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Modification of Plant Architecture in Chrysanthemum by Ectopic Expression of the Tobacco Phytochrome *B1* Gene

Zhi-Liang Zheng[1]
Department of Horticulture and Crop Science, The Ohio State University, Columbus, OH 43210

Zhenbiao Yang[2]
Departmen of plant Biology and Plant Biotechnology Center, The Ohio State University, Columbus, OH 43210

Jyan-Chyan Jang[3] **and James D. Metzger**[4]
Department of Horticulture and Crop Science, The Ohio State University, Columbus, OH 43210

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Abstract. Height control is a major consideration during commercial production of chrysanthemum [*Dendranthema ×grandiflora* Kitam. (syn. *Chrysanthemum ×morifolium* Ramat.)]. We have addressed this problem by a biotechnological approach. Plants of 'Iridon' chrysanthemum were genetically engineered to ectopically express a tobacco (*Nicotiana tabacum* L.) phytochrome B1 gene under the control of the CaMV 35S promoter. The transgenic plants were shorter in stature and had larger branch angles than wild type (WT) plants. Reduction in growth caused by the ectopic expression of the tobacco phytochrome B1 gene was similar to that caused using a commercial growth retardant at the recommended rate. Another morphological effect observed in the leaves of the transgenic plants was more intense green color that was related to higher levels of chlorophyll. The transgenic plants appeared very similar to WT plants grown under a filter that selectively attenuated far red wavelengths. Furthermore, when plants were treated either with gibberellin A_3 (which promoted growth) or 2-chlorocholine chloride, an inhibitor of gibberellin biosynthesis (which inhibited growth) the difference in the average internode length between the transgenic plants and WT plants was the same in absolute terms. This suggests that reduction of growth by the expressed *PHY-B1* transgene did not directly involve gibberellin biosynthesis. The commercial application of this biotechnology could provide an economic alternative to the use of chemical growth regulators, thereby reducing production costs.

Height control is a major consideration during production of many horticultural crops. Reduction of height by genetic means or cultural practices often provides economic advantages to the producer. Cultivars of many crops that exhibit shorter stems frequently have higher harvest indices. The physiological basis for this is the redirection of assimilate partitioning such that there is an increase in the proportion of shoot biomass accumulating in the harvested unit, thereby resulting in increased yields (Loomis and Conner, 1992). Control of excessive stem elongation is also essential for prevention of lodging in some crops such as vegetable transplants (Garner and Bjorkman, 1996). In addition, shorter stems increase the quality and value of floriculture and other greenhouse-grown crops and therefore height control is an economic necessity for growers (Gianfagna, 1995).

Height control in greenhouse crops is achieved by several approaches. The most common practice is the use of chemical growth regulators (Dole and Wilkins, 1999). Most commercial growth retardants reduce stem growth by inhibiting gibberellin (GA) biosynthesis, thereby reducing the endogenous levels of one or more biologically active GAs (Gianfagna, 1995). However, this method necessitates significant economic inputs by the grower in terms of both the cost of chemicals and the labor required for application. Furthermore, in many countries, synthetic chemical growth regulators for height control are not labeled for use in crops destined for human consumption.

Cultural methods are also used to control the height of greenhouse crops. For example, manipulation of the daily greenhouse thermoperiod by maintaining the night temperature higher than the day temperature (DIF) is an effective cultural alternative to the use of chemical growth regulators for many crops (Dole and Wilkins, 1999). However, negative DIF can not be exploited in certain geographical regions, or at certain times of the year when it is impractical or deleterious to plant growth to raise the greenhouse night temperature higher than the day temperature (Dole and Wilkins, 1999). Mechanical stimulation (brushing or impedance) of plants can also be used to limit undesirable stem elongation, but implementing this method of height control is often impractical on a commercial scale (Garner and Bjorkman, 1996). A promising new method to control excessive internode elongation in many greenhouse crops is use of photoselective filters that selectively attenuate far red (700 to 800 nm) wavelengths (McMahon and Kelly, 1995; Rajapakse et al., 1999).

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[1]Former graduate research assistant. Currently postdoctoral research associate, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521.
[2]Assistant professor, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521.
[3]Assistant professor.
[4]Professor and corresponding author; e-mail metzger.72@osu.edu.

The third approach of stem growth control is the development of cultivars with reduced stature by conventional breeding (Loomis and Conner, 1992). While this approach lowers grower economic inputs compared to the use of cultural methods or growth regulators, the development of new cultivars with shorter stems is a slow and expensive process. As a result, the breeding approach to the control of plant stature has been exploited primarily for agronomic crops.

Biotechnology provides an attractive alternative to conventional breeding in that traits controlled by single genes can be incorporated quickly in existing cultivars, which can greatly reduce the time and costs of developing new cultivars. Genetic engineering of phytochrome genes has provided a potential means to control vegetative growth and reproductive development (Robson and Smith, 1997). The ectopic expression of a *PHYA* gene in tobacco (*Nicotiana tabacum* L.) and potato plants (*Solanum tuberosum* L) significantly inhibited stem elongation and increased the harvest index through hypersensitivity to far red light (FR) (Robson et al., 1996). Likewise, ectopic expression of *Arabidopsis* or rice (*Oryza sativum* L.) *PHYB* gene in *Arabidopsis* led to reduced extension growth of light-grown seedlings (McCormac et al., 1993; Wagner et al., 1991). In addition, ectopic expression of *Arabidopsis PHYB* in tobacco and potato inhibited stem elongation of mature plants as well (Halliday et al., 1997; Thiele et al., 1999).

Recently, a *PHYB*-like phytochrome encoded by the *HLG*-locus in *Nicotiana plumbaginifolia* Viv. has been shown to be a sensor for red light (R) rather than the ratio of R to FR (R/FR) as is the case for *PHYB* in *Arabidopsis*, rape (*Brassica rapa* L.) and cucumber (*Cucumis sativum* L.) (Hudson et al., 1997; Smith and Whitelam, 1997). In *hlg* mutants, the response to low R/FR is not attenuated but the response to R is altered (Hudson et al., 1997). The *HLG*-encoded *PHYB* shares 97% homology with the *PHY-B1* in *N. tabacum* (Adam et al., 1997). Since light rich in red wavelengths inhibits stem elongation in many species (Smith, 1994), we hypothesized that the ectopic expression of the tobacco *PHY-B1* would increase the sensitivity to R, resulting in the inhibition of stem elongation. Therefore, the main objective of the research was to test this hypothesis using chrysanthemum as target species for transformation.

Materials and Methods

Plant materials and growth conditions. Rooted stem cuttings of 'Iridon' chrysanthemum were obtained from Yoder Brothers, Inc. (Pendleton, S.C.) and were maintained in the greenhouse as stock plants from which plants for experiments were generated by vegetative propagation. Plants were fertilized with a complete fertilizer (20N–8.6P–16.6K) at a concentration of 200 $mg \cdot L^{-1}$ of N (Scotts-Sierra Horticulture Products Co., Marysville, Oh.) three times a week. The proximal portions of terminal stem cuttings from vegetative stock plants were dipped in a 1 $mg \cdot mL^{-1}$ solution of napthaleneacetic acid (NAA), and then planted in 500-mL pots containing a commercial soilless medium (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, Ohio). The resulting plants were grown in a greenhouse with a temperature setpoint of 25 °C. Natural photoperiods were extended to 17 h long days (LD) using light from 1000 W high-pressure sodium vapor lamps [80 to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR)] from 0600 to 1000 HR and 1600 to 2300 HR. Short day (SD) conditions were achieved by covering the plants daily with black shade cloth from 1630 to 0830 HR.

Construction of *35S::Nt-PHY-B1*. A 4 kb BamHI/ClaI fragment of a full length *Nt-PHY-B1* cDNA (Kern et al., 1993) was blunt-ended and ligated in the sense orientation into SmaI site in the pJIT117D2 vector. The expression vector contained both the cauliflower mosaic virus (CaMV) 35S promoter and the poly A terminator sequences as well as the dual enhancer sequences minus the chloroplast-targeting peptide sequence (Guerineau et al., 1988). The resulting pJB was cut by KpnI, and a 5.5 kb KpnI fragment containing the *Nt-PHY-B1* cDNA, 35S promoter and terminator was subcloned into the KpnI site of the binary vector pBIN19. The resulting plasmid pESB was then transferred into the hypervirulent strain of *Agrobacterium tumefaciens* Conn., EHA105, using the freeze–thaw method (An et al., 1993).

Plant transformation. Plants were regenerated from leaf discs and young internode segments on Murashige and Skoog (MS) medium (An et al., 1993) containing benzyladenine (0.5 $mg \cdot L^{-1}$) plus naphthalene acetic acid (NAA) (1.0 $mg \cdot L^{-1}$). The plant transformation procedure was modified from Sherman et al. (1998). *Agrobacterium* cells harboring pESB were grown in 200 mL of YEP liquid medium (An et al., 1993) containing 50 $mg \cdot L^{-1}$ kanamycin and 25 $mg \cdot L^{-1}$ rifampicin. After 2 days when the optical density at 600 nm was about 2, the cultures were centrifuged at 4000 g_n for 5 min and the pellet suspended with 10 mL of sterile MS medium. Leaf discs (1.0 cm^2) were immersed in the *Agrobacterium* suspensions for 5 min and then placed on regeneration medium containing no antibiotics for 3 d. The leaf discs were then transferred to the regeneration medium supplemented with 50 $mg \cdot L^{-1}$ kanamycin and 500 $mg \cdot L^{-1}$ carbenicillin. Putative kanamycin-resistant shoots were regenerated in 4 to 5 weeks. The shoots were excised and transferred onto MS medium with 0.1 $mg \cdot L^{-1}$ of NAA and containing both kanamycin and carbenicillin at the same concentrations used in the shoot regeneration medium. After about 3 weeks, plants with well-developed root systems were planted in pots and transferred to the greenhouse as described previously.

PCR and RNA blot analyses. Polymerase chain reaction (PCR) was used to amplify the tobacco *PHY-B1* cDNA to identify the putative transgenic plants. Plants selected for kanamycin resistance were grown in the greenhouse for several weeks. Genomic DNA was extracted from young leaves using the CTAB-method (McGarvey and Kaper, 1991). Two primers were used to amplify a 1.2 kb fragment. The sense primer SB1 (5' GAA TGG TAT ACG CTT TAC AA 3') was specific for *Nt-PHY-B1* cDNA, while the antisense primer SB2 was based on the pBIN19 vector sequence: 5' TCC AGC CGA ATT CCC CGA TA 3'. PCR was conducted under the following conditions for 30 cycles: 94 °C, 1 min.; 55°C, 1 min.; 72 °C, 1 min. An additional 7 min extension at 72 °C was performed after the last cycle.

Total RNA was extracted from young leaves of chrysanthemum plants (Logemann et al., 1987), fractionated on a 1.2% formaldehyde agarose gel, and then transferred onto a nylon membrane. The probe was labeled by PCR (Sambrook et al., 1989). A 0.3 kb *PHYB-1* cDNA fragment was amplified using the primer SB1 described above and an antisense primer BP2 (5' ACC TGT AGT ATT CTC ACT TG 3'). PCR labeling was carried out using the same condition as described above except a(^{32}P)-dATP was incorporated. The hybridization was performed as described by Sambrook et al. (1989).

Characterization of the transgenic plants. Heights of single-stemmed plants were measured from the medium surface to the terminal bud. The branching pattern of shoots was analyzed in plants in which apical dominance was released by removing the

apical 1 cm of the shoot (pinched) 3 weeks after transplanting the cuttings. The pinched plants were grown under LD for 3 weeks and then transferred to SD conditions. After 2 months, the angles between the main stem and the three oldest branches were measured using a protractor. Canopy diameter was determined by measuring the maximum horizontal extension of the branches. The canopy height was measured using a ruler from the medium surface to the top of the plants. Leaf areas were determined using a leaf area meter (model 3100; LI-COR Inc., Lincoln, Nebr.).

Chlorophyll levels of leaves from wildtype (WT) and transgenic plants were compared. Two leaf discs, 0.26 cm^2 each, were removed from the lamina of leaf number 1 (the youngest leaf at least 2 cm long) and leaf number 5 and placed in 5 mL *N, N*-dimethylformamide. After 2 d in the dark at 4 °C, 1 mL of the extract was removed and absorbance at 664 and 647 nm was measured. Total chlorophyll levels were determined using the method of Moran (1982). Five plants from each genotype were analyzed, and the experiment was repeated once.

Growth response to far red attenuated light. Effect of reducing FR relative to visible wavelengths on the growth of wildtype (WT) and transgenic plants was compared. Plants from 3-week-old stem cuttings were grown in 500 mL pots in the greenhouse under a plastic film containing a dye that selectively absorbs radiation of 700 to 800 nm (Mitsui Chemical Corp., Osaka, Japan). This filter resulted in an increase in the R/FR ratio, defined as the ratio of photon flux at 655 to 665 nm to that at 725 to 735 nm, at noon on a clear day in January from 1.20 to 1.73 as measured by a portable spectroradiometer (LI-1800; LI-COR). Control plants were grown under a clear plastic film that provided a photosynthetic photon flux nearly the same as the FR filter, but did not affect the R/FR ratio. Temperatures were also nearly the same in both treatments. Stem heights were measured every 3 d following transfer. Each treatment consisted of five plants, and the experiment was repeated once.

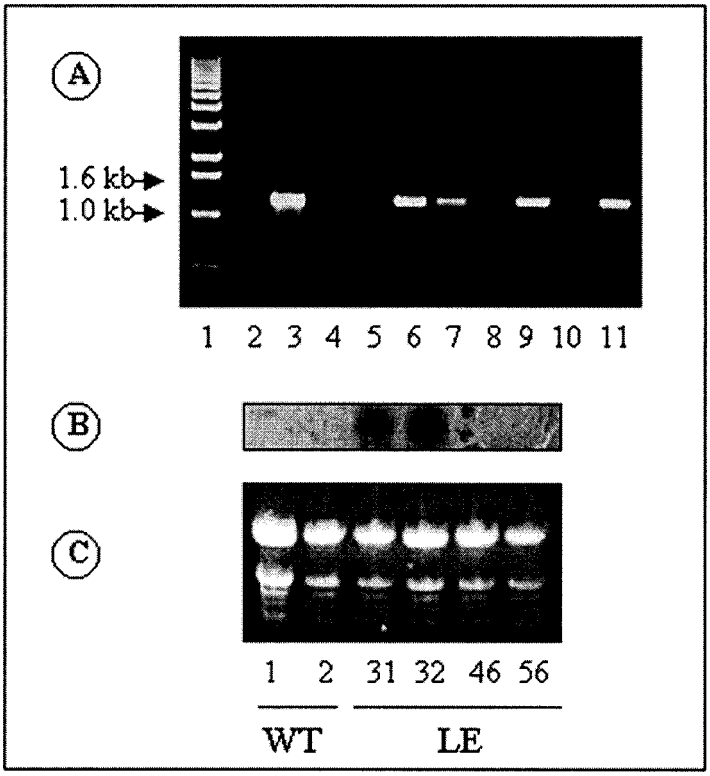

Fig. 1. Identification and molecular characterization of transgenic chrysanthemum plants. (**A**) PCR amplification of the *Nt-PHY-B1* transgene from kanamycin-resistant lines: lane 1, 1kb ladder marker; lanes 2 and 3, without and with plasmid pESB, respectively; lane 4, wild type; lanes 5–11, transgenic lines regenerated from leaf explants mediated by the EHA105 strain of *Agrobacterium*, LE11, 31, 32, 45, 46, 50, 56. (**B**) RNA blot analysis. A fragment of *Nt-PHY-B1* cDNA was used as a probe to detect the expression of the transgene. Shown are LE31 and LE32 with high levels of expression of *PHY-B1* gene. (**C**) Loading of 5 mg total RNA (15 mg for WT1) as shown by ethidium bromide staining.

Response to growth regulator applications. The role of GA in the phytochrome control of stem elongation was investigated by comparing the effects of 2-chlorocholine chloride (CCC) and exogenous GA_3 on stem elongation in transgenic and WT plants. Stem cuttings of the various genotypes were rooted and grown in the greenhouse under LD as described previously. After 3 weeks, the pots were drenched with 100 mL of a 9.5 mM solution of a commercial preparation of CCC. After 10 d the CCC drench was repeated. A 10 mL solution containing 10 mg GA_3 dissolved in 10% (v/v) aqueous acetone plus 0.01% (v/v) Tween 20, was applied to the shoot tips of plants every 2 d. Stem heights of the plants were measured every 3 d for 15 d. The number of nodes was counted from the node closest to the medium surface to the node subtended by the youngest 1 cm long leaf. Each treatment consisted of five plants.

Flowering responses. The effects of a night break (NB) during an otherwise inductive long night on flowering of WT and transgenic plants were compared. Eight plants of each genotype were transferred to SD conditions as described previously, and a low irradiance NB (1.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant level) of R was provided from 0000 to 0030 HR. The R was obtained from two 20-W cool-white fluorescent lamps wrapped with one layer of red cellulose film that provided a R/FR of 3.34. The number of days to the appearance of visible flower buds and the first open flower were recorded.

All experiments were completely randomized design. Data were analyzed using one-way analysis of variance and significantly different means separated using Tukey's multiple comparison test.

Results and Discussion

Generation and molecular characterization of transgenic chrysanthemum plants. A total of 32 kanamycin-resistant plants were obtained; each of these was examined further for integration of the *Nt-PHY-B1* cDNA into the chrysanthemum genome by PCR analysis of genomic DNA from young leaves of greenhouse-grown plants. Four independently transformed lines, LE31, LE32, LE46 and LE56, were identified (Fig. 1A). No band was amplified from WT plants since the antisense primer was based on the vector sequence. The low percentage of the kanamycin-resistant lines exhibiting a positive PCR response is consistent with other work on transformation of chrysanthemum and appears to be due, at least in part, to greater kanamycin tolerance than many other plant species (Sherman et al., 1998).

Our goal was to express the tobacco phytochrome B1 gene at high levels and thus a strong CaMV 35S promoter with dual enhancers (Guerineau et al., 1988) was chosen to constitutively drive the expression of the *Nt-PHY-B1* transgene. Northern blot analysis showed high expression of the *Nt-PHY-B1* transgene in two of the four transgenic lines, LE31 and LE32 (Fig. 1B). No *Nt-PHY-B1* message was detected in WT plants, suggesting low sequence homology between the tobacco and chrysanthemum B-type phytochrome genes. The growth habits of these two transgenic lines were characterized further.

Table 1. Effect of ectopic expression of the tobacco *PHYB-1* gene on heights of chrysanthemum plants grown under both LD and SD conditions.[z]

Photoperiod	Genotype	Plant ht (cm)	Nodes (no.)	Internode length (mm)
LD	Wildtype	11.4 a[y] (1.2)	19.8 a (0.4)	6.1 a (0.6)
	LE31	8.5 b (0.8)	19.2 a (0.8)	4.6 b (0.3)
	LE32	9.4 b (0.3)	19.4 a (0.6)	5.1 b (0.2)
SD	Wildtype	13.7 a (1.0)	22.5 a (1.2)	6.4 a (0.3)
	LE31	11.0 b (1.4)	22.1 a (1.6)	5.2 b (0.4)
	LE32	11.9 b (0.8)	23.0 a (1.1)	5.4 b (0.4)

[z]Plants were propagated vegetatively from stem cuttings under LD. After 3 weeks, half of the plants were transferred to SD. Plant heights, number of nodes, and average internode lengths were determined after 15 and 28 d for LD- and SD-treated plants, respectively. Values represent means ± SD of five and eight plants for LD- and SD-treated plants, respectively.
[y]Mean separation within columns for a photoperiodic treatment by Tukey's multiple comparison test at $P = 0.05$.

Table 2. Effect of ectopic expression of the tobacco *PHYB-1* gene on total leaf chlorophyll levels in chrysanthemum plants.[z]

Leaf age	Genotype	Total chlorophyll ($mg \cdot g^{-1}$ fresh wt)	Leaf area (cm^2)
Young	Wildtype	0.91 a[y] (0.13)	4.46 a (0.64)
	LE31	1.28 b (0.14)	4.47 a (0.88)
	LE32	1.28 b (0.15)	4.69 a (0.61)
Mature	Wildtype	1.39 b (0.11)	15.61 b (1.38)
	LE31	1.70 c (0.26)	15.26 b (1.38)
	LE32	1.67 c (0.22)	15.08 b (1.97)

[z]Plants were propagated vegetatively from stem cuttings and grown in a greenhouse for 5 weeks under LD. Young and mature refer to the first and fifth youngest leaves, respectively. Values represent the means ± SD of five plants.
[y]Mean separation within columns by Tukey's multiple comparison test at $P = 0.05$.

Table 3. Effect of ectopic expression of the tobacco *PHYB-1* gene on shoot architecture in chrysanthemum plants.[z]

Genotype	Branch angle (degrees)	Canopy diam (cm)	Canopy ht (cm)	Diam to ht ratio
Wildtype	33.5 a[y] (7.2)	36.5 a (3.2)	32.6 a (1.1)	1.12 a (0.13)
LE31	62.6 b (3.3)	43.2 b (3.9)	29.1 b (2.6)	1.50 b (0.27)
LE32	65.6 b (3.9)	40.8 b (2.4)	29.2 b (1.3)	1.40 b (0.08)

[z]Young plants were pinched to release apical dominance and grown under LD for 3 weeks before being transferred to SD. The diameter, height and branch angles of flowering plants were measured after 2 months grown under SD. Values represent the means ± SD of four or five plants.
[y]Mean separation within columns by Tukey's multiple comparison test at $P = 0.05$.

Transgenic plants have shorter internodes and contain higher levels of chlorophyll. Plants of the transgenic lines LE31 and LE32 were shorter than WT plants with a 13% to 25% reduction of stem height (Table 1). The observed height reduction in the transgenic plants resulted from shorter internodes and not from fewer nodes being produced; average internode lengths in plants of both transgenic lines varied from 16% to 28% less than WT plants (Table 1).

The leaves of LE31 and LE32 appeared slightly greener than WT plants. Comparative analysis of young and mature leaves of the three genotypes showed that LE31 and LE32 had roughly 40% and 22% higher total chlorophyll levels, respectively than WT plants (Table 2). The increase of leaf chlorophyll in transgenic plants is consistent with observations that chlorophyll levels are reduced in *PHYB* mutants of *Arabidopsis* (Reed et al., 1993), and *PHY-B1* antisense potato plants (Jackson et al., 1996).

Since there was no difference in leaf areas among the three genotypes, individual leaves of the transgenic plants contained higher absolute amounts of chlorophyll. Recently, Thiele et al. (1999) have shown quantitatively similar increases in leaf chlorophyll levels resulting from heterologous expression of *Arabidopsis PHYB* in potato. In this case the higher chlorophyll levels resulted in higher rates of photosynthesis, and as a consequence, higher tuber yields.

Transgenic plants exhibit altered shoot architecture. The light environment controls many aspects of shoot development and canopy structure. Most of these responses are caused by changes in R/FR, and are therefore collectively termed the shade avoidance response (Smith, 1994; 1995). One manifestation of the shade avoidance response in many species is the orthotropic growth habit of leaves when plants are subjected to light environments with low R/FR ratios; this presumably represents an adaptation to maximize the interception of light in crowded conditions (Whitelam and Johnson, 1982). However, the ectopic expression of the *Nt-PHY*-B1 gene did not affect leaf angles in plants grown under thin or dense spacing (data not presented). This is consistent with the observation that changes in leaf angle in response to the R/FR ratio were unaltered in *hlg* mutants of *N. plumbaginifolia* deficient in a phytochrome closely related to *PHY-B1* of *N. tabacum* (Hudson and Smith, 1998).

Phytochrome is also involved in the control of lateral branching, and indeed, a decrease in lateral branching is another important component of the shade-avoidance response in many species (Smith, 1994; 1995; Smith and Whitelam, 1997). However, we observed no difference in the number of lateral branches between the transgenic and WT plants (data not presented), indicating apical dominance is unaffected by the ectopic expression of the *PHY-B1* gene. Nevertheless, we did observe one distinct alteration in the growth of lateral branches in the transgenic plants: larger branch angles (Table 3). Moreover, canopy diameters of both LE31 and LE32 were greater than WT plants, while canopy heights were less. The ratio of the diameter to canopy height of LE31 and LE32 was significantly higher than that of WT plants (Table 3). The morphological basis for the increase in canopy diameter in the transgenic plants was the larger angles between the lateral branches and the main stem.

Shoot architecture is often altered as competition for light increases (Harper, 1977). In general, branching patterns are optimized to reduce both self-shading and shading by neighbors. Reduced lateral branching and/or an orthotropic growth habit of the branches can minimize shading by neighbors. A more plagiotropic growth habit of the branches, which is characteristic of many plants growing in open communities, maximizes the area available for the interception of light. To our knowledge, there are no reports demonstrating involvement of specific phytochrome(s) in the regulation of branch angles in dicots. However, the action of phytochrome has been demonstrated in the control of shoot zenith angles (the angles of tillers with respect to the vertical) in

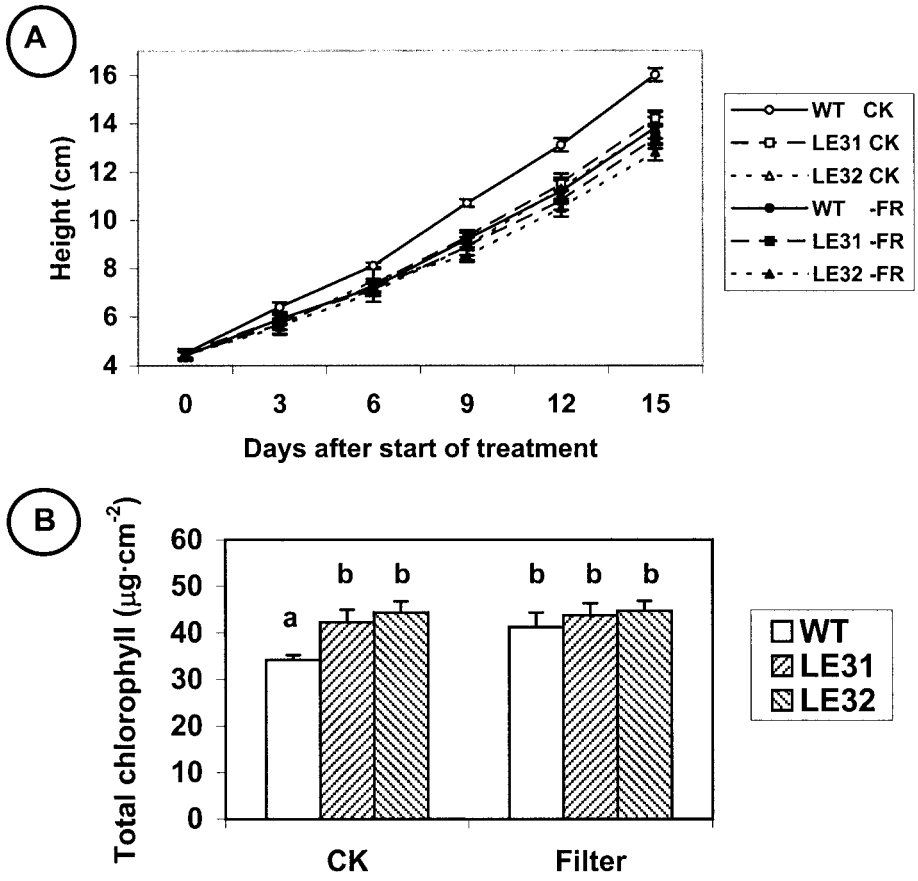

Fig. 2. Transgenic chrysanthemum plants phenocopy wildtype plants grown under FR-attenuated sunlight (**-FR)**. Plants were transferred to boxes covered with clear plastic (**CK**, control) or a plastic photoselective filter 3 weeks after vegetative propagation. Plants were maintained in a greenhouse in which the natural photoperiod was extended to 17 h. (**A**) Stem heights measured every 3 days. Each symbol represents the mean ± SE of five plants. (**B**) Total chlorophyll levels measured 15 d after transfer to the photoselective filters. Values represent means ± SD. Columns with the same letter are not statistically different at $P = 0.05$. **WT**, wildtype. **CK**, control.

two grass species, *Lolium multiflorum* Lam. (annual ryegrass) and *Paspalum dilatatum* Poir. (Dallis grass). In these two species, shoot zenith angles increase with higher R/FR ratios (Casal et al., 1990; Gibson et al., 1992). When grown in the absence of nearby neighbors, these grasses have shoots with a more plagiotropic growth habit than plants growing in crowded communities. As a consequence, the plants are able to intercept a higher proportion of incident radiation per plant (Casal et al., 1986). Therefore, one possible function of the tobacco phy-B1 may be to control branch angle in response to changing light environments.

TRANSGENIC CHRYSANTHEMUMS PHENOCOPY WT PLANTS GROWN IN FR-ATTENUATED LIGHT. To investigate the mechanism of inhibition of stem growth in transgenic chrysanthemums, plants were grown in a light environment in which the R/FR of sunlight at noon was increased from 1.20 to 1.73 using a spectrally selective plastic filter. As in the previous experiment, plant heights of both LE31 and LE32 were less than WT plants under control conditions (clear plastic film), but also phenocopied WT plants grown in FR-depleted light (Fig. 2A). However, no further reduction in the growth of the transgenic plants was achieved by subjecting them to FR-depleted light, suggesting that the tobacco phy-B1 is not a sensor for the shade avoidance response.

Another characteristic of the shade avoidance response in many species is retardation of leaf development including leaf area growth, chloroplast development, and chlorophyll synthesis as the R/FR decreases (Smith, 1994; 1995). Conversely, transfer of plants of many species, including chrysanthemum, to FR-attenuated light environments results in a higher level of chlorophyll per unit area (McMahon and Kelly, 1995; Rajapakse et al., 1999). We also observed that the leaves of WT chrysanthemum plants grown under the spectral filter contained roughly 20% higher chlorophyll levels than when grown under control conditions (Fig. 2B). However, the higher chlorophyll levels in WT leaves in response to the increase in R/FR were not statistically different from those observed in the transgenic plants grown under the clear plastic. Moreover, subjecting LE31 and LE32 to a FR-attenuated light environment did not result in any further increases in chlorophyll levels (Fig. 2B).

Results herein suggest that the tobacco phy-B1 is a sensor for R and its ectopic expression in transgenic chrysanthemum plants increased sensitivity to R only, resulting in inhibition of stem elongation. Similarly, unlike phytochromes from the *PHYB*–subgroup in *Arabidopsis*, the closely related phytochrome in *N. plumbaginifolia* encoded by *HLG* is not the sensor for low R/FR, but rather responds exclusively to R (Hudson et al., 1997; Smith and Whitelam, 1997). A similar conclusion has been drawn for phy-B1 homolog in tomato (*Lycopersicon esculentum* Mill.), a closely related species (van Tuinen et al., 1995).

EXPRESSION OF *PHY-B1* REDUCES GROWTH BY A MECHANISM THAT DOES NOT DIRECTLY INVOLVE GA BIOSYNTHESIS. We have shown that both LE31 and LE32 plants behave as WT plants grown under FR-depleted light. GA has been proposed to partially mediate the regulation of chrysanthemum plant height under different R/FR (Rajapakse and Kelly, 1991; Rajapakse et al., 1999). Supporting this hypothesis are observations that transgenic tobacco plants overexpressing an oat (*Avena sativum* L.) *PHYA* gene had reduced levels of endogenous GA (Jordan et al., 1995). Higher endogenous GA levels have been observed in the ma_3^R mutant of sorghum (*Sorghum bicolor* L.) deficient in phyB (Beall et al., 1991; Childs et al., 1997). Therefore, we investigated whether the action of the ectopically expressed tobacco phy-B1 in chrysanthemum involved GA biosynthesis.

Endogenous GA levels were reduced by treating with CCC, an inhibitor of GA biosynthesis (Sponsel, 1995). This treatment resulted in a reduction of internode length of both WT and the transgenic plants (Table 4). However, the mean internode length of LE31 and LE32 plants treated with CCC was still shorter than CCC-treated WT plants (Table 4). Moreover, absolute differences in mean internode length between transgenic and WT plants were nearly the same (1.3 and 1.5 mm for LE31, and 0.9 and 1.1 mm for LE32) in both control and CCC treatments (Table 4). Treatments with GA_3 fully restored growth in both WT and transgenic plants to that of plants treated with GA_3 alone. Nevertheless, exogenous GA_3 failed to reverse the reduction in stem

Table 4. Comparison of the effect of exogenous GA_3 and 2-chlorocholine chloride (CCC) on internode length of transgenic and wild-type chrysanthemum plants.[z]

Treatment	Genotype	Mean internode length (mm)	Mean difference from WT plants (mm)
Control	Wildtype	6.0 a[y] (0.6)	---
	LE31	4.7 b (0.3)	1.3
	LE32	5.1 b (0.2)	0.9
GA	Wildtype	11.1 c (0.6)	---
	LE31	9.9 d (0.3)	1.2
	LE32	9.9 d (0.6)	1.2
CCC	Wildtype	4.8 b (0.2)	---
	LE31	3.3 e (0.3)	1.5
	LE32	3.7 e (0.5)	1.1
GA+CCC	Wildtype	11.2 c (0.7)	---
	LE31	9.9 d (0.3)	1.3
	LE32	9.9 d (0.4)	1.2

[z]Plants were propagated vegetatively from stem cuttings and grown in the greenhouse under LD. After 3 weeks plants received either a soil drench of a 9.5 mM solution of CCC, 10 mg of GA_3 applied to the shoot tips every 2 d for a total of 7 treatments, or a combination of the CCC and GA_3 treatments. Values represent means ± SD of five plants. Internode lengths were determined 15 d after the treatments began.
[y]Mean separation within columns by Tukey's multiple comparison test at $P = 0.05$.

Table 5. Effect of ectopic expression of the tobacco *PHYB-1* gene on night-break sensitivity in chrysanthemum plants.[z]

Photoperiod treatment	Genotype	Days to appearance of flower buds	Days to flower opening
SD	Wildtype	25.1 a[y] (1.4)	60.4 a(1.3)
	LE31	25.3 a (1.4)	63.5 a (1.9)
	LE32	25.5 a (1.7)	64.8 a (3.2)
NB-R	Wildtype	28.4 b (0.7)	76.0 b (3.3)
	LE31	33.1 c (2.2)	95.6 c (6.9)
	LE32	32.3 c (1.5)	93.3 c (5.3)

[z]Plants were propagated vegetatively from stem cuttings and grown in the greenhouse under LD. After 3 weeks plants were transferred to SD or SD plus a 30-min night-break of red light from 0000 to 0030 HR. The number of days from the start of the treatments to the appearance of visible flower buds and to the opening first flower was recorded. Values represent means ± SD of eight plants per treatment.
[y]Mean separation within columns by Tukey's multiple range test at $P = 0.05$.

length caused by expression of tobacco *PHY-B1* gene (Table 4). Similarly, exogenous GA_3 did not fully restore hypocotyl growth in *Arabidopsis PHYB* overexpressors (Wagner et al., 1991).

Regardless of the treatment, the average internode length of plants of both transgenic lines was always about 1.2 mm less than WT plants subjected to the same treatment (Table 4). In other words, the combined effect of transgenic expression and growth retardant treatment on stem growth was the sum of their effects when applied individually. One interpretation of this observation is that GA does not mediate the R-induced inhibition of stem elongation in the transgenic plants. A similar rationale was used to argue against a possible role of GA in mediating phytochrome control of hypocotyl growth in mustard (*Sinapis alba* L.) (Mohr and Appuhn, 1962).

There are other independent lines of experimentation providing corroborating evidence for the aforementioned conclusion. First, endogenous GA levels were not altered in either the cucumber (*Cucumis sativus* L.) *lh* mutant or the pea (*Pisum sativum* L.) *lv* mutant which are deficient in B-type phytochromes (Lopez-Juez et al., 1995; Weller et al., 1994) or the *PHYB* mutant of *Arabidopsis* (Reed et al., 1996). Second, it has been shown in several instances that GA and phytochrome regulate distinct cellular processes contributing to growth. In *Thlaspi arvense* L. (field pennycress), most of GA-induced petiole growth and stem growth can be attributed to an increase in cell number, while phytochrome regulation of growth in these tissues appear to be restricted to controlling the ultimate length of cells (Metzger, 1988; Metzger and Dusbabek, 1991).

EXPRESSION OF *Nt-PHY-B1* HAS ONLY A SLIGHT EFFECT ON NIGHT-BREAK SENSITIVITY. Chrysanthemum is a short-day plant (SDP) that flowers when the length of the dark period is > 9.5 h (Larson, 1992). For commercial production of chrysanthemums, a certain amount of vegetative growth is required before flowering is induced. Vegetative conditions are maintained by providing a night break (NB) of low irradiance light in the middle of an otherwise inductive long night. The most effective wavelengths for NB inhibition of flowering SDP are R, and phytochrome B is primarily responsible for mediating the NB-R response (Thomas and Vince-Prue, 1997). Indeed, the ectopic expression of *PHYB* in a short day tobacco cultivar greatly increased the sensitivity to a 30 min NB using light from fluorescent lamps (Halliday et al., 1997). On the other hand, suppression of *PHYB* expression through antisense RNA in potato disrupted the inhibition of tuber formation caused by a NB (Jackson et al., 1996). Based on these effects of altering phytochrome B levels on the response to NB, we also examined whether expression of the tobacco *PHY-B1* would also increase the sensitivity to NB in chrysanthemum, and thus have the practical effect of reducing the minimum duration of the NB required to maintain the plants in a vegetative state. Since we demonstrated previously that the tobacco phy-B1 mediated R-induced inhibition of stem elongation, we then tested whether the ectopically expressed phy-B1 also participates in mediating the NB-R inhibition of flowering in the SDP chrysanthemum. This was examined using NB-R of sufficient duration (30 min) to delay, but not completely block flowering.

As shown in Table 5, ectopic expression of phy-B1 did not affect flower formation or development under SD conditions. However the delay in flowering caused by suboptimal NB-R was greater in the transgenic plants than WT; there was an additional 4 to 5 and 17 to 20 d delay in the time to appearance of visible flower buds and flower bud opening, respectively (Table 5). The slight delay in the time to appearance of visible flower buds observed in the transgenic plants indicates that the initiation of flower primordia occurred about the same time as WT. This coupled with the much greater effect on the time to flower bud opening suggests that the primary effect of the ectopic expression of phytochrome-B1 on flowering is on flower development rather than floral induction. This is not unexpected since flower and inflorescence development are growth processes, and we have shown previously that stem growth is reduced in the transgenic plants. Nevertheless it is still possible that under different NB conditions (e.g., higher photon flux and/or longer duration), an effect on inductive processes would be more apparent.

BIOTECHNOLOGICAL IMPLICATIONS. Genetic engineering of phytochrome genes has significant potential applications in both fundamental plant research and crop production (Robson and

Smith, 1997). Ectopic expression of oat *PHYA* in tobacco resulted in suppression of the shade avoidance response, thereby increasing the leaf-harvest index (Robson et al., 1996). Thiele et al. (1999) also found that the heterologous expression of *Arabidopsis* phytochrome B in potato reduced stem elongation and increased chlorophyll levels. This latter effect resulted in an increase in photosynthetic performance and subsequent higher tuber yields (Thiele et al., 1999). We demonstrated that plant architecture can be modified in other agriculturally useful ways by ectopic expression of a B-type phytochrome gene.

Management of plant stature is a significant activity during production of many greenhouse crops. Currently, control of plant height is achieved through the use of chemical growth retardants or cultural methods such as negative DIF and mechanical stimulation, but all of these methods have distinct disadvantages (Dole and Wilkins, 1999; Garner and Bjorkman, 1996; Gianfagna, 1995). The ectopic expression of the tobacco *PHYB-1* gene in crops provides an alternative technology that could potentially reduce economic inputs required for management of plant height. Using this strategy, we were able to generate plants that exhibited a reduction in stature comparable to that using a growth regulator at recommended rates (Tables 1 and 4).

In addition, since no synthetic chemical growth regulators for height control are labeled for use in crops destined for human consumption, the extension of this technology into such crops may have applications in other areas of agricultural production as well. In many cases, shorter stems result in higher harvest indices (Loomis and Conner, 1992; Robson et al., 1996).

The branching patterns of crops are also important components in controlling yield and crop quality. Commercially desirable changes in branching patterns are usually achieved by manual pruning, so it is likely that genetic approaches to the control of branching patterns would also provide economic advantages for growers. For example, monocultures of tomato plants with a prostrate growth habit (larger branch angles) have increased total and marketable yields and are notably easier to harvest compared with plants of upright growth habit (Ozminkowski et al., 1990). Therefore, transgenic plants having a canopy architecture characterized by shorter stems and larger branch angles than WT plants could result in plants with a more open canopy area that are more efficient in capturing sunlight.

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