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Hairy root transformation in alfalfa (*Medicago sativa* L.)*

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Summary. The widely cultivated forage legume alfalfa (*Medicago sativa* L.) was transformed with the agropine type *Agrobacterium rhizogenes* NCPPB 1855. Sterile root and callus cultures were derived from tumorous hairy roots which were easily obtained independent of the plant variety or genotype. Plant regeneration, via somatic embryogenesis, was achieved only when a selected alfalfa line, characterized by high regenerative capability, was utilized. Genetic transformation was confirmed by the presence of agropine and T-DNA. Phenotypic alterations, mainly affecting the root system, were observed in transformed plants. The possibility that T-DNA-induced variations could be useful in the improvement of *M. sativa* is discussed.

Key words: Hairy root – *Medicago sativa* L. – Phenotypic variation – *Agrobacterium rhizogenes* – Genetic transformation

Introduction

Agrobacterium rhizogenes induces hairy root disease in most dicotyledonous plants (Elliot 1951). This syndrome is characterized by the proliferation of adventitious roots at the site of bacterial infection.

Rhizogenic ability is conferred to plant cells by a fragment of DNA (T-DNA) which is transferred from the large root-inducing (Ri) plasmid, harboured by the bacterium, to the plant genome, where it is stably integrated and expressed (Chilton et al. 1982; White et al. 1982; Willmitzer et al. 1982; Spanò et al. 1982; Durand-Tardif et al. 1985; Taylor et al. 1985). T-DNA is also responsible for the synthesis of unusual amino acid derivatives, called opines, in the transformed cells (Tepfer and Tempé 1981; Petit et al. 1983).

Hairy root transformation thus shares common features with the insurgence of crown gall tumours induced by *Agrobacterium tumefaciens* (for a review see Nester et al. 1984). Contrary to crown gall tumorigenesis, hairy root transformation does not hinder the ability of transformed cells to regenerate whole plants (Ackermann 1977; Chilton et al. 1982; Spanò and Costantino 1982; Tepfer 1984; Ooms et al. 1985a). Hairy root regenerants are fertile and transmit T-DNA to their progeny (David et al. 1984; Costantino et al. 1984; Tepfer 1984; Ooms et al. 1985a).

Phenotypic alterations related to the transformed state and not merely due to somaclonal variation have been observed in hairy-root plants. Such alterations could be of interest for breeding programs.

To date few papers have been published on genetic transformation by *Agrobacteria* in crop plants (Ooms et al. 1983; Mariotti et al. 1984; Ooms et al. 1985a, b; Owens and Cress 1985; Webb 1986) and it would be of interest, therefore, to extend basic studies on these species.

Medicago sativa L. is the most widespread forage legume found in temperate regions, where it represents one of the major protein sources for livestock feeding. In this paper we report experiments concerning the transformation of this legume with *A. rhizogenes*, the regeneration of transformed plants and their phenotypic characterization.

Materials and methods

Plant material and bacterial strains

Medicago sativa L. cvs. 'Europe' and 'Adriana' and plants regenerated from the P1 cell line were utilized in this study. P1, an alfalfa cell line obtained within the cv. 'Adriana', is characterized by a high regenerative capability via somatic

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embryogenesis (Arcioni et al., unpublished results). *Agrobacterium rhizogenes* strains NCPPB 1855, harbouring the agropine type Ri plasmid pRi 1855, was maintained on solid YMB medium (Hooykaas et al. 1977).

Hairy root induction, culture and plant regeneration

Alfalfa plants, either seed or callus derived, were grown in aseptic conditions on hormone-free MS medium (Murashige and Skoog 1962), ¼ strength, at 23 °C, 1500 lux, 14 h photoperiod. Actively growing *A. rhizogenes* cultures were inoculated onto stems of 1–2 month-old plants after cutting, or directly injected using a hypodermic syringe. Adventitious hairy roots which developed after one month were explanted and cultured in liquid MS medium supplemented with 1 mg/ml carbenicillin (Geopen, Pfizer) to allow further growth. Roots were dedifferentiated on UM solid medium (Uchimiya and Murashige 1974) containing 2 to 5 mg/l 2,4 D [1] and 0.25 mg/l Kinetin. Carbenicillin concentration was gradually reduced during subsequent subculturing and sterile actively growing calli were obtained within 2 months time. Plantlets were regenerated from transformed calli via somatic embryogenesis in solid hormone-free MS medium with a reduced level of sucrose (2% w/v). They were further grown in hormone-free MS medium, ¼ strength solidified with agar (1.2% w/v) and then transferred to soil.

Opine detection

Agropine analysis by high voltage paper electrophoresis was carried out as described by Petit et al. (1983).

DNA isolation

Plant DNA was isolated according to the procedure described by Chilton et al. (1982). Recombinant pBR 322 plasmids were isolated essentially as described by Ish-Horowicz and Burke (1981). Elution of DNA from agarose gels was carried out by the freeze-thaw technique described by Locker (1979).

Gel electrophoresis, Southern blotting and hybridizations

Plasmid and plant DNA were digested with the appropriate restriction endonucleases, Eco RI, Hind III and Bam HI (New England Biolabs) according to the suppliers recommendations. Gel electrophoresis, transfer to nitrocellulose membrane (Southern 1975), in vitro labelling by nick-translation (Rigby et al. 1977) and hybridizations were carried out as described by Costantino et al. 1984.

Results and discussion

Hairy root induction and plant regeneration

Adventitious hairy roots occurred in almost all the infected plants, independent of the alfalfa genotype or cultivar utilized. When young plantlets – 4 to 5 weeks old – were infected, a small callus was initially formed from which abundant root proliferation took place. This initial callus formation was less evident or completely absent when older plants – 10 weeks old or more – were infected (Fig. 1 a).

Explanted hairy roots grew in vitro on hormone-free liquid media (UM or MS) (Fig. 1 b). They showed macroscopic differences in comparison to control root cultures, the former being larger in size and faster in growth.

The addition of auxin (2,4 D or NAA, 2 mg/l) inhibited root growth and promoted callus formation. Single roots were induced to form friable fast growing calli in solid UM or B5 (Gamborg et al. 1968) basic media, supplemented with 2 to 5 mg/l 2,4 D, 0.25 mg/l kinetin and 2 mg/l 2,4 D, 0.1 mg/l kinetin, respectively. No culture clone showed hormonal autotrophy.

Contrary to what has been observed in other plant species (Tepfer 1984), *M. sativa* hairy root cultures were unable to spontaneously regenerate whole plants. Various levels and combinations of phytohormones were not successful in promoting shoot regeneration (data not shown).

A previous paper (Mariotti et al. 1984) reported the suppression of regeneration ability in crown gall transformed cell lines obtained from tumours induced by various *A. tumefaciens* strains on several *M. sativa* cultivars. The loss of regeneration ability by crown gall tumours, well-documented in several plant species, is due to an unbalanced production of hormones (Ooms et al. 1981; Leemans et al. 1982; Akiyoshi et al. 1983). In contrast, hairy roots have been shown to easily regenerate whole plants.

Even though plant regeneration has been obtained in various *M. sativa* cultivars, morphogenesis can be considered routinely achievable only in very few cases as this process is strongly conditioned by plant genotype (Pezzotti et al. 1984).

These observations have been confirmed in our experiments. As a matter of fact, by screening more than 50 genotypes we did not manage to obtain plant regeneration from hairy root calli of *M. sativa* cvs. 'Europe' or 'Adriana'. On the other hand, plant regeneration was easily achieved when hairy root calli derived from P1 plants were utilized. P1 plants were obtained from an alfalfa cell line, P1, characterized by high regenerative capability (Arcioni et al., unpublished results). Transformed calli, grown in UM medium supplemented with 2 to 5 mg/l 2,4 D and 0.25 mg/l kinetin, were transferred to hormone-free MS medium containing 2% w/v sucrose, where large amounts of embryos were formed (Fig. 1 c). Normal plants developed from the embryos (Fig. 1 d).

On the contrary, hairy root calli induced and grown on B5 medium showed the tendency to differentiate roots. Occasionally teratomata-like structures occurred on the morphogenetic areas of the calli; this was probably due to abortive embryo production.

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[1] *Abbreviations:* 2,4 D = 2,4-dichlorophenoxyacetic acid; NAA = α-naphtaleneacetic acid

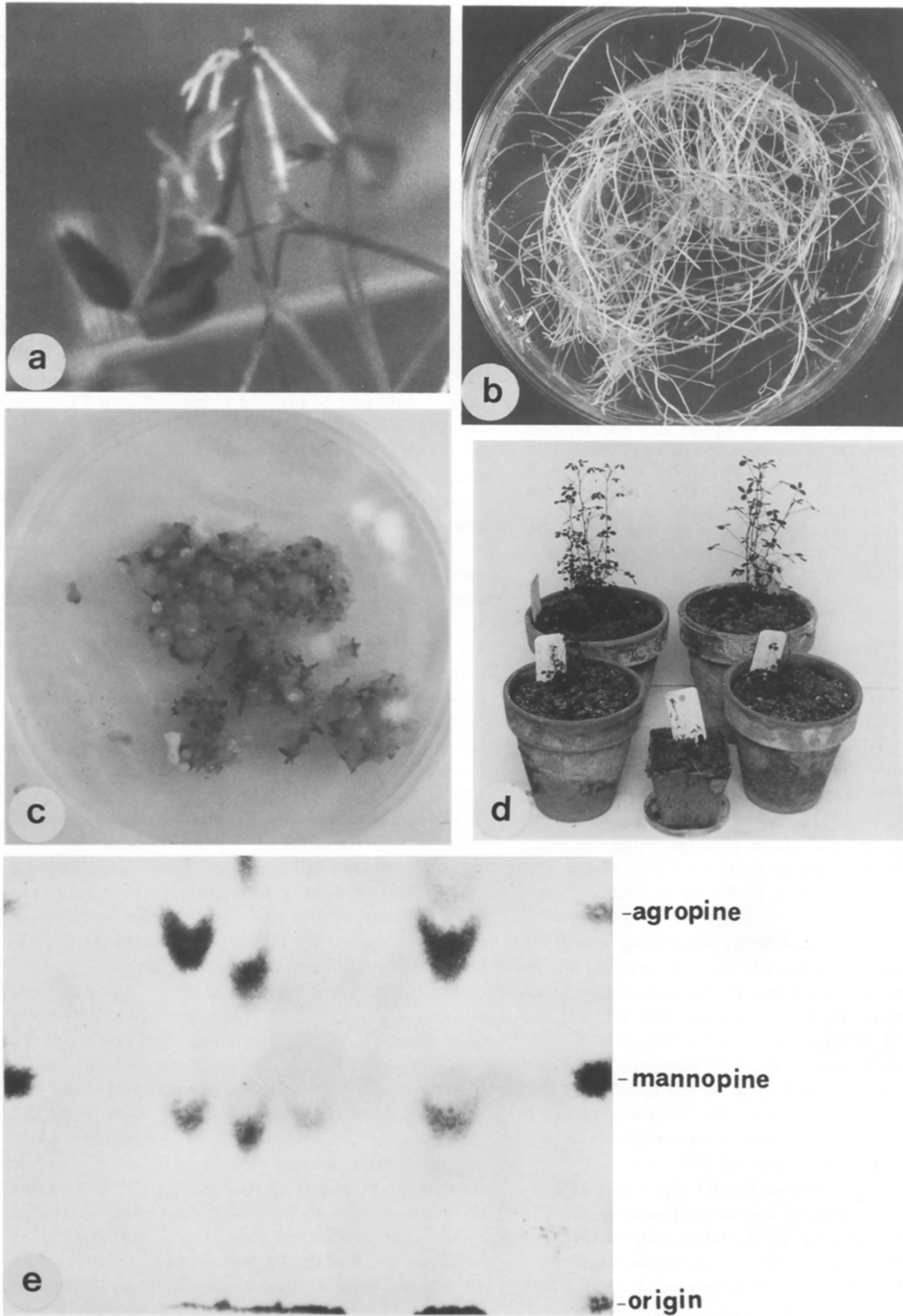

Fig. 1. **a** Hairy roots induced by *A. rhizogenes* 1855 on stems of aseptically grown plantlets of *M. sativa* 3 weeks after infection. **b** Hairy root culture actively growing in liquid hormone-free MS medium. **c** Somatic embryogenesis of hairy root P1 callus. **d** Hairy root transformed plants. **e** High voltage paper electrophoresis of extracts from 10 independent clones. The migration of opine standards is shown in the lateral tracks

These findings confirm that the various steps of embryogenesis which are genetically controlled are not affected by hairy root transformation.

Opine analysis

Hairy root cultures, as well as regenerated plantlets, were tested for the presence of agropine, the opine whose synthesis is specifically induced by the *A. rhizogenes* strain utilized in this study (Tepfer and Tempé 1981).

Out of more than forty independent clones analysed, only 50% were found to be positive (see Fig. 1 e).

The lack of opine synthesis, in spite of the typical hairy root appearance, has already been reported by Petit et al. (1983) for hairy root cultures induced by agropine type Ri plasmids. These findings can be explained by the fact that agropine synthetic functions are localized on the TR-DNA of pRi 1855 (De Paolis et al. 1985). So far there is no evidence for a coordinate transfer of TR and TL-DNA during transformation with *Agrobacteria* containing two distinct regions of transforming DNA, i.e. the octopine/agropine type Ti plasmids (Tomashow et al. 1980) and the agropine type Ri plasmids (Petit et al. 1983, 1986).

T-DNA analysis

One of the regenerated plantlets, clone 12 (C12), that has previously been shown to contain agropine, was further analyzed to assess the extension and structure of T-DNA.

pRi 1855 T-DNA consists of two distinct segments, TL (left) and TR (right) DNA, separated on the plasmid map by a long stretch of non-transferred DNA (De Paolis et al. 1985). In this study we utilized the recombinant pBR 322 clone pMP 66 (Pomponi et al. 1983), which encompasses more than 90% of the TL-region of pRi 1855, as a probe in a Southern blot analysis of C12 DNA.

As shown in Fig. 2 a, bands co-migrating with Hind III restriction fragments 19, 23, 37 b, 38 and 45 in pMP 66 (lane A) are present in the autoradiogram of C12 DNA (lane B), thus indicating that the internal structure of TL-DNA is maintained in this plant.

The reduced size of Hind III fragment 38 in the pMP 66 digest, indicated by a starred number in Fig. 2 a lane A, is consistent with the extension of clone pMP 66, which overlaps only a short segment of this fragment (see also the map in Fig. 2 b).

Southern analysis of an Eco RI digest of C12 confirms the maintenance of the internal structure of TL-DNA. In this case, the hybridization pattern (Fig. 2 a, lane B') shows bands with the same electrophoretic mobility as Eco RI restriction fragments 15, 36, 37 a and 40 in pMP 66 (lane A' in Fig. 2 a).

It should be noted that the probe utilized in this study does not include the right end of the TL-region of pRI 1855. Therefore, in order to localize the right border of TL-DNA in plant C12 we utilized Eco RI fragment 37 b purified from an Eco RI digest of clone pMP 83 (see Fig. 2 b). These results (not shown) allowed us to locate the right border within the Eco RI fragment 37 b. It was found to be rather close to the Hind III site, which is in good agreement with the preexisting data on the extension of the TL-region of pRi 1855 and with the presence of the Hind III fragment 38 as the internal segment in the genome of plant C12 (Fig. 2 a, lane B).

Additional bands that do not co-migrate with any of the restriction fragments in the pMP 66 digest are evidenced by this clone in C12 DNA (Fig. 2 a, lanes B,B'). These bands represent junction fragments, containing only part of plasmid sequences joined to plant DNA. In particular, they all account for the TL-DNA left border. Their multiplicity should therefore give a rough estimate of the copy number of TL-DNA in transformed C12 plant. This number can be reasonably assessed in the range of 3–5.

Control experiments in which DNA purified from untransformed alfalfa plants, isogenic to plant C12, was probed with clone pMP 66, showed a localized and significant sequence homology between the transforming DNA of pRi 1855 and the genome of normal plants. The autoradiographic pattern of normal DNA partially overlaps that of plant C12 (data not shown).

Analogous sequences, referred to as 'cellular T-DNA' (cT-DNA), have already been described in several plant species other than *M. sativa* (White et al. 1982, 1983; Spanò et al. 1982; Costantino et al. 1984). cT-DNA might represent the physiological counterpart of T-DNA, endogenous plant genes involved in cell growth and differentiation, somewhat analogous to retroviral oncogenes. Alternatively, they could consitute the memory of a past *Agrobacterium* infection, as recently suggested by Furner et al. (1986) for the cT-DNA found in different *Nicotiana* species.

Phenotypic traits of plant C 12, i.e. agropine production, strongly pointed to the presence of TR-region derived sequences in its genome. Agropine synthetic function (*ags*) have in fact been mapped on the TR segment of pRi 1855 T-DNA. Southern analysis of C12 DNA has been carried out utilizing clone pMP 137, encompassing the right end of TR-region, where the *ags* locus is located (see Fig. 2 b). In Fig. 2 a the autoradiogram of a Hind III digest of C12 DNA (lane D) is compared with the restriction pattern generated by the same enzyme on pMP 27, a recombinant clone which covers all of the TR-region of pRi 1855 (Fig. 2 b). As can be seen a band comigrating with Hind III fragment 16 b in pMP 27 (lane C) is present in the hybridization pattern of C12 DNA (lane D). Two further bands of different molecular weight and varying intensity are also evidenced by this probe. Since the

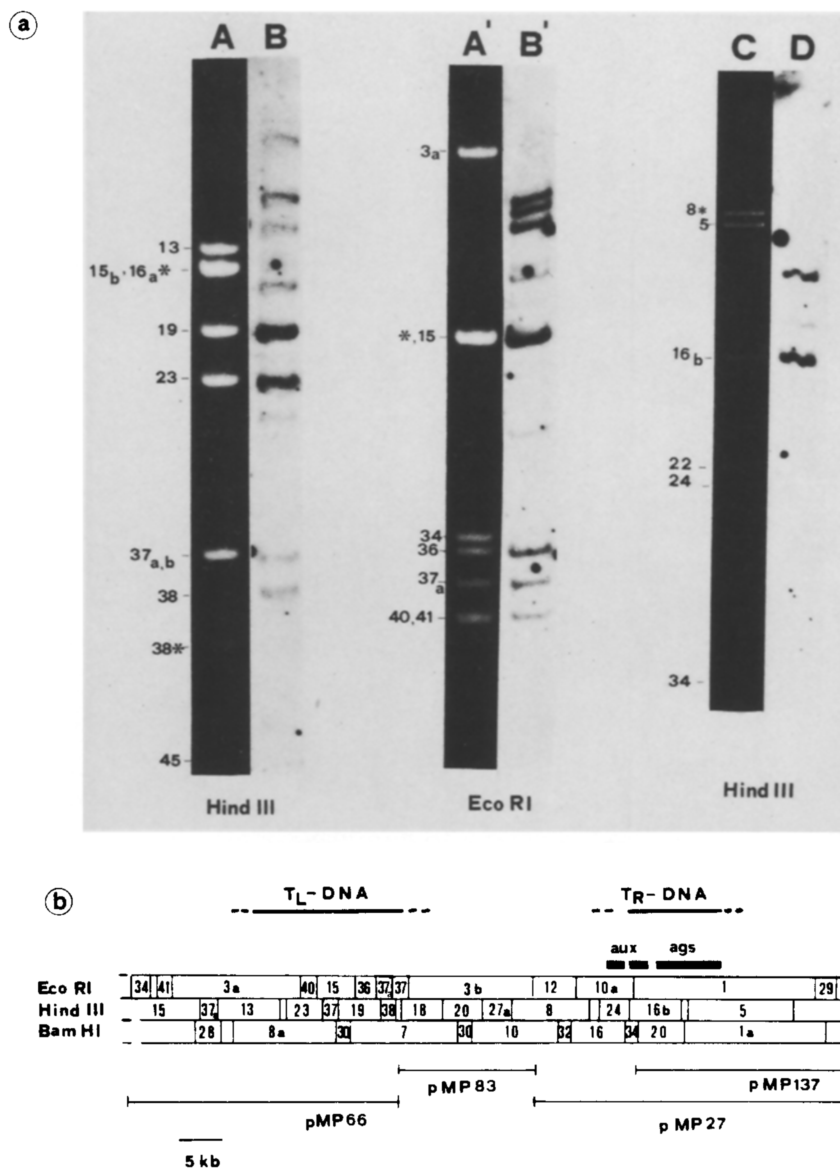

Fig. 2. a Southern blot analysis of DNA from hairy root regenerant C12. A,A' = restriction profiles of the probe, pMP 66, digested with Hind III and Eco RI, respectively. B,B' = autoradiograms of plant DNA digested with the same enzymes. C = restriction profile and clone pMP 27 digested with Hind III. D = autoradiogram of C12 DNA digested with the same enzyme and probed with clone pMP 137 (see also Fig. 2b). The numbers on the left refer to restriction fragments. Starred numbers indicate fragments joined to pBR 322 moieties. **b** Restriction map of the T-region of pRi 1855. The extension of TL and TR-DNA in plant C12 is indicated by the black line on the top. *ags* = agropine synthesis; *aux* = auxin synthesis

probe covers only the right end of the TR-region, these results suggest that there are at least two copies of TR-DNA in the C12 genome, and that the extension of the right border within Hind III fragment 5 is variable.

In order to better define the extension of the left end of TR-DNA, Eco RI fragment 10a was purified from an Eco RI digest of clone pMP 27 and used to probe a Bam HI digest of C12 DNA. The results of this analysis (data not shown) indicated that the left border of TR-DNA falls within Hind III fragment 24, close to the Bam HI site. Since the auxin synthetic genes identified in the TR of all the agropine type Ri plasmids (Huffmann et al. 1984; Cardarelli et al. 1985) encompass both Hind III fragments 16b and 24 (Fig. 2b), these results indicate that only the rightmost gene (*aux* 1) is present in the genome of plant C12. This was also found in *N. plumbaginifolia* regenerants (Hoge et al., pers. commun.). Therefore, it might be possible that only clones containing TR-DNA deleted of auxin synthetic genes (or containing only part of the *aux* locus) are able to regenerate whole plants.

Phenotype of transformed plants

Transformed plantlets grown in vitro exhibited a more developed root system characterized by plagiotropic behaviour in comparison to normal regenerants. Moreover, individual roots showed distinctive traits. They were larger and showed a marked development of lateral roots.

Fig. 3. a Hairy root transformed alfalfa plant (right) and isogenic, untransformed plant (left). Comparison of the root apparatus (**a**) and of the aereal part (**b**)

In order to avoid misleading in vitro effects, phenotypic variations were evaluated in well established soil-grown plants and compared to isogenic, callus-derived plants. Modifications in the number of primary roots have been reported in various plant species transformed by *A. rhizogenes.* However, in *M. sativa,* the presence of T-DNA completely changes the structure of the root apparatus. The root system, tap-root shaped in normal plants, was fasciculate in the transformants (Fig. 3 a). This alteration makes the transformed plants similar to the annual *Medicago* species growing in semiarid areas. Ri T-DNA transformation could therefore switch the *M. sativa* plants from perenial to annual or biannual behaviour.

On the other hand, cuttings of alfalfa show fasciculate roots during the first period of growth while the main root appears later. The possibility thus arises that this phenomenon could also take place in transformed plants, even though in this case the regeneration process, somatic embryogenesis, seems to rule out this hypothesis.

Further modifications affecting internode length and number, leaf size and stem number were also observed.

While internode length and leaf size were reduced, the number of stems and internodes was increased in comparison to normal alfalfa plants at the same developmental stage (see Fig. 3 b). These variations lead to an increase of the overall leafiness of the plants. Since this character is related to plant productivity and nutritive value, it could be of great interest in plant breeding.

Leaf wrinkling, characteristic of hairy root regenerants in other plant species, was negligible in alfalfa transformants.

The appearance of these modified phenotypic traits is not merely due to somaclonal variation, as demonstrated by the absence among more than 200 untransformed P1 regenerants of variants comparable to hairy root plants (Arcioni et al., in prep.).

The above mentioned modifications do not affect the reproductive apparatus of the regenerated plants

which were fertile and set seed. Moreover, as Ri T-DNA has been shown to be transmitted through meiosis in a Mendelian manner, these alterations should in principle be heritable.

The results of this study thus indicate that Ri T-DNA transformation can be utilized to stably modify the morphology of alfalfa plants. Further work is in progress in order to assess the possible agronomic value of these variants.

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