Gene for a protein capable of enhancing lateral root formation

Yoji Mikami¹, Goh Horiike²,a, Masanori Kuroyanagi¹,a,b,¹, Hiroshi Noguchi¹,a, Masanori Shimizu¹, Yasuo Niwa², Hirokazu Kobayashi³,c,¹

¹Laboratory of Pharmacognosy, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan
²School of Biosources, Hiroshima Prefectural University, 562 Nanatsuka, Shobara-shi, Hiroshima 727-0023, Japan
³Laboratory of Plant Cell Technology, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

Received 11 November 1998; received in revised form 9 March 1999

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 metallo-carboxypeptidase 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. © 1999 Federation of European Biochemical Societies.

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 Tt-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 over-express 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 niconicin 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 di-oxygenyl 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 (5.5×10⁶ plaque forming units) was prepared from vigorously growing hairy roots of H. niger infected with A. rhizogenes strain 15834 using an 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 32P-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 excised and self-ligated in vitro to obtain pBluescript II SK⁺ phagemid vectors, 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 MboI at 37°C for 1 h. The MboI digest was ligated with Lambda FIX II vector arms (Stratagene) and subsequently packaged using a Gigapack II packaging extract (Stratagene). Approximately 9×10⁵ plaques were subjected to screening by hybridization with the 32P-labelled HR7 cDNA under the conditions described for Northern blot analysis. A positive clone isolated after three rounds of screening was digested with HindIII. The resulting 2.6 kbp fragment was subcloned into the EcoRV 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 (PHR7::uidA), the genomic clone pBSHR7G1 was cleaved at position +7 of

0014-5793/99/$20.00 © 1999 Federation of European Biochemical Societies. All rights reserved.
PHI: S0014-5793(99)00489-5
the HR7 cDNA with SfalNI and at 1378 bp upstream with SmaI. The resulting fragment was blunt-ended and inserted into the SmaI 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<sub>HR7</sub>-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 NaCIO₄ 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 (pBI121-v<sub>uidA</sub>) in which the SmaI-SacI fragment of <i>uidA</i> 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 <i>Agrobacterium tumefaciens</i> LBA4404 (Clontech) by the freeze-thaw method [15]. Sterile leaf disks of <i>H. niger</i> were incubated for 2 days with <i>A. tumefaciens</i> harboring either pBI35SHR7 or pBIN19 on MS agar medium [10] supplemented with 3% sucrose. 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 <i>H. niger</i> 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 (ReverseScript 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'-CACGGGGACCTC-TAGAGGT-3') complementary to a region starting at the nucleotide +2 to the transcription initiation site of the CaMV 35S promoter and another oligonucleotide (5'-GTTGACTTACGAC-GAGAA-3') were used for PCR of exogenous HR7 to amplify a 1.5 kb fragment.
336 bp DNA fragment. For RT-PCR of endogenous HR7, an oligo-
nucleotide (5'-TTTCTATTTTTACATTACCC-3') complementary to
a region beginning at the nucleotide '+9' near the transcription ini-
tiation site of endogenous HR7 (see Fig. 2A) paired with another
oligonucleotide (5'-GTTGTACTTAGCACGAAGAA-3') was em-
ployed 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 expres-
sion 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 se-
quenced (Fig. 2A). Primer extension analysis revealed only
one primer extension product, indicating that the transcrip-
tion initiation site was an adenine residue 36 nucleotides up-
stream of the ATG translation initiation codon (data not
shown). A putative TATA box sequence, TATAAAT, was
identified 33 nucleotides upstream of the transcription initia-
tion site. Sequence comparison of the genomic and cDNA
clones revealed the presence of one 356 bp intron £anked
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. Fol-
lowing 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 com-
parison using BLAST [21] and FASTA [22] analysis indicated

![Fig. 3. Histochemical analysis of HR7 expression in hairy roots of H. niger. Expression of PHR7-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.](image-url)
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 metallo-carboxypeptidase 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. 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.

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.
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 HR7 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.

Acknowledgements: The authors are grateful to the Ministry of Education, Science, Sports and Culture of Japan for a Grant-in-Aid for Exploratory Research (Number 08877317).

References