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研究报告

纤维植物罗布麻发根的诱导及植株再生

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摘 要: 利用 3 种发根农杆菌(LBA9402、R601、和 R1000)转化纤维植物罗布麻无菌种子苗的根茎叶不同外植体部位, 首次诱导其生成发根并实现了直接由发根途径的植株再生。罗布麻发根诱导与所用的发根农杆菌菌株, 外植体部位及光周期密切相关。发根农杆菌 LBA9402 感染罗布麻的根外植体, 实现了最高转化率达 100%。与 LBA9402 及 R601 相比,被发根农杆菌 R1000 感染的根外植体适合在黑暗环境下培养, 其诱导生成的发根密度可达平均每个外植体 22 条。在不加激素的 1/2 MS 培养基上, LBA9402 和 R601 诱导产生的发根可以诱导生成不定芽, 不定芽诱导率达 20%。不定芽切下后, 在不加激素的 1/2 MS 培养基上 2 周内可以诱导生根。通过聚合酶链式反应(PCR)对发根及再生植株进行了鉴定, 证明发根农杆菌的 T-DNA 插入了植物的基因组。为罗布麻的分子育种建立了稳定的转化及再生体系, 为下一步通过转入外源基因改善其农艺性状奠定了基础。

关键词: 罗布麻, 发根农杆菌, 发根, 植株再生, 转化

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 by 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 the hairy root lines and regenerated plants were induced by *A. rhizogenes*.

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

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Introduction

Apocynum venetum L is belonging to the family *Apocynaceae* and widely distributed throughout central and northwestern China. The plant is of interest due 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 many kinds of amino-acids with health-care effects which don't 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* which possesses desirable characteristics for industry production.

As is 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 work 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 plants, which were less sensitive to plants 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 been used to produce transgenic plants in many species[4]. Regeneration of whole plants from hairy roots by adventitious shoots was also reported in several plant species[5].

Aimed at 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 in this work. 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 28°C, 200 r/min, and then suspensions of *A. rhizogenes* were propagated for 4 hours followed 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)°C on a rotary shaker. Morphological characterization of root clones is 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. 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, primers respectively were: *rolB* Forward (5′-3′) CTTATGACAAACTCATAGATAAAGGTT and *rolB* Reverse (5′-3′) TCGTAACTATCCAACTCACATCAC; *rolC* Forward (5′-3′) GATATATGCCAAATTTACACT AG and *rolC* Reverse (5′-3′) GTTAACAAAGTAGGA AACAGG.

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

2 Results and discussion

2.1 Hairy root induction

Hairy root appeared on the cut surfaces of explants 7~10 days after infected 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 transformation frequency and density of hairy roots were calculated by the following formulas:

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

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

Root explants exhibited the highest transformation frequency of all three *A. rhizogenes* strains examined (Fig. 1), as compared with leaf blade and stem explants, this high transformation frequency of root explants, may be due to the fact that root explants are more competent for transformation than leaf blade or stem explants in *A. venetum*.

Photoperiod affected the transformation frequencies and density of hairy roots (Fig. 2). Although the transformation frequencies of all three strains were higher in the 16 h/8 h (light/dark) photoperiod (Fig. 2A), but the density of hairy roots was markedly different under two conditions (Fig. 2B). Thus the product of the transformation frequency and the density of hairy roots may be a more reliable parameter for evaluation of the transformation efficiency. To R1000, the density of hairy roots induced from the root explants in the dark was approximately three times of root explants cultured under 16 h/8 h (light/dark) photoperiod.

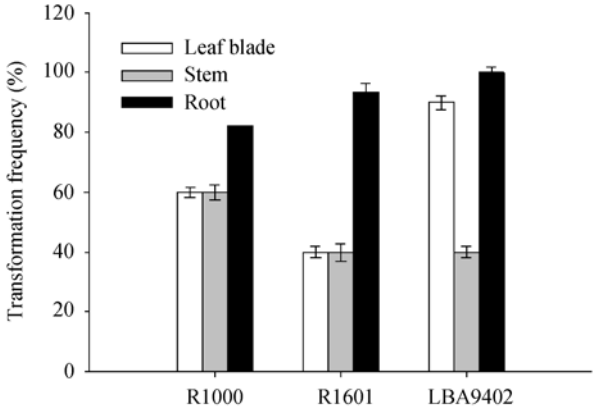

Fig. 1 Transformation frequencies of *A. venetum*
Different explants (leaf blades, stems and roots), and *A. rhizogenes* strains (R1000, R1601and LBA9402) were examined. The data were recorded after one month of culture. Data were means of four replicates, each with 50 explants. Bars represent S.E. of the means. Evaluated by t-test, values in the figure are significantly different ($P < 0.05$)

In contrast, the density of hairy roots induced by R1601 was considerably less in the dark. As for LBA19402, the effect was not so significant ($P>0.05$). Those results suggested that photoperiod had an important effect on the density of hairy roots induction from *A. venetum* using different strains. We inferred that the effect of photoperiod on hairy roots induction may depend on the characteristics of *A. rhizogenes* and the source of the explants.

Many parameters related to Ri-mediated transformation efficiency were studied in the past such as plant species, *A. rhizogenes* strains, and sources of explants. *A. rhizogenes* strains play an important role in the transformation process, as they are responsible for the efficiency of gene transfer and the virulence of *A. rhizogenes* strains vary widely among plant species[7].

The Growth and morphology characteristics of hairy roots are recorded. These transformed hairy root lines were characterized by frequently branching (Figs. 3A, 3B, 3C) and fast growth rates. There was enormous variability in the morphology and growth patterns among individual root clones, because each root clone arose from a separate transformation cell. Based on growth-pattern, type of branching and number of lateral hairy roots, the hairy root clones could be classified

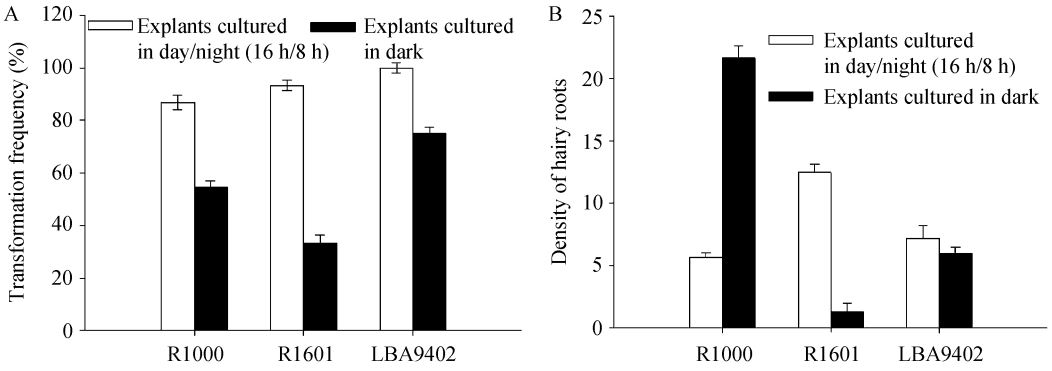

Fig. 2 Effect of photoperiod on hairy roots induction of different strains of *A. rhizogenes*

(A) transformation frequency under different photoperiod; (B) density of hairy roots under different photoperiod. The data were recorded after one month of culture. Data were means of four replicates, each with 50 root explants. Bars represent SE of the means

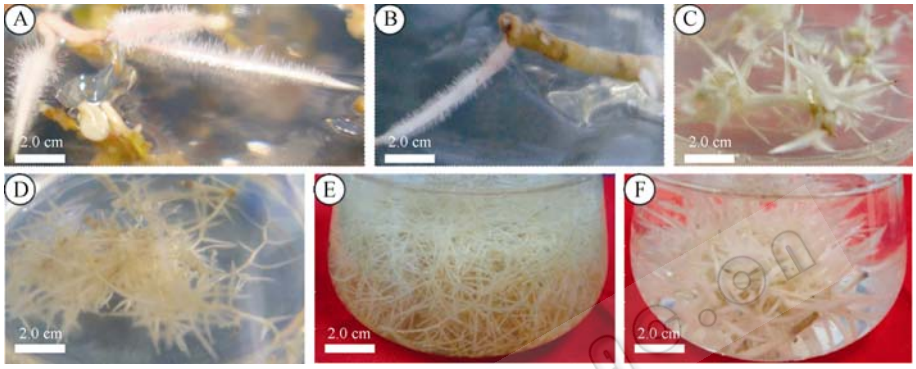

Fig. 3 Induction of hairy root from *A. venetum*

A~B: adventitious hairy roots formation from cut surface of leaf blade and root explants inoculated with *A. rhizogenes* R1601 for 20 days after inoculation; C: hairy roots with abundant branches; D~F: different morphological categories of hairy roots clones of *A. venetum* cultured in liquid 1/2 MS medium. D: less branched, and moderately growing hairy roots type (LBM); E: profusely branched and fast growing hairy roots type (PBF); F: callusing type and slow growing hairy roots type (CSG)

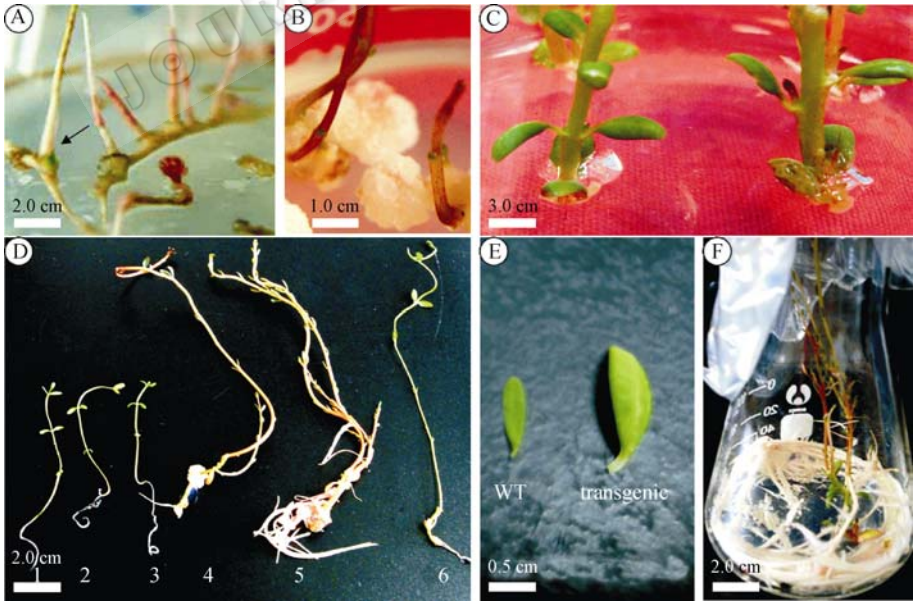

Fig. 4 Plant regenerated from hairy-root cultures of *A. venetum*

A: adventitious shoots formed directly from a hairy-root, an arrow indicates shoot primordium; B: adventitious shoot formation from calli derived from hairy roots cultured on the hormone free 1/2 MS solid medium, after 4 weeks of culture; C: the regenerated shoots were cut down from the hairy roots and induced for root; D: aseptic seedlings (1~3) and hairy roots derived plants (4~6); E: the leaf blade of the aseptic seedlings and the regenerated transgenic plant; F: the root mass of the regenerated plant

into three categories (Figs. 3D, 3E, 3F): (1) less-branched, moderately-growing type (LBM); (2) profusely branched, fast-growing type (PBF); (3) callusing and slow-growing type (CSG). We selected putative transformed root lines on the basis of active growth and formation of lateral roots, and these lines were confirmed by PCR analysis.

2.2 Plant regeneration from hairy roots

Regenerated shoots were formed from hairy root type PBF induced by R1601 and LBA9402, after 4~5 weeks cultured on hormone free 1/2 MS solid medium, under normal light at (25 ± 1)°C. The adventitious shoot induction frequency was 20%. However, hairy root type LBM, CSG and the hairy roots induced from R1000 were unable to produce regenerated shoots. We found that there were two ways for hairy roots regenerating shoots in *A. venetum*. Most shoots arose primarily from the branching sites of the hairy roots through direct organogenesis (Fig. 4A), but there were also some shoots formed from the calli of hairy roots (Fig. 4B).

Adventitious shoots induced from calli derived from hairy roots were obtained in *Crotalaria juncea*[8], *Panax ginseng*[9], *Catharanthus roseus*[10], and other plant species. And direct organogenesis shoots derived from hairy roots also achieved in *Gentiana cruciata*[11] and *Brassica oleracea*[12]. However, we found no significant differences in the shoots originating from the two ways and the regenerated plants from those shoots in *A. venetum*.

In our experiment, we have tried to use different plant growth regulators to improve the adventitious shoot induction frequency (data not show). However, we found that when added plant growth regulators, such as benzylaminopurine or kinetin, hairy roots tended to form callus and was not beneficial for the induction of regenerated shoots from direct organogenesis, and therefore adventitious shoot induction frequency was decreased. In many plant species, plants can be regenerated from hairy roots when they are cultured on medium with growth regulators, while in some plant species adventitious shoots are formed from hairy roots cultured on growth regulator-free medium. Besides species differences, it is known that the rol genes affect hormonal contents and response of transgenic plants, thus the variation in the regeneration ability could be caused by the expression levels of the different integrated rol genes and their interactions with complex pathways of the plant[5].

When 2~3 cm long, regenerated shoots were excised and transferred on hormone-free 1/2 MS solid medium for root induction (Fig. 4C). Excised adventitious shoots were rooted at a frequency of 100% within 2 weeks. Plantlets produced well developed root systems within 5 weeks in the rooting medium (Figs. 4D 4~6). Finally, we obtained 30 regenerated plants induced from the hairy roots in about 2 months.

Comparing with aseptic seedlings (Figs. 4D 1~3), we observed 3 representative phenotypes of regenerated plants in *A. venetum*: (1) long internodes, thick and red stem, and fleshy leaf blades (Fig. 4D 4); (2) short internodes, thick and red stem, and small leaf blades (Fig. 4D 5); and (3) long internodes, thick and green stem, and fleshy leaf blades (Fig. 4D 6).

Although approximately 1/3 regenerated plants shared reported characteristic phenotypes of plants derived from hairy roots in some plant species[13], including shortened internodes, and prolific root mass with extensive lateral branching (Figs. 4D 5 and Fig. 4F), we observed no plants with wrinkled leaves as reported in other plant species. Moreover, we noted that there were new phenotypes which have not been reported. Some regenerated plants were stronger, which was reflected by the thick stems (Figs. 4D 4~6) and fleshy leave blades (Fig. 4E), and all regenerated plants had a relatively fast development procedure than the aseptic seedlings, when the both were cultured for 1 month on the hormone free 1/2 MS solid medium, the height of regenerated plants (Figs. 4D 4~6) were nearly twice of the aseptic seedlings (Figs. 4D 1~3).

2.3 Confirmation of transformation by PCR analysis

Ri-mediated transformation was confirmed by PCR analysis. PCR analysis was performed on hairy root lines of HR1000, HR1601, and HR9402, and the plants derived from hairy roots of R1601 and LBA9402. The PCR analysis revealed the presence of the *rolB*, *rolC* and *aux1* genes in all three hairy root lines and the regenerated plants (Figs. 5A, 5B, 5C). These results confirmed independent insertion of TL and TR-DNA into plant genome[14]. It is well known that TR-DNA is not essential for hairy root phenotype, but that *aux1* gene from this segment provides an additional source of auxin to the transformed cells[15]. However, *rol* genes from TL-DNA play the major role in induction of hairy roots formation.

3 Conclusions

In conclusion, this study demonstrated that cultured hairy roots of *A. venetum* are capable of producing adventitious shoots that subsequently developed into whole plants. An efficient transformation and plant regeneration system were successfully established, which provides an excellent platform for gene

transformation in *A. venetum*. Further studies on introduction of foreign genes into plant genomes, alteration of the branching properties of *A. venetum* by gene manipulation, are now in progress.

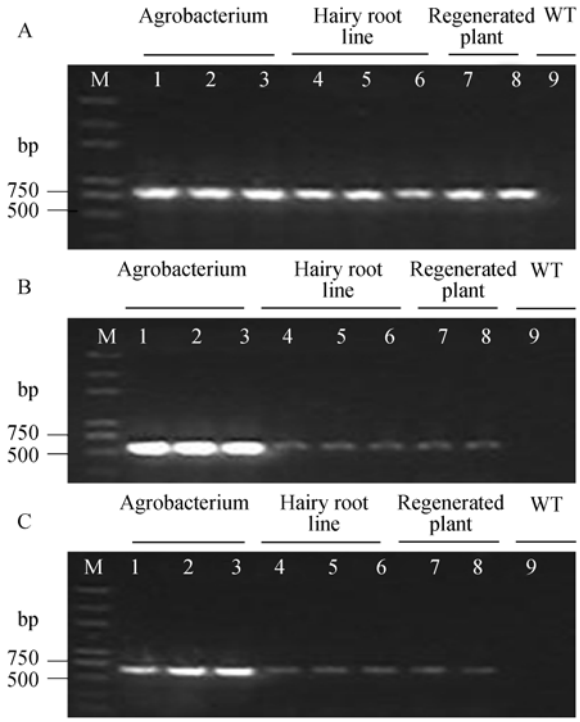

Fig. 5 PCR analysis of the *rolB*, *rolC* and *aux1* gene in hairy roots and regenerated plants

(A) *rolB* (860 bp); (B) *rolC* (574 bp); (C) *aux1* (656 bp) M: DNA marker; 1~3: *Agribacterium* strains of R1601, R1000, and LBA9402 as positive control; 4~6: hairy root lines of HR1000, HR1601, and HR9402; 7,8: plants derived from hairy roots of HR1601 and HR9402; 9: wild type plant as a negative control

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