Molecular analysis of adventitious rooting in Fagaceae species

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Abstract

The molecular basis of the maturation-related loss of adventitious root (AR) formation in woody plants is still poorly understood. In this chapter we describe the use of an in vitro experimental system and a stepwise approach used to study rooting related genes in European chestnut (Castanea sativa) and English oak (Quercus robur) that are regulated by auxin. The system is based on differences in rooting ability, in response to auxin, of juvenile-like and mature shoot cultures derived from basal shoots and crown branches of the same mature tree. Differential display of mRNA was used to isolate auxin-regulated genes differentially expressed in cultures of both origins during the first days after root induction treatment. Expression analysis of five cDNA fragments isolated from chestnut showed four of the five genes were up-regulated by auxin in juvenile-like shoots. In oak, the expression analysis of the QrCPE gene that encodes a small putative cell wall protein rich in glycine and histidine residues showed this gene is induced by auxin in juvenile-like shoots. This suggests a possible role for the gene in AR formation in oak microshoots. The results obtained for molecular markers of rooting in other studies, and in other Fagaceae species, are also briefly discussed.

Introduction

Maturation greatly affects many phenotypical and physiological characteristics that negatively affect the regenerative ability of plant material [1]. As trees grow older, cuttings obtained from mature trees gradually lose the competence to form adventitious roots (ARs) [2]. Loss of rooting ability associated with maturation is a major drawback in the vegetative propagation of mature selected trees, since adventitious rooting is a prerequisite for the successful production of viable plants, either by conventional propagation or by the application of in vitro micropropagation [3]. Although the vegetative propagation of mature trees may be achieved by use of juvenile-like material, i.e. stump sprouts and epicormic shoots, this material is not always available. Rejuvenation methods may partially restore juvenile traits, but it is very difficult to achieve rooting rates comparable to those displayed by the juvenile material [4]. Several studies have focused on the role of auxins in the adventitious rooting in woody plants [5,6,7]; however the process is still not completely understood. Rooting ability is highly species-dependent and is greatly influenced by genotype and by the maturation stage of the plant material, amongst other factors [8]. Changes in morphological and physiological traits during the maturation process, such as the age-related loss of rooting ability, are probably associated with changes in gene expression [9]. Although different
approaches have been used to analyze changes in maturation during this transitional phase in woody plants, the molecular mechanisms underlying these changes are still unknown. The first reports on the identification of phase-change related genes in woody plants showed the gene coding for light harvesting chlorophyll a/b-binding protein (CAB) was expressed at higher levels in juvenile than in mature needles of Eastern larch (Larix laricina) [10] and at higher levels in juvenile than in mature petioles of English ivy (Hedera helix) [11]. Accumulation of dihydroflavonol reductase (DFR) mRNA was not detected in mature-phase lamina tissue of English ivy, which also lacked anthocyanin, whereas the DFR gene was expressed in juvenile tissue, containing also anthocyanin pigment [12]. In contrast, the expression of a proline-rich protein (PRP) gene in mature English ivy petioles, forming no roots, was greater than in juvenile petioles, which formed roots in response to auxin [11]. Moreover, the gene expression pattern, analyzed by in situ hybridization, showed the PRP mRNA preferentially accumulated in the mature petioles in the specific cell types involved in root initiation. This indicates an inverse relationship between rooting competence and gene expression [13]. Several auxin-regulated genes identified in loblolly pine (Pinus taeda) are highly expressed in hypocotyl cuttings in response to auxin during the early stages of induction of AR formation [14,15]. The auxin inducible (5NG4) gene isolated from loblolly pine, which encodes a putative transmembrane protein, was differentially expressed in juvenile and mature shoots [16]. Moreover, the high and specific expression of this gene in the auxin-treated juvenile shoots prior to AR formation suggests the gene may be involved in lateral root (LR) or AR formation. Using microarray analyses, Brinker et al. [17] demonstrated changes in the mRNA levels of genes during the auxin-induced development of roots in lodgepole pine (Pinus contorta) hypocotyls. Recently, Sánchez et al. [18] reported the isolation of a Monterey pine (Pinus radiata) SCARECROW-LIKE (PrSCL1) gene induced by auxin in hypocotyl cuttings that may play a role during the earliest stages of AR induction. Although other studies have been carried out on rooting related genes in forest trees, especially gymnosperms, there is little information available as regards other woody species, such as species in the Fagaceae. In this chapter, we describe the current state of the research on genes related to adventitious rooting in shoot cultures of European chestnut (Castanea sativa) and English oak (Quercus robur), as well as other related species.

Comparative analysis in juvenile-like and mature shoot cultures of European chestnut and English oak
Adventitious rooting related genes in European chestnut and English oak were analyzed using an in vitro experimental system with juvenile-like and mature shoots, previously developed for both species [7,19]. This system is based on the different morphogenetic capacity of both types of microshoots. In both species, shoot cultures of juvenile-like and mature origin were initiated, respectively, from basal shoots and crown branches of the same mature trees [20,21], and were maintained in vitro. Cultures of the chestnut clone P2 (Fig. 1A) and the oak clone Sainza (Fig. 1B) derived from basal shoots (juvenile-like tissues) are more vigorous and show greater proliferation rates, as well as higher rooting ability, than crown branch-derived shoots (mature tissues). These experimental model systems enable the study of age-related differences in morphogenetic capacity, such as the rooting ability, in materials at different ontogenetic
stages, independent of the genotype effect. These model systems have been used at anatomical, physiological, biochemical, and molecular levels to study phase-change related differences, either during the proliferation phase [22-24] or during root induction [7,19,25,26]. Detailed descriptions of the model systems used, as well as the most important results achieved regarding anatomical and physiological events during rooting are presented in the chapter by Ballester et al. in this book. Although these studies have provided valuable information concerning the different behaviour of juvenile-like and mature tissues and have allowed potential phase-change markers to be defined, further studies at the molecular level are needed for a better understanding of the mechanisms regulating the maturation-related decline in AR formation.

Isolation of auxin regulated cDNAs

Auxins play an important role in adventitious rooting and, in many plants, the application of exogenous auxin is a prerequisite to induce ARs [27]. Although various auxins may be used to induce rooting, such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphtaleneacetic acid (NAA), IBA is the most commonly used [28]. Root formation in microshoots of chestnut (P2 clone) and oak (Sainza clone) is also induced by treatment with either 4.9 mM IBA for 1 min [20] or 123 μM IBA for 24 h [7]. However, rooting rates are greater in microshoots derived from juvenile-like tissues (juvenile-like microshoots) than those derived from mature tissues (mature microshoots): 74% compared with 2% in oak and 94% compared with 12% in chestnut. Differences in rooting response of microshoots derived from juvenile-like and mature tissues to the IBA treatment allow us to study rooting related genes that are differentially regulated by auxin.

Differential display analysis and cloning of cDNAs

To identify rooting related genes in our model systems we used the mRNA differential display technique, performed with the RNAimage™ kit (Genhunter Corp., Nashville, TN) according to the manufacturer’s instructions. This methodology is a sensitive approach for identifying genes differentially expressed at two stages in a given tissue at the mRNA level. Total RNA was isolated from the basal segments (1 cm) of microshoots

Figure 1. Shoot multiplication of European chestnut (*Castanea sativa*) clone P2 (A) and English oak (*Quercus robur*) clone Sainza (B) originated from basal shoots (left) and crown branches (right).
harvested at the end of the multiplication cycle and 15 min, 1 h and 24 h after the start of
IBA treatment, as described by Dong and Dunstan [29]. The DNA-free total RNA was
used for differential display experiments, which were carried out according to Liang and
Pardee [30]. cDNA was synthesized with 200 ng of total RNA and the three one-base-
anchored oligo-dT primers, and the reverse-transcribed mRNAs were subsequently
amplified with the same set of anchored primers, in combination with short arbitrary
primers. After amplification, the PCR labeled products were separated on denaturing
polyacrylamide gels in adjacent lanes. Bands of interest corresponding to auxin-induced
genes were selected and excised from the gel by comparison of the cDNA profiles
generated from auxin-treated shoots and those obtained from control shoots harvested at
the end of the proliferation cycle. Two representative differential display patterns were
obtained with primers HT11C and H-AP12 (Fig. 2A) or H-T11G and H-AP14 (Fig. 2B)
and with RNA from mature and/or juvenile-like chestnut shoots harvested at the end of
the proliferation cycle, and after 15 min, 1 h, or 24 h of auxin treatment.

Figure 2. Differential display of total RNA from in vitro shoot cultures derived from
basal shoots (Bs) (juvenile-like tissue) and crown branches (C) (mature tissues) of a
P2 European chestnut (Castanea sativa) tree, harvested at the end of the proliferation
cycle (0) or after 15 min, 1 h or 24 h of auxin treatment. RNA was reverse transcribed
with the 3´anchored primers H-T11C (A) and H-T11G (B) and the resultant cDNAs
were amplified in the presence of α-32P-dATP with the same 3´primer and either
H-AP12 (A) or H-AP14 (B) as the 5´ primer. The gel was dried and exposed to X-ray
film. Arrows mark the differentially amplified products.

As expected most of the cDNA bands visualized in the polyacrylamide gels were present
in equal abundance in lanes of PCR products derived from different samples. However,
there were some quantitative and qualitative differences in the band patterns. Only bands
consistently showing differential amplification after auxin treatment were selected for
further analysis. Among auxin treated and untreated microshoots from the experiments
with European chestnut and English oak, several bands with different intensity were
isolated. The cDNA fragments of interest obtained by reamplification of the excised and
eluted bands were cloned in PCR-Trap vector (Genhunter Corp.), according to the
manufacturer’s instructions, and were checked for expression by the Northern blot procedure. The cDNA fragments obtained were relatively short (375 to 550 bp) and correspond to the 3’ untranslated region (3’UTR).

**Molecular markers of AR formation in oak microshoots**

To date, there are very few reports of molecular markers for adventitious rooting in oak. Discovery of these molecular markers will contribute to a better understanding of the rooting process. Two developmental phase-specific molecular markers in oak have been identified by comparing the cDNA profiles from mRNA isolated from juvenile or mature oak trees [31]. Although the expression of both cDNA fragments was higher in material derived from mature trees than in material from juvenile trees, no further details of the expression analysis of these cDNAs during adventitious rooting was reported.

On the basis of an *in vitro* oak plant system similar to the model system described above, Racchi et al., [32] found no differences between the catalase and superoxide dismutase activity profiles in two lines of oak microcuttings, despite their different ontogenetic origins and rooting abilities. Instead, the authors found a high level of catalase activity (CAT-2) in both the basalledus and roots of rooted microshoots, irrespectively of the line origin, indicating this isoform is specifically related to rooting. However, CAT-2 cannot be used to predict the rooting ability of a cutting, since this isoform is equally active in both lines, despite their different rooting capacities. In a previous report, we described the identification by differential display of the *QrCPE* cDNA, which is differentially expressed during *in vitro* culture of juvenile-like and mature microshoots of English oak [25]. The deduced amino acid sequence showed *QrCPE* encodes a small putative cell wall protein rich in glycine and histidine residues. Accumulation of *QrCPE* mRNA was greater in mature microshoots than in juvenile-like microshoots at the end of the multiplication period. The highest accumulation of *QrCPE* mRNA was in roots, and preferential accumulation was detected in specific organs of ontogenetically older shoots. Additionally, expression analysis of *QrCPE* in juvenile, juvenile-like and mature material from soil-grown oak plants indicated this gene is expressed from embryonic to mature phases, and is progressively down-regulated during plant maturation [25]. These results suggest regulation of the *QrCPE* expression in a phase-dependant manner in the *in vitro* conditions.

Expression of the *QrCPE* gene was also investigated in juvenile-like microshoots during the *in vitro* rooting process. Northern blot analysis revealed induction of *QrCPE* expression by auxin in the basal segments of the rooting competent microshoots during the early stages of AR formation (Fig. 3).

The steady-state level of accumulation of *QrCPE* mRNA was higher in juvenile microshoots treated with auxin than in untreated microshoots harvested at the end of proliferation cycle, with the highest amount of transcripts being detected after 24 h of auxin treatment. Preliminary experiments also showed greater expression of *QrCPE* in juvenile-like microshoots treated with auxin than in auxin-treated mature microshoots during the first days of AR induction.
Figure 3. Expression of QrCPE during adventitious root (AR) formation in juvenile-like microshoots of English oak (Quercus robur). Total RNA was isolated from the base of microshoots sampled at the end of the multiplication cycle (0) or 1 (1+), 2 (2+), and 3 (3+) d after a 24-h treatment with 123 μM indole-3-butyric acid (IBA). 20 μg of RNA from each sample was loaded in each lane, subjected to electrophoresis in a 1.2% agarose gel containing formaldehyde, transferred to nitrocellulose filter and hybridized with the corresponding 32P radioactive probe. The 18S and 25S rRNA bands stained with ethidium bromide in the corresponding gels prior to blotting are shown as loading controls.

The steady-state level of accumulation of QrCPE mRNA was higher in juvenile microshoots treated with auxin than in untreated microshoots harvested at the end of proliferation cycle, with the highest amount of transcripts being detected after 24 h of auxin treatment. Preliminary experiments also showed greater expression of QrCPE in juvenile-like microshoots treated with auxin than in auxin-treated mature microshoots during the first days of AR induction. Because the in vitro rooting capacity of oak juvenile-like microshoots is always greater than that of mature microshoots, the greater expression of QrCPE in rooting competent shoots treated with auxin may also be correlated with the greater ability of these shoots to produce roots. Similarly, the 5GN4 gene isolated from loblolly pine was also found to be differentially expressed in juvenile loblolly pine shoots and mature shoots prior to AR formation, and was induced by auxin in rooting competent shoots [16]. Moreover, the analysis of the deduced amino acid sequences of both genes (5GN4 and QrCPE) showed homology with the nodulins involved in the early stages of root nodule development in European alder (Alnus glutinosa) [33] and barrel medick (Medicago trunculata) [34].

Previously reported results on the QrCPE gene [25] indicate QrCPE expression is regulated by specific cell types and dependent on ontogenetic stage. Our results presented here demonstrate the auxin-mediated induction of QrCPE in rooting competent shoots. Together these results suggest QrCPE may play a role in adventitious rooting. However, a more extensive analysis of gene expression must be carried out to confirm the differential regulation of this gene by auxin in juvenile-like and mature microshoots.

Additional studies are also necessary to determine the specific localization of the QrCPE mRNA during AR formation.

Gene expression analysis of rooting related genes in European chestnut microshoots
We selected and cloned five cDNA bands from the differential display gels and named them Cs114F2A, Cs714F2G, Cs410A0C, Cs312A0C and Cs914F2G. As expected, database searches for these cDNAs, which corresponded to 3’UTR, did not reveal any significant homology with known sequences, since homologous genes differ dramatically in their 3’UTR and also, this region is frequently not included in GenBank data [35].

Expression of the above-mentioned cDNA clones was analyzed in juvenile-like and mature microshoots of chestnut after treatment with or without auxin for 1 min and then transferred onto the auxin-free medium to determine if any of these cDNAs were differentially expressed in the two phases during the first stages of AR induction.

Initial expression patterns of Cs114F2A and Cs714F2G cDNAs were similar in the analyzed shoots (Fig. 4). The mRNA levels of both cDNAs in juvenile-like and mature microshoots, whether treated with auxin or not, increased notably 1 h after the shoots were transferred onto the auxin-free medium, and then decreased and remained at a constant level (Cs114F2A; Fig. 4A), or became undetectable (Cs714F2G; Fig. 4B). Twelve hours after treatment, the expression of both cDNAs was induced by IBA in juvenile-like shoots and reverted to basal levels in untreated shoots and IBA-treated mature shoots. The relatively large amounts of Cs114F2A and Cs714F2G transcripts detected only in juvenile-like shoots 12 h after IBA treatment may be related to the first cytological events of de-differentiation of specific cells, leading to root primordia formation and thus to the competence of these shoots to form roots in response to auxin. Histological analysis of root induction in juvenile-like microshoots of chestnut showed certain cells in the cambial zone were activated 12 h after IBA treatment. Those cells stained more densely and their nuclei and nucleoli became more prominent than before treatment [19].

The mRNA levels for Cs410A0C were highest in the juvenile-like shoots treated with IBA (Fig. 5A). An abundance of Cs410A0C transcript above basal levels was first detected 12 h after transfer onto the auxin-free medium in juvenile-like and mature shoots, with or without IBA treatment, and the levels increased in juvenile-like shoots between 12 to 72 h after IBA treatment. Some induction was also observed in untreated juvenile-like and mature shoots, where Cs410A0C was expressed at low levels from 12 to 72 h after transfer with the lowest mRNA accumulation in untreated mature shoots. Thus, Cs410A0C was up-regulated by auxin after 12 h of treatment, and auxin induced effect was greater in juvenile-like shoots than in mature shoots.

The expression pattern of Cs312A0C was also analyzed in juvenile-like and mature microshoots of chestnut, with or without IBA treatment. Expression of Cs312A0C was greatest in juvenile-like shoots treated with IBA for all the times analyzed. The greatest amount of Cs312A0C was in juvenile-like shoots treated with IBA (Fig. 5B). Juvenile-like shoots also contained high levels of Cs312A0C mRNA at the end of the proliferation cycle, and these levels fell drastically between 12 to 24 h after auxin treatment, and then increased again to the highest levels from 48 h onwards. In the untreated juvenile-like and mature shoots, expression was reduced to basal levels, which were almost undetectable, for all the times analyzed (15 min, 1, 12, 24, 48 and 72 h after transfer). Moderately high amounts of transcripts were also detected in mature shoots sampled 15 min and 1 h after IBA treatment, however the levels of Cs312A0C mRNA were lower than those detected in IBA-treated juvenile-like shoots at the same time. One hour after
IBA treatment, the amount of $Cs312A0C$ mRNA in juvenile-like shoots fell, and increased again after 48 h of treatment.

**Figure 4.** Expression of the $Cs114F2A$ (A) and $Cs714F2G$ (B) cloned fragments in shoot cultures derived from juvenile-like basal shoots (Bs) or mature crown branches (C) of a P2 European chestnut (*Castanea sativa*) tree. Total RNA was isolated from the base of shoots at the end of the multiplication cycle (0) or from shoots treated with (+) or without (-) 4.9 mM indole-3-butyric acid (IBA) for 1 min and sampled at the time intervals indicated for RNA isolation and gel blot analysis. For each sample, 20 $\mu$g of total RNA was subjected to Northern analysis under the same conditions as described in Fig. 3. The ethidium bromide-stained gels indicate RNA integrity and uniform loading before blotting.
Figure 5. Expression of the *Cs410A0C* (A) and *Cs312A0C* (B) cloned cDNAs in shoot cultures derived from juvenile-like basal shoots (Bs) or mature crown branches (C) of a P2 European chestnut (*Castanea sativa*) tree. Total RNA was isolated from the base of shoots at the end of the multiplication cycle (0) or from shoots treated with (+) or without (-) 4.9 mM indole-3-butyric acid (IBA) for 1 min and sampled at the time intervals indicated for RNA isolation and gel blot analysis. For each sample, 20 μg of total RNA was subjected to northern blot analysis under the same conditions as described in Fig. 3. The 18S and 25S rRNA bands stained with ethidium bromide in the corresponding gels prior to blotting are shown as loading controls.

Our results indicate expression of *Cs410A0C* and *Cs312A0C* reached the highest levels in auxin-treated juvenile-like shoots 48 and 72 h after IBA treatment. The trend observed with *Cs410A0C* was similar to the response reported for the expansin gene during the early stages of AR induction in hypocotyl stem cuttings of loblolly pine in response to auxin [14]. Peak expression of the expansin was detected between the 24 to 48 h after auxin induction, which coincides with the earliest events in the adventitious rooting in the rooting competent cells within the stem. Increased levels of five AUX/IAA genes were also detected in hypocotyl cuttings of loblolly pine after auxin treatment [15]. However, the abundance of all five mRNAs in loblolly pine increased, even when
cuttings were not treated with auxin; the highest levels occurred 1 d following cutting preparation and declined to basal levels after 3 d. The expression of *Cs914F2G cDNA in juvenile-like and mature shoots* treated with IBA (Fig. 6) revealed a different pattern compared to *Cs410A0C* and *Cs312A0C*. Auxin induction was greater in mature than in juvenile-like shoots, and this induction was detected in mature shoots as early as 15 min after IBA treatment. Expression of *Cs914F2G* in juvenile-like shoots treated with IBA and sampled at different times showed similar changes in mRNA levels as for the *Cs114F2A* and *Cs714F2G* cDNAs. However, the greater expression in mature shoots, which do not form roots in response to auxin, than in juvenile-like shoots suggests the differential expression of this cDNA may be negatively related to rooting ability. Woo et al. [11] also reported the isolation of a PRP gene from English ivy that is expressed at higher levels in mature English ivy than in juvenile petioles, after auxin treatment. As already mentioned, we cannot discuss the putative biological function of these five chestnut cDNAs in relation to their sequences, since the relevant information is still not available. The 5´RACE experiments are being carried out to obtain the full sequences of these cDNAs, which will provide valuable information for elucidating their possible functions. Moreover, *in situ* localization of those genes will be able to provide further insight into their functional significance.

![Figure 6](image_url)

*Figure 6.* Expression of the *Cs914F2G* cDNA in shoots cultures derived from juvenile-like basal shoots (BS) and from mature crown branches (C) of a P2 European chestnut (*Castanea sativa*) tree. Total RNA was isolated from the base of shoots at the end of the multiplication cycle (0) or from shoots treated with 4.9 mM indole-3-butyric acid (IBA) (+) for 1 min and sampled at the time intervals indicated for RNA isolation and gel blot analysis. For each sample, 20 μg of total RNA was subjected to northern blot analysis under the same conditions as described in Fig. 3. The 18S and 25S rRNA bands stained with ethidium bromide in the corresponding gels prior to blotting are shown as loading controls.

The expression pattern of the cloned *QrCPE cDNA* in juvenile-like European chestnut microcuttings was examined to test the effect of auxin during the first stages of the AR
induction process. This analysis was carried out on the basal (Fig. 7A) and apical portions of the same microshoots (Fig. 7B). Northern blot analysis with QrCPE insert as a probe revealed homologous mRNA species were more abundant in IBA-treated than non-treated shoots. Although auxin induction of QrCPE in the basal portion of shoots was greater 12 h after IBA treatment than 24 h after treatment (Fig.A), in the apical portion of shoots, auxin had a greater effect on QrCPE induction 24 h after treatment (Fig. 7B). The level of QrCPE induction reverted to basal levels in the apical portion of untreated shoots 24 h after transfer (Fig. 7B).

These data from European chestnut support the results obtained with English oak microshoots and indicate the QrCPE gene is up-regulated by auxin in juvenile-like microshoots of chestnut and oak during the first stages of adventitious rooting. The ARRO-1 gene isolated from apple (Malus spp.) during the induction of rooting in stem discs is also up-regulated by auxin during the induction phase of AR formation [36], and may have potential as a molecular marker for AR formation in woody plants. Induction of ARRO-1 is sustained in the apple disc after removal onto an auxin-free medium. Similarly, the induction of QrCPE does not appear to depend on the presence of the auxin stimulus, as its expression is also sustained in the chestnut shoots treated with IBA for 1 min and then transferred onto the auxin-free medium.

**Figure 7.** Expression of QrCPE in shoot cultures derived from juvenile-like basal shoot cultures of a P2 European chestnut (Castanea sativa) tree. Total RNA was isolated from the base (A) and from the apical portion (B) of shoots at the end of the multiplication cycle (0) or from shoots treated with (+) or without (-) 4.9 mM indole-3-butyric acid (IBA) for 1 min and sampled at the time intervals indicated for RNA isolation and gel blot analysis. For each sample, 20 μg of total RNA was subjected to northern blot analysis under the same conditions as described in Fig. 3. The 18S and 25S rRNA bands stained with ethidium bromide in the corresponding gels prior to blotting are shown as loading controls.

Three genes isolated from grapevine (Vitis vinifera) encoding an actin depolymerizing factor (ADF) protein [37] and two proline-rich cell wall proteins [38] were also found to be induced during rooting in stem cuttings of grapevine. Only the VvADF gene was
induced by auxin during the first 48 h of rooting suggesting the three genes may play an important role in the initiation of new roots on grapevine cuttings either by modifying organization of the actin cytoskeleton in the initial cells of roots or by altering the cell wall properties to enable root emergence. With the *QrCPE* gene, there was correlation between the induction of gene expression in auxin-treated juvenile-like shoots during the first events of de-differentiation of certain cells in the cambial zone prior to root initiation and the ability of these shoots to form roots after the auxin treatment.

We have also characterized two *SCARECROW-LIKE* genes (*PrSCL1* and *CsSCL1*) induced by auxin in rooting competent cuttings of Monterey pine and European chestnut [18]. The presence of these *SCARECROW-LIKE* genes may play a role in the early stages of AR formation in these species.

The amount of *CsSCL1* transcripts increased in response to IBA within the first 24 h of the root induction process, which coincides with cell reorganization, before the activation of cell divisions leading to the production of AR meristems. The *CsSCL1* gene belongs to the GRAS protein family, which are involved in auxin signaling and root development [39]. Moreover, *Arabidopsis SCR* gene (*AtSCR*) plays an essential role in the determination and maintenance of the root apical meristem identity and root patterning [40,41] and is expressed during the AR formation in excised hypocotyls from *Arabidopsis* plants [42]. Parallels between *AtSCR* and the expression data of *CsSCL1* suggest a putative role for *CsSCL1* in AR formation from stem cuttings.

**Molecular markers for adventitious rooting in other Fagaceae species**

To date, there are few reports concerning molecular markers of adventitious rooting in Fagaceae species other than those described above for English oak and European chestnut. The temporal expression of a B-type cyclin gene associated with the cell cycle during AR induction is reported to occur during adventitious rooting of cork oak (*Quercus suber*) microshoots [43]. Expression of this gene increased after 48 h of treatment with auxin, probably in association with the first cell divisions. The expression detected in the control shoots appeared to be related to the unorganized cell divisions associated with callus formation.

A few biochemical markers for adventitious rooting in the Fagaceae have been suggested. Changes in peroxidase activity and peroxidase isoform patterns have been proposed as biochemical markers of adventitious rooting in different species [44-47]. The study of peroxidase profile patterns during the *in vitro* rhizogenesis of Antarctic beech (*Nothofagus antarctica*) microshoots also showed qualitative and quantitative changes in isoperoxidase expression during adventitious rooting [48]. These authors found four acidic peroxidases appeared during the induction phase of rooting and variation in the number and activity of isoperoxidases was a good indicator of adventitious rooting ability. Differences in the activity of soluble peroxidases and the electrophoretic patterns of soluble anionic peroxidase isoforms were also shown for rooting and non-rooting genotypes of Cretan ebony (*Ebenus cretica*) cuttings [49]. Exogenous auxin treatment can also increase the level of peroxidase activity in the rooting zone of teak (*Tectona grandis*) shoot cuttings [50]. Moreover, polyamines may be possible markers of the rooting process in woody species [51-54]. In cork oak and grapevine microshoots the concentration of free putrescine increased in response to root
induction treatment, and thus may also be used as a reliable marker of in vitro root induction in both species [55]. The role of polyamines in AR formation is more deeply discussed in the chapter by Kevers et al. in this book.

In the near future, we hope more biochemical and molecular markers will be identified in relation to adventitious rooting in woody species and more specifically in Fagaceae species. We are currently working to obtain the full sequences of the five above mentioned cDNAs of European chestnut. Moreover, the spatial and temporal expression of genes is being carried out to localize the mRNAs at the cellular level during the adventitious rooting process of European chestnut and English oak. We also developed a genetic transformation system in chestnut [56] that we will use in the near future to investigate the functional analysis of candidate genes. To enhance our knowledge of gene function during AR formation more efficient methods of regeneration and genetic transformation are required for woody species. Alternatively, Arabidopsis can be used for complementation of mutants and transformation analysis.

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References