Theor Appl Genet (1985) 70:440–446

THEORETICAL AND APPLIED GENETICS

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Genetic modification of potato development using Ri T-DNA

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Received January 5, 1985; Accepted January 23, 1985
Communicated by R. Riley

Summary. Forty-two potato plants were regenerated from a hairy-root line obtained after infection of a shoot of *Solanum tuberosum* cv 'Desiree' with *Agrobacterium rhizogenes* strain LBA 9402 (pRi1855). Transformed plants were uniform and had a distinct phenotype and development compared with untransformed controls. Their growth was vigorous, especially early in their development, their roots were abundant and showed reduced geotropism, their leaves were slightly crinkled and glossy and they produced longer tubers with more frequent, prominent eyes. Cytological examination showed that ten of the forty-two transformed plants had either 47 or 49 chromosomes instead of the normal 48. In two of these aneuploids structural changes were observed.

Key words: *Agrobacterium rhizogenes* – Potato – Somaclonal variation – Genetic manipulation

Introduction

Infection of wounded dicotyledonous plants with *Agrobacterium rhizogenes* normally produces localised profuse root development (hairy-root, Riker et al. 1930).

This differentiation process is very similar to the induction of crown galls (Smith and Townsend 1907) by the related *A. tumefaciens* (see for reviews Hooykaas and Schilperoort 1983; Nester et al. 1984; Binns 1984). A segment of a bacterial Ri- or Ti-plasmid is stably integrated into a plant chromosome (Chilton et al. 1977; White et al. 1982) and this transferred DNA, or T-DNA, is root-inducing in the case of the Ri plasmid of *A. rhizogenes* and tumour-inducing in the case of the Ti-plasmid of *A. tumefaciens*. Both Ri and Ti T-DNA contain genes for synthesis of transformed-cell specific opines (Petit et al. 1983) and for hormonal changes (Ooms et al. 1981; Garfinkel et al. 1981; Akiyoshi et al. 1983). The latter are thought to be the genetic basis of hairy-root and crown-gall development.

Success in regenerating whole plants from Ri and Ti-transformed cells has been limited to a few plant species, most notably tobacco (Turgeon et al. 1976; Wood et al. 1978; Wullems et al. 1981; Ackermann 1977; Tepfer 1984). This has been followed by the isolation of "disarmed" *Agrobacterium* strains that have been used to introduce other genes into morphologically apparently normal plants (De Block et al. 1984; Horsch et al. 1984). Alternatively it has led to the prospect of studying the expression of Ti and Ri T-DNA genes in various differentiated plant tissues and their effect on plant development, not only in tobacco but in any plant in which they can be introduced.

Tobacco plants transformed with intact Ti T-DNA normally failed to form roots. In contrast, Ri T-DNA caused abundant root development and crinkling of the leaves. Both observations illustrate the prospect of using Ti and Ri T-DNA genes to genetically and specifically change plant growth and development. We have explored the feasibility of this approach for studying potato development, in particular the effect of specific hormone-related genetic factors on tuberisation, by isolating Ti and Ri T-DNA transformed potato plants.

In a previous study we demonstrated the isolation of Ti T-DNA transformed potato (Ooms et al. 1983) and subsequently we showed that such plants tuberised strongly, presumably because of an extreme photoperiodic response arising from increased endogenous cytokinins (Ooms and Lenton, submitted).

In the present study we report on the isolation of potato plants genetically transformed with Ri T-DNA and their modified leaf, root and tuber development. Because potato plants regenerated from protoplasts or explants show varying degrees of phenotypic and chromosomal changes (somaclonal variation, Larkin and Scowcroft 1981) we also examined how much variation our plant regeneration procedure induced among the regenerated transformed plants.

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Materials and methods

Shoot cultures of cultivar 'Desiree' and of transformed potato shoots were propagated as described previously (Ooms et al. 1983) at 25 °C, 12 h daylength. *Agrobacterium* strain LBA9402, obtained from the Molbas *Agrobacterium* collection in Leiden, The Netherlands, was grown at 29 °C on YMB media (0.5 g/l K2HPO4; 0.2 g/l MgSO4 · $7H_2O$; 0.1 g/l NaCl; 10 g/l mannitol; 0.4 g/l yeast extract; pH 7.0). LBA9402 is a rifampicin resistant derivative of NCPPB1855.

Stems of potato shoots were punctured with a drawn out, broken Pasteur pipette and the wound sites were infected with *A. rhizogenes.* Two-four weeks after infection transformed roots were excised and grown on agar solidified Murashige and Skoog (1962) media plus 2% sucrose (MS20) without hormones, but usually with carbenicillin (200 μg/ml) or cephatoxim (200 μg/ml) to suppress bacterial growth. Once bacteria free, the roots were placed on MS20 with 0.12 mg/l 2.4-D and 2 mg/l zeatin for 8 weeks which caused callus development and then, to induce shoots from the calli, on MS30, with 2.25 μg/l BAP and 10 mg/l GA_3 for an additional 6 weeks. The regenerated shoots were micropropagated at 3–4 week intervals and underwent at least two cycles of micropropagation before potting up. The growth conditions in growth chambers was 18 °C 12 h day length.

The isolation of plant DNA, hybridisation conditions and experimental details concerning T-DNA analysis are described elsewhere (Burrell et al., in preparation). In short, 10 μg plant DNA was used for analysis and in 1× and 5× reconstruction experiments 1.56 ng and 7.80 ng, respectively total *A. rhizogenes* DNA per 1 μg potato DNA was taken as the equivalent of one Ri plasmid molecule per potato genome. These well-defined amounts of restriction endonuclease treated bacterial DNA, present on the same Southern blot filter as restriction endonuclease treated DNA from transformed tissues, are helpful in determining the structure of T-DNA in transformed tissues (hybridising fragments in transformed DNA that comigrate with hybridising bacterial fragments are likely to be identical) and it provides a basis for the estimated copy number of the introduced T-DNA per potato genome (comparisons are made between the intensity of bands on the autoradiogram that represent hybridising fragments of identical size in digested plant DNA and reconstruction DNA). The final hybridisation conditions between Southern blots of Biodyne A filter and in vitro ^{32}P-labelled plasmid DNA were 20 °C below Tm indicating the allowance of approximately 15% mismatch between the T-DNA hybrids.

Chromosome numbers in root tips were determined as described elsewhere (Karp et al. 1982).

Results

Transformation and regeneration

The stem of a shoot culture of the commercial Dutch cultivar 'Desiree' was infected with *A. rhizogenes* strain LBA9402. After 2–3 weeks, roots developed profusely from the site of infection (Fig. 1 A). Some of these roots were excised and propagated in vitro as bacteria-free root cultures on agar solidifed Murashige and Skoog medium (MS20). From 14 independently induced calli on one root culture, DES-9402-X (D9X), we isolated 42 shoots (three shoots from each callus; Fig. 1 C; see also "Materials and methods"). Forty-two shoot cultures were established and maintained by in vitro micropropagation. At least one shoot from each culture was potted up in soil, grown in a growth chamber and 3–4 months later, harvested for tubers.

Although the transformed potato plants appeared uniform throughout their development, they differed distinctly in morphology from untransformed potatoes. Shoot cultures of transformed potatoes produced abundant, frequently branching roots (Fig. 1 C, D). Some of these roots grew on or above the agar surface (Fig. 1 D) suggesting reduced geotropism. Distal and proximal root-tips plus the side roots excised from these cultures grew vigorously (average growth rate on MS20 was 3 mm/day/root-tip). In contrast, excised untransformed roots usually grew only from distal root tips, albeit at approximately the same rate (3 mm/day/root-tip). The leaves of the transformed shoot cultures were also different. They were sometimes compound and usually slightly more crinkled than the leaves of untransformed shoots.

Three weeks after subculturing, when stems and leaves were harvested, transformed shoots invariably weighed more than untransformed shoots. The roots of transformed shoot cultures also weighed more. It was observed that freshly propagated transformed and untransformed meristem segments started to form new shoots and roots from their axillary buds, both approximately 5–7 days after subculturing (Fig. 1 E). The growth rate of the fastest growing roots, developing from these new transformed and untransformed shoots did not differ markedly either (Fig. 1 E) but the transformed roots branched more frequently and were more abundant (Fig. 1 E). When grown in soil in a growth chamber, the transformed plants established themselves more quickly and grew more vigorously than the untransformed controls (Fig. 1 F). However, after a further growth period of three months they reached similar size at maturity (Fig. 1 G). Likewise, tuber yields were similar although a few plants were exceptional, as will be discussed later. Transformed tubers differed significantly in shape. They were longer with more frequent and prominent eyes (Fig. 1 H). The leaves of mature transformed plants were more glossy and crinkled than those of untransformed plants.

Cytological variation

To establish as near as possible that Ri T-DNA transformed potato plants can be isolated with a normal chromosome complement and to evaluate to what extent variation was induced by the regeneration procedure used, we examined a minimum of five well spread chromosome preparations of roots from all 42 regenerated plants. Nine of the 14 calli regenerated from

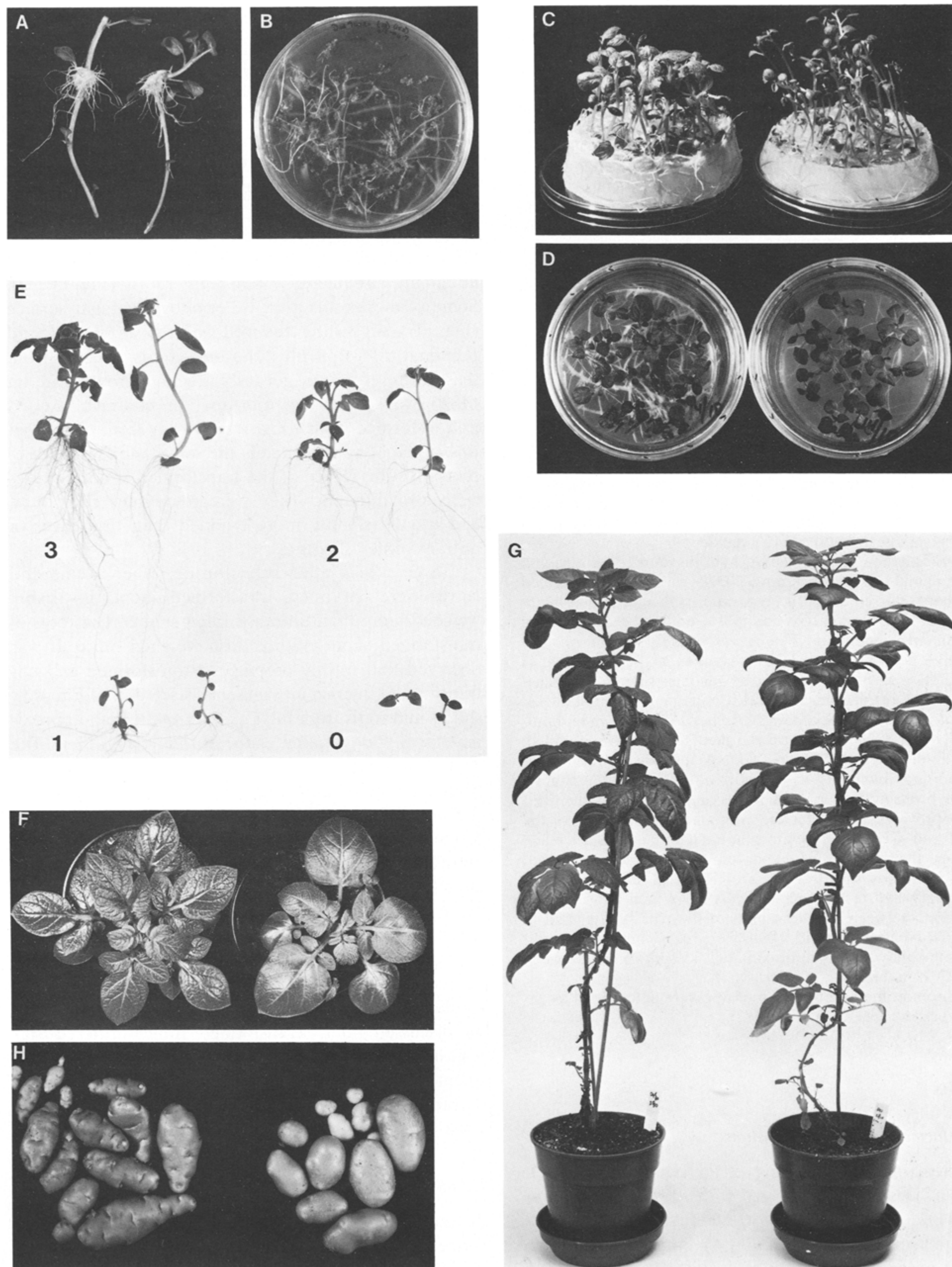

Fig. 1 A–H. Hairy-roots and Ri-transformed potatoes. **A** shoots of cultivar 'Desiree', three weeks after infection with *Agrobacterium rhizogenes* strain LBA 9402. **B** Regeneration of transformed shoots from root culture D9X. **C, D** Shoot-culture of transformed (D9X8a; *left*) and untransformed potato ('Desiree'; *right*). Note the slightly reduced geotropism (**C**) and tendency to branch (**D**) of the transformed roots. **E** Transformed- (*left*) and untransformed shoots (*right*) immersed in water. The shoots were grown for 3, 2, 1 and 0 weeks after subculturing. **F** Three weeks old shoot cultures as in (**E**) potted up and grown in a growth chamber for an additional three weeks. Transformed plants (*left*) established themselves quickly and firmly during this initial period. **G** Mature transformed – (D9X8a; *left*) and untransformed potato plants ('Desiree'; *right*). **H** Transformed tubers (D9X8a; *left*) are longer with more frequent, prominent eyes than untransformed tubers ('Desiree'; *right*)

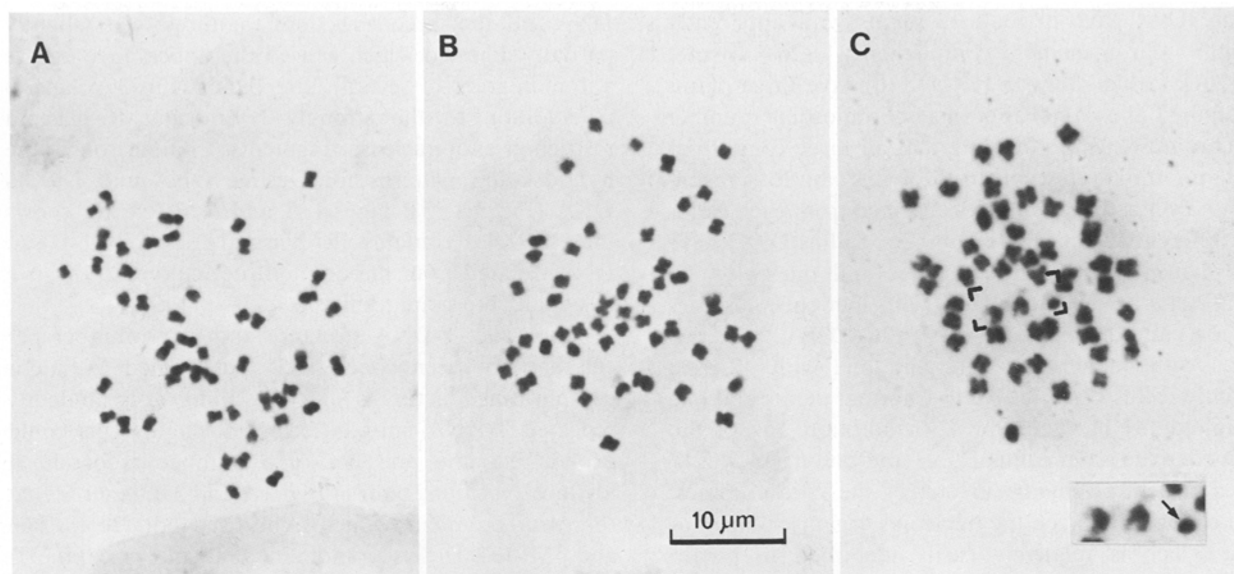

Fig. 2 A–C. Chromosomes in root-tip cells of Ri-transformed potato plants; **A** D9X6c, euploid (2n=4x=48); **B** D9X9b, aneuploid (2n=49); **C** D9X17c, euploid with a deletion of part of the long arm of one of the nucleolus organiser chromosomes. The enlarged area shows two normal and the one deleted nucleolus organiser chromosome

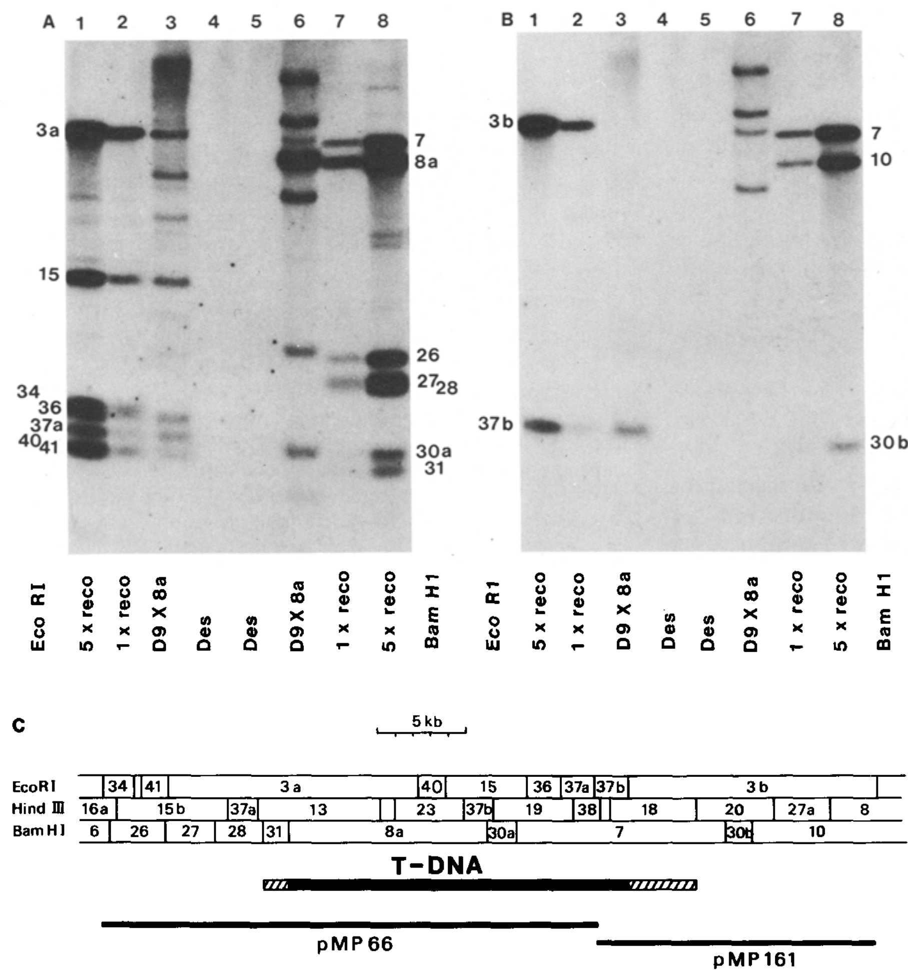

Fig. 3 A–C. T-DNA banding patterns and structure of T-DNA in transformed potato D9X8a. **A** An autoradiogram illustrating the T-DNA banding pattern obtained after exposure of a Southern blot which contained DNA, treated with restriction endonucleases EcoRI and BamHI, from transformed (D9X8a) and untransformed potato ('Desiree') that was probed with ^{32}P-labelled plasmid pMP66. **B** as **A**, but with pMP161 instead of pMP66. **C** Restriction endonuclease map of part of the Ri-plasmid used in this study (Pomponi et al. 1983; *top*); the most likely extent of T-DNA in D9X8a (*middle*); graphic representation of the cloned Ri-plasmid fragments that formed part of the plasmids pMP66 and pMP161 (Pomponi et al. 1983; *bottom*)

culture D9X gave in total 27 plants with apparently normal chromosome complements (2n=4x=48; Fig. 2A). Callus number 16 (D9X16) gave three plants which had the same abnormal chromosome number (47 chromosomes), indicating that all three came from the same initiation event. In the remaining four regeneration events the three shoots derived from each callus had different chromosome numbers. Callus D9X3 gave two plants with 47 chromosomes and one with 46. D9X9 gave two aneuploids with 49 chromosomes (Fig. 2B) and one euploid (48). Callus D9X12 gave two plants with 48 chromosomes and one with 47, and similarly callus D9X17 gave two normal plants and one aneuploid (47 chromosomes). In addition one of the shoots derived from callus D9X3 and one from D9X17 had a structural change in one of the chromosomes. Both changes involved the nucleolus organiser chromosome which is relatively easily identified in potato (Fig. 2). In one case (D9X3e) this chromosome had an additional segment (the result of translocation or amplification) and in the other (D9X17c) a portion of the long chromosome arm had been deleted (or translocated to another chromosome) (Fig. 2C). In summary, 76% of the plants were euploid, 24% aneuploid and the aneuploidy was of very limited range (46–49).

The minimal variation in chromosome numbers is in accordance with the uniformity of the regenerated Ri-transformed plants. Potato is tetraploid and small changes in chromosome number normally have little or no obvious effect on the phenotype. Two of the aneuploids, however, (D9X3e, three plants tested and D9X16a, one plant tested) were exceptional in failing to form tubers or producing very small tubers.

T-DNA

Direct proof that root culture D9X and the plants regenerated from this culture contained Ri T-DNA was obtained from Southern-blot analysis. Total high molecular weight DNA was isolated from regenerant D9X8a and from root culture D9X. Aliquots of 10 μg each were digested with restriction endonucleases BamHI and EcoRI, separated in 0.7% agarose gels and blotted as described using Biodyne A membrane filter. DNA from an untransformed 'Desiree' plant and 5× and 1× reconstruction mixtures were controls. The filter was probed first with ^{32}P-labelled clone pMP66 giving a first autoradiogram (Fig. 3A). Then the filter was washed in low salt at elevated temperature to denature the DNA hybrids with the first probe and it was reprobed with clone pMP161 to obtain a second autoradiogram (Fig. 3B). The two probes cover entirely one of at least two possible Ri-plasmid derived T-DNA's that have been detected in other hairy-root tissues (White et al. 1982; Huffman et al. 1984). The bacterial DNA in the reconstruction mixtures was slightly partially digested which caused the appearance on the autoradiogram of several faint bands (Fig. 3A, lane 1) in addition to the strongly hybridising Ri-plasmid restriction endonuclease fragments. Comparison of the hybridisation patterns for Desiree DNA and D9X8a DNA (Fig. 3A, 3B lanes 4, 3 and 5, 6) clearly shows that D9X8a contains Ri-plasmid derived T-DNA. DNA isolated from the original root culture D9X gave essentially the same results.

The exact T-DNA structure and copy number per cell has not been conclusively determined. A major complicating factor is the possibility of tandemly repeated T-DNA units differing in length. This could explain e.g. the presence of a number of bands at identical positions both in Fig. 3A and 3B, lanes 6, that represent fragments which hybridised both to pMP66 and pMP161. However, more detailed analysis of the T-DNA structure using additional HindIII digests confirms the presence of T-DNA in D9X8a and suggests that the most likely extent of T-DNA is as indicated in Fig. 3C.

Discussion

Whole plant genetic modification experiments require suitable transformation and plant regeneration techniques. In tobacco, transformed plants have been regenerated from transformed protoplasts, transformed cells in explants and from tumours and hairy-root tissues. Mostly, the initial transformation event was done by *Agrobacterium* infection, although direct PEG induced DNA uptake by protoplasts has also been demonstrated (Krens et al. 1982).

Tobacco is a particularly suitable 'model' plant because it is very amenable to tissue culture techniques and plants regenerated via tissue culture are largely uniform. Potato on the other hand has the scientifically interesting and agronomically important developmentally controlled process of tuberisation but it is less amenable to tissue culture techniques. Perhaps, more importantly, the potato plants regenerated from protoplasts and explants, especially from tetraploid cultivars, showed considerable phenotypic and genotypic variation (Shepard et al. 1980; Thomas et al. 1982; Karp et al. 1982). Obviously, this latter characteristic, although in its own right interesting, is undesirable if specific genetic changes are to be introduced. Some control, however, over at least the numerical chromosomal variation is possible by the choice of the starting material and the culture methods (Karp et al. 1982; Wheeler et al. 1985; Sree Ramulu et al. 1983). Plants from protoplasts show a high percentage of aneuploids, mostly with a wide range of numerical chromosome variation. High chromosome numbers were found invariably in plants with gross phenotypic abnormalities (Karp et al. 1982; Sree Ramulu et al. 1983). In contrast, most potato plants from explants are similar or identical to the parental plant and consistently show less frequent and less extensive chromosome variation (Wheeler et al. 1985).

Therefore, it could be expected that plants regenerated from transformed cells in explants, like from transformed roots, would show relatively minimal variation. Our present results show that this is indeed the case. The 42 hairy-root derived plants showed little phenotypic variation despite some numerical variation (24% were aneuploid) which was within a narrow range (46–49 chromosomes per cell).

The demonstration of plant regeneration from hairy-roots and the limited variation among regenerated plants indicates that in principle, Ri plasmids can be used as vectors to introduce any gene via Ri T-DNA into potato. This would enable expression studies of these genes in various potato tissues, including tubers. Because the full evaluation of other ways of obtaining transformed potato plants has not yet been completed, e.g. via protoplast or explant transformations using disarmed *Agrobacterium* strains, it is too early to compare the use of Ri plasmids with the full range of other potential potato transformation systems. Compared with shoot inducing Ti T-DNA, however, the only system for which some information is available, it has the advantage that undesirable chromosomal changes can be scored relatively easily in roots of Ri transformed plants. Note that Ti T-DNA transformed plants normally are impaired in root formation. On the other hand, the Ti T-DNA transformed shoots tuberise quickly, which may turn out to be convenient, particularly in gene expression studies in tubers. Moreover, tubers transformed with Ti T-DNA are morphologically similar or identical to untransformed tubers. Whether this is of any consequence for tuber gene expression studies remains to be investigated.

Perhaps the most important conclusion from the present results, however, is that specific genetic changes can be brought about in potato development and tuberisation. This demonstrates the feasibility of using specific hormone-related T-DNA genes, possibly with specifically modified control sequences, to study the complex process of tuberisation in a novel way by combining molecular approaches with cell biological and physiological studies.

Ri T-DNA in potato has caused some developmental changes similar to those in Ri T-DNA transformed tobacco plants, like crinkling of the leaves and extensive root development at or near soil level. We found that although the growth rates of the fastest growing Ri T-DNA transformed- and untransformed roots were very similar the transformed shoot initiated new roots more frequently. Whether this is unique for potato remains to be determined. Particularly relevant were the changes in tuber development: transformed tubers were longer and had more frequent prominent eyes (Fig. 1). To determine more conclusively that this is because of the Ri T-DNA genes, we are regenerating additional Ri T-DNA transformed plants from independently induced root cultures of different potato cultivars. This will also determine whether the current regeneration procedure applies equally well to other varieties.

It is noted that the morphology of transformed tubers has some characteristics in common with that of tubers from potato plants infected with potato spindle tuber viroid (PSTV). They are both elongated, but PSTV tubers have a normal number of sunken eyes rather than many swollen eyes. The limited knowledge of the molecular changes underlying both phenomena, however, limits a further evaluation at this moment.

Finally, it is emphasised that further research is required to demonstrate specifically which of the Ri T-DNA genes causes the changes in plant and tuber development. This is emphasised even more by the initial results of RNA analysis of the Ri T-DNA transformed potato plants. These suggest that the introduced T-DNA is differentially expressed in various potato tissues (roots, shoots, tubers). Therefore, the abnormal tuber development may be caused either directly by T-DNA gene(s) being expressed in tubers or indirectly by the effect on tuber development of other plant tissues with modified physiology resulting from differential T-DNA expression.

Acknowledgements. We thank Dr. P. Costantino (Italy, Rome) for generously providing clones pMP66 and pMP161. Part of this work was supported by research contract No. 471 of the Biomolecular Engineering Programme of the Commission of the European Communities. We thank our colleagues of the Biochemistry Department for stimulating discussions and continuing interest.

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