Molecular Cloning and Characterization of Auxin-regulated Genes from Mungbean Hypocotyls during Adventitious Root Formation

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Abstract. Adventitious root formation (rooting) in 'Berken' mungbean [Vigna radiata (L.) R. Wilczek.] cuttings is stimulated by indole-3-acetic acid (IAA). To understand the molecular events that occur during IAA-induced adventitious root initiation, a λgt11 cDNA library was made from mungbean hypocotyls treated with 500 μM IAA for 3 hours and differentially screened. Two cDNAs MII-3 and MII-4 were isolated. Southern analysis revealed that both cDNAs are encoded by different genes. Expression studies showed different patterns for both genes. Both MII-3 and MII-4 were highly expressed in IAA treated hypocotyls, whereas MII-4 was also induced in IAA treated epicotyls. There was no expression of either MII-3 or MII-4 in control or IAA treated leaves. With increasing concentrations of IAA from 100 to 1000 μM there was an increase in the average root number per cutting as well as a stimulation in MII-3 and MII-4. Both MII-3 and MII-4 showed a stimulation in expression 4 hours following treatment with 500 μM IAA reaching a maximum from 4 to 8 hours followed by a decline thereafter. Basal expression of MII-3 was evident between 2 and 8 hours, whereas a high degree of basal expression was found with MII-4 from 1 to 8 hours followed by a sharp decline. Cycloheximide (50 μM) dramatically reduced rooting and MII-3 expression, whereas MII-4 was only slightly affected.

Adventitious roots in stems and leaves originate in the phloem parenchyma and are therefore, not associated with primary or secondary roots (Lovell and White, 1986). Consequently, stem cuttings, under appropriate conditions, produce adventitious roots resulting in a new root system and a complete plant. Adventitious roots may arise spontaneously or be induced by environmental factors and chemicals (Barlow, 1986). The study of adventitious root formation is important for understanding and controlling root development, and vegetative propagation. Most research on adventitious rooting has focused on the physiological and biochemical events involved in root initiation and development. It has been shown that nucleic acid and protein synthesis are required during rooting (Blazich and Heuser, 1981; Haissig, 1986). Environmental factors, such as light, CO2, O2, pH, water, temperature, and nutrients also influence rooting (Andersen, 1986; Davis et al., 1988). The effects of plant hormones on rooting were examined extensively in many plant species (Davis et al., 1988; Jarvis, 1986). It is now generally accepted that auxins play a central role in the initiation and development of adventitious roots. Both natural and synthetic auxins promote rooting, while cytokinins are generally inhibitory (Blakesley et al., 1991). Although past studies have dealt with physiological and biochemical changes during adventitious root formation, information on the molecular mechanism associated with it is limited (Haissig et al., 1992).

Auxin-induced gene expression in intact tissues and cultured cells has been studied for nearly a decade (Guilfoyle, 1986; Key, 1989; Theologis, 1986). Although many auxin-induced or repressed genes have been isolated and characterized in different plants and different responses such as: cell division (van der Zaal et al., 1987; Takahashi et al., 1989), cell elongation (Ainley et al., 1988; Conner et al., 1990; Hagen et al., 1984; McClure and Guilfoyle, 1987; Theologis et al., 1985; Yamamoto et al., 1992a and b), fruit ripening (Reddy et al., 1990; Reddy and Poovaliah, 1990), induction of ACC synthase genes (Botella et al., 1992; Kim et al., 1992; Nakagawa et al., 1991), and lateral root initiation (Taylor and Scheuring, 1994), there are presently no reports on the isolation of auxin-responsive genes during adventitious rooting.

To begin the investigation on the molecular control of adventitious rooting, an understanding of the transcriptional events during the initiation of root primordia is needed. This requires the identification of genes whose transcription is necessary under optimal rooting conditions. The mungbean adventitious rooting system is highly responsive to auxins and has been used as a model system for physiological and biochemical studies (Blazich and Heuser, 1979b). The major objective of this study was to identify and characterize genes associated with adventitious rooting in mungbean.

Materials and Methods

Plant materials. Vigna radiata, mungbean seeds were surface-sterilized in 10% (v/v) clorox for 10 min, rinsed in distilled water and aerated for 24 h in tap water before sowing 1 cm deep in styrofoam trays containing perlite. The seeds were then grown under continuous cool white fluorescent lights with an irradiance of about 35 μmol·s⁻¹·m⁻² at 25°C for 7 days. Seedlings were watered with tap water every other day and on alternate days with a salt solution consisting of Ca(NO3)₂·4 H₂O (85 mM) and H₂BO₃ (1.6 mM). Uniform cuttings from 7-day-old seedlings were produced by removing a portion of the hypocotyl and attached roots 3-cm below the cotyledonary node (Blazich and Heuser, 1979b). Each cutting consisted of a 3-cm hypocotyl, epicotyl, two primary leaves, and an unexpanded trifoliolate bud.

Plant treatment and rooting assay. The cuttings were initially placed in distilled water. Ten cuttings were then selected at random and placed in a 19×65-mm shell vial containing 1 ml of treatment solution that contained various chemicals as indicated.
in each experiment. The vials were arranged at random in a growth room under the same temperature and light conditions described for seedling growth. The treatment solution was absorbed within 3 h, after which a sufficient amount of distilled water was added to bring the level of liquid in the vial to the cotyledonary nodes of the cuttings. Additional distilled water was added every 24 h to maintain that level for the duration of the experiment. After a 120-h incubation period, unless otherwise indicated, the number of roots per cutting was recorded and the mean from 30 cuttings for each treatment taken. Partially elongated roots appearing as bumps below the hypocotyl epidermis and elongated roots above the surface were included in the root counts. Hypocotyls, epicotyls, and primary leaves used for expression studies were harvested from 7-day-old mungbean cuttings treated with 500 µM IAA in 5 mM potassium phosphate buffer, pH 6.2, or buffer alone for 3 h. Sections 3 cm long from primary roots taken from 7-day-old mungbean seedlings were treated in the same manner as the other plant parts. Treatments involving foliar applications to the primary leaves was accomplished by spraying them to drip with the same solutions previously mentioned with the addition of a drop of Tween 20. Leaves were harvested 3 h following treatment.

Construction of cDNA library. Total RNA was extracted according to Chirgwin et al. (1979). Briefly, 12.5 g of hypocotyl tissue was ground to a fine powder and extracted in 25 ml of extraction buffer (25 mM sodium citrate buffer, pH 7.0 containing 4 M guanidine thiocyanate, 0.5% sodium sarcosine, 1.5 M CsCl, 50 mM EDTA, and 100 mM β-mercaptoethanol). The clarified supernatant was layered onto a 5.7 M cesium chloride cushion and spun for 16 h at 37000 rpm. The RNA pellet was resuspended in 100 µg·ml–1 denatured salmon sperm DNA at 42°C, then hybridized in the same solution with cDNA probes (5 × 10⁶ cpm/ml) overnight at the same temperature. Membranes were washed 2 times for 15 min with 2× SSC plus 0.1% SDS at room temperature, twice for 15 min with 2× SSC plus 0.1% SDS at 62°C, and twice for 15 min with 0.2× SSC plus 0.1% SDS at 62°C. Membranes were exposed to X-ray film with two intensifying screens at –80°C overnight. Blots were stripped and rehybridized with a pea ribosomal gene (Jorgenson et al., 1982), which was expressed constitutively and did not respond to IAA to ensure that equal amounts of RNA were loaded in each lane.

Genomic southern analysis. Genomic DNA was extracted from leaves of 7-day-old mungbean seedlings by the phenol/ chloroform method and purified by CsCl centrifugation (Sambrook et al., 1989). Leaves (10 g) were frozen in liquid nitrogen, ground to a fine powder, and then extracted in 50 ml of buffer containing 100 mM Tris-HCl, 50 mM EDTA (pH 8.0), 250 mM NaCl, 1% Sarkosyl and 2% β-mercaptoethanol. The mixture was then centrifuged for 15 min at 16000g, the supernatant extracted twice with phenol : chloroform : isoamyl alcohol (25:24:1), and the nucleic acids recovered by ethanol precipitation. The pellet was resuspended in 10 ml of TE buffer (pH 8.0) and CsCl (1 g·ml–1) was added before ultracentrifugation at 300000g for 20 h. The DNA band was collected and ethanol precipitated. The DNA pellet was resuspended in 500 µl of TE buffer. About 10 µg of DNA was digested with restriction endonucleases (Eco RI, Bam HI, Hind III), size fractionated on a 0.6% agarose gel, and transferred to nylon membranes by capillary transfer (Sambrook et al., 1989). Prehybridization and hybridization conditions were the same as described for the northern analysis. The washing steps were two times for 10 min with 2× SSC plus 0.1% SDS at room temperature, 1 time for 30 min with 0.1× SSC plus 0.1% SDS at room temperature, and 1 time for 30 min with 0.1× SSC plus 0.1% SDS at 65°C. Air dried membranes were exposed to X-ray film with two intensifying screens at –80°C overnight.

Results and Discussion

Sequence analysis. The complete nucleotide and deduced amino acid sequences of the DNA fragments were aligned using the DNA sequence analysis program in the Intelligenetics software. The percentage homology was determined using FASTDB program in the Intelligenetics software.

DNA sequence analysis. DNA sequences were determined by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using Sequenase version 2.0 with universal and reverse M13 sequencing primers (USB, Cleveland, Ohio). DNA sequence analysis was facilitated by the use of the Intelligenetics molecular biology software. The percentage homology was determined using FASTDB program in the Intelligenetics software.

Northern blot analysis. Total RNA was extracted from roots, hypocotyls, epicotyls, and leaves of 7-day-old seedlings or cuttings treated with various chemicals frozen in liquid nitrogen and stored at –80°C until used as previously described by Chirgwin et al. (1979). Total RNA (10 µg) was fractionated on a 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis, the RNA was transferred to a nylon membrane by capillary transfer according to standard procedures (Sambrook et al., 1989). Probes were labeled with [32P]dCTP by the random-primer method with Amersham’s Multiprime DNA labeling system. The membranes were first prehybridized for 4 h in a solution containing 50% formamide, 6× SSC, 5× Denhardt’s reagent, 0.5% SDS, and 200 µg·ml–1 denatured salmon sperm DNA at 42°C, then hybridized in the same solution with cDNA probes (5 × 10⁶ cpm/ml) overnight at the same temperature. Membranes were washed 2 times for 15 min with 2× SSC plus 0.1% SDS at room temperature, twice for 15 min with 2× SSC plus 0.1% SDS at 62°C, and twice for 15 min with 0.2× SSC plus 0.1% SDS at 62°C. Membranes were exposed to X-ray film with two intensifying screens at –80°C overnight. Blots were stripped and rehybridized with a pea ribosomal gene (Jorgenson et al., 1982), which was expressed constitutively and did not respond to IAA to ensure that equal amounts of RNA were loaded in each lane.

The insert cDNA from the positive plaques was excised with Eco RI and ligated into pBluescript (SK+) (Stratagene, La Jolla, Calif.) The ligation mixtures were used to transform E. coli DH5α. Transformants were selected on LB plates containing ampicillin (50 mg ml–1) and X-gal (0.33 mg·ml–1). Plasmid DNA was isolated using the alkaline lysis method (Sambrook et al., 1989).

Genetic linkage analysis. The genetic linkage map of the mungbean was constructed using the DH lines of the DH population described above with the RAPD markers. The markers were scored as presence (1) or absence (0) using the software package MAPMAKER (Lincoln et al., 1992). The genetic linkage map of the mungbean was constructed using the DH lines of the DH population described above with the RAPD markers. The markers were scored as presence (1) or absence (0) using the software package MAPMAKER (Lincoln et al., 1992).
Fig. 1. The complete nucleotide and deduced amino acid sequences of auxin-induced mungbean cDNA clones MII-3 (A) and MII-4 (B).
Fig. 2. Southern blot analysis of mungbean genomic DNA digested with Eco RI, Bam HI, and Hind III and probed with \[^{32}\text{P}]\text{dCTP-labeled MII-3 or MII-4 cDNA. A partial restriction map for MII-3 or MII-4 cDNA is located below its respective blot.}

1991) were 80%, 79%, and 52%, respectively. Similarity between MII-4 with other auxin-induced genes including: Aux (Ainley et al., 1988, Conner et al., 1990), SAAU (McClure and Guilfoyle, 1987), \textit{par} (Takahashi et al., 1989), PS-IAA4/5 and PS-IAA6 (Oeller et al., 1993; Theologis et al., 1985), ARG (Yamamoto et al., 1992a, b), ISAR (Reddy et al., 1990), and RSI (Taylor and Sheuring, 1994) was <35%.

Genomic southern analysis. When digested mungbean genomic DNA was probed with MII-3, there were two bands in the Eco RI lane, two bands in the Hind III lane, and three bands in the Bam HI lane (Fig. 2A). In the MII-3 cDNA there are no Eco RI or Hind III sites and two Bam HI sites. The additional bands may have been due to sites found in the introns of this gene or due to cross hybridization with closely related genes. However, the possibility exists that additional copies of this gene may be present in the mungbean genome. When digested DNA was probed with MII-4 there were two bands found in the Eco RI lane, while in the Hind III and Bam HI lanes three bands were found (Fig. 2B). In the MII-4 cDNA there is one Bam HI site, one Hind III site, and no Eco RI site present. The two bands found in the Bam HI lane are in agreement with cDNA sequence information, however, the additional bands found in the Eco RI lane may have been due to sites found in introns of this gene or due to cross hybridization with closely related genes. But, the possibility also exists that additional copies of this gene may be present.

Expression of MII-3 and MII-4 in different plant parts. To evaluate if MII-3 and MII-4 expression was tissue specific, total RNA from roots, hypocotyls, epicotyls, and leaves from 7-day-old mungbean seedlings with or without IAA treatment were probed. Northern blot analysis revealed that without auxin treatment MII-3 was not present in hypocotyls, roots, epicotyls, and leaves (Fig. 3). Low levels of MII-4 were present in hypocotyls and epicotyls but not detectable in roots or leaves. Three hours following treatment with 500 \mu M IAA, MII-3, and MII-4 were dramatically stimulated in mungbean hypocotyls. IAA had no effect on MII-3 levels in epicotyls, leaves, and roots, however, slightly promoted MII-4 levels in epicotyls (Fig. 3).

Adventitious roots arise from the hypocotyl region of the mungbean cuttings. Without exogenous IAA treatment, roots only come from the very bottom of the hypocotyl. With IAA treatment roots develop in four longitudinal rows along the whole hypocotyl (Blazich and Heuser, 1979a). We also found that the epicotyl region formed adventitious roots especially when the hypocotyl was excised from the cutting or was damaged (data not shown). Thus, the major expression of MII-3 and MII-4 in the hypocotyl and the low levels of MII-4 in the epicotyl of mungbean cuttings...
after IAA treatment is correlated with rooting potential. Following IAA application to the base of the cutting (Fig. 3). To evaluate if this was caused by an IAA concentration gradient formed in the cutting, the same concentration of IAA was sprayed directly on the leaves attached to cuttings. To be sure that IAA was taken up by the leaf, IAA-induced ethylene production was evaluated and shown to increase dramatically in response to foliar applications (data not shown), thereby providing evidence that IAA was taken up by the leaf. However, foliar application of IAA had no effect on MII-3 and MII-4 expression 3 h following treatment (data not shown). Although MII-3 and MII-4 are auxin-inducible and exhibit strong tissue-specificity, further investigation is necessary to evaluate if they are involved in the initiation of roots in mungbean.

Effects of auxin on MII-3 and MII-4 expression and rooting. Basal levels of MII-3 and MII-4 were found in mungbean hypocotyls and there was a dose-dependent increase in the expression of MII-3 and MII-4 following the initiation of IAA treatment. Expression of both MII-3 and MII-4 increased as the concentration of IAA increased (Fig. 4). The IAA dose-dependent rooting response shown in Fig. 4 coincides with increases in MII-3 and MII-4 expression in the hypocotyls following IAA treatment. NAA (100 µM), 2,4-D (500 µM) or IAA (500 µM) promote adventitious root formation in mungbean cuttings as well as MII-3 and MII-4 expression that corresponded with the increase in rooting observed (Fig. 5).

Kinetics of MII-3 and MII-4 expression. Over a time course, MII-3 in control hypocotyls was detected within 4 h after initiation of the experiment, reached a maximum at 6 h, and declined thereafter (Fig. 6). Two hours following the initiation of IAA treatment (500 µM) MII-3 levels increased, reaching a peak from 4 to 8 h, followed by a decline (Fig. 6). Basal levels of MII-4 in the control remained fairly constant through 8 h followed by a sharp decline 10 h after treatment initiation, and by 12 h was not detected. Two hours following the initiation of IAA (500 µM) treatment, there was a dramatic increase in MII-4 levels, reaching a peak from 4 to 8 h followed by a decline (Fig. 6). It has been reported that the first cell divisions of the phloem parenchyma leading to adventitious root initiation occur 20–24 h following the initiation of auxin treatment (Blazich and Heuser, 1979a). Tripepi et al. (1983), using 3H-uridine and 3H-thymidine uptake as indicators of RNA and DNA synthesis, respectively, observed that nuclei in the potential rooting zone cells were labeled...
with \(^3\)H-uridine (nucleolus) within 2 h following the initiation of auxin treatment. \(^3\)H-thymidine was incorporated 14–16 h later. They suggested that \(^3\)H-uridine was incorporated into mRNA in the nucleoli and that the initial transcriptional events occurred within 24 h in the rooting zone parenchyma.

In the presence of IAA, cycloheximide dramatically inhibited adventitious rooting and \(MII-3\) expression, while having an observable, but slight inhibition of \(MII-4\) (Fig. 7). Cycloheximide treatment had no observable effect on basal levels of either \(MII-3\) or \(MII-4\) (Fig. 7). The inhibitory effect of cycloheximide on the expression of the auxin-induced \(MII-3\) and \(MII-4\) is contrary to its effect on other reported auxin-induced genes where it has been shown to dramatically promote expression (Franco et al., 1990; Theologis et al., 1985).

In summary, we have identified and characterized 2 cDNA clones corresponding to auxin-induced mRNAs by differentially screening a \(\lambda\) gt11 cDNA mungbean library. The data presented here show that auxin induced the expression of \(MII-3\) and \(MII-4\) in the hypocotyl of mungbean cuttings, which corresponded with an increase in adventitious root initiation. Although there was a correlation of inducibility of \(MII-3\) and \(MII-4\) with adventitious rooting, further investigation is necessary to evaluate whether or not they are directly responsible for the initiation of roots in mungbean.

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Vigna radiata (or Vigna unguiculata) hypo-