

Studies of de Novo Flower Initiation from Thin Cell Layers of Tobacco

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Abstract. The potential for in vitro floral photoinduction of epidermal and subepidermal thin cell layers (TCLs) taken from the short-day plant *Nicotiana tabacum* L. 'Maryland Mammoth', the long-day plant *Nicotiana glauca* L., and the day-neutral plant *Nicotiana tabacum* L. 'Samsun', in both the floral and vegetative states, was examined. Whether cultured under long days (16 hr) or short (8 hr), only TCLs from the flowering day-neutral species flowered in vitro. These were termed responsive TCLs. TCLs from photoperiodic plants yielded only vegetative buds; these were termed nonresponsive. Vegetative bud formation in nonflowering TCLs generally was greater than in flowering TCLs but did not approach the number of flower buds on flowering TCLs. In vitro grafts of responsive TCLs to nonresponsive TCLs resulted in flowering only in the responsive portion, regardless of the position of the graft. Just as the nonresponsive TCLs were not induced to flower by some graft-transmissible substance, responsive TCLs were not inhibited from flowering when grafted to nonresponsive TCLs.

The culture of 3 to 6 layers of cytologically differentiated epidermal and subepidermal cells (thin cell layers, hereafter TCLs) on a nutrient medium in vitro has been described as an ideal system for the study of the control of flowering in plants (15). A range of organogenetic phenomena can be induced from TCLs of tobacco by manipulation of the culture medium. Although roots, callus, and vegetative shoots may differentiate in vitro from TCLs of both day-neutral and photoperiodic cultivars of tobacco, flower buds have been restricted to the former (6, 15).

De novo flowers develop on TCLs of day-neutral species of tobacco if the cell layers are taken from the inflorescence and cultured on a Murashige and Skoog (10) basal medium containing 30 g/liter glucose, 1 μ M 1*H*-indole-3-butanoic acid (IBA), and 1 μ M *N*-(2-furanylmethyl)-1*H*-purin-6-amine (kinetin). In vitro flowering is maximized if TCLs are taken from distal portions of the inflorescence; a decreasing capacity for in vitro flower formation occurs with increasing distance from the inflorescence (13). An endogenous gradient of floral promoters or inhibitors or both along the stem and inflorescence has been hypothesized (1).

An unidentified substance or group of substances involved with flower initiation has been demonstrated to be graft transmissible between tobacco plants in vivo (7). By grafts between whole plants, flowering day-neutral plants can induce nonflowering short-day plants, flowering short-day plants can induce nonflowering long-day plants and vice versa, and flowering short-day plants can induce long-short-day plants (7).

Several daylength-sensitive species have been induced to flower through in vitro photoperiodic induction. However, this induction has not been possible with any photoperiodic species of *Nicotiana* (12). Hillman (5) found that whole plants of *Lemna* could be induced to flower in vitro when subjected to short days. Harada (3) induced vegetative shoot apices of *Pharbitis* and *Chrysanthemum* to flower through in vitro photoinduction, and Rossini and Nitsch (11) accomplished the same using leaf disks of *Streptocarpus nobilis*. Stem internodes, hypocotyl sections,

and root segments also have been reported on several species to flower through in vitro photoinduction (12). In these experiments, the explants taken from vegetative plants responded to photoperiodic induction; hence, reception of the photoperiodic stimulus does not depend upon the presence of leaves, meristems, or intact plants.

The relationship between photoperiodism in a source plant and organogenetic capacity of its TCLs cultured under 2 photoperiods has been examined in the present report. The possibility of a graft-transmissible substance from TCLs of nonresponsive tobacco also has been studied with paired cultures.

Materials and Methods

Seeds of *Nicotiana tabacum* 'Samsun' (day-neutral plant), *N. tabacum* 'Maryland Mammoth' (short-day plant), and *N. glauca* (long-day plant) were obtained from the USDA-ARS Tobacco Research Laboratory in Oxford, N.C. Two plants of each cultivar were grown in the greenhouse (24°C) under both long days (16 hr) and short days (8 hr) from March to July of 1983. Long days were provided by incandescent lighting and short days by shading with black cloth. Every 10 days, a new set of plants was started under each photoperiod to ensure that plants of each species would be simultaneously in the appropriate stage of development.

TCLs in all experiments were cut to about 2 x 10 mm and cultured in Bellco glass tubes (150 x 25 mm) containing 30 ml of a medium (pH 5.6), consisting of the Murashige and Skoog (10) macro- and microelements, 100 mg/liter myo-inositol, 30 g/liter glucose, 0.4 mg/liter thiamine-HCl, 9 g/liter agar, 1.0 μ M IBA, and 1.0 μ M kinetin. Cultures were incubated at 24° to 26°C under 60–75 μ mol s⁻¹m⁻² light produced by a combination of cool-white fluorescent and incandescent bulbs. A photoperiod of 16 hr per day was used unless otherwise stated.

De novo flower buds and vegetative shoots visible under x10 magnification were counted on TCLs after 28 days in culture. Data were analyzed by SAS GLM procedure with mean separation by the Student-Newman-Keuls' test (2).

Expt. 1. Influence of the physiological state of the mother plant on de novo organogenesis in vitro. TCLs were removed from all 3 plant types in both the vegetative and floral states. TCLs were taken from flowering plants in the green fruit stage

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of development. For disinfestation, flowers and fruit were removed from the inflorescence, and the peduncles were cut into 6 cm lengths. Peduncle sections were disinfested by a wash in distilled water, followed by a 5 sec dip in 70% ethanol and 5 min in 2.1% sodium hypochlorite. Plant sections remained in a sterile distilled water rinse until cultured. On vegetative plants, TCLs were taken from internodal sections along the top 20 cm of the plant. They were disinfested as described previously.

Half of the TCLs in each treatment were cultured under short days (8 hr) and half under long days (16 hr). The entire experiment was conducted twice with 12 replications in each of the 12 treatments, using a randomized complete block design.

Expt. 2. In vitro grafting experiments. TCLs from the distal portion of an inflorescence (floral-responsive) of 'Samsun' were grafted to TCLs taken from the stem of the same plant, 3 nodes below the inflorescence (nonfloral responsive). De novo flowers have not been observed in vitro on cultures of the latter (14). Stem-stem and inflorescence-inflorescence grafts as well as ungrafted TCLs from stem or inflorescence (controls) were compared. The 6 treatments were replicated 12 times in a randomized complete block design and the experiment was conducted twice.

Floral-responsive TCLs of 'Samsun' were grafted to nonre-

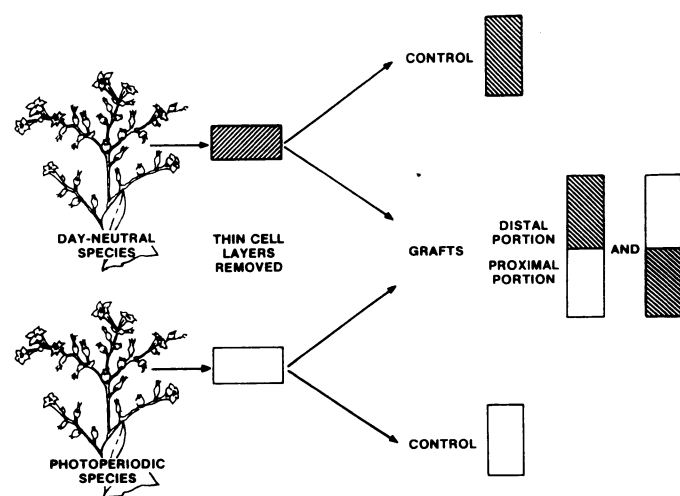


Fig. 1. Positioning of thin cell layers in grafting experiments.

sponsive TCLs from inflorescences of 'Maryland Mammoth' or *N. sylvestris*. Neither of the latter species has exhibited de novo flower formation from TCLs (6). Each pair of TCLs was cultured reciprocally, i.e., with the floral-responsive member in either the acropetal or basipetal position (Fig. 1). Ungrafted TCLs of all 3 species, as well as 'Samsun'-'Samsun' grafts, were used as controls. Careful positioning of freshly cut TCLs in the culture tube, so that parenchyma cells of both TCLs were closely aligned, was sufficient association for graft union formation. The 8 graft treatments were replicated 18 times in a randomized complete block design, and the experiment was completed twice.

Results

Expt. 1. In vitro flowering was observed only on TCLs from flowering plants of *N. tabacum* 'Samsun', regardless of the photoperiod under which they were cultured (Table 1). TCLs from *N. tabacum* 'Maryland Mammoth' and *N. sylvestris* did not flower, regardless of the physiological state of the mother plants or the photoperiod under which they were cultured.

De novo vegetative shoot formation was observed on all TCLs. However, fewer vegetative shoots were formed on 'Samsun' TCLs from flowering plants and cultured under short days than on any other TCL. The number of vegetative shoots produced by TCLs of the 2 photoperiodic species did not differ with the explant source or photoperiod during culture.

Expt. 2. When TCLs from the inflorescence of 'Samsun' were grafted to TCLs from stem tissue of the same plant, flowering was observed only on the explant taken from the inflorescence (Table 2). TCLs from stem tissue did not flower in any treatment, whereas flowering of inflorescence-derived TCLs was equivalent in all treatments. De novo vegetative shoot formation on TCLs from the inflorescence was less than that of TCLs from stem tissue.

When reciprocal grafts were made between TCLs from inflorescences of *N. tabacum* 'Samsun' to TCLs of *N. tabacum* 'Maryland Mammoth' or *N. sylvestris*, only the explant from 'Samsun' flowered in vitro (Table 3). TCLs from inflorescences of *N. sylvestris* and 'Maryland Mammoth' did not form de novo flowers when cultured singly or in graft combination. The average number of vegetative shoots per TCL was less for TCLs from 'Samsun' than from 'Maryland Mammoth'.

Table 1. Influence of photoperiod on flower and vegetative shoot production from tobacco thin cell layers.

Explant source	Daylength of source plant (hr)	Avg no. of flower buds per TCL ^{z,y}		Avg no. of vegetative buds per TCL ^{z,y}	
		Short days ^x	Long days ^w	Short days ^x	Long days ^w
<i>Nicotiana tabacum</i> ‘Samsun’ (day neutral plant)					
Flowering	8 or 16	10.7 a	10.3 a	0.4 b	0.9 a
Vegetative	8 or 16	0.0 b	0.0 b	1.1 a	1.3 a
<i>Nicotiana tabacum</i> ‘Maryland Mammoth’ (short-day plant)					
Flowering	8	0.0 b	0.0 b	1.3 a	0.9 a
Vegetative	16	0.0 b	0.0 b	1.4 a	1.4 a
<i>Nicotiana sylvestris</i> (long day plant)					
Flowering	16	0.0 b	0.0 b	1.5 a	1.5 a
Vegetative	8	0.0 b	0.0 b	1.6 a	1.4 a

^zTCL = thin cell layer.

^yMean separation within columns by the Student-Newman-Keuls' test, 1% level.

^xShort days = 8 hr.

^wLong days = 16 hr.

Table 2. Flower and vegetative bud production from grafted thin cell layers from inflorescence and stem tissues of *Nicotiana tabacum* 'Samsun'.

Graft	Avg no. of flower buds per TCL ^{z,y}	Avg no. of vegetative buds per TCL ^{z,y}
Inflorescence TCL (control)	10.0 a	0.6 c
Stem TCL (control)	0.0 b	1.1 ab
Inflorescence TCL grafted acropetally to Stem TCL		
Inflorescence portion	9.8 a	0.7 bc
Stem portion	0.0 b	0.8 ab
Stem TCL grafted acropetally to inflorescence TCL		
Stem portion	0.0 b	1.2 ab
Inflorescence portion	9.9 a	0.6 c
Inflorescence TCL grafted to inflorescence TCL		
Inflorescence ₁ portion	9.8 a	0.6 c
Inflorescence ₂ portion	9.8 a	0.6 c
Stem TCL grafted to stem TCL		
Stem ₁ portion	0.0 b	1.2 ab
Stem ₂ portion	0.0 b	1.3 a

^zTCL = thin cell layer.

^yMean separation within columns by Student-Newman-Keuls' test, 1% level.

Discussion

Although genotypic control of flowering is well-established, specific environmental conditions, such as temperature and photoperiod, are often prerequisites to floral evocation (7). In vivo flowering of *N. tabacum* 'Maryland Mammoth' and *N. sylvestris* requires short and long days, respectively. Because leaves are not the sole plant organs responsive to photoinduction (7), and because in vitro photoinduction of daylength-sensitive species other than *Nicotiana* has been reported (3, 5, 11, 12), TCLs of 'Maryland Mammoth' and *N. sylvestris* tobacco were examined for their response to photoinductive conditions in vitro. However, under the experimental conditions reported, TCLs from short-day 'Maryland Mammoth' and long-day *N. sylvestris*, in either the floral or vegetative state, could not be induced

to flower through in vitro photoperiodic manipulations. Only TCLs from inflorescences of 'Samsun' responded by in vitro flowering.

These results indicate that there is a substance or condition responsible for flowering present in TCLs of 'Samsun' and not in the others. If, as hypothesized, flowering is evoked in vivo by reaching some critical balance of hormones (17), the differential response of TCLs from day-neutral and photoperiod-sensitive species of tobacco may reflect a difference in the period of time, during which that critical balance is maintained. Previous experiments have demonstrated that manipulations of the growth regulators in the medium do not affect this internal balance of photoperiodic tobacco (14, 15). A sequential derepression of flowering genes also has been hypothesized to explain the ontogeny of flowering (4). The combination of culture conditions and physiological state of the explant results in evocation of flowering of 'Samsun' TCLs; however, the physiological state of TCLs from daylength-sensitive tobacco does not permit floral evocation under the same conditions.

Recent work has demonstrated that the concentration of gibberellins increases and the amount of abscisins decreases in *N. sylvestris* during long-day induction; on the other hand, the amount of gibberellins decreases and abscisins increases in Mammoth tobacco during short-day induction (8). The critical balance of hormones required to evoke flowering in vitro may well differ for different photoperiodic species.

Grafts between whole plants of induced and noninduced *Nicotiana* have successfully transferred floral promoters from the induced plants to the noninduced (9, 16). We attempted to do the same using responsive and nonresponsive TCLs on the hypothesis that the substance(s) responsible for floral-induction in plants of different response types is/are identical physiologically and chemically (7). The hypothesis also contends that these response types do not differ with respect to the presence or absence, and the nature of, the photoperiodic control of flowering substance(s). However, our work suggests that neither promoters nor inhibitors were transferred between TCLs. Stimulation of flowering was not observed in nonresponsive TCLs

Table 3. Flower and vegetative bud production on individual thin cell layers (TCL) of *Nicotiana tabacum* 'Samsun', *Nicotiana sylvestris*, and *Nicotiana tabacum* 'Maryland Mammoth'.

Graft	Avg no. of flower buds per TCL ^z	Avg no. of vegetative buds per TCL ^z
<i>Nicotiana sylvestris</i> (long-day plant)	0.0 b	1.5 ab
<i>Nicotiana tabacum</i> 'Samsun' (day-natural plant)	9.7 a	0.5 cd
<i>Nicotiana tabacum</i> 'Maryland Mammoth' (short-day plant)	0.0 b	1.6 a
'Samsun' TCL grafted acropetally to 'Mammoth' TCL		
'Samsun' portion	9.4 a	0.4 cd
'Mammoth' portion	0.0 b	1.4 ab
'Mammoth' TCL grafted acropetally to 'Samsun' TCL		
'Mammoth' portion	0.0 b	1.2 abc
'Samsun' portion	8.7 a	0.5 cd
'Samsun' TCL grafted to 'Samsun' TCL		
'Samsun' ₁ portion	9.1 a	0.4 cd
'Samsun' ₂ portion	9.0 a	0.3 d
'Samsun' TCL grafted acropetally to <i>N. sylvestris</i> TCL		
'Samsun' portion	8.6 a	0.5 cd
<i>N. sylvestris</i> portion	0.0 b	1.0 abcd
<i>N. sylvestris</i> TCL grafted acropetally to 'Samsun' TCL		
<i>N. sylvestris</i> portion	0.0 b	0.8 bcd
'Samsun' portion	9.4 a	0.3 cd

^zMean separation within columns by the Student-Newman-Keuls' test, 1% level.

grafted to responsive ones, nor was the inhibition of flowering observed in the responsive TCLs. These results seem to support Zeevaart's (17) critical balance hypothesis. The results also suggest that *in vitro* flower production may involve processes, conditions, and/or substances that are quite different from those involved in whole plant graft-transmissible floral induction.

It may be possible that a floral inhibitor or promoter was present in the TCLs and was transported through the graft, but not in sufficient quantity to exert an effect on morphogenesis in the receiving TCL. It also may be possible that this substance was present but was not transported through the grafts into the epidermal and subepidermal cells. The majority of evidence indicates that transmission through grafts occurs only after tissue union, and specifically, the union of phloem (16); phloem is not present in TCLs. However, the substance that is transported in the phloem also moves in continuous, living tissue with no preferential movement acropetally or basipetally (7). On removal of pairs of cocultured TCLs, the original explant, along with *de novo* organs formed *in vitro*, always were united into a single unit; this was taken as evidence of graft union sufficient for transmission. The substance or group of substances responsible for flowering in day-neutral TCLs apparently is not transportable through epidermal and subjacent cells.

The 2nd experiment also supports the idea that flowering is a developmental process, where the history of the individual cells is relevant to their future functional capacities. Cells of responsive TCLs may have undergone developmental changes while attached to the mother plant which enable them to form flowers; nonresponsive TCLs may not have achieved the developmental state or may not have as stable a developmental state. If this is the case, the growth regulator concentrations in the medium are a minor consideration.

The number of vegetative shoots on nonflowering TCLs, although generally greater than on flowering TCLs, did not approach the number of flower buds on flowering TCLs. Therefore, there was no preexisting limit to the number of meristematic centers that could develop on a given TCL. Vegetative and floral meristems appear to have arisen independently. Floral meristems appeared to exert limited suppression on the development of vegetative meristems.

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