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Promotion of Adventitious Root Formation of Difficult-to-Root Hardwood Tree Species

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ABBREVIATIONS

ABA	abscisic acid
AGO1	ARGONAUTE1
ARF	adventitious root formation
ARF17	auxin response factor 17
EST	expressed sequence tags
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
JA	jasmonate
K-IBA	indole-3-butyric acid-potassium salt
LRF	lateral root formation
NAA	naphthalene acetic acid
NO	nitric oxide
PCR	polymerase chain reaction
PIN	proteinase inhibitor
QTL	quantitative trait loci
Rol	root loci
SA	salicylic acid
SAM	S-adenosylmethionine synthase
SCL	SCARECROW-LIKE
SCR	SCARECROW
SHR	SHORT-ROOT
WRC	wound-related compounds

I. INTRODUCTION

North American hardwood tree species, such as alder (*Alnus* spp.), ash (*Fraxinus* spp.), basswood (*Tilia* spp.), beech (*Fagus* spp.), birch (*Betula* spp.), black cherry (*Prunus serotina*), black walnut (*Juglans nigra*), black willow (*Salix nigra*), elm (*Ulmus* spp.), hackberry (*Celtis occidentalis*), hard maple (*Acer* spp.), hickory (*Carya* spp.), oak (*Quercus* spp.), pecan (*Carya* spp.), sassafras (*Sassafras albidium*), sweetgum (*Liquidambar styraciflua*), sycamore (*Platanus* spp.), and yellow poplar (*Liriodendron tulipifera*), are important resources for the forest products industry worldwide and to the international trade of lumber and logs. The economic market for these tree species can be very high. Many of these hardwood species are also planted in the urban landscape, plantations, or orchards for seed or nut production. Timber, sawlog, and veneer log production of many of these fine hardwood species provides material for the manufacture of residential and commercial structures and furnishings and numerous specialty products, as contrasted with other hardwoods used for fuel or pulp (Pijut et al. 2007). Hardwood trees are also important for ecological reasons, such as wildlife habitat, mast production, riparian buffers, windbreaks, erosion control, watershed protection, agroforestry, conservation, land reclamation, native woodland restoration, and aesthetics (MacGowan 2003; Jacobs 2006).

Many economically and ecologically important hardwood tree species have a low genetic or physiological capacity for adventitious root formation and are considered recalcitrant to routine, commercial-scale vegetative propagation via rooted cuttings. Why this phenomenon exists for many tree species (such as ash, beech, oak, and walnut) and not for others is not fully understood. Propagation of forest or urban tree planting stock by rooted cuttings can: circumvent problems with seed viability, germination, and storage; help overcome complex dormancy issues; shorten the time to flowering or encourage consistent flowering; maintain superior genotypes; and contribute to the genetic uniformity of tree plantations (Macdonald 1986). Clonal reproduction (resulting in progeny genetically identical to the original plant material) via adventitious rooting that can be easily adapted for many species, genotypes, or cultivars will allow for the production of clones of elite, pest-, or disease-resistant trees or genetically improved trees (e.g., high-value wood) for planting and breeding programs. Some disadvantages associated with clonal reproduction and adventitious root formation can be less branched roots, more horizontal orientation, poor adventitious root distribution around the stem, or too few roots (D. Struve, person. commun.). In a tree improvement or breeding program, the genetic gains

(additive and nonadditive) associated with clonal reproduction can be substantially greater than those associated with seed- or seedling-based forestry production systems (Zobel and Talbert 1984; Ritchie 1994).

A. Adventitious Root Formation

Adventitious root formation (ARF) as used in this review can be distinguished from lateral root formation (LRF), as the development of roots on excised aerial plant parts (shoots or stems), or from an unusual point of origin on the plant (old roots that have undergone secondary growth) (Esau 1977). Hence, LRF can be defined as the development of roots in a typical succession from the primary tap root of a tree and its laterals still in the early stage of growth. The fundamental mechanism(s) that trigger or regulate the initiation and development of adventitious roots on stem cuttings from woody species is a complex physiological, genetic, and environmental process and is still largely unknown. The endogenous origin and development (division of parenchyma cells) of adventitious roots close to the vascular tissue closely resemble the process of LRF (Esau 1977). De Klerk et al. (1999) summarized the successive phases in rooting of apple microcuttings as dedifferentiation, induction, outgrowth in the stem, and outgrowth from the stem. The initial phase, dedifferentiation, was the activation of cells (to become competent) by wounding related compounds and auxin. The induction phase was the initiation of cell division (to become committed) where auxin stimulates the formation of root meristemoids. During outgrowth in the stem phase, meristemoids develop into typical dome-shape root primordia (to become determined), and at this stage auxin (exogenously applied) then becomes inhibitory. Root primordia elongate and develop during the differentiation phase and finally grow out of the stem during the last phase of the adventitious rooting process.

B. Types of Root Formation in Stem Cuttings

There are two major types of root formation in stem cuttings: preformed and wound induced. Preformed or latent root initials are present during stem development and lie dormant until stem cuttings are made and placed in the proper environmental conditions favorable for further development and emergence as adventitious roots (Hartmann et al. 2002). Examples include willow (*Salix*) and poplar (*Populus*). Root initials often are established by the end of the season in the current year's wood and will develop from cuttings made the following season.

Species with preformed root initials generally root easily from stem cuttings (Hartmann et al. 2002).

Wound-induced roots are the major type of ARF in stem cuttings. Once the stem (or shoot) is removed from the plant (wounding), a series of wound responses occur and de novo adventitious root regeneration proceeds (Hartmann et al. 2002). At the wounded sites, sealing off of the wound (protection from desiccation and pathogens) occurs by the production of suberized, protective cells. Cells begin to divide, and a layer of parenchyma cells (callus) then forms at the wound site. The use of auxin during adventitious rooting enhances the formation of callus in addition to inducing the formation of roots. Cells in the vicinity of the vascular cambium and phloem (near the source of hormones and carbohydrates) begin to divide and initiate adventitious roots (Hartmann et al. 2002). The developmental phases that occur in the stem cutting follow the stages described previously for apple microcuttings.

The maturation-related loss of adventitious rooting competence in hardwood tree species is a major limiting factor to the clonal propagation of these woody species. Successful vegetative propagation via ARF in cuttings of hardwood tree species can be achieved if numerous factors are considered carefully. This chapter concentrates on what is known or largely unknown about the genetics and physiological basis of ARF, various factors that affect rooting of cuttings from some valuable North American hardwood tree species, individual case studies of horticultural versus forestry species, and future challenges and opportunities in understanding ARF of woody species. A comprehensive review of the literature was not possible, but we hope that we have captured or highlighted many important species and research studies over the last 12 years (1997–2009).

The *Rol* (root loci) genes *rolA*, *rolB*, *rolC*, and *rolD* are plant oncogenes carried on root-inducing plasmids of the plant pathogen *Agrobacterium rhizogenes*. Natural infection of plants by *A. rhizogenes* causes hairy-root disease characterized by a massive growth of adventitious roots at the site of infection. Transformation of species with *A. rhizogenes* or with its *rol* genes produce hairy roots from which adventitious shoots (whole plants) can be regenerated, but typically with the characteristic hairy-root phenotype. The phenotypic alterations caused by the expression of these genes vary in degree but can include reduced apical dominance in both stems and roots, dwarfism, shortened internodes, abundant lateral branching, wrinkled and wider leaves, smaller leaves, adventitious root production, altered flowering and morphology, and reduced pollen and seed production (Welander and Zhu 2006). Each *rol* gene or combination of *rol* genes produces specific developmental alterations in transformed

plants, but the biochemical functions of *rol* genes are not yet fully understood. The altered phenotypes can be advantageous in some ornamental and horticultural crop species (Casanova et al. 2005), but may not prove very advantageous in the genetic improvement and clonal propagation of hardwood tree species for timber, sawlog, and veneer log production. For example, silver birch (*Betula pendula*) plants transformed with *rolC* and *rolD* genes or all the *rol* genes were significantly shorter, had smaller leaves, and had a bushy growth habit (Piispanen et al. 2003). Therefore, *rol* genes in the context of producing adventitious roots with hardwood tree cuttings will not be addressed in this chapter.

II. GENETICS AND PHYSIOLOGY OF ADVENTITIOUS ROOT FORMATION

A. Herbaceous and Woody Models of Root Formation

Progress understanding the fundamental biology of ARF in woody plants has been slow, and recent important advances have been based primarily on investigations using model systems such as *Arabidopsis*. Because of the advantages of herbaceous models and because of the importance of LRF, research on LRF outpaces research into ARF (which is most important in woody perennials), even in species where both types of root formation can occur or be induced. While research into LRF potentially informs ARF (Butler and Gallagher 1998; Brinker et al. 2004), the full extent to which these two processes overlap has emerged only recently. It remains to be seen if it will be possible to translate what is learned about LRF and ARF in herbaceous species to practical use in woody species. Caution is warranted because it is unlikely that the biology of ARF is univocal in plants, and it is likely that some woody species will differ substantially biologically (De Klerk et al. 1999; Sanchez et al. 2007; Negi et al. 2008). There has been some progress in understanding ARF in woody species, and the apple 'Jork 9' is probably the closest to a woody model for ARF (Butler and Gallagher 1998; Sedira et al. 2007).

1. Poplar as a Model System. Poplar might also be useful as a model, since its genome was fully sequenced and a variety of *Populus* genomics tools are available. Comparison of poplar clones or mutants that are difficult to root would be an important step in the exploitation of this species for basic research in ARF (Haissig et al. 1992). Zhang et al. (2009) exploited an approach based on mapping root extension–related

quantitative trait loci (QTL) in a pseudo–test cross between the difficult-to-root *P. deltoides* (eastern cottonwood) and easily rooted *P. euroamericana*. Mean root length and total number of roots were found to be under strong genetic control, and the traits were strongly correlated with each other. They identified five QTLs related to root growth trajectory; four of the QTLs were derived from *P. euroamericana* and one from *P. deltoides*. By matching their findings with QTL and gene expression studies in *P. trichocarpa*, they hoped to identify conserved genomic regions affecting root formation that can be used for improvement.

2. Herbaceous Models of Phase Change. After germination, flowering plants develop through additional distinct stages or phases, including juvenile, mature (or adult), and reproductive phases (Willmann and Poethig 2005). The shift from juvenile to mature phases is economically significant to plant propagators because, in many species, it is difficult or impossible to induce adventitious roots from mature tissues. Phase change from mature to reproductive is economically important in both herbaceous annuals and woody perennials, but it differs in an important way for the two groups. Herbaceous plants typically make a once-in-a-lifetime decision to flower; perennials develop in a pattern of seasonal vegetative and floral development that repeats over many years (Hsu et al. 2006). The ability of meristems to switch between vegetative and floral phase may be regulated by a small number of genes that also affect growth habit and life span (Melzer et al. 2008). Phase change from juvenile to mature can be observed and studied in a number of tractable herbaceous model systems, including *Arabidopsis* and maize (Willmann and Poethig 2005). The same herbaceous models may also provide insight into the practical problem of rejuvenating mature tissues (Orkwiszewski and Poethig 2000).

B. Crucial Elements: Auxin and Competent Tissue

The two indispensible factors in nearly every example of ARF were auxin (De Klerk et al. 1999) and a tissue that was predisposed to initiate roots (Haissig et al. 1992), since mature and juvenile tissues from the same source plant often have completely different responses to auxin treatment (Geneve and Kester 1991; Vielba et al. 2008). The list of environmental and endogenous factors shown to influence ARF includes nearly everything that can affect plant growth (e.g., hormones, light quantity, light quality, oxygen, carbon dioxide, nitric oxide [NO], free radicals, relative humidity, pH of the growth media, physical structure of the growth media, antioxidants, wounding, polyamines,

and concentration and types of nutrients in the media, etc.). For most of these factors, the biological role in ARF is uncertain; some may enhance ARF simply by keeping shoots or microshoots healthy while the process of ARF takes place. Others, such as polyamines, may act more directly by affecting the production or distribution of endogenous factors, such as auxin (Castillo and Casas Martinez 2008; Naija et al. 2009); or, as in the case of NO, by acting as an intermediary in auxin signaling (Pagnusset et al. 2003). Thus, the most parsimonious explanation for the action of all other factors shown to influence ARF is that they interact with, enhance, or suppress the action of auxin or that they "juvenilize" the shoot. The physiology and molecular genetics of ARF can thus be reduced to the genetics (of the parent plant material) and physiology of auxin (regulation) and of maturation, the former subject being better researched and understood than the latter. Li et al. (2009) has reviewed exogenous and exogenous factors in ARF signaling and auxin response genes.

1. Operational Model of ARF. The developmental stages of ARF in apple were summarized by De Klerk et al. (1999). The first phase (0–24 hr after indole-3-butyric acid [IBA] treatment) involves dedifferentiation of shoot tissues, and was marked by the accumulation of starch grains and wound-related compounds (WRCs). The second phase, induction (24–96 hr), was marked by the initiation of cell division, usually at about 48 hr, and the degradation of starch grains; at the end of this phase, meristemoids of about 30 cells can be observed. Meristemoid cells were typically small with relatively large nuclei and dense cytoplasm. During the second phase, hormone treatment with cytokinins or treatment with salicylic acid (SA) inhibited ARF. In *Petunia*, the first macroscopic anatomical events associated with ARF could be observed 72 hr after treatment with IBA (Ahkami et al. 2009). The third phase was root outgrowth within the stem (96–120 hr). By this time, auxin was no longer required and can even become inhibitory. Typical dome-shape primordia can be observed, and sensitivity to cytokinins and SA decreases. In the final phase, after 120 h, new roots have grown through the epidermis of the stem.

2. Role of Wounding and Mobilization of Resources. The model by De Klerk et al. (1999), while reductionist and not generalizable to all species (Brinker et al. 2004), can be useful for integrating disparate observations. For example, the role of WRC early in ARF was highlighted by the association between ARF and ethylene, abscisic acid (ABA), jasmonate (JA), NO, peroxide, peroxidases, and other WRCs during the dedifferentiation and initiation in phases (De Klerk et al. 1999; De Klerk 2002;

Pagnussat et al. 2002; McDonald and Wynne 2003; Husen 2008; Tartoura 2008; Ahkami et al. 2009). Although wounding is inescapable in the production of woody cuttings, the induction of WRCs may be more than incidental to subsequent ARF. Brinker et al. (2004) suggested that a defense barrier may be initiated during the early stages of ARF. In *Petunia*, wounding (at the time when cuttings were collected) set off a predictable cascade of JA signaling and subsequent activation of both JA responsive and JA biosynthetic genes (Ahkami et al. 2008). The jasmonate and salicylate pathways were thought to act in concert to regulate downstream defense responses (Schenk et al. 2000). A separate physiological role for JA in the establishment of a tissue competent for ARF is described below. Ethylene, a hormone associated with wounding in many species, was shown to both enhance and inhibit rooting (Biondi et al. 1990; Mori et al. 2008; Negi et al. 2008). The significance of this observation was complicated, and perhaps explained by the fact that auxin itself stimulates production of ethylene in at least some plants (Biondi et al. 1990; Woeste et al. 1999), and that ethylene can up-regulate auxin biosynthesis to affect root development (Swarup et al. 2007). The coordination of cell repair, DNA replication, cell division, and cell elongation processes necessary for ARF requires the investment of considerable energy and structural carbohydrates (Ahkami et al. 2008; Husen 2008), and the production of proteins (Hutchison et al. 1999). In *Petunia*, Ahkami et al. (2008) found that sugars, starch, and the concentration of enzymes in the pentose phosphate and glycolytic pathway were maximal after meristem formation. This, according to the authors, indicated more of a supportive role of root growth rather than initiation of ARF. This conclusion was at least conceptually in agreement with the findings of Li and Leung (2000), Takahashi et al. (2003), and Ruedell et al. (2008). Ahkami et al. (2008) suggested that wounding induces JA, which in turn activates enzymes that degrade sucrose in the apoplast to hexoses. Sucrose was transported for use in cell repair and cell division, establishing the wounded tissue as a sink. The importance of transporters in the early phases of ARF was also a significant finding of Kohler et al. (2003).

3. Genomic Analysis Reveals Molecular Details. The capacity to describe changes in gene expression during plant development has been considerably enhanced by the use of transcript profiling methods, such as differential messenger RNA display, microarray, and quantitative real-time polymerase chain reaction (PCR) technologies. Although transcript abundance is at best an indirect assessment of physiological activity, changes in transcript abundance that correspond with cellular processes

can shed light on the regulation of development and reveal similarities that underlie apparently unrelated developmental events. Microarrays in particular can produce a large amount of data, but it can be difficult to determine which of the observed changes in transcript abundance are most important (i.e. up- or down-regulations of some genes may shed light on the process of tissue building, but may not be specifically related to ARF). The use of new techniques to isolate specific cells, the use of certain controls, and the analysis of tissues from early phases of ARF can help avoid pitfalls (Schnable et al. 2004). Experiments that include genotypic differences (comparing hard-to-root and easy-to-root genotypes of the same species, and comparing wild-type plants with near-isogenic mutants) or differences related to maturation (juvenile versus mature tissues from the same plant) also add power to analyses of messenger RNA abundance (Haissig et al. 1992). The most complete microarray analyses of ARF to date were published by Brinker et al. (2004) for *Pinus contorta* and by Kohler et al. (2003) for poplar. Lindroth et al. (2001a,b) had previously identified *PSTAIRE CDC2*, a gene putatively involved in cell division competence, and two apparently root-specific S-adenosylmethionine synthase (SAM) genes (the key regulatory enzyme of ethylene biosynthesis for many species) associated with ARF. By using a microarray of 2,178 cDNAs, they identified 220 genes that were differentially expressed during root development, with most of the genes (121) differentially expressed within 3 days of wounding and initial auxin treatment. Transcriptional profiling of the first 3 days after auxin treatment showed an increase in expression of genes needed for protein synthesis and decreases in genes for protein degradation; the opposite pattern appeared later as roots formed and elongated. They found that an ATP-binding cassette (ABC) transporter, a gene typically repressed by auxin, was up-regulated during the phase when meristems were being formed, possibly indicating that auxin was actively transported to the site of meristem formation during this phase. Kohler et al. (2003) generated 7,013 expressed sequence tags (ESTs) from the adventitious roots of hybrid cottonwood at various stages of development, focusing on aquaporins and transporters that were differentially expressed during the time course of ARF. They found that several were regulated in a stage-specific manner, but made no explicit connection between their data and current models of ARF.

4. Cell Building. Coordinated DNA replication and cell division was clearly necessary for the development of new meristems (Sedira et al. 2007). Brinker et al. (2004) identified a member of the PSTAIRE class of cyclin-dependent kinases, CDC2, which was up-regulated

during ARF in *Pinus contorta*. Cyclins and cyclin-dependent kinases such as CDC2 cooperate to regulate cell cycle progression. Brinker et al. (2004) speculated that CDC2 may have a role in establishing cell division competence in preparation for organogenesis. In this respect, the cyclin *CycB1* may prove an important marker of cell division since it was expressed early in the pericycle in cells that will develop into lateral roots (Beeckman et al. 2001) and adventitious roots (Ahkami et al. 2009), but the gene itself was not expressed in primary roots (Porceddu et al. 1999). In the same paper where they describe the regulation of CDC2 in association with ARF in *Pinus contorta*, Brinker et al. (2004) also identified the up-regulation of a *PINHEAD-ZWILLE*–like gene closely related to a gene from *Arabidopsis*, *ARGONAUTE1* (*AGO1*), that was first identified as essential for normal leaf development (Bohmert et al. 1998). Since its discovery, *AGO1* has emerged as a critical factor regulating development in plants and animals (Peters and Meister 2007).

5. Auxin and Cytokinin. In the model of ARF just described, cytokinins strongly inhibited auxin-induced organogenesis in the second phase, from 24–48 hr after IBA treatment. It has been demonstrated by several researchers, however, that cytokinin can increase ARF in woody plants (Huetteman and Preece 1993; Ricci et al. 2005; Van Staden et al. 2007). The explanation for this apparent paradox is almost certainly found in the details of the interplay of auxin and cytokinins. Laplaze et al. (2007) and Pernisova et al. (2009) have shown that cytokinins play an integral role in auxin-induced organogenesis. In the model proposed by Laplaze et al. (2007), cytokinins interfere with polar auxin transport, the establishment of an auxin gradient, and the subsequent coordination of cell-cycle progression and cell type respecification that produces organized lateral root primordia. Pernisova et al. (2009) were able to demonstrate that cytokinin was necessary for modulating the organogenic signal of auxin. They showed that auxin-induced organogenic development was accompanied by endogenous cytokinin production and the localized induction of cytokinin signaling pathways. In agreement with Laplaze et al. (2007), they found that cytokinin inhibits proteinase inhibitor (PIN) auxin efflux carriers, and the inhibition was independent of ethylene effects, but they concluded that endogenous cytokinin was necessary for producing auxin differentials that are required for de novo organogenesis. The interaction between cytokinin and PIN auxin carriers may be tissue and PIN gene specific, so species-specific details may yet emerge.

6. Light and Auxin. For at least some species, light quality and quantity clearly influence ARF (Fett-Neto et al. 2001; Wynne and McDonald 2002;

Takahasi et al. 2003; Fuernkranz et al. 2006; Pinker et al. 2008; Ruedell et al. 2008). The effects of light on ARF may be mediated through several downstream pathways, but the best evidence documented to date may be the interaction of *GH3* class II family genes and *AGO1*, mentioned earlier. *AGO1* mutants (*ago1*) were defective in light-regulated hypocotyl elongation and ARF, but not LRF, indicating that a properly functioning *AGO1* was necessary for auxin homeostasis and for processes specific to ARF (Sorin et al. 2005). *AGO1* was a critical element in micro-RNA-mediated regulation of gene silencing, because *AGO1* was a principal component of RNA-induced silencing complex (Hammond et al. 2001). *AGO1* has been shown to assist in the regulation of ARF by influencing the expression of *AUXIN RESPONSE FACTOR 17* (*ARF17*) and through *ARF17*, *GH3* genes (Sorin et al. 2005). *GH3* genes were one of three major classes of auxin early response genes that can be regulated by both light and auxin (Hsieh et al. 2000), and the accumulation of GH3 was positively correlated with ARF (Sorin et al. 2005). One possible mechanism for this observation was that GH3 can adenylate hormones, including indole-3-acetic acid (IAA).

7. MicroRNAs. As mentioned previously, the GH3 class of auxin early response genes was probably regulated by *ARF17*. *ARF17*, in turn, appears to be negatively regulated by microRNAs *MIR160* and *MIR167* working in concert with *AGO1*. These two microRNAs also *positively* regulate the auxin response factors *ARF6* and *ARF8* (Gutierrez et al. 2008), possibly setting up a system where *ARF17* and *ARF6/8* are maintained in a dynamic balance to modulate auxin homeostasis appropriate for local cell fate determination. Mutations in two other auxin response factors, *NPH4*/*ARF7* and *ARF19*, also led to loss of ARF (Wilmoth et al. 2005). Auxin response factors such as *ARF6/8* were regulated by light, and can regulate other auxin response factors at both the transcriptional and the posttranscriptional level by affecting the maturation of *MIR160* and *MIR167* (Gutierrez et al. 2008). A second microRNA, *MIR164*, responds to auxin induction and participates in the regulation of NAM/ATAF/CUC (NAC) domain transcription factor proteins. *MIR164* interacts with NAC1 mRNA to down-regulate auxin signals related to LRF (Guo et al. 2005), providing a mechanism of homeostatic balance and preventing overproliferation of roots.

C. Regulation of Radial Pattern Formation

A second group of regulatory molecules implicated in ARF in both conifers and *Arabidopsis* were the members of the GRAS family of genes,

specifically SCARECROW-LIKE (SCL) and SHORT-ROOT (SHR) (Hochholdinger and Zimmerman 2008). Both SCL and SHR act within the first 24 h after auxin treatment, during dedifferentiation but before cell division. In *Arabidopsis*, these genes have been shown to regulate radial pattern formation, are required when ground tissues such as the endodermis and cortex must be separated by asymmetric cell divisions, and to function in endodermal cell fate specification (Miyashima et al. 2009). SHR moves from the stele outward where it induces SCR (SCARECROW) expression. SCR can enhance its own expression and interact with SHR to activate additional downstream targets (Hochholdinger and Zimmerman 2008). In *Pinus radiata*, *PrSCL1* was induced by exogenous auxin, but expression of *PrSHR* was auxin independent (Gutierrez et al. 2008). A similar pattern of SCL induction was observed in chestnut (*Castanea sativa*), although root primordia do not originate from the same location in *Pinus* and *Castanea* (Sánchez et al. 2007), possibly indicating that the roles of key genes were conserved even when the spatial or temporal details of ARF were not. Vielba et al. (2008) reported that SCL was induced by auxin treatment even in explants of mature stems of chestnut, indicating that the block in root formation in mature explants may occur downstream of *GRAS* gene interactions. The SHR/SCR pathway apparently regulates root pattern formation independently of the previously mentioned ARGONAUTE1-regulated pathway (Shunsuke et al. 2009).

D. Juvenility and Maturation

1. Methylation as a Model. Although auxin signaling (Lau et al. 2008) and the role of auxin in the regulation of ARF are beginning to be unraveled, the regulation of maturation and phase change is more complex. Specifically, what are the cellular and biochemical hallmarks of maturation and phase change, and how does maturation reconfigure the process of auxin response such that ARF is inhibited in mature tissues? As in the regulation of auxin responses, the genetics of phase change probably will require research using tractable herbaceous models (Poethig 1990; Hunter et al. 2006). A prevalent theory of maturation is that it reflects changes in DNA methylation. By comparing the DNA methylation of samples from juvenile and mature chestnut cuttings, Hasbun et al. (2007) found that aging implied a progressive increase of methylated 5-deoxycytidines (5-dmC), although flowering shoots showed a slight decrease in methylation compared to mature vegetative shoots. Baurens et al. (2004) found the opposite trend to prevail in microshoots of *Acacia mangium*; their results indicated that DNA from

shoots with juvenile leaf morphology was more methylated than DNA from shoots with mature leaves. They also identified three sites with $C^{5m}CGG$ modification that were exclusive to juvenile samples and three similarly modified sites exclusive to mature samples. In maize, it was found that the methylation patterns at a locus called *purple-plant1*, which codes a transcription factor that modifies anthocyanin biosynthesis, were affected by vegetative phase. Methylation of an allele of the *purple-plant1* gene called *Pl-Blotched* increased during the juvenile-to-adult transition, was maximal in mature leaves, and its methylation state was reset each generation (Hoekenga et al. 2000). Irish and McMurray (2006) investigated the same gene using an in vitro system, and found that rejuvenated shoot apices were hypomethylated, indicating that reversion of phase was associated with loss of methylation.

2. Epigenetic Regulation of Phase Change. The connection between phase change and epigenetic gene regulation was further solidified when it was determined that a number of vegetative phase change mutants in *Arabidopsis* were also implicated in the genesis of small RNAs (19- 24-nt RNAs that include both microRNAs [miRNA] and short interfering RNA [siRNA]). *Trans*-acting siRNAs (ta-siRNA) were a subclass of siRNA (Willmann and Poethig 2005). It appears now that small RNAs have an important role in determining meristem boundaries and in meristem initiation (Chuck et al. 2009), perhaps pointing to the connection between the ability to form adventitious roots and developmental phase change. This connection was reinforced by the observation that ta-siRNAs appear to regulate the maturation of auxin response factor mRNA, and this function appears to be conserved across diverse lineages (Axtell et al. 2007). In maize, levels of the ta-siRNA that target auxin response factor genes decreased in some mutants that were defective in meristem maintenance and the siRNA pathway; mutations in the same genes in *Arabidopsis* were defective in phase change (Chuck et al. 2009). Studies of the developmental defects associated with the misexpression of *MIR156* microRNA in *Arabidopsis* and *MIR172* of maize permitted Chuck et al. (2009) to suggest a converse regulatory relationship between *MIR156* and *MIR172*. When the level of *MIR156* was high (during the juvenile phase), the level of *MIR172* was low; vice versa during the mature phase. Although the most tractable herbaceous models provide hints of connections between maturity of vegetative tissues, auxin metabolism, and organogenesis, the genetic and physiological connections between phase change and competence to form adventitious roots remains as an opportunity for exploration.

III. CONTROLLABLE FACTORS THAT AFFECT ROOTING OF CUTTINGS

A. Type of Stem Cutting and Season of Collection

1. Hardwood. Hardwood (dormant; leafless) stem cuttings are obtained from the previous season's growth. Leaves can be removed without peeling the bark from the cutting, if cuttings are taken late in the growing season. Cuttings are usually prepared in late fall, winter, or early spring. Healthy stems free of disease or damage with at least two nodes are collected. Hardwood cuttings are not readily perishable but are prone to desiccation, and can be stored for several months and still retain high ARF potential. Prior to sticking, cuttings should be kept cool, out of direct sunlight, or can be stored for a few days to months (0 °C) if not stuck the same day. Successful rooting of hardwood stem cuttings of forest tree species usually requires wounding (basal cut just below a node), the use of an auxin treatment to the basal end of the cutting, warm soil temperature (bottom heat), cool ambient air temperature, light, and intermittent mist or fog when propagated in the greenhouse (Table 6.1).

Pijut and Moore (2002) reported hardwood stem cuttings (cut to 20–23 cm in length) of 5- and 6-year-old *Juglans cinerea* rooted (9%) when treated with a basal dip (10–15 s) in 29 mM indole-3-butyric acid-potassium salt (K-IBA), placed in a moist medium (perlite:peat), under intermittent mist (15 s every 18 min), with bottom heat (27 °C), 12 h of supplementary lighting (60 $\mu mol\, m^{-2}\, s^{-1}$), and a greenhouse temperature of 22 ± 2 °C. Swamy et al. (2002) reported hardwood stem cuttings (cut to 17–19 cm in length) of 2- and 15-year-old *Robinia pseudoacacia* rooted ($\sim$40% or $\sim$50%) when treated with a basal dip (24 hr) in 1.3 mM naphthalene acetic acid (NAA) or 3.7 mM IBA, placed in a sterilized medium (sand:vermiculite), under intermittent mist (10 s every 30 min), with bottom heat (25 °C), 10 h of normal light (50 $\mu E\, cm^{-2}\, s^{-1}$), and a greenhouse temperature of 25 °C.

2. Semi-Hardwood. Semi-hardwood (greenwood) stem cuttings are usually prepared in late summer or early fall. Stems with partially matured wood (lower region of the stem becoming lignified) are collected. Semi-hardwood cuttings are prone to desiccation and wilting; therefore, these should be collected in the cool, early morning when leaves and stems are turgid and should be stuck as soon as possible. Prior to insertion in media, cuttings should be kept cool, moist, and out of direct sunlight. Successful rooting of semi-hardwood stem cuttings, much like hardwood cuttings, usually requires wounding, the use of an auxin

Table 6.1. Recent advances (1997–2009) in vegetative propagation of some important North American hardwood tree species.

Tree species	Age of tree or cutting type	Propagation parameters	Treatment of cuttings[a]	Rooting (%)	References
Acer rubrum	Forced softwood shoots	Mist; perlie: vermiculite	4.9 IBA in talc	59	Henry and Preece 1997
A. rubrum 'Autumn Flame'	Softwood; greenhouse plants	Perlite; subirrigation temperature	Hormodin® 3	69	Zhang et al. 1997
A. rubrum 'Autumn Flame'	Softwood in July	Perlite or pumice; subirrigation	Dip'N Grow solutions	poor	Regan and Henderson 1999
A. rubrum 'Franskred', 'Red Sunset'	Semi-hardwood in June	Perlite or pumice; subirrigation	Dip'N Grow solutions	poor	Regan and Henderson 1999
A. rubrum 'Bowhall', 'Franksred'	Softwood; semi-hardwood	Fog; peat:perlite: sand; shading	24.6 IBA	27–87	Zaczek et al. 1997, 1999, 2000
A. rubrum 'Franskred'	Not stated	Perlite; subirrigation temperature	Dip'N Grow solutions	31–80	Owen et al. 2003
Acer saccharum	Forced softwood shoots	Mist; perlie: vermiculite	4.9 IBA	15	Henry and Preece 1997
A. saccharum 'Legacy'	Juvenile; softwood	Fog; peat:perlite: sand; shading	49.2 IBA	0–13	Zaczek et al. 1997
A. saccharum	8-yr-old; during rapid shoot elongation and shoot lignification	Mist: peat: vermiculite; phenology	Stim-Root® #2	25–75	Tousignant et al. 2003
A. saccharum (Caddo)	Eight mature trees; softwood	Mist; wounding; strong-lite high porosity mix	12.3 or 24.6 IBA; 13.4 or 26.9 NAA; IBA + NAA	<30	Alsup et al., 2004

A. saccharum	2- and 9-yr-old; during rapid shoot elongation until end of shoot lignification	Mist; peat: vermiculite; light; etiolation; phenology	Stim-Root®; IBA; NAA	70–96	Richer et al. 2003, 2004
Betula spp.	Juvenile; greenhouse; field	Mist; wounding; bottom heat	Dip'N Grow; 4.9 IBA	24–100	Barnes 2002
Castanea dentata	Forced softwood shoots	Mist; perlite	20.7 K-IBA	3	Preece et al. 2001
Fagus grandifolia	Shoots from exposed roots	Mist; wounding; bottom heat	9.8 IBA + 5.4 NAA	25	Barnes 2003
Fraxinus americana	Semi-woody epicormic sprouts	Mist	Dip'N Grow solutions	>80	Van Sambeek and Preece 1999
Juglans cinerea	5- to 6-yr-old; hardwood; softwood	Mist; peat:perlite; bottom heat; pruning	0–74 IBA; 0–62 K-IBA	6.3–88	Pijut and Moore 2002; Pijut, 2004
Liquidambar styraciflua	3- to 5-yr-old; softwood; severely pruned	Mist; peat:perlite; fertilization	Hormodin® 2	76–86	Rieckermann et al. 1999
Platanus occidentalis	Hardwood	Hot-beds in winter	no PGRs	>80	Arene et al. 2002
Prunus serotina	9-yr-old; hardwood; softwood	Mist; peat:perlite	0–74 IBA; 0–62 K-IBA	50–54	Pijut and Espinosa, 2004
Quercus alba, *Q. bicolor*, *Q. macrocarpa*, *Q. Rubra*	45-day-old; 3-yr-old; softwood	Etiolation; banding; girdling; *agrobacterium rhizogenes*; peat: perlite; mist; stoolbeds	39.4 IBA talc	10–48	Ferrini and Bassuk 2002
Q. alba, *Q. palustris*	4-yr-old;18-yr-old; softwood	Fog; peat:perlite: sand; shading	49.2 IBA	0–30	Zaczek et al. 1997

(*Continued*)

Table 6.1. (*Continued*).

Tree species	Age of tree or cutting type	Propagation parameters	Treatment of cuttings[a]	Rooting (%)	References
Q. bicolor	10- to 15-yr-old; softwood; forced; stumps; hedges	Mist; pro-mix bx®	41.4 K-IBA	0.6–2.5	Fishel et al. 2003
Q. bicolor	3- to 5-yr-old	Mist; perlite; light; banding; etiolation; age; severe cutback	29.5 IBA	88–91	Amissah and Bassuk 2007
Q. bicolor	8-yr-old	Mist; perlite; light; banding; etiolation; severe cutback	29.5	0–79	Amissah and Bassuk 2007
Q. bicolor, *Q. macrocarpa*	2- to 6-yr-old	Peat:perlite; etiolation; age; solvents; shoot position; modified container layering	39.4–49.2 IBA	0–100	Amissah and Bassuk 2004
Q. bicolor, *Q. macrocarpa*	8-yr-old	Field & air layering; severe cutback; etiolation	49.2 IBA basal paint	62–83	Amissah and Bassuk 2005
Q. bicolor, *Q. macrocarpa*	9-yr-old	Mist; perlite; light; banding; etiolation; severe cutback	29.5	6–81	Amissah and Bassuk 2007
Q. bicolor, *Q. macrocarpa*	8-yr-old	Perlite; mist; light; severe; cutback; etiolation; banding; stem anatomy	29.5 IBA	0–85	Amissah et al. 2008

Q. bicolor, *Q. macrocarpa*, *Q. palustris*	3- to 5-yr-old	Peat:perlite; severe cutback; incandescent light; age; root restriction & pruning; modified stoolbed; etiolation	39.4 IBA basal paint	24–100	Hawver and Bassuk 2000
Q. nigra	Juvenile; semi-hardwood	Fog; peat:perlite: sand; shading	49.2 IBA	20–77	Zaczek et al. 1999, 2000
Q. palustris	20-yr-old; semi-hardwood	Fog; peat:perlite: sand; shading	49.2 IBA	10–40	Zaczek et al. 1999, 2000
Q. rubra	Mature; grafted plants; softwood	Fog; peat:perlite: coarse sand	49.2 IBA	9–67	Zaczek and Steiner 1997; Zaczek 1999
Q. rubra	2-yr-old grafts; semi-hardwood	Fog; peat:perlite: sand; shading	49.2 IBA	33–48	Zaczek et al. 1999, 2000
Q. rubra	10- to 15-yr-old; softwood; forced; stumps; hedges	Mist; pro-mix bx®	41.4 K-IBA	15–43	Fishel et al. 2003
Q. rubra	Juvenile; mature; serial grafting	Fog; peat:perlite: coarse sand	59.0 IBA	55–91	Zaczek et al. 2006
Robinia pseudoacacia	2- and 15-yr-old; softwood; hardwood	Mist; sand: vermiculite; age; bottom heat; season	1.3–4.0 NAA; 1.2–3.7 IBA	35–83	Swamy et al. 2002a,b

[a]Concentration of auxins tested reported in millimolar (mM) unless indicated otherwise; Indole-3-butyric-acid (IBA); Indole-3-butyric acid-potassium salt (K-IBA); Naphthalene acetic acid (NAA); Plant growth regulators (PGRs); Dip'N Grow: http://www.dipngrow.com/; Hormodin®: http://www.ohp.com/; Stim Root®: http://www.plant-prod.ca/index_e.php.

treatment, ambient light or shading, and intermittent mist or fog when propagated in the greenhouse (Table 6.1). Leaves on semi-hardwood stem cuttings are usually trimmed to reduce the leaf surface area, in order to lower transpirational water loss, reduce leaf rotting, and save space in the propagation house.

Regan and Henderson (1999) reported semi-hardwood cuttings of *Acer rubrum* 'Franksred' and 'Red Sunset' desiccated quickly when collected in June in Oregon and rooted poorly in a perlite medium when cuttings received a 10 s dip in Dip'N Grow (49.2 mM IBA and 26.9 mM NAA) with subirrigation. Semiwoody leafy cuttings of *Fraxinus americana* obtained from epicormic sprouts forced from mature branch segments rooted (>80%) when treated with Dip'N Grow solutions (1:24 or 1:99) (Van Sambeek and Preece 1999). Semi-hardwood oak and maple shoots were rooted [*Quercus nigra* (20–77%), *Q. palustris* (10–40%), *Q. rubra* (33–48%), and *A. rubrum* 'Bowhall'(53–80%)], when treated with 49.2 mM IBA (oaks) and 24.6 mM IBA (maple), placed in a moist medium of peat:perlite:sand, maintained under low irradiance (93% shading), and intermittent cool fog (Zaczek et al. 1999, 2000). Oak shoots had all but the uppermost three leaves removed, whereas maple shoots were pruned to single-node cuttings. Semi-hardwood cuttings of *J. cinerea* rooted (11–46%) when treated with 29 or 62 mM K-IBA and 34 or 74 mM IBA (Pijut and Moore 2002). All but two leaflets were removed from these butternut cuttings, and rooting data were collected after 5–6 weeks. *Acer saccharum* semi-hardwood cuttings collected at 0800, treated with Stim-Root® #2 (19.7 mM IBA), and placed in a peat:vermiculite medium under mist rooted 65–75% after 12 weeks (Tousignant et al. 2003).

3. Softwood. Softwood stem cuttings are obtained from the current season's soft, succulent, new growth. Cuttings are usually prepared in late spring through early summer, during the period of active growth, but before extensive stem lignification. Several weeks exist where softwood cuttings can be collected and rooted, but the best time period needs to be determined for each individual species. Softwood cuttings generally root easier than other types of cuttings and have the highest rooting potential. Softwood cuttings are extremely perishable, stress easily, desiccate quickly, and therefore should be collected in the early-morning hours and kept moist, cool, and turgid at all times. Softwood cuttings need to be stuck as soon as possible the same day as collected. Successful rooting of softwood stem cuttings of forest tree species usually requires some sort of stock plant manipulation, certain cutting length, wounding or auxin treatment, the use of various rooting substrates, and several

different greenhouse parameters, such as for light and air temperature, shading, mist, fog, humidity, or fertilization. In summary, there is a large environmental affect on ARF in cutting propagation. Leaves on cuttings are usually reduced in size to lower transpiration rates when propagated in the greenhouse. Cutting yield is the important factor in cutting propagation (i.e., the percentage of plantable cuttings obtained from the propagation system). A large factor in cutting yield is overwintering success. Cuttings from many taxa can be rooted, but few can be successfully overwintered and outplanted. Table 6.1 illustrates recent advances in vegetative propagation of several difficult-to-root hardwood tree species using softwood cuttings. A few will be highlighted here and in the next sections dealing with other factors that affect rooting of cuttings, since no one protocol works best for all species or genotypes.

Zaczek et al. (1997) found that adventitious rooting of maple and oak cuttings was influenced by shade and auxin treatment. Softwood cuttings of *A. rubrum* 'Bowhall', 'Franskred', and 'Red Sunset' treated with 24.6 mM IBA rooted 67%, 87%, and 87%, respectively, when inserted in a peat:perlite:sand medium with intermittent cool fog and kept under 91% shade (control, 83% shade). Softwood oak cuttings (*Q. alba* and *Q. palustris*) only rooted at 30% and 23%, respectively, when treated with 49.2 mM IBA under the same fog and shade conditions (Zaczek et al. 1997). Softwood cuttings from nine Caddo sugar maples (10- to 30-year-old) exhibited variability in rooting percentages among individual trees, but the type and concentration of auxin used made no significant difference (Alsup et al. 2004). Rooting was <30% when 13–46 cm cuttings (bottom leaves removed and wounded on each side of the base of the cutting) were treated with IBA, NAA, or IBA plus NAA and placed in a high-porosity rooting medium under mist with natural photoperiod (Alsup et al. 2004). *Betula* (*B. alleghaniensis*, *B. lenta*, and *B. nigra*) tip cuttings with two to four leaves rooted 87–100% when collected in late spring to early summer and treated with Dip'N Grow (1:10) or 4.9 mM IBA (Barnes 2002). Early-season softwood cuttings were effective for vegetative propagation from 5- and 6-year-old *J. cinerea* trees when treated with 62 mM K-IBA (77% rooting) or 74 mM IBA (88% rooting) (Pijut and Moore 2002). Butternut rooted cuttings survived overwintering in cold storage and acclimatization to the field (Pijut 2004). Sweetgum (*Liquidambar styraciflua*) succulent sprouts, from severely pruned 3- to 5-year-old stock plants previously established in the field from rooted cuttings, rooted well when treated with Hormodin® 2 (14.8 mM IBA) (Rieckermann et al. 1999); 86% of all cuttings rooted, 76% survived, and 58% were deemed plantable when terminal and subterminal cuttings, with the lower leaves removed (leaving at least two leaves) and the

remaining lobes of leaves cut in half, were placed in a peat: perlite medium under 55% shade with intermittent mist for 10 weeks (Rieckermann et al. 1999).

B. Stock Plant Maturation and Manipulation

1. Juvenile → Adolescent → Mature. The maturation-related loss of adventitious rooting competence in hardwood trees becomes a major limiting factor in clonal forestry, since many desirable traits or characteristics (e.g., wood quality or seed production) are not expressed until the tree matures. Therefore, in these already difficult-to-root species, ARF from cuttings declines with the chronological age of the tree; cuttings from seedling (juvenile) trees root more readily than cuttings from mature (reproductive) trees. Phase change (juvenile → adolescent → mature) results from the expression of certain genes at specific times in the life cycle of a plant, or in response to environmental or internal signals (Hartmann et al. 2002). The length of the juvenile period in hardwood trees can range from 5 to 40 years, therefore making ARF in cuttings from selected mature trees extremely difficult and timely. A juvenile → mature gradient ("cone of juvenility") also exists in juvenile (seedling) trees, making ARF from cuttings variable (Hartmann et al. 2002). The area near the base of the tree is considered the oldest chronologically, but youngest in terms of ontogenetic age (Hartman et al. 1997). The stems and branches, therefore, are oldest in maturity (capable of reproduction), but youngest in chronological age. Therefore, rooting potential decreases with increasing distance from the ground.

2. Pruning, Hedging, Forcing, or Serial Grafting. Several horticultural or forestry-related practices or techniques can be utilized to help promote physiological juvenility (improve ARF from cuttings) in difficult-to-root tree species. Pruning or hedging is the process of cutting back the original stock plant from which cutting material is taken, in order to produce new physiologically invigorated (juvenile) shoots or stump sprouts that root more readily (Macdonald 1986; Hackett 1988; Howard 1994). Pijut and Moore (2002) reported that it was necessary to prune back 2 years of growth after hardwood stem cutting collection of 5- to 6-year-old *J. cinerea*, in order to encourage sprouting of softwood stems for collection and rooting (77–88%). This same protocol was also important to encourage sprouting of softwood stems for collection and rooting (50–54%) from 9-year-old *Prunus serotina* field trees (Pijut and Espinosa 2004). Amissah and Bassuk (2005) reported significantly higher rooting percentages (~77% for *Q. bicolor* and ~70% for *Q. macrocarpa*) in

layered propagules arising from 8-year-old field trees cut back to a 0.04 m stump above the soil surface.

Forcing shoots from latent buds formed during primary growth, but remaining dormant throughout secondary growth under the bark (Hartmann et al. 2002), of dormant trees cut into sections and placed in a greenhouse, has been used successfully for cutting propagation of maple, white ash, and oak. Red maple stem sections (>50%) produced shoots, and softwood cuttings taken from these sections rooted at 60%, compared to shoots produced (20%) on sugar maple stem sections with rooting at 15% (Henry and Preece 1997). However, there may be unintended consequences; the genotypes that readily produce shoots from latent buds are the genotypes that provide the most cuttings. Therefore, if these good "shoot producing" individuals are propagated, one will produce genotypes that have a high potential for forming undesirable shoots (D. Struve, person. commun.). Semi-woody epicormic sprouts forced from branch segments of white ash were rooted (>80%) and field planted, but cuttings of forced black walnut or white oak did not root (Van Sambeek and Preece 1999). Semi-hardwood cuttings from 10- to 15-year-old forced northern red oak rooted on average 40%, but the main boles exhibited a vertical gradient in the number of shoots produced for cutting propagation (Fishel et al. 2003).

Serial grafting of mature scion wood (with subsequent sequential regrafting) from difficult-to-root species onto seedling (juvenile) rootstock has improved the ARF of cuttings from these grafted stock plants of several woody species (Howard 1994; Hartmann et al. 2002). Zaczek et al. (2006) performed serial grafting over three consecutive years in order to study the effects of ontogeny, genotype, and grafting on rooting performance of northern red oak cuttings. Grafting tended to increase rooting and the number of roots per cutting, but the effect was not progressive with increasing serial grafting (Zaczek et al. 2006). Rootstock maturation did not significantly affect rooting potential. From the results, the authors hypothesized that northern red oak buds are predetermined in the developmental fate relative to rooting parameters and are only minimally influenced by serial grafting (Zaczek et al. 2006).

3. Light, Etiolation, Banding, or Shading. Light or the exclusion of light can be a major factor that influences the physiological and anatomical status of the stock plant (photosynthesis, levels of endogenous hormones, carbohydrate levels, and cell formation and development (root primordia, lignification, etc.), and subsequent ARF of cuttings collected

from these plants (Davis et al. 1988; Davis and Haissig 1994; Hartmann et al. 2002). Reducing light levels (irradiance), modifying light quality (red and far-red), and the use of supplemental lighting to extend the photoperiod (fluorescent, metal halide, or high-pressure sodium) on stock plants prior to cutting collection are several ways to increase the rooting potential of cuttings (Moe and Anderson 1988; Howard 1994; Hartmann et al. 2002). Etiolation (forcing new shoot growth under conditions of heavy shade), banding (excluding light from that portion of the stem that is to become the cutting base), and shading (reduced light) have been shown to improve the rooting potential of cuttings taken from treated stock plants (Maynard and Bassuk 1988; Howard 1994; Hartmann et al. 2002).

Three- to 5-year-old oak seedlings (*Q. bicolor*, *Q. macrocarpa*, and *Q. palustris*) cut back to a 2- to 4-cm stem stump and subjected to ~98% light exclusion produced etiolated shoots that were then subjected to an IBA treatment (basal paint), and modified stoolbed technique (Hawver and Bassuk 2000). Etiolation used in conjunction with this modified stoolbed technique improved ARF of all three oak species from 29%, 52%, and 25% to 64%, 83%, and 100%, respectively (Hawver and Bassuk 2000). A 30-min application of far-red light or incandescent light at 22:30 hr every evening encouraged ARF from these stock plants (Hawver and Bassuk 2000). Amissah and Bassuk (2004), using this same etiolation plus container layering technique, evaluated the use of gibberellin (GA_{4+7}) on stock plant budbreak, and also evaluated the effect of stock plant age, shoot origin, and IBA solvents on ARF in *Q. bicolor* and *Q. macrocarpa*. GA_{4+7} at 500 mg l^{-1} applied every fourth day increased budbreak in stock plants, but stock plant age (2- to 6-year-old) had no negative effect on ARF (Amissah and Bassuk 2004). ARF was greater (36%) in shoots from stock plants cut back to a 3–4 cm stump above the soil and etiolated, compared to shoots from intact tall plants (1.8%) of *Q. bicolor* (Amissah and Bassuk 2004). Rooting percentages were highest when IBA was dissolved in less toxic solvents, such as 50% or 100% acetone or 98% ethanol (Amissah and Bassuk 2004). Amissah and Bassuk (2007) investigated the effect of light and etiolation with or without stem banding on ARF in *Q. bicolor* and *Q. macrocarpa* cuttings. Light and stem banding had no significant influence on ARF using stock plants grown in the greenhouse, whereas etiolation enhanced rooting in field-grown cuttings (Amissah and Bassuk 2007). Amissah et al. (2008) analyzed the relationship of stem anatomy to ARF in stem cuttings of *Q. bicolor* and *Q. macrocarpa* using etiolation and age of the stem at time of cutting as a pretreatment. The overall conclusion was that the difference in rooting was more related to the ease of adventitious

root initiation rather than to restrictions on root emergence (Amissah et al. 2008). Partial or total light exclusion (blanching, etiolation, and a combination of both) on 9-year-old *A. saccharum* trees did not stimulate ARF from cuttings (Richer et al. 2003, 2004). Maynard and Bassuk 1996 also came to this same conclusion (i.e., it is cell competency, not anatomical barriers) when they studied the effects of etiolation, shading, and banding on shoot development of *Carpinus betulus* L. *fastigiata* cutting propagation.

C. Treatment of Cuttings

The length of stem cuttings collected for ARF varies from 7.5 to 76 cm, with at least two or more nodes, depending on the type of cutting (hardwood, semi-hardwood, or softwood) and species (Hartmann et al. 2002). Spethmann (2007) reported improved rooting success, survival, and further growth when long cuttings (50–150 cm) were used to propagate roses and several tree species. All cuttings were treated with 24.6 mM IBA, stuck in a peat:sand (3:1) mixture (pH 4.5), and rooted in a high-pressure fog system (Spethmann 2007). Differences also exist in rooting success when lateral, terminal, flowering, or vegetative shoots were used in a clonal propagation system (Rieckermann et al. 1999; Hartmann et al. 2002).

Wounding of stem cuttings occurs when the material is first collected from the stock plant, but additional basal stem wounding can be beneficial to rooting success with certain woody species (Macdonald 1986; Hartmann et al. 2002).

Many plant growth regulators, such as auxins, cytokinins, ethylene, abscisic acid, gibberellins, polyamines, and brassinosteroids, to name a few, influence ARF in cuttings (Macdonald 1986; Davis et al. 1988; Davis and Haissig 1994; Hartmann et al. 2002), but auxins usually have the greatest effect on ARF. Various concentrations (depending on the species and type of cutting) of the auxins IAA, IBA, or NAA are most commonly employed in ARF studies of North American hardwood tree species (Table 6.1). These auxins can be applied singly or in combination as a basal dip or soak, in talc or lanolin paste, or as a basal paint. A recent review by Blythe et al. (2007) discusses the methods of auxin application in cutting propagation over the last 70 years; therefore, it will not be discussed further in this chapter. Auxin application as a foliar spray has recently been reported to work well for rooting several woody plant species (Drahn 2007). Commercial formulations, solvents, and carriers used in auxin application can be reviewed in Hartmann et al. (2002).

D. Rooting Medium

No one rooting medium works best for ARF in cuttings of North American hardwood tree species. Typically, peat or sphagnum moss, perlite, vermiculite, or sand was used singly or in various ratios (Table 6.1). Peat is usually included because of its large total pore space and its ability to hold water. Perlite, vermiculite, or sand improved drainage of the rooting medium. Occasionally commercial soil mixes have been used successfully for ARF in cuttings. Regardless of the medium used, it is important to steam sterilize the rooting medium before use in order to rid it of any pathogens that could be detrimental to the cuttings.

E. Greenhouse Parameters

Greenhouse parameters can be an important factor in the success or failure of ARF in cuttings. Intermittent mist or fog systems are regularly employed to control water loss of leaves. Intermittent mist systems minimize water vapor in the leaves by lowering the leaf-to-air vapor pressure gradient slowing down leaf transpiration, and also reduce leaf temperature (Loach, 1988a; Hartmann et al. 2002). Fog systems maximize water vapor in the air by raising the ambient humidity (Hartmann et al. 2002). Unlike mist, the very fine water droplets from a fog system remain suspended in the air longer, do not condense on the leaf surface of the cutting, and can minimize physiological stress of leafy cuttings of some woody species (Mateja et al. 2007).

Greenhouse air temperatures of approximately 21 °C to 27 °C (day) and approximately 15 °C (night) are usually employed for rooting cuttings of most temperate species (Hartmann et al. 2002), but higher day and night temperatures are also successfully employed in some greenhouses that are maintaining more than propagation benches. The temperature of the rooting medium can also be a crucial factor. Bottom heat (usually 18 °C to 25 °C), by the use of heating pads or circulating hot water tubing or pipes below the rooting medium, was employed when rooting hardwood cuttings, but has also been used successfully with other types of cuttings (Loach, 1988; Hartmann et al. 2002).

Irradiance (relative amount of light), light duration (length of the photoperiod), and light quality (wavelength) can also influence ARF of cuttings (Hartmann et al. 2002). Light is necessary for photosynthesis of leafy cuttings, but each of these parameters must be determined to fit the particular species being propagated. Light or the absence of light has been studied extensively during in vitro rooting (plant tissue culture), but unfortunately, many times this important greenhouse parameter was

not mentioned in a research report. Zaczek et al. (1997, 1999, 2000) reported that the duration of exposure (0–119 days) to low solar irradiance (93% shade) during the rooting phase was beneficial to several oak and maple taxa that are considered difficult to root.

Mineral nutrition of cuttings during the rooting phase may also be an important factor in ARF in cuttings, but optimal nutrition levels for many North American hardwood tree species have yet to be determined (Rieckermann et al. 1999). It is difficult to determine the effect of applied fertilization during the rooting process on root primordia initiation versus root primordia elongation (Hartmann et al. 2002).

IV. CASE STUDY OF HORTICULTURAL VERSUS FOREST TREE SPECIES

In the past, many horticultural and forest tree species were grafted as a result of either recalcitrance to vegetative propagation by rooted cuttings or because of the desire to impart attributes of a particular rootstock to the scion, mostly in the case of the horticultural trees. With some species, because of delayed graft incompatibility issues that started to plague the commercial nursery industry or because of the desire to have a particular cultivar grown on its own roots, research was pursued in the past several decades to improve rooted cutting success. We will use a case study to explore recent successes and continued difficulties with two forest species, *Quercus rubra* and *Acer rubrum* in comparison to the horticultural species *Malus spp.* and *Pyrus communis*.

A. Horticultural Species

The breakthroughs in improvement of rooted cutting success of *Malus* spp. and *Pyrus communis* came when researchers started testing greenwood cuttings taken early in the growing season, increasing the length of cuttings compared to what had been traditionally employed (Spethmann 2007), and providing shading to the base of the cutting. Even with the best treatments, both *Malus* and *Pyrus* cultivars still vary in rooting percentage. Currently, *Malus* spp. are generally propagated in May or early June as softwood cuttings, although cuttings taken later in the season can be successfully rooted, but much less so than early softwood cuttings (Dirr and Heuser 1987). In general, 12 to 49 mM IBA was used as the rooting stimulant with the 10–16 cm cuttings placed in peat:perlite under mist. Savaci et al. (2007) found etiolation of hardwood cuttings improved rooting success, and after biochemical analysis the

improvement was found to be associated with a decrease in chlorophyll a and b, carotenoids, and anthocyanin.

Pyrus communis was also vegetatively propagated as leafy softwood cuttings, and IBA was usually supplied at 39–49 mM. In various studies, the addition of heat to the cutting as a prepropagation treatment, altered day-length, controlled environments, fertilizer, shading, and other root promoting chemicals have been tested. In addition, research on hardwood cuttings continues. Barbosa et al. (2007) found with 10 mM IBA, rooting was the best (83%) in growth chamber conditions with short days (8 h), 90% relative humidity, and temperature at 25 °C. Mbabu and Spethmann (2005) studied the effect of slow-release fertilizer, substrate pH, and cutting length on rooting success of various *P. communis* cultivars. The highest rooting percentage occurred at pH 5.7 with 2 kg m^{-3} of Plantacote Mix 4 M. In their study, the length of cutting did not make a difference in rooting. Baraldi et al. 1993 were able to shed light on the rooting differences between cultivars when they compared easy- and difficult-to-root cultivars of *P. communis*. They found that the easy-to-root cultivar 'Conference' was able to metabolize IBA to IAA faster and at a higher rate than the difficult cultivar 'Doyenne d'Hiver'. Wang (1991) found shading the base of *Pyrus* 'PB10030' rootstock microcuttings increased rooting. In addition, phloroglucinol (0.125–1.0 mM) along with darkness for 5 days promoted rooting. Turovskaya (1988) found that with two rootstocks, rooting was reduced by 50% when leaves on the cuttings were not left intact. Hardwood cuttings were possible to root, but required a heat treatment in storage for 30 days or more before placing the cuttings in outdoor beds. Barbosa et al. (2007) had some success with rooting hardwood cuttings (25 cm) of 'Limeira' pear in the greenhouse and growth chamber after treatment with 10–30 mM IBA. El-Shazly and El-Sabrout (1994) studied hardwood cuttings of 'Le Conte' pear taken throughout the season and found with 20 mM IBA cuttings taken in April (in the Middle East) had the highest rooting percentage (30%).

B. Forest Species

In contrast to the two horticultural species just discussed, physiologically mature *Quercus rubra* is nearly impossible to root from either softwood or hardwood cuttings, and the most success has occurred with using material with juvenile physiology. In comparison, much more progress has been made with *Acer rubrum* to the point that rooted cutting propagation is now a routine commercial practice. Although not thoroughly studied, the difference in success with these two species may well be the variation in the length of the juvenile period for that species.

Red oak has a longer juvenile period than red maple, so one would think that there is additional time where red oak could be propagated (D. Struve, person. commun.). Most red maples are from rooted microcuttings.

In studies with both species, Zaczek et al. (1999) theorized that low irradiance (7%) during fog-humidified propagation could improve rooting success of semi-hardwood cuttings. The treatment significantly increased both the percentage rooting and number of roots per cutting of *Q. rubra* as well as *A. rubrum*. Later, rejuvenation was attempted by grafting cuttings with juvenile to mature physiology on rootstocks, before attempting to root cuttings from those grafts (Zaczek et al. 2006). Researchers found that *Q. rubra* cuttings were only marginally affected by grafting, and those cuttings were predetermined to respond to rooting whether cuttings had been grafted or not. Earlier Zaczek and Steiner (1997) had found that cuttings from shoots stimulated to grow from axillary buds on grafted plants had a lower percentage rooting (25%) than cuttings with buds that were not stimulated to elongate (66.7%). Ferrini and Bassuk (2002) found that rooting decreased with the increasing age of the mother plants, which had been seen as early as 1939 (Thimann and Delisle 1939). Sanchez et al. (1996) confirmed the importance of juvenility by demonstrating good rooting capacity of shoots derived from basal epicormic sprouts that had been placed in vitro.

In one of the earliest reports of cutting propagation of *A. rubrum*, it was found that anthocyanin in leaves was the most important factor that led to root initiation over other factors, such as continuous light, 16-h photoperiod, IBA dips, or application of sucrose or riboflavin (Bachelard and Stowe 1962). Even earlier, the use of IBA as a necessary rooting treatment to the cut ends of softwood cuttings was established in the literature (Afanasiev 1939). Struve and Arnold (1986) found IBA to be the superior auxin source for rooting of *A. rubrum* when it was compared to K-IBA, *N*-phenyl-indole-3-butyramide (NP-IBA), phenyl indole-3-thiobutyrate (P-ITB), and phenol indole-3-butyrate (P-IBA), although the aryl esters improved rooting quality.

Most of the recent work has been performed with particular horticultural cultivars of *A. rubrum*, but the particular cultivars will not be the focus of this section. In addition, despite the use of cultivars, these studies still shed light on rooted cutting propagation for this species. The work that will be discussed has focused on use of subirrigation, various substrate temperatures, and fertilization in addition to cutting types and seasonal effects.

In a study by Owen et al. (2003), *A. rubrum* cuttings were subjected to subirrigation. In addition, three auxin concentrations were evaluated

along with three substrate temperatures. With the intermediate substrate temperature (23 °C), root length (156 mm) and rooting percentage (69%) increased, although the 20 °C treatment resulted in the greatest number of cuttings that rooted (15). Henry and Preece (1997) forced softwood shoots from excised stem sections of *A. rubrum*, and those shoots had a 60% rooting percentage. Zaczek et al. (1997) rooted *A. rubrum* under 91% and 97% shade, and those cuttings resulted in faster rooting, higher number of roots per cutting, and higher percentage rooting. Zhang et al. (1997) studied transpiration rate, survival, and rooting of unmisted *A. rubrum* softwood cuttings. The highest rooting percentage (74%) occurred at the lowest medium temperature treatment (24 °C). The addition of subirrigation improved rooting percentage. Zhang and Graves (1995) added nitrogen fertilizer (3.6 and 7.2 mol N m^{-3}) to subirrigation, and rooting was improved (95%) over subirrigation alone. Without subirrigation, fertilizer did not have an effect on rooting (Lane and Still 1984). Wilkins et al. (1995) tested rooting of single-node stem cuttings. Cuttings taken in May and dipped in 3 or 8 g kg^{-1} IBA supplied as Hormodin® No. 2 or 3 had a range of rooting percentage from 22% to 100%, depending on the genotype. Dehgan et al. (1989) studied the effect of season on *A. rubrum* semi-hardwood cuttings. They found that cuttings taken in May and July–September had the highest rooting percentage (up to 88%).

Even after numerous attempts to root cuttings of *Q. rubra* by manipulating the pre- and posttreatment physiology of the material used for cuttings, for the most part, the species is still recalcitrant to vegetative propagation by traditional rooted cutting methods. However, improvements in rooting success with *Malus* spp., *P. communis*, and *A. rubrum* continue to be made while new knowledge is being gained about the underlying biochemical mechanisms that are leading to those improvements. For the most part, except for particular cultivars, published rooting procedures can guide commercial applications of these technologies.

V. CONCLUDING REMARKS

For many North American fine hardwood species, treatments to enhance adventitious rooting that manipulate environmental factors, rooting medium, and exogenous chemical stimuli have been extensively explored, and probably only minor improvements in ARF would be gained by expending much more effort in this line of research. The fundamental factors that influence ARF and the cofactors that subtly

influence the endogenous or exogenous effects have been identified. What remains to be understood are the biochemical underpinnings of the observed biology of ARF. An improved understanding of the regulation of auxin metabolism, transport, and action, and the regulation of tissue differentiation and maturation probably will be the most important and productive areas of ARF research in the future. Model plant systems, including rooting mutants within a species with varied rooting capacity, will be useful tools for gaining insight into the genetic basis for ARF.

Comparisons of gene regulation between juvenile and mature phenotypes within the same species might also provide some useful insight into inhibition of rooting that occurs with the transition to maturity. Until recently, the costs associated with large-scale analyses of the genes, proteins, and metabolites shown to affect plant development have prohibited their application to all but a few model species. The advent of new (pyro)-sequencing technologies and metabolomics has placed these types of analyses within reach of woody-plant researchers. Molecular tools and genetic data are available now for us to begin to identify genes and (epi)genetic regulators that are influencing ARF in fine hardwoods. Once we increase our understanding of the underlying genetic controls, we can begin to develop novel means of overcoming this inhibition.

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