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Floral induction in woody angiosperms

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Application. Methods for floral induction will enable breeders to more rapidly improve populations. They are particularly important to tree improvement and reforestation programs because genetically superior individuals can be identified and bred more rapidly. Early flowering techniques will also allow genetic engineers to demonstrate stability of transgene inheritance and expression, and efficiently study effects of gene constructs designed to induce reproductive sterility. Sterility will enhance environmental containment of transgenes and may stimulate increased Abstract. The long juvenile period of trees is a severe impediment to study of reproductive biology and genetic inheritance, and is a serious constraint for traditional breeding programs. Very little effort has been directed toward the development of practical methods for inducing early flowering in most woody angiosperms, particularly members of the genus Populus. This review is intended to stimulate interest in, and provide direction for, future research in this area. An emphasis is placed on techniques that can be applied easily and inexpensively. Inductive treatments discussed include: phytohormones (gibberellins, cytokinins, abscisic acid, ethylene and auxin); growth retardants (paclobutrazol, uniconazole, daminozide, chlormequat and cimeethacarb); physical constraints (girdling, root restriction and shoot training); cultural conditions (photoperiod, mineral nutrition, moisture stress and temperature); and grafting. Recent reports on stimulation of flowering by genetic transformation with floral homeotic genes are also described. Several research avenues which appear promising are proposed for near-term study.

Introduction

This review was motivated by our ongoing studies on genetic engineering of reproductive sterility in poplars (genus Populus). Recently we transformed various poplar clones with genes that should be capable of causing male and female sterility. Currently we must wait several years for these trees to reach maturity so that the effects of these genes can be confirmed. If reproductive sterility is to serve as a means for risk reduction during deployment of transgenic trees (reviewed in Strauss et al. 1995), it is necessary to be able to test several kinds of genes and observe repeatable effects over several years. Likewise, rapid generational turnover is needed to speed traditional breeding,

genetic analysis, and character dissection using genome mapping techniques. Surprisingly, no reliable methods or systematic studies for inducing precocious flowering in the genus Populus are known. This review is therefore based primarily on results from other genera of woody angiosperms, with occasional references to conifers where relevant. It tends to focus on those treatments that can be used on a practical basis in operational breeding and experimental programs (i.e. labor-intensive methods or those requiring chemicals that are prohibitively expensive or difficult to obtain were excluded). Finally some recently developed molecular approaches to floral induction are included.

Biology of flowering

Juvenility is generally defined as the period during which a plant cannot be induced to flower (Zimmerman et al. 1985). The duration of the juvenile phase in woody plants is quite variable, and can be quite lengthy. For example, seedlings in the genus *Rosa* flower when 20-30 days old, whereas *Fagus sylvatica* L. doesn't flower until it is 30-40 years old. This is a serious problem in forestry, where many of the commercially important species don't flower until they are at least 15-20 years old (Hackett 1985). Research on flowering in forest trees has historically been done for two primary purposes: to accelerate progeny testing and to speed the production of genetically improved seed (Bonnet-Masimbert and Zaerr 1987).

There are many exceptions, however, to this stringent definition of juvenility. *Betula* seedlings can initiate male and female catkins within eight to 30 months of germination. Two-year-old western red cedar *(Thuja plicata* D. Don) seedlings also flower in response to ringing (girdling) and gibberellic acid treatments. They were considered to be immature based on their juvenile leaf character. In addition, cuttings taken from a seven-month-old tropical tree, *Triplochiton scleroxylon* K. Schum., were induced to flower in under two years when seedlings were decapitated and grown at a 45° angle. Although the age at which this species normally flowers was not given, it was assumed that the seedlings were immature (Longman 1976). These examples have led Longman to question whether some species even have a juvenile period.

Phase change describes the period during which a plant undergoes the transition from juvenility to maturity. This transition is a gradual and continuous process. Phase change is said to have occurred if a plant flowers, no matter how flowering is induced, *and* flowering continues under normal conditions without the application of the stimulus that originally induced flowering (Zimmerman et al. 1985).

Juvenility is often characterized by a period of rapid vegetative growth, which slows considerably after maturity is reached. Obviously, it is advantageous for a plant to delay reproductive growth, not only to compete for light and other resources but to produce sufficient photosynthetic capacity to support seed and biomass production. Certain treatments that retard growth have been demonstrated to promote flowering of reproductively mature trees of the same species. However, there is little evidence that such treatments promote earlier flowering in immature seedlings. Where such treatments are effective, seedlings are probably in transition rather than in a juvenile phase (Hackett 1985).

The ability to flower is regarded as a stable condition; once maturity is achieved, plants will continue to flower provided that the normal flower-inducing conditions are imposed (Hackett 1985). The absence of flowering does not necessarily imply immaturity. Flowering only indicates that maturity has been reached, not when it occurred (Zimmerman et al. 1985). Maturity can be reversed by sexual or apomictic reproduction; adventitious bud/embryo formation; and by various nutritional regimes, hormonal treatments or environmental conditions (Hackett 1985; Wareing 1987).

The juvenile and mature growth phases are often distinguishable by other morphological characteristics. Depending on the species, these can include: thorniness, leaf shape and size, phyllotaxy, ability to form adventitious roots/buds, and pigmentation (reviewed by Hackett 1985). In this regard, Wareing (1959) drew a distinction between maturation and aging. The former describes transition from juvenility to maturity; the latter includes loss of vigor associated with increased complexity. Maturation only occurs in seedling plant development, whereas plants propagated vegetatively from sexually mature plants, unless rejuvenated, only undergo aging (Hackett 1985).

In assessing ability to flower, it is important to use treatments that are known to induce flowering but do not promote maturation. Confusion arises when the same treatments promote sexual maturity and flower initiation. In many of the studies cited in this review, it is not possible to distinguish between the two during the juvenile or transitional period. Accordingly, the use of precocity will be restricted to those cases where the plants that flowered were thought to be immature.

Chemical methods

Phytohormones

Gibberellic acid

Gibberellins (GAs) are plant growth regulators which are produced in the roots and fruit of higher plants (Frydman and Wareing 1973b; Luckwill 1980). It has also been postulated that GA precursors produced in leaves and buds are converted to other forms in the roots (Crozier and Reid 1971). Although they are primarily known for their roles in seed germination and internode elongation, they have also been used to stimulate precocious flower production in gymnosperms (Pharis et al. 1987; Pharis and King 1985; reviewed in Sedgley and Griffin 1989). However, they appear to have little effect on floral initiation in woody angiosperms. This was evident in a recent report on work with cherry *(Prunus avium L.;* Oliveira and Browning 1993), but is true for a number of other species as well (see references in Pharis and King 1985). In general, chemical treatments that stimulate precocious flowering in gymnosperms do not tend to work with woody angiosperms, and *vice versa* (Hackett 1985; Zimmerman et al. 1985; Philipson 1990).

The notable exception to this is apple *(Malus sylvestris* Mill.). There appears to be a critical threshold below which GA inhibits flowering. For example, Luckwill (1970) showed that foliar sprays of GA_3, applied at a rate of 50 mg/1, suppressed apple flower bud formation, whereas 500 mg/l enhanced it. It has also been shown that the type of GA produced (or applied), the duration of its movement, and the rapidity with which it is converted to inactive or less active products all influence its effects (Pharis and King 1985; Looney et al. 1985).

A further consideration is the flowering habit of the plant under study. Much of the work with apple has been focused on cultivars which flower biennially. This is important because seeds are a rich source of GAs, which are thought to diffuse out of the developing fruit to inhibit the initiation of floral buds for the following season. Maximum GA activity in developing apple seeds is 3,000 times higher (on a fresh weight basis) than in the flesh (Dennis 1976). The amount of $^{3}H\text{-}GA_3$ diffusing out of the seeds of alternately cropping "Laxton's Superb" is significantly greater than from the steadily cropping variety "Cox's Orange Pippin" (Road and Donaldson 1977, Hoad 1978). Dennis (1970) also showed that hand pollination methods that caused ordinarily seedless clones to produce seeds also resulted in both greater fruit set in the current year and a decrease in the number of blossoms on spur shoots the following year.

Gibberellic acid produced in the roots may also promote and maintain juvenility. This hypothesis is based on work with English *ivy (Hedera helix L.)* which showed that:

- apical buds from juvenile tissue had higher levels of GAs than buds from mature tissue,

- roots contained high levels of GAs, and

- aerial parts of derooted seedlings and cuttings contained lower levels of GAs than the corresponding tissues in intact plants (Frydman and Wareing 1973a, b; 1974).

Wareing and Frydman (1976) concluded that as the distance between the roots and the shoot apex increases, the amount of GAs arriving at the shoot tip decreases, resulting in a loss of juvenility.

A number of treatments that cause early flowering influence endogenous GA levels. Many of the growth-inhibiting chemicals that promote flowering also inhibit GA biosynthesis (see below). In addition, root confinement (described below), often leads to a more densely branched root system and consequent multiplication of root tips (Bravdo et al. 1992). Assuming that GAs are produced in root tips, this strengthens the theory that GAs play a role in the flowering of woody plants. Finally, phytohormones that antagonize the effects of GAs tend to promote flowering (discussed below).

Cytokinins

As with GAs, root tips are thought to be the site of cytokinin biosynthesis (Skene 1975; Van Staden and Davey 1979); therefore, cytokinins have been implicated in the stimulation of flowering by treatments that increase root-tip density (Oslund and Davenport 1987). Ramirez and Head (1979) showed that zeatin (a naturally occurring cytokinin) promotes flower initiation in apple. Srinivasan and Mullins (1978, 1979) reported that treating grape *(Vitis vinifera L.)* apices with PBA (a synthetic cytokinin) caused inflorescence and fruit development in four-week-old seedlings; without treatment, flowering did not occur until three to five years of age. There is also some evidence that cytokinin treatment can affect the gender of the flowers produced (Galoch 1980).

Abscisic acid

Abscisic acid (ABA) is generally associated with the control of dormancy induction, seed development, seed germination, root geotropism, and water stress (Walton 1980). It has also been linked to the control of flowering. For example, reducing the daylength under which blackcurrant *(gibes nigrum L.,* a short-day plant) was grown led to enhanced flowering (Nasr and Wareing 1958). This same treatment also caused a reduction in the endogenous

levels of GA-like substances and an increase in the levels of vegetative growth inhibitors (possibly ABA; see Jackson and Sweet 1972). Abscisic acid has also been shown to stimulate flowering of blackcurrant grown under non-inductive conditions (El-Antably et al. 1967) and of stem-girdled birch seedlings (Arshad 1980; as reported in Philipson 1990). In the latter study, it was shown that ABA accumulated above the girdle. Hillman et al. (1974) reported the juvenile *Hedera helix* leaves contained five times more ABA than did mature leaves. In general, these observations suggest that GAs suppress flowering while growth inhibitors (anti-gibberellins) promote flowering in woody angiosperms (see below). In the above cases, ABA treatment probably did not result in a phase change because some of the control plants also flowered.

Ethylene

Ethephon is the generic term for an ethylene-releasing agent sold under the trade name of Ethrel® (Amchem Products, Inc., Ambler, PA). Development of this "liquid ethylene" has helped overcome some of the difficulties of working with a gaseous phytohormone.

Excessive shoot growth is believed to have an adverse affect on fruit bud initiation (Luckwill 1970). Ethephon, which can be used as a growth retardant, has been used to induce fruit drop to control apple crop sizes. However, when ethephon was applied alone or in combination with succinic acid-2,2dimethylhydrazide (SADH, Alar®, see below) after the normal period of fruit drop, it caused an increase in flower bud initiation the following growing season (Greene et al. 1976; Lord et al. 1974, 1975). In their controversial article, Volz and Knight (1986) showed that ethephon could significantly increase bud formation on apple without adversely affecting shoot growth. Elfving (1984) has also reported that ethephon treatment can result in greater flower bud production without a concomitant decrease in shoot growth. More recently, however, Jones et al. (1989) showed that increases in flowering in response to ethephon alone or in conjunction with Alar® were always correlated with a reduction in vegetative growth. Despite the uncertainty surrounding its affect on shoot growth, it is clear that ethephon can stimulate floral bud production in apple.

Endogenous ethylene levels have been correlated with flowering in apple. For example, spurs with flower buds produced three times more ethylene than one-year-old branches with vegetative buds. In addition, summer pruning, which has been shown to induce flowering, causes increased ethylene synthesis (Klein and Faust 1978).

Auxin

The role auxins play in the control of flowering is very poorly understood, and the data is both contradictory and circumstantial. Auxin export from the seeds of biennially flowering cultivars of apple is nearly 60% greater than for cultivars which bear fruit annually (Grochowska and Karaszewska 1976). In addition, a substance with auxin-like activity is transported through apple pedicels to a much greater extent in a biennially bearing cultivar than for a cultivar which produces an annual crop (Hood 1978). In contrast, foliar applications of an auxin (IBA) completely inhibit flowering in blackcurrant (Schwabe and Al-Doori 1973).

The effect of auxin on flowering may be manifest through its effects on ethylene synthesis (Yu and Yang 1979) and other physiological processes. Auxin may indirectly affect flowering by improving nutritional status and mobilizing carbohydrates. Auxin stimulates the differentiation of vascular tissue, thus increasing the supply of nutrients and hormones to developing organs and hastening their development (Bruinsma 1974). Also, treatments which reduce carbohydrate levels, such as low light or high temperature, have been shown to prolong the juvenile phase (Hackett 1976; Schwabe 1976), and auxin is known to increase a tissue's sink strength (Sachs 1977).

Growth retardants

Paclobutrazol

Of all the treatments used to induce flowering in woody angiosperms, paclobutrazol (pp333, Bonzi®; Uniroyal, Inc.; Middlebury, CT), a triazole, is used most commonly. Initially it was used as a means of chemically pruning fruit trees in orchards. It soon became apparent that a secondary effect was flower induction, expressed during the following growing season. It acts by inhibiting GA biosynthesis (Rademacher 1989), consistent with the theory that GAs produced in the seeds of the current year's fruit can inhibit flower bud production for the following year.

Paclobutrazol is used extensively by apple growers. For example, when Williams and Edgerton (1983) treated 25-year-old apple trees (cv. Red Delicious) with the equivalent of 2 g of pp333/m^2 as a root drench to control vegetative growth, they achieved a reduction in vegetative growth and also observed a dramatic increase in yield (from 7.1 to 15.4 35-lb. boxes/tree). Both effects were most pronounced two years after treatment. Part of the increase in yield caused by pp333 appeared to be due to increases in fruit set and frost resistance.

In the year following treatment, Tukey (1983) observed a three-fold increase in apple yield in response to pp333 applied as a foliar spray or as a root drench. Yields were proportional to the concentration of pp333

used, with the highest yields from the 2,000 ppm treatment. As with the previous study, part of the increase at the highest levels appeared to be due to an increase in spring frost resistance. Similarly, Volz and Knight (1986) found that pp333, applied as a foliar spray at a rate of 250 ppm, resulted in a significant increase in the yield of Bramley and Cox varieties of apple the year following a triple application of pp333 (evenly spaced throughout the growing season).

Paclobutrazol has been used with other fruit tree crops. Williams and Edgerton (1983) observed increased yields with pear *(Pyrus communis L.* cv. Anjou), but only when the pp333 treatments were followed by GA treatment. Root drenches with paclobutrazol (0.2 gm/cm stem diameter) caused increased flowering in the following year with peach *(Prunus persica (L.)* Batsch), sweet cherry *(Prunus avium L.)* and sour cherry *(Prunus cerasus,* Edgerton 1986). Cobianchi (1989) obtained an increase in flowering in pear the year after a trunk drench treatment (75 mg/cm2 trunk area) was applied. Paclobutrazol applied at a rate of 1.2-1.5 g/tree as both a root drench and foliar spray induced inflorescence formation on 2 ½-year-old clove trees *(Syzygium aromaticum (L.)* Merr. & Perry), and greatly enhanced yield in nine-year-old trees. Clove trees normally begin flower production at five years of age, and require at least 20 years to reach full bearing (Martin and Dabek 1988).

In their review on triazole plant growth regulators, Davis et al. (1988) summarized what was known about pp333 at that time. Paclobutrazol has a low solubility in water (30 ppm) and its uptake is enhanced by wetting agents (e.g. Tween 20), but one must be careful to select surfactants and solvents that are not phytotoxic. In general, pp333 treatments will result in more consistent annual bearing, but can also induce precocious flowering. Repeated applications throughout the growing season are better than one large dose, and root or stem applications are much more effective than foliar sprays. Paclobutrazol is transported in the transpiration stream and is most effective when it is applied directly or transported to the shoot apex. That which is absorbed by mature leaves is not translocated to the stems or shoot tips (Quinlan and Richardson 1986). Finally, pp333 is readily adsorbed by organic material and soils with high cation exchange capacity. Because it is highly immobile in soil, root uptake will depend upon the chemical's relative proximity to the roots. Its half-life in soil varies considerably, but usually is between three and 12 months (Lever 1986).

Results with pp333 similar to those in fruit crops have been reported with *Eucalyptus.* Eucalypts are woody angiosperms that flower either biennially or sporadically. It was recently reported that pp333, when applied as a collar drench, foliar spray, or trunk injection could induce flower bud initiation in *E. globulus* Labill. and *E. nitens* (Dean & Maid.) ex. Maid. trees ranging

between 19 months and 17 years of age (Griffin et al. 1993; Hasan and Reid 1995). As with fruit trees, the inhibition of vegetative growth was apparent in the year the treatments were applied, whereas the effect on flowering was not manifested until the following year. However, the increase in flower bud initiation persisted for six years. Equally promising results were obtained by Moncur et al. (1994) with eucalypts grown as espaliers (branches horizontally trained along wires). In the latter study the authors also measured endogenous GAs and showed an inverse relationship between GA levels and reproductive activity. Interestingly, when paclobutrazol-treated E. *nitens* grafts were maintained in a warm greenhouse over winter they did not produce flower buds, although there was a reduction in endogenous GA concentrations in response to paclobutrazol. In contrast, paclobutrazol-treated grafts that overwintered outside did flower (Moncur and Hasan 1994), suggesting that temperate eucalypts have a chilling requirement for floral bud development.

Other triazoles

Uniconazole (S-3307 or XE-1019) is a compound closely related to paclobutrazol, but can be more effective at floral induction. Cobianchi (1989) found that a given amount of S-3307 will inhibit vegetative growth to the same extent as pp333 in cherry, whereas a lower dosage of S-3307 was effective at inhibiting growth in pear (cv. Cornice). S-3307 was more effective than pp333 in stimulating floral bud initiation in pear when equivalent amounts of active ingredient were used.

Daminozide

The first compound used to control shoot growth in fruit trees was daminozide (SADH, B-995, B-9A or AlarA; Uniroyal, Inc.; Batjer et al. 1963). It was effective in controlling vegetative growth and stimulating floral bud initiation, but very high application rates are required. For example, Volz and Knight (1986) were unable to increase floral bud production even with triple applications of Alar® at a rate of 1,000 ppm. In contrast, with a treatment of 2,000 ppm Alar®, Luckwill and Silva (1979) were able to increase yield of apple (cv. Golden Delicious) in the year of application by increasing fruit set, and also increased yield in the following year by increasing floral bud initiation. Treatment with 0.5 to 1 % (5,000-10,000 ppm) Alar® was informally observed to stimulate flowering in aspen (B. Li, pers. comm.).

CCC

Chlormequat (Cycocel&, (2-chloroethyl)trimethylammonium chloride; American Cyanamid Inc., Princeton, NJ) has been shown to increase yield but, at least in pears and apples, this is largely a result of decreased fruit drop rather than an enhancement of floral bud initiation (Varga 1969). When

applied at a rate of 10 mg per plant, CCC also promoted flowering to a small extent in non-girdled birch seedlings but caused significant flowering in girdled birch seedlings (Arshad 1980, as reported in Zimmerman et al. 1985). In addition, cuttings taken from 60-day-old *Salix pentandra* L. seedlings flowered in response to 1mM foliar sprays of CCC. This effect was antagonized by GA_3 (Junttila 1980). Finally, grape seedlings grown in nutrient solutions containing 100 mg/l CCC led to increased levels of cytokinin in the bleeding sap (Skene 1970). As noted above, treating grape apices with cytokinin led to flower initiation.

Cimecthacarb

A relatively new GA biosynthesis inhibitor, Primo® (Ciba-Geigy, Greensboro, NC), has yielded some promising results as a stimulator of precocious flowering in woody angiosperms (R.P. Pharis, pers. comm.). However, it has not been used extensively.

Manipulation of growth environment

Accelerated growth

It is still unclear what controls the transition from juvenility to maturity in plants. In the late 1950s it was thought that a tree develops the capacity for reproduction either as a result of attaining a certain size or by undergoing a certain number of cycles of growth and dormancy. In birch *(Betula verrucosa* Ehrh.), which usually doesn't produce catkins until it is five to 10 years old, seedlings exposed to long days grew rapidly and flowered when less than a year old (Longman and Wareing 1959). These findings led the authors to conclude that the onset of flowering is size-dependent.

In a subsequent study, half of the Japanese larch *(Larix leptolepis* (Sieb. & Zucc.) Gord.) seedlings that had been grown from seed for 21 months under continuous long days were positioned horizontally; the rest were grown vertically (Robinson and Wareing 1969). Three and one-half years after sowing, two-thirds of the trees grown horizontally produced catkins, whereas none of the vertically-grown seedlings flowered. Because the buds were initiated before the shoot training had begun, it was assumed that horizontal training only allowed for the initiation of cones on seedlings that had already become sexually mature as a result of an earlier treatment (i.e. long days).

In the same study (Robinson and Wareing 1969), blackcurrant seedlings were grown under long days prior to being transferred to a short-day regime, a treatment combination known to initiate flowers. The results from their initial trials seemed to indicate that blackcurrant seedlings are unable to initiate

flowers until they attain a certain minimum stem height. In a follow-up experiment, however, seedlings grown under long days were decapitated and the cuttings were rooted before the plants were allowed to reach the height at which they were known to flower. Because the rooted cuttings flowered prior to reaching the previously established minimum height needed for flowering to occur, it was concluded that height growth per se was not the determining factor in the control of flowering. It was then postulated that the transition from the juvenile to the adult condition was mediated by the number of cell divisions the apical region undergoes.

This theory is not consistent with observations made with other species, though, and may result from different ecophysiological responses of species adapted to contrasting environments. For example, *Eucalyptus occidentalis* Endl., which normally flowers when it is three to five years old (Blakely 1955), flowers precociously as seedlings (less than one year old) when grown under a photoperiod of at least 16 hours (Bolotin 1975). Moreover, the response to photoperiod is modified by other environmental factors. Flower bud formation in Camellia *(Camellia japonica)* is greater when plants are exposed to long photoperiods and high temperatures than to elevated temperatures alone (Bonner 1947).

Nutritional status

Improved mineral nutrition is often considered a flower-inducing treatment. Zimmerman (1971), in one of the first such reports, was able to reduce the time to first flowering in tea crabapple *(Malus hupehensis* Rehd.) seedlings from three years to 92 months by growing them continuously under "favorable conditions" (extended photoperiods) in a greenhouse. This also included weekly treatment with 20-20-20 (N-P-K) water soluble fertilizer. Aldwinckle (1975) obtained nearly identical results with apple. In the latter study, the plants were watered weekly with 15-6.4-12.4 fertilizer supplemented with trace elements, in addition to the incorporation of a slow release 17-10.8-7.3 fertilizer (Osmocote) in the upper layer of the soil every two to three months.

In his subsequent review article, Zimmerman (1972) considered this flower induction response to be largely a result of faster growth. In other words, if, as Robinson and Wareing (1969) suggested, the onset of sexual maturity is controlled by the number of cell divisions the apex undergoes, the faster a tree grows the sooner this requirement can be met. Nitrogen is usually considered to be the most important nutrient, and the form in which it is supplied appears to be influential (Philipson 1990). For example, Grasmanis and Edwards (1974) showed that when ammonium was supplied to apple in liquid culture there was a significantly greater flowering response than when trees received nitrate as their sole nitrogen source. In contrast, Edwards and Allsopp (1956)

found that heteroblastic development (differences in leaf size and fonn, which indicate that phase change has occurred) in *Marsilea drummondii* A.Br. was stimulated by substituting nitrate for ammonium salts or urea in their nutrient solution. These same authors also showed reduced levels of nitrogen caused a reversion to juvenile condition.

There are many early reports concerning the role endogenous carbohydrate status plays in the control of floral induction in fruit trees (reviewed by Davis 1957). Treatments that elevate carbohydrate levels promoted flowering, especially in plants propagated vegetatively from scions of mature plants (review by Zimmerman et al. 1985). In addition, Allsopp (1953) found that feeding glucose, fructose or sucrose promoted heteroblastic development in *Marsilea.* Mannitol, which is used as an osmoticum but is not readily metabolized, was ineffective at influencing leaf characteristics (Allsopp 1954), implying that the effect was nutritional and not osmotic.

Moisture stress

There is considerable evidence that water stress can enhance flower initiation in conifers, and that hot, dry summers induce abundant seed crops in both conifers and broadleaf species (Philipson 1990). Moisture stress is also known to cause large increases in the endogenous levels of ABA (Walton 1980), which, as stated previously, is considered to be an anti-gibberellin. Water stress has recently been used to stimulate flowering of aspen in the greenhouse. Potted plants were periodically allowed to dry down to pre-dawn moisture stress readings in the range of -100 to -140 p.s.i. (-6.9 to -9.7 bars) before they were rewatered. This treatment appears to be effective only when applied during the period of shoot elongation (B. Li, pers. comm.).

Temperature

Low temperatures have been used to induce precocious flowering in a number of tree species (reviewed by Jackson and Sweet 1972). For example, Hield et al. (1966) induced precocious flowering in seven-month-old grapefruit *(Citrus paradisi* Macf.) seedlings grown using day and night temperatures that were low enough to induce dormancy. Other examples of woody angiosperms requiring low temperatures for floral induction include: peach (Lammerts 1943); olive *(Olea europaea L.;* Hackett and Hartmann 1963); sweet orange *(Citrus sinensis* L. Osbeck; Moss 1976); and lychee *(Litchi chinensis* Sonn.; Menzel 1983). In most cases, these cold treatments were applied indoors during the winter. More recently, Moncur (1992) showed that transferring *Eucalyptus lansdowneana F.* Muell. & J Brown seedlings, grown under a 16-hr photoperiod, from a heated greenhouse (24/19 °C, day/night temper-

atures) to a cold regime (15/10°C) for five to 10 weeks before returning them to warm conditions (24/19 °C) was sufficient to induce floral buds. This effect was enhanced when seedlings were transferred to the cold under highlight conditions. This is consistent with the findings of King et al. (1992), who reported that exposing *Pimelea ferruginea* Labill. and P. *rosea* R.Br. to mean temperatures below 15 °C for more than five weeks caused precocious flowering. Thus, flowering in perennial plants appears to benefit from cold treatments much as annual species require vernalization for rapid flowering.

Effects of in vitro culture on maturation and flowering

Results from a wide range of studies suggest that adventitious bud and embryo formation are rejuvenating processes, and that mature meristems cannot be formed *de novo* (reviewed by Hackett 1985). However, there is mounting evidence that significant maturation sometimes occurs during tissue culture. For example, loblolly pine plantlets generated from immature juvenile embryonic tissue exhibited mature characteristics after growing two years in the field (McKeand 1985). Scorza and Janick (1980) reported that callus derived from leaf, stem and tendril explants of passionvine *(Passiflora suberosa L.)* produced shoots that flowered in as few as 21 days. Embryoids derived from callus on mature ginseng roots *(Panax ginseng* C.A. Meyer) flowered *in vitro* within one month of subculture; it normally has a three-year juvenile period (Chang and Hsing 1980). Similarly, somatic embryos derived from callus of zygotic embryos of date palm *(Phoenix dactylifera)* flower in the embryoid stage, whereas plants derived from sexual embryos do not flower for several years (Hackett 1987). Finally, adventitious buds formed on callus derived from early flowering lines of *Betula pendula* Roth retained their ability to flower early when grown under continuous light in the greenhouse (Huhtinen 1976). Thus, *in vitro* culture can sometimes be a powerful means for accelerating, or maintaining, reproductive competence.

Physical methods

Vascular restriction

Treatments that restrict vascular movement cause the accumulation of shootproduced metabolites (e.g. carbohydrates, ABA and auxin) above the restriction, while root-supplied metabolites or nutrients (e.g. cytokinins, GAs and nitrogen) accumulate below the restriction. As described above and elsewhere (Zimmerman et al. 1985; Hackett 1985) these compounds can influence flowering. Below are the most common ways in which vascular movement has been impeded.

Girdling. Circumferential girdles (removal of a swath of the bark, down to the phloem, around the entire stem) have been effective in a number of tree species. For example, Wesoly (1985) demonstrated that complete stem girdles not only induced flowering in Scots pine (Pinus silvestris L.), it also led to elevated levels of GA-like activity within 48 hours of treatment. Eris and Barut (1993) showed that complete stem girdles alone or in combination with GA treatment resulted in an increase in panicles/shoot, flowers/panicle and fruit set/panicle during "on" and "off" years in olive.

Overlapping girdles

A less severe treatment, overlapping partial girdles, has also proven to be useful for stimulating flowering. There are many early reports in which overlapping girdles were used to stimulate flowering in conifers (e.g. Bilan 1960). Overlapping girdles in conjuction with hormone treatment have been even more successful. Male strobili production was greatly enhanced in loblolly and slash pines that were partially girdled and treated with a combination of NAA and GA_{4+7} (Hare 1979). More recently, Pharis et al. (1986) found it to be the most successful adjunct treatment for promoting flowering in GAtreated white spruce (Picea glauca (Moench) Voss), a species which flowers only sporadically and doesn't ordinarily respond well to GA treatment. It has also been used to enhance female flower production in loblolly pine (Pinus taeda L.) seed orchards (Wheeler and Bramlett 1991).

Scoring

A related technique, scoring (cutting completely through the bark around the trunk with a sharp knife, without removing any bark), has also been used to stimulate flowering. In apple, this treatment was even more effective than a foliar spray of 2,000 ppm Alar® (Veinbrants 1972).

Wire girdles

Recently, wire girdling has been used with some success to stimulate flowering in aspen. This method involves encircling selected branches or the main stem with a steel wire, which is twisted "tightly". The wire is adjusted as the plant grows to avoid killing parts distal to the wire. The best time to apply this technique is in the spring or early summer (B.Li, pers. comm.). There are numerous examples of wire girdles being used to stimulate flowering in conifers (reviewed in Hoekstra and Mergen 1957 and Bilan 1960; Greenwood and Schmidtling 1981).

Root growth control

Trickle irrigation

Proebsting et al. (1977) reported that trickle-irrigated apple trees bloomed in their third and fourth years, with substantially greater yields than from trees that were sprinkled. They attributed this response to restricted root growth, and noted that the yield enhancement afforded by trickle irrigation was comparable to the effects induced by other management practices, such as plant nutrition, growth regulators and physically controlling root size.

Fertigation

The practice of applying fertilizer through trickle irrigation has also resulted in significant increases in spur, axillary and terminal flower bud initiation in apple in the second, third and fourth years beyond the commencement of treatment, as compared to broadcast fertilization and irrigation alone (Hipps 1992). This effect was achieved along with faster growth rates. Unlike the previous study, it was not possible to separate the effects of nutrition from those of restricted root growth. However, these results are consistent with the results of Lever (1986), who showed that the efficiency of pp333 treatment could be enhanced by irrigation patterns that produce a concentration of superficial roots. This also supports the conclusion drawn by Bonnet-Masimbert (1982; as reported by Hackett 1985) that any treatment which inhibits root growth tends to promote flowering.

Confinement and root pruning

In addition to limiting root growth by irrigation practices, growth in confined soil volumes has also led to dwarfing, precocious flowering and higher fruiting efficiency with peach trees (Richards and Rowe 1977). Copper treatment of pots or herbicide treatment of fabric surrounding roots prevents root circling and penetration, leading to the development of more root tips. As discussed above, this appears to promote flowering, especially in conjunction with nitrogen fertilization (Proebsting pers. comm.). The same effect may be achieved by root pruning or wrenching, which can also lead to a more densely branched root system. Aspen was informally observed to flower after growing seedlings in small pots and cutting the roots of potted plants with a large knife in two or three directions tangential to the stem (B. Li, pers. comm.).

Shoot training

Fruit tree growers have induced early flowering by training shoots to grow horizontally. Longman et al. (1965) demonstrated that it was possible to stim-

ulate flowering by growing apple trees horizontally. They also reported that tying upwardly growing branches of young Japanese larch trees horizontally or downward led to dramatic increases in male cone initiation. Denby et al. (1988) have tested a variety of shoot training techniques and have shown that two treatments, angle training and spindle training, are useful in elevating yield and inducing precocious flowering in pear (cv. Anjou). Ethylene may be responsible for this effect; tying branches in a horizontal position leads to increased ethylene production (Robitaille and Leopold 1974; Robitaille 1975).

Topworking

Recently, a system was developed to induce early flowering in loblolly pine (Bramlett and Bums 1995; Bramlett et al. 1995). Immature scions, ranging in age from one to five years, were grafted onto branches in the lower and upper crown of mature trees. Pollen strobili did not occur on any of the one-year old grafted scions. However, scions of all other age classes produced pollen cones within 13 months. In the lower crown, the percent of grafts with pollen ranged from 15% for two-year-old scions to 75% for five-year-old scions. Pollen production was less frequent in the upper crown, and only occurred on three- to five-year-old material.
In the same study, female strobili were produced in the upper crown on scions of all age classes. Success ranged from 21 % for one-year-old scions to 80% for four-year-old scions. A total of 247 female strobili were produced on 53 grafts in the upper crown. No female cones were produced on scions grafted in the lower crown. As with the grafts on which male cones were initiated, scions bearing female strobili maintained their juvenile characteristics in all other respects.

It is widely known that female flowers on conifers are concentrated on the upper branches, and male flowers are predominantly found on the lower branches (Kramer and Kozlowski 1979). This is thought to facilitate crosspollination. In the work of Bramlett (1995), some of the branches on which grafting was done were wire- and saw-girdled several days prior to grafting. These branch treatments had no effect on pollen initiation. This implies that the pollen induction stimulus is not transported in phloem, though it is possible that this signal is translocated via the xylem. As mention earlier, cytokinins, which are produced in the roots and transported in the xylem, are thought to be involved in gender determination. Hence, a concentration gradient along the length of the stem could account for the floral gender localization.

Transfer of flower-inducing genes

Recently, a number of floral-meristem-identity genes have been isolated from *Arabidopsis thaliana* and other model plant species. One such gene, *LEAFY (LFY),* is involved in controlling the transition from an inflorescence to a floral meristem (Weigel et al. 1992). A second floral-meristem-identity gene, *APETALAI* (API), is required for sepal and petal development and is also involved in controlling the inflorescence to floral meristem switch (Mandel et al. 1992). When either API or *LFY* are expressed constitutively, transgenic Arabidopsis plants flowered in vitro in just ten days (Weigel and Nilsson 1995; Mandel and Yanofsky 1995). Furthermore, when the Arabidopsis-derived *LFY* gene is expressed constitutively in hybrid aspen (Populus tremula x P. *tremuloides* Michx.), it flowered *in vitro* within five months (Weigel and Nilsson 1995). We have recently repeated this work on a male clone of hybrid aspen in our laboratory (unpublished results). A gene of unknown function isolated from rice (Oryza sativa L.; OsMADSl), similar to API, caused precocious flowering when ectopically expressed in transgenic tobacco (Chung et al. 1994). Because transgenes behave as dominant traits, they can also be incorporated into breeding lines to stimulate early flowering. However, present versions of these constructs cause the conversion of all vegetative meristems to reproductive meristems, leading to premature cessation of flower development and plant death. Work is under way to develop genetic constructs that cause less severe flowering phenotypes, as well as inducible systems for controlling expression (D. Weigel, pers. comm.). By shortening the generation time from years to weeks, such constructs could have a revolutionary impact on genetic analysis and tree breeding. The early flowering trait could be readily removed by genetic segregation, enabling normal development to resume.

Molecular techniques for inducing transgenic plantlets to flower *in* vitro can greatly accelerate our ability to engineer altered flower development in trees. We plan to introduce gene constructs designed to render trees sterile into the early flowering lines of hybrid aspen described above. This will circumvent the need for lengthy field trials to observe the constructs' effects on flowering. It will also obviate the need to take special precautions to contain transgenic pollen and seed during the trials, a serious logistical problem with fast growing species such as poplars.

Conclusions

It would appear that the definitions of juvenility and phase change are in need of refinement. Precocious flowering has now been induced on seedlings in a

number of species, all of which bear classically defined juvenile characteristics. Examples include: Eucalyptus globulus (Hasan and Reid 1995), loblolly pine (Bramlett et al. 1995) and Betula spp. (Longman 1976). Furthermore, flowers have been observed on in vitro-grown Populus cultures (Weigel and Nilsson 1995). These data indicate that factors which control vegetative and reproductive phase change can be independently manipulated.

To maximize the chances of finding a reliable way to induce reproductive phase change, it would be best to know more about the underlying mechanism of control. No matter what triggers the onset of reproductive growth, control will ultimately be exerted at the genetic level. It has recently been suggested that acquisition of the competence to flower occurs at the cellular level, rather than at an organismal level (Weigel 1995; Weigel and Nilsson 1995). Therefore, use of a molecular framework, similar to that presented in a recent review by Weigel (1995), will provide the most direct approach to solving this problem.

Due to their long juvenile periods, secondary metabolite accumulations and recalcitrance with respect to transformation and regeneration, woody angiosperms are not ideal model systems for basic floral research. However, rapid progress is being made with a number of herbaceous species (e.g. *Arabidopsis, Antirrhinum* and *Nicotiana;* Kelly and Meeks-Wagner 1993). Many of the floral genes isolated so far are highly conserved and function in heterologous systems. Therefore, genes cloned in herbaceous species can be used as probes to find their homologs in woody plants.

Research into woody plant floral development requires an experimental system in which the transition to maturity is rapid and can be readily induced. Two woody plants have recently emerged as candidate model systems for researching floral development: cranberry and eucalyptus. Both can be transformed and regenerated easily and can be chemically induced to flower precociously (Serres and McCown 1993; Griffin et al. 1993). Now that transgenic poplar can be induced to flower *in vitro,* it too is an attractive model system. Genes thought to control the vegetative-to-floral transition can easily be tested in these species. While this basic research is being done, and even after it is achieved, there will still be a need to induce flowering chemically or culturally, especially in traditional breeding programs. Therefore, we cannot abandon our efforts on this front.

Despite the work of Wareing and others, it does appear that the distance between the roots and shoots is critical to maintaining juvenility. In general, phytohormones are produced in either the shoot or root tips, they occur in concentration gradients along the length of the plant, and they play an important role in phase change. Gibberellic acid appears to be the key regulator, whereas the other hormones either enhance or antagonize its effect. By taking

cuttings, the supply of root-produced hormones is temporarily interrupted, thus disrupting the endogenous hormone balance, possibly explaining the results of Robinson and Wareing (1969).

Considering the important role played by GAs in reproductive development, it is not surprising that the most successful flower-inducing treatments are inhibitors of GA biosynthesis, especially paclobutrazol. However, GAs are not only involved in floral initiation but they also control internode length. Applying growth retardants early in a plant's life results in severe stunting. When the plant recovers from chemical treatment, the distance separating the roots and shoots is greatly reduced. Techniques that favor rapid vegetative growth, so that plants rapidly attain a certain minimum size, tend to promote flowering. Hence, it may be prudent to allow a year of establishment and growth before applying treatments, especially in the case of fast growing species like cottonwoods.

Research efforts should not be confined to paclobutrazol, though. Currently, there is a movement toward the use of more environmentally friendly chemicals for floral induction. There have even been suggestions that paclobutrazol and other chemicals be banned from U.S. and European markets. It appears that the primary concern is chemical longevity in the soil.

Regardless of the method used for floral induction, timing of treatments is critical. This is especially true in the field as plants undergo a dormant period after which they have limited morphogenic ability to respond. For now, it is unclear whether plants are capable of responding to inductive signals throughout the year or just prior to bud differentiation. Observing responses to treatments applied at different stages in development will provide valuable insight into the floral development control mechanism.

Trees grown in the greenhouse respond to treatments differently than those grown in the field. Because genetic engineers may not always be granted the permits needed to allow their trangenic plants to flower outdoors, it would be helpful to have flower induction techniques that are effective on trees grown in the greenhouse as well as in the field. Special facilities are needed for greenhouse studies when working with trees to avoid the necessity for pruning, which often results in the maintenance of or a reversion to juvenility (Hackett 1985), but the same methods could be used in either location. Apart from height constraints, though, it is often more desirable to conduct this sort of research indoors because environmental conditions can be controlled more easily.

This is an exciting time in flower biology. We now have the tools at our disposal to unlock the secrets of what has been a very elusive problem. Considering the current demand for forest products, the potential improvements to be derived from tree breeding and the plethora of flower-related genes that

need to be tested, methods for inducing flowering take on a much greater importance than they have in the past.

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