RESEARCH PAPER

A method for the isolation of root hairs from the model legume Medicago truncatula

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Abstract

A new method for the isolation of root hairs from the model legume, Medicago truncatula, was developed. The procedure involves the propagation of detached roots on agar plates and the collection of root hairs by immersion in liquid nitrogen. Yields of up to 40 μg of root hair protein were obtained from 50–100 root tips grown for 3 weeks on a single plate. The high purity of the root hair fraction was monitored by western blot analysis using an antibody to the pea epidermis specific protein PsRH2. Sequence analyses revealed that the protein homologous to PsRH2 in M. truncatula, MtRH2, is identical to the root protein MtPR10-1. The MtRH2 protein proved to be a useful endogenous marker to monitor root hair isolation since it is also specifically expressed in the root epidermis.

Key words: Green fluorescent protein (GFP), legume, Medicago truncatula, PR10 defence protein, root hairs.

Introduction

Leguminous plants are able to establish a symbiosis with soil bacteria collectively known as rhizobia. This interaction culminates in the formation of root nodules and in the differentiation of bacteria into bacteroids, which are capable of reducing atmospheric nitrogen into ammonia (for a review see Mylona et al., 1994). Nodule formation involves responses of the host in various root tissues. For example, cell divisions occur in the cortex, whereas deformation and curling of root hairs occur in the epidermis. Both processes are set in motion by signal molecules, the so-called lipochito-oligosaccharides or Nod factors, which are secreted by the bacteria (Dénarie and Cullimore, 1993).

Studies on the very early stages of the plant–rhizobial interaction are facilitated if root hairs can be isolated as this would allow biochemical analysis. Root hairs are tubular extensions of epidermal cells that are formed on specialized protoderm cells called trichoblasts (Peterson and Farquhar, 1996). Roots are indeterminate growing organs and, as a consequence, root hairs at successive stages of development are lined up along the root. The elongation of root hairs takes places at their tip, and their normal growth as well as Nod factor-induced root hair deformation require a functional actin skeleton (de Ruijter et al., 1999). Root hairs can be classified by their position on the roots, their cytoarchitecture, and their response to Nod factors (Heidstra et al., 1997). Zone I of roots contains growing young root hairs that do not deform upon treatment with Nod factors and that have, at their tip, a relatively large zone devoid of organelles but rich in vesicles. Zone II consists of almost full-grown root hairs, which are able to deform upon the application of Nod factors. Zone III contains mature root hairs that lack the vesicle-rich zone and do not deform upon exposure to Nod factors.

Studies on the responses of root hairs to Nod factors has focused on the model legume Medicago truncatula. This species has considerable advantages for molecular genetic studies as compared to crop legumes. For example, it has a small diploid genome, self-fertile flowers, and a short generation time (Barker et al., 1990). In addition, powerful genetic tools have been developed for this legume species, including the efficient transformation of several ecotypes.
by *Agrobacterium tumefaciens* and *A. rhizogenes* (Boisson-Dernier *et al*, 2001), and the production of BAC libraries, detailed genetic maps, and numerous nodulation and mycorrhizal mutants (Pennmetsa and Cook, 1997). In this paper, a highly efficient method is described for the isolation of root hairs of *M. truncatula* and the isolation procedure is monitored in detail by using molecular markers.

### Materials and methods

#### Plant material

Plants of *Medicago truncatula* Gaertn. R108-1 (c3) were used for these studies because this line is most efficiently transformed (Hoffmann *et al*, 1997). Seeds, kindly provided by Dr Trinh (Gif-sur-Yvette, France), were soaked in concentrated H2SO4 for 5 min to disrupt the seed coat, rinsed five times with water, surface-sterilized for 2 min with a 1:1 mixture (v/v) of 3% H2O2 and 96% ethanol, and finally washed five times with sterile water. Seeds were then kept in water for 4 h at room temperature and plated on a solid growth medium containing 1% agar and the following mineral nutrients: 2.72 mM CaCl2.2H2O, 1.95 mM MgSO4.7H2O, 2.2 mM KH2PO4, 1.26 mM Na2HPO4.12H2O, and 80 μM Fe-citrate.2H2O (Vincent, 1970). After incubation at 4 °C for 72 h, plates were placed vertically at 15 °C in the dark for 1 d to allow the seeds to germinate.

#### Growth of explants

A 1.5 cm portion of the main root, including the tip, was cut from the seedling and placed on the surface of SH medium (Schenk and Hildebrandt, 1972) containing 1.5% Phytagar (Life Technologies, Paisley, UK) in a 10×10 cm square Petri dish. This high agar concentration prevents growth of the roots under the surface. The plates were covered with aluminum foil to shield them from light and were incubated in a growth chamber at 21 °C. After 7–10 d, when the detached root was growing actively and lateral roots had developed, five pieces of 1 cm, containing at least a complete lateral root, were cut from the explant root and placed about 5 cm apart on a new Petri dish. Plates were then incubated under the same conditions as described above. A week later, when the roots had covered the surface of the plate, the lateral roots were sliced 3–6 mm above the tip with a scalpel blade and the older tissue was removed. The roots were allowed to regrow for another week.

#### Root hair collection

To collect root hairs, the Petri dish (without lid) was immersed in a stainless steel tank (12×15×4 cm) with liquid nitrogen. When the nitrogen had stopped boiling and the plate was fully frozen, root hairs were gently brushed from the agar surface with a number 2 paintbrush. The brush was kept continuously inside liquid nitrogen, otherwise water condensation may occur resulting in the breakage of the brush hairs. After brushing, the liquid nitrogen containing the root hairs was poured through a 400 μm nylon mesh into a plastic 50 ml tube placed on ice. Liquid nitrogen was allowed to evaporate until 10 ml were left, then the tube was closed with a perforated cap and stored at −80 °C.

#### Reverse transcription-PCR (RT-PCR) of total root and root hair RNA

RNA was isolated using the VS total RNA isolation kit (Promega, Madison, WI). For extraction of root hair RNA, the 50 ml tube (see above) was rinsed with 100 μl of the VS kit extraction buffer to collect the root hairs. For extraction of total root RNA, four 10 mm long lateral roots (including the root tips), grown as described above, were used. A fraction of the extracted root hair RNA (50%) and total root RNA (5%) was used for cDNA reverse transcription using Moloney murine leukemia virus reverse transcriptase (Life Technologies) with a poly-A’ primer. The amounts of cDNA to be used as templates were standardized with ubiquitin. Primers (forward: GTTGAAGGAAACGTTG; reverse: GTAACCTTCAAGGCCCCTT) were designed based on homologous regions of *MiPR10-I* (Gamers *et al*, 1998; accession number Y08641) and *PsRH2* (Mylona *et al*, 1995; accession number S74512). PCR conditions were an initial denaturation step of 5 min at 95 °C; 12–20 cycles of 45 s at 95 °C, 45 s at 54 °C and 45 s at 72 °C; and a final elongation step of 10 min at 72 °C. PCR products were separated by electrophoresis, transferred to nylon membranes at alkaline pH and hybridized with a 32P-labelled probe generated by PCR using the same primers. Autoradiographs were analysed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

#### Protein extraction from roots and root hairs

For extraction of hair root protein, the 50 ml tube containing the root hairs (mentioned above) was placed on ice and rinsed with 100 μl of cytoskeleton stabilizing buffer (Morelli *et al*, 1998) containing 5 mM HEPES, pH 7.5, 10 mM Mg-acetate, 2 mM EGTA, 2 mM phenylmethylsulphonyl fluoride, 15 mM β-mercaptoethanol, 0.5% polyoxyethylene-10-tridecyl ether, and a cocktail of protease inhibitors (Complete; Boehringer-Mannheim, Mannheim, Germany). An aliquot was analysed by microscopy to check whether the root hair preparation was contaminated with other plant material. Samples were then centrifuged at 550 g for 30 s and the supernatant was used for analysis.

For extraction of total root protein, four 10 mm long lateral roots (including the tips) were ground in liquid nitrogen. The powder was resuspended in cytoskeleton stabilizing buffer, left to stand at 4 °C for 20 min and centrifuged at 15 500 g for 1 min. Protein was determined in both supernatants by the Bradford assay using bovine serum albumin as the standard (Bio-Rad, Hercules, CA).

#### Western blot analysis

Proteins (5 μg) were separated in 12% (w/v) acrylamide SDS-gels and blotted to polyvinylidene difluoride membranes with a Mini-Trans Blot apparatus (Bio-Rad, Hercules, CA, USA) according to standard protocols. RH2 antibody was prepared in the laboratory. 400 μg of pea root hair protein was separated by 2D gel electrophoresis and the spot representing RH2 was cut from the blot (Mylona *et al*, 1994). Subsequently, from this material the antibody was raised in a New Zealand rabbit (H Franssen, unpublished results). The blot was blocked by incubation for 1 h with 5% (w/v) skimmed milk in TRIS-buffered saline (10 mMTRIS-HCl, pH 7.8; 150 mM NaCl) containing 1% (v/v) Tween 20. The primary antibody (RH2) was used at a dilution of 1:10 000. After 1 h incubation, the secondary antibody (goat anti-rabbit antibody conjugated to horseradish peroxidase; Boehringer-Mannheim) was added at a dilution of 1:5000 and incubated for a further 1 h. After each incubation, blots were washed three times for 10 min in TRIS-buffered saline. Horseradish peroxidase was detected using the ECL luminiscence kit (Amersham Biosciences, Uppsala, Sweden) with X-ray film.

#### Vectors and plant transformation

The vector used for plant transformation, pBinSL, is a derivative of pBin101 (Clontech). pBinSL contains the *PsRH2* promoter (from the *DRRG49-c* gene; Chiang and Hadwiger, 1990), between the restriction sites HindIII and BamHI, and the GFP sequence (Davis and Vierstra, 1998), between the restriction sites BamHI and SalI.

The pBinSL construct was introduced into *A. tumefaciens* strain GV3101 by electroporation. Transgenic plants of *M. truncatula* were grown on SH plates co-infiltrated with 100 μl of the root hair and root hair mutant suspension.
(R108-1) were obtained essentially as described by Hoffmann et al. (1997) and analysed by PCR and Southern blots to determine whether the construct was properly inserted.

**Fluorescence microscopy**

The roots of transgenic plants transformed with pBinSL were observed and photographed with a confocal microscope, Zeiss LSM510 Axiocam 100, using a plan-neofluar 10×0.3 objective and the LSM-FCS version 2.8 SPI software of ZEISS.

**Southern blot analysis**

Genomic DNA was extracted from leaves and flowers of *M. truncatula* following the hexadecyltrimethyl ammonium bromide method (Stewart and Le, 1993). DNA (7 μg) was digested with Hind III, electrophoresed, blotted and hybridized as described for RT-PCR. The probe was the same as that used for the detection of MtRH2 by RT-PCR.

**Results**

**Root hair isolation procedure**

Initially an attempt was made to adapt a method for root hair isolation that was previously used successfully for pea (Röhm and Werner, 1987; Gloudemans et al., 1989). However, the root hair fraction obtained with this method was heavily contaminated with other plant material. The roots of *M. truncatula* are considerably thinner than those of pea, and hence it is likely that fragmentation of roots into small pieces occurred before the root hairs were released, resulting in contamination and poor recovery of root hairs. Western blot analysis confirmed that this procedure did not lead to an enrichment of root hair protein (data not shown).

To increase root hair yield while minimizing root fragmentation, a procedure was developed to grow roots of *M. truncatula* (R108-1) on the surface of agar plates, so that the root hairs stick out. Root explants were grown on 1.5% agar and 1.5% sucrose in SH medium. The main root of a seedling was detached from the shoot and placed on the agar medium. After 7–10 d, the explant had elongated and developed lateral roots. These lateral roots were subsequently used to create new explants and were moved onto a new plate (Fig. 1A). A week later the explants had formed lateral roots and covered a larger area of the plate (Fig. 1B). The older parts of the roots were removed and only a 3–6 mm long piece from each lateral root tip was left on the plate (Fig. 1C). If only young root hairs (zones I and II) were to be collected, the plates were used at this time. Otherwise, roots were allowed to grow for another week before collecting the root hairs (Fig. 1D). The roots stayed alive for several months in these conditions, eventually completely covering the plate.

To collect the root hairs, the entire Petri dish was immersed in liquid nitrogen and the surface gently brushed while the plate was submerged in the nitrogen. The root hair suspension was collected and passed through a 400 μm nylon mesh to remove the larger particles. Light microscopy confirmed the high purity of the root hair preparation. However, the weight of the collected root hairs could not be measured, as condensed water would make it unreliable. The amount of total root hair protein produced by the method of Röhm and Werner (1987) from 40 3-d-old seedlings of *M. truncatula* was always <1 μg, whereas, with this method, 25–40 μg of root hair protein was usually obtained from a single 3-week-old plate, containing 50–100 root tips.

The ability of root explants to grow in the absence of hormones was also examined to determine whether the method is specific for *M. truncatula* (R108-1) or can be used for other cultivars (e.g. Jemalong) or species (e.g. *Lotus japonicus* and *M. sativa*). At the initial stage, root explants of Jemalong behaved like those of line R108-1, growing and developing lateral roots; however, about 2 weeks after detachment from the plant, growth had almost ceased and older tissue was withering and dying. Root tips were able to remain alive on the plates for several weeks, even months, but they did not grow further. However, hairy root explants produced in *M. truncatula* (Jemalong) (Boisson-Dernier et al., 2001; Ramos, unpublished data), *L. corniculatus* (Petit et al., 1987), or *L. japonicus* (Stiller et al., 1997) after infection with *A. rhizogenes* behaved similarly to *M. truncatula* (R108-1); that is, they were able to grow indefinitely. Likewise, detached roots of untransformed *M. sativa* (Aragón) plants were able to propagate in vitro.
MtRH2 is a useful marker to monitor root hair isolation

To test the efficiency of the root hair isolation procedure, advantage was taken of the specific expression of *PsRH2* in the root epidermis (Mylona *et al*., 1995). Constructs were made of the *GFP* gene under the control of the *PsRH2* promoter and these were used to transform *M. truncatula* plants (R108). Fluorescence microscopy showed that GFP expression was confined to the root epidermis and that GFP accumulates in the root hairs of zones I and II (Fig. 2). Thus, in *M. truncatula* transgenic plants, GFP-driven expression by the *PsRH2* promoter is a convenient marker to monitor root hair isolation and to determine the enrichment. Further, roots of *M. truncatula* plants transformed transiently by *A. rhizogenes* (Boisson-Dernier *et al*., 2001) harbouring a *PsRH2::GUS* construct confirmed this expression pattern and the lack of expression at the root tip (data not shown).

To find out whether the RH2-like *M. truncatula* protein or mRNA can be detected in root hairs, immunoblots and RT-PCR were used. Protein extracts from roots and root hairs of untransformed plants were analysed by western blotting using the anti-PsRH2 antibody. A single immunoreactive protein band was observed, corresponding to the expected molecular mass (12 kDa) of PsRH2 (Fig. 3). This protein, tentatively identified as MtRH2, was far more abundant in root hairs than in whole root preparations, which provides strong evidence that MtRH2 is localized specifically in the epidermis, as occurs with PsRH2 (Mylona *et al*., 1995). The expression of the corresponding gene was analysed by RT-PCR with primers based on the *PsRH2* sequence. Figure 4 shows that the transcript was far more abundant in root hairs than in total root RNA.

![Fig. 2.](image)

Fig. 2. Fluorescence (A) and white light (B) microphotographs of a 3-d-old seedling root. Note that GFP accumulation is localized in the root epidermis.

![Fig. 3.](image)

Fig. 3. Western blot analysis of proteins from total roots (10 mm including root tips) and root hairs. In both cases, a single immunoreactive protein band was observed, which corresponded to the expected molecular mass (12 kDa) of PsRH2. Lanes were loaded with 5 μg of protein.

![Fig. 4.](image)

Fig. 4. Expression analysis of the MtRH2 gene by RT-PCR using primers based on the *PsRH2* sequence. The amounts of cDNA template from root hairs or total roots were standardized with ubiquitin. The number of PCR cycles is indicated for each lane to show that the signal is below saturation. The transcript was clearly more abundant in root hair than in total RNA root preparations.
isolated. Genomic DNA was digested with Southern blot analysis of the indicating that there is one copy of the was detected (at approximately 6 kb; data not shown), indicating that there is one copy of the MtRH2 gene in the M. truncatula (R108-1) genome. However, the possibility cannot entirely be ruled out that two copies, in an inverted tandem arrangement, are present.

Discussion

A new method for root hair isolation has been developed, with high yield and purity, in the model legume, M. truncatula. The method is especially suitable in those cases where plant material is a serious limiting factor. For instance, transgenic plants can be generated and roots can be used well before seed becomes available, thus providing relatively large amounts of homogeneous material in less than one generation time (approximately in 3 weeks). Another advantage of the method is the possibility to select different root hair populations by simply removing the undesired parts of the root with a scalpel blade before root hair collection. By exploiting the advantages of A. rhizogenes-mediated transformation, this method can be expanded to other legume species and allows high amounts of homogeneous root (hair) material to be obtained in a short period. A drawback of the method is, however, that detached roots, whether or not induced by A. rhizogenes, do not form nodules.

In this method, use is made of a specific protein marker of root epidermis, RH2, to monitor the root hair isolation process. A construct of the reporter gene GFP under the control of the PsRH2 promoter was made and introduced into M. truncatula (R108-1). Transgenic plants expressed GFP specifically in the root epidermis and this property was exploited to verify the high purity and yield of these root hair preparations. In the course of this work, it was also shown that there is a protein (and gene) homologous to PsRH2, MtRH2, in M. truncatula. This was proven by analyses of western blots (immunoreactive band with identical molecular mass) and RT-PCR and Southern blots (nucleotide identity and single gene copy). All these analyses are also consistent with a root epidermis-specific expression of MtRH2. Thus, the isolation of root hairs can be readily monitored in untransformed plants by using MtRH2 as a marker. cDNA sequence analysis showed that MtRH2 is identical to MtPR10-1, which is known to encode a root-specific protein (Gamas et al., 1998). Expression of transgenic proteins can, therefore, be specifically targeted to M. truncatula root hairs with the use of the endogenous MtRH2 promoter or homologous promoters such as PsRH2. This should be a promising tool for root hair research.

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References


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