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BRIEF COMMUNICATION

Indole-3-acetic acid accumulation during poplar rhizogenesis revealed by immunohistochemistry

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Abstract

Poplar hybrid 741 [*Populus alba* × (*P. davidiana* + *P. simonii*) × *P. tomentosa*] leaves were rooted within 8 d when cultured *in vitro* on 1/2 Murashige and Skoog (MS) medium. The spatial distribution of endogenous indole-3-acetic acid (IAA) in the rhizogenesis was investigated, using an immunohistochemical approach. In addition, the effect of 2,3,5-triiodobenzoic acid (TIBA) on IAA distribution was also analyzed. The results showed that a strong IAA signal was detected in the vascular bundles of the basal regions of the petioles 3 d after root induction. Furthermore, the signal in vascular bundles of the basal regions of the petioles was stronger than that of the middle regions of the petioles. Application of TIBA on lamina delayed both the accumulation of IAA in the vascular bundles and rhizogenesis. These data indicate that an endogenous IAA rise in vascular bundles is among the first signals leading to the rhizogenesis, and that it results from transportation of the hormone from the lamina of the leaf to the base of the petiole, rather than by *in situ* IAA generation.

Additional key words: adventitious root, immunohistochemical localization, auxin, cytokinin, *in vitro* culture, TIBA.

The relation between auxin and rhizogenesis has been studied for many years. Indole-3-acetic acid (IAA) was the first plant hormone used to stimulate the rhizogenesis of cuttings (Cooper 1935). There have been a number of recent studies addressing the role of auxin in the rhizogenesis (Ludwig-Muller *et al.* 2005, Ahmad *et al.* 2006, Gangopadhyay *et al.* 2010, Ma *et al.* 2010). These researches focused on the quantitative analysis of auxin using different analytical methods and on the observations of the biological characteristic of rhizogenesis after application of exogenous auxin. While researches provided important information on actions of the auxin, the auxin mechanism in the rhizogenesis has not been clearly established. Firstly, results obtained from these studies were more or less interfered by the absorption or transportation of applied auxin. Secondly, the concentration of auxin only in the target site, rather than in the whole tissue, could reflect its active level. Furthermore, little was known about the site of IAA production or its transport path.

With the application of immunology on the botany, it has become possible to detect *in situ* auxin in plant tissues. Immunohistochemical localization technique has been previously used in maize (Shi *et al.* 1993), *Arabidopsis* (Avsian-Kretchmer *et al.* 2002), the shoot apexes of strawberry (Hou and Huang 2005) and tobacco

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Abbreviations: 6-BA - 6-benzyl aminopurine; BSA - bovine serum albumin; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; EDC - 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; MS - Murashige and Skoog; TBS - Tris buffer solution; TIBA - 2,3,5-triiodobenzoic acid.
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(Chen *et al.* 2010). However, many aspects of IAA distribution and its mechanism of action in rhizogenesis remain unknown. In the present research, an immunohistochemical approach was used to study the spatial distribution patterns of endogenous IAA and its dynamic changes in the rhizogenesis of poplar leaves. Additionally, the effect of TIBA on the distribution of IAA in the rhizogenesis was also analyzed. This study provides a substantial base not only for the further research on the mechanism of IAA action during rhizogenesis, but also for the vegetative propagation in woody plants.

Poplar 741 [*Populus alba* × (*P. davidiana* + *P. simonii*) × *P. tomentosa*] was cultured aseptically on proliferation medium (full-strength MS supplemented with 0.3 mg dm^{-3} 6-benzylaminopurine, 6-BA, and 0.1 mg dm^{-3} indole-3-butyric acid, IBA). Subculturing was performed every 20 d. Shoots, 4.5 - 5.0 cm in length, were then cultured in rooting medium (growth regulator free 1/2 MS). After 7 - 10 d of cultivation, *in vitro* plantlets, 6.5 - 7.0 cm in length, were obtained. For observation of the rhizogenesis, the leaves in the middle of each plantlet were used for root induction. Shoot proliferation and rooting were performed in 350-cm^3 cylindrical bottles. Each bottle contained 15 leaves and the leaf petiole was inserted into the rooting medium to a depth of 3 mm. The cultures were incubated at 25 ± 3 °C with a 16-h photoperiod (irradiance of 53 μmol m^{-2} s^{-1}) provided by cool-white fluorescent tubes. The number of leaves used was not less than 30 for each treatment. The petioles were examined daily and root formation was scored.

For immunohistochemical localization of IAA, 3-mm thick basal regions of the petioles at different points of development were excised. All excised samples were prefixed immediately in a 2 % (m/v) aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, *Sigma*, St. Louis, USA) and post-fixed overnight in a solution containing 4 % (m/v) paraformaldehyde and 2.5 % (v/v) glutaraldehyde at 4 °C. The fixed tissues were then dehydrated through a graded ethanol series, embedded in paraffin, sectioned at 8-μm and submitted to the immunolocalization of IAA performed as described by Holgate *et al.* (1983) with slight modifications. Briefly, sections were incubated for 45 min in blocking solution [0.05 M Tris buffer (TBS), pH 7.6, 0.3 % (v/v) *Triton X-100*, 10 % (v/v) normal goat serum, and 5 % (m/v) bovine serum albumin] and incubated overnight at 4 °C with anti-IAA antibodies (*Agdia*, Elkhart, IN, USA) diluted 1:200 in a TBS/BSA solution. Subsequently, the sections were incubated for 4 h at room temperature with gold-labelled goat anti-mouse IgG (12 nm in diameter) diluted 1:50 in TBS/BSA. After washing, the sections were stained with a silver staining solution (0.1 M citrate buffer, pH 3.5, 1.7 % (m/v) hydroquinone, and 0.1 % (m/v) silver nitrate). As the colour developed (15 min) on the sections, they were rinsed with water, dehydrated, mounted, observed and photographed. To verify the specificity of the anti-IAA antibodies, three negative controls were included: no EDC prefixation, no anti-IAA antibodies, and the substitution of normal mouse serum for the anti-IAA antibodies.

For the effect of TIBA on the rhizogenesis, TIBA was dissolved in absolute ethanol and added to autoclaved agar to yield a variety of concentrations (2.5, 5, 10, and 50 μM), and then was applied locally to the lamina of the leaves. The leaves were then cultured in rooting medium. The culture conditions were the same as above. The petioles were examined daily and root formation was scored. The immunolocalization of IAA in the basal regions of the petioles treated with 5 μM TIBA was determined as described above.

To illustrate the IAA distribution quantitatively, the density of silver particles in various tissues was measured as follows: 30 visual fields were observed under an oil lens (100× objective lens, 10× ocular lens) for each tissue. The number of silver particles in each visual field was counted and the labelling density was presented as the number of silver particles per 100 μm^2.

All treatments were repeated at least three times and all samples were analyzed three times. Analysis of variance (*ANOVA*) was conducted on the data, and significant differences between the means were determined using multiple-range test. Statistically significant differences were assumed at a P-value < 0.05.

On 1/2 MS medium without exogenous growth regulators, poplar 741 leaves produced adventitious roots easily. Adventitious roots were observed directly at the 3-mm basal regions of the petioles 6 d after induction. The rooting rate, which was 42.8 % on day 6, increased to 100 % on day 8. The average number of roots on a leaf was between 3 and 4. The fact that no exogenous auxin is required for the rhizogenesis makes this system very suitable for the study of the internal dynamics of IAA and poplar is a good model of woody plant. Various poplar cultivars, cultured *in vitro* in our laboratory, show no significant difference in the characteristic of rhizogenesis.

A monoclonal antibody raised against IAA was used to localize IAA in the petioles during rhizogenesis of poplar leaves. The primary antibody (from *Agdia*) was raised against IAA-BSA conjugate (IAA-17-II-A), originally prepared and described. Its specificity was proved by analysis of its cross-reactivity against 34 IAA-related compounds (Mertens *et al.* 1985). The cross-reactions of the antibody with some IAA analogues, such as indole-3-butyric acid (IBA) and indole-3-acetyl-myo-inositol ester were only 1.3 and 0.2 %, respectively, and with IAA precursor tryptophan was less than 0.1 %, which indicated high specificity of this antibody for IAA.

Before root induction, a weak signal was detected throughout the basal region of the petioles (Fig. 1*A*). The densities of silver particles in the cortex and vascular tissues were 4.80 and 5.02 per 100 μm^2, respectively. Three days after induction, a strong IAA signal was

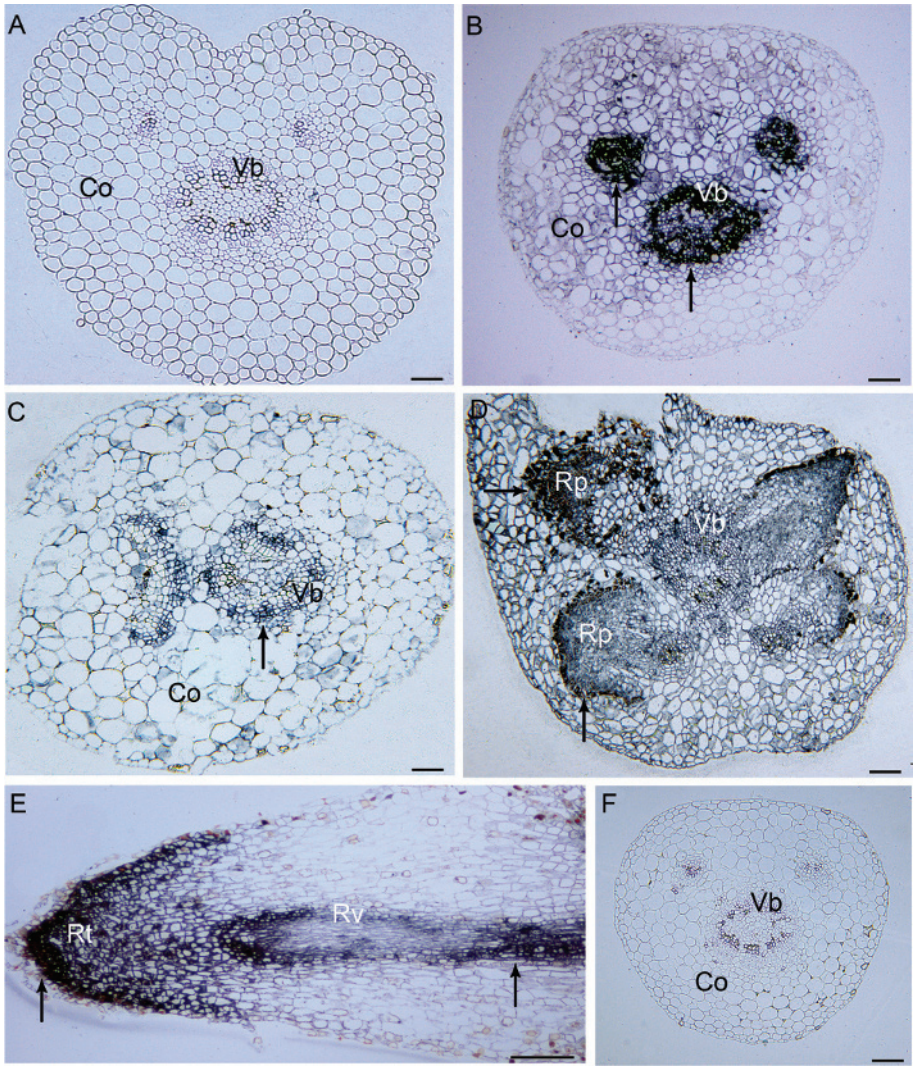

Fig. 1. Immunohistochemical localization of IAA in the basal regions of poplar petioles during rhizogenesis. The black colour on sections was the IAA signal. *A* - The transverse section of the basal region of poplar petiole before root induction. Little IAA was detected. *B, C* - The transverse sections of the basal region and middle region of poplar petioles after 3 d of *in vitro* culture. IAA was detected in vascular bundles (*arrows*). *D* - The transverse section of the basal region of poplar petiole after 5 d of *in vitro* culture. IAA was mainly distributed in root primordium (*arrows*). *E* - The longitudinal section of adventitious root. IAA was mainly distributed in root tip and root vascular cylinder (*arrows*). *F* - The transverse section of the basal region of poplar petiole after 3 d of culture when the primary antibody was omitted. Little IAA signal was detected. Sections of the following controls also resulted in no signal, similar to *F*: without EDC pre-fixation and incubation with substitute normal mouse serum. Co - cortex, Vb - vascular bundle, Rp - root primodium, Rt - root tip, Rv - root vascular cylinder. *Bars*: *A*, *B*, *C*, *D*, *F* - 50 μm, *E* - 200 μm

observed in the vascular bundles of the basal regions of the petioles (Figs. 1*B*). The density of silver particles in the vascular bundles increased sharply from 5.02 to 216.84 per 100 μm^2 ($P < 0.01$), indicating accumulation of IAA in vascular bundles at the onset of adventitious roots. Additionally, the vascular bundles of the middle regions of the petioles also showed an IAA signal, which was weaker than that of the basal regions of the petioles (Fig. 1*C*). Five days after induction, the cells of the vascular cambium dedifferentiated and developed into visible root primordia, which bore a strong IAA signal (Fig. 1*D*). The density of silver particles in the root primordia was statistically higher than in the other tissues ($P < 0.01$). As the root primordia grew and developed into adventitious roots, the IAA signal was detected in root tip and root vascular cylinder (Fig. 1*E*). The densities of silver particles in the root tip and root vascular cylinder were 228.93 and 31.77 per 100 μm^2, respectively. All controls showed little or no signal (Fig. 1*F*), indicating that our immunohistochemical localization technique was reliable and the antibody used was highly specific. The present observation suggests a correlation between the accumulation of IAA and the induction of a novel morphogenic program.

The inhibition of adventitious root formation was observed when varying concentrations of TIBA were applied onto the lamina of the leaves. Following treatment with 2.5 μM TIBA, the rooting rate dropped from 100 to 52.2 % after 8 d of induction ($P < 0.01$). After treatment with 5 μM TIBA, the rooting rate was only 5.6 % at the same time ($P < 0.01$). When treated with 10 μM TIBA, the basal petiole had no roots and formed callus. When the concentration of TIBA increased to 50 μM, the leaves were damaged and the lamina was discolored. These results are in agreement with the hypothesis that auxin and its polar transport are critical for the adventitious root formation (Zhou *et al.* 2003, Xu *et al.* 2005, Ramirez-Carvajal *et al.* 2009, Negi *et al.* 2010).

The spatial distribution patterns of IAA in petioles of the leaves treated with 5 μM TIBA were similar to that without TIBA, but the accumulation of IAA in the vascular bundles was delayed for 2 - 3 d. Three days after the application of TIBA, no obvious IAA signal was found throughout the basal regions of the petioles. After 5 d of culture, IAA signal was present in the vascular bundles and the density of silver particles was 143.73 per 100 μm^2, which was higher than in the other tissues ($P < 0.01$). After 8 d of culture, the IAA signal was observed mainly in the root primordia, in which density of silver particles was higher than in the surrounding tissues ($P < 0.01$). After 10 d of culture, the IAA signal became localized in the root tip and root vascular cylinder.

In present study, the IAA was accumulated more in the vascular bundles of the basal regions of the petioles than in the middle region of the petioles (Fig. 1*B*,*C*). Moreover, application of TIBA on lamina prevented the accumulation of IAA in the vascular bundles and delayed the rhizogenesis. These results suggested that IAA in the vascular bundles of the basal regions of the petioles initiated adventitious rooting. It is probably transported there through vascular bundle from lamina, rather than generated *in situ*. The results are consistent with the hypothesis that IAA could be exported from the leaves of pea plants into the phloem, and then re-directed into the basipetal polar auxin transport pathway (Cambridge and Morris 1996). Taken together, the present observation shows the spatial distribution patterns of IAA and its dynamic changes during the rhizogenesis of poplar leaves for the first time.

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