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Genetic manipulation in cultivars of oilseed rape (*Brassica napus*) using *Agrobacterium*

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Summary. The response of oilseed rape cultivars to infection with *Agrobacterium tumefaciens* and *A. rhizogenes* and the possibility of regenerating genetically transformed oilseed rape plants were examined. The frequency at which *Agrobacterium* induced galls or hairy-roots on in vitro cultured plants ranged from 10% to 70%, depending on the cultivar. From galls induced by the tumorigenic strain T37, known to be strongly shoot inducing on tobacco, roots developed frequently. Occasionally, shoots formed and some of these produced tumour cell specific nopaline. Attempts to grow the transformed shoots into plants have so far been unsuccessful. Whole plants transformed with Ri-T-DNA, however, were regenerated. These had crinkled leaves and abundant, frequently branching roots that showed reduced geotropism, similar to previously isolated Ri T-DNA transformed tobacco and potato plants. The transformed oilseed rape plants flowered, but failed to form seeds.

Key words: *A. tumefaciens* – *A. rhizogenes* – *Brassica napus* – Plant development

Introduction

Infection of wounded dicotyledonous plants with *Agrobacterium tumefaciens* or *A. rhizogenes* can cause tumour development (crown gall; Smith and Townsend 1907) or prolific root formation (hairy-root disease; Riker et al. 1930) respectively at the site of infection.

Both types of newly developing tissues are characterised by the acquisition of a bacterial DNA fragment from a large tumour inducing (or Ti-) plasmid of *A. tumefaciens* (Chilton et al. 1977) or a large root inducing (or Ri-) plasmid of *A. rhizogenes* (White et al. 1982). The transferred DNA or T-DNA fragments are stably integrated in plant chromosomes (Chilton et al. 1980; Willmitzer et al. 1980; Thomashow et al. 1980) and carry different sets of eukaryotic genes. The T-DNA gene expression products are responsible for tumour- and hairy-root development by endogenously altering plant hormone related processes (Ooms et al. 1981; Garfinkel et al. 1981; Leemans et al. 1982). In some plant species whole transformed plants have been regenerated from both crown gall cells and hairy-root cells. Species for which Ti T-DNA transformed plants or shoots have been regenerated include tobacco (Braun 1959; Turgeon et al. 1976; Wullems et al. 1981), *Arabidopsis thaliana* (Pavingerova et al. 1983). *Bidens alba* (Norton and Towers 1984), *Medicago sativa* (Marriotti et al. 1984) and various cultivars of the agronomically important food crop potato (Ooms et al. 1983). Regenerated plants transformed with Ri T-DNA include carrot (Chilton et al. 1982), convolvulus (Tepfer 1984) tobacco (Ackermann 1977; Tepfer 1984) and potato (Ooms et al. 1985).

Here we report that it is also possible to transform cultivars of one of the major oil crops in the western hemisphere, oilseed rape, and demonstrate that whole transformed plants can be regenerated.

Materials and methods

Seeds of a number of oilseed rape cultivars ('Willi', 'Brutor', 'Jet Neuf', 'Rafal', 'Victor') were dipped in 96% ethanol (30 s) then surface sterilised in 8% household bleach (Domestos, 15 min) and finally washed 6–7 times with sterile water. They were germinated (2–5 days: 25 °C dark) on 0.9% agar solidified Murashige and Skoog medium (1962) without hormones but with 2% sucrose (MS20). Sterile seedlings were transferred into glass jars with MS20 and kept at 25 °C, 16 h day length, for an additional 2–3 weeks before infection with *Agrobacterium, A. tumefaciens* strain T37 was grown at 29 °C on 1.8% agar solidified TY medium (5 g/l tryptone, 3 g/l yeast extract)

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and *A. rhizogenes* strain LBA9402 on YMB medium (0.5 g/l K_2HPO_4; 0.2 g/l $MgSO_4 \cdot 7\ H_2O$; 0.1 g/l NaCl; 10 g/l mannitol; 0.4 g/l yeast extract; pH 7.0). LBA 9402 is a rifampicin resistant derivative of NCPPB1855.

Stems of oilseed rape shoots were punctured with a drawnout and broken Pasteur pipette and the wound sites were immediately infected with either T37 or LBA9402. Hairy-roots were excised from the shoots approximately one month after infection and grown on agar solidified MS20 supplemented with 200 mg/l cephotaxime to suppress bacterial growth. Regeneration of plants transformed with Ri T-DNA was obtained by prolonged culturing of a hairy-root line first on a dedifferentiation medium (6 weeks) and then on a regeneration medium (12 weeks). The first medium consisted of MS medium with 4% sucrose, supplemented with 2.0 g/l $CaCl_2 \cdot 2\ H_2O$; 0.18% mg/l NAA; 2.5 mg/l 6-BAP and 0.1 g/l thiamine. The second medium was also based on MS medium but with 3% sucrose, 2.25 mg/l 6BAP and 10 mg/l GA_3.

T-DNA analysis, opine assays and chromosome counts were done as described elsewhere (Burrell et al. subm; Otten and Schilperoort 1978; Petit et al. 1983; Karp et al. 1982).

Results and discussion

Infections of a number of cultivars of oilseed rape (with *A. tumefaciens* or *A. rhizogenes*) proved most optimal when in vitro cultured shoots were used. The success of infection was judged by the induction of clearly visible tumour or hairy-root formation four weeks after infection. Depending on the cultivar, the percentage of successful transformations induced by *A. rhizogenes* strain LBA9402 (pRi 1855) varied from 70% (for winter rape cv. 'Rafal') to 10% (for spring rape cv. 'Willi'), intermediate values of 50% were found for winter rape cv's 'Jet Neuf' and 'Brutor' (Fig. 1 A). Similarly, *A. tumefaciens* strain T37, known to induce shoot formation on tobacco (Braun 1959), also caused only 50% of infected 'Jet Neuf' shoots to form galls. However, in contrast with T37 tobacco tumours only around 20% of the T37 oilseed rape tumours formed shoots (Fig. 1 B). Approximately 10% formed shoots plus roots and from the remaining tumours (about 70%) only roots developed. T37 transformed tumours produced nopaline and nopaline was also detected in some but not all of the shoots that developed from the galls (not shown). This indicated that, as for shoots derived from galls on similarly infected tobacco plants, differentiated transformed shoots can develop from undifferentiated tumour tissue. So far, however, we have been unsuccessful in growing such transformed shoots into whole plants even when attempting to graft them onto normal root stocks. Normal, untransformed plants on the other hand were reproducibly regenerated from the galls. Particularly after growing the galls on media with 1–5 mg/l GA_3 which stimulated elongation and made subsequent handling easier.

To obtain further insight in the conditions required for the isolation of genetically manipulated, whole-plant derivatives of oilseed rape cultivars, we attempted to regenerate plants transformed with T-DNA from *A. rhizogenes*. First we established a bacteria free hairy-root line, JN9A, from a single root, induced by infection of a cultured shoot of 'Jet Neuf' with *A. rhizogenes* strain LBA9402. Direct proof that JN9A was indeed transformed and contained Ri T-DNA was obtained when high molecular weight JN9A DNA was isolated, digested with restriction endonucleases *Eco*RI, *Bam*HI and *Hind* III and Southern blots were prepared following standard procedures. These were probed with ^{32}P-labelled plasmids containing Ri-plasmid fragments as described by Burrell et al. (submitted). The Southern blot analysis showed that Ri-plasmid derived T-DNA was present (not shown). The exact T-DNA structure however, appeared complicated and probably consisted of at least five T-DNA copies of which at least some appeared linked together. It is noted that no T_R-DNA (Huffmann et al. 1984) was found in line JN9A and in agreement with this, JN9A did not produce agropine or mannopine (not shown). Having established that JN9A is indeed a transformed line, a range of media were tested to regenerate plants from JN9A. In these experiments only occasionally, leaf-like structures developed (Fig. 1 C) and only two shoots grew into mature plants (Fig. 1 D). In these instances two successive media were required and a long incubation period (more than 16 weeks). The regenerated shoots were multiplied in vitro by meristem culture and two morphologically identical transformed shoot culture lines were established (JN9A1 and JN9A2). After some of the JN9A1 shoots were transferred to soil (Fig. 1 D) and then vernalized (6 weeks at 4 °C) flowers developed (Fig. 1 E). In total it took ca 36 weeks from initial shoot formation in culture to flowering.

Striking differences were observed in growth and development between JN9A1 and 'Jet Neuf'. Cultured shoots of JN9A1 formed abundant roots of which many branched frequently and tended to grow with reduced geotropism near the agar surface or even grew upwards. The leaves of JN9A1, both when cultured in vitro as well as when grown in soil, were more crinkly than those of untransformed 'Jet Neuf' (Fig. 1 D). Frequently, the leaf tips of in vitro cultured JN9A1 shoots turned black, resembling tip burn of lettuce where it is thought to be a calcium related disorder (Collier and Tibbits 1982). In sharp contrast to transformed tobacco, where second generation Ri T-DNA transformed plants have been obtained (Tepfer 1984), we found that JN9A1 failed to form any seeds (Fig. 1 F). Because JN9A1 contained the normal 38 chromosomes per cell (Fig. 1 G) it is likely that all these changes are caused by the introduced T-DNA genes and certainly not by any gross chromosomal changes. This is supported further by a comparison of phenotypic changes in JN9A1 with

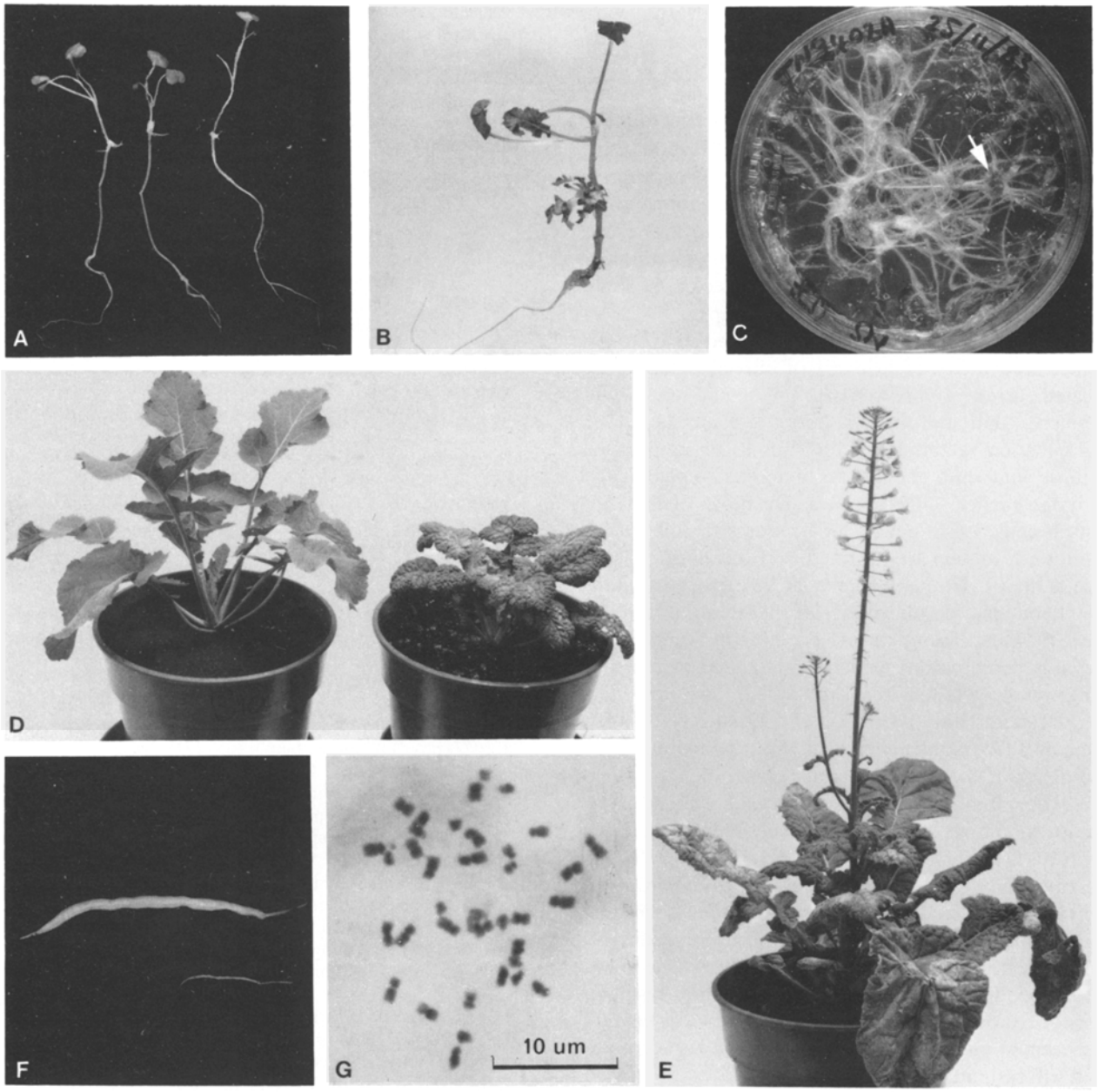

Fig. 1 A–G. Transformation of oilseed rape by *Agrobacterium.* **A** Hairy-root formation on stems of cultured shoots of *Brassica napus* cv. 'Jet Neuf', one month after infection with *A. rhizogenes* strain LBA9402 (pRi 1855). **B** Crown gall with shoots induced by *A. tumefaciens* strain T37 on in vitro grown oilseed rape cv. 'Victor'. **C** Differentiation of a shoot from hairy-root line JN9A. **D** Untransformed oilseed rape cv. 'Jet Neuf' (*left*) and a derivative JN9A-1 transformed with Ri T-DNA (*right*) grown in pots. **E** JN9A-1 flowering. **F** 'Jet Neuf' forms normal seeds (*above*); whilst JN9A1 does not form seeds (*below*). **G** Root-tip squash preparation of JN9A-1 showing the normal 38 chromosomes per cell

similarly changed phenotypes in tobacco, carrot, convolvulus and potato plants transformed with T-DNA of similar origin (Ackermann 1977; Tepfer 1984; Ooms et al. 1985). They all showed crinkly leaves and frequently branching abundant roots, typically with reduced geotropism. The leaves of oilseed rape plants were affected more severely by the introduced Ri T-DNA genes than, for instance, leaves of Ri T-DNA transformed potato plants isolated in our laboratory (Ooms et al. 1985). Whether this is due to plant specific different T-DNA expression patterns or to differences in responsiveness of the host plants remains to be investigated.

It is noted, that we only regenerated a few transformed plants from a single transformed root-line, thus it cannot be categorically concluded that oilseed rape

cultivars transformed with natural Ri T-DNA genes, never form seeds. In tobacco, plants regenerated from independant transformation events and also from a single transformed root culture sometimes showed small phenotypic differences for instance in the number of seeds produced per pod. Whether these differences are correlated with variation in T-DNA gene expression or due to additional variation in the expression of host genes is as yet unknown. The current results however, may be taken as an indication that natural Ri T-DNA perhaps is not suitable as a vector to study seed specific gene expression in oilseed rape.

The present results have established that transformed derivatives of cultivars of oilseed rape can be isolated using *Agrobacterium*. It can be concluded therefore, that the use of more advanced genetic manipulation vectors based on Ti or Ri plasmids, such as those with antibiotic resistance markers replacing the hormone related T-DNA genes (De Block et al. 1984; Horsch et al. 1984; Bevan 1984), seems feasible in attempting to obtain transformed derivatives of oilseed-rape cultivars. These plants would be expected to have no phenotypic peculiarities. Furthermore, it can be learned from the present experiments and from previous tissue culture experiments aimed at regenerating whole plants from individual *Brassica* cells (Kartha et al. 1974; Thomas et al. 1976; Lu et al. 1982; Xu et al. 1982; Kohlenbach et al. 1982; Glimelius 1984) that *B. napus* has a low natural regeneration capacity. The latter studies showed that careful evaluation of culture conditions and cultured tissues did considerably improve the efficiency of plant regeneration. This is therefore likely to be possible for the regeneration of transformed oilseed rape plants. Without such improvements, direct *Agrobacterium* infections, leading to transformed oilseed rape plants not only will be genotype dependent, and sometimes infrequently result in transformation, but also the time required to regenerate transformed plants will be prolonged and regeneration itself will be inefficient.

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