Isolation of mannose 6-phosphate reductase cDNA, changes in enzyme activity and mannitol content in broomrape (Orobanche ramosa) parasitic on tomato roots

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We are interested in developing a control strategy efficient at the early stages of subterranean development of Orobanche in the inhibition of mannose 6-phosphate reductase (M6PR, EC 1.1.1.224), the key enzyme of mannitol production in the parasite. We examined M6PR gene expression during pre-conditioning, germination, procambium growth, underground shoot development and emergence of Orobanche ramosa L. attached to tomato roots, the enzyme activity at each of the above stages and the level of stored mannitol in the parasite. A 1266-bp length cDNA isolated by 3' and 5' RACE was identified as a M6PR sequence by cDNA expression in E. coli and M6PR activity measurement. Only one M6PR gene was detected in O. ramosa following southern blot analysis. M6PR expression, analysed by RT-PCR, was constant from the pre-conditioned seed to the emergence of broomrape, i.e. M6PR expression is constitutive in Orobanche. M6PR activity was also detected in pre-conditioned seeds and attachment to tomato roots resulted in a two-fold increase in enzyme activity during tubercle enlargement and crown root formation. Hexitol and mannitol accumulation was strongly enhanced in the attached parasite, with accumulation primarily in the shoot. This result supports the prospect of utilizing M6PR inhibitors as early applied herbicides to control this parasite in the early stages of its development.

Introduction

Orobanche (broomrape) is one of the most important agricultural pests in numerous cropping systems (faba bean, sunflower, tobacco, tomato...) in many parts of the world (Musselman 1980). Orobanche is an obligate root-parasite, completely devoid of chlorophyll and consequently dependent on its host for a heterotrophic supply of resources. Germination of the pre-conditioned seed, induced by host-root exudates, ends in the development of a root-like organ, known as the germ tube or procambium. After attachment to the host root, it invades it and intimately connects the conducting tissues of the host root. The connecting organ, called a haustorium, serves as a bridge for water and nutrient uptake from host to parasite.

Infection inevitably results in reduced growth, yield and quality of crops and damage becomes noticeable even before the parasite emerges from the soil (Graves 1995). When chemical control of Orobanche can be applied, it remains expensive and not selective enough (Foy et al. 1989, Eplee and Norris 1995). Indeed, none of the developed practices is applicable in any crop. New control methods, more selective and active at early stages of subterranean development of Orobanche, need to be developed for the rapid destruction of the parasite before there is irreparable damage to the host plants.

Orobanche accumulates high amounts of mannitol, lowering its osmotic potential to a value much more negative than the host one (Harloff and Wegmann 1987). This osmoregulation process, essential to broomrape for water and nutrient uptake from host, is absent from the major host crops. Therefore herbicides preventing this sugar alcohol production could be efficient against the parasite whatever the infested crop. Our interest is focused on the identification of specific inhibitors of mannose 6-phosphate reductase (M6PR, EC 1.1.1.224), the key enzyme of the mannitol biosynthetic pathway (Har-
loff and Wegmann 1987). This enzyme has been purified and characterized in the aerial shoot of O. ramosa (Harloff and Wegmann 1993, Robert et al. 1999a). Some M6PR inhibitors have been recently identified (Robert et al. 1999b) and might be used against Orobanche. However, M6PR activity in the very early stages of the parasite development has yet to be demonstrated. This study begins characterization of potential developmental regulation of M6PR during pre-conditioning, germination and growth of O. ramosa after attachment to tomato roots. Based on the only available characterized M6PR sequence (Everard et al. 1997), an enzyme from celery, M6PR cDNA was successfully cloned using RACE strategies, then sequenced. RT-PCR was used to examine the accumulation of M6PR transcripts at different specific developmental stages of the parasite as previously characterized by Linke et al. (1989). Changes in total protein level, M6PR activity and soluble carbohydrate concentration, including mannotiol, were also determined.

Materials and methods

Plant material

Broomrapes used for tomato infestation were harvested from greenhouse cultures in September 1997. O. ramosa L. (Orobanchaceae) was grown on tomato (Lycopersicon esculentum Mill. Solanaceae) in the greenhouse under controlled conditions, i.e. 13 h photoperiod, 300 μmol quanta m⁻² s⁻¹ photosynthetically active radiation (Phytolamp, 400 W, Comptoir Electrique Français, Lyon, France), 20/25 °C (night/day). Two 7-day-old tomato plants were placed in pots containing parasite seeds pre-conditioned as described by Reid and Parker (1979). Cultures were then watered three times per week. Following one and a half months of culture, broomrapes were regularly harvested and divided into developmental stages (Table 1) as defined by Linke et al. (1989). The average FW per Orobanche was measured for each developmental stage. Tubercles and shoots were separately analysed at stages V and VI (Table 1).

Some Orobanche seeds were previously surface sterilized by 5 min in sodium hypochlorite (1%) and rinsed five times with sterile distilled water. They were pre-conditioned in Petri dishes containing glass fibre filter papers moistened with sterile distilled water for a week in darkness. Half of the pre-conditioned seeds (stage PS) were immediately used for experimental purposes. Germination of the second set of seeds was induced in Petri dishes containing glass fibre filter papers moistened with GR24 (1 ppm), an analogue of the natural germination stimulant, strigol. Germinated seeds (stage GS) with 3–5 mm proalumelles were harvested after a week in darkness at 21°C. The observed germination rate was 90%. Analyses were carried out with all these samples, except stage I because of limited fresh material.

Nucleic acid isolation

Total DNA and total RNA were isolated from frozen tissues using the DNeasy and RNeasy Plant kits from Qiagen (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

Isolation of the M6PR cDNA by 3’ and 5’ RACE

Using the 3’ RACE system according to the manufacturer’s instructions (Gibco BRL, Life Technologies, Rockville, MO, USA), the 3’ end of the cDNA was isolated from the tubercle of emerged broomrape. Total RNA (1 μg) was used as a template for the reverse transcription reaction using the anchored oligo(dT) primer supplied with the kit. A first PCR amplification was carried out using a M6PR-specific primer (M6PR1, 5’-TTTACACWSCGCGTTYAAAATGC-3’) and the adapter homologous primer (AUAP) supplied with the kit. A re-amplification was done using the M6PR4 nested primer (5’-GGNTATCNGCNAYTTTGAYTTGTGC-3’), the AUAP primer and 5 μl of the first amplification product diluted to 1/100. PCR-amplified products were cloned in pCR2.1 plasmid from the Original TA cloning kit (Invitrogen, Groningen, The Netherlands) and inserts were sequenced by the sequencing service from the ESGS-Cybergene Company (Ervy, France). Sequence analysis was carried out using the MacDNASIS software system (Hitachi Software Engineering Co., Ltd, San Bruno, CA, USA). The 5’ end of O. ramosa M6PR cDNA was determined using the 5’ RACE system. The gene-specific primer and

Table 1. Average FW of the harvested broomrapes. ND: not determined.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Average FW (mg per Orobanche)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young fixations</td>
<td>ND</td>
</tr>
<tr>
<td>Stage I. Proalumine attachment to the host root surface</td>
<td></td>
</tr>
<tr>
<td>Stage II. Young tubercle formation</td>
<td>1.22 ± 0.03</td>
</tr>
<tr>
<td>Stage III. Formation of crown roots on the tubercle</td>
<td>9.75 ± 1.25</td>
</tr>
<tr>
<td>Stage IV. Development of the shoot bud (3–6 mm in length) from the tubercle</td>
<td>45 ± 15</td>
</tr>
<tr>
<td>Underground Orobanche (Stage V) with a slowly growing shoot (2–3 cm in length)</td>
<td>356 ± 94</td>
</tr>
<tr>
<td>Tubercle</td>
<td>82 ± 24</td>
</tr>
<tr>
<td>Shoot</td>
<td>274 ± 70</td>
</tr>
<tr>
<td>Emerged Orobanche (Stage VI) with a rapidly growing flowering shoot (10–16 cm in length)</td>
<td>1681 ± 417</td>
</tr>
<tr>
<td>Tubercle</td>
<td>323 ± 109</td>
</tr>
<tr>
<td>Flowering shoot</td>
<td>1358 ± 308</td>
</tr>
</tbody>
</table>
the nested gene-specific primer were M6PR6 (5'-GCGCTCAACACAGGACGATG) and M6PR7 (5'-ACACATGACCTGACGATG), respectively. PCR products were cloned and sequenced as above. A cDNA (Accession Number AF055910) containing the entire M6PR coding sequence was obtained using the 3' RACE system and the gene-specific primer, M6PROA (5'-ATGATGATTACACTCAAACATG).

Southern blot analysis
Total DNA (10 μg) was digested overnight with restriction enzymes, fractionated on a 0.7% agarose gel and transferred to Quiabrane nylon plus membrane (Quiglen) using an alkaline transfer method (Reed and Mann 1985). A 32P labelled M6PR cDNA probe was made using a random priming kit (DNA labelling beads - dCTP, Amersham Pharmacia Biotech, Uppsala, Sweden). Filter hybridization was performed at 42°C in 5x SSC, 1% Nonidet N-40, 0.1% dextran sulphate, 50% formamide and 100 μg ml⁻¹ of denatured salmon sperm DNA. Filter was washed twice in 2x SSC, 0.1% SDS at room temperature and then in 0.1x SSC, 0.1% SDS at 65°C prior to autoradiography.

RT-PCR analysis
The first-strand cDNA synthesis was carried out with the SuperScript first-strand synthesis system for RT-PCR from Gibco BRL using 1 μg of total RNA as template. After the reverse transcription step, the total cDNA served as template in the PCR amplification reaction using 2 μM of M6PR-specific primers (M6PR1OR-3', 5'-AACAATGGCTTCAAAATGCC and M6PR3-3', 5'-CTTTCACAAGACCCAGTTTGC). As an internal control, a fragment of the actin cDNA was amplified concomitantly with M6PR by adding 2 μM of actin-specific primers (actine5OR-5', 5'-ACCCCTTGGTTGCG ACAAT and actine4-3', 5'-GCCYTGRATVGCRCAT-ACAT) to the PCR reaction. PCR amplification was done with 2 μl of first-strand cDNA and 2.5 units of Taq DNA polymerase (Appligene-Oncor, Illkirch, France) in a reaction volume of 50 μl. The cycling conditions were: 95°C for 1 min, 46°C for 1 min, and 72°C for 2 min for 25 cycles and a final extension of 72°C for 7 min. The PCR products were separated on a 1.5% agarose gel, transferred to a nylon membrane and hybridized with a mixture of DIG-labelled M6PR and actin probes. The M6PR and actin PCR products were both labelled by random-primed incorporation of digoxigenin-labelled deoxyuridine triphosphate (DIG DNA labelling kit, Roche, Meylan, France) and detected with the DIG luminescent detection kit (Roche).

Expression of M6PR cDNA in E. coli and enzyme purification
An NcoI restriction site was inserted at the first ATG codon of the cDNA using PCR with the following oligo-nucleotide (CGGCCATATGGTATTACACTC; CC are the substituted nucleotides). PCR DNA was cloned in pBluescript vector, giving pNcoIM6PR. It was partially digested with NcoI and BamHI restriction enzymes because an inner NcoI site was present in M6PR cDNA, giving after cleavage, two products (700 and 400 bp). After gel electrophoresis, the 1.1 kb NcoI/BamHI product, corresponding to the full length M6PR cDNA, was excised from the gel and ligated to pEF-21d (+) expression vector digested by NcoI and BamHI. The ligation product was used to transform BLR E. coli strain (Novagen, Madison, WI, USA). The resulting clone was named BLR/pEF21d (+)+M6PR. A control clone corresponding to BLR transformed with the plasmid without insert (BLR/pEF21d (+)) was also obtained. BLR/pEF21d (+)+M6PR and the control BLR/pEF21d (+) were assayed for M6PR activity. Duplicate 100 ml cultures were grown at 37°C in LB + ampicillin (50 μg ml⁻¹). Once cultures reached 0.6 A600, expression was induced by addition of IPTG to a final concentration of 0.4 mM and the cultures were collected at 1 A600. Non-induced cultures were maintained at 37°C and harvested at 1 A600. After centrifugation (3200 g, 4°C, 5 min), pellets were resuspended in 10 ml of Tris-HCl buffer A (100 mM, pH 7.0) containing 5 mM EDTA, 2 mM DTT, 15 mM 2-mercaptoethanol and 6 M urea. The cell lysates were centrifuged at 15000 g for 5 min at 4°C and supernatants were dialysed twice against the buffer A minus urea. After concentration with 10 kDa molecular mass cut-off Vivaspin concentrators (Sartorius, Goettingen, Germany), the dialysates were used for enzyme purification on a Reactive Yellow 86-linked Sephadex affinity gel (Robert et al. 1999a). The samples were submitted to activity measurement and SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 according to BioRad’s instructions (Heracles, CA, USA).

M6PR extraction and protein content measurements
Pre-conditioned and germinated seeds (0.15-0.3 g FW) were homogenized using a potter-Elvihjem (Fischer Scientific, Illkirch, France) whereas the other samples (0.2-1 g FW) were homogenized with a Waring blender (Ultra-Turrax T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) in 5 volumes of Tris-HCl buffer (100 mM, pH 7.5) containing 15 mM 2-mercaptoethanol, 5 mM EDTA and 50% polyvinylpyrrolidone (PVPP, g/g FW). Homogenates were squeezed through gauze then centrifuged at 12 000 g for 15 min at 4°C. Supernatant were desalted using PD-10 columns (Sigma, St. Quentin Fallavier, France) pre-equilibrated with the extraction medium minus the PVPP. An aliquot of each desalted crude extract was taken for protein determination (Bradford 1976).

Enzyme assays
Assays were performed as previously described by Robert et al. (1999a). The barium salt of mannose 6-P (M6P
SIGMA) was converted to K salt. Optimal M6PR activity was spectrophotometrically (340 nm) determined at 40°C in a reaction mixture (1 ml total volume) containing 31 mM Tris-HCl pH 7.0, 50 mM M6PR, 0.3 mM NADPH and 100-300 µl of desalted crude extract.

Soluble carbohydrate analysis

Analysis was carried out as described by Fer et al. (1993). Soluble carbohydrates were extracted from the harvested samples (0.2-1 g FW) in 80% ethanol, and separated from cationic and anionic compounds using ion exchange chromatography. Mannitol, sucrose, glucose and fructose (hexoses) were quantified using the KONTRON HPLC system (model 400) (Bio-Tek instruments, Vermont, USA) equipped with a guard column (SUPELCOSIL™Ca, 5 cm × 4.6 mm, SUPELCO Inc., St. Quentin Fallavier, France), a SUPELCOSIL™Ca carbohydrate HPLC column (30 cm × 7.8 mm, SUPELCO Inc.) kept at 80°C and a differential refractometer (WATERS 410, St. Quentin en Yvelines, France). The isocratic system was operated at 0.8 ml min⁻¹ using degassed ultrapure water as the mobile phase.

Results

Isolation of a O. ramosa M6PR cDNA

Using 3' and 5' RACE strategies, a 1120-bp length cDNA was isolated. This cDNA contained a 933-bp coding sequence sharing 72% identity with the Apium graveolens M6PR cDNA and 77% with the two putative M6PR sequences from Arabidopsis thaliana. The deduced O. ramosa M6PR protein was 310 amino acid long and shared significant identity with the characterized A. graveolens M6PR protein (71%) and those of Arabidopsis (84 and 83%) (Fig. 1). This protein also exhibited a 68% identity with the NADP-dependent D-sorbitol 6-phosphate dehydrogenase (S6PDH), also called aldose 6-phosphate reductase from Malus domestica, a key enzyme in sorbitol biosynthesis (Kanayama et al. 1992). The absence of a serine residue at position 2 in O. ramosa, and in other plant M6PR proteins, indicated that the sequence determined in this study should encode for the M6PR protein.

Clone confirmation: M6PR expression in E. coli

Expression experiments in E. coli led to the production of a major 37 kDa polypeptide only with BLR/pET-21d (+)-M6PR after IPTG induction (Fig 2A). The corresponding enzyme crude extract exhibited a M6PR specific activity of 8.22 mMg⁻¹ proteins. E. coli M6PR purification resulted in a 40% yield and 24-fold purification factor. It should be noted that no S6PDH activity was detected in the purified protein fraction. The observed 37 kDa polypeptide corresponds to the lower band of the O. ramosa purified protein fraction (Fig 2B).

Southern blot analysis

Estimation of M6PR copy number in O. ramosa was carried out by gel blot analysis of total genomic DNA. Fig.
Fig. 2. Expression of M6PR in E. coli and enzyme purification. (A) SDS-PAGE of E. coli crude extracts before and after IPTG induction. Lanes 1 and 2, 80 μg proteins of crude extracts from non-induced BLR/pET-21d(-) and BLR/pET-21d(-) M6PR clones, respectively. Lanes 3 and 4, 80 μg proteins of crude extracts from IPTG induced cultures. (B) SDS-PAGE of purified M6PR expressed in E. coli. Lane 1, 5 μg of E. coli purified M6PR, lane 2, 3 μg of O. ramosa native M6PR. M6PR was purified as described by Robert et al. (1999a). The extreme right lanes contain molecular weight markers in kDa.

Fig. 3. Southern analysis of M6PR gene in O. ramosa. Total genomic DNA was digested with EcoRI, HindIII and BamHI as noted above lanes. The migration positions of the molecular weight standards are indicated on the right (kb).

2B shows the hybridization pattern obtained by using a probe synthesized from a 1.15-kb fragment of M6PR cDNA. This cDNA clone contained an EcoRI site and no HindIII and BamHI sites. One or two bands per lane were observed and these results are consistent with the existence of only one M6PR gene in O. ramosa. The

![RT-PCR detection of M6PR expression in O. ramosa growing on tomato. RT, without reverse transcription step; PS, pre-conditioned seeds; GS, germinated seeds; Tub., tubercle; Sh., shoot.](image)

BamHI pattern could be explained by the occurrence of a restriction site in an intron sequence.

**RT-PCR analysis**

Amplification of an actin gene product was used as a control of the quality of the extracted RNA and of the RT-PCR reactions. The RT-PCR reactions, using actin specific primers, gave the predicted single product of 397 bp. Likewise, M6PR-specific primers gave the predicted single product of 187 bp (Fig. 4). No amplification was observed in experiments without the reverse transcription step (RT).

M6PR gene was definitely expressed and remained almost stable throughout the development of O. ramosa, even before attachment to the host root, i.e. in the pre-conditioned and germinated seeds. Indeed, seed germination by GR24 did not result in an induction of M6PR gene expression. Likewise, this gene exhibited an identical expression pattern in tubercle and shoot of subterranean and emerged Orobanche.

**Changes in total protein concentration and M6PR activity**

Pre-conditioned seeds contained a relatively high concentration of proteins that decreased greatly during the germination and the procambium growth (Fig. 5). The difference is mainly due to water imbibition and absorption, which occur during procambium growth and ex-

**Table 2. Changes in M6PR specific activity in O. ramosa growing on tomato**

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Specific activity (mmol mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-conditioned seeds</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>Germinated seeds</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>Young fixations</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>1.77 ± 0.10</td>
</tr>
<tr>
<td>Stage III</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td>Stage IV</td>
<td>1.16 ± 0.06</td>
</tr>
<tr>
<td>Underground Orobanche (Stage V)</td>
<td></td>
</tr>
<tr>
<td>Tubercle</td>
<td>2.00 ± 0.08</td>
</tr>
<tr>
<td>Shoot</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>Emerged Orobanche (Stage VI)</td>
<td></td>
</tr>
<tr>
<td>Tubercle</td>
<td>1.80 ± 0.03</td>
</tr>
<tr>
<td>Flowering shoot</td>
<td>1.38 ± 0.03</td>
</tr>
</tbody>
</table>

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plains also the 5-fold higher specific activity, measured in the germinated seeds (Table 2).

After attachment to tomato root, total protein concentration and M6PR activity increased 2-fold between stage II and stage III, corresponding to the tubercle enlargement and the crown root formation (Fig. 5). In later developmental stages, protein concentration and M6PR activity did not change. M6PR activity, expressed as nkat g⁻¹ FW, was similar in underground and aerial organs, but the specific activity of the enzyme was almost 2-fold higher in the tubercle than in the shoot, especially in the subterranean broomrape (Table 2).

Changes in soluble sugar concentration

In pre-conditioned and germinated seeds, total soluble carbohydrate content was low but mannitol could be detected (Fig. 6). Hexoses (glucose and fructose in similar concentration, data not shown) were the major soluble sugars in all the developmental stages. Hexoses and mannitol concentrations progressively increased following the broomrape attachment to tomato roots, whereas sucrose concentration did not show significant changes. As soon as the underground shoot grows, mannitol and hexoses are preferentially accumulated in that organ. Then 80–90% of the mannitol, when expressed as mg per Orobanche (Table 1, Fig. 6), is confined in the shoot before and after emergence.

Discussion

O. ramosa contains a unique M6PR gene

In the biosphere, sugar alcohols account for as much as 30% of the global primary photosynthetic production. In higher plants, mannitol is by far the most abundant polyal as it has been reported in over 70 higher plant families, including autotrophic plants like Rubiaceae (coffee), Oleaceae (Olive), Apiaceae (celery) and harmful parasitic plants such as Orobanchaceae and Scrophulariaceae (Striga) (Stewart et al. 1984, Harloff and Wegmann 1987, Loeschter 1987). While M6PR is known as the key enzyme of mannitol production in higher plants (Loeschter et al. 1992), only the celery M6PR mRNA sequence was entirely determined before this study.

RACE and genomic Southern blot experiments strongly suggest the occurrence of only one sequence in O. ramosa, sharing identity with celery mRNA and putative Arabidopsis genes (Fig. 1). Expression in E. coli of the corresponding cDNA as well as S6PDH and M6PR activity measurements definitively confirm the identity of the M6PR sequence isolated in this study. Indeed the theoretical molecular weight value (35 kDa) of this protein is compatible with that of the corresponding E. coli produced protein (37 kDa) determined after separation on acrylamide gel (Fig. 2B). Moreover, M6PR is a member of the ald-o-keto reductase super family whose peptide sequences contain two conserved domains probably involved in NADPH binding (Bohren et al. 1989). In the O. ramosa predicted protein, these two domains are well conserved (Fig. 1).

It should also be noted that comparison between the M6PR nucleotide sequence and those available in the databank revealed that several ESTs of other plant origins (Arabidopsis, barley, cotton, Medicago trunculata, potato, rice, tomato, wheat) have significant similarity with M6PR sequences. Among these species, tomato,
M6PR activity before and after broomrape attachment

M6PR activity and mRNAs were detected as early as the pre-conditioning stage (Fig. 4 and Fig. 5). Low M6PR activity was also detected in the desalted crude extracts of the dry seeds (1 wt % g⁻¹ DW), 10-fold lower than in the pre-conditioned seeds. The increase in M6PR activity during pre-conditioning could be due to rehydration and/or protein synthesis. Following GR24-induced germination and proculume growth, no change in M6PR activity was observed (Fig. 5 and Table 2). Thus before broomrape attachment to the host root, M6PR activity has already increased significantly from the pre-conditioned seed. This is an efficient developmental strategy after attachment, rapidly allowing the conversion of the host-derived sucrose into mannitol.

The sink strength of the attached parasite increases throughout its development. This results in an increase of nutrient uptake from the host, likely used for protein synthesis which seems to be very active from the stage 3 (1.33 ± 0.4 and 3.33 ± 1.06 mg protein per Orobanche at stages II and III, respectively; Table 1 and Fig. 5). M6PR activity increases 2-fold between stage II and stage III (Fig. 5). Everard et al. (1997) reported a correlation between M6PR activity and photosynthetic capacity in celery leaf based on a developmental regulation of M6PR expression at the transcriptional level. A similar link between M6PR activity and rate of nutrient uptake from the host could occur after attachment of broomrape, but this remains to be demonstrated. However, as very short-term changes in carbon flux are required to maintain the balance in partitioning, the occurrence of a post-translational control of M6PR was also suggested (Everard et al. 1994, 1997). Several data support M6PR post-translational regulation through the redox activation of the four cysteine groups of the enzyme (Everard et al. 1997). Existence of six cysteine groups in the Orobanche enzyme (Fig. 1) and its inhibition by high DTT levels (data not shown) could suggest also a post-translational regulation of M6PR. In any case, enzyme activity is already maximal at stage III and remains at this high level in all the later developmental stages.

Therefore M6PR mRNA and protein (Fig. 4 and Fig. 5) are present in all of the broomrape organs and all of their developmental stages. M6PR expression is actually constitutive in O. ramosa as compared to celery in which M6PR activity and gene expression are limited to photosynthetic tissues (Evert et al. 1997).

Mannitol and hexoses are strongly accumulated in shoot

Identity of the major mannitol-producing organ should also be discussed. Conversion of the host-derived sucrose into mannitol in the tubercle is known but, taking into account the M6PR activity measured in the shoot (Fig. 5), mannitol synthesis could also occur in that organ, before and after emergence of the parasite. Indeed, the two other enzymes involved in the mannitol biosynthetic pathway, mannose-6-phosphate isomerase (EC 5.3.1.8) and mannitol-1-phosphate phosphatase (EC 3.1.3.22), have been also detected in young and old flowering shoots of O. ramosa (Harloff and Wegmann 1987, 1993). However, that the tubercle would be more efficient in mannitol synthesis is in agreement with its higher M6PR specific activity (Table 2).

Mannitol and hexoses were detected in broomrape before its attachment to the host root (Fig. 6). However, their accumulation is strongly enhanced following the haustorial connection to the host root. While considered to be synthesized in tubercle from the host-derived sucrose, mannitol and hexoses are mainly accumulated in shoots (Fig. 6), before and after emergence, as already reported by Harloff and Wegmann (1987). This supports the occurrence of a long-distance transport of these sugars in broomrape. However, nothing is known about the mechanism and the pathway involved in this transport (Nouraud et al. 2001). High mannitol and hexose concentrations are maintained in shoots during all the parasite development, supporting the essential role of these sugars in the high sink strength for water and sucrose of the parasite.

This study clearly shows that M6PR, the key enzyme of the biosynthesis of a major osmoticum in O. ramosa, is constitutively expressed in O. ramosa and this even before its attachment to the host. This supports the essential role of constitutive M6PR expression in the carbon metabolism specific to this parasitic way of life. Thus selective M6PR inhibitors might be interesting tools to control broomrape at any, even early, stages of development.

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