

Transformed callus does not necessarily regenerate transformed shoots

Mark C. Jordan and Alan McHughen

Crop Development Centre, University of Saskatchewan, Saskatoon, S7N 0W0, Canada

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Abstract

Agrobacterium tumefaciens carrying a disarmed Ti plasmid vector was used for the transformation of flax tissue. Transformed callus was obtained from inoculated hypocotyl segments and healthy green shoots were regenerated from this callus. Nopaline assays on shoot tissue were positive for nopaline content if carried out soon after removal of the shoot from the callus but negative if carried out 2-3 weeks after removal. All of these shoots gave rise to kanamycin sensitive progeny and were most likely escapes arising from non-transformed cells protected from the selective agent by transformed cells in the callus. Careful analysis of regenerated shoots from transformed callus is necessary in order to distinguish escapes from true transgenics.

Introduction

Noncogenic strains of Agrobacterium tumefaciens carrying Ti plasmid vectors with plant selectable markers (often kanamycin resistance and opine production) have been used to produce transgenic plants of an increasing number of non-Solanaceous crop species (Pua *et al.* 1987; Deak *et al.* 1986; Umbeck *et al.* 1987). In these reports the transgenic nature of the plants was unambiguously proven by Southern blot analysis of the transformed shoots themselves and/or by demonstration of the inheritance of the transgenic trait. In some species (e.g. flax), the definitive Southern test is impractical due to the small genome and plant size, requiring possible sacrifice of the whole organism to obtain enough DNA to run the test. In such cases alternate methods of ascertaining the transgenic state of the shoots are necessary. There are reports on the production of callus (Garcia *et al.* 1986) or callus and shoots (Basiran *et al.* 1987) from inoculated tissue where the definitive analyses for transformation are carried out only on the callus. In this paper we report that without careful analysis of shoots regenerated from such transformed callus or their progeny, false positive results can be observed leading to the mistaken belief that shoots from transformed callus are actually transformed themselves.

Materials and Methods

Plant material: Linum usitatissimum cultivars McGregor and STS-II (McHughen and Swartz 1984) were surface sterilized by a 2 minute treatment in 75% ethanol followed by two 10 minute treatments with 25% commercial bleach and two rinses with sterile distilled water. The seeds were placed on Murashige and Skoog (MS) (1962) medium with 3% sucrose and 0.8% agar. After 5-7 days in the dark 5-10 mm hypocotyl segments were excised and used as host tissue.

Agrobacterium tumefaciens: The vector used is described by Fraley *et al.* (1985), containing a chimeric neomycin phosphotransferase (NPT) II gene for kanamycin resistance and a wild type nopaline synthase gene integrated into the disarmed plasmid pTiB6S3-SE.

Inoculation: Hypocotyl segments were inoculated by either dipping the cut ends in a bacterial colony on a 3-day-old streaked plate or by swirling them in a culture (made by growing overnight a single bacterial colony from a 3-day-old streaked plate in Luria-Bertani medium containing the appropriate antibiotics) for approximately 5 seconds followed by blotting between two pieces of sterile Watman No. 1 filter paper. The inoculated segments were placed on MS medium plus 1 mg/l benzyladenine, 0.02 mg/l naphthalene acetic acid, 3% sucrose and 0.08% agar (MS-1 medium). After 3 days of cocultivation the segments were placed on MS-1 medium plus 600 mg/l kanamycin sulfate (Sigma), 250 mg/l Cefotaxime (Calbiochem) and 500 mg/l carbenicillin (as Pyopen, Ayerst) for 4-6 weeks. As green shoots arose and reached the top of the plates a leaf piece was removed and assayed for nopaline while the shoot was transferred to a vial containing MS medium plus 500 mg/l carbenicillin for rooting. Care was taken that no callus tissue was transferred with the shoot. In some cases the shoots were placed in MS medium plus 400 mg/l kanamycin and 500 ml/ carbenicillin. Rooted shoots were placed in a mist chamber for 2-3 weeks before transfer to the greenhouse. Nopaline assays were carried out on leaf tissue 10 days and 21-30 days after transfer to vials. Callus from the hypocotyl pieces was subcultured to fresh selection medium and shoots regenerated from this callus were also treated as above.

In some cases leaf pieces were removed from shoots and placed on selection medium for leaf callus assays.

Nopaline assays: Assays were conducted as per Rogers *et al.* (1986) with the exception that no preculture of the tissues on arginine was used. The tissue was squashed directly onto the electrophoresis paper.

Progeny analysis: Progeny seeds from regenerated green shoots were germinated as above and hypocotyl pieces were excised and placed on selection medium.

Results and Discussion

Non-inoculated hypocotyl pieces became swollen on selection medium but did not turn dark green or produce shoots. Inoculated hypocotyl pieces turned dark green, produced callus on the cut ends and shoots from the entire length of the segment (Fig. 1). Shoots arising from the middle of the segment tended to be bleached white or green/white streaked. Most of the completely green shoots regenerated from or near the callus formed at the cut ends.

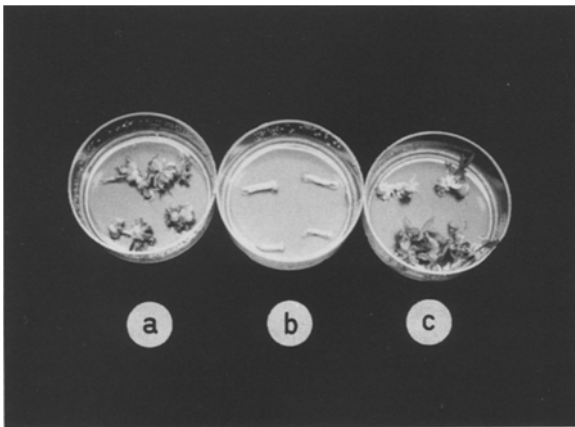


Fig. 1. Flax hypocotyl segments on MS-1 medium with or without kanamycin (600 mg/l) after 4 weeks. (a) Non-inoculated hypocotyls on MS-1 medium without kanamycin. (b) Non-inoculated hypocotyls on MS-1 medium plus kanamycin. (c) Inoculated hypocotyls on MS-1 medium plus kanamycin.

Nopaline assays performed on leaf tissue from shoots arising from callus showed a strong positive result on all but one of the 38 STS-II shoots tested. The intensity of the nopaline spots was comparable to that obtained by testing callus tissue alone (Fig. 2a). This is similar to results reported by Basiran *et al.* (1987). However when nopaline assays were done 10 days after transfer of shoots to vials the nopaline spots were much weaker (Fig. 2b) and when assays were performed 3-4 weeks after transfer, all the shoots tested negative for the presence of nopaline in the leaf.

Rooting in selection medium has also been used as a criterion for demonstrating transformation (Basiran *et al.* 1987; Rogers *et al.* 1986). No shoots from non-inoculated control hypocotyls on non-selection medium or from inoculated hypocotyls on selection medium ever rooted in media containing 400 mg/l kanamycin. However, if any basal callus tissue was present on the shoots then rooting sometimes did occur in selection medium.

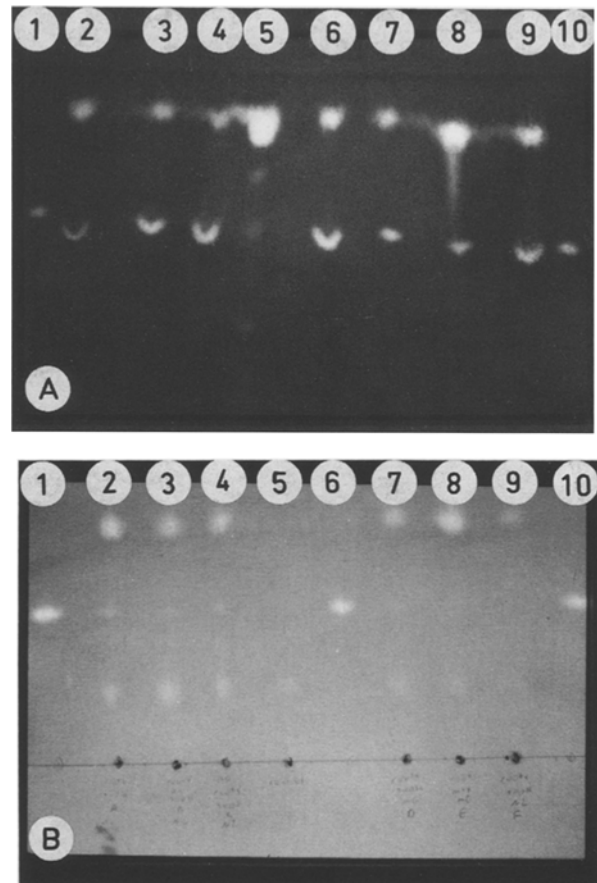


Fig. 2. Nopaline assays of kanamycin resistant flax callus and regenerated shoots. A. Lanes 1 and 10, nopaline standards; Lanes 2, 3, 4, 6, 9, callus; Lane 5, control callus from non-inoculated hypocotyl segment; Lanes 7 and 8, leaves from regenerated shoots. B. Lanes 1, 6, 10, nopaline standards; Lanes 2, 3, 4, 7, 8, 9, leaves from regenerated shoots 10 days after removal from callus; Lane 5, control shoot (regenerated from control callus (from a non-inoculated hypocotyl segment)).

Hypocotyl segments from the progeny of shoots from inoculated hypocotyls reacted the same as control segments did on selection medium (Fig. 1). Thus the original shoots were unlikely to be transformed as it is doubtful that T-DNA expression would be lost in the progeny of all the shoots.

Regeneration from hypocotyl explants occurs primarily from the epidermis (Murray *et al.* 1977) while the transformed cells tend to occur and proliferate around the cut ends of the explant. Thus the majority of regenerated shoots arise from non-transformed epidermis tissue. It is possible that transformed tissue does regenerate but the efficiency would be very low and an extremely large number of shoots would have to be rigorously analyzed in order to identify any transformants. None of the shoots we analyzed from this inoculation technique was transformed.

A modification of the above inoculation technique in which epidermal strips are peeled off prior to inoculation does yield transgenic shoots and these shoots pass the resistance trait on to their

progeny (Jordan and McHughen 1988). This strongly suggests that the critical factor in obtaining transgenic flax plants is decreasing the number of shoots produced from non-transformed tissue and by simultaneously increasing the number of transformation events per explant.

Although we have demonstrated that the production of transformed tissue on flax hypocotyl explants is possible by disarmed Agrobacterium tumefaciens carrying plant selectable markers this does not mean that transformed plants can easily be obtained by inducing shoot regeneration from the inoculated explants. The explant consists of both transformed and non-transformed cells with the transformed cells cross-protecting the non-transformed cells from the selection agent. Regeneration is primarily from non-transformed epidermal cells while regeneration from transformed cells has not been observed using the inoculation technique.

The nopaline assay data indicate that nopaline produced by transformed cells in the callus can be transported to shoots derived from kanamycin sensitive cells. Thus nopaline synthase activity should not be used as a criterion for selecting transformants unless the shoots have been removed from the callus for at least 3 weeks, in the case of flax; other species might require more or less time.

Rooting in selection medium is likely a better criterion for selection than nopaline assays but it is essential that all basal callus tissue be removed before transfer to selection medium. We have shown that transformed callus does not always give rise to transformed shoots and that it is easy to be misled into concluding transformed plants have been obtained if caution is not exercised in selection between transformants and escapes. Southern blots on shoot material or

progeny tests could be used in addition to nopaline and rooting assays for unequivocal demonstration of shoot transformation, but we have found a simple leaf callus assay on kanamycin to be a good indicator. This test consists of taking 3 leaf segments from each shoot and plating them on standard callusing medium, supplemented with 100 mg/l kanamycin. In our lab, callus will develop only from leaf segments from transgenic shoots. This is a particularly fast and useful assay for testing putative transformants but also should be supplemented with Southern and/or progeny analysis.

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