Quantification of indole-3-acetic acid in untransformed and Agrobacterium rhizogenes-transformed pea roots using gas chromatography mass spectrometry

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Abstract. Root segments of Pisum sativum L. were transformed by several strains of Agrobacterium rhizogenes. The resulting hairy roots, as well as apical segments from untransformed pea roots, were used to initiate root lines cultured in vitro. Levels of free IAA were quantified in the sub-cultured lines by gas-chromatography coupled to mass spectrometry, using selected ion monitoring. For most of the cultured untransformed and transformed root lines the IAA content was very small, compared with levels in untransformed intact primary roots. However, an agropine-type hairy root line (incited by strain 15834) contained significantly higher amounts of IAA. The peculiar phenotype of this root line (abundant production of calli) appears to be associated with an increased IAA level, as opposed to most of the hairy root lines, where the extensive secondary root proliferation associated with the hairy-root disease cannot be merely attributed to a markedly enhanced IAA content.

Key words: Agrobacterium - Auxin - Pisum (hairy roots) - Root (auxin levels) - Transformation (root)

Introduction

The hairy-root disease of plants (Riker et al. 1930), characterized by the abundant proliferation of transformed roots at the site of infection, is caused by the transfer of genetic material from Agrobacterium rhizogenes to the infected plant cells (see Gelvin 1990). The transferred DNA (T–DNA), located on a large root-inducing plasmid (the Ri plasmid), becomes stably integrated in the plant genome, and controls the growth and the differentiation of the transformed tissues. Hairy roots generally grow better than untransformed roots when cultured in vitro without plant hormones, and can readily be distinguished by their typical phenotype (see Tepfer 1984), i.e. extensive lateral root proliferation, synthesis of opines, lack of gravireaction and unusually high density of root hairs.

While crown-gall tumorigenesis proceeds by means of a de-novo synthesis of IAA, leading to a markedly enhanced IAA level, hairy-root proliferation appears to be controlled in other ways than by de-novo IAA over-production. Using different plant materials, Shen et al. (1988, 1990) have shown that hairy roots are 100 to 1000 times more sensitive to applied auxin than normal roots. The degree of hypersensitivity has been evaluated through long-term (root elongation), medium-term (proton excretion) and short-term (transmembrane-potential modification in protoplasts) responses to auxin. The brevity of this latter short-term response (less than 2 min) indicates that such hypersensitivity might be related to early events in the auxin reception/transduction pathway (Shen et al. 1988). Thus, the hypersensitivity to IAA could be a major determinant of the substantial proliferation of lateral roots in transformed roots. The question might, however, be raised whether responses to applied auxin are relevant to features (such as root proliferation) which are controlled – among others – by endogenous IAA. Also, there is no difference in the effects of applied auxin on cell division of protoplasts from normal and rol-transformed leaves (Maurel et al. 1991). Recent studies (Estruch et al. 1991a, b) have assigned a physiological role to two of the rol genes of A. rhizogenes. These genes are responsible for the release of active IAA (rolB) and of active cytokinins (rolC) from intracellular pools of inactive conjugates. Their concerted action might therefore modulate the intracellular concentrations of these hormones (Estruch et al. 1991b).

It has previously been demonstrated that transformation of the pea root was achievable with high rates of success using a variety of Agrobacterium strains (Schaerer and Pilet 1991). The aim of the present work was to determine whether (i) modifications of IAA level occur in sub-cultured root-derived hairy roots and
whether (ii) this can be associated with the hairy-root phenotypes observed.

Materials and methods

Bacterial strains and plant material. All the strains used were a kind gift of Dr. M. Dubois (Elf-BioRecherches, Castanet-Toloson, France). Agrobacterium rhizogenes 8196 (mannopine strain, harboring only its T-DNA and lacking the auxin-biosynthesis gene cluster borne on the T-DNA), A4 and 15834 (agropine strains harboring both the T1- and the T2-DNA, and hence the auxin-biosynthesis genes tms 1 and 2) were maintained at 4°C on solid YEB medium (1 g l−1 yeast extract (Difco laboratories, Detroit, Mich., USA), 5 g l−1 Difco bacto-pectone, 5 g l−1 Difco beef extract, 5 g l−1 sucrose, 2 ml of 1 M MgSO4, 16 g l−1 Difco bacto-agar). The day before transformations, single colonies of each strain were transferred into liquid medium and left to grow overnight (shaking incubator, 28°C).

Seeds of Pisum sativum L. cv. Glore du Midi (wrinkle-seeded; Agence agricole D. Roulin, Lausanne, Switzerland) were surface-sterilized and germinated under sterile conditions as previously described (Schaerer and Pilet 1991).

Transformations and in-vitro root cultures. Root segments were transformed as described elsewhere (Schaerer and Pilet 1991). Roots emerging from segments transformed by A. rhizogenes were individually cultivated in liquid hormone-free B5 medium (Gamborg 1970) containing 250 mg l−1-1 ampicillin to prevent further bacterial growth. Cultures showing vigorous growth were checked for the presence of opines according to Dahl et al. (1983). The opine-containing lines were further grown in B5 medium (100 ml in 300-ml Erlenmeyer flasks) containing ampicillin, with sub-cultures taking place every five weeks. After several sub-cultures, 18 hairy root lines were selected (6 for each A. rhizogenes strain) on the basis of their steadiness of growth. Sub-culture took place every three to four weeks by transfer of small external fragments sliced off the root tissues (see also Robins et al. 1991), the shoots grown in the absence of plant hormones developed poorly; their steadiness of growth were shown to contain the opines related to the inducing bacterial strain (data not shown), confirming thereby their transformed state. The sub-culture technique allowed root growth to be fairly high, since a 9- to 22-fold increase in the dry weight was scored at every sub-culture (data not shown). In contrast, non-transformed pea root lines grown for several months in the absence of plant hormones developed poorly; their elongation was rather limited and lateral roots were never obtained. Six hairy root lines were selected for each bacterial strain used. Mannopine was consistently detected in sub-cultured roots incited by strain 8196 and therefore proved to be a reliable transformation marker. This was not the case for passaged agropine-type roots, since only 3 (out of 12) lines contained mannopine and/or agropine after about one year. Reasons for the loss of T2-DNA markers have already been discussed (Schaerer and Pilet 1991).

Results

Hairy root lines. The co-cultivation method allowed the transformation of pea at high and reproducible rates (see Schaerer and Pilet 1991). Though no attempt was made to show the presence or the absence of T-DNA by Southern analysis, all the hairy-root cultures exhibiting appreciable growth were shown to contain the opines related to the inducing bacterial strain (data not shown), confirming thereby their transformed state. The sub-culture technique allowed root growth to be fairly high, since a 9- to 22-fold increase in the dry weight was scored at every sub-culture (data not shown). In contrast, non-transformed pea root lines grown for several months in the absence of plant hormones developed poorly; their elongation was rather limited and lateral roots were never obtained. Six hairy root lines were selected for each bacterial strain used. Mannopine was consistently detected in sub-cultured roots incited by strain 8196 and therefore proved to be a reliable transformation marker. This was not the case for passaged agropine-type roots, since only 3 (out of 12) lines contained mannopine and/or agropine after about one year. Reasons for the loss of T2-DNA markers have already been discussed (Schaerer and Pilet 1991).

Phenotype of the transformed root lines. Some phenotypic traits easily differentiated the hairy roots from their untransformed counterparts. Unlike normal roots, growth in hairy roots was mostly due to the extensive proliferation of laterals (Fig. 1A). The fastest growing hairy roots showed a 18- to 22-fold increase in their dry weight at every sub-culture. The abnormally high production of root hairs in liquid medium was not observed in normal roots, which were almost totally devoid of root hairs (Fig. 2A, B).

The gravireactivity of the different root lines was also periodically checked on solid culture medium (see Capone et al. 1989; Schaerer 1991). A clear positive response was recorded for untransformed roots, but this was never the case in transformed roots (data not shown). In addition to these typical hairy-root traits, one 15834-type root line was also characterized by the continuous proliferation of small calli (Fig. 1B), the frequency and the size of which increased with the root age. This feature was never shown to occur spontaneously in the other hairy root lines. However, when sub-culture was delayed by at least two weeks, agropine-type roots...