Gene for a protein capable of enhancing lateral root formation

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Abstract Analysis of genes preferentially expressed in hairy roots caused by infection with *Agrobacterium rhizogenes* has provided insights into the regulation of lateral root formation. A hairy root preferential cDNA, *HR7*, has been cloned from hairy roots of *Hyoscyamus niger*. *HR7* encodes a novel protein partially homologous to a metallocarboxypeptidase inhibitor and is expressed exclusively in the primordium and base of lateral roots in hairy roots. Overexpression of *HR7* in transgenic roots of *H. niger* dramatically enhances the frequency of lateral root formation. The results of this study indicate that expression of *HR7* plays a critical role in initiating lateral root formation.

Key words: Peptidase inhibitor; Hairy root; Lateral root formation; *Hyoscyamus niger*

1. Introduction

The formation of lateral roots is an important event in plant morphogenesis. Although lateral root formation has been correlated with auxin levels [1–5], the precise mechanisms involved in this process have not been clearly identified. Hairy roots induced by infection with *Agrobacterium rhizogenes* are thought to be lateral roots which grow abnormally following integration of the T-DNA of the Ri plasmid of *A. rhizogenes* into the plant genome. Hairy roots are characterized by rapid growth, formation of numerous lateral roots and a lack of geotropic response in phytohormone-free medium [6]. Shen et al. have demonstrated that hairy roots have a 100–1000 times greater sensitivity to auxin than uninfected roots [7]. It has recently been reported that the *rolB* gene on the T_L-DNA modifies auxin signal reception or its subsequent transduction pathway in plant cells [8,9]. This change likely affects auxin-regulated gene expression and could thus lead to hairy root formation.

Identification of genes expressed specifically in hairy roots may provide insights into the signal transduction events leading to lateral root formation in uninfected plants. In this study, we have cloned a gene, designated *HR7*, that is preferentially expressed in hairy roots of henbane, *Hyoscyamus niger*. We have generated transgenic roots of *H. niger* that overexpress *HR7*, resulting in an enhanced lateral root formation characteristic of the hairy root phenotype.

2. Materials and methods

2.1. Plant materials

H. niger was grown axenically in vitro on MS agar medium [10] supplemented with 3% sucrose at 25°C under a 16 h light/8 h dark cycle. Hairy roots were induced by infection with *A. rhizogenes* strain 15834 by the leaf disk method [11]. Hairy roots were cultured at 25°C on rotary shakers at 70 rpm in Gamborg B5 liquid medium at pH 5.7 [12] containing 2% sucrose, 10 μg/ml thiamine-HCl, 1.0 μg/ml nicotinic acid, 1.0 μg/ml pyridoxine-HCl and 100 μg/ml myo-inositol. For other experiments, *H. niger* was grown on soil at 25°C under a 16 h light/8 h dark cycle.

2.2. Northern blot analysis

Total RNA was prepared as described [13]. Denatured total RNA was electrophoresed in 1% agarose gels containing 2.2 M formaldehyde and transferred to a Hybond N+ nylon membrane (Amersham). Hybridization was performed according to standard procedures [14] and membranes were washed under high stringency conditions. Colorimetric detection using digoxygenin-labelled DNA probes was performed with a DIG DNA labelling and detection kit (Boehringer, Mannheim) according to the manufacturer's instructions.

2.3. Screening of a cDNA library

A cDNA library (5.5×10^5 plaque forming units) was prepared from vigorously growing hairy roots of *H. niger* infected with *A. rhizogenes* 15834 using a Uni-Zap XR vector (Stratagene) according to the manufacturer's instructions. Preliminary screening was carried out by hybridization with poly(A)$^+$ RNA fractions prepared from hairy roots, which were ^{32}P-labelled as first strand cDNA using a ZAP-cDNA Synthesis kit (Stratagene). Dot blot hybridization was used for a second round of screening on a Hybond N+ nylon membrane. Hybridization, washing and detection were carried out as described above. Clones hybridizing preferentially with the cDNA from hairy roots were excized and self-ligated in vitro to obtain pBluescript II SK+ phagemids, then sequenced using an Automatic Sequencer System 373A (Applied Biosystems) after processing with a Taq Dye Primer Cycle Sequencing kit (Applied Biosystems).

2.4. Isolation and analysis of a genomic clone for HR7

Genomic DNA of *H. niger* was partially digested with *Mbo*I at 37°C for 1 h. The *Mbo*I digest was ligated with Lambda FIX II vector arms (Stratagene) and subsequently packaged using a Gigapack II packaging extract (Stratagene). Approximately 9×10^5 plaques were subjected to screening by hybridization with the ^{32}P-labelled *HR7* cDNA under the conditions described for Northern blot analysis. A positive clone isolated after three rounds of screening was digested with *Hin*cII. The resulting 2.6 kbp fragment was subcloned into the *Eco*RV site of a pBluescript II SK+ phagemid vector, designated pBSHR7G1. Nucleotide sequences of clones were determined as described above.

2.5. Expression and detection of GUS in hairy roots

To create a *HR7* promoter-*uidA* gene fusion construct (P*HR7-uidA*), the genomic clone pBSHR7G1 was cleaved at position +7 of

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the *HR7* cDNA with *Sfa*NI and at 1378 bp upstream with *Sma*I. The resulting fragment was blunt-ended and inserted into the *Sma*I site of the binary vector pBI101 (Clontech). This plasmid, pBIHR7GUS, was directly transferred into *A. rhizogenes* strain 15834 by the freeze-thaw method [15]. Transformants of *A. rhizogenes* were selected on YEB agar plates [16] with 400 μg/ml kanamycin. Hairy roots carrying P*HR7-uidA* were generated from leaf disks of *H. niger* by incubation with *A. rhizogenes* strain 15834 harboring pBIHR7GUS [11]. The tips of a hairy root were excized and transformants were selected on Gamborg B5 agar medium containing 50 μg/ml kanamycin. After staining to detect GUS activity [17], roots were bleached for 1 h in $NaClO_4$ solution (1% active Cl) and examined on a Nikon DIA-PHOT-TMD dissecting microscope.

2.6. Transformation of roots to overexpress HR7

The entire open reading frame (ORF) of the *HR7* cDNA was introduced into a modified binary vector (pBI 121-*ΔuidA*) in which the *Sma*I-*Sac*I fragment of *uidA* of pBI121 (Clontech) was deleted. This resulting construct (pBI35SHR7) contained the CaMV 35S promoter-*HR7* ORF in the sense orientation harbored in the pBI121 backbone. As a control, the binary vector pBIN19 [18] was used. Each of the plasmids was directly introduced into *Agrobacterium tumefaciens* LBA4404 (Clontech) by the freeze-thaw method [15]. Sterile leaf disks of *H. niger* were incubated for 2 days with *A. tumefaciens* harboring either pBI35SHR7 or pBIN19 on MS agar medium [10] supplemented with 3% sucrose. Inoculated leaf disks were subsequently cultured on MS agar medium containing 2 μg/ml 1-naphtylacetic acid, 0.2 μg/ml 6-benzylaminopurine, 50 μg/ml kanamycin, 200 μg/ml carbenicillin and 3% sucrose. Calli generated from different parts of the leaf disks were transferred to Gamborg B5 agar medium [12] supplemented with 50 μg/ml kanamycin, 200 μg/ml carbenicillin and 2% sucrose to induce adventitious roots. The tips of these roots were isolated and selected on Gamborg B5 agar medium containing 50 μg/ml kanamycin. To determine the number of lateral roots, 2 cm long root tips were cultured on Gamborg B5 liquid medium without kanamycin at 25°C on rotary shakers at 70 rpm. After 14 days, the length of primary roots was measured and the number of lateral roots was counted.

2.7. Reverse-transcribed (RT)-PCR for transgene expression

Total cellular RNA prepared from *H. niger* roots was treated with RNase-free DNase I (Boehringer, Mannheim) at 37°C for 15 min. RT-PCR was performed with 5 μg of total RNA and an oligonucleotide (5′-GTTGTACTTAGCACGAAGAA-3′) complementary to +320–+339 of the *HR7* cDNA using reverse transcriptase (Rever-Script I, Wako, Japan) for cDNA synthesis. An aliquot from the reaction mixture was further subjected to PCR with *Taq* polymerase (NipponGene, Japan). An oligonucleotide (5′-CACGGGGGACTC-TAGAGGAT-3′) complementary to a region starting at the nucleotide '+2' close to the transcription initiation site of the CaMV 35S promoter and another oligonucleotide (5′-GTTGTACTTAGCAC-GAAGAA-3′) were used for PCR of exogenous *HR7* to amplify a

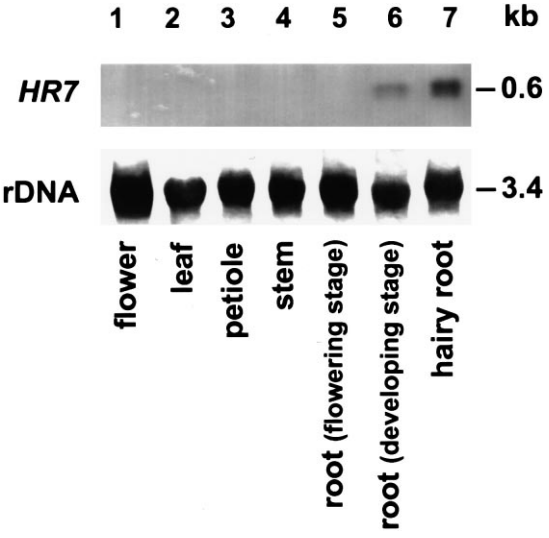

Fig. 1. Root-specific expression of *HR7*. Total RNA (10 μg each) from flowers, leaves, petioles, stems and roots of *H. niger* at flowering stage, roots at a developing stage 3 weeks after germination and from hairy roots, were electrophoresed on 1% agarose denaturing gels, transferred to a nylon membrane and hybridized with DIG-labelled *HR7* cDNA or cDNA encoding 25S rDNA.

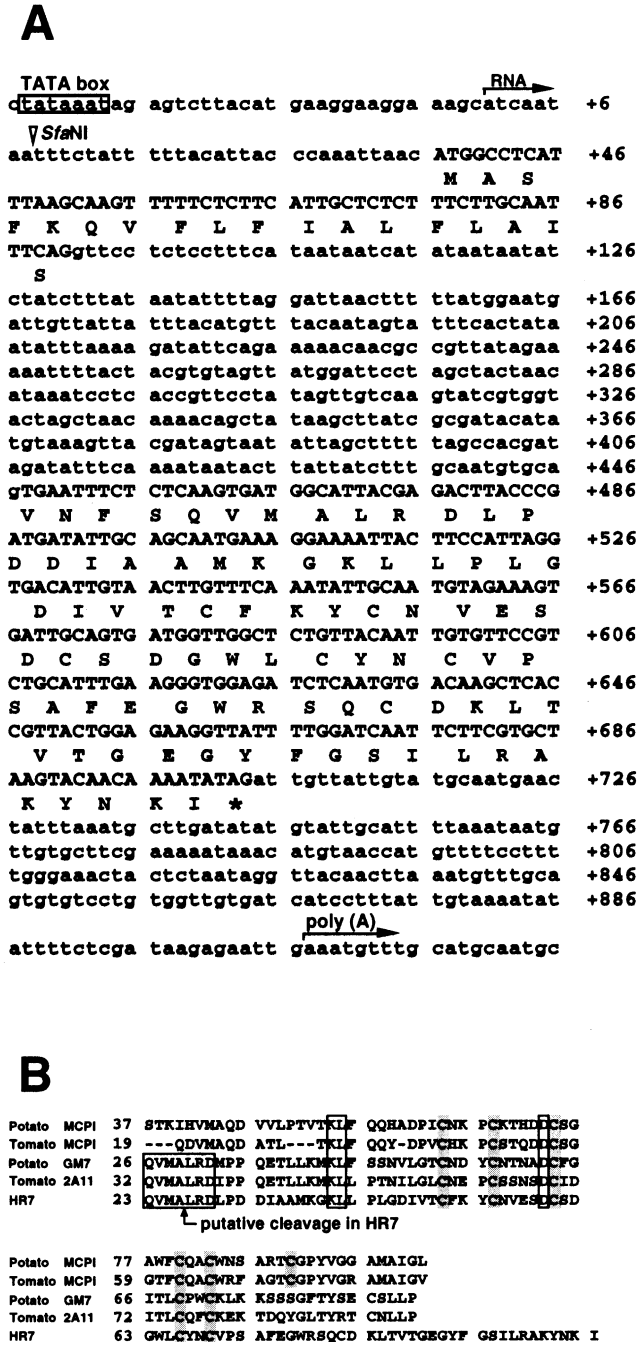

Fig. 2. Sequence analysis of the *HR7* gene. A: Nucleotide and deduced amino acid sequences of the *HR7* gene. The exons are shown in uppercase. A putative TATA box, a transcription initiation site determined by primer extension and a poly(A) addition site are indicated. B: Homology of *HR7* to other reported sequences. The shadowed boxes indicate the Cys residues which form disulfide bonds in MCPI. Conserved residues among five peptides and the region including a putative cleavage site in *HR7* are enclosed with squares. The arrow shows the putative cleavage site of *HR7* predicted by von Heijne's rule [20]. The DDBJ/GenBank/EMBL database accession number for the *HR7* sequence is AB016808.

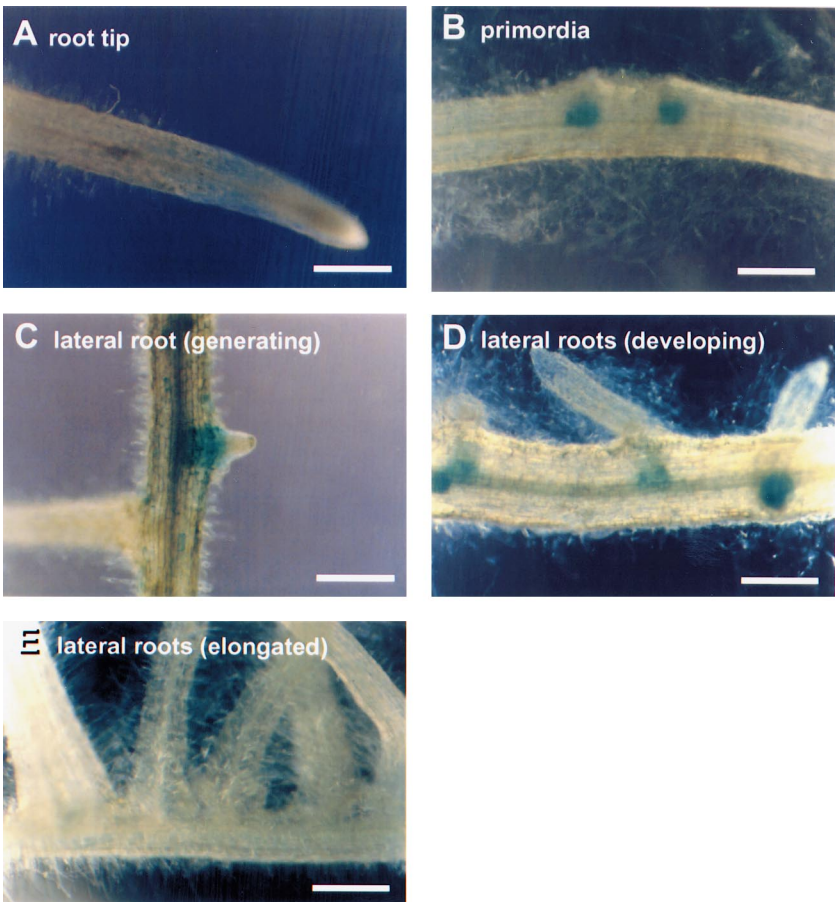

Fig. 3. Histochemical analysis of *HR7* expression in hairy roots of *H. niger*. Expression of P*HR7-uidA* was monitored by observing GUS activity. Root primordia (B) and the base of lateral roots (C, D) were stained blue. No GUS activity was detected in lateral roots longer than approximately 1 cm (E) or in root tips (A). All scale bars indicate 500 μm.

336 bp DNA fragment. For RT-PCR of endogenous *HR7*, an oligonucleotide (5′-TTTCTATTTTTACATTACCC-3′) complementary to a region beginning at the nucleotide '+9' near the transcription initiation site of endogenous *HR7* (see Fig. 2A) paired with another oligonucleotide (5′-GTTGTACTTAGCACGAAGAA-3′) was employed to amplify a 333 bp DNA fragment.

3. Results and discussion

3.1. Isolation of genes expressed preferentially in hairy roots of *H. niger*

288 clones that were strongly expressed in hairy roots were selected by hybridization from the hairy root cDNA library, followed by a second screening by dot blot hybridization. Seven of the 45 individual clones obtained by the second screening exhibited strong hybridization signals with RNA from hairy roots by Northern blot analysis. Expression levels of the transcripts for these seven cDNA clones (designated *HR1–HR7*) were determined by Northern blot analysis in leaves, stems and roots of *H. niger* at flowering, as well as in hairy roots. Among the seven genes examined, the expression of *HR7* was the highest overall and the most localized in hairy roots. We have, therefore, analyzed the structure and expression of *HR7* in more detail. The levels of transcription of *HR7* in other organs were analyzed by hybridization (Fig. 1). The *HR7* transcript was detected in hairy roots but was expressed weakly in developing roots of 3 week old plants. No evidence for transcription of *HR7* was obtained from other organs.

3.2. Nucleotide sequence of the HR7 gene

Genomic DNA fragments for *HR7* cDNA were examined in total DNA of *H. niger* by Southern blot analysis. One copy of *HR7* appeared to be present in the haploid genome (data not shown). The genomic DNA fragment including *HR7* was selected from a genomic DNA library of *H. niger* and sequenced (Fig. 2A). Primer extension analysis revealed only one primer extension product, indicating that the transcription initiation site was an adenine residue 36 nucleotides upstream of the ATG translation initiation codon (data not shown). A putative TATA box sequence, TATAAAT, was identified 33 nucleotides upstream of the transcription initiation site. Sequence comparison of the genomic and cDNA clones revealed the presence of one 356 bp intron flanked with consensus eukaryotic intron donor and acceptor sites [19]. The ORF of *HR7* encoded a protein consisting of 103 amino acids with a calculated molecular weight of 11.5 kDa. The protein was enriched with Leu (10.7%). One-fourth region on the N-terminal side of the protein was hydrophobic. Following von Heijne's rule [20], 26 of the N-terminal amino acids of this region were predicted to be a signal sequence that would be cleaved out (Fig. 2B). Sequence homology comparison using BLAST [21] and FASTA [22] analysis indicated

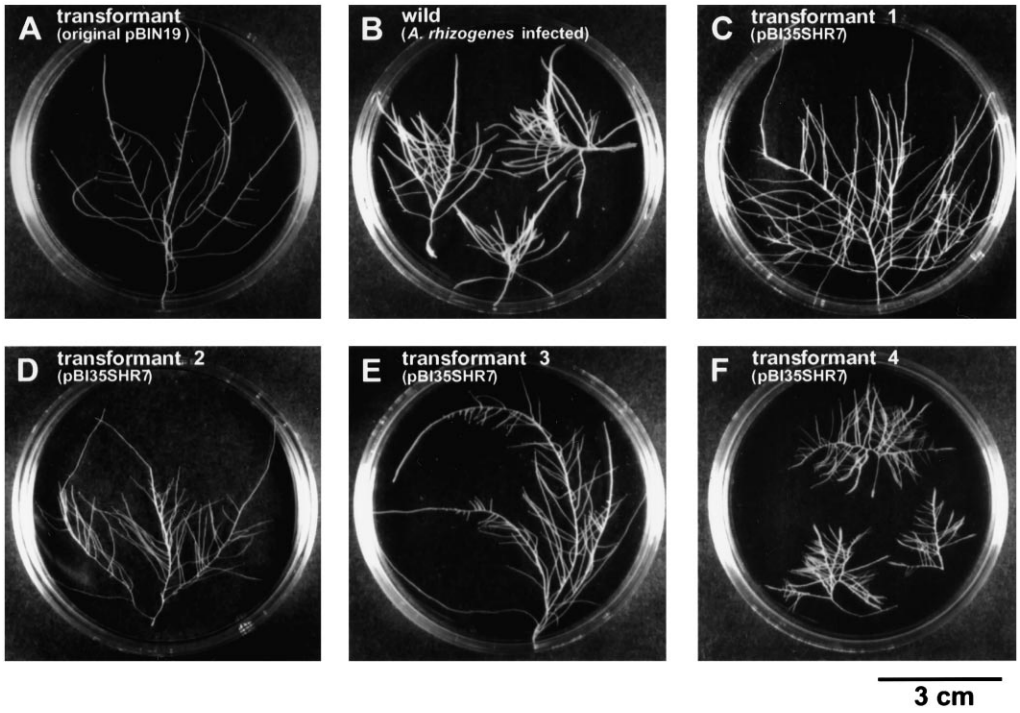

Fig. 4. Morphological changes induced by constitutive expression of *HR7* in transgenic roots of *H. niger*. Roots were transformed with the CaMV 35S promoter-*HR7* ORF construct (pBI35SHR7). Transgenic roots exhibited a greatly increased lateral branching (strong phenotype) (F). Transformed roots having an intermediate phenotype (C–E), control roots (A) and hairy roots (B) are shown for comparison.

limited similarities to the potato tuber-specific cDNA GM7 [23] (37% identity at the amino acid level and 60% at the nucleotide level) and the tomato fruit-specific cDNA 2A11 [24] (38% at the amino acid level and 61% at the nucleotide level). Both of these clones are partially homologous to a metallocarboxypeptidase inhibitor (MCPI) (Fig. 2B). It is noteworthy that the region including a putative cleavage site in HR7, and five Cys residues scattered from residue 50 to 69 in HR7, are well-conserved among HR7, potato GM7 and tomato 2A11. The six Cys residues of MCPI form three disulfide bonds that are thought to play a crucial role in the direct interaction between the inhibitor and carboxypeptidase [25].

3.3. *HR7 expressed at the primordium and base of lateral roots in hairy roots*

Hairy roots harboring a P-*HR7-uidA* (GUS) fusion gene were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). All transgenic lines had very similar staining patterns. GUS activity was detected in lateral root primordia (Fig. 3B) and at the base of lateral roots (Fig. 3C and D). GUS activity became weaker or disappeared in lateral roots longer than approximately 1 cm (Fig. 3E). In other parts of hairy roots, such as root tips or vascular systems, no GUS staining was observed (Fig. 3A). These data indicate that *HR7* is expressed in lateral root primordia and in early stages of developing lateral roots in hairy roots of *H. niger*.

3.4. *Overexpression of HR7 resulting in lateral root formation*

Using *A. tumefaciens*, we introduced *HR7* under the control of the cauliflower mosaic virus (CaMV) 35S promoter for overexpression into *H. niger*. We have obtained 24 independent transgenic root lines resistant to the selectable marker kanamycin with the CaMV 35S promoter-*HR7* ORF construct and 18 lines with the binary vector pBIN19 as a control. Among 24 lines, nine exhibited a significantly increased frequency of lateral root formation compared with control lines. Two of the nine lines showed a greatly enhanced lateral branching with 11–18 lateral roots per cm (Fig. 4F). Lines

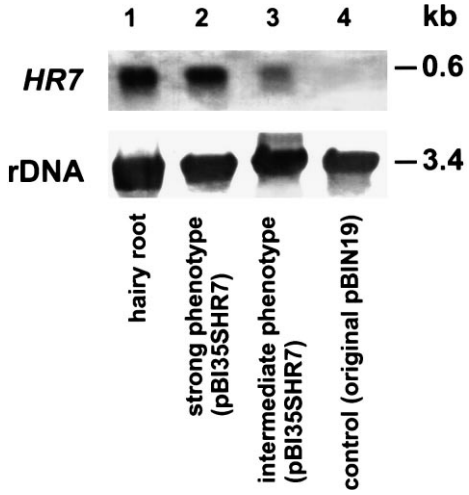

Fig. 5. Transcript levels in *H. niger* roots transformed with the CaMV 35S promoter-*HR7* ORF construct (pBI35SHR7). Total RNA (10 μg per lane) from hairy roots (lane 1), transgenic roots showing the strong phenotype (lane 2) and intermediate phenotype (lane 3) and control normal roots transformed with original pBIN19 (lane 4), all that were cultured in Gamborg B5 medium, were electrophoresed in a 1% denaturing agarose gel, transferred to a nylon membrane and hybridized with DIG-labelled *HR7* cDNA. These RNA samples were also hybridized with the DIG-labelled cDNA encoding rDNA.

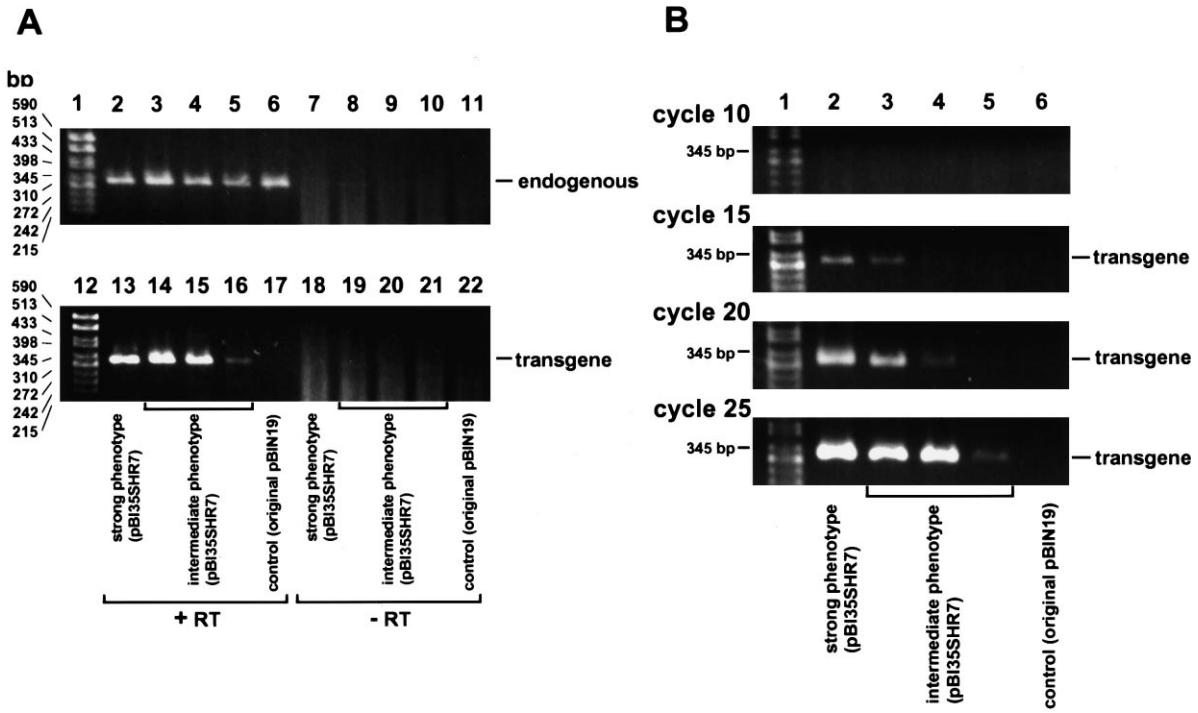

Fig. 6. (A) Expression of transgene *HR7* in *H. niger* roots. Total RNA from *H. niger* roots transformed with the CaMV 35S promoter-*HR7* ORF construct (pBI35SHR7) (lane 2–5, 7–10, 13–16 and 18–21) and transformed with original pBIN19 (lane 6, 11, 17 and 22) were treated with RNase-free DNase I and further subjected to RT-PCR (lane 2–6 and 13–17) after reaction with reverse transcriptase (+RT) or PCR (lane 7–11 and 18–22) without reverse transcriptase reaction (−RT). PCR products from transcripts for endogenous *HR7* are shown in the upper panel and those for exogenous *HR7* are presented in the lower panel. Reaction mixtures were electrophoresed in a 3% agarose gel (Agarose X, NipponGene) and stained with ethidium bromide. (B) Levels of transcripts for transgene *HR7* in *H. niger* roots transformed with pBI35SHR7. RT-PCR products of transcripts for exogenous *HR7* that were taken from the reaction mixtures after indicated PCR cycles were electrophoresed in a 3% agarose gel (Agarose X, NipponGene) and stained with ethidium bromide.

with an intermediate degree of blanching (Fig. 4C–E) had 5.7 ± 1.1 lateral roots per cm (mean ± S.D.). Control lines (Fig. 4A) had 2.0 ± 1.9 lateral roots per cm (mean ± S.D).

Northern blot analysis was used to examine the relationship between the phenotype and the expression of *HR7* (Fig. 5). Roots with the strong phenotype exhibited relatively high levels of *HR7* transcripts, almost equaling levels in hairy roots. In roots with the intermediate phenotype, the levels of *HR7* transcripts decreased. Roots without an increased lateral blanching did not exhibit the increased levels of *HR7* transcripts.

Transcripts for transgenic and endogenous *HR7* were detected distinguishably by RT-PCR with RNA from roots and primers specific to their 5′-untranslated regions: the exogenous species possessed the 5′-untranslated region derived from the CaMV 35S gene and the endogenous species had the same 5′-untranslated region as that of cDNA for *HR7*. No PCR products were confirmed to be amplified from contaminating DNA with or without reaction using reverse transcriptase (Fig. 6A). The RT-PCR was as sensitive as to amplify endogenous transcripts from all samples to make strong bands on an ethidium bromide-stained agarose gel (lanes 2′6, Fig. 6A). Northern blot analysis indicated that the endogenous transcripts were undetectable in control roots (Fig. 5), suggesting that the contents of endogenous ones are less than 1/100 of the transgene transcripts. Higher levels of exogenous *HR7* transcripts were detected only in roots transgenic with the CaMV 35S promoter-*HR7* ORF that showed enhanced lateral branching, medium levels of the transcripts were found in the transgenic roots with an intermediate degree of blanching and no exogenous *HR7* transcripts were amplified from roots transformed with the binary vector without *HR7* insert (Fig. 6B). Therefore, we may conclude that the frequency of lateral root formation in the transgenic roots depends on the level of *HR7* expression.

3.5. Perspective

The HR7 protein shares conserved characteristic sequences with the metallocarboxypeptidase inhibitor (Fig. 2). Therefore, we suggest that HR7 has a domain that interacts with another protein, possibly functioning as a peptidase inhibitor or acting to transduce signals by protein-protein interaction. It was recently noted that activation and inhibition of proteolysis are involved in a signal transduction cascade leading to cellular functions [26]. A signal protein has been reported to trigger the proteolytic activation of a developmental transcription factor in *Bacillus subtilis* [27]. In contrast, we suggest that HR7 inhibits the proteolysis of a protein involved in lateral root formation, resulting in the development of lateral roots.

We have observed that expression of *HR7* in normal roots is induced by auxin (data not shown). Previous reports that have demonstrated a correlation between lateral root formation and auxin [1–5] support the hypothesis that *HR7* expression is controlled by auxin signaling. Although no known auxin responsive elements have been found in the *HR7* promoter, our results may reasonably be explained if the expression of *HR7* is regulated by factors which are themselves

directly controlled by auxin signaling. Further analysis of HR7 may lead to a more complete understanding of the events regulating lateral root formation.

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