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Regeneration of flax plants transformed by *Agrobacterium rhizogenes*

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Abstract

Regeneration of flax (*Linum usitatissimum*) following transformation by either *Agrobacterium tumefaciens* carrying a disarmed Ti-plasmid vector, or *Agrobacterium rhizogenes* carrying an unmodified Ri plasmid, was examined. Hypocotyl and cotyledon explants inoculated with *A. tumefaciens* formed transformed callus, but did not regenerate transformed shoots either directly or via callus. However, cotyledon explants inoculated with *A. rhizogenes* formed transformed roots which did regenerate transformed shoots. Ri T-DNA encoded opines were detected in the transformed plantlets and Southern hybridization analysis confirmed the presence of T-DNA from the Ri plasmid in their DNA. Transformed plantlets had curled leaves, short internodes and some had a more developed root system characterized by plagiotropic behaviour.

Introduction

Flax (*Linum usitatissimum* L.) is agronomically important as a source of natural fibres and industrial oil, and may become important as a source of edible oil and protein [11]. It is also scientifically important because of the well characterized genetic control of host-parasite interaction between flax and its rust (*Melampsora lini* [Ehrenb.] Lév.), elucidated by the pioneering work of Flor (see [9] for a review). Genetic manipulation of flax may be useful both in improving the agronomic qualities of flax and in elucidating the molecular basis of the genetic interaction between flax and its rust.

Flax is a dicotyledonous plant amenable to transformation by *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942 or *Agrobacterium rhizogenes* (Riker, Banfield, Wright, Keitt and Sagen 1930) Conn 1942, which induce tumours and hairy roots, respectively, on dicotyledonous plants. In both cases, defined regions of DNA (T-DNA) from Ti (tumour-inducing) and Ri (root-inducing) plasmids, respectively, are transferred into plant cells and integrated into plant DNA [4, 5, 32]. A variety of vector systems based on the Ti or Ri plasmid have already been used to introduce DNA into various plants [15, 23, 31].

Flax is easily regenerated from hypocotyl explants [10, 16, 18], and with less ease from cotyledon explants [18, 25], so it should be possible to regenerate flax plants that have been transformed by *Agrobacterium*. Recently, Basiran *et al.* [2] reported the regeneration of transformed flax plants from transformed callus on hypocotyl explants inoculated with a disarmed strain of *A. tumefaciens*. Unfortunately, no data showing *npt*-II activity or the presence of T-DNA in transformed shoots are presented. In this paper we examine the regeneration of flax after transformation with *A. tumefaciens* and *A. rhizogenes*.

Materials and methods

Plant materials

Six cultivars of flax were used, five of which were cultivars used or developed by H. H. Flor. These were Bison (CI 389, an American seed flax cultivar [7]), Stewart (CI 1072 [8], derived from J. W. S. CI 708-1, a fibre flax cultivar [7]), Akmolinsk (CI 515-1, a Russian seed flax cultivar [7]), Abyssinian (CI 701, an Abyssinian seed flax cultivar [7]) and Bombay (CI 42, an Indian seed flax cultivar [7]). These cultivars were chosen not only because they have been characterized with respect to the rust resistance genes they carry [9], but also because they have diverse geographic or varietal backgrounds, and so, presumably diverse genetic backgrounds, a factor which we found to be important in shoot regeneration (unpublished data). The sixth cultivar was Precederia which, although uncharacterized with respect to rust resistance, has shown a ready ability to regenerate from protoplast-derived callus [1]. Seeds of these cultivars were surface-disinfected, then germinated, and seedlings were grown on hormone-free Murashige and Skoog (MS) medium [20] at 25 °C under a 16 h light (500 lux)/8 h dark cycle.

Bacterial strains and plasmids

The *Agrobacterium* and *Escherichia coli* strains and plasmids used in this work are summarized in Table 1. *Agrobacterium* strains were grown at 25 °C in Luria broth (LB) [19] without salt. *E. coli* strains were grown at 37 °C in LB containing 40 μg/ml ampicillin.

Transformation and regeneration using A. tumefaciens.

To transform and regenerate flax using *A. tumefaciens*, we used strain C58C1 carrying the disarmed Ti plasmid pGV3850::pLGV2103 [12]. This plasmid

Table 1. Bacterial strains and plasmids.

Species	Strains	Relevant plasmids	Opines encoded	Other relevant characters	Source	Reference
A. rhizogenes	A4	pRiA4	agropine and deoxy-mannityl-glutamine[a]	non-polar[b]	L. Moore	33
	1855	pRi1855	agropine and deoxy-mannityl-glutamine	non-polar	J. De Ley	27
	TR7	pRiTR7	deoxy-mannityl-glutamine	polar	ICPB[c]	21
A. tumefaciens	C58C1	pGV3850:: pLGV2103	nopaline	chimeric *npt*-II gene and pBR322 sequences in T-DNA	P. Zambryski	12
E. coli	HB101	pMP27	-	*Eco*RI fragments of pRi1855 cloned	P. Costantino	22
	HB101	pMP66	-	in pBR322	P. Costantino	22
	-	pBR322	-	-	BRESA[d]	3

[a] Agropine is encoded by the TR-DNA and deoxy-mannityl-glutamine by the TL-DNA of the Ri plasmid.

[b] Non-polar strains of *A. rhizogenes* contain TL and TR-DNA on their Ri plasmids whereas polar strains only carry TL-DNA. TR-DNA encodes auxin biosynthesis, which confers auxin independence to the root induction process i.e. roots are induced independently of the polarity of auxin transport in an inoculated explant (see [26] for a discussion).

[c] ICPB = International Collection of Phytopathogenic Bacteria.

[d] BRESA = Biotechnology Research Enterprises South Australia.

was derived from pTiC58 and contains between the T-DNA borders; a chimaeric *npt*-II gene, which was used as a selective marker conferring kanamycin resistance to transformed plant tissue; the nopaline synthase gene, which was used as a non-selective marker; and pBR322 sequences, which were used as targets in Southern hybridization analysis.

Our approach to the use of hypocotyls was based on two observations, first, that regeneration of shoots from hypocotyls is very efficient [10, 16, 18], and second, that most of these shoots arise directly from epidermal cells of the hypocotyl [16]. Our main strategy was therefore to attempt to transform epidermal cells of the hypocotyl without altering their capacity for direct regeneration.

Hypocotyl sections, 0.8–1 cm in length, from 7-day-old seedlings of Bison, Stewart and Akmolinsk were inoculated at room temperature with an overnight liquid culture of *A. tumefaciens* that had been incubated either with or without 100–200 μM sinapinic acid. This compound specifically activates the Vir region of the Ti plasmid [29] and was used in an attempt to maximize transformation efficiency and as a possible means of effecting the transformation of epidermal cells even if they were not wounded.

Several different methods of inoculation were employed.

1. Explants were submerged and shaken in the bacterial culture at 50 rpm for 1–2 h.
2. Explants were gently rubbed with a mixture of the bacterial culture and an abrasive powder (celite), to remove the cutin layer of the hypocotyl and injure epidermal cells.
3. Explants were placed in the bacterial culture, then vacuum infiltrated at −85 kPa for 3–5 minutes in an attempt to stress epidermal cells and to drive bacteria into the stomata, so that bacteria might gain access to epidermal cells from within stomata.

After inoculation, the explants were blotted dry. Half the explants for each treatment were transferred to MS medium containing 0.02 μg/ml naphthalene acetic acid (NAA), 1 μg/ml 6-benzylaminopurine (6-BA) and 20 μg/ml adenine (NBA medium), and the other half were transferred to NBA medium containing 200 μM sinapinic acid. All explants were incubated at 25 °C under a 16 h light (1000 lux)/8 h dark cycle for 2 days. Then the explants were transferred to NBA medium containing 400–500 μg/ml cefotaxime and 100 μg/ml kanamycin (NBACfKm medium) and incubated as above. Green buds that formed on the explants were cut off when they reached about 1 cm in length and placed on MS medium containing 400–500 μg/ml cefotaxime and 100 μg/ml kanamycin (CfKm medium) to allow root formation. Several thousand hypocotyl explants were used in these experiments.

Cotyledon explants, 0.5 cm × 0.5 cm, from 7-day-old seedlings of Akmolinsk and Precederia were submerged in an overnight liquid culture of *A. tumefaciens* for 1–2 hours. After blotting dry, they were transferred to MS medium containing 1–2 mg/l 6-BA and incubated as above for 2 days. Then, explants were transferred to MS medium containing 1–2 μg/ml 6-BA, 500 μg/ml cefotaxime and 100 μg/ml kanamycin (BCfKm medium), and incubated as above. About 30 days later, calli developing from the cut edges of each piece were cut into small pieces (2–3 mm in diameter), and put on BCfKm medium again for regeneration. About a thousand cotyledon explants were used in these experiments.

Transformation and regeneration using A. rhizogenes.

Cotyledons from 7-day-old seedlings of Precederia, Bombay, Akmolinsk and Abyssinian were detached, cut into halves, and submerged in an overnight liquid culture of *A. rhizogenes* for about 2 hours. Then they were blotted dry, transferred to MS medium, and incubated as above for 2 days, except that diffuse light was used during the light phase of the cycle. They were then transferred to MS medium containing 500 μg/ml cefotaxime and incubated as above with diffuse light. Roots, 0.5–1 cm in length, which developed at the cut ends of cotyledons, were excised and placed on NBA medium containing 500 μg/ml cefotaxime (NBACf medium) and incubated as above (with strong light) to allow shoot formation.

DNA extraction

Total plant DNA was extracted using a method de-

rived from that of Rogers and Bendich [24], as follows. Tissue was frozen in liquid nitrogen and pulverized with a mortar and pestle. One ml of 2× CTAB extraction buffer (2% [w/v] cetyl-trimethylammonium bromide, 100 mM Tris pH 8.0, 20 mM disodium EDTA, pH 8.0, 1.4 M NaCl) was added to each gram of tissue. The slurry was incubated at 65 °C for 5 minutes. An equal volume of chloroform was added and the suspension was gently but thoroughly mixed. Then the emulsion was centrifuged at 11 000 *g* at 4 °C for 5 minutes. The upper phase was transferred to another tube, one volume of 1% CTAB was added, and the suspension gently mixed, then incubated at room temperature for 30 minutes. The suspension was then centrifuged as above. The pellet was washed once with 0.1 M sodium acetate in 75% ethanol, once with ice-cold 75% ethanol, dried briefly in air, and dissolved in 0.1 × TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0).

Plasmid DNA was prepared by alkaline lysis and purified by CsCl gradient centrifugation in the presence of ethidium bromide as described by Maniatis *et al.* [17].

Gel electrophoresis, Southern transfer and DNA hybridizations.

Plasmid and plant DNA was digested to completion with *Hind* III (Pharmacia) according to the manufacturer's instructions, then separated by electrophoresis in a 0.8% agarose gel as described by Maniatis *et al.* [17], and transferred to a GeneScreen Plus membrane (Du Pont) according to the manufacturer's instructions. Probe plasmids were labelled with (^{32}P)dCTP using a nick translation kit (BRESA) and hybridization carried out as described by Maniatis *et al.* [17]. Since pRi1855 is almost identical to pRiA4 [14], pMP27 and pMP66, which contain most of the TR and TL-regions of pRi1855, respectively [22], were used as probes in Southern hybridization analysis of plantlets transformed by both 1855 and A4. pBR322 [3] was used as a probe in Southern hybridization analysis of plant tissues transformed by *A. tumefaciens* carrying pGV3850::pLGV2103.

Opine assays

Opine analysis by high-voltage paper electrophoresis was carried out as described by Yamada and Itano [34] to detect nopaline, and by Ryder *et al.* [26] to detect agropine and deoxymannitylglutamine.

Results

Transformation and regeneration using A. tumefaciens

Ten to 15 days after inoculation, callus appeared on the cut ends of inoculated hypocotyl and cotyledon explants in all treatments on media containing kanamycin. These calli continued growing vigorously on media containing 100 μg/ml kanamycin. Large amounts of nopaline were detected in these calli and Southern hybridization analysis showed they were transformed (data not shown). In a few cases, buds appeared on kanamycin-resistant callus, but they all remained small and failed to develop on medium containing kanamycin.

Seven to 10 days after inoculation many buds appeared along the surface of hypocotyl segments cultured on NBACfKm medium. Most buds were white and of the few green buds that did appear most bleached out gradually. In contrast, only a few white buds appeared on uninoculated hypocotyl segments on the same medium. After about 4 weeks, some buds on inoculated hypocotyl sections, including both white and green buds, had grown to 1–2 cm in length. Small amounts of nopaline were detected in both white and green shoots (data not shown).

Green shoot tips about 1 cm in length were excised and transferred to CfKm medium where some of them developed very short roots. However, when they were repeatedly subcultured on the same medium, nopaline could no longer be detected (data not shown), the shoots became bleached and no roots formed. Southern hybridization analysis of DNA from two of the shoots which initially produced short roots showed that they were not transformed (data not shown). In all, about 100 green buds were regenerated on inoculated hypocotyl sections, but none was transformed.

The attempt to injure epidermal cells by rubbing with an abrasive powder may have assisted transformation, but it also stimulated callus formation and suppressed shoot regeneration. No transformed shoots were regenerated from epidermal cells around vacuum infiltrated stomata, and the addition of sinapinic acid had no noticeable effect.

Transformation and regeneration using A. rhizogenes

About 20 days after inoculation, numerous adventitious roots appeared at the cut end of almost all cotyledon explants inoculated with strains 1855 and A4, but those inoculated with TR7 produced far fewer roots. Buds regenerated on roots 20–35 days after they were excised and transferred to NBACf medium. Roots induced by *A. rhizogenes* showed a much lower frequency of shoot regeneration compared to untransformed roots (Table 2). For the cultivar Bombay, the differences in bud regeneration between untransformed roots and those induced by 1855, A4 or TR7 were all statistically significant ($\chi^2_1 = 11.65$, $p<0.01$; $\chi^2_1 = 9.39$, $p<0.01$; and $\chi^2_1 = 6.22$, $0.01<p<0.02$, respectively). Furthermore, the data for the two non-polar strains, 1855 and A4, were homogeneous ($\chi^2_1 = 0.16$, $0.5<p<0.7$). These data were pooled and compared with those for the polar strain TR7, by Fisher's exact test, and no significant difference was found ($p = 0.26$).

Analysis of plants transformed by A. rhizogenes

Opine analysis showed that 7 regenerated plants were opine-positive (data not shown). Four of these were chosen for further study. These comprised Pre-1 and Bom-5, both transformed by 1855 and derived from Precederia and Bombay, respectively, and Bom-1 and Bom-3, both transformed by A4 and derived from Bombay. Bom-1, Bom-3 and Bom-5 lost their ability to synthesize opines after several subcultures (Fig. 1), despite the presence of T-DNA (Fig. 2), whereas Pre-1 retained this ability.

Southern hybridization analysis showed hybridizing bands comigrating with *Hin*d III restriction fragments 19, 23, 37b and 45 of pMP66 present in DNA from plantlets Pre-1, Bom-3 and Bom-5, and similarly for Bom-1 with the exception of fragment 19, which was missing (Fig. 2A). Additional hybridization bands of various fragment sizes and intensities, that did not co-migrate with any of the restriction fragments in pMP66 were also evident (Fig. 2A). One of these corresponds to internal TL-DNA restriction fragment 38 which is not wholly contained within pMP66 (Fig. 2C). The others presumably represent junction fragments containing TL-DNA joined to flax DNA or to itself in tan-

Table 2. Number of *A. rhizogenes*- or hormone-induced roots that formed buds, and the number of buds from *A. rhizogenes*-induced roots that produced opines.

Flax cultivar	*A. rhizogenes* strains or hormone	Number of roots placed on NBACf medium	Number of roots forming buds	Number of buds producing opine[a]
Akmolinsk	1855	300	1	nt
Abyssinian	1855	420	0	0
Precederia	1855	240	1	1
Bombay	1855	98	5	2
	A4	192	12	2
	TR7	48	2	2
Bombay	0.5 μg/ml NAA	80	16	na

[a]nt, not tested; na = not applicable.

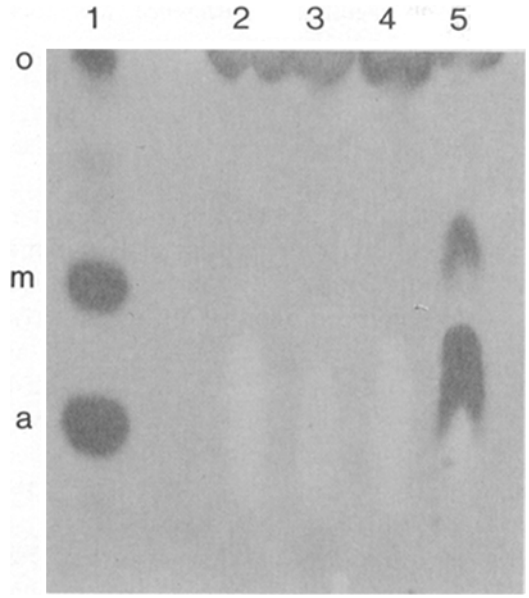

Fig. 1. Opine assays of plants regenerated from hairy roots and subcultured on MS medium containing 500 μg/ml cefotaxime. 2 μl of supernatant from 30 mg of tissue homogenized in 30 μl of 0.1 M Tris-HCl pH 8 were applied in each lane. Lane 1, opine mixture standard containing 10 μg agropine (a) and 10 μg deoxymannitylglutamine (m); lane 2, extract from Bom-3: lane 3, extract from Bom-1; lane 4, extract from Bom-5; lane 5, extract from Pre-1. The migration of opines was retarded in plant extracts compared to the opine standards. o = the origin.

dem repeats. Since pMP66 does not include the right side of the TL-region of pRi1855 (Fig. 2C), then only junction fragments involving the left side of the TL-DNA would have been detected. From the multiplicity of these fragments, it would appear that multiple copies of the TL-DNA may have been integrated into Pre-1 and Bom-3.

An intense hybridizing band co-migrating with *Hind* III restriction fragment 16b in pMP27 was present in DNA from Pre-1. Bands co-migrating with fragment 24 and another small fragment in pMP27 were also present in DNA from Pre-1, but the hybridization was much less intense compared to the band co-migrating with 16b. Several other hybridization bands of various fragment sizes and intensities were also detected, but none of these appeared to correspond to *Hind* III restriction fragments of pMP27 (Fig. 2B). Since pMP27 includes both sides of the TR-DNA of pRi1855 (Fig. 2C), these bands presumably represent junction fragments containing both sides of the TR-DNA joined to flax DNA or themselves in tandem repeats. From the multiplicity of these fragments, it would also appear that multiple copies of the TR-DNA may have been integrated into Pre-1. There was no evidence for insertion of TR-DNA into the DNA of Bom-1, Bom-3, or Bom-5 (Fig. 2B).

These data indicate that one of the four plantlets, Pre-1, was transformed by both TL-DNA and TR-DNA and that the other three were transformed only by TL-DNA, and in the case of Bom-1 by only part of the TL-DNA.

Morphology of plants transformed by A. rhizogenes

Transformed plants grown *in vitro* exhibited curled leaves, short internodes, and some had a more developed root system characterized by plagiotropic behaviour, compared to normal regenerants (Fig. 3). In at least two cases some side shoots recovered normal morphology during subculture of transformed plants.

Discussion

Initially, we hoped to regenerate transformed shoots from hypocotyl segments inoculated with *A. tumefaciens* containing a disarmed Ti-plasmid, because direct regeneration of buds from the epidermal cell layer of hypocotyls is very efficient [10, 16, 18]. However, this was not achieved. The differentiation on inoculated hypocotyls of buds which initially appeared green in the presence of kanamycin was presumably due to inefficient kanamycin selection, perhaps allowing transformed callus to cross protect untransformed shoots. The nopaline detected in these shoots presumably diffused from the transformed callus. Similar problems with the occurrence of false positives have also been reported for attempts to regenerate transformed plants from cotton hypocotyls inoculated with *A. tumefaciens* [6].

We also obtained no transformed shoots from callus on inoculated hypocotyls or cotyledons,

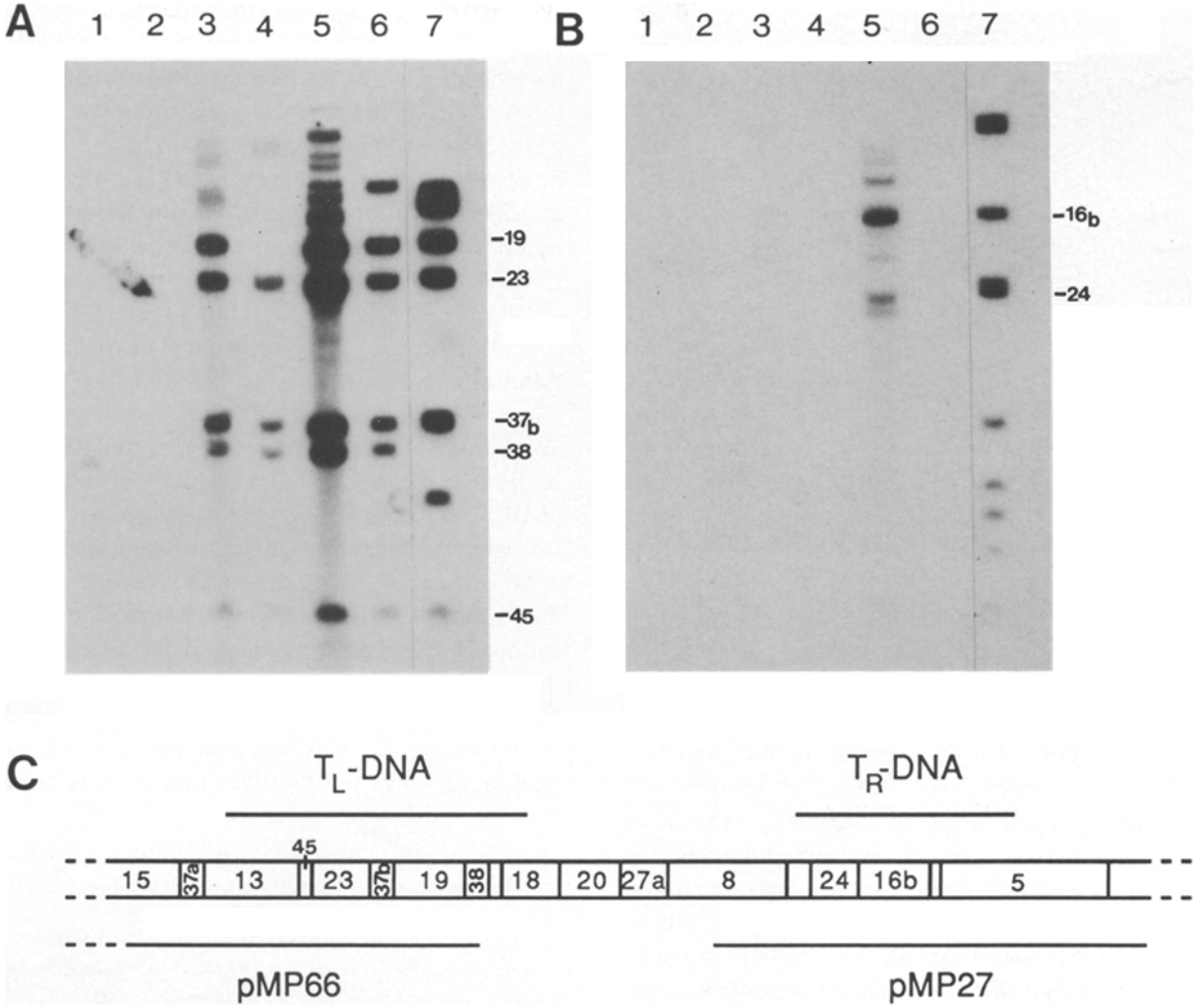

Fig. 2. A and B. Southern hybridization analysis of *Hin*d III digested DNA from hairy root regenerants, Pre-1, Bom-1, Bom-3, Bom-5, and their untransformed progenitors, Precederia and Bombay. Approximately 15 μg of plant DNA were loaded in each lane. Lane 1, untransformed Bombay; lane 2, untransformed Precederia; lane 3, Bom-3; lane 4, Bom-1; lane 5, Pre-1; lane 6, Bom-5; lane 7, 1–2 copy reconstruction using *Hin*d III digested probe DNA. A. Probed with pMP66. B. Probed with pMP27. The numbers on the right correspond to restriction fragments in C. The arrow in B indicates the position of a small fragment in lane 5 which co-migrates with a small fragment of *Hin*d III digested pMP27 in lane 7. C. *Hin*d III restriction map of the T-DNA regions of pRi 1855 based on the map of Spanò *et al.* [28].

presumably because the regeneration rate of transformed callus was too low in the cultivars we used. However, it should be possible, although probably difficult, to obtain transformed plants by this means.

In contrast to the results for *A. tumefaciens*, transformed plants were obtained readily from hairy roots induced by *A. rhizogenes*. To the best of our knowlege this is the first report of the regeneration of flax transformed by *A. rhizogenes*. These results show that the transformation by *A. rhizogenes* is an effective alternative to transformation by disarmed strains of *A. tumefaciens* for the genetic engineering of plants, especially when it is not possible to regenerate shoots readily from callus but is possible from roots.

In transformed plants obtained from hairy roots, the spontaneous disappearance of opines and the recovery of normal plant morphology without the loss of T-DNA has also been reported previously

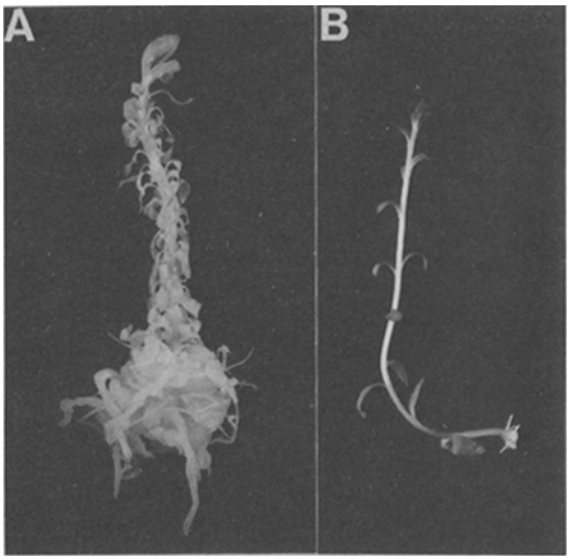

Fig. 3. A. Transformed flax plantlet Bom-5 with short internodes, prolific root system and curled leaves; B. Untransformed flax plantlet of the cultivar Bombay.

[30], and is perhaps due to cytosine methylation of inserted T-DNA [13]. Considering this fact, together with the fact that opines diffuse from transformed to untransformed tissue, we conclude that the opine assay is not always a reliable measure of transformation, since tissue without opines may be transformed, while tissue with small amounts of opine may not be transformed. So, the use of opine assays alone to judge whether a tissue or plant is transformed must be treated with caution.

These results also show that the frequency of buds regenerated from roots induced by either polar or non-polar strains of *A. rhizogenes* was significantly lower than that for untransformed roots. Since the Ri plasmid in polar strains contains only TL-DNA, one interpretation is that expression of TL-DNA in roots interferes with shoot regeneration. In addition, Southern hybridization analysis showed that three of the four transformed plants analysed did not have insertions of TR-DNA, although all four were transformed by non-polar strains of *A. rhizogenes*. Furthermore, in Pre-1 the hybridization intensity for the left part of the TR-DNA, viz. the band corresponding to *Hin*d III restriction fragment 24 in pMP27, was much lower than that for the right part, viz. the band corresponding to fragment 16b in pMP27 (Fig. 2B). This suggests that deletions or rearrangements of the left part of the TR-DNA may have occurred, leading to the left part of the TR-DNA, which contains *aux*-2, an auxin biosynthesis gene [28], being under-represented. These data may be consistent with the suggestion made by Spanò *et al.* [28] that expression of *aux*-2 in transformed roots hinders bud regeneration, so that only roots or root tissue deficient in TR-DNA regenerate shoots.

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