sorter (FACS IV, Becton Dickenson, Sunnyvale, USA) according to the method of Galbraith et al. (1983) with some modifications (Hänisch ten Cate et al. 1986).

Cytological analysis

Chromosome numbers of regenerated plants were analysed in mitotic metaphase cells in root tips stained by Feulgen reagent (Sree Ramulu et al. 1985).

Agropine and mannopine tests

The presence of agropine and mannopine in various tissues was established by paper electrophoresis and silver-staining according to Petit et al. (1983).

Results

Plant regeneration

Upon infection of potato tuber discs with the two *A. rhizogenes* strains, many hairy roots (hereafter called transformed roots) developed randomly on the surface of the discs without visible callus formation. From infected leaf segments, transformed roots developed at the wounded borders only from the leaf veins. The primary transformed roots showed variation in phenotype and growth rate. No differences were observed between the effects of the two bacterial strains used.

Healthy looking, vigorously growing roots were excised and subcultured on MS medium. After 3 to 5 subcultures, the transformed roots appeared free of bacteria.

After the onset of induction, shoots appeared on transformed roots, particularly on compact green callus, within 3 weeks. The majority of this callus type produced shoots. Shoot formation directly from roots was also observed. The frequencies of shoot-producing root lines and the number of shoots per root line were similar in the two potato cultivars (Table 1). The high frequency of shoot-forming root lines from leaf segments of cv Bintje infected with AR 15834 was based on a number too low to permit conclusions.

Characterization of regenerated plants

Normal cv Bintje shoots grown in jam jars were slender with single leaves only. On the other hand, 24 of the 26 Ri-Bintje lines, and all of the shoots of the Ri-Desirée lines, were bigger and grew more vigorously in vitro. Among the Ri-Bintje shoot lines several phenotypes could be distinguished. The normal, slender plant type had single round to oblong leaves, and the robust type had single leaves as well as compound crinkled leaves, showing axillary buds with excessive rooting (Fig. 1 A).

In cv Bintje, 7 root lines generated 26 shoot lines (1 to 8 shoot lines per root line) (Table 2). The shoot characteristics were similar within the shoot lines but different between the root lines. One root line (*d*) produced two types of shoot lines, i.e., a transformed type (*d1*) and a control type resembling cv Bintje (*d2*) (Fig. 1 A).

In cv Desirée, 3 root lines generated 13 shoot lines (3–5 shoot lines per root line) of a similar phenotype showing robust, vigorously growing plants with single and compound leaves. During propagation, 3 of the shoot lines segregated for another type of shoot, which was even more robust but with only single leaves (Fig. 1 B).

Presence of opines

All the in vitro shoot lines showed the presence of mannopine, which proves the transformed condition of the plants. As inferred from the spot sizes on the electropherograms, the amounts of agropine were lower than those of mannopine in the majority of lines, and not detectable in about 30% of the plants investigated (Tables 2 and 3). The in vivo-grown shoots showed great variation in spot sizes; the ratio of mannopine/

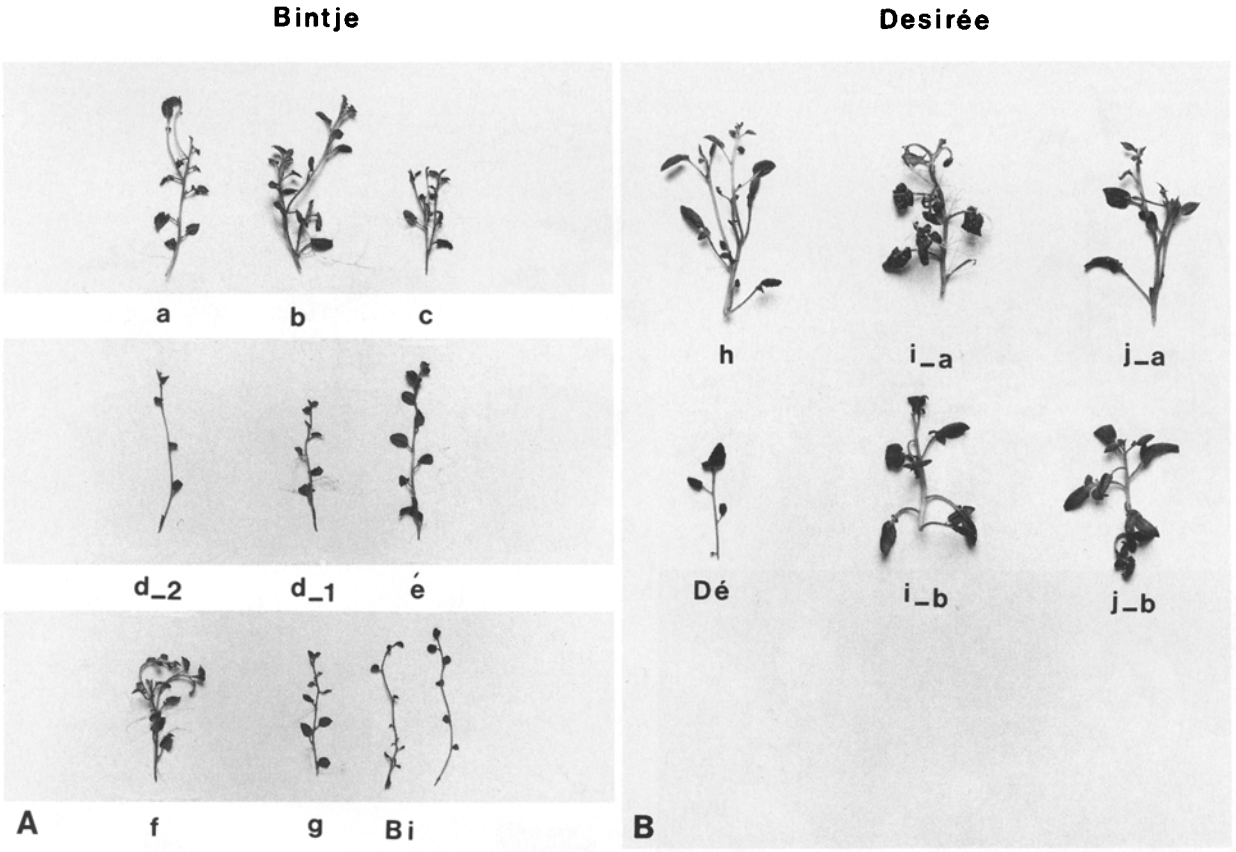

Fig. 1. In vitro grown Ri-shoots of the various root lines in the cv Bintje (**A**) and cv Desirée (**B**). The code of shoots *corresponds with* the root lines in Tables 2 and 3

Table 3. Characteristics of Ri-Desirée plants regenerated from root lines

Root line	*h*	*i*	*j*
No. of shoot lines	5	5[a]	3[a]
No. of subshoot lines	–	1 (a, b)	2 (a, b)
Opines in in vitro shoots:[b]			
agropine	±	+	±
mannopine	++	++	±
Tuber form:			
oblong	5	6	1
round	–	–	4
Tuber weight (g/plant):	6.5±4	7.6± 5[c] 60 ±23[d]	20± 5[c] 53±25[d]
Flowering	2	–	1

[a] One and two shoot lines, respectively, segregated in two subshoot lines with the phenotypes a and b (Fig. 1 B)
[b] ±: low level; +: average; ++: high level
[c] Yield of "a" type shoot line
[d] Yield of "b" type shoot line

Table 4. Relative nuclear DNA content and chromosome number in plants regenerated from root lines induced by *A. rhizogenes* in potato cvs Bintje and Desirée

	DNA content				Chromosome counts	
	C-value	% nuclei	C-value	% nuclei	No. of plants	Chromosome no.
Control Bintje	3.8±0.1	85	7.6±0.3	15	10	48
Ri-Bintje	4.0±0.2	80	7.8±0.3	20	19	48
Ri-Desirée	4.2±0.3	75	8.2±0.5	25	13	48
					1	96

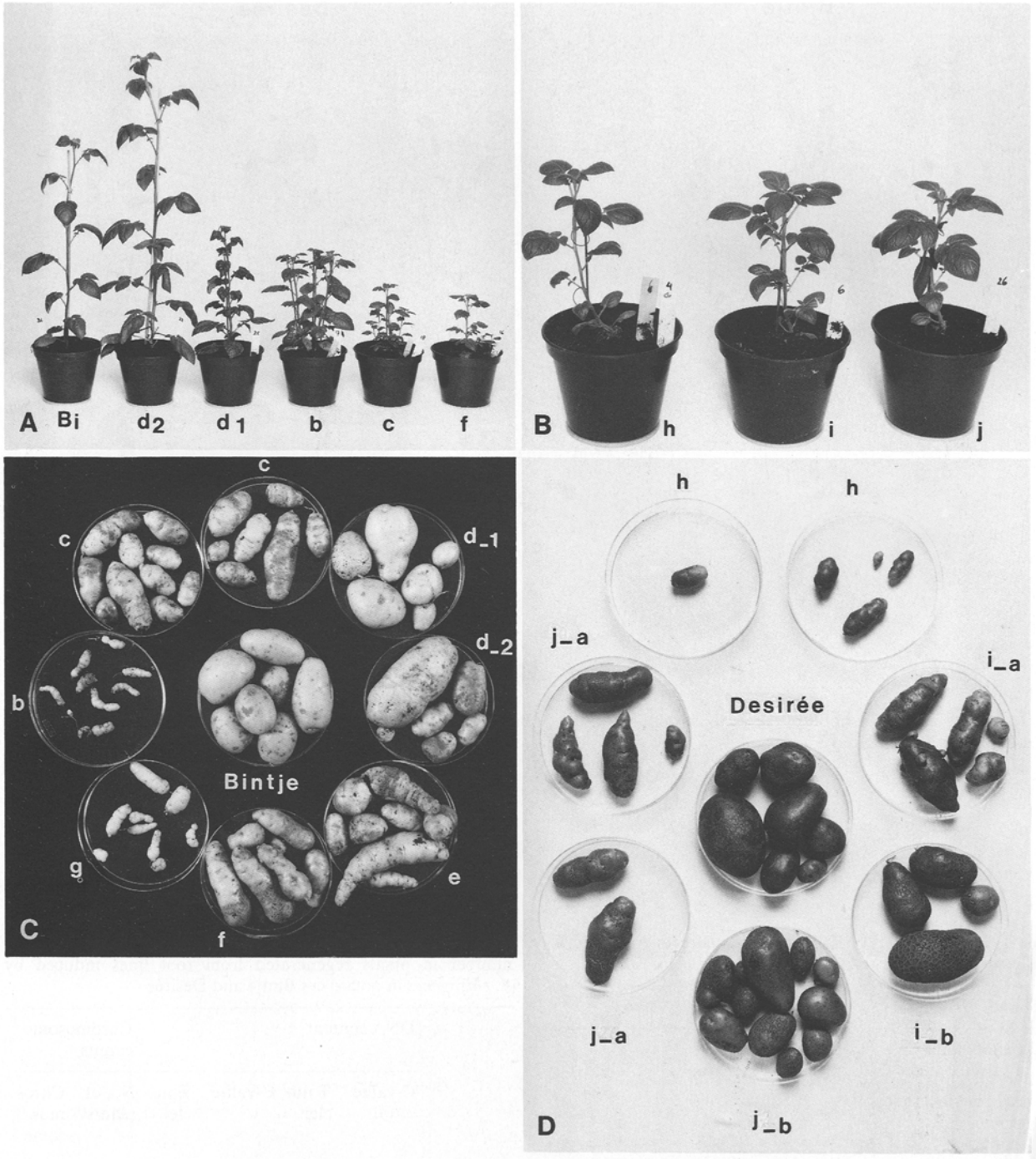

Fig. 2. In vivo grown Ri-plants of various root lines in the cv Bintje (**A**) and cv Desirée (**B**), and their respective tubers (**C** and **D**). The code of plants and tubers *corresponds with* the root lines in Tables 2 and 3

agropine content in shoots is presumed to be root clone dependent.

Nuclear DNA content and chromosome number

In cv Bintje, all Ri-plants had nuclei with approximately 4C and 8C DNA content corresponding to G1 and G2 nuclear phases of tetraploid cells and the normal chromosome number ($2n=4x=48$). In cv Desirée, 13 Ri-plants were tetraploid and one octoploid (Table 4). Apparently, one shoot line of root line *j* (Table 1) was a mixoploid which segregated into sub-shoot lines during in vitro shoot multiplication.

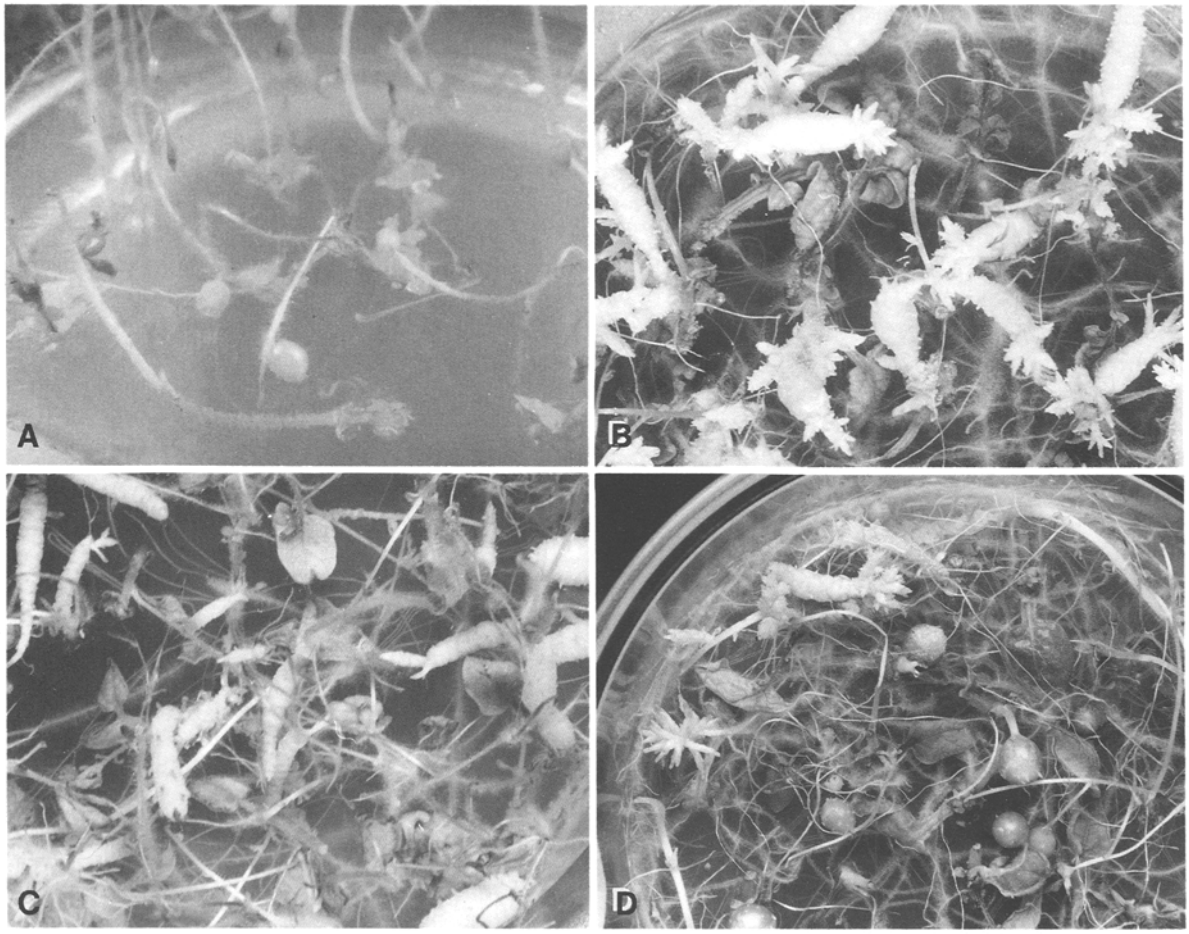

Fig. 3. Tuber formation in vitro on Ri-Bintje and Ri-Desirée shoots, after 2 months of growth. **A** Round Bintje tubers; **B** Oblong Ri-Desirée tubers of root line *h;* **C** Oblong Ri-Bintje tubers of root line *g;* **D** Oblong and round tubers of Ri-Desirée from rootline *j*

Phenotypic traits of in vivo grown plants

Ri-Bintje plants. After transfer to pots, Ri-Bintje plants showed variation which was not visible during in vitro growth. Plants of 1 line died soon after transfer (Table 2). Most of the variation in height of plants and tuber yield and form appeared between root lines (Fig. 2 A, C). Variation in apical dominance was also root line dependent, but less stringent, since some variation was also found between shoot lines. More variation between and within shoot lines was observed in leaf habitus, rooting and flowering (Table 2).

Root line *d* appeared heterogeneous and segregated for height of plants and tuber yield and form (sublines *d1* and *d2*; Fig. 2 C). Furthermore, 2 shoot lines of root line *b* segregated for leaf habitus and rooting, and 2 shoot lines of root line *g* segregated for leaf habitus only (Table 2).

Leaf form varied considerably from normal (resembling the control Bintje) to curly and undulated, highly crinkled. Deviations (light and dark green) from normal leaf colour occurred at random in young leaves among the 4 root lines with more than 1 shoot. Sparse hairs were observed in all the plants of 1 shoot line derived from root line *c*.

Flowering occurred occasionally in Ri-Bintje plants grown in the greenhouse. The flowers were male sterile, like those of normal plants. The control Bintje plants did not flower under these conditions.

Tuberization occurred in vitro and in vivo. Abnormal tuber form (oblong and deep-set eyes) was observed under both conditions (Figs. 2 C and 3 C).

Ri-Desirée plants. In contrast to the Ri-Bintje plants, the phenotype of all Ri-Desirée plants was approximately uniform. The plants were tall, with strong apical dominance and normal leaves (Fig. 2 B). Flowering occurred in only a few plants (Table 3).

Among the Ri-regenerants, variation was observed in tuber form and yield, both in vivo and in vitro (Table 3, Fig. 2 D, Fig. 3 B, D), and appeared mainly between the root lines. Root line *j* generated 3 shoot

lines with 2 sub-shoot lines, 1 producing oblong tubers, 2 round tubers and 1 oblong as well as round tubers in vitro.

Discussion

The results obtained in the present study show that approximately 10% of the transformed root lines generated shoots, irrespective of the potato cv and *Agrobacterium rhizogenes* strain used. The transformed nature of the regenerants was established by the general occurrence of opines, specific products from T-DNA gene expression. These results are in agreement with those of Trulson et al. (1986) with transformed and normal roots of cucumber.

Remarkably, the Ri-plants obtained in the present study showed high tetraploid chromosomal stability with one exceptional octoploid. Recently, Ooms et al. (1985) reported plant regeneration from a Ri-transformed root line of cv Desirée, and found 25% of the regenerants to be aneuploid. The higher frequency of abnormal chromosome number might be attributed to a longer duration of the callus phase preceding shoot formation, i.e., 7 weeks instead of 4 weeks in this study. The genetic stability during the regeneration procedure, developed for Ri-regenerants, is quite different from the genetic instability seen during plant regeneration from protoplasts in potato. Although a considerable part of instability may be attributed to early genetic events in the protoplast culture (Sree Ramulu et al. 1984), the callus phase can also contribute to the high proportion of aberrant regenerated plants. It is not known to what extent the early genetic events causing instability proceeds in the callus phase, but it is evident that callus tissue is in itself unstable (Hänisch ten Cate and Sree Ramulu 1987).

The shoot-producing callus obtained from the root lines was different from that formed from shoot-derived protoplasts by its hard, compact, lobed structure and green colour. Moreover, the callus cells remained in tetraploid condition (Ottaviani and Hänisch ten Cate 1987). Apparently, the factors causing variation among Ri-regenerants have not affected genetic stability. It is likely that specific gene expression for root structure and function is the underlying factor. About one-third of the nuclei isolated from normal and transformed roots contained 8C and 16C DNA (Hänisch ten Cate et al. 1987). The tetraploidy of the regenerants suggests that they developed from meristematic parts of the root tissue.

The vitality of Ri-shoots in vitro was reflected in robust branching plants with larger leaves and shorter internodes than the slender control shoots of cvs Bintje and Desirée. However, the greater vigour was not maintained in vivo. Little is known about the physiological characteristics of Ri-plants as compared to normal plants. Preliminary results indicate that in vitro grown Ri-Bintje plants have a greater fresh and dry weight than normal plants (Helder et al. 1987). These results are in agreement with those of Ooms et al. (1986). Apparently, Ri-plants have a greater capacity of growth than normal plants in a rich in vitro medium.

The general characteristics of Ri-transformants appearing in each root line of both cvs Bintje and Desirée were oblong tuber form with reduced yield and decreased dormancy in vitro. In addition, height of plants was generally reduced in cv Bintje. The other characteristics of transformed plants were not generally present. All the characteristics observed in the transformed plants were similar to the deviations observed in the corresponding organs of transformed tobacco and other species (Tepfer 1984).

Apart from variation between root lines and shoot lines, segregation of characteristics and reversion to normal phenotype was observed in both phases of the regeneration process and in both cultivars. Segregation of phenotypic characteristics has also been observed in tobacco after transformation with *A. tumefaciens* and *A. rhizogenes* strains (Peerbolte 1986); the segregants contained different lengths and parts of T-DNA, and remained stable for long periods of culture. Segregation was attributed to deletions of T-DNA occurring during and soon after integration of T-DNA into the plant genome. It is likely that segregation in our potato material occurred at various periods of the regeneration process. The switch points of differentiation are candidates for the onset of segregation. T-DNA hybridization analysis in our potato Ri-plants is in progress to investigate length and copy number of T-DNA inserts, as well as the differential role of TL and TR DNA (Vilaine and Casse-Delbart 1987).

Although only three independent root lines were available from cv Desirée, it is obvious that variation in plants from cv Bintje root lines is much larger. As mentioned in the "Introduction", cv Bintje is highly heterozygous, which might make this cultivar prone to stronger interaction with the expression of the exogenous T-DNA genes. Such interaction effects may also be responsible for the uncoupling of expressivity of the various plant characteristics typical for Ri-transformants. Since most of the transformant characteristics are submitted to hormonal regulation, this is a likely pathway of the interaction of the cv Bintje genome with T-DNA.

The high frequency of segregation to approximately normal cv Bintje plants suggests that *A. rhizogenes* Ri-plasmids might be suitable as virulence vectors in the binary plant transformation system (Simpson et al. 1986; Hoekema et al. 1984; Sukhapinda et al. 1987),

with the additional advantage of genetic stability of the regeneration process.

Acknowledgements. The authors are much indebted to Drs. F. Heidekamp, W. J. Stiekema and L. van Vloten-Doting for the critical reading of the manuscript, and to Mrs. J. Claes for experimental work.

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