Enhanced Secretory Activity of Atropa belladonna Hairy Root Culture Over-expressing ADP-Ribosylation Factor Gene

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Monomeric GTP-binding proteins are involved in regulating numerous essential functions of eukaryotic cells, such as cell differentiation, intracellular vesicle transport, and cytoskeleton organization. Based on the amino acid homology and the deduced functions, the low molecular weight GTP-binding proteins are generally classified into several families. It has been well known that activation of these small GTP-binding proteins is strictly controlled by the two regulatory proteins, GDP/GTP exchanging factor (GEF) and the GTPase-activating protein (GAP). GEP interacts with inactive small G-proteins to covert them to GTP-bound active form, while small G-protein-GTP complex is changed to the inactive form by the hydrolysis of GTP to GDP by the action of GTPase-activating protein (GAP). ARF is a member of the Arf/Sar family of small G-proteins, and several lines of evidence suggest that these proteins function in vesicular transport from endoplasmic reticulum to the plasma membrane via the Golgi apparatus. As well as other small G-proteins, the functions of this class of are regulated by ARF-specific GEF and GAP. It has been demonstrated that over-expression of ARF-specific GEF restores the inhibitor-induced defect of secretory activity. On the other hand, it has been recently found that over-expression of ARF-specific GAP appreciably inhibits the protein-trafficking processes from the endoplasmic reticulum to Golgi apparatus. Therefore, the effects of unphysiologically high concentrations of the two ARF-regulator proteins GEF and GAP have been examined in detail. In contrast, however, only very limited information is available on the biochemical properties of the transformed cells over-expressing ARF protein itself.

We have reported previously that genes encoding ARF proteins of carrot are organized as a small or multi-gene family in the genome, and arf-001 (GeneBank accession no. AY874441) has been isolated as a carrot ARF gene. The primary amino acid sequence of arf-001 appears to show significant homology to ARF proteins from various biological sources. In order to understand the physiological functions and effects of over-accumulation of the products of ARF genes in higher plant cells, in the present experiments, we attempted to prepare the transformed cells of Atropa belladonna over-expressing arf-001. The newly developed vectors, pBCR82 and pBCR90, have been employed for over-expression of arf-001 and as the empty vector control, respectively, and the transformed cells were obtained as hairy root tissues by co-expression of rol cluster in these vectors.

Materials and Methods

Materials

Seeds of A. belladonna L. were surface-sterilized in 70% (v/v) ethanol and 2% (v/v) sodium hypochlorite, successively, and, after several washings with autoclaved water, they were placed on Murashige and Skoog’s agar medium (M&S) at 26 °C under constant illumination for germination. The sterilized seeds were then incubated at 26 °C under constant illumination for germination, and the leaf segments prepared from the seedlings were used in the transformation experiments.

Transformation of Belladonna

The coding region of carrot arf-001 was subcloned into Gateway cassette downstream of cauliflower mosaic virus (CMV) 35S promoter in pBCR82 by the action of LR clonase (Invitrogen). Agrobacterium tumefaciens 4404 (Invitrogen) was transformed with this constructed vector, pBCR82-arf-001, and was cultivated in LB liquid medium at 26 °C for 2 d. Then, cut surfaces of the leaves of belladonna seedlings were immersed in the bacterial suspension for 2—3 s for infection. In a parallel experiment, the seedlings were also infected with A. tumefaciens in LB liquid medium at 26 °C for 2 d.
on a reciprocal shaker (Taitec, NR-150, 110 strokes/min) at 26 °C under darkness.

**Expression of arf-001 in Belladonna** The transformed hairy root tissues of *A. belladonna* (100 mg fresh weight) were harvested and homogenized in liquid N₂ with a mortar and pestle, and total RNA was prepared with an RNeasy Plant Mini Kit (Qiagen) according to the instruction manual. The RNA samples were immediately used for reverse-transcription (RT) and PCR amplification employing a OneStep RT-PCR Kit (Qiagen). The RT reaction was carried out with 1 μl RNA preparation, and PCR amplification of DNA fragments was performed with 5'-GGA CAA GAT CCG TTC TTT -3' as the forward primer and 5'-GCC TTA GCG ATA TTG TTG GAA AGC C-3' as the reverse primer, respectively.

**Immuno-Blot Analysis of ARF Protein** Immuno-blot analysis of ARF protein in belladonna root cultures was carried out essentially according to the method described previously. In brief, the homogenized hairy root tissues (1 g fresh weight) were fractionated by centrifugation, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were blotted onto a nitrocellulose membrane (0.4 μm) on a semi-dry transfer cell (Bio-Rad, Transblot SD), and, after blocking with 1% (w/v) dry fat milk, the membrane was incubated with anti-rabbit ARF (Santa Cruz Biotechnology Inc.) in 5 ml Tris-buffered saline at 4 °C overnight. The membrane was then incubated with Protein A conjugated with alkaline phosphatase (Bio-Rad) at 25 °C for 2 h, and was washed several times with the buffered saline. Primary antibody bound to ARF protein was visualized by color development reaction.

**Determination of Secretory Activity of Transformed Belladonna** Approximately 0.5—1.5 g of tissues of hairy roots transformed by pBCR82-arf-001 or pBCR90 were inoculated into 70 ml of Murashige and Skoog’s liquid medium. In some experiments, the transformed cultures were similarly inoculated in the presence of 10 μg/ml of monensin (MP Biochemicals). At regular intervals, the root tissues were weighed and 2 ml aliquots of the culture medium were removed for determination of protein concentrations.

**RESULTS AND DISCUSSION**

**Transformation of Belladonna Plants** Two newly developed vectors, pBCR82, an expression vector for activation tagging in transformed hairy roots, and pBCR90, an empty vector control of pBCR82 lacking in Gateway cassette, were employed for transformation of belladonna cells. Leaf segments of belladonna seedlings germinated under sterilized conditions were infected by *A. tumefaciens* which had been transformed with pBCR82 or pBCR90. The formation of hairy root tissues was observed after incubation of the infected tissues for 2—4 weeks (Fig. 1a). It appeared that the root-forming ratio of the leaf segments was dependent on the age of the belladonna seedlings. It is likely that the leaf tissues detached from 3-week-old seedlings after germination were most efficiently transformed with pBCR82-arf-001 (more than 60%), and the root-forming ratio of pBCR82-arf-001 and pBCR90 was almost similar. These results also imply that the novel rol cluster-containing expression vector pBCR82 would be applicable to a variety of medicinal and useful plants for over-expression of foreign genes as well as in several 'model' plants such as *Arabidopsis thaliana* and *Oryza sativa*. After cultivation of the root tissues on cefotaxime-containing agar medium for several passages, they were transferred into liquid medium without the antibiotic (Fig. 1b). The hairy root cultures with a relatively rapid growth rate were selected and two cell lines of the transformed belladonna were established for pBCR90 (designated as 90-1 and 90-2) and pBCR82-arf-001 (82-1, and 82-2), respectively, for further analyses.

**Expression of arf-001 Gene in Transformed Belladonna** Expression of carrot *arf-001* gene in the transformed belladonna was confirmed by RT-PCR analysis. As shown in Fig. 2, the primer pair designated for carrot *arf-001* amplified the DNA fragments of the expected size (330 mer) in pBCR82-arf-001-transformants, 82-1 and 82-2. In contrast, no band was detected when RT-PCR was carried out for the control belladonna cultures transformed with pBCR90 (90-1 and 90-2). Accumulation of *arf-001* product in pBCR82-arf-001-transformed belladonna was confirmed by immuno-blot analysis (Fig. 2). Obvious signals corresponding to the position of carrot ARF protein (approximately 22 kDa) were observed in the *arf-001*-transfornants, however, in contrast, no or only very scant signals, if any, were detected in the hairy roots obtained by the transformation with pBCR90. This result suggests that anti-ARF raised against rabbit ARF is capable of cross-reacting with carrot ARF protein probably because of the highly conservative structures of this class of proteins. In contrast, the concentration of constitutive ARF of control belladonna cells was very low or almost negligible under the present experimental conditions although the possibility cannot be excluded that the commercially
available ARF-antibody does not cross-react with the endogenous belladonna protein. This set of results strongly suggests that carrot arf-001 was specifically and appreciably expressed in belladonna hairy root cultures transformed with pBCR82-arf-001, and high concentrations of carrot ARF protein had accumulated in the root tissues.

Biochemical Properties of Transformed Belladonna

In the next experiments, the differences in biochemical properties between pBCR90- and pBCR82-arf-001-transformants were examined. As shown in Fig. 3a, the growth rates of the cultures transformed with pBCR90 varied to some extent, and the fresh weights of 90-1 and 90-2 reached approximately 2.5—4.5 g after 12 d incubation. On the other hand, the growth rates of 82-1 and 82-2 were somewhat slower than those of the controls, and the final fresh weights were about 1.0—2.3 g. Although the final weights of the hairy root cultures depended on the initial inoculum sizes, in repeated experiments, a 2.5—4 fold increase in the fresh weights after 12 d incubation was reproducibly observed in the control cultures, while in the arf-001-transformants, the increase was about 2—2.5 fold. In order to examine the possible change in the secretory activity by over-expression of arf-001, we determined the protein concentrations in the culture medium of the transformed belladonna root tissues (Fig. 3b). The amounts of the extracellular proteins of the root cultures, 82-1 and 82-2, appreciably increased after the transfer of the tissues into fresh medium, and it reached the maximum level after 6 d incubation (approximately 2—3.5 mg protein/g fresh weight). However, it decreased thereafter to the low level about 2.5—4.5 mg protein/g fresh weight. It has been demonstrated that the antibiotic at this concentration did not affect the viability of plant cells, and the destruction of cellular structures was also not observed. In the presence of monensin, the growth of the root cultures appeared to be reduced to some extent (Fig. 3a), and protein concentrations in the culture medium were markedly reduced (Fig. 3b). These results strongly suggest that the transient increase in the extracellular protein concentration specifically observed in the arf-001-transformants was due to the enhancement of the secretory activities but not the leakage of intracellular components caused by certain damage to membrane structures and/or cell death.

It has been demonstrated that accumulation of non-physiological concentrations of two ARF-regulator proteins, GEF and GAP, results in the enhancement and inhibition of the normal secretory processes, respectively. In the present study, it has been demonstrated that over-expression of arf-001 and accumulation of ARF protein in belladonna root cultures caused marked enhancement of the secretion activity via Golgi body. Therefore, over-accumulation of unphysiological concentrations of ARF protein alone should be capable of stimulating the secretion processes without artificial manipulation of the ARF-regulators in plant cells. Recently, many attempts have been made to employ higher plant cells as a chamber for mass production of useful biological polymers, such as antibodies, vaccines and cytokines. If translatable region(s) of appropriate gene(s) encoding ARF proteins (usually about 500—600 bp) can be co-expressed together with desired genes, this could activate the secretion of the products into culture medium. Although some targeting signal(s) might be required at the top or the end of the coding region of the desired genes, secretion of the products into the medium should be a great advantage for the recovery and purification of the proteins synthesized in higher plant cells. Further characterization of transformed root cultures over-expressing ARF gene is in progress in our laboratory.
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REFERENCES