Root Hair Specific Proteins in *Glycine max*

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In root hairs from seedlings of *Glycine max* cultivars, isolated from the root system and compared with the complete organ, specific soluble proteins have been found. By FPLC chromatography and SDS gel electrophoresis root hair specific proteins with molecular weights of 13, 21, 34, 38 and 42 kDa were separated. Additionally, proteins with molecular weights of 12, 20, 69 and 74 kDa were significantly enriched in root hairs compared to roots without root hairs. By using CNBr activated Sepharose with antibodies against the root system without root hairs, the presence of root hair specific proteins was confirmed in extracts from isolated root hair cells. Enrichment of Fe and Ca in some of the proteins from the root hairs is demonstrated. The present knowledge of the biochemical specificity of legume root hairs, the target cells of *Rhizobium* and *Bradyrhizobium* infection, is discussed.

Introduction

The geometry of the root system of higher plants is to a large extent affected by the development of root hairs. The ratio of the surface of root hairs to the other parts of the root system can vary between 0.2 in *Allium cepa* and up to 3.8 in *Salsola kali.* The average length of root hairs is also species specific, with figures of 350 μm for *Medicago sativa,* 900 μm in *Zea mays* and 1100 μm in *Lolium perenne.* The average number of root hairs per mm root in the root hair zone for the same species is 105, 160 and 88 respectively [1, 2]. Specific signal molecules that trigger the growth of the root hairs from trichoblasts are unknown. Kinetin can increase the length of the root hairs in *Raphanus sativus* [3] and low concentrations of ethylene have been reported to stimulate root hair formation in cereals [4].

The function of root hairs in increasing the radius of the active root zone is established for several nutrients such as phosphorus [5] and potassium [6, 7]. The significance of root hairs for nutrient uptake may also be related to the frequency of plasmodesmata between the rhizodermis cells and the neighbouring cortical cells. In *Trianaea bogotensis* trichoblasts had 20 times more plasmodesmata than the hairless cells [8], in *Rhaphanus sativus* there was only a factor of two [9].

Characterization of root hairs by specific biochemical components and concentrations of metabolites and nutrients has only recently begun, after the development of methods to separate root hairs from the other parts of the root system on a preparative scale [10–13]. In root hairs of soybean a 7- to 10-fold accumulation of calcium, cobalt and iron has been found compared to the other parts of the root system [11]. This accumulation is of special interest, since the microsymbiont of soybean, *Bradyrhizobium japonicum,* has a high requirement for these nutrients. In peas, root hair specific soluble proteins [10] and membrane bound proteins [12] have been characterized. The functions of these proteins for nutrient uptake or recognition of the symbionts are unknown so far. In this communication, we report on proteins specific or quantitatively enriched in root hairs of soybean (*Glycine max* cultivars) and on ion accumulation in root hair proteins.

Materials and Methods

Germination of seedlings and separation of root hairs

Seeds of *Glycine max* cv. Maple arrow were sterilized and grown as previously described [11]. The best stage of development to separate the root hairs from the other parts of the root system is a 5–8 cm long primary root without secondary roots. The roots were separated from the seedlings and transferred to a metal plate frozen in liquid nitrogen

Abbreviations: PAA, poly-acryl-amide; TCE, Trichloracetic acid; TES, N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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in a "Styropor" container. Root hairs, fragile as ice cones after this treatment, could be separated from the main root with thin brushes, collected in liquid nitrogen and stored as also the roots without root hairs, at −80 °C.

Protein extraction

The separated root hairs and the residual root system without root hairs were homogenized in TES-buffer (50 mM with 10 mM glutathion, pH 7.5) in a mortar with sand. After a centrifugation at $400 \times g$ for 10 min the supernatant was stored and the pellet was reextracted with buffer after sonication for five times 30 s. After another centrifugation for 10 min with $400 \times g$, the supernatants were pooled. A centrifugation for 30 min with $32{,}000 \times g$ followed. The supernatant of this centrifugation was dialyzed against distilled water at 3 °C over night. The dialysate was concentrated by freeze-drying or ultrafiltration to a concentration of 5 μg protein · μl^{-1}. Protein concentrations were determined by the method of Bradford [14].

FPLC-separation

The crude extracts from root hairs and from the residual root system were separated on a molecular sieve column (Serva SI 200 Polyol 500×7.1) with an ammonium phosphate buffer (0.2 M, pH 6.0) as a solvent, using a LKB-FPLC system with two pumps. The flow rate was 0.25 ml · min^{-1}, the absorption of the eluate was recorded at 280 nm. For each separation about 500 μg of protein was used.

Gel-electrophoresis

SDS-PAA-gel electrophoresis was performed according to Stegemann [15] and Laemmli [16, 17]. Protein samples and marker proteins were mixed with 2% SDS, 2% sucrose and 0.01% bromophenol blue and heated for 2 min at 100 °C. The gels were stained in a solution with 30 g TCE, 200 ml methanol, 800 ml distilled water and 0.4 g Coomassie blue R 250. The gels were destained in a solution with 29% methanol, 66.2% distilled water and 4.8% acetic acid.

Silver staining of gels was performed according to Merril *et al.* [18] with 0.1% $AgNO_3$ for 40 min followed by 2.5 M Na_2CO_3 containing 0.02% formaldehyde for 5 to 10 min.

Native PAA gel electrophoresis was performed in the same way without SDS.

Antibodies against root proteins

Rabbits (variety Deutsche Großsilber) were immunized with 0.2 mg root (without root hairs) protein after day 1, 2, 3, 7, 14, 28 and 42 together with 0.1 ml Freund's adjuvant. 10 days later, 20 ml of blood was taken. The antiserum was purified as previously described [19]. Antibodies were bound to CNBR activated sepharose 6MB by mixing the sepharose gel in 0.1 M $NaHCO_3$ + 0.5 M NaCl buffer, pH 8.5 (buffer A) in a handmixer for 2 h at room temperature or at 4 °C over night. The unbound antibodies were removed by repeated washing with 0.1 M acetate buffer (pH 4.5 + 0.5 M NaCl). Unreacted groups were saturated with 0.2 M glycine in buffer A. Protein extract from root hairs was calibrated on the column for 20 min and the unbound proteins washed with 20 × the column volume buffer (6 mM, pH 7.5 + 0.5 M NaCl). The root hair proteins bound to the antibodies were finally extracted with citrate buffer (0.1 M, pH 2.8) and collected. For a further analysis by SDS-gel electrophoresis and FPLC, the protein samples were concentrated by freeze-drying after dialysis.

Determination of Fe and Ca in protein fractions from native PAA gels by atomic absorption spectrophotometry (AAS)

After separation of 100 μg protein each from root extracts and root hair extracts on native PAA gels, control samples were stained with Coomassie blue as previously described to identify the position of the proteins. The other samples were developed but remained unstained to exclude a contamination by the staining reagents. Gel pieces (1 cm^2) were cut out at those positions with especially pronounced protein bands. As blanks, gel samples with no obvious protein concentration were used. The gel samples were solubilized in 3 ml 30% H_2O_2 for 1 week in highly purified glass vessels. The iron and calcium concentration in the various protein samples was determined with a Pye Unicam SP 19100 instrument with a Massmann cuvette at a temperature of 2800 °C for 15 s. The sample volume for each determination was 5 μl.

Results and Discussion

In order to compare the protein composition of soybean root hairs with that of the residual root system, crude extracts of both components were first separated on a molecular sieve by FPLC (Fig. 1). The elution profile gave quantitative differences in the area of 20 to 28 min, 30 to 35 and 65 to 80 min elution time (Fig. 1). These samples were pooled into three fractions and used for further separation on SDS-PAA-gel electrophoresis with silver staining (Fig. 2). Most obvious is an enrichment of certain proteins in root hairs, compared to the other parts of the root system in fraction 2. Significantly enriched are proteins with 74, 42, 34 and 21 kDa. In fraction 3, a protein with a molecular weight of 13 kDa is present only in root hairs but not in the other parts of the root system. On the other side, there is also a number of protein bands significantly enriched in the other parts of the root system compared to the root hairs. This is very obvious with proteins of 31 kDa and 19 kDa in fraction 2 (Fig. 2). The comparison of native proteins from root hairs and roots shows even more striking differences (Fig. 3). A very significant part of the root hair protein is concentrated in two bands with molecular weights of 140 and 150 kDa. Two bands of 12 and 13 kDa are present only in the root hair preparation.

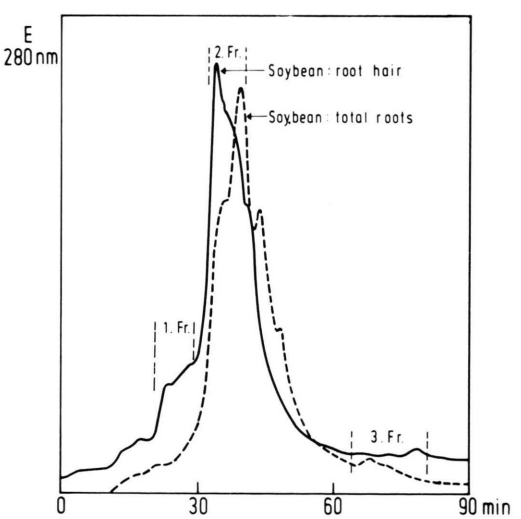

Fig. 1. Separation of soluble proteins (500 µg) from root hairs and roots of *Glycine max* cv. Maple Arrow by FPLC on Polyol Si G 200. Flow rate: 25 ml·min^{-1}. 1., 2. and 3. Fr.: fractions used for SDS gel electrophoresis in Fig. 2.

The existence of root hair specific or enriched proteins was further studied by using antibodies against root proteins (without root hairs) and FPLC separation of these proteins not bound to the antibodies.

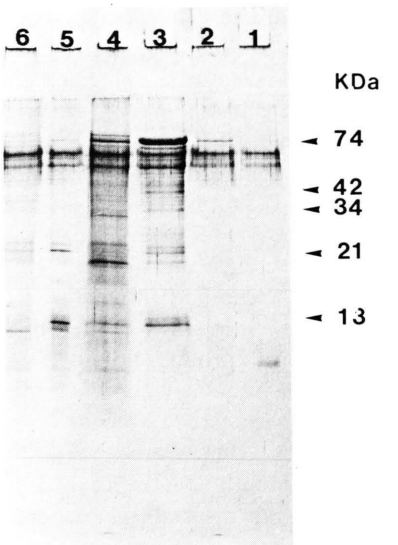

Fig 2. SDS-polyacrylamide gel-electrophoresis with silver-staining on discontinuous 1 mm gels of the fractions 1, 2 and 3 from Fig. 1. Root hair specific or significantly enriched proteins are indicated by arrows, molecular weights are given in Kilodalton (kDa). Lane 1: root hair, fraction 1; lane 2: root, fraction 1; lane 3: root hair, fraction 2; lane 4: root, fraction 2; lane 5: root hair, fraction 3; lane 6: root, fraction 3.

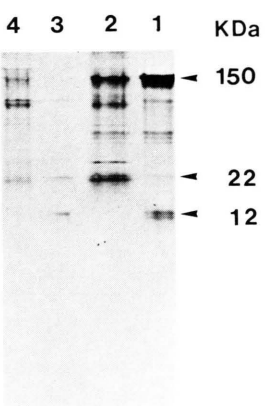

Fig. 3. Native polyacrylamide gel-electrophoresis of fractions from the FPLC-G 200 separation from root hairs and roots of *Glycine max* cv. Maple Arrow. 10 µg protein per lane. Lane 1: root hair, fraction 2; lane 2: root, fraction 2; lane 3: root hair, fraction 3; lane 4: root, fraction 3. Molecular weight marker proteins were trypsin inhibitor, ovalbumin and bovine albumin.

The elution profile shows some marked peaks of proteins from root hairs not bound to the antibodies against the proteins from the roots. However, the presence of those peaks is no proof, that these proteins are root hair specific, since the antiserum most likely does not contain antibodies against all residual root proteins and therefore, would also not react with some root proteins. The root hair proteins not bound to the antibodies against the root proteins were further separated on SDS-PAA-gels (Fig. 4). The characteristic root hair specific protein bands from Fig. 2 can be also seen on this gel with a molecular weight of 14, 19, 34, 43 kDa. Due to the high concentration of proteins on this gel, the 75 kDa protein cannot be discriminated. However, a 84 kDa protein is additionally obvious in this root hair preparation. As a control, proteins bound to the antibodies against the root proteins were also separated on SDS-PAA-gel. As expected, there is a large number of proteins present, which are apparently very similar in root hairs as well as in roots.

One of the few already known specific characteristics of root hairs from soybeans is the accumulation of iron, calcium and also cobalt compared to the roots [11]. Therefore the iron concentration of the separated root hair fractions was analyzed (Fig. 5). Iron was significantly enriched in protein bands with a molecular weight of more than 200 kDa, of about 140, 75 and 25 kDa, respectively. It is important to mention that the blanks for these analyses had to be taken always from the same gel preparation (without proteins) since the Fe-concentrations in the gels are relatively large. The enrichment of calcium is most obvious in the 140 kDa protein (Fig. 6).

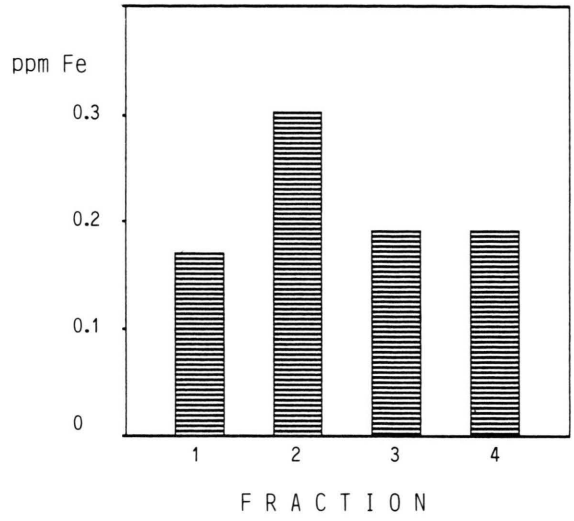

Fig. 5. Fe content in root hair protein fractions of *Glycine max* cv. Maple Arrow after separation on native gels (minus concentrations from pure gel samples). 1 = proteins with a molecular weight of more than 200 kDa, 2 = 140 kDa, 3 = 75 kDa, 4 = 25 kDa.

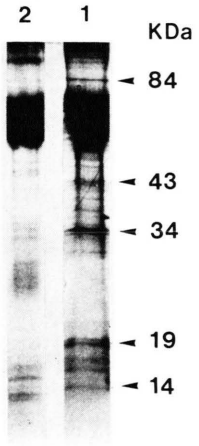

Fig. 4. SDS-polyacrylamide gel-electrophoresis with silver-staining on discontinuous 1 mm gels of root hair proteins not binding (lane 1) and binding (lane 2) to antibodies against proteins from the root system (without root hairs) of *Glycine max* cv. Maple Arrow.

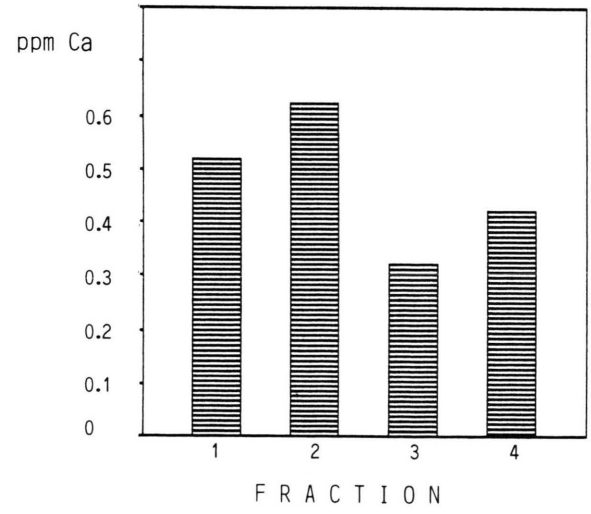

Fig. 6. Calcium content in root hair protein fractions of *Glycine max* cv. Maple Arrow after separation on native gels (minus concentrations from pure gel samples). 1 = protein bands with a molecular weight of more than 140 kDa, 2 = 140 kDa, 3 = 60–75 kDa, 4 = 12 kDa.

Root hairs of higher plants are one of the few examples where a multicellular organism develops cell types with a predominantly "unicellular" habitus. The geometry of these very elongated cells however will not imply that the protein composition is different from other cell types in the roots. These cells represent also the surface layer of the root system responsible for the communication with the environment. Two functions of legume root hairs are of main interest: 1. uptake of nutrients and water as in all other root systems. 2. the recognition and uptake of symbionts, *Rhizobium* and *Bradyrhizobium* species. After root hair specific proteins have been detected in pea [10, 12], this is the first report that such proteins exist also in soybean. Therefore we can now assume that in legumes in general root hair specific proteins exist. The enrichment of calcium and iron in certain fractions of these proteins does not allow any conclusion about the function of these proteins *e.g.* in transport or storage of iron. Further biochemical studies with larger quantities of these isolated proteins will allow to analyze their functions.

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