Plant Physiol. (1992) 98, 166–173
0032-0889/92/98/0166/08/$01.00/0

Received for publication January 10, 1991
Accepted August 5, 1991

Rejuvenation of *Sequoia sempervirens* by Repeated Grafting of Shoot Tips onto Juvenile Rootstocks *in Vitro*[1]

Model for Phase Reversal of Trees

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ABSTRACT

Repeated grafting of 1.5-centimeter long shoot tips from an adult *Sequoia sempervirens* tree onto fresh, rooted juvenile stem cuttings *in vitro* resulted in progressive restoration of juvenile traits. After four successive grafts, stem cuttings of previously adult shoots rooted as well, branched as profusely, and grew with as much or more vigor as those of seedling shoots. Reassays disclosed retention for 3 years of rooting competence at similar levels as originally restored. Adventitious shoot formation was remanifested and callus development was depressed in stem segments from the repeatedly grafted adult. The reversion was associated with appearance and disappearance of distinctive leaf proteins. Neither gibberellic acid nor N^6-beneyladenine as nutrient supplements duplicated the graft effects.

Development of plants usually progresses from a strictly vegetative juvenile phase to the sexually competent adult phase. The process of maturation is achieved within a single growing season among annuals and over several years in trees. When lacking reproductive structures, juvenile and adult phases may still be distinguishable by leaf phyllotaxy, shape, size, and color; stem branching and tropism; thorniness; and other vegetative characteristics. Tissue culturists and plant propagators regard as most significant the diminished competence for organogenesis and plant regeneration in explants or cuttings obtained from mature plants. Adventitious root and shoot formation is substantially reduced, often absent, in explants or cuttings from flowering-age trees.

Maturation of the shoot apical meristem has been reversible. Juvenile growth has been reobserved in adult plants or tissues after applications of GA_3, *e.g. Hedera helix* (20), *H. canariensis* (24), and *Acacia melanoxylon* (4), or cytokinin, *e.g. Vitis vinifera* (14) and *Picea abies* (5); prolonged subculturing of shoots *in vitro, e.g. Eucalyptus* spp. (9) and *Sequoia sempervirens* (26); and grafting of scions with shoot apices onto juvenile rootstocks, *e.g. H. helix* (8), *H. canariensis* (24), *Hevea brasiliensis* (17), *Persea americana* (19), *S. sempervirens* (9), *Sequoiadendron giganteum* (13), and *Thuja plicata* (12).

Investigators of phase change have used *H. helix,* a vine when juvenile, as the experimental model because reversion is quickly achieved, particularly by gibberellin treatments. The degree to which the *H. helix* meristem is rejuvenated has depended on gibberellin dosage (20), and the rejuvenating effects of gibberellin have been preventable with ABA (21). Unfortunately, some of the more significant results from *H. helix* experiments, particularly those implicating gibberellin, have not been entirely reproducible with trees. At best, only transient characteristics have been restorable by gibberellin treatment, *e.g.* a few cycles of juvenile leaves (4). Furthermore, with some tree species gibberellin has caused precocious flowering (18).

We now present an alternative model for trees, based on reversion of *Sequoia* shoot tips after their repeated grafting onto juvenile rootstocks *in vitro.* Morphogenic competence and related biochemistry are restored progressively and incrementally in successive grafts; transmission of juvenile traits occurs relatively quickly; and restored traits are retained for 3 years. The species is also easily established and manipulated in tissue culture, and grafts are easily performed.

MATERIALS AND METHODS

The procedure of repeatedly grafting an adult shoot tip onto fresh juvenile rootstocks *in vitro* is illustrated in Figure 1. In using the procedure with *Sequoia sempervirens* (D. Don.) Endl., protocols had to be developed for maintenance of shoots of varying levels of maturity of rejuvenation, preparation of rejuvenating rootstocks, preparation of scions, grafting and caring for grafts, and appropriate bioassays.

Protocol for Shoot Cultures

Cultures of continuously proliferating shoots were initiated from stem terminals of freshly germinated seedlings and of newly emerged laterals on cone-bearing branches of mature

[1] Research supported in part by grants NSC-77-0201-B001-16 and NSC-78-0211-B001-08 to L.-C.H. from the Republic of China National Research Council and INT-8711887 to T.M. from the U.S. National Science Foundation.

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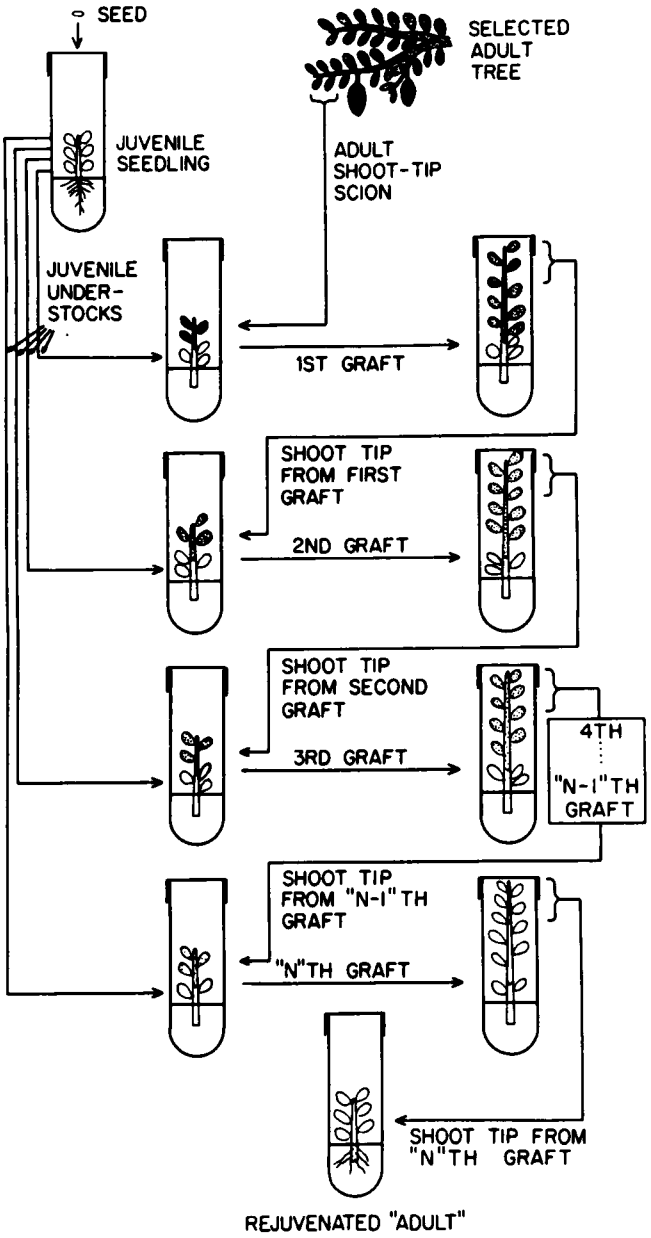

Figure 1. Scheme for repeated grafting of a mature tree shoot tip onto fresh juvenile rootstocks *in vitro*.

trees, and from remnant scion growth after regrafting of their shoot tips. The seedlings provided the juvenile material and the mature trees furnished the initial adult explants.

Seeds were disinfested by immersion in 0.5% sodium hypochlorite for 20 min, rinsed with autoclaved water and sown on filter-paper platforms in 250-mL DeLong flasks. The flask contained 10 mL of autoclaved distilled water. After 3 to 5 d at 27°C and under low intensity (22.5 μmol $m^{-2}s^{-1}$, 16 h daily, Gro Lux) illumination, uninfected seeds were transferred to new flasks and allowed to continue their germination. After an additional 2 weeks, the seedlings were transferred to nutrient medium in 25- × 150-mm glass culture tubes. The medium contained Murashige and Skoog salts (16), 3% sucrose, 0.2% Gelrite, and, in μM; 555 *i*-inositol, 3 thiamine HCl, 2.4 pyridoxine HCl, 4.1 nicotinic acid, and 26.6 glycine. Each tube contained 25 mL of medium, was capped with polypropylene closure (Bellco kaput), and was autoclaved at 121°C for 10 min. After a further 6 weeks, 1- to 2-cm long stem terminals were excised and cultured in the above nutrient medium.

For initial culture of adult shoots, 3-cm long terminals were washed with household detergent, disinfested with 0.5% sodium hypochlorite and rinsed, and their 1-cm tips were severed and transferred to nutrient tubes, as above.

Stocks of all shoots were established by subculturing 2- to 3-cm long terminal growths at 6-week intervals. They were maintained at constant 27°C and under 16-h daily exposure to 22.5 μmol $m^{-2}s^{-1}$ Gro Lux light.

Protocol for Preparing Rootstocks

Rootstocks were prepared from stock-cultured seedling shoots. Terminal segments, approximately 2 cm long, were rooted as follows: first, they were placed 1 per tube in 25- × 150-mm glass culture tubes containing 12.5 mL of root-inducing medium. This medium contained no mineral salts, but 1.5% sucrose, 0.2% Gelrite, and, in μM: 555 *i*-inositol, 3 thiamine HCl, 2.4 pyridoxine HCl, 4.2 nicotinic acid, 26.6 glycine, and 492 indole-3-butyric acid. After 2 weeks, they were transferred to root-emergence medium, the composition of which included the above ingredients, but without the indole-3-butyric acid, the sucrose raised to 3%, and the addition of Murashige and Skoog salts. The root-emergence step employed 25 mL of medium per tube. In both steps, the cultures were positioned vertically and illuminated 16 h daily with 22.5 μmol $m^{-2}s^{-1}$ Gro Lux light. After 1 week in root-emergence medium, the shoots were transferred to Petri dishes lined with moist Whatman No. 42 filter paper and prepared for insertion of scions. Roots had not yet emerged at this time. Their terminals were severed, leaving 1-cm long basal segments, and the segments were slit 2 to 3 mm longitudinally down the middle.

Protocol for Preparing Scions

Scions were obtained from stock-cultured adult shoots and from emerged shoots of grafted shoot tips. Terminals, 1.5 cm long, were found to be optimum. Their basal ends were cut obliquely into wedges to facilitate insertion into rootstocks.

Grafting and Care of Grafts

Grafting was done by simply inserting the scion wedge into the longitudinal incision of the rootstock. The grafted stems were transferred to nutrient tubes containing freshly prepared root-emergence medium and incubated upright under 16-h daily illumination. Shoots that originated in rootstock were removed as they emerged. Over 90% of grafts showed successful union after 4 weeks. Samples of freshly and successfully grafted plants can be seen in Figure 2. Regrafts onto fresh rootstocks were performed after 8 weeks.

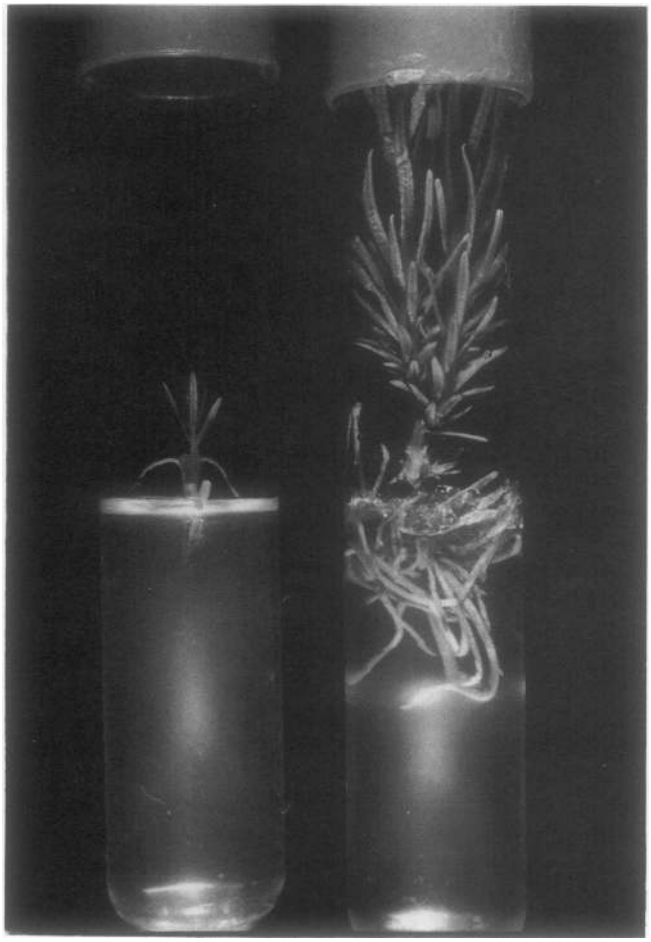

Figure 2. Freshly (left) and successfully grafted (right) *S. sempervirens in vitro*. The latter was photographed after 3 months and is therefore further developed than the experimental graft.

Criteria of Phase Reversal and Bioassays

The main criterion of reversion was the restoration of rooting competence. Uniform, 2-cm long cuttings of graft-derived shoots were rooted through the two-step procedure employed in preparing rootstocks. The incidence of rooted shoots and the number of roots were recorded after 8 weeks in the root-emergence medium. A second criterion was shoot vigor, observable as increased stem elongation and lateral branching rates in the root-emergence medium. Still another criterion of rejuvenation was the capacity of stem segments to produce adventitious shoots. For this, segments with their lateral buds removed were cultured in a medium supplemented with 10 μM each of IAA and kinetin. Finally, callus development was examined in stem sections and subcultured callus, using supplements of 10 μM IAA and 1 μM kinetin. The adventitious shoots were allowed to develop under illumination, and the callus in constant darkness.

GA_3 and BA[3] Effects

Possible duplicatability of grafting effects by gibberellin or cytokinin treatments was examined by preculturing adult and once-grafted shoots in nutrient media supplemented with 10^{-6} to 10^{-4} M GA_3 or BA for 4 weeks, then assaying for rooting competence.

Protein Analysis

Leaves were flash frozen in liquid nitrogen and their proteins extracted by TCA precipitation, following the method of Damerval *et al.* (7). Protein concentrations were measured according to the procedure of Bradford (6). The two-dimensional PAGE protocol of Beckstrom-Sternberg (2) was used for separations. Ampholines for isoelectric focusing were used in a 4:1 ratio of Servalyte (pH range 5–7) and Pharmalyte (pH range 3–10). Isoelectric gels were focused for 30 min at 200 V, then 90 min at 300 V. One microgram of carbonic anhydrase pI markers was loaded prior to 20 μg of protein sample. The second-dimension gels contained 12.5% acrylamide and employed Gelcode molecular weight standards. Electrophoresis was carried out in this sequence: 1 h at 300 V, 18.5 h at 600 V, and 1 h at 1200 V. Gels were color silver-stained according to the procedure of Beckstrom-Sternberg (2).

Statistical Analysis

Statistical significance was determined by computing standard errors of means or obtaining 95% confidence limits from tables of binomials (11, 23).

RESULTS

Rooting Competence

Rooting occurred in 100% of seedlings and up to 30% of adult stem sections (Fig. 3). Other experiments disclosed that

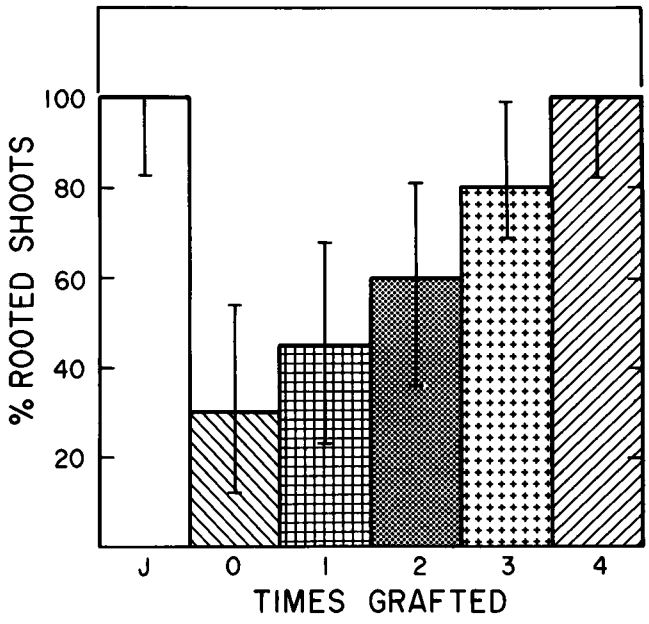

Figure 3. Incidence of rooting in stem cuttings from repeatedly grafted *S. sempervirens* shoot tips. In all figures, J = juvenile, or seedling stem, and 0 = ungrafted adult. Also, bars = ± SE.

[3] Abbreviations: BA, N^6-benzyladenine; pI, isoelectric point.

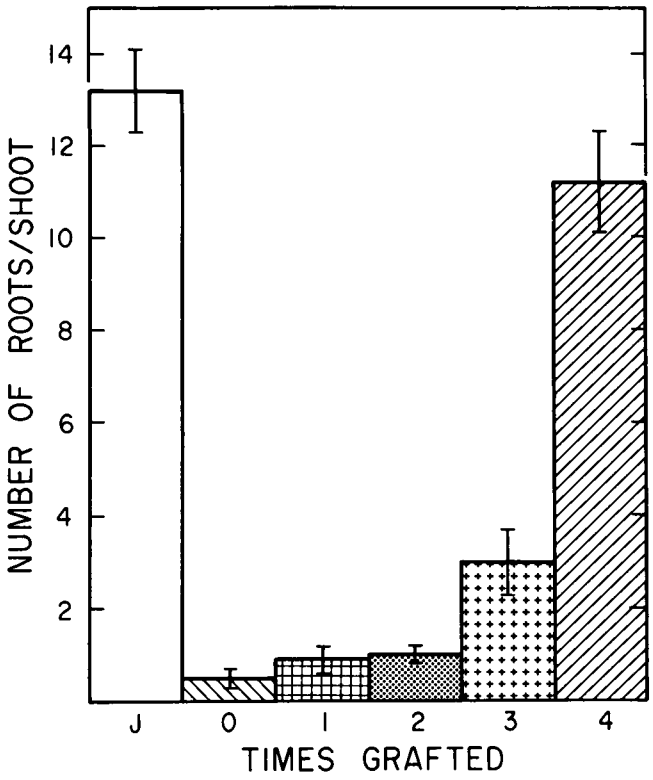

Figure 4. Number of roots produced on cuttings from repeatedly grafted *S. sempervirens* shoot tips.

adult stems freshly excised from trees usually rooted at substantially lower frequencies, or below 10%. The rooting incidence increased linearly with successive regrafts of the adult shoot tip onto fresh juvenile rootstock, with 100% rooting being attained after four grafts. The number of roots per cutting increased less steeply over the first three grafts, then rose sharply from the third to the fourth graft (Fig. 4). Juvenile shoots produced 13 roots per cutting and ungrafted adults averaged fewer than 1. Significant increase of root number required three repeated grafts; essentially complete restoration of root number needed four grafts. Representative cultures of grafted and ungrafted shoots in root-emergence medium can be seen in Figure 5.

Elongation and Branching of Stem

Juvenile stems elongated 1.5 cm after 4 weeks in root-emergence medium, whereas ungrafted adult stems elongated only slightly more than 0.5 cm (Fig. 6). Stem elongation of the adult improved linearly over three successive grafts, eventually equaling that of juvenile stems after the third graft. Elongation of four-times-grafted *Sequoia* was, unexpectedly, about twice that of the juvenile.

Lateral branching increased markedly with successive grafts (Fig. 7). Ungrafted adult shoots showed less branching than juvenile shoots. Repeatedly grafted adult shoots, on the other hand, displayed substantially more branching than juvenile shoots.

Leaves acquired increasingly juvenile-appearing characteristics (Fig. 5). Those of the ungrafted adult were rigid and relatively orthotropic, whereas leaves of the juvenile and repeatedly grafted adults appeared flaccid and tended to

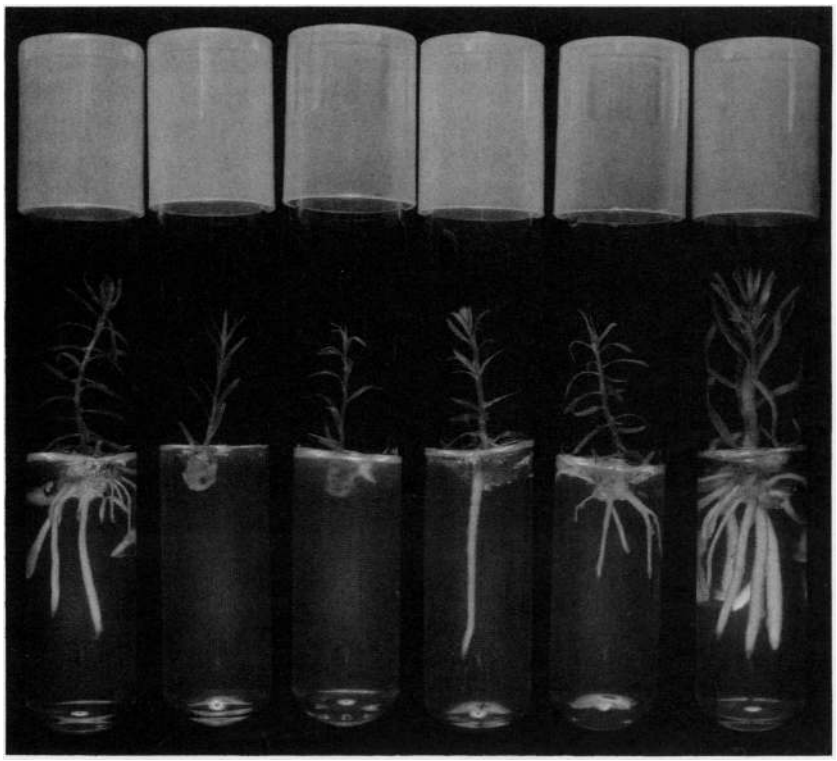

Figure 5. Representative cultures of shoot terminals from grafted and ungrafted *S. sempervirens* shoot tips. Left to right: J, ungrafted, and one-, two-, three-, and four-times-grafted adult. Note flaccid and drooping leaves of juvenile and rejuvenated shoots, and their thicker, softer-appearing stems.

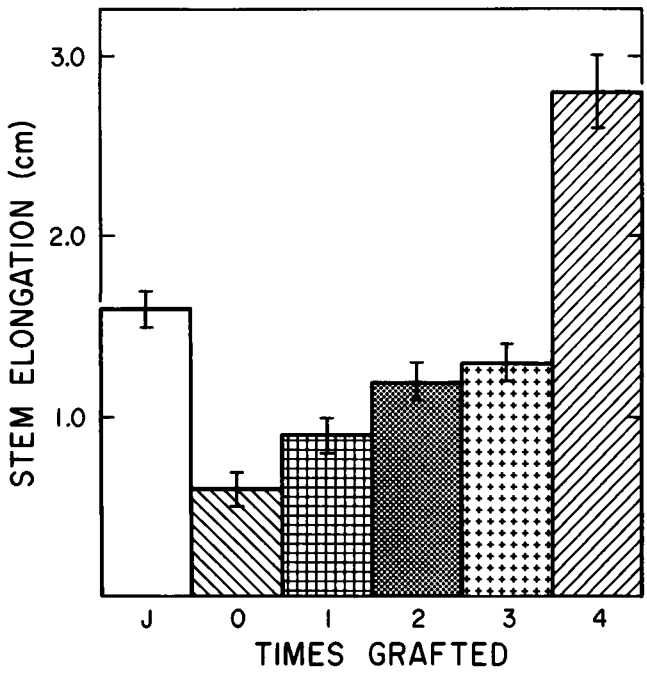

Figure 6. Elongation of stem terminals from repeatedly grafted *S. sempervirens* shoot tips. Note that elongation of the four-times-grafted adult exceeded that of the juvenile stem.

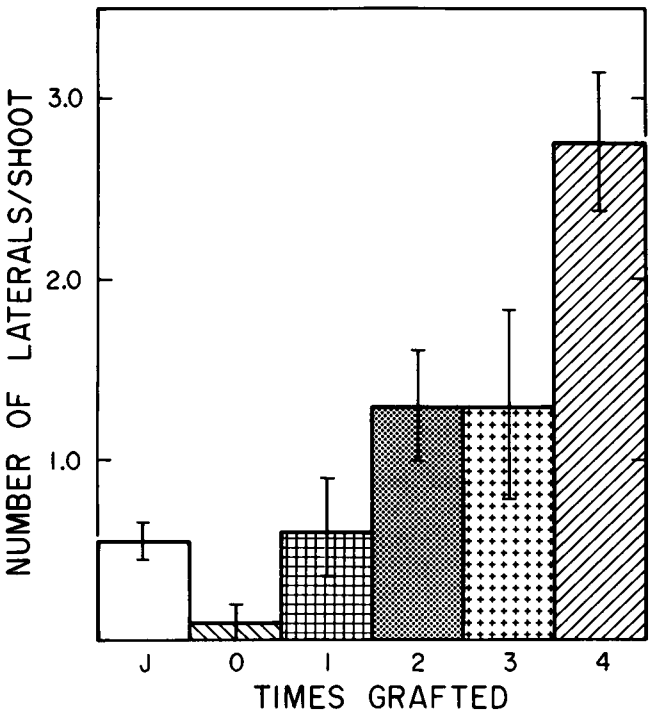

Figure 7. Effects of repeated grafting of adult shoot tips from mature *S. sempervirens* onto juvenile rootstock on restoration of branching competence in adult stem. Also note that branching of the repeatedly grafted adult exceeded that of juvenile stems.

Table I. *Adventitious Shoot Formation in Stem Segments from Seedling, Adult, and Repeatedly Grafted Adult S. sempervirens Shoots*

Shoot Source	% Cultures with Adventitious Shoots	Adventitious Shoots/Segment	% Dead Shoots
Seedling	80 (56–94)[a]	2.6 ± 0.7[b]	5 (0–25)
Adult tree	0 (0–17)	0	100 (83–100)
One ×-grafted adult	10 (0–25)	0.1 ± 0.1	90 (69–99)
Two ×-grafted adult	25 (9–49)	0.3 ± 0.1	75 (51–91)
Three ×-grafted adult	50 (27–73)	0.8 ± 0.2	45 (23–68)
Four ×-grafted adult	75 (51–91)	2.0 ± 0.3	15 (3–38)

[a] 95% confidence limits in parenthesis. [b] ± SE.

droop. Stems of the juvenile and repeatedly grafted adults were also less rigid. Again unexpectedly, four-times-grafted adult shoots developed distinctly larger leaves and thicker stems than seedling shoots.

Adventitious Shoot Formation

Adventitious shoots differentiated in 80% of the juvenile stem segments and none of the ungrafted adult stem segments (Table I). Repeated grafting increased the incidence of adult stems that formed adventitious shoots. The incidence equaling that of juvenile shoots was attained after four grafts. The number of adventitious shoots per explant also increased progressively in successive grafts, with the maximum being resultant after four grafts.

Further apparent was an improved survival of stem sections when cultivated in shoot differentiation medium (Table I). Nearly all juvenile, but no adult, explants survived. The mortality rate among adult explants was lowered proportionately with repeated grafts.

Callus Development

Table II contains callus growth data of seedling, adult, and grafted adult tissues. Increase of callus fresh weight occurred more slowly in juvenile than in adult tissue. Repeated grafting

Table II. *Fresh Weight Yields of Callus, in mg, from Stem Segments of Seedling, Adult, and Repeatedly Grafted S. sempervirens Shoots (Inoculum Weighed 200 mg)*

Shoot Source	Days in Culture		
	7	14	21
Seedling	285 ± 10[a]	379 ± 25	473 ± 46
Adult tree	279 ± 12	422 ± 37	810 ± 66
Three ×-grafted adult	263 ± 9	403 ± 17	695 ± 62
Four ×-grafted adult	296 ± 13	322 ± 21	402 ± 40

[a] ± SE.

of the adult onto juvenile rootstock resulted in progressively depressed callus yields; a yield comparable to that of the juvenile was observed after four grafts.

Protein Patterns

Readily reproducible two-dimensional PAGE patterns of proteins were obtained for extracts from repeated samples of leaves. Photographs of typical gels of adult, juvenile, and repeatedly grafted adult can be seen in Figure 8. Figure 8B also includes the gel pattern of adult shoots that had been partially rejuvenated by prolonged subculturing; the medium contained no cytokinin. That of the more recently initiated culture of adult shoots is seen in Figure 8A.

Careful examination of gels disclosed several proteins, some of which are indicated by arrows, that were evident in leaves of adult shoots but not in juvenile and repeatedly grafted shoots, and vice versa. Leaves of adult *Sequoia* contained six proteins with relative pIs of −7.6 and −9.0 and approximate mol wts of 36,400, 35,700, and 35,000. Those of juvenile and grafted adult displayed proteins that migrated with these relative pIs and mol wts, respectively: −8.5 and 37,800; −6.25 and 27,400; −4.25 and 27,400; −6.25 and 25,400; −6.25 and 25,000; and −4.25 and 25,000.

Retention of Rooting Competence

Table III presents the rooting incidence data, obtained from reassays performed 6 months after all grafting had been terminated and again after 3 years of stock culture. There was a slight increase in rooting competence during stock culture, particularly of shoots that had been restored only to perhaps threshold levels, *e.g.* 3×-grafted shoots.

GA_3 and BA Effects

Preculturing adult, once-grafted adult, and juvenile shoots for 4 weeks in media supplemented with GA_3 or BA resulted only in depression of rooting and shoot growth, and more notably at the two higher dosages, 10^{-5} and 10^{-4} M. The depressions continued through three passages in phytohormone-free medium. Bioassays performed on shoots precultured in the lowest growth-regulator concentration, 10^{-6} M, and on those that had recovered from the inhibitory effects of the higher doses, revealed no evidence of reversion.

DISCUSSION

H. helix has been the classic model for phase-change investigations. But it may not be the best source of information for

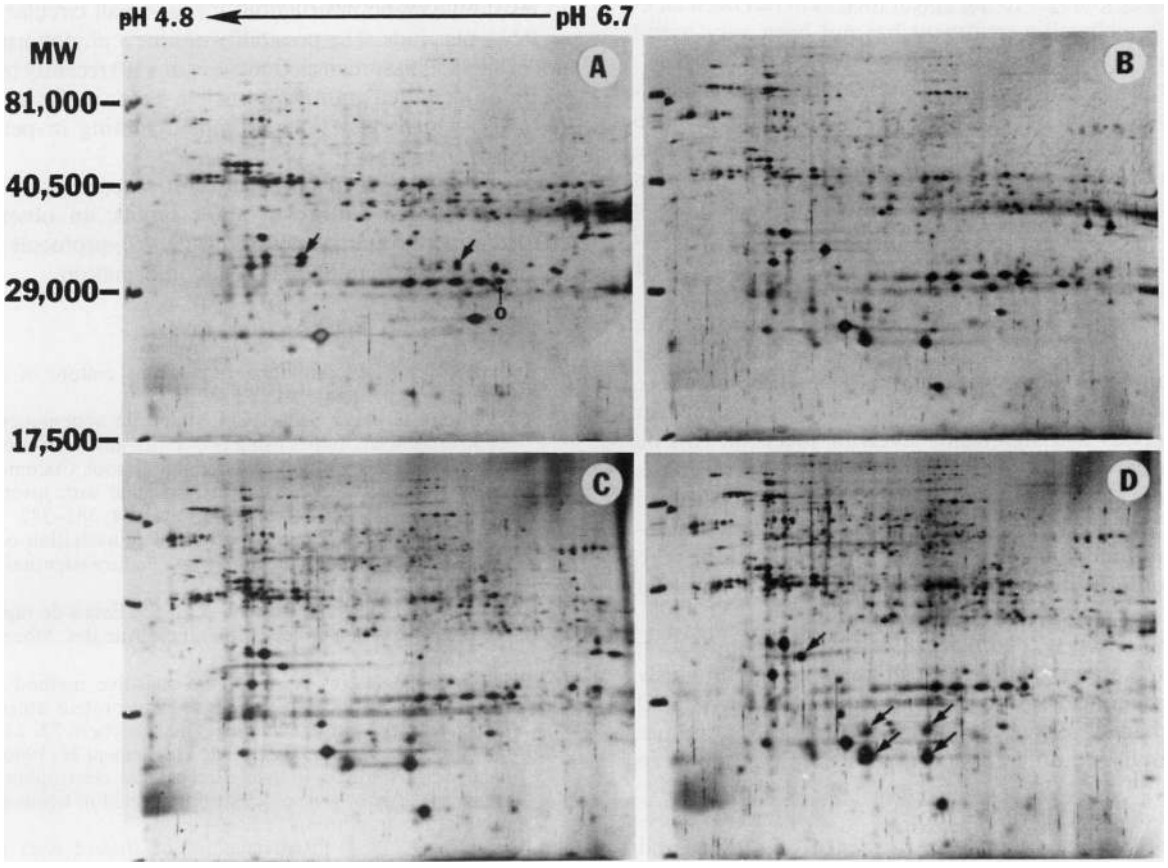

Figure 8. Two-dimensional PAGE patterns of leaf proteins of adult, juvenile, and adult that had been rejuvenated by repeated grafts. A, Newly established culture of adult shoots; B, adult shoots that had been in stock culture over 3 years; C, repeatedly grafted adult; and D, juvenile, or seedling, shoots. Arrows indicate proteins that occur in adult or seedling leaves, but not both.

Table III. *Retention of Relative Levels of Restored Rooting Competence in Successively Grafted Adult S. sempervirens Shoots*

Initially assayed and reassayed in 1985, and again reassayed in 1988

Shoot Source	% Rooted Shoots		
	Initial assay	First reassay	Second reassay
Seedling	100 (78–100)[a]	93 (68–100)	100 (83–100)
Adult tree	33 (12–62)	33 (12–62)	20 (6–44)
One ×-grafted adult	40 (16–68)	40 (16–68)	40 (19–64)
Two ×-grafted adult	53 (27–79)	47 (21–73)	50 (27–73)
Three ×-grafted adult	67 (38–88)	53 (27–79)	85 (56–94)
Four ×-grafted adult	100 (78–100)	100 (78–100)	100 (83–100)

[a] 95% confidence limits in parentheses.

the broad range of plant species. Some fundamental findings, particularly the critical responses to giberellin, have not been duplicated with tree species. Gibberellin treatment has elicited some organ modifications, *e.g.* reappearance of juvenile leaves for a few cycles (4). But evidence of profound and lasting morphogenetic changes in the apical meristem has been lacking. Clearly, gibberellin treatment has not been very useful for exploring phase reversal of trees.

In contrast, rejuvenation has been more generally achievable when scions from mature plants were grafted onto juvenile rootstocks. In fact, the first evidence of reversion by grafting was demonstrated with *H. helix* (8). Nevertheless, whereas rejuvenation of trees occurs incrementally with successive grafts, that of *H. helix* is achieved after grafting only once. Progressive changes that are associated with the gradual shift in phases are, thus, not as easily observed in *H. helix.*

Grafting for rejuvenation can be performed *in vitro* (12, 13, 19) as well as *in vivo* (8, 9, 17, 24). Grafting in the former instance can be done with substantially smaller and more juvenile rootstocks; hence, regrafting can be performed at shorter intervals and significant levels of reversion can be attained more quickly. With *S. sempervirens,* regrafting is possible every 8 weeks and key juvenile traits can be observed after the four successive grafts. Furthermore, unless controlled-environment chambers are used *in vivo,* the *in vitro* procedure ensures more readily reproducible developmental conditions.

S. sempervirens was chosen because an earlier investigation had shown the ease with which cultures of continuously proliferating shoots could be established, both juvenile and adult, and had developed a rooting bioassay (25). Another study had disclosed differentiation of adventitious shoots in tissue culture (1).

The graft-associated rejuvenation of *S. sempervirens* is evident as progressive increases in incidence of rooting and number of roots, elongation and branching of stems, and vigor of roots and stems; restoration of juvenile leaves and competence for adventitious shoot differentiation; diminishment of callus growth; and reduction in mortality of explants. None of these changes are effected by nutrient medium addenda of gibberellin or cytokinin. The repression, especially of rooting, by GA_3 and BA is not unexpected. Even at much lower dosages, both gibberellin and cytokinin have been established as inhibiting rooting in most species (15, 22). The rejuvenated states, based on increased rooting competence, were retained by stock-cultured shoots for more than 3 years, and at intensity levels that corresponded to the number of successive grafts. Unfortunately, none of the plants were established in soil to observe their further development, especially their cone-bearing characteristics.

Of considerable significance was the absence of some distinctive proteins and appearance of others in the leaves of rejuvenated *S. sempervirens* when compared with proteins from adult leaves. The two-dimensional PAGE patterns were essentially identical for repeatedly grafted adult and juvenile. Bon (3) has been able to correlate a 16 kD shoot apex protein with juvenility of *Sequoiadendron giganteum,* but the precise role of this protein remains unestablished. The protein observations, nevertheless, suggest a logical basis for studying phase change at the molecular level. Additional information now available includes the transmissibility of the signal through cells of the graft union; this probably implies a small sized molecule(s). Prolonged retention of restored traits, at least 3 years *in vitro,* suggests self-replicating molecule(s), perhaps nucleic acid(s). If it is nucleic acid, then it must be constructed to resist enzymatic destruction, *e.g.* as small circular DNA- or RNA-plasmids. The possibility of a new phytohormone is not excluded, inasmuch as Geneve *et al.* (10) recently reported on an unidentified graft transmissible factor in the lamina of *H. helix,* which over time promoted rooting in petiole of mature *H. helix.*

In view of the slight but significant reversion that occurs during prolonged culture of adult shoots, an observation consistent with other investigations (9, 26), protocols should be followed closely to obtain precise information.

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