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RESEARCH PAPER

Agrobacterium rhizogenes-mediated Transformation and Regeneration of the *Apocynum venetum*

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Abstract: A system for the *Agrobacterium rhizogenes*-mediated transformation and plant regeneration of *A. venetum* has been developed. The highest transformation frequency was 100%, achieved using strain LBA9402 with root explants. The highest density of hairy roots reached 22 when root explants transformed by R1000 cultured in the dark. Adventitious shoots were obtained from profusely branched, fast-growing (type PBF) hairy roots, and the adventitious shoot induction frequency was 20%. Regenerated shoots rooted easily on hormone-free 1/2 MS solid medium in 2 weeks. Approximately 1/3 regenerated plants derived from hairy roots exhibited prolific roots with shortened internodes, whereas other regenerated plants showed another phenotype: long internodes, strong stems, and fleshy blades. However, all regenerated plants displayed a relatively fast development procedure and stronger than the aseptic seedlings. Polymerase chain reaction (PCR) analyses confirmed that the hairy root lines and regenerated plants were induced by *A. rhizogenes*.

Keywords: *Apocynum venetum*, *Agrobacterium rhizogenes*, hairy roots, plant regeneration, transformation

Introduction

Apocynum venetum L belongs to the family *Apocynaceae* and is widely distributed throughout central and northwestern China. The plant is of interest owing to its diversified medicinal values, such as treatments of heart disease, hypertension, nephritis, and neurasthenia[1]. Meanwhile, *A. venetum* is an excellent kind of raw material for the textile industry[2]. It contains cardiac glycoside and several kinds of amino-acids with health-care effects, which do not exist in other fiber plants. However, cultivated *A. venetum* has too many branches to harvest high-quality fiber. Therefore, we plan to apply transgenic technology to realize genetic control of shoot branching and improve the fiber qualities of *A. venetum*, and breed a new cultivar of *A. venetum* that possesses desirable characteristics for industry production.

As already well known, the application of genetic modification technology in plants is closely related to an efficiently transformable genetic system and regeneration protocol. However, an efficient regeneration is still a bottleneck in *A. venetum*. Only one preliminary study resulting in a few regenerated plantlets from calli of *A. venetum* was reported before[3], however, the experimental results were not reappeared in our lab. We found that *A. venetum* is a kind of recalcitrant plant, which is less sensitive to plant growth regulators and difficult to obtain somatic embryos and regenerated nonchimeric shoots through tissue culture.

Agrobacterium rhizogenes can transfer T-DNA from binary vectors, enabling production of transgenic plants containing foreign genes. Ri-mediated transformation has

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been used to produce transgenic plants in several species[4]. Regeneration of whole plants from hairy roots by adventitious shoots was also reported in several plant species[5].

Aimed at the establishment of stable transformation and plant regeneration system of *A. venetum*, we studied the induction of hairy roots through Ri-mediated transformation, production of regenerated plants from hairy roots of *A. venetum*, and the phenotypes of the regenerated plants. This is the first report on plant regeneration of *A. venetum* from Ri-mediated hairy roots.

1 Materials and methods

1.1 Plant material and bacterial strains

The seeds of *A. venetum* were germinated for 30 days on the MS solid medium; aseptic seedling of *A. venetum* was well developed with 3–4 pairs of leaf blade, and the seedling with the length of 3.0–4.0 cm was most suitable for transformation. Different explants of *A. venetum*, such as leaf blade, stem, and root, were inoculated with suspensions of three different *A. rhizogenes* strain (R1000, R1601, and LBA9402) cells.

1.2 Inoculation and hairy root culture

Leaf blades, stems, and roots were cut into about 0.5–1 cm in length, and were pre-cultured on MS solid medium for 2 days. After activated twice on the plate with solid yeast extract-peptone medium, single colonies of *A. rhizogenes* were propagated in liquid medium with yeast extract-peptone for 24 h at 28ºC, 200 rpm, and then suspensions of *A. rhizogenes* were propagated for 4 hours following the 1:50 inoculated proportion. Pre-cultured explants were immersed in suspensions of *A. rhizogenes* cells (OD_{600} = 0.5–1.0) for 30–60 min, and the explants infected by the bacteria were incubated in the dark for co-cultivation on MS solid medium. After 3 days, the bacteria were washed away from the explants with sterile water, and the explants were then transferred onto MS solid medium supplemented with 500 mg/L cefotaxime sodium (cef) for sterilizing the *A. rhizogenes*. In order to examine the effect of photoperiod on hairy root induction, root explants were cultured in the dark and under a 16 h/8 h (light/dark) photoperiod, respectively. 7 to 10 days after infection, hairy roots emerged from the infection site. After 3 subcultures, single transformed hairy root tips of 2–3 cm in length were excised and put in 40 mL hormone free 1/2 MS liquid medium, with one hairy root tip per flask. The hairy root cultures were maintained under a 16 h/8 h photoperiod at (25±1)ºC on a rotary shaker. The morphological characterization of root clones was recorded. Root clones were characterized into different morphological categories based on their growth habit (slow, fast, and moderately growing) and branching patterns (less or profusely branched and callusing types).

1.3 Plant regeneration from hairy roots

To induce adventitious shoots, hairy-root cultures were cut into 2-cm-long segments and cultured on hormone free 1/2 MS solid medium. Each treatment consisted of five explants per flask with 8 replicates. After 4–5 weeks, regenerated shoots formed from hairy roots. The adventitious shoot induction frequency was recorded. When 2–3 cm long, regenerated shoots were excised and transferred on hormone free 1/2 MS solid medium for root induction.

1.4 Evidence for transformation

Total genomic DNA was extracted from transformed root and regenerated plants using the CTAB DNA isolation method[6]. In order to prove the integration of TL-DNA and TR-DNA of *A. rhizogenes* in the transformed roots and regenerated plants, segments from both TL-DNA and TR-DNA regions were amplified using the gene-specific primers. The primers used for amplification of the *aux1* sequence (656 bp) from TR-DNA were: *aux1* Forward (5′-3′) CTCAAGAGC GCTACTCCTTCAAGTG; and *aux1* Reverse (5′-3′)TCT CCCGCTTTCCAGATATATTGAC. For amplifications of *rolB* and *rolC* sequences (862 bp and 574 bp) from TL-DNA, the primers respectively were: *rolB* Forward (5′-3′) CTTATGACAAACTCATAGATAAAGGTT and *rolB* Reverse (5′-3′) TCGTAACTATCCAACTCACATCAC; *rolC* Forward (5′-3′) GATATATGCCAAATTTACACTAG and *rolC* Reverse (5′-3′) GTTAACAAAGTAGGA AACAGG.

The amplification involved 35 cycles of PCR with 50 ng of DNA template. The cycling conditions consisted of a 1 min denaturation at 94ºC, a 30 s annealing at 55ºC followed by a 30 s extension at 72ºC, and a final extension at 72ºC for 10 min. PCR products were examined by electrophoresis on 1% agarose gels (*W*/*V*) in 1×TAE buffer and staining with ethidium bromide.

2 Results and discussion

2.1 Hairy root induction

Hairy root appeared on the cut surfaces of explants 7–10 days after infection with *A. rhizogenes*. After 30 days, the transformation frequencies were apparently varied. These *A. rhizogenes* strains were not equally efficient in hairy root induction. Both the transformation frequency and the density of hairy roots were calculated by the following formulas:

$$\text{Transformation frequency} = \frac{\text{Final number of explants forming roots}}{\text{Initial number of infected explants}} \times 100\% \quad (1)$$

$$\text{Density of hairy roots} = \frac{\text{Final number of hair roots}}{\text{Final number of explants forming roots}} \quad (2)$$