Histology of Adventitious Root Formation in Four Woody Species and Effect of a Synthetic Auxin[1]

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Abstract

Cutting propagation provides uniform plants that retain the original characteristics of the donor. This method requires forming adventitious roots. Our previous studies indicate that semi-lignified stem cuttings of American chestnut [*Castanea dentata* (Marshall) Borkh.] and yellow camellia (*Camellia nitidissima* Chi.) exhibit low levels of the rooting-promoting hormone indole-3-acetic acid and high levels of other hormones that inhibit rooting induction and development. Additionally, their hormone distribution between leaves and stems differs from that of easy-to-root poplar. This unfavorable endogenous hormone profile may contribute to the recalcitrance of rooting in American chestnut and yellow camellia cuttings. In this study, we found that the vascular cambium region in American chestnut cuttings was the least active and most condensed, with clusters of active cell division not presenting until 48 days after excision and auxin induction. American chestnut stems possess a closed sclerenchyma ring, while sclerenchymata in yellow camellia, poplar hybrids (*Populus* spp.), and weeping willow (*Salix babylonica* L.) cuttings are dispersed. Promoter analysis of an adventitious root-promoting gene, *AINTEGUMENTA*, showed that the number of auxin-responsive *cis*-elements in poplar is at least three times greater than in American chestnut, likely contributing to the contrasting rooting response with auxin between the two species. Compared to 1-naphthaleneacetic acid, a slow-release synthetic auxin, 4-chlorophenoxyacetic acid-l-tryptophan-OMe, induced more adventitious roots in American chestnut cuttings.

Species used in this study: American chestnut [*Castanea dentata* (Marshall) Borkh.], poplar hybrids '*Ogy*' [*Populus* × *euramericana* (Dode) Guinier] and 717-1B4 (*P. tremula* L. × *P. alba* L.), weeping willow (*Salix babylonica* L.), yellow camellia (*Camellia nitidissima* Chi.).

Chemicals used in this study: 1-Naphthaleneacetic acid (NAA), 4-chlorophenoxyacetic acid–l-tryptophan-OMe.

Index words: Adventitious root formation, auxin, clonal propagation, vegetative cuttings.

Significance to the Horticulture Industry

Plant propagation is a vital part of the agricultural, forestry, and horticultural industries. Among the many forms of plant propagation, cuttings remain preferred for many species due to their simplicity and the ability to maintain desirable genetic traits. Although adventitious root formation in cuttings is a prerequisite step in clonal propagation, the process is not well understood, particularly in trees. Furthermore, some economically important plant species, including those used in horticulture, ornamentals, forestry, or medicine, are recalcitrant to rooting. Our study provides new insights into adventitious root induction, which could benefit other recalcitrant woody species. The impact is of great significance in the propagation of elite genotypes for nurseries, research, and conservation.

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Introduction

Adventitious roots (ARs) are roots that arise from any part of a plant other than the radicle (embryonic) root. Some species, such as corn (*Zea mays* L.), banyan trees (*Ficus benghalensis* L. 1753), and mangroves (*Rhizophora* spp.), naturally grow adventitious roots as part of their normal development to provide additional support, improve access to nutrients and water, or enable vegetative propagation (Steffens and Rasmussen 2015). Adventitious roots can also be induced in response to stress conditions, such as wounding. Taking advantage of this feature, propagation through cutting (excision) has become one of the most cost-effective and simplest methods of vegetative propagation in horticulture and forestry.

In cutting propagation, a section of a plant, commonly a shoot or leaf, is stimulated to develop roots and grow into a new plant. As an asexual method, it ensures uniformity in terms of traits such as disease resistance and flower color, which is advantageous for commercial growers and researchers aiming for consistency. Moreover, plants propagated by cutting often retain the same mature tissue characteristics of donor plants, allowing them to bypass the juvenile growth phase and reach reproductive maturity faster than seedlings. For cutting propagation to succeed, the cuttings must form adventitious roots. While some species, such as willow and poplar, readily form adventitious roots, developing these roots is a bottleneck for recalcitrant species, limiting the effectiveness of propagation by cuttings.

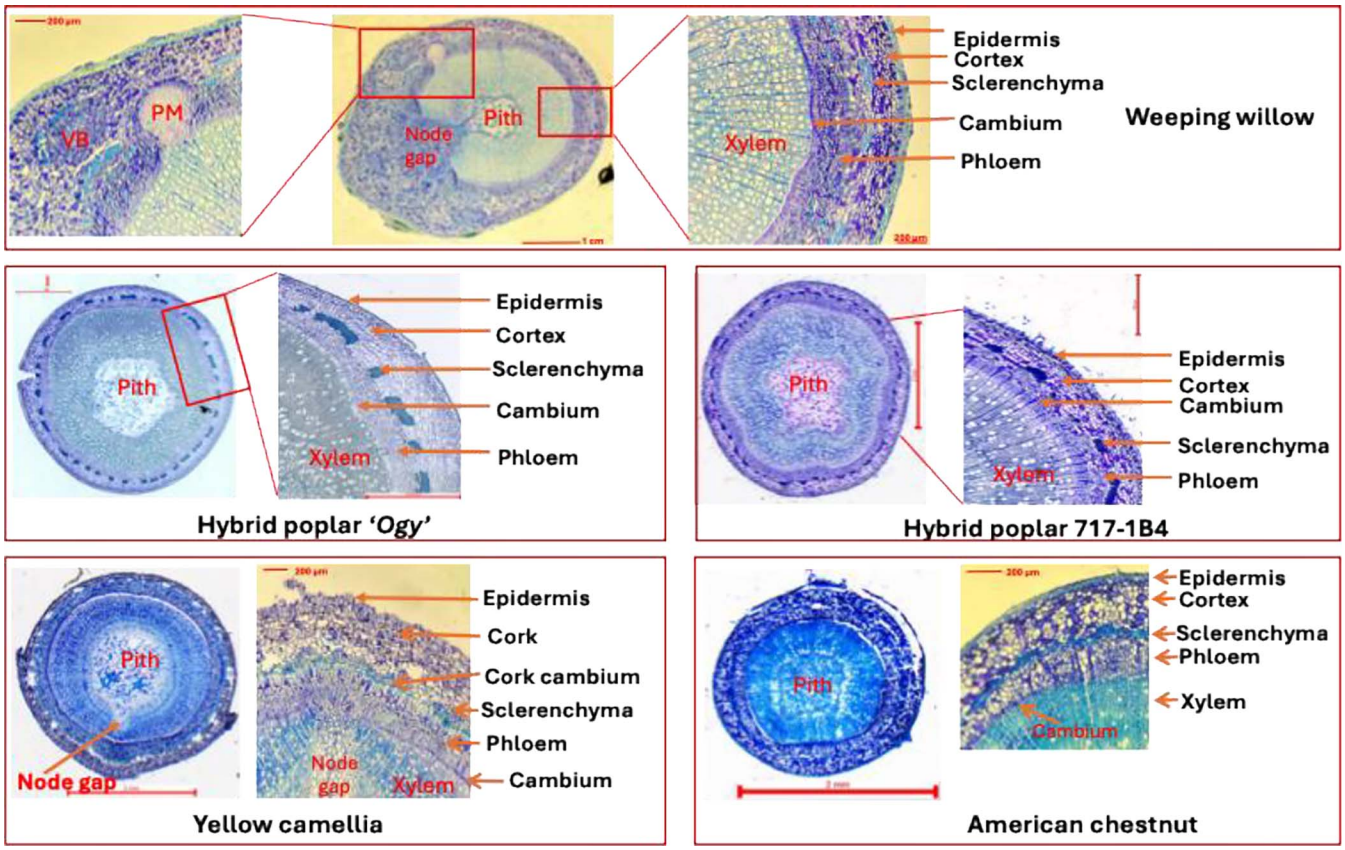

Fig. 1. Examples of stem cross sections of semi-lignified cuttings from willow, poplar, yellow camellia, and American chestnut before the rooting experiments. The staining dye was toluidine blue.

Adventitious root formation is a complex developmental process affected by both endogenous factors, such as growth regulators and the anatomical structure of the cutting, and exogenous factors, including humidity, and light. For adventitious roots triggered by wounding or stress, the rooting process is heavily governed by auxin levels, which often requires cells to dedifferentiate and regain their ability to form new root structures (Roussos 2023). In this study, we employed histological analysis to examine adventitious root development in easy-to-root species, including weeping willow (*Salix babylonica* L.) and two poplar hybrids with differing rooting capacities, as well as difficult-to-root species, yellow camellia (*Camellia nitidissima* Chi.) and chestnut (*Castanea spp*.). We also investigated the effect of a slow-release synthetic auxin (4-chlorophenoxyacetic acid–l-tryptophan-OMe) on rooting of chestnut cuttings. This compound, related to indole-3-butyric acid (IBA), has recently been found to be remarkably effective in inducing root formation in species traditionally resistant to rooting, such as *Eucalyptus* species, apple (*Malus domestica* Borkh.), and argan trees (*Argania spinosa* L.) (Roth et al. 2024).

Yellow camellias, also referred to as golden camellias, are species within the genus *Camellia*, family Theaceae. While white, pink, and red camellia are common, yellow flowers are rare. Currently, fifty-two species of yellow flowering camellias have been described in southern China and Vietnam (Manh et al. 2019). In addition to their prized flower color, yellow camellias are well known for their high concentrations of bioactive compounds such as polysaccharides, polyphenols, saponins, and flavonoids. Their leaves and flowers have long been used in traditional medicine to improve health. The dry flowers are highly valued, with costs reported to range from 320 to 700 USD per kilogram (Manh et al. 2019), underscoring the economic significance. Research indicates that auxin induction is necessary for adventitious root formation in yellow camellia cuttings (Wei et al. 2010, Wang et al. 2019). For example, *C. impressinervis* C. & L. exhibited a 91.4% rooting success rate after 4 months of growth when treated with 0.5% (by weight) IBA (Van Do et al. 2020).

Chestnut trees belong to the genus *Castanea* in the beech family (Fagaceae), with major species including the American chestnut [*C. dentata* (Marshall) Borkh.], European chestnut (*C. sativa* Mill.), Chinese chestnut (*C. mollissima* Blume), and Japanese/Korean chestnut (*C. crenata* Siebold & Zucc.), reflecting their geographical distribution. These trees are valued for both their edible nuts and timber. However, species in *Castanea* are notoriously difficult to develop adventitious roots (Vielba et al. 2020), limiting the commercial potential of the elite trees. Currently, most rooting experiments are conducted with tissue-cultured microshoots, while studies involving cuttings have largely focused on suckers and stool-bed layering (Vielba et al. 2020). Our previous studies (Lu et al. 2023, 2024) show that both American chestnut and yellow camellia cuttings have low levels of the rooting-promoting hormone indole-3-acetic acid (IAA) and high levels of other hormones that do not favor rooting induction and development. Furthermore, the distribution of hormones between leaves and stems

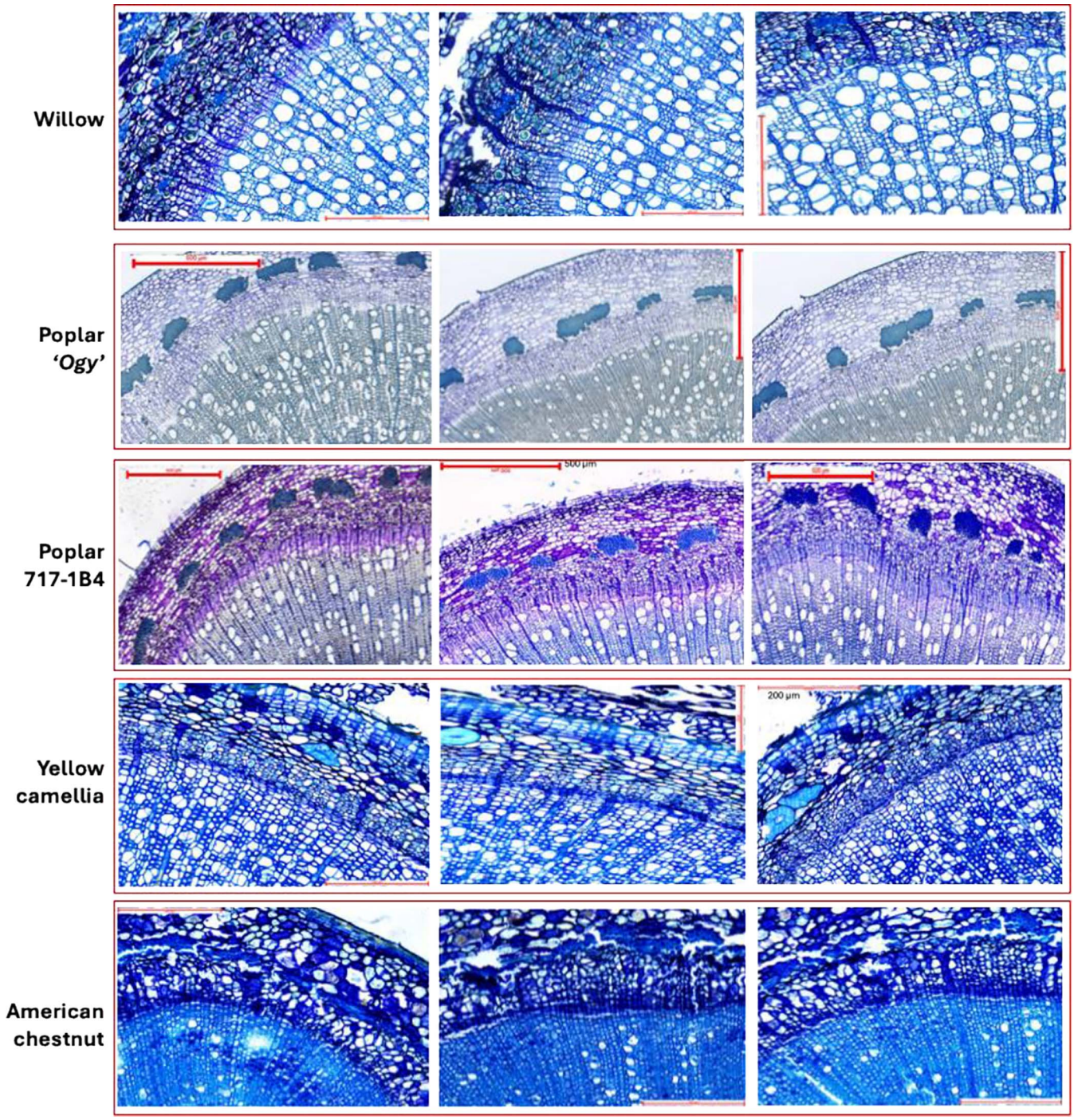

Fig. 2. Examples of vascular cambium of semi-lignified cuttings from willow, poplar, yellow camellia, and American chestnut before the rooting experiments. The staining dye was toluidine blue.

also differed from that of poplar, which may explain the recalcitrance of American chestnut and yellow camellia *C. nitidissima* cuttings to rooting. This study provides further insight into the mechanisms of AR induction and development in difficult-to-root species.

Materials and Methods

Adventitious root induction and growth conditions. Rooting experiments utilized current-year, semi-lignified shoots measuring 10-15 cm (3.9-5.9 in) in length. Two or three leaves were retained per cutting and trimmed to approximately 5 cm (5.9 in) in length. Cuttings of weeping willow were sourced from a mature tree in the South Carolina Botanical Garden, Clemson, SC. Poplar cuttings were taken from 1-year-old, cutting-propagated hybrid *'Ogy'* (*Populus* × *euramericana*) and 717-1B4 (*P. tremula* × *P. alba*) plants. Yellow camellia cuttings were collected from four-year-old grafted *C. nitidissima* plants, while American and Chinese chestnut cuttings came from 4-year-old seedlings. Chestnut hybrid cuttings were obtained from mature trees donated by breeders, including hybrids between American and Japanese chestnut species and among Japanese, American, and Chinese species.

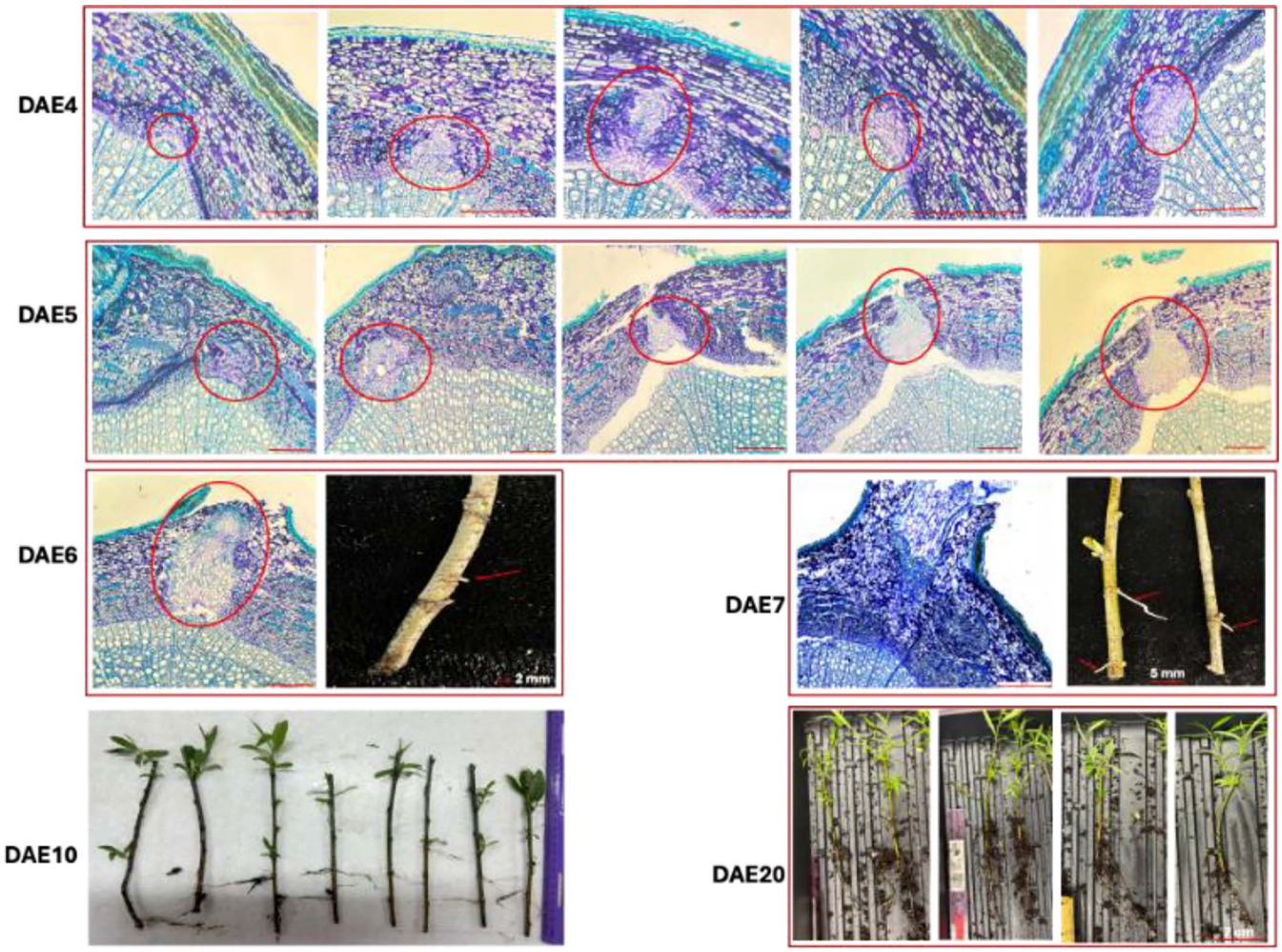

Fig. 3. Examples of adventitious roots of various days after excision (DAE) in semi-lignified willow cuttings. The staining dye was toluidine blue for cross sections. Root primordia are circled.

No auxin treatment was applied to hybrid poplar *'Ogy'* or weeping willow cuttings. For auxin induction, the basal (~2 cm, 0.8 in) of each cutting, including one or two nodes, was dipped in a 100 mg·L^{-1} 1-naphthaleneacetic acid (NAA) solution for two hours (for cuttings of yellow camellia *C. nitidissima* and American chestnut) or a 3000 mg·L^{-1} IBA with 25 mM 4-chlorophenoxyacetic acid–l-tryptophan-OMe for one minute (for chestnut cuttings). Poplar hybrid 717-1B4 cuttings were treated with Hormex Rooting Powder #16. After treatment, cuttings were placed in a rooting medium consisting of 4 parts peat moss, 0.4 part vermiculite, and 2 parts perlite (by weight). The cuttings were then placed in a transparent box with small holes on the bottom. For yellow camellia cuttings, the rooting box was covered with a transparent lid with ventilation holes. Chestnut cuttings were partially covered with sphagnum moss and the box was covered with window screen mesh. All boxes were placed in a temperature-controlled mist room at 22–23 C (71.6–73.4 F) with high humidity. The mist cycle was set to 14 minutes off and 6 seconds on, maintaining 98–99% humidity. Each species had at least 20 cuttings per experiment.

Histology. Stem samples were fixed in a 10:50:5 (v/v/v) formaldehyde/ethanol/acetic acid (FAA) solution or 10% neutral buffered formalin for 24 hours at room temperature. After fixation, samples were dehydrated through an ethanol series (70, 85, 95, and 100%), infiltrated with xylene, and embedded in paraffin. For harder stems, such as those of willow, a softening step with 20% hydrochloric acid for 24 hours was performed prior to fixation. Transverse sections, 15 µm-thick, were cut with a rotatory microtome (Leica RM2155, Nussloch, Germany) and stained with toluidine blue.

For histochemical beta-glucuronidase (GUS) staining, freshly hand-cut stem sections or adventitious roots were first incubated in a reaction buffer (14 mM NaH_2PO_4, 36 mM Na_2HPO_4, 10% Triton, 2 mM $K_4[Fe(CN)_6]$, 2 M $K_3[Fe(CN)_6]$, 10% Triton) without substrate X-Gluc (ThermoFisher Scientific, catalog number B1691) under vacuum for 5 min at room temperature (RT). After releasing the vacuum, the samples were allowed to sit at room temperature for 20–30 min. The stem sections or adventitious roots were then transferred into a fresh reaction buffer containing X-Gluc, and the vacuum step was repeated. The GUS staining was carried out at a 37 C (98.6 F) incubator for 15 hours, followed by chlorophyll removal with 80% ethanol. GUS-stained materials were stored in 70% ethanol. Microscopic images were captured using either an upright microscope equipped with a built-in digital camera (Leica

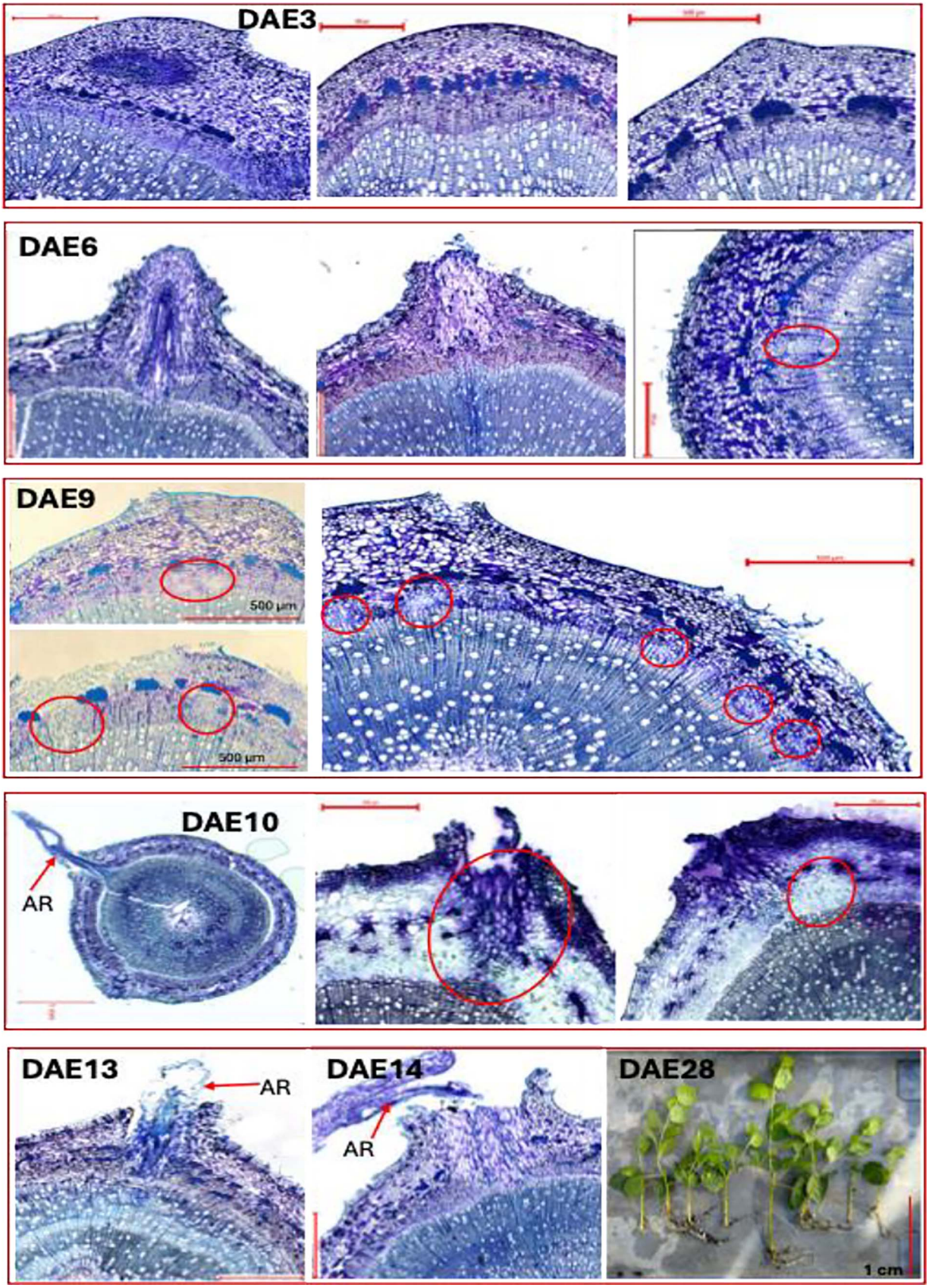

Fig. 4. **Examples of adventitious roots of various days after excision (DAE) in semi-lignified poplar hybrid *'Ogy'* cuttings. The staining dye was toluidine blue for cross sections. Root primordia are circled.**

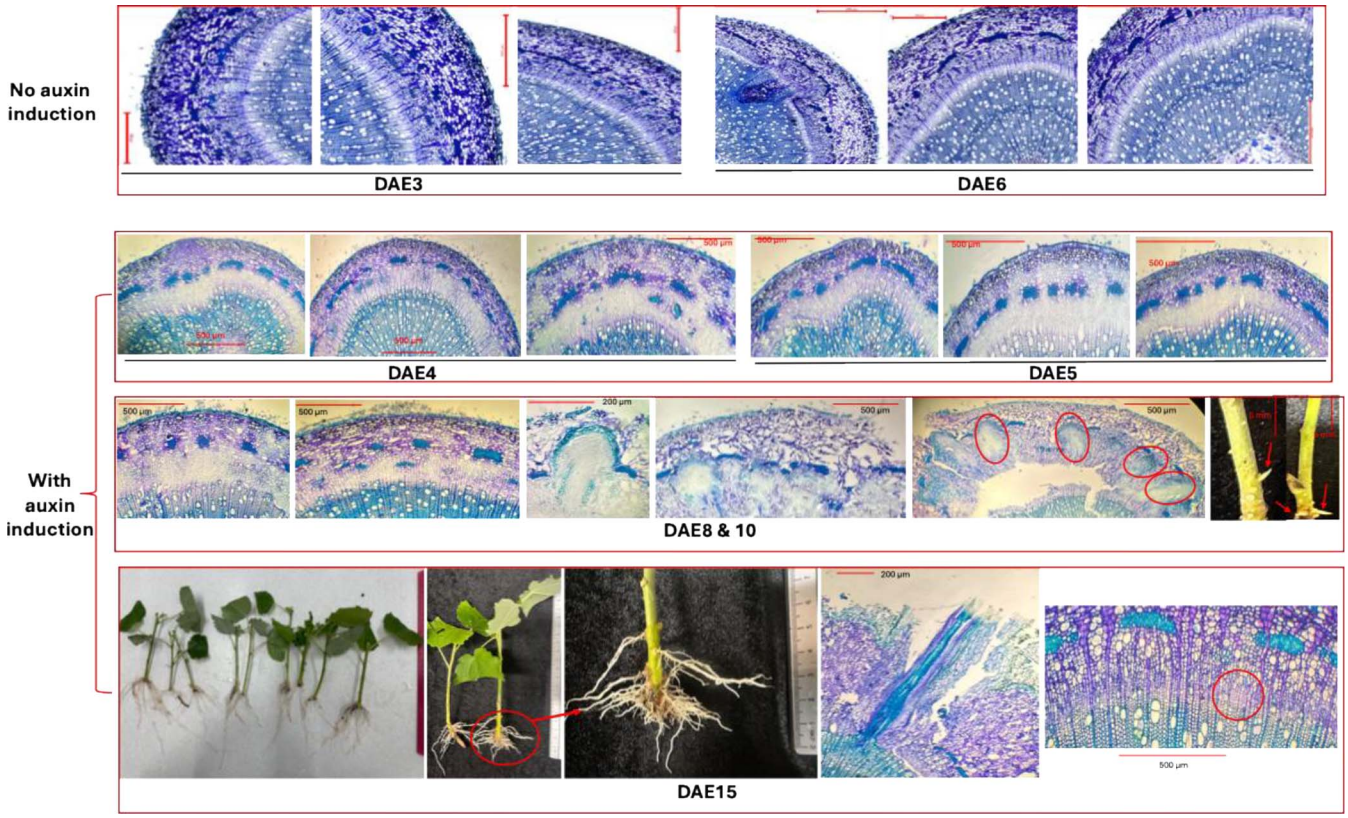

Fig. 5. Examples of adventitious roots of various days after excision (ADE) in semi-lignified poplar hybrid 717-1B4 cuttings. The staining dye was toluidine blue for cross sections. Some root primordia are circled. The rooting hormone was Hormex Rooting Powder #16.

LMD6500, Nussloch, Germany) or Meiji MT-51 (Meiji Techno America, Campbell, CA) with an iPhone 14 Pro (Apple, Cupertino, CA).

Promoter motif analysis. The promoter sequence of the *AINTEGUMENTA* (*ANT*) gene, 3kb region upstream of the start codon, was retrieved from Phytozome 13 (https://phytozome-next.jgi.doe.gov/) for all available *Populus* (*P. deltoides* Mash., *P. nigra* L. × *P. maximowiczii* Henry, *P. tremula* L. × *P. alba* L., *P. trichocarpa* Torr. & A. Gray ex Hook) and *Castanea* species [*C. mollissima* Blume Mahogany, *C. mollissima* Blume Nanking, *C. dentata* (Marshall) Borkh.)]. Promoter motifs were identified with New PLACE (A Database of Plant *Cis*-acting Regulatory DNA Elements, https://www.dna.affrc.go.jp/PLACE/?action=newplace) (Higo et al. 1999), following the methodology outlined by Lei et al. (2021).

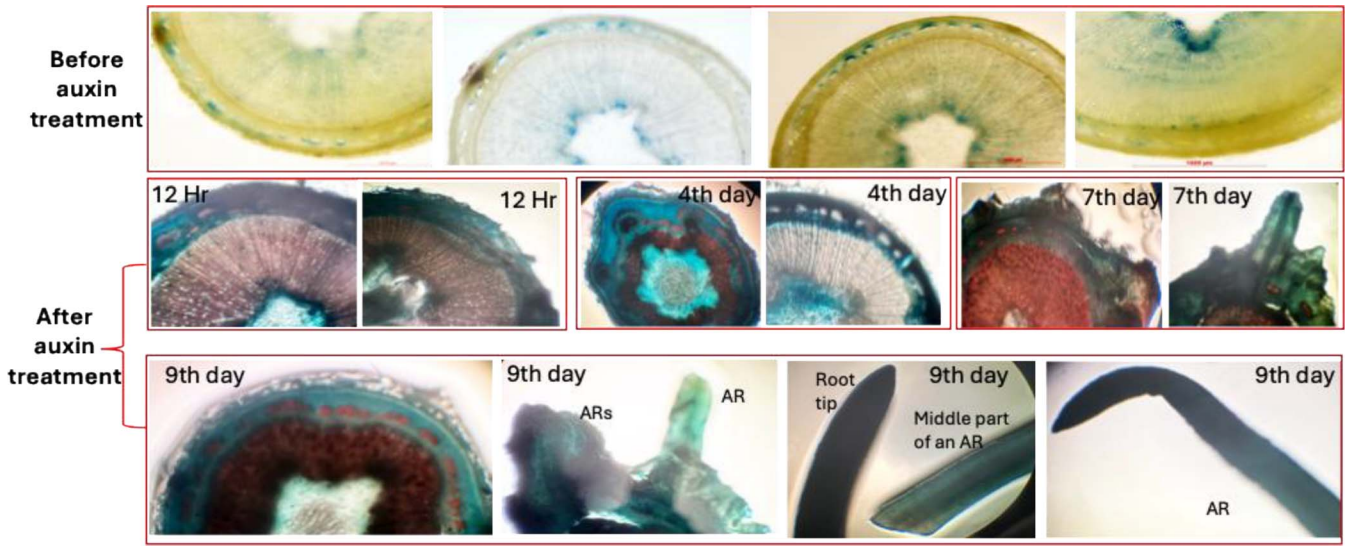

Fig. 6. Histochemical beta-glucuronidase (GUS) staining of cross sections and adventitious roots (ARs) of poplar hybrid 717-1B4 carrying *DR5::GUS*. *DR5* is an auxin-responsive promoter. Samples were incubated at 37 C for 15 hours. The cuttings were dipped with a commercial rooting powder (Hormex Rooting Powder #16).

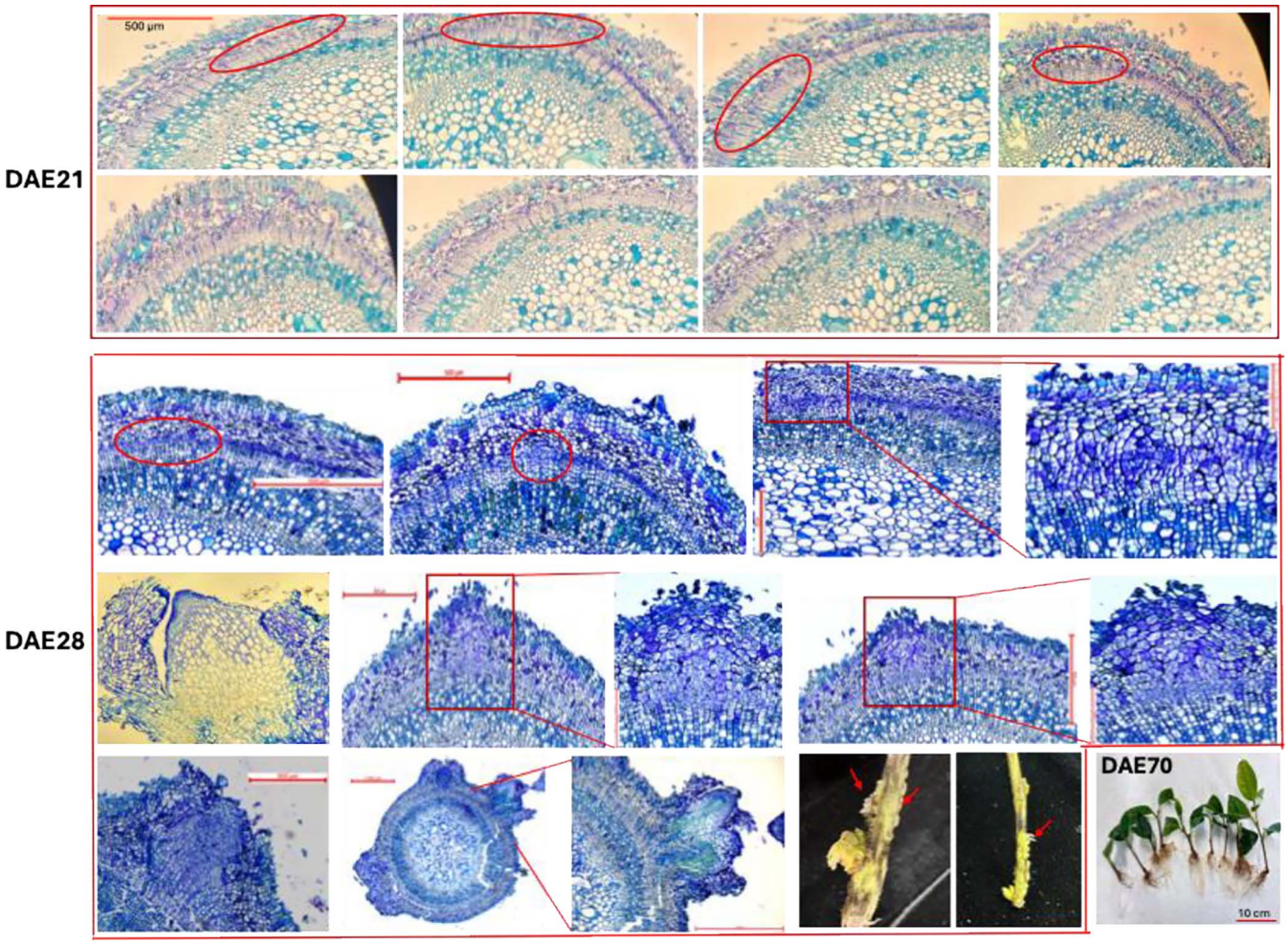

Fig. 7. Examples of adventitious roots of various days after excision (DAE) in semi-lignified yellow camellia cuttings. The cuttings were treated with 100 ppm 1-Naphthaleneacetic acid (NAA). The staining dye was toluidine blue for cross sections. Some root primordia are circled.

Results and Discussion

There is a closed sclerenchyma ring in American chestnut cuttings. Semi-lignified cuttings of all studied species share the same general stem structures: from inward to outward, there are pith, xylem, vascular cambium, phloem, cortex, and epidermis (Fig. 1). Notably, yellow camellia cuttings uniquely contained a cork cambium layer, which was not apparent in the other species. In American chestnut cuttings, we observed a closed sclerenchyma ring, a feature absent in the other species, where sclerenchymas were discontinuous. The closed sclerenchyma ring in American chestnut cuttings could hinder the emergence of adventitious roots, as adventitious root primordia must force apart the sclerenchyma before roots can outgrow. In addition, American chestnut cuttings were more lignified, as indicated by a stronger blue coloration on cross-sections, reflecting toluidine blue staining of lignified vessels and fibers (Retamales and Scharaschkin 2014). Among the studied species, the vascular cambium region appeared most condensed in American chestnut cuttings and least condensed in weeping willow and hybrid poplars (Fig. 2). A latent primordium was also observed in a weeping willow stem section (Fig. 1), suggesting a more active vascular cambium in easy-to-root cuttings.

Weeping willow cuttings exhibit rapid rooting, typically forming adventitious roots within a week. As seen in Figure 1, primordia are readily present in willow cuttings, indicating active cell division in the vascular cambium region. Without the need for auxin treatment, weeping willow cuttings achieve near-perfect rooting success, with a rooting rate approaching 100% (Lu et al. 2024). Root primordia were observed as early as the 4th day after excision (DAE4), with roots beginning to emerge from the stem by DAE6 (Fig. 3). By DAE20, a well-developed adventitious root system was established, with vascular connections between the adventitious root and the cutting stem. The high rooting efficiency is attributed to the presence of IBA and salicylic acid (SA) within the species (Rehman et al. 2018). As a result, willow water is commonly used as a natural hormone. For example, willow bark extract significantly increased the rooting percentage of olive (*Olea europaea* L.) cuttings, and extracts from willow shoots, leaves, and bark similarly enhanced the average number of roots per olive cutting (Al-Amad and Qrunfleh 2014). In apple cuttings, willow extract increased the rooting rate to 97.2%, with an average of 14.1 roots per cutting (Rehman et al. 2018). A study by Salih et al. (2024) reported that white willow (*Salix alba* L.) shoots collected in March had the highest total phenols (57 mg·L^{-1}) and IAA (365.17 mg·L^{-1}), while shoots

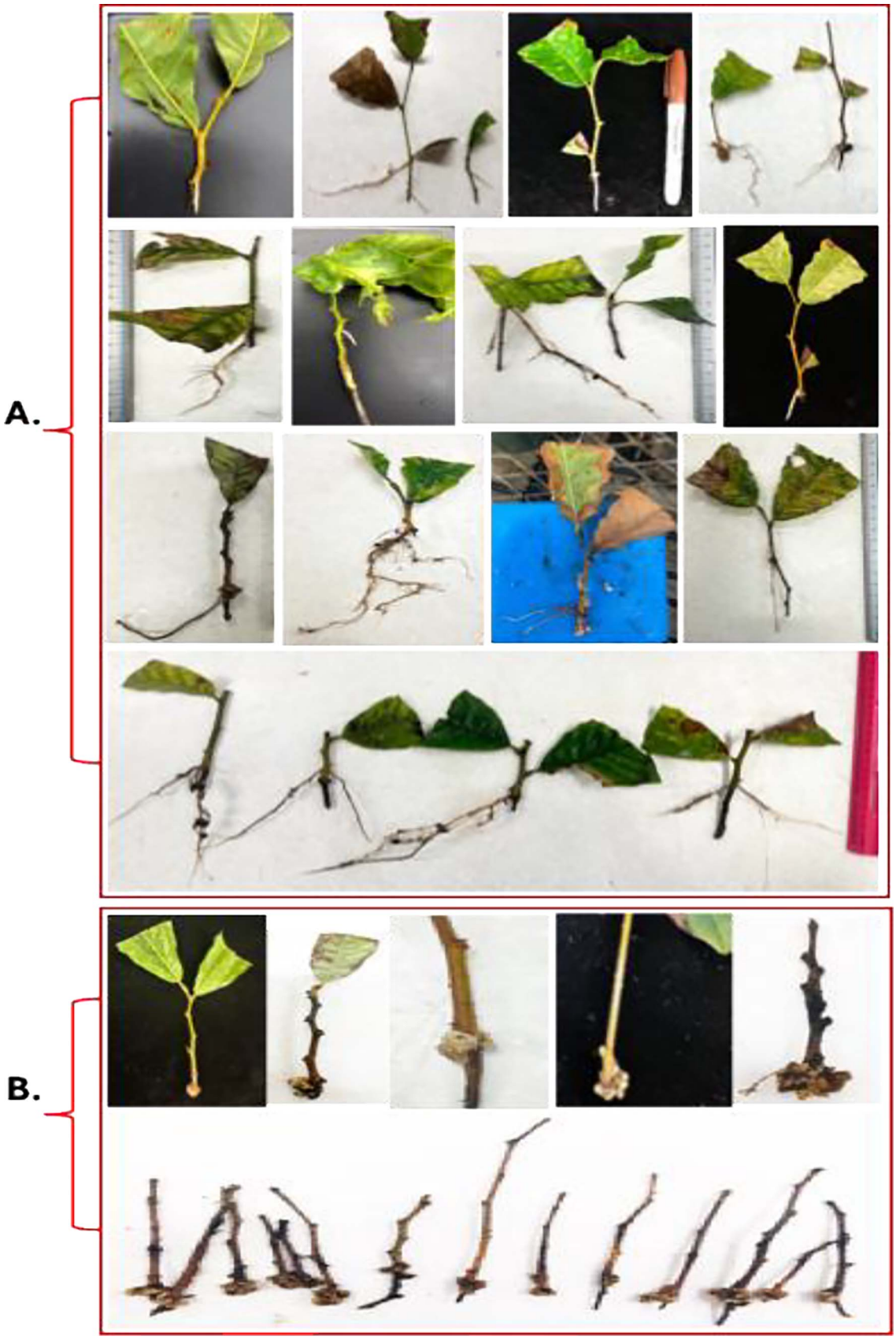

Fig. 8. **Examples of chestnut cuttings with adventitious roots (A) or calli (B). The cuttings were treated with 100 ppm 1-Naphthaleneacetic acid (NAA). Cuttings included in the experiments included American chestnut, Chinese chestnut, and hybrids.**

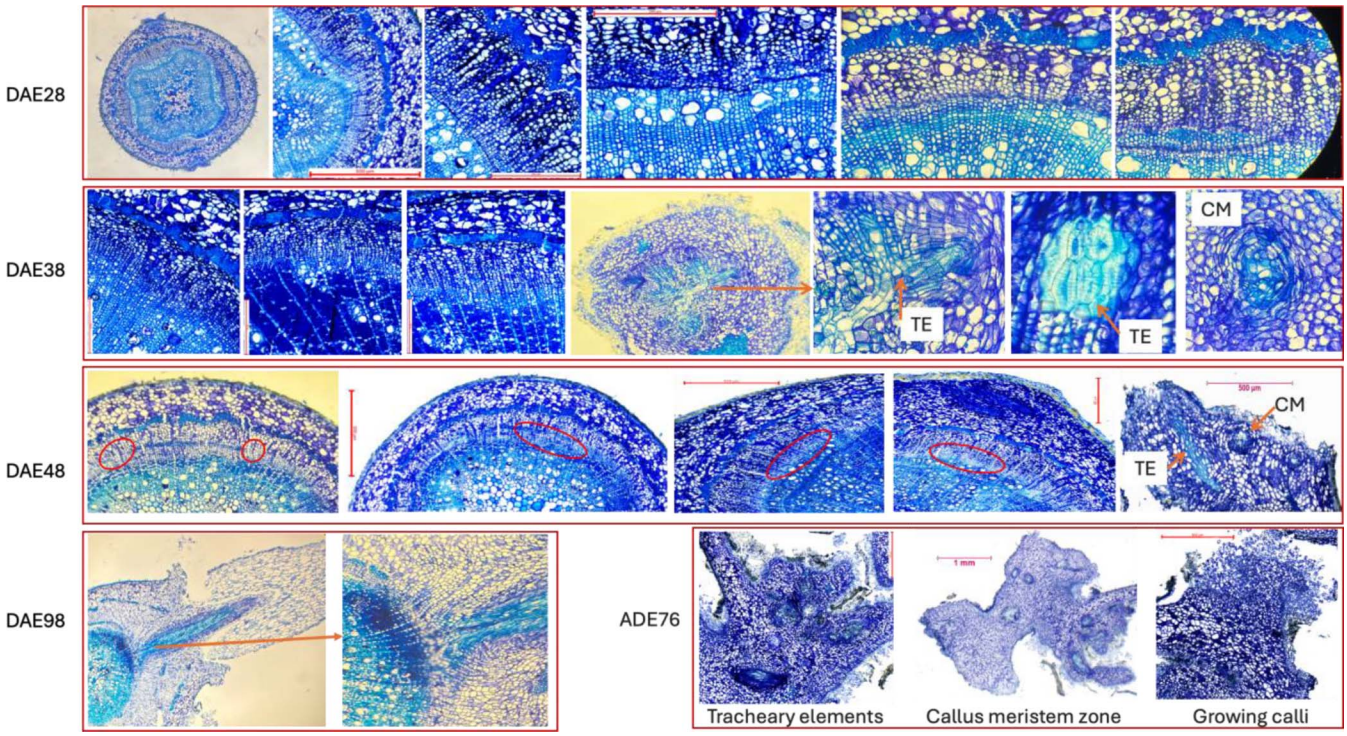

Fig. 9. Examples of adventitious roots of various days after excision (DAE) in semi-lignified American chestnut cuttings, which were treated with 100 ppm 1-Naphthaleneacetic acid (NAA). The staining dye was toluidine blue for cross sections. Some potential root primordia are circled. CM: callus meristem zone; TE: tracheary elements.

collected in April contained the highest concentration of flavonoids (44.96 mg·L^{-1}) and salicylic acid (492.61 mg·L^{-1}).

Rooting capacity varies in poplar genotypes. Poplar (*Populus*) is generally considered easy to root. However, rooting ability varies among different genotypes in *Populus*: species within the *Populus* sections Turanga, Leucoides, Tacamahaca, and Aigeiros are typically easy to root, while species in the section *Populus* tend to be more difficult (Ye et al. 2011). The hybrid *'Ogy'*, derived from *P. deltoides* Mash. and *P. nigra* L., both of which belong to the easy-to-root section Aigeiros, demonstrated a high rooting capacity. Without auxin induction, we achieved an average rooting rate of 92.6% in *'Ogy'* cuttings (Lu et al. 2024). Similar to weeping willow, adventitious roots started to emerge from *'Ogy'* cuttings within 6 days after excision (DAE6, Fig. 4). By DAE3, the vascular cambium region appeared wider compared to its initial state at DAE0 (Fig. 2). Each cutting developed multiple primordia, and the vascular tissues of the newly formed adventitious roots were successfully connected to those of the cuttings.

Hybrid 717-1B4 (*P. tremula* L. × *P. alba* L., section *Populus*) cuttings are known to form roots at a relatively low efficiency (Doty et al. 2007). Without auxin induction, the rooting rate was less than 50%, and it took ~3 weeks for the few adventitious roots to emerge from the stems. As shown in Figure 5, no adventitious root primordia were observed by DAE6. However, with auxin induction, cells in the vascular cambium region were stained lightly, suggesting thin cell walls and active cell division, by DAE4 and DAE5, with adventitious root primordia and roots forming by DAE8 and DAE10. Within two weeks, a rooting rate of 90% was achieved, and multiple adventitious roots were established, all of which were connected to the vascular tissues of the stems (Fig. 5). These results suggest that 717-1B4 cuttings respond effectively to auxin induction, significantly improving their rooting capacity.

Using an auxin-responsive promoter (DR5) to drive the expression of the beta-glucuronidase (*GUS*) reporter gene (*DR5::GUS*), Chen et al. (2013) treated hybrid poplar 717-1B4 stems for 1, 2, 4, 8, 12, 20 and 24 h with 10 μM NAA. They found that GUS induction could be detected as early as 1 h and continued to increase throughout the studied period. In the enzymatic reaction, GUS hydrolyzes the colorless substrate X-Gluc into the product 5,5'-dibromo-4,4'-dichloro-indigo (diX-indigo), which appears blue and can be seen using light microscopy (Guivarc'h et al. 1996). In the current study, we found spotty GUS staining in the protoxylem, cortex, and vascular cambium before auxin treatment (Fig. 6). By 12 hours post auxin treatment, GUS staining in the cortex became more intense. By the fourth day, the staining in the vascular cambium region became more obvious. By the seventh and ninth days, adventitious roots showed obvious GUS staining. These results indicate that 717-1B4 stems responded quickly to auxin treatment, explaining the genotype's effective rooting response. Lastly, adventitious root tips displayed a darker GUS staining than the middle region, indicating a higher accumulation of auxin in root tips.

Yellow camellia cuttings require auxin induction. Without auxin treatment, yellow camellia exhibits minimal rooting. However, with auxin application, a rooting rate as

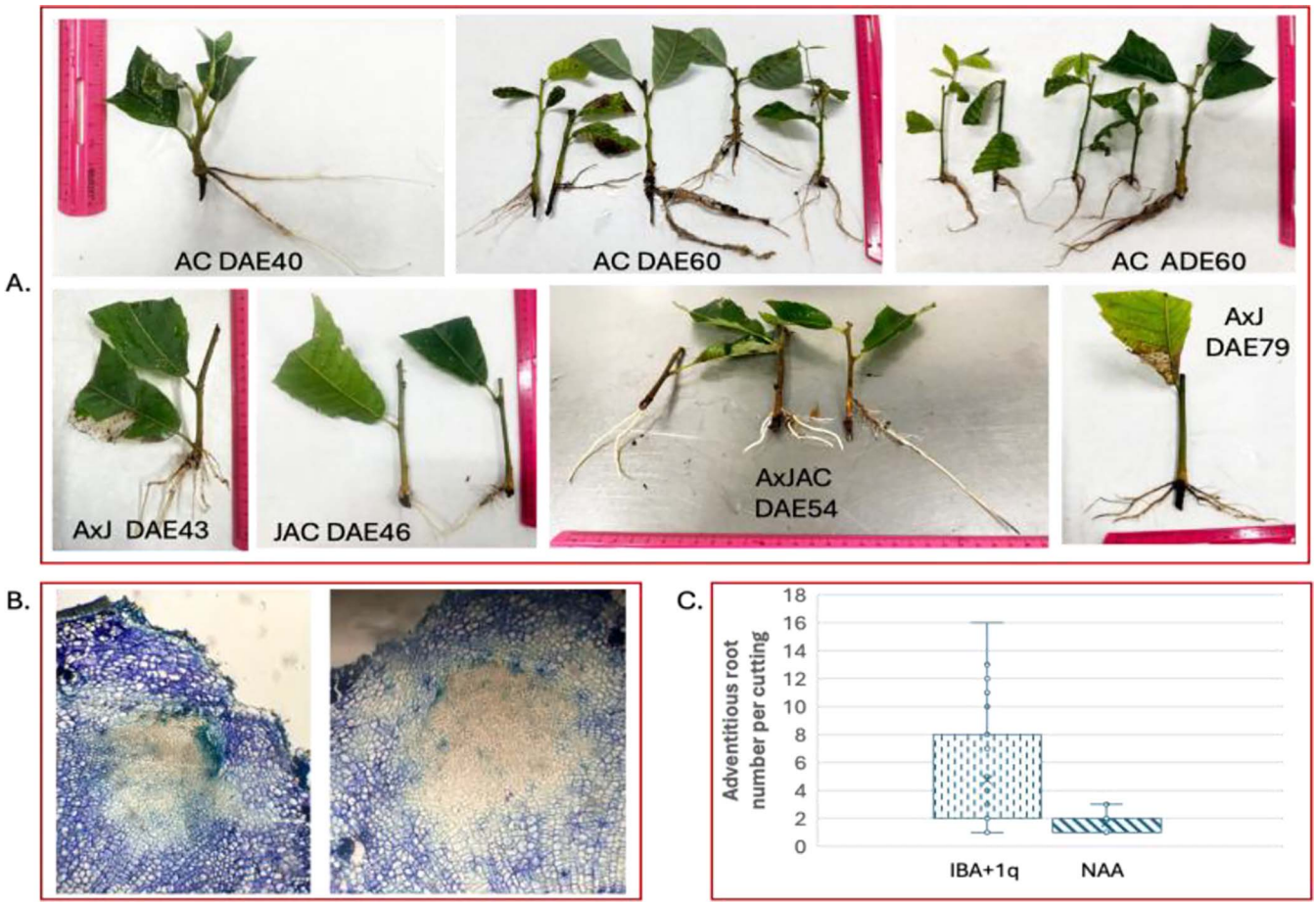

Fig. 10. Rooting induction of chestnut cuttings with a combination of IBA (3 g L^{-1}) and 25 mM 4-chlorophenoxyacetic acid–l-tryptophan-OMe. Treatment time was one minute. A: Rooted chestnut cuttings; B: AR primordia; C: Adventurous root number per cutting, n (IBA+1q)= 29, n (NAA)=25. AC: American chestnut; A×J: hybrid between American and Japanese chestnut species; AJC: hybrid among Japanese, American, and Chinese species; A×AJC: a cross between American and AJC.

high as 90% has been reported (Huang et al. 2017). In our study, we achieved an average rate of 86.3% (±5.3% SD) (Lu et al. 2024). The histological observations showed that by DAE21, the vascular cambium region became more active, with some cells appearing less condensed (Fig. 7). By DAE28, we observed the emergence of adventitious roots outside the stems, alongside adventitious root primordia and clusters of actively dividing cells inside the stems. By DAE70, well-developed adventitious root systems with multiple roots were established (Fig. 7).

Chestnut cuttings are the most recalcitrant among the species studied regarding root formation. With 100 ppm NAA application, very few adventitious roots were induced, ranging from 1 to 3 roots (Fig. 8A). Oftentimes, callus formation was observed (Fig. 8B). As reported by Lu et al. (2023), an average rooting rate of 14.7%±4.9% (standard deviation, SD) was obtained for adventitious roots without calli, while a rooting rate of 10.8% (±2.8%) was recorded for adventitious roots with calli. Moreover, 70.5% (±14.5%) of American chestnut cuttings formed calli only. Histology uncovered a thin ring of vascular cambium cells at DAE28 (Fig. 8). At DAE38, multiple layers of vascular cambium cells were formed, along with tracheary elements and callus meristem zones. At DAE48, clusters of actively dividing cells were present, giving rise to adventitious root primordia. Tracheary elements and callus meristem zones were also present at this stage. With NAA induction, it takes at least 1.5 months for ARs to emerge (Lu et al. 2023). As shown in Figure 9, a well-developed American chestnut adventitious root exhibited its vascular tissue continuous with that of the stem, and callus tissues had callus meristem zones providing disorganized clumps of cells.

A slow-release synthetic auxin (4-chlorophenoxyacetic acid–l-tryptophan-OMe, 1q) can enhance adventitious root formation in chestnut cuttings. This synthetic auxin has proven highly effective in inducing root formation in species that are traditionally difficult to propagate (Roth et al. 2024). For instance, in *Eucalyptus x trabutii*, a hybrid of *E. camaldulensis* Dehn. and *E. botryoides* Sm. known for its low rooting capacity, induction with IBA alone failed to generate adventitious roots. However, when combined with 1q, a rooting rate of 45% was achieved. For *Eucalyptus grandis* (Hill) Maiden, root regeneration was improved from 17% with IBA alone to nearly 40% when IBA was combined with 1q. Likewise, supplementation of 1q increased the rooting rate by ∼2-fold compared with IBA alone in apple rootstock clone CG41 and in two out of three clones of argan tree (Roth et al. 2024). Metabolic and functional analyses revealed that the enhanced adventitous root-promoting activity of 1q is due to prolonged auxin

signaling, facilitated by rapid initial uptake followed by slow release and clearance of the free auxin 4-chlorophenoxyacetic acid. When treated with this compound, American chestnut cuttings rooted by DAE40 (Fig. 10A). Adventitious root primordia became obvious by DAE30 (Fig. 10B). By DAE60, an average of 4.8 adventitious roots per cutting was achieved, as compared to 1.7 with NAA treatment (Fig. 10C). The rooting rate % was 65.8% (±10.2%).

Chestnut species have fewer auxin-responsive elements in the promoter of AINTEGUMENTA (ANT) gene. The *ANT* gene encodes a transcription factor that directly regulates genes involved in auxin signaling and cell proliferation (Krizek et al. 2020). Overexpression of the poplar *ANT* gene led to an increased number of adventitious roots, whereas RNA interference-mediated down-expression of its expression caused a delay in adventitious root formation (Rigal et al. 2012). When comparing hormone motifs in the promoter region of *ANT* gene between poplars and chestnut species, we found that poplars possess 4 to 7 times more auxin *cis*-regulatory elements than chestnuts (Table 1). This difference may explain the rapid and efficient response of poplar clone 717-1B4 to adventitious root induction with auxin, as well as the superior rooting results achieved in chestnut cuttings with the slow-release synthetic auxin. These results underscore the significance of auxin and auxin signaling in efficient adventitious root induction. Additionally, the promoter analysis also indicates that the chestnut *ANT* promoters have fewer total *cis*-regulatory elements (769.8 ± 3.5 vs. 825.0 ± 35.4) and abscisic acid-responsive elements (8.2 ± 0.4 vs. 16.7 ± 3.9) compared to poplar. In contrast, both poplar and chestnut species exhibit similar numbers of wounding-responsive elements (12.3±1.3 vs. 12.8±0.4) (Table 1).

Our study highlights the significance of genetic adaptability in adventitious root formation, an organogenic process that involves cell fate reprogramming to initiate a root meristem. Willows have a strong genetic predisposition to root from cuttings, with readily available primordia and preformed root initials in intact shoots (Fig. 1 and Shang et al. 2024). While hybrid poplar 717-1B4 cuttings do not root as easily as hybrid poplar '*Ogy*', the former genotype responds efficiently to auxin for rooting induction. Similarly, yellow camellia cuttings respond well to NAA treatment. In contrast, chestnut species are highly recalcitrant to rooting. Galic et al. (2014) reported a rooting rate range of 46% to 65% in American chestnut juvenile softwood and semi-hardwood cuttings treated with 1% IBA; however, few of these cuttings leafed out, and only 15% survived the winter. In our study, induction with 200 ppm NAA generated 1-3 adventitious roots per chestnut cutting. In combination with 4-chlorophenoxyacetic acid–l-tryptophan-OMe, we achieved 4.8 ARs per chestnut cutting. A well-developed adventitious root system is crucial for the survival and establishment of cuttings, as high rooting percentage and root number are the key markers of successful rooting (Roussos 2023).

In a comprehensive study of 38 *Populus* germplasm resources, Zhang et al. (2024) identified 1,944 positive selection regions related to adventitious root development,

Table 1. Numbers of hormone signal motifs present in the 3kb-promoter of *AINTEGUMENTA* (*ANT*) genes in *Populus* and *Castanea* species. Sequences were retrieved from the species' sequenced genomes in Phytozome 13.

	Species	# Total signals	Wounding response	Auxin	Abscisic acid	Ethylene	Gibberellin	Salicylic acid	Jasmonic acid	Cytokinin
Populus (poplar)	*P. deltoides* (Podel.02G125500)	862	14	13	22	3	41	6	5	1
	P. deltoides (Podel.05G160900	850	11	20	14	3	52	12	0	6
	P. nigra x P. maximowiczii (NM6_Poman.05G123800)	865	12	14	17	6	60	14	0	3
	P. nigra x P. maximowiczii (NM6_Poman.02G103000)	817	12	16	21	2	38	5	3	1
	P. *tremula x P. alba* (717-1B4_HAP1 _PtXaTreH.02G0)	852	10	14	17	7	34	7	1	0
	P. tremula x P. alba (717-1B4_HAP1_PtXaTreH.05G1)	758	12	23	15	5	39	11	1	0
	P. tremula x P. alba (717-1B4_HAP2_PtXaAlbH.02G0)	819	14	12	19	4	32	6	4	3
	P. tremula x P. alba (717-1B4_HAP2_PtXaAlbH.05G1)	830	13	14	11	4	54	16	3	3
	P. trichocarpa (Potri.005G148400)	820	13	14	20	2	42	5	2	1
	P. trichocarpa (Potri.002G114800)	777	12	13	11	5	56	15	2	4
Castanea (chestnut)	*C. mollissima* Mahogany (CmolMahoganyHAP2)	771	13	3	8	4	38	7	3	3
	C. mollissima Mahogany (CmolMahoganyHAP1)	771	13	3	8	4	38	7	3	3
	C. mollissima Nanking (CmolNankingHAP1)	773	13	3	8	4	38	7	3	3
	C. mollissima Nanking (CmolNankingHAP2)	774	13	3	8	4	38	7	3	3
	C. dentata (Caden.12G083100)	760	12	3	9	4	40	6	1	2

as well as 3,426 specific single nucleotide polymorphisms (SNPs) and 687 specific insertions/deletions (Indels) in the clones with good adventitious root development, and 3,212 specific SNPs and 583 specific Indels in the clones with poor adventitous root development, respectively. Moreover, the authors also revealed eight major putative genes associated with poplar adventitious root development, which hold the potential for improving adventitous root formation in recalcitrant species. These genetic findings provide promising targets for future research aimed at enhancing rooting capacity, particularly in species that are difficult to root.

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