Analysis of indole-3-butyric acid-induced adventitious root formation on Arabidopsis stem segments

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Abstract

Root induction by auxins is still not well understood at the molecular level. In this study a system has been devised which distinguishes between the two active auxins indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA). IBA, but not IAA, efficiently induced adventitious rooting in Arabidopsis stem segments at a concentration of 10 μM. In wild-type plants, roots formed exclusively out of calli at the basal end of the segments. Root formation was inhibited by 10 μM 3,4,5-triiodobenzoic acid (TIBA), an inhibitor of polar auxin transport. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in trp1, a tryptophan auxotroph of Arabidopsis with a bushy phenotype but no demonstrable reduction in IAA levels. By contrast, two mutants of Arabidopsis with measurably higher levels of IAA (trp2, amt1) show root induction characteristics very similar to the wild type. Using differential display, transcripts specific to the rooting process were identified by devising a protocol that distinguished between callus production only and callus production followed by root initiation. One fragment was identical to the sequence of a putative regulatory subunit B of protein phosphatase 2A. It is suggested that adventitious rooting in Arabidopsis stem segments is due to an interaction between endogenous IAA and exogenous IBA. In stem explants, residual endogenous IAA is transported to the basal end of each segment, thereby inducing root formation. In stem segments in which the polar auxin transport is inhibited by TIBA, root formation does not occur.

Key words: Adventitious root formation, Arabidopsis, auxin, auxin-inducible proteins, differential display, indole-3-butyric acid, protein phosphatase 2A, TIBA.

Introduction

Root development in Arabidopsis thaliana has been the subject of many studies employing mutant screens during the last few years (for a review see Casson and Lindsey, 2003). While development of the primary root from the embryonic stage has received a lot of attention and the processes involved are beginning to unravel, the formation of lateral and adventitious roots is less well understood. Lateral and adventitious roots are formed post-embryonically. While lateral roots typically form from the root pericycle, adventitious roots form naturally from stem tissue. Adventitious roots are less predictable in their cellular site of origin than lateral roots. They may form from the cambium or, in the case of detached stem cuttings, from calli. Therefore it appears that adventitious roots can be formed by two different pathways: (i) direct organogenesis from established cell types or (ii) from callus tissue following mechanical damage (Casson and Lindsey, 2003, and references therein).

Adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species that are difficult to root. (Davies et al., 1994; Kovar and Kuchenbuch, 1994). The auxin indole-3-acetic acid (IAA) was the first plant hormone to be used to stimulate rooting of cuttings (Cooper, 1935). At that time it was discovered...
that a second, ‘synthetic’ auxin indole-3-butyric acid (IBA) also promoted rooting and was even more effective than IAA (Zimmerman and Wilcoxon, 1935). IBA is now used commercially worldwide to root many plant species (Hartmann et al., 1990). Since its introduction more than 50 years ago, IBA has been the subject of many experiments, mostly involving trial and error studies to achieve optimum rooting conditions for the plant species in question. Application of IBA to cuttings of many plant species results in the induction of adventitious roots, in many cases more efficiently than IAA (Epstein and Ludwig-Müller, 1993). For example, in Vigna radiata the induction of adventitious roots was observed after IBA, but not IAA application (Riov and Yang, 1989). The greater ability of IBA to promote adventitious root formation compared with IAA has been attributed to the higher stability of IBA versus IAA both in solution and in plant tissue (Nordström et al., 1991). The effective concentration of IBA in these kinds of studies was also dependent on the pH of the medium. It was shown that, at lower pH values, lower IBA concentrations in the medium were sufficient to induce rooting of apple cuttings (Harbage and Stimart, 1996).

Differences in the ability to form adventitious roots have been attributed to differences in auxin metabolism (Alvarez et al., 1989; Blazkova et al., 1997; Epstein and Ludwig-Müller, 1993). It was shown, for example, that a difficult-to-root cultivar of Prunus avium conjugated IBA more rapidly than an easy-to-root cultivar (Epstein et al., 1993). Only in the easy-to-root cultivar was the appearance of free IBA observed after several days and the authors concluded that the difficult-to-root cultivar was not able to hydrolyse IBA conjugates during the appropriate time points of adventitious root development. Interestingly, it was possible to induce rooting of the difficult-to-root cultivar after application of an inhibitor of conjugation (Epstein et al., 1993). It has been shown that IBAsp is even more active than free IBA in the promotion of adventitious roots in mung bean, possibly due to its higher stability during the rooting process (Wiesman et al., 1989). However, other differences such as uptake and transport can also account for the differences in rooting behaviour (Epstein and Ludwig-Müller, 1993).

The physiological events leading to root initiation may be revealed by using targeted or untargeted molecular approaches to identify genes that may be involved in adventitious rooting. IBA has been identified as a natural substance in Arabidopsis thaliana (Ludwig-Müller et al., 1993) and there are indications that at least part of the action of IBA is not through IAA in this species (Poupart and Waddell, 2000; Zolman et al., 2000). Therefore a system has been devised for adventitious root formation on stems of the model plant Arabidopsis under sterile conditions, where roots are specifically induced after the application of IBA but not of IAA. The results have shown that (i) IBA is one important factor in Arabidopsis to induce adventitious roots, (ii) the timing of auxin application is important to distinguish between callus and root formation, and (iii) this system is suitable for identifying genes involved in adventitious root formation. Finally, the effect of an auxin transport inhibitor, TIBA, on IBA-induced adventitious root formation has been investigated and IAA-deficient mutants were used to analyse the interplay between IAA and IBA during adventitious rooting.

Materials and methods

Plant material

Arabidopsis plants were grown aseptically on Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) in Magenta® boxes at 24 °C under constant illumination with cool-white fluorescent lights, approximately 40 μmol m⁻² s⁻¹. The seeds were surface-sterilized with 5% (v/v) commercial bleach (Clorox: a 5% solution of sodium hypochlorite) for 20 min, washed thoroughly, planted on 1% agar, and vernalized for 24 h at 4 °C. Inflorescences from 4–8-week-old-plants were used because, during this period, the age of the stems did not influence callus/root formation, although on stem segments of older plants no root formation could be observed; data not shown). The inflorescences were cut into 0.5 cm node-free segments and incubated in the dark or under constant illumination in Petri dishes containing full-strength MS agar containing the appropriate concentrations of IAA or IBA with or without different concentrations of 3,4,5-triiodobenzoic acid (TIBA). In the light, the plates were covered with yellow plastic to prevent photo-oxidation of auxins (Campanella et al., 1996). Starting at 5 d, plates were examined daily and the proportion of segments showing callus or root formation was scored.

For the differential display experiments, segment length was reduced to 3 mm to increase the number of ends per fresh weight. For the subsequent treatments, segments were transferred under sterile conditions to fresh Petri dishes containing either plain MS agar or MS agar with the appropriate hormone supplement.

For histology, stem segments were fixed for at least 24 h in FAA (5% formaldehyde, 5% acetic acid, 50% ethanol), then dehydrated through a series of ethanol steps (70%, 80%, 95%) before infiltration with JB-4 resin (Polysciences, Inc., Niles, IL). Sections of 2–4 μm were stained with toluidine blue.

Evaluation of the rooting process

On each Petri dish for the different treatments 10–12 Arabidopsis stem segments were placed. Each experimental condition consisted of at least two Petri dishes. All experiments were performed at least three times, resulting in a minimum of 60 segments which were scored per treatment. Mean values of the three independent experiments are given. After the different treatments the Arabidopsis stem segments were inspected for callus or root formation and the number of segments exhibiting the respective organs counted.

RNA extraction and differential display

Isolation of total RNA was performed using TRIzol reagent (Gibco BRL, now marketed by Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions using 300 mg fresh weight of treated and control segments. Reverse transcription followed by PCR using anchored VNT11 3’-primers and 10-mer OPA 5’-primers (both Operon Technologies) was performed essentially as described by Liang and Pardee (1992). 35S-Radiolabelled amplification products were resolved on 6% acrylamide sequencing gels and detected by autoradiography. The experiment was repeated to show reproducibility of fragment induction. Fragments induced only under condition C.
were excised, re-amplified with the same primer combination, the PCR products purified (QiAquick® gel extraction kit, Qiagen), ligated into pBSK vector, and sequenced from both ends at The Institute for Genomic Research.

Northern blot analysis

Total RNA was isolated as described above. The synthesis of the biotinylated (bio-dUTP, Boehringer Mannheim) cDNA probe used for northern hybridization was performed by PCR. Template was cDNA prepared from total RNA of Arabidopsis stems induced with IBA. For amplification of the phosphatase 2A-like protein subunit as a probe, the following primer pair was designed according to the sequence information obtained: forward 5'-GATCATGTGATA-GAAGATAAAATTAGTGCCT-3'; reverse 5'-TCTTCTATCACATGATCTGTCAGGGACCA-3'. PCR was performed according to standard procedures using the following programme: initial denaturation at 96 °C for 5 min, followed by 30 cycles of 96 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s. Equal sample loading (20 μg total RNA) was confirmed by hybridization with an actin 2 (At3g18780) probe amplified with the following primers: forward 5'-GAAGATTAGGTCGTTGCACCACCTG-3'; reverse 5'-ATTAACATTGCAAAGAGTTTCAAGGT-3'. Non-radioactive northern blots were performed according to Löw and Rausch (1994), with the Northern-Light™-kit from Tropix (Serva) for detection.

Results

Indole-3-butyric acid can induce adventitious roots on Arabidopsis stem segments

Several reports deal with the better performance of IBA versus IAA during the rooting process. This was attributed to parameters such as stability, transport, or metabolism. Therefore a protocol was devised which would induce adventitious roots on Arabidopsis stems by one of the auxins but not the other. This study’s experiments showed that several parameters influenced adventitious root induction and helped to discriminate between the actions of IAA and IBA. These were: (i) concentration of the hormone, (ii) duration of treatment, (iii) priming event, and (iv) second hormone treatment.

In a first set of experiments, 0.5 cm explants of Arabidopsis stems were incubated for 7 d on MS medium containing either IAA or IBA at different concentrations and the phenotype was recorded (Fig. 1A). Since the explants looked similar when they were cultivated on hormone plates for 7 d, only the explants on different IBA concentrations are shown. The induction of adventitious roots was always preceded by callus formation. Root induction was seen at 1 μM and 10 μM IBA and IAA, and at 100 μM hormone the roots looked stunted with more root hairs produced (Fig. 1A). Similarly, root induction by IAA or IBA was also possible using excised leaves (Fig. 1B). The concentration dependence was also comparable with that for stem segments.

On stem segments treated with IBA, adventitious roots clearly arose from the cambium, which first de-differentiates to form a callus (Fig. 2B). This is followed by the formation of roots (Fig. 2C) that subsequently elongated and by the formation of additional callus areas, which gave rise to new adventitious roots (Fig. 2D). In the controls without IBA such structures were never visible (Fig. 2A).

Timing of hormone requirement for adventitious rooting

To determine the period of IBA exposure required for adventitious root induction, the stem segments were incubated on 10 μM IAA or IBA for different time periods up to 48 h and then transferred to hormone-free MS medium for the remaining time. Callus and root formation was scored at 7 d (Fig. 3). The proportion of explants forming callus increased up to 100% after 48 h on auxin-containing medium (Fig. 3A). While callus formation was comparable on IAA- or IBA-containing MS agar, root formation was found only when IBA was in the medium. After a 6 h exposure, a response was already found, but optimum rooting was observed with a treatment of 48 h (Fig. 3B). After longer incubation periods the difference between IAA and IBA treatment became less pronounced (data not shown). The inset in Fig. 3B shows a picture of stem segments incubated for the respective time on either 10 μM IAA or 10 μM IBA.

A two-stage treatment was developed to distinguish between callus and root induction by IBA (Fig. 4). In stage I,
explants were incubated for 24 h on 10 μM IBA, a treatment that resulted in callus formation. In stage III, explants were given a second 10 μM IBA treatment of variable duration after a period of 24 h on hormone-free medium (stage II). The explants were transferred to hormone-free medium after the second IBA treatment for the remainder of the experiment (stage IV) and root formation was scored 14 d after the start of the second treatment. The second treatment resulted in the formation of adventitious roots on 60–95% of the explants, provided that it was at least 48 h long (Figs 4A, 5A). In addition, it was shown that the highest rooting efficiency was found with treatments that involved two exposures to IBA separated by a time without hormone (Fig. 5A). Increasing the incubation time of the second treatment on IBA also resulted in more segments showing adventitious root formation. Interestingly, in the experiments using only one long IBA treatment (Fig. 5B–D), more roots were formed when the treatment started with MS medium alone.

The auxin concentration was also important for the second treatment in which the explants were incubated for 48 h with different concentrations of IBA. Again with 1 μM and 10 μM IBA good induction of adventitious rooting was found with up to 95% of the segments showing roots (Fig. 5B). Callus formation without subsequent root formation was observed at concentrations <0.1 μM IBA.

Identification of transcripts expressed during adventitious rooting using differential display

The treatments of Arabidopsis stems described above were used to test this system for its suitability to isolate differentially expressed genes during adventitious rooting. Since the experimental procedure allowed the difference between callus formation and adventitious rooting to be distinguished, the comparison of control stems with stems treated to form callus or adventitious roots should provide transcripts which are specific for the rooting process. The

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**Fig. 2.** Development of adventitious roots on Arabidopsis stems without (A) and after treatment with 30 μM IBA (B–D). Sections were taken 3 d (B), 5 d (C), and 9 d (D) after placing the segments on rooting medium. Sections of 2–4 μm were stained with toluidine blue. Adventitious roots are marked by arrows.
following three tissue samples were compared: (i) untreated segments; (ii) segments exposed to 10 μM IBA for 24 h, which will induce callus but not root formation; and (iii) segments given 24 h 10 μM IBA, 24 h no IBA, 48 h 10 μM IBA/MS. The segments were placed either on MS medium or MS supplemented with IBA after the indicated time periods (see time scale; one white bar segment represents 24 h). Percentage of root formation under the respective treatment conditions is given to the right of the bar. The stages mentioned in the text are indicated above the respective bar in Roman numerals: I: first IBA treatment; II: first period on MS; III: second IBA treatment; IV: remaining time until roots are visible on MS. The flash indicates a discontinuous time scale.

Fig. 3. Callus (A) and root (B) formation after continuous treatment with 10 μM IAA (hatched bars) or IBA (black bars) for different times on MS medium. The photograph shows the phenotype of rooted stem segments incubated for different periods on 10 μM IAA or IBA.

Fig. 4. Adventitious root formation is increased by a two-stage treatment and does not require continuous exposure to IBA. Different treatments with IBA are marked as follows: (dotted section) MS only, (black section) MS+10 μM IBA. The different variations tested are: (A) 24 h IBA/24 h MS/48 h IBA/MS; (B) 72 h IBA/MS; (C) 24 h MS/72 h IBA/MS; (D) 48 h MS/72 h IBA/MS. The segments were placed either on MS medium or MS supplemented with IBA after the indicated time periods (see time scale; one white bar segment represents 24 h). Percentage of root formation under the respective treatment conditions is given to the right of the bar. The stages mentioned in the text are indicated above the respective bar in Roman numerals: I: first IBA treatment; II: first period on MS; III: second IBA treatment; IV: remaining time until roots are visible on MS. The flash indicates a discontinuous time scale.

were amplified from the 3' -end. Therefore, all sequences are 3'-UTRs of the respective cDNAs. Since the completion of the Arabidopsis genome sequencing project, identification of gene sequences has been much facilitated. One 390 bp fragment (01-a) was homologous to a regulatory subunit B of protein phosphatase 2A (At3g54930). A second 340 bp fragment (01-b) was found to be derived from At1g29470 which was annotated as similar to the early-responsive dehydration stress protein, ERD3 that contains a putative methyltransferase motif. A third 300 bp fragment (06-a) was derived from At5g48545, a gene encoding an unknown protein of the histidine triad family protein with a HIT domain (http://www.tigr.org/tigr-scripts/TrEMBL/ath1/). Expression analysis confirmed the presence of the PP2A homologous mRNA specifically in tissues after IBA-induced adventitious root formation (Fig. 6B).

The polar auxin transport inhibitor TIBA inhibits adventitious root formation

Factors important for the effect of auxins during rooting might be (i) synthesis, (ii) metabolism, and (iii) transport. The latter was tested by using the polar auxin transport inhibitor 3,4,5-triiodobenzoic acid (TIBA) concomitantly with the IBA treatment leading to adventitious roots. Inhibition of root formation was observed when varying
Concentrations of TIBA were added together with a fixed concentration of IBA (10 μM) in the medium (Fig. 7). While 0.1 μM and 1 μM TIBA had no inhibitory effect, 10 μM TIBA was already inhibitory and 100 μM TIBA completely prevented adventitious root formation. With lower TIBA concentrations there even seemed to be a small promoting effect after longer incubation times.

Arabidopsis mutants with altered adventitious root formation

IBA is an important factor for adventitious root formation if applied exogenously. However, endogenous auxins may also play a role in the rooting process. Therefore three mutants with altered auxin levels were investigated for their ability to form adventitious roots after IBA treatment. The mutant *amt1* (Kreps and Town, 1992) has no altered phenotype compared with the wild type when grown under normal conditions. However, if *amt1* was grown on 10 μM IBA, the roots looked more stunted with a higher number of lateral roots and, at higher concentrations, less root growth than the wild type was observed. *amt1* also showed altered levels of IAA and IBA (Ludwig-Müller et al., 1993). It was therefore of interest to test whether this mutant behaved differently concerning adventitious rooting and so at the same time two other mutants with defects in the tryptophan biosynthesis pathway, *trpl* and *trp2* (Last et al., 1991; Rose et al., 1992) were included. Since adventitious root formation was shown to be concentration-dependent in *Arabidopsis*, several IBA concentrations were tested on wild-type and mutant stem segments. At intermediate IBA concentrations (3–10 μM), root induction was less efficient in *trpl*, a tryptophan auxotroph of *Arabidopsis* with a bushy phenotype but no demonstrable reduction in IAA levels, compared with wild-type Columbia (Fig. 8). The two other mutants (*amt1* and *trp2*) with measurably higher levels of IAA show root induction characteristics very similar to the wild type.

Discussion

*Arabidopsis* has been used for the investigation of lateral root development (Neuteboom et al., 1999) because of its relatively simple organization of both primary and lateral roots (Dolan et al., 1993). Lateral root formation in root cultures of *Arabidopsis* was initiated by exogenous auxin. Differential screening of a cDNA library from roots treated with 1-NAA and the inactive analogue 2-NAA led to the isolation of four cDNAs clones coding for proteins putatively active outside the cell such as subtilisin-like serine protease (Neuteboom et al., 1993). *Arabidopsis* mutants exhibiting more lateral roots (*sur1, sur2*) were linked to an overproduction of IAA (Boerjan et al., 1995; Delarue et al., 1998). However, other genes regulated independently of auxin induction are also involved in lateral root development, such as the nuclear-localized protein ALF4 (DiDonato et al., 2004).

Evidence for the involvement of IBA, but not IAA, in lateral root development was recently reported for lateral root induction in rice (Wang et al., 2003). While IBA was
able to induce lateral roots, the same response was found only at 20-fold higher concentrations of IAA (Chhun et al., 2003, 2004). In addition, a rice lateral rootless mutant Lrt1 could be rescued by IBA but not IAA treatment (Chhun et al., 2003). The mutated gene has yet to be described.

In contrast to lateral root development, adventitious root formation has significant practical implications because of the many plant species that are difficult to root. IBA is now used commercially worldwide to root many plant species (Hartmann et al., 1990). However, Arabidopsis as a model to study adventitious rooting has so far been neglected. The aim of this study was 2-fold: (i) to analyse the process leading to adventitious roots on Arabidopsis stems and to find out which of the two auxins known to be present in Arabidopsis are involved in the process and to devise an experimental system which could be used to distinguish between callus and root formation and between IAA and IBA in the rooting process; (ii) to test this system for its use in the isolation of differentially expressed transcripts specifically involved in the rooting process. These transcripts could allow a more detailed analysis of adventitious rooting at the molecular level and help to identify candidate genes important for this process. The possible function of the transcripts isolated in this study for the rooting process will be briefly discussed. Furthermore, this system is also suitable for the analysis of available Arabidopsis mutants or chemical inducers or inhibitors of the rooting process.

It was shown that IAA and IBA were able to induce adventitious roots on cuttings of Arabidopsis stems if the segments were not removed during the treatment (Fig. 1), whereas removal of the segments from auxin-containing medium to MS medium only resulted in the production of calli with about the same efficiency for both hormones. Callus formation preceded adventitious rooting (Fig. 2). After shorter incubation times only IBA treatment resulted in the formation of roots (Fig. 3), indicating that IBA is an important factor for rooting. Several possibilities exist to explain the better performance of IBA versus IAA (summarized in Epstein and Ludwig-Müller, 1993): (i) higher stability, (ii) differences in metabolism, (iii) differences in transport, and (iv) IBA is a slow release source of IAA.

There is now a great deal of evidence that IBA occurs naturally in plants. The higher stability of IBA, in contrast to IAA, during rooting assays was reported by Nordström et al. (1991) which affected both degradation and metabolism. It was therefore suggested that IBA may be a very simple ‘conjugate’ of IAA and must be converted to IAA by β-oxidation to have an auxin effect. The conversion of IBA to IAA occurs in many plant species, such as Malus pumila (Alvarez et al., 1989), Pinus sylvestris (Dunberg et al., 1981), Populus tremula (Merkelbach et al., 1991), Pyrus communis (Baraldi et al., 1993), and Vitis vinifera and Olea europaea (Epstein and Lavee, 1984). However, in microcuttings of Malus it was found that IBA was converted to IAA only at very low levels (1%), but IBA itself induced more roots than IAA. This led the authors to suggest that either IBA itself is active or that it modulates the activity of IAA (van der Krieken et al., 1992, 1993).
The transport hypothesis is supported by recent findings that IBA and IAA are differently transported in Arabidopsis (Rashotte et al., 2003). These experiments are in agreement with this study’s results using polar auxin transport inhibitors.

Several lines of evidence are now emerging which suggest that part of the effects of IBA are the direct action of the auxin itself (Ludwig-Müller, 2000; Poupart and Waddell, 2000), although other functions may be modulated by the conversion of IBA to IAA via β-oxidation (Zolman et al., 2000; Bartel et al., 2001). For example, drought and osmotic stress induced the synthesis of IBA and, consequently, the endogenous content of IBA was increased, whereas IAA was less affected (Ludwig-Müller et al., 1995). In addition, IBA but not IAA was induced after the inoculation of maize roots with an arbuscular mycorrhizal fungus (Ludwig-Müller et al., 1997; Kaldorf and Ludwig-Müller, 2000). In this paper a system was established for the induction of adventitious roots on sterile-grown stem sections of Arabidopsis thaliana where IBA induced adventitious roots under conditions where IAA was ineffective (Fig. 3). There was a desire to dissect the rooting process and therefore different time and concentration schemes were used for the optimization of adventitious root formation (Fig. 4), which allowed callus and subsequent root formation to be distinguished (Fig. 5).

The second goal of this research was the identification of differentially expressed transcripts during the rooting process. For this, the differential induction of callus and root on Arabidopsis stem segments were used and those treatments were compared with the controls (Fig. 6). Only those transcripts which showed up under treatment C (Fig. 6) were analysed further.

Initial studies on the hydrolytic enzymes found during root formation after IBA treatment in cuttings of mung bean revealed the induction of endo-β-1,4-glucanase (Shoseyov et al., 1989), whereas the activities of β-1,3-glucanase and α-amylase were not affected. It was shown by in situ hybridization that the genes for endo-β-1,4-glucanase were expressed in the area of adventitious root primordia formation and in the cortex, where maceration of the cell walls was in progress in order to enable root emergence through the hypocotyl. To detect the induction of genes during adventitious root formation in loblolly pine (Pinus taeda) after treatment with IBA, a non-targeted approach via differential display reverse transcription-polymerase chain reaction was carried out (Hutchison et al., 1999). One of the clones isolated by this method showed strong similarity to the α-expansin gene family of angiosperms and the differential gene expression after IBA treatment was confirmed by RNA blot analysis. Expansins are thought to be responsible for acid-induced cell wall loosening and are expressed in rapidly growing tissues (Cosgrove and Li, 1993; McQueen-Mason, 1995). They were reported to be induced in loblolly pine in non-growing regions of the stem prior to the resumption of cell division leading to the appearance of adventitious roots (Hutchison et al., 1999).

One fragment differentially expressed during the adventitious rooting process in Arabidopsis (Fig. 6B) was identified as a regulatory subunit B of protein phosphatase 2A. In plants, type 2A serine/threonine protein phosphatases (PP2As) are critical in controlling the phosphorylation state of proteins involved in such diverse processes as metabolism, cell–cell communication, response to hormone, and auxin transport (Smith and Walker, 1996). The specificity, activity and subcellular targeting of PP2A is modulated by its association with the A and B subunits (Kamibayashi et al., 1994). In Arabidopsis, three families of B-type regulatory subunits were identified, each consisting of more than one member (Corum et al., 1996; LaTorre et al., 1997; Rundle et al., 1995; Sato et al., 1997). Expression analysis indicated that, in plants, every B subunit shows a widespread, but fine-tuned, expression pattern in different organs (Thakore et al., 1999). The function of PP2A during polar auxin transport has recently received more attention (Muday and DeLong, 2001, and references therein). One Arabidopsis mutant that provided insight into the regulation of auxin transport is called roots curl in NPA1 (rcn1). This mutant was isolated using an assay for alterations in differential root elongation in the presence of the auxin transport inhibitor NPA aimed at isolating genes encoding proteins involved in auxin transport or its regulation. The RCN1 gene encodes a regulatory A subunit of PP2A and the rcn1 mutant exhibits reduced PP2A activity in extracts (Deruère et al., 1999). The phenotypic alterations in this mutant are consistent with reductions in PP2A activity because treatment of wild-type plants with the phosphatase inhibitor cantharidin produces a phenocopy of rcn1. The RCN1 gene is expressed in the seedling root tip, the site of basipetal transport, in lateral root primordia, and in the pericycle and stele, the likely site of acropetal transport (Muday and DeLong, 2001). It can be hypothesized that other PP2A subunits are co-ordinately expressed and that polar auxin transport also plays a role in adventitious root formation in Arabidopsis. This assumption is supported by the observation here that the auxin transport inhibitor TIBA inhibited adventitious root formation. Deduced from the findings summarized above a role can be proposed for PP2A in the regulation of auxin transport during adventitious rooting by altering the phosphorylation status of proteins involved in these processes thus most likely acting upstream of auxin transport. Auxin transport itself might be important for adventitious rooting by increasing local auxin concentrations.

A second fragment was identified as derived from an early-responsive dehydration stress ERD3 with otherwise unknown function (http://www.tigr.org/tdb/e2k1/ath1/). The sequence contains also a methyltransferase motif. Protection against dehydration may result in an increase
of lateral or adventitious root formation. It was shown that IBA synthesis was increased under drought stress in maize (Ludwig-Müller et al., 1995) and the root system under these conditions was shorter, but with considerably more lateral roots. Drought rhizogenesis is an adaptive strategy that occurs during progressive drought stress and is characterized in Arabidopsis and other Brassicaceae and related families by the formation of short tuberized hairless roots (Vartanian et al., 1994). These roots are capable of withstanding a prolonged drought period and give rise to a new functional root system upon rehydration. IBA might play a role during this process by inducing new roots. This protein might therefore play a more general role in IBA-induced root formation. As long as the function of ERD3 is unclear, this has to remain a hypothesis.

The Histidine Triad (HIT) motif identified in the third gene product, His-phi-His-phi-His-phi-phi (phi, a hydrophobic amino acid), was identified as being highly conserved in a variety of organisms (Seraphin, 1992). The crystal structure of rabbit Hint (histidine triad nucleotide-binding protein), purified as an adenosine and AMP-binding protein, showed that proteins in the HIT superfamily are conserved as nucleotide-binding proteins (Brenner et al., 1997). Hint homologues hydrolyse adenosine 5′-monophosphoramidate substrates and function as positive regulators of Cdk7/Kin28 in vivo (Bieganski et al., 2002), and Phi (fragile histidine family) homologues related to the HIT family are diadenosine polyphosphate hydrolases (Barnes et al., 1996). Therefore, the role of this protein during adventitious root formation might be in the regulation of the cell cycle or in signal transduction pathways.

In conclusion, it has been shown that it was possible to dissect the adventitious root formation process in Arabidopsis in such a way as to distinguish between the action of the two auxins IAA and IBA and to establish conditions where one hormone treatment arrests the process at the callus formation stage, whereas a second hormone treatment induces the formation of roots from these calli. In addition, it has been shown that the experiments presented here are a promising method to identify IBA-induced transcripts during adventitious root formation in the model plant Arabidopsis thaliana. To study the process of adventitious root formation further, several experiments can be envisioned: (i) the isolation of additional differentially expressed fragments from this screen, or using the now available microarrays to increase the number of cDNAs; (ii) using this screening method to identify Arabidopsis mutants impaired in adventitious root formation; and (iii) using known Arabidopsis mutants to investigate their response to IBA in this system. The gene sequences identified can then be used to probe the adventitious rooting pathway in horticulturally important species that are difficult to root.

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