14

Poplar (*Populus* spp.)

Richard Meilan and Caiping Ma

Summary

Although species within the genus *Populus* are, in general, easier to transform and regenerate in vitro than most other trees, many poplar species are very recalcitrant. Many protocols that previously have been reported were developed for a specific genotype or species. Thus, it has often been necessary to re-optimize a protocol each time research is initiated with a new genotype. The method presented in this chapter has been effective for a wide variety of poplar genotypes.

Key Words: Aspen; cottonwood; *Populus*; regeneration; transformation.

1. Introduction

Unlike many other trees, species within the genus *Populus* (including aspens and cottonwoods) can be regenerated in vitro via direct organogenesis (as opposed to embryogenesis). Whereas reliable transformation systems have been developed for pure species and hybrids within the section *Populus*, the genotypes in other sections within the genus *Populus* ***(1)*** have been found to be recalcitrant. To date, only a limited number of genotypes in sections *Tacamahaca* and *Aigeros* have been successfully transformed ***(2–7)***. We have developed a protocol that works well with numerous *Populus* genotypes and that is the basis for this chapter ***(8)*** (summarized in **Table 1 and Fig. 1**). Over the past 10 yr, we have used this protocol to transform 16 different constructs into 13 genotypes (pure species and hybrids) of cottonwood (e.g., *Populus deltoides*, *P. trichocarpa* × *P. deltoides*, *P. deltoides* × *P. nigra*, and *P. nigra* × *P. maximowiczii*) and have produced 705 lines (independent transgenic events). The transformation efficiency (i.e., the number of explants from which a rooted plant is recovered, expressed as a percent of the number of explants that were co-cultivated) varies from 0.4 to 5.0%, depending mostly on the construct being inserted and the genotype

From: *Methods in Molecular Biology, vol. 344:* Agrobacterium *Protocols, 2/e, volume 2*
Edited by: Kan Wang © Humana Press Inc., Totowa, NJ

Table 1
Chronology for the Production of Transgenic Poplar

Subheading	Frame	Description	Materials needed	Time
3.1.1.	A	Poplar pre-growth	Propagation medium	1 mo
3.3.6.	E	Co-culture explants in liquid medium	Overnight *Agrobacterium* culture ($OD_{600\ nm}$ = 0.4 to 0.6)	1 h
3.3.9.	F	Co-cultivation on agar	Callus-induction medium, no antibiotics	2–3 d
3.4.1.		Decontamination	Sterile water and washing solution with 200 mg/L timentin	2 h
3.4.2.		Callus induction	Callus-induction medium with kanamycin and timentin	2–3 wk
3.4.4.	G	Shoot regeneration	Regeneration medium with kanamycin and timentin	4 mo
3.4.6.	H	Shoot elongation	Elongation medium with kanamycin and timentin	1 mo
3.5.1.	I	Rooting shoots	Root-induction medium with kanamycin and timentin	1 mo
		PCR analysis	Transgene-specific PCR primers	1 d
3.5.3.	J, K	Propagation	Propagation medium with kanamycin and timentin	2 mo
3.6.		Transplanting and hardening	Pots, soil, plastic bags	3 wk
		Total time to obtain fully hardened transgenic plants		10–12 mo

Note: "Subheading" refers to the heading in the text under which the procedure is described, "Frame" refers to the appropriate image in **Fig. 1**.

used. We have also generated over 9600 lines by transforming 165 various transgenes into two aspen clones: 717-1B4 (female, *Populus tremula* × *P. alba*) and 353-38 (male, *P. tremula* × *P. tremuloides*). Transformation efficiency for these hybrid aspens has ranged from 5 to 37%, depending on the construct and the strain of *Agrobacterium* used, but the average is generally from about 15 to 20%.

One- to two-month-old in vitro-grown plants are good sources for starting material. Fresh, young shoots from plants grown in a greenhouse can also be used, but those tissues must be surface-sterilized before inoculation with *Agrobacterium*. Explants (leaf discs or stem and petiole segments) are first pre-cultured in the dark to initiate the formation of calli from which shoots will eventually regenerate. Explants from the pre-cultured plant material are then inoculated with an

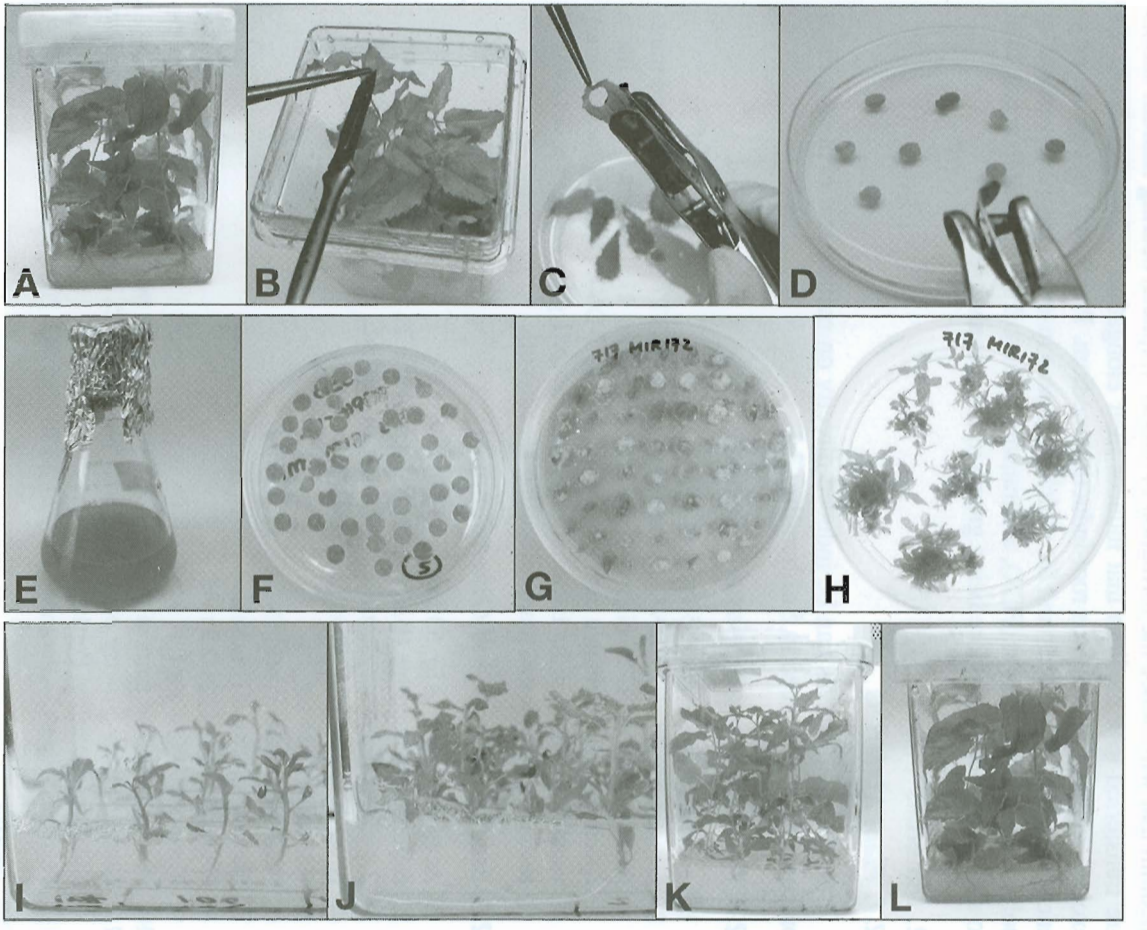

Fig. 1. Steps involved in the production of transgenic poplar.

Agrobacterium cell suspension and co-cultivated before being transferred to a medium that will induce the formation of shoots. Explants bearing nascent shoots are transferred to a medium that induces their elongation before the shoots are excised and then placed on a root-induction medium. Plants that root in the presence of the selection agent, and for which transgene insertion has been verified by polymerase chain reaction (PCR), are transferred to soil and gradually acclimated to ambient conditions before being grown in the greenhouse.

2. Materials

2.1. Plant Materials

Shoot organogenesis can be induced directly on mature leaf explants. Plant preparation is described in **Subheading 3.1.**

2.2. Agrobacterium *Strains and Vectors*

1. *Agrobacterium* strain: C58/pMp90 ***(9)***.
2. Genetic constructs: T-DNA is assembled in the pART7 shuttle vector and transferred to the pART27 binary vector ***(10)*** (*see* **Note 1**). The latter vector has a spectinomycin selectable marker gene outside the T-DNA (to select for the binary vector's presence in its bacterial host) and an *npt*II gene within the T-DNA (to select for transformed plant cells using kanamycin).

2.3. Stock Solutions and Other Supplies

1. Acetosyringone (AS): 50 m*M*.
2. 6-Benzylaminopurine (BA): 0.5 mg/mL (*see* **Note 2**).
3. 2,4-Dichlorophenoxyacetic acid (2,4-D): 1.0 mg/mL.
4. N^6-(2-isopentenyl)adenine (2iP): 5 m*M*.
5. Kanamycin: 25 mg/mL
6. Naphthaleneacetic acid (NAA): 1.0 mg/mL
7. Spectinomycin: 50 mg/mL
8. Thidiazuron (TDZ): 0.5 m*M*
9. Timentin: 100 mg/mL
10. Potting soil: Two parts of perlite mixed with one part peat moss
11. Containers for growing plants ex vitro: "Rose Pots": 5.7 × 8.3 cm (Anderson Die and Manufacturing, Portland, OR).
12. Fertilizer: Peters (Allentown, PA), 20/20/20 (N/P/K), 200 ppm.

2.4. Media

1. Propagation media: 1/2-strength Murashige-Skoog (MS) (***11***), pH 5.8, 0.7% agar (Sigma).
2. Callus-induction medium (CIM)
   a. For cottonwood (CIM1): MS salts, 0.5 μ*M* BA, 0.5 μ*M* zeatin, 5 μ*M* NAA, 5 μ*M* 2,4-D, and 1.28 m*M* 2-morpholinoethanesulfonic acid (MES), adjust to pH 5.8 with 1 *N* NaOH. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.

   b. For aspen (CIM2): MS salts, 10 μ*M* NAA, 5 μ*M* 2iP, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
3. Bleach solution: 20% solution of commercial bleach (5.25% sodium hypochlorite) containing 0.1% of Triton X-100.
4. Luria–Bertani (LB) media (*12*): 16 g/L tryptone, 8 g/L yeast extract, and 5 g/L NaCl; adjust to pH 7.0. For solid medium, add 15% agar.
5. Induction medium (IM): 1/2-strength MS salts, MS vitamins, 200 mg/L L-glutamine, 100 mg/L myo-Inositol, 1.28 m*M* MES, and 1.8 g/L D(+)-galactose; adjust to pH 5.0.
6. Rooting medium: 1/2-strength MS containing 0.1 mg/L indole-3-butyric acid (IBA) adjusted to pH 5.8. Add 0.7% agar as solidify agent.
7. Shoot-induction medium (SIM)
   a. For cottonwood (SIM1): MS salts, 10 μ*M* BA, 10 μ*M* zeatin, 1 μ*M* NAA, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
   b. For aspen (SIM2): MS salts, 0.2 μ*M* TDZ, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
8. Shoot-elongation medium (SEM): MS salts, 0.2 mg/L BA, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
9. Washing solution: 1/2-strength MS salts, MS vitamins, 1 μ*M* NAA, 1 μ*M* BA, 1 μ*M* 2iP, 250 mg/L ascorbic acid, and 400 mg/L timentin (*see* **Note 3**); adjust to pH 5.8.

2.5. Growth Conditions

1. Tissue culture growth room: 25°C (continuous) with a 16-h photoperiod; the light is provided by fluorescent tubes (TL70, F25T8/TL735; Philips) at a photon flux density of 45 $\mu E/m^2/s$.
2. Head house: 22–25°C (continuous) with a 24-h photoperiod; the light is provided by fluorescent tubes (cool white, 95 W, F96T12/CW/HO/SS, Sylvania) at a photon flux density of 18 $\mu E/m^2/s$ (*see* **Note 4**).
3. Greenhouse: 25–27°C with a 16-h photoperiod; the light is provided by metal halide bulbs at a photon flux density of 45 $\mu E/m^2/s$.

3. Methods

3.1. Plant Preparation

3.1.1. From In Vitro-Grown Plants

1. Excise shoot tips (3 to 4 leaves) from 1- to 2-mo-old plants growing in Magenta GA-7 boxes containing propagation media (*see* **Fig. 1A,B**).
2. Transfer shoot tips into propagation medium with 0.1 mg/L IBA (*see* **Note 5**).
3. Separate remaining shoot tips into leaves, stems, and petioles (*see* **Note 6**).

3.1.2. From Ex Vitro-Grown Plants

1. Excise shoot tips (8 to 10 cm) from 3- to 6-mo-old greenhouse-grown plants.
2. Gently wash shoot tips in warm, soapy water for 3 to 5 min (*see* **Note 7**), followed by two rinses with sterile, deionized, double distilled (ddH_2O) water.

3. Separate leaves, stems, and petioles.
4. Soak plant parts in 70% ethanol for 1 to 3 min.
5. Gently rock explants in bleach solution for 10 to 12 min.
6. Rinse plant material 3 to 5 times with sterile ddH_2O.

3.2. Explant Preparation and Pre-culture

1. Place explants in sterile ddH_2O in sterile Petri dish (*see* **Note 8**).
2. Use an autoclaved single-hole paper punch in a laminar flow hood to cut discs from leaves (*see* **Fig. 1C**) (*see* **Note 9**).
3. Use a scalpel to cut stems and petiole into segments 5-to 8-mm long (*see* **Note 10**).
4. Wound stem and petiole sections by gently and repeatedly drawing the scalpel blade across their surfaces.
5. Align leaf disks and/or stem explants on CIM and leave Petri dishes in the dark for 1 to 2 d (*see* **Note 11**).

3.3. Inoculation With Agrobacterium and Co-cultivation

1. Streak *Agrobacterium* from frozen glycerol stock (*see* **Note 12**) on solid LB media; grow 1 to 2 d at 28°C.
2. Select an individual colony to inoculate 50 mL LB media containing appropriate antibiotics for the strain of *Agrobacterium* being used (*see* **Fig. 1E**) (*see* **Note 13**).
3. Grow *Agrobacterium* culture overnight on an incubating shaker set at 250 rpm and 28°C. Approximate $OD_{600\ nm}$ at harvest: 1.5 to 2.0.
4. Pellet bacterial cells by spinning for 30 min at 1992*g* (3500 rpm).
5. Dilute cell suspension to $OD_{600\ nm}$ of 0.4 to 0.6 with liquid IM containing 25 μM acetosyringone (AS) (*see* **Note 14**).
6. Place 30 to 40 explants (leaf discs and wounded stem segments) in capped, 50-mL disposable polypropylene, conical centrifuge (Falcon) tube containing 30 to 40 mL of diluted *Agrobacterium* culture.
7. Shake (orbitally) tubes containing *Agrobacterium* suspension and explants at 150 to 200 rpm for 1 h.
8. Decant *Agrobacterium* culture into a flask (*see* **Fig. 1E**) and remove excess *Agrobacterium* from explants by blotting them on sterile paper toweling using sterile forceps (*see* **Note 15**).
9. Align explants on fresh CIM (30–40 explants per plate). Incubate plates (*see* **Fig. 1F**) in the dark for 2 d (*see* **Note 16**).

3.4. Callus Induction and Shoot Regeneration

1. Transfer inoculated explants into Falcon tube; rinse four to five times with 30–40 mL sterile ddH_2O and once with washing solution.
2. Blot explants dry on sterile paper toweling and place onto CIM containing selection agent such as 25 mg/L kanamycin and 200 mg/L timentin (*see* **Note 17**).
3. Incubate explants in the dark for 2–3 wk.
4. Transfer explants to SIM containing 100 mg/L kanamycin and 200 mg/L timentin, culture sealed plates in growth room under lights (*see* **Fig. 1G**).

5. Subculture explants onto fresh SIM every 2–3 wk until shoots form.
6. Transfer explants with multiple small shoots to SEM containing 100 mg/L kanamycin and 200 mg/L timentin (*see* **Fig. 1H**).

3.5. Rooting Regenerants

1. For further selection, excise shoots at 0.5–1.0 cm down from growing tip and place individual shoots on rooting medium containing 25–50 mg/L kanamycin and 100 mg/L timentin. Between 10 and 12 shoots can be placed in each Magenta box (*see* **Fig. 1I**) (*see* **Note 18**).
2. Place Magenta boxes in growth room until roots emergence, usually 1 to 2 wk (*see* **Note 19**).
3. Propagate authentic transgenic plantlets by repeating **steps 1** and **2** of **Subheading 3.5.** (*see* **Fig. 1J,K**).

3.6. Transplanting into Soil

1. Select plants that are 1- to 2-mo post-propagation (about 5 to 6 cm tall).
2. Remove plants from Magenta boxes and rinse away excess agar gently with cold tap water (*see* **Note 20**).
3. Insert plant roots into moist potting mix in a 5.7 × 8.3 cm Rose Pot.
4. Place pots into Zip-Lok® sandwich bags containing about 100 mL of tap water.
5. Transfer plants to growth room, set at 25 ± 1°C and 16-h photoperiod, for about 3 wk.
6. Open bags for progressively longer intervals each day to allow acclimation to ambient conditions (*see* **Note 21**).
7. After 2 to 3 wk, plants can be removed from bags, transferred to greenhouse, and maintained with fertilizer once every 2 to 3 wk (*see* **Note 22**).

4. Notes

1. The binary vector can affect the efficiency with which transgenic plants are recovered. We have found the pART27 backbone to work well. We select for the presence of this binary vector in the bacterial host by using 200 mg/L spectinomycin.
2. Solutions 1–7 are stored at –20°C and filter-sterilized before use; solution 8 is stored at 4°C and no sterilization is needed because it is dissolved in 100% methanol.
3. Ascorbic acid and timentin should be added to the wash solution just before use.
4. In this room we equilibrate our plants (as described in **Subheading 3.6.**) before they are transferred to the greenhouse period.
5. This is a propagation step (so plants will be available for future use). In general, cottonwoods require an exogenous supply of auxin to stimulate root development, whereas aspens do not.
6. Use only fully expanded, healthy leaves.
7. Use a couple drops of standard dishwashing detergent/100 mL water in a 200-mL beaker.
8. This is done to keep the explants from desiccating and browning while being manipulated.
9. Try to include leaf veins when cutting the leaf discs, because a preponderance of shoots arise in the vicinity of the veins *(13)*.

10. Only use internodes and avoid nodes because the lateral buds frequently give "false-positive" shoots.
11. This step is known as pre-culture and may not be necessary for all poplar genotypes.
12. The stock is made by growing a liquid *Agrobacterium* culture to its stationary phase, mixing an aliquot 1:1 with glycerol in a 2-mL screw-cap freezer vial, and plunging the sealed tube into liquid nitrogen. When stored at –80°C, the cells in these stocks will remain viable for many years.
13. *Agrobacterium* strain C58/pMp90 (***9***) is effective for a wide range of *Populus* genotypes. Other useful strains include: AGL1, EHA105, and LBA4404.
14. Acetosyringone elicits the expression of *Agrobacterium vir*-region genes (***14***). It is best to add the AS immediately before use.
15. Sterilize paper towels by wrapping in aluminum foil and autoclaving for 20 min at 121°C.
16. This step is commonly referred to as co-cultivation. Do not allow severe *Agrobacterium* overgrowth. Normally, 2 to 3 d of co-cultivation is sufficient; after 4 to 7 d, the bacterium will overgrow the explant, making it difficult to control.
17. The selection agent used will depend on the selectable marker present on the T-DNA of your binary vector. Only those plant cells containing the T-DNA bearing the selectable marker gene will survive in the presence of the selection agent to which the marker provides resistance. The *npt*II gene is the most commonly used selectable marker; it imparts resistance to kanamycin. The concentration of kanamycin typically used with poplar is 50 mg/L. The optimum concentration of kanamycin or any other selection agent (e.g., an herbicide) will need to be determined empirically for the particular genotype of *Populus* with which you are working. The goal is to minimize the number of "escapes" (nontransformed cells that regenerate into plants) but avoid overwhelming the transgenic cells with too much antibiotic. The timentin is incorporated into the medium to kill the *Agrobacterium*.
18. It is important to record the date, the plate, and the explant from which the shoot was taken, and not to select shoots that are connected to the same callus mass. The goal is to have shoots that regenerated from cells representing independent transformation events (also known as "line").
19. Shoots that root in kanamycin-containing media are likely to be transformed. However, it is necessary to verify the presence of the transgene in the regenerated plants via PCR, and eliminate the possibility of escapes.
20. Media removal should be thorough in order to avoid fungal or bacterial contamination during plant acclimation.
21. The bag should be opened for 10 to 20 min on the first day. During this time, the plants must be checked frequently. If the plants begin to wilt, mist them with water and close the bag immediately. On successive days, the bags can be left open for progressively longer intervals (try doubling the time the bag is left open each day). It is very important not to stress the plants to the point that they wilt.
22. Plants can be maintained in vitro, in the greenhouse, or in the field. If the latter is done, a permit will need to be obtained from the USDA Plant and Animal Health Inspection Service (APHIS; *http://www.aphis.usda.gov/brs/index.html*).

References

1. Eckenwalder, J.E. (1996) Systematics and evolution of *Populus*. In: *Biology of Populus and its Implication for Management and Conservation* (Stettler, R.F., Bradshaw, H.D., Jr., Heilman, P.E., and Hinckley, T.M., eds.), National Research Council Canada, Ottawa, ON, pp. 201–222.
2. DeBlock, M. (1990) Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of hybrid aspen and poplar clones. *Plant Physiol.* **93,** 1110–1116.
3. Confalonieri, M., Balestrazzi, A., and Bisoffi, S. (1994) Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*. *Plant Cell Rep.* **13,** 256–261.
4. Confalonieri, M., Balestrazzi, A., Bisoffi, S., and Cella, R. (1995) Factors affecting *Agrobacterium tumefaciens*-mediated transformation in several black poplar clones. *Plant Cell Tissue. Org. Cult.* **43,** 215–222.
5. Huang, F.H. (1994) Putatively *Agrobacterium tumefaciens*-mediated transformation of glyphosate tolerant hybrid poplar strain NC 5331. *Q. J. Chin. For.* **27,** 43–50.
6. Wang, G., Castiglione, S., Chen, Y., et al. (1996) Poplar (*Populus nigra* L.) plants transformed with a *Bacillus thuringiensis* toxin gene: Insecticidal activity and genomic analysis. *Transgen. Res.* **5,** 289–301.
7. Heuchelin, S.A., McNabb, H.S., and Klopfenstein, N.B. (1997) *Agrobacterium*-mediated transformation of *Populus* x *euramericana* "Ogy" using the chimeric CaMV 35S-*pin2* gene fusion. *Can. J. For. Res.* **27,** 1041–1048.
8. Han, K.-H., Meilan, R., Ma, C., and Strauss, S.H. (2000) An *Agrobacterium* transformation protocol effective in a variety of cottonwood hybrids (genus *Populus*). *Plant Cell Rep.* **19,** 315–320.
9. Koncz, C. and Schell, J. (1986) The promoter of the T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204,** 383–396.
10. Gleave, A. (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20,** 1203–1207.
11. Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15,** 473–496.
12. Luria, S.E., Adam, J.N., and Teng, R.C. (1960) Transduction of lactose utilizing ability among strains of *Escherichia coli* and *Shigella dysenteriae* and the properties of the transducing phage particles. *Virology* **12,** 348–390.
13. Lee-Stadelmann, O.Y., Lee, S.W., Hackett, W.P., and Read, P.E. (1989) The formation of adventitious buds in vitro on micro-cross sections of hybrid *Populus* leaf midveins. *Plant Sci.* **61,** 263–272.
14. Stachel, S.E., Messens, E., van Montagu, M., and Zambryski, P. (1985) Identification of the signal molecules produced by wound plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. *Nature* **318,** 624–629.