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*Research Article*

# Phenotypic Characterization of Transgenic *Miscanthus sinensis* Plants Overexpressing *Arabidopsis* Phytochrome B

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Phytochromes are dimeric pigment proteins with reversible photochromism between red and far-red light-absorbing forms. They are photoreceptors that regulate various aspects of plant growth and development and have been used for biotechnological applications to improve agricultural performance of crops. *Miscanthus* species have been suggested as one of the most promising energy crops. In this paper, *Arabidopsis* phytochrome B *(PHYB)* gene was introduced into *Miscanthus sinensis* using *Agrobacterium*-mediated transformation method that we developed recently, with the herbicide resistance gene *(BAR)* as a selection marker. After putative transgenic plants were selected using the herbicide resistance assay, genomic integration of the transgene was confirmed by genomic PCR and Southern blot analysis, and transgene expression was validated by Northern blot analysis. Compared to nontransformed control plants, transgenic plants overexpressing *PHYB* showed phenotypes with increased phytochrome B function, which includes increased chlorophyll content, decreased plant height, and delayed flowering. Therefore, these results suggest that *Arabidopsis* phytochrome B is functional in *M. sinensis* and provide a method to develop *Miscanthus* varieties with enhanced agricultural performance using phytochromes.

## 1. Introduction

Phytochromes are photoreceptors that regulate various aspects of plant growth and development in response to red and far-red light signals from the environment [1, 2]. They are dimeric chromopeptides with each monomer (120–130 kDa) possessing a covalently linked open-tetrapyrrole chromophore called phytochromobilin via a thioether linkage to a cysteine residue [3]. The most important characteristic of phytochromes is its reversible photochromism: the property of changing color on photon absorption and of reverting to the original form on the absorption of another photon. Phytochromes are synthesized in red light-absorbing Pr form ($\lambda_{max}$ = 660 nm) which can be phototransformed into far-red light-absorbing Pfr form ($\lambda_{max}$ = 730 nm) upon exposure to red light. The functional activities of the phytochromes are modulated by the photochromic transformation between these two forms [4, 5]. Generally, the Pr-to-Pfr phototransformation of phytochrome is known to induce the highly regulated signaling network for photomorphogenesis in plants [6–8].

Phytochrome-mediated photomorphogenic responses include germination, stem and leaf growth, chloroplast development, biosynthesis of chlorophylls and other pigments, shade avoidance, circadian rhythm, and flowering [9]. In the aspect of biotechnological applications of phytochromes, physiological analysis of plants overexpressing phytochrome transgenes has previously revealed the potential of transgenic approaches to modifying the architecture of crop plants [10]. As an example, tobacco

plants expressing oat phytochrome A transgene (*PHYA*) exhibit proximity-conditional dwarfing which leads to up to 20% improvement in harvest index [11]. In principle, phytochromes mediate plant shade avoidance responses in which plants react to far-red radiation reflected from neighbors and induce elongation growth to prevent overtopping by competitors growing alongside [12]. If the shade avoidance responses are induced, the reallocation of resources into elongation growth might result in reduced leaf and storage organ production with a decrease in crop yield [13]. Thus, the shade avoidance responses can be detrimental in agriculture because it limits the planting density. Therefore, it has suggested that plant productivity can be enhanced by reducing perception of the neighbors (i.e., suppression of shade avoidance responses) through the manipulation of phytochrome red/far-red light perception [14].

*Miscanthus* species are tall perennial rhizomatous grasses with C4 photosynthesis which tend to give high biomass yields annually over a wide range of climates [15, 16]. Thus, they are suggested as one of the most promising energy crops for biomass production [17]. So far, the triploid hybrid *Miscanthus* × *giganteus* between the diploid *M. sinensis* and the tetraploid *M. sacchariflorus* is currently the commercially grown species in the genus [18, 19]. However, this hybrid is sterile and lacks genetic variation, so the necessity for seed-propagated varieties in genetically stable and fertile species, such as *M. sinensis,* has been raised [20].

The genetic improvement by conventional breeding has been used to improve traits of many species, but its success has been limited by barriers such as sexual reproduction and the relatively long time periods required for breeding programs. More recently, genetic engineering methods with the help of plant transformation technologies have been used to improve many species more efficiently, whereby useful traits have been introduced from a broader range of sources within an economically viable time frame [21]. In the case of *Miscanthus* species, the genetic transformation systems have been established very recently using particle bombardment-mediated and *Agrobacterium*-mediated methods [22, 23]. However, there is no report thus far of transgenic Miscanthus plants with a useful gene other than selective marker genes. Therefore, genetically engineered Miscanthus plants need to be further developed by introducing a useful gene using the established transformation method.

The objective of this study was to develop transgenic *M. sinensis* plants overexpressing *Arabidopsis* phytochrome B gene (*PHYB*) using the *Agrobacterium*-mediated genetic transformation method that we developed recently. In addition, the phenotypes of the transgenic plants were investigated in terms of phytochrome function, which includes chlorophyll content, plant height, and flowering time. In the present study, we successfully obtained transgenic *M. sinensis* plants with a vector harboring *PHYB* as well as the herbicide resistance gene (*BAR*) as a selection marker and found that the overexpression of *PHYB* increased chlorophyll content, reduced plant height, and delayed flowering in *M. sinensis* plants. Therefore, the present work provides a method for the development of genetically engineered *Miscanthus* varieties with enhanced agricultural performance using phytochromes.

## 2. Materials and Methods

*2.1. Preparation of Recombinant Arabidopsis Phytochrome B Proteins.* Chromophore-assembled holoproteins of *Arabidopsis* phytochrome B (phyB) were expressed, reconstituted, and purified as previously described [24, 25]. Full-length *Arabidopsis PHYB* gene was subcloned into a *Pichia* expression vector pPIC3.5K (Invitrogen). Ten amino acid streptavidin affinity tag from pASK75 vector (Biometra) was attached to the 3′ end of the *PHYB* gene. The primers 5′-CTCCCCGGGTACGTAACCATGGTTTCCGGAGTC-GGGG-3′ (forward, *Sma*I/*Sna*BI) and 5′-TCGCAGCGC-TATATGGCATCATCAGCATCATG-3′ (reverse, *Eco*47III) were used for the subcloning. The pPIC3.5K construct bearing *PHYB* was then transformed into *Pichia* cells by means of a Micropulser Electroporation apparatus (Bio-Rad). Recombinant phytochrome proteins were expressed in the *Pichia* expression system (Invitrogen) and purified by streptavidin affinity chromatography (IBA), according to the procedure described by the manufacturer. Phycocyanobilin (PCB) was prepared from *Spirulina platensis* extracts by methanolysis as described [25] and used as chromophores for the holo-phytochrome assembly. Crude extracts were prepared by breaking *Pichia* cells in liquid nitrogen using a homogenizer (Nihonseiki Kaisha). The phytochrome samples were then precipitated by adding 0.23 g/L ammonium sulfate and resuspended in a buffer (100 mM Tris, pH 7.8, 1 mM EDTA), the PCB chromophores in DMSO were added to the samples at a final concentration of 10 $\mu$M, and the mixture was incubated for *in vitro* reconstitution on ice for 1 h. After dialysis to remove free chromophores, the samples were loaded onto affinity chromatography columns and holo-phyB proteins were purified.

*2.2. $Zn^{2+}$ Fluorescence and Spectroscopic Analysis.* For $Zn^{2+}$ fluorescence assay to assess the chromophore ligation, the protein samples were analyzed on a 10% SDS-PAGE gel and the gel was soaked in 20 mM zinc acetate/150 mM Tris-HCl (pH 7.0) for 5–30 min at room temperature with gentle shaking. Zinc fluorescence of holo-phytochromes was visualized under UV light (312 nm). For the spectroscopic analysis of recombinant phyB, absorption spectra were recorded by a diode array UV/VIS spectrophotometer (Cary) after red or far-red light irradiation. All the spectroscopic experiments were carried out under the green safety light condition, which consisted of a white fluorescent lamp equipped with a plastic filter (Rosco) with a maximal transmittance at 500 nm, and a fiber optic illuminator system (Cole-Parmer) equipped with 656 and 730 nm interference filters (Oriel) was used as a light source. The light intensity was 8 W/m$^2$ for red light and 6 W/m$^2$ for far-red light. The samples were illuminated with red for 15 min or far-red light for 10 min. A difference spectrum was obtained by subtracting the Pfr spectrum

from the Pr spectrum and used to determine the interpeak distance between Pr and Pfr absorption wavelength maxima ($\Delta$ of maxima) and the ratio of Pr and Pfr absorbance peaks ($A_{Pfr}/A_{Pr}$).

*2.3. Plasmid and Agrobacterium Strain Used for Genetic Transformation.* *Arabidopsis PHYB* gene (AT2G18790) was subcloned into the binary vector pCAMBIA3301 with *Bam*HI/*Sma*I under the control of maize ubiquitin promoter ($P_{ubi}$) and *Agrobacterium tumefaciens NOS* gene terminator. pCAMBIA3301 carries *BAR* gene for herbicide resistance as a selectable marker, and the *BAR* gene encodes a phosphinotricin acetyltransferase that confers resistance to the herbicide, phosphinotricin (PPT). The binary vector DNA was then used to introduce into *A. tumefaciens* strain EHA105 by the freeze-thaw method [26].

*2.4. Generation of Putative Transgenic M. sinensis Plants.* SNU-M-045 germplasm seeds of *M. sinensis* that have been maintained in Seoul National University were used for the transformation, and tissue culture and genetic transformation of *M. sinensis* were performed as described [23]. Mature seeds-derived embryogenic calli and *A. tumefaciens* EHA105 harboring pCAMBIA3301 with *PHYB* were used for the genetic transformation. Embryogenic calli were induced and cultured on the callus induction medium (MS salts and vitamins, 3% sucrose, 3 mg/L 2,4-D, 750 mg/L $MgCl_2 \cdot 6H_2O$, 25 mM L-proline, 0.2 g/L Gelrite, pH5.7) for 8 to 10 weeks. The embryogenic calli were then immersed in *Agrobacterium* suspensions and incubated for 15 min with gentle shaking followed by removal of excess bacteria and air-drying on filter paper. Infected calli were then transferred onto cocultivation medium (MS salts and vitamins, 2% sucrose, 1% glucose, 3 mg/L 2,4-D, 400 $\mu$M acetosyringone, 3 g/L Gelrite, pH 5.7) and incubated in the dark at 25°C for 5 days. During the transformation process, GUS staining assays were performed to check whether or not the embryogenic calli were transformed. After transformation, transgenic calli were selected on the selection medium containing 5 mg/L PPT, and transgenic shoots were induced on the regeneration medium with 3 mg/L PPT. Plantlets with well-developed roots were established in soil and grown for 2 weeks, prior to herbicide treatment. Herbicide resistance assay was then performed by spraying 0.4% (v/v) BASTA (which contains 18% glufosinate ammonium), and the herbicide resistance of putative transgenic plants was determined after 14 days.

*2.5. Molecular Analyses of Transgenic M. sinensis Plants.* Genomic PCR, Southern blot, and Northern blot analyses of transgenic plants were performed as previously described [27, 28]. For genomic PCR analysis, total genomic DNA was isolated from the leaves of greenhouse-grown plants, and the coding region for the *PHYB* or *BAR* transgene was amplified by PCR from either genomic DNA or a positive-control vector, using the following sets of oligonucleotide primers: 5′-TCGGCGTTCCGGTCCATGGTTAG-3′ (forward) and 5′-GCAGCGCTCAGTGTCTGCGTTCTCAAA-ACG-3′ (reverse) for *PHYB*, 5′-CTACCATGAGCCCAG-AACGACG-3′ (forward) and 5′-CTGCCAGAAACCCAC-GTCATGCCAGTTC-3′ (reverse) for *BAR*. The actin gene (*ACT*) of *M. sinensis* was also amplified using the same template and the primers 5′-AACTGGGATGATATGGAG-AA-3′ (forward) and 5′-CCTCCAATCCAGACACTGTA-3′ (reverse) and then run as a loading control of genomic DNA. The PCR products of *PHYB*, *BAR,* and *ACT* were expected to be 1106, 421, and 1,046 bp, respectively.

For the Southern blot analysis, the genomic DNA was digested with either *Hin*dIII or *Eco*RI, and hybridizations were carried out with the *BAR* gene probe that was labeled with [$\alpha^{32}$P] dCTP using the Radiprime II Random Prime Labeling System (Amersham Biosciences). For Northern blot analysis, total RNA was extracted from leaves using Trizol reagent (Invitrogen), and hybridizations were carried out with [$\alpha^{32}$P] dCTP-labeled *PHYB* probe.

*2.6. Measurement of Chlorophyll Content.* Chlorophyll (chl) content was measured with the third leaf of the tillers harvested on DOY (day of year) 273. The leaves were extracted with 80% acetone and the solution absorbance was measured at 645 nm ($A_{645}$) and 663 nm ($A_{663}$). Total chlorophyll content was calculated from the equation: total chlorophyll ($\mu$g/mL)= $20.2 \times A_{645} + 8.02 \times A_{663}$. The relative chlorophyll content was then calculated by setting the chlorophyll content of nontransgenic control plant to 100%.

*2.7. Phenotypic Analyses of Transgenic M. sinensis Plants.* *M. sinensis* plants in soil were grown and vegetatively propagated in a culture room (24–26°C with a 16 h photoperiod). For the investigation of phenotypes, the plants were then transferred and planted from the culture room onto an isolated LMO greenhouse of Seoul National University in Suwon, Korea (N 37° 16′ 15.08″, E 126° 59′ 22.02″). Plant heights were measured as the lengths from the ground to the top of each plant. Heading and flowering dates were defined as the emergence of the panicle tip from the flag leaf sheath and the opening of the first flower, respectively.

*2.8. Statistical Analysis.* The results of the physiological parameters were analyzed using ANOVA with IBM SPSS statistics 20 software. Significant difference from the control value(s) was determined at $P < 0.05$ level. All of the data represented the mean ± SD or SE of at least three independent experiments.

## 3. Results and Discussion

*3.1. Photochemical Properties of Arabidopsis phyB.* There are five isoforms of plant phytochromes (phyA–phyE) in the dicot model plant *Arabidopsis thaliana*, among which phyA and phyB have been known to play major roles in plants [29, 30]. For example, phyA regulates seed germination and seedling growth in response to far-red light (FR), whereas phyB regulates seed germination in response to red light (R),

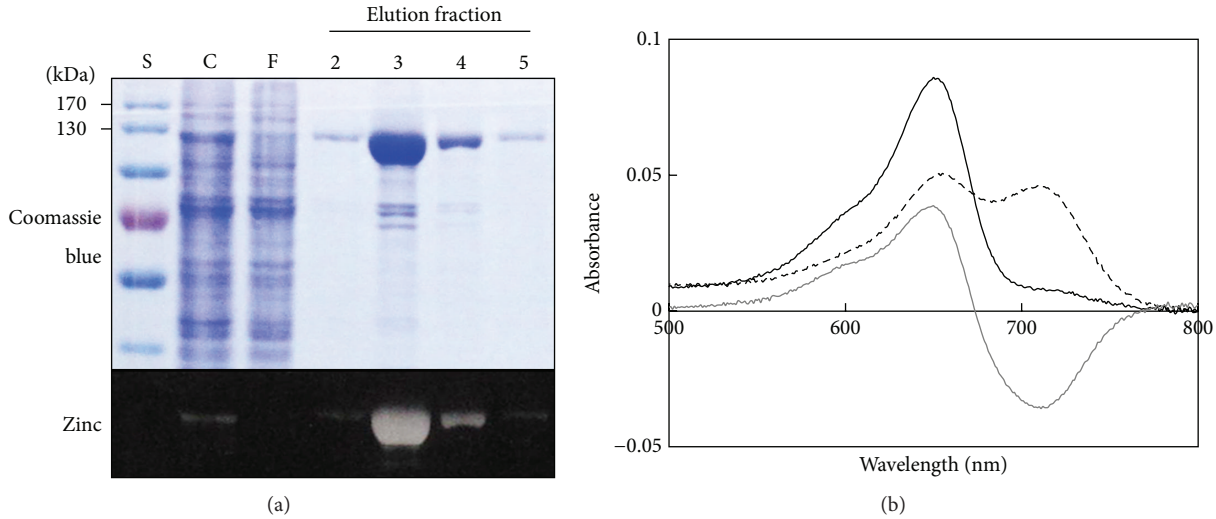


Figure 1: Photochemical analysis of purified *Arabidopsis* phyB proteins assembled with phycocyanobilin (PCB). (a) Purification of PCB-assembled phyB proteins by streptavidin affinity chromatography. SDS-PAGE gel was shown with Coomassie blue staining. Lane S: Fermentas prestained protein ladder; lane C: crude extract; lane F: flow-through from the affinity column; lanes 2–5: elution fractions of holo-phyB. Zinc-induced fluorescence assay (Zinc) was shown to confirm chromophore-assembled phyB proteins. (b) Absorption and difference spectra of the purified PCB-assembled phyB proteins. Absorption spectra of Pr (solid line) and Pfr (dotted line) were shown, and the difference spectrum (gray line) was obtained by subtracting the Pfr absorption spectrum from the Pr absorption spectrum.

plant growth and shade avoidance in response to R/FR ratio, and flowering time [4]. Especially, it has been well known that phyB-deficient mutant (*hy3*) shows phenotypes of constitutive shade avoidance and early flowering [31], suggesting that phyB is necessary to suppress the shade avoidance responses and flowering in plants, which is necessary to improve plant productivity. Therefore, we planned to introduce *Arabidopsis* phyB into *M. sinensis* and to investigate the phenotypes of the monocot crop overexpressing dicot phyB.

Before the genetic transformation, we first performed the photochemical analysis of *Arabidopsis* phyB using purified recombinant proteins, because phyB has not been characterized well due to its low abundance in native tissue [32]. In contrast, the spectral properties of phyA have been well characterized because of the relatively high abundance of this photoreceptor in dark-grown tissue (~100 times more than phyB). In the present study, we expressed and purified recombinant phyB proteins successfully using the *Pichia* protein expression system and streptavidin affinity chromatography (Figure 1(a)). Purified *Arabidopsis* phyB showed ligation with the chromophore phycocyanobilin (PCB) and normal patterns of absorbance and difference spectra (Figure 1). Spectroscopic analysis of the purified phyB proteins revealed that the absorbance wavelength maxima of Pr ($\lambda_{Pr}$) and Pfr ($\lambda_{Pfr}$) forms are 650 nm and 714 nm, respectively. Compared with the photochemical properties of oat phyA in a previous report [25], *Arabidopsis* phyB showed similar properties to phyA including $\Delta$ of maxima (64 m) and $A_{Pfr}/A_{Pr}$ (1.09) obtained from the difference spectrum. The only observed difference was that the absorbance wavelengths of phyB were slightly blue-shifted (4–6 nm) compared with the absorbance peaks of oat phyA (654 nm for Pr and 720 nm for Pfr). Collectively, the results demonstrate that PCB-assembled recombinant *Arabidopsis* phyB has a typical photochromism of plant phytochromes.

*3.2. Production of Transgenic M. sinensis Plants Overexpressing Arabidopsis PHYB.* Embryogenic calli derived from mature seeds and the binary vector pCAMBIA3301 harboring the *PHYB* gene (Figure 2(a)) was used for transformation by following *Agrobacterium*-mediated transformation method that we recently developed [23]. Since the binary vector used for the transformation contained the *BAR* herbicide resistance gene, putative transgenic creeping bentgrass plants were identified by herbicide resistance assay. The *in vitro* regenerated putative transgenic lines were grown in a greenhouse for two weeks and then sprayed with 0.4% (v/v) BASTA. Following herbicide treatment, nontransformed wild-type control plant (NT) died within 14 days, whereas all of the putative transgenic plants were resistant to the herbicide (Figure 2(b)). These results indicate that the *BAR* gene was expressed in these transgenic plants. When the *Agrobacterium*-mediated transformation efficiency was calculated as a percentage of herbicide-resistant transgenic lines obtained from all infected calli, a transformation efficiency of 0.71% (4 independent lines out of 567 infected calli) was obtained, which is similar to the reported efficiencies (0.84~0.20%). These putative transgenic lines were used further for molecular and phenotypic analyses.

*3.3. Molecular Analyses of Transgenic M. sinensis Plants with Arabidopsis PHYB.* To verify the insertion of *BAR* and *PHYB* transgenes in the putative transgenic plants, PCR assays on genomic DNA extracted from the leaves were performed.

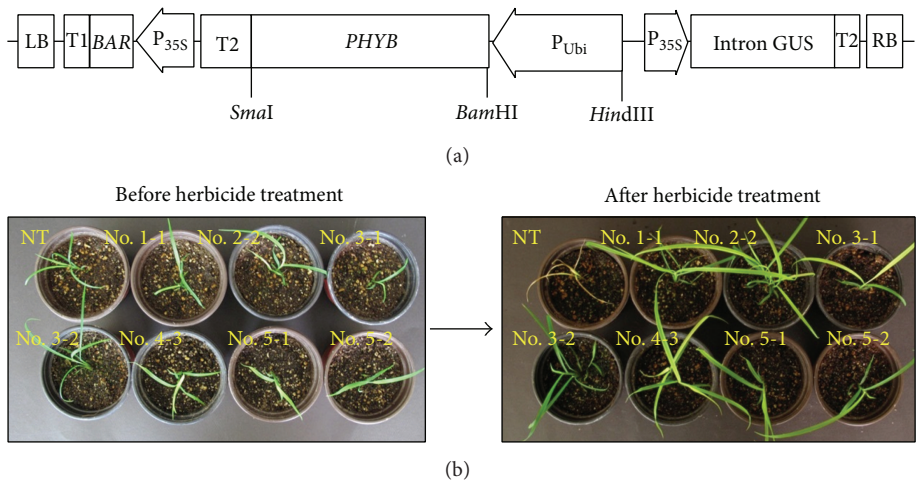


Figure 2: Construct for the transformation and herbicide resistance assay of putative transgenic plants. (a) T-DNA region of the binary vector plasmid pCAMBIA3301 harboring *Arabidopsis PHYB* gene. RB: right border; LB: left border; $P_{35S}$: CaMV 35S promoter; $P_{ubi}$: ubiquitin promoter; intron GUS: GUS coding region with catalase intron insertion; *PHYB*: full-length phyB gene of *Arabidopsis*; *BAR*: phosphinotricin acetyltransferase gene coding region; T1: CaMV 35S gene terminator; T2: *A. tumefaciens NOS* gene terminator. Arrows indicate directions of transcription. (b) Herbicide resistance assay. Numbers in lanes represent putative transgenic plants selected for this analysis. 0.4% BASTA was sprayed onto nontransformed wild-type control plant (NT) and the transgenic plants, and the herbicide resistance of the plants was determined 14 days later.

Integration of both transgenes was observed in all herbicide-resistant plants (Figure 3(a)), whereas no amplified band was observed in the PCR of the total DNA from nontransformed control plant (NT). The integration of foreign DNA into the plant genome was further confirmed by Southern blot hybridization using the *BAR*-specific probe (Figure 3(b)). The 1 and 3 transgenic lines showed the same band patterns in the blots, suggesting that they were the transgenic lines originated from the same callus (data not shown). Thus, two independent transgenic lines (1/3 and 4) contained single gene integration, whereas line 2 had a gene copy number of two and line 5 had a gene copy number of three. No hybridization signal was observed when probing DNA samples derived from nontransformed control plant (NT).

The expression of *PHYB* transgene was analyzed by Northern blot analysis (Figure 3(c)). While all of the transgenic plants expressed the integrated transgenes, no hybridization was detected in the nontransformed control plant (NT). However, the levels of transgene expression differed among transgenic lines. 1-1, 3-1, 3-2, and 4-3 lines with single gene integration expressed similar levels of *PHYB* transcripts, while the 2-2 and 5-1 lines with two and three gene copy number showed reduced *PHYB* transcript levels. Since it has been known that transgenic plants with multiple integrated genes expressed lower transcript levels of the transgenes than those with single gene integration, the reduced expression of *PHYB* transgene in 2-2 and 5-1 lines was consistent with the previous findings that multiple gene copies frequently lead to cosuppression and gene silencing [33, 34]. Collectively, we obtained several independent transgenic lines with single transgene integration, which were used for further phenotypic analyses.

*3.4. Phenotypic Analysis of Transgenic M. sinensis Plants Overexpressing Arabidopsis PHYB.* Phytochromes regulate photomorphogenic responses in plants, which include stem and leaf growth, biosynthesis of chlorophylls, and flowering. Especially, loss-of-function mutant of phyB shows dramatically enhanced elongation growth and induces flowering early [31]. In contrast, overexpression of phyB has been shown to induce a short hypocotyl phenotype in *Arabidopsis* and tobacco plants [35, 36], and to influence photosynthetic performance with increased pigmentation in potato [37]. More recently, overexpression of *Arabidopsis* phyB in cotton has been reported to cause pleiotropic effects such as semi-dwarfism, decreased apical dominance, and increased yield [38]. Based on these previous reports, we investigated the chlorophyll content, plant height, and flowering time of the transgenic *M. sinensis* plants compared with nontransformed control plant.

The results of chlorophyll content measurements showed that all the transgenic plants had increases in chlorophyll content relative to control plant, but increased levels were somewhat varied among transgenic lines (Figure 4). The 1-1 and 4-3 lines with single gene integration showed approximately 28 and 26% increases in chlorophyll content, respectively, while the 2-2 and 5-1 lines with two and three gene copy number showed about 18 and 6% increases. These results were well corelated with the transcript levels of *PHYB* expression (Figure 3(c)), suggesting that chlorophyll biosynthesis is increased in the transgenic lines with higher expression of functional phyB. The results are also consistent with the previous reports of strong dependency of phytochrome function on the photoreceptor amounts [39, 40]. Transgenic *M. sinensis* plants overexpressing *PHYB* also showed shorter plant height than the control plant

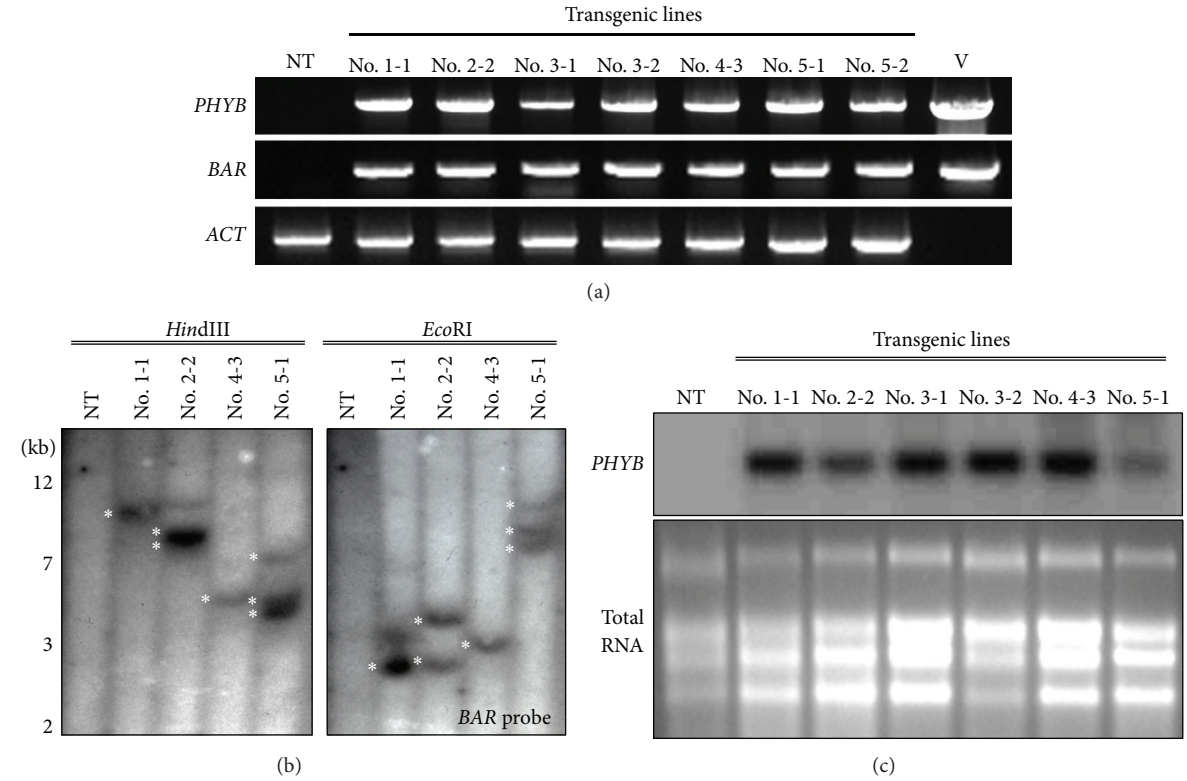


Figure 3: Molecular analyses of representative transgenic *Miscanthus* plants with *PHYB*. (a) Genomic PCR analysis of transgenic lines. The coding regions of the *PHYB* and *BAR* transgenes were amplified by PCR from genomic DNA. The actin gene (*ACT*) of *M. sinensis* plants was shown as a loading control of the genomic DNA. NT: nontransformed control plant; V: pCAMBIA3301 harboring *PHYB* gene that was used for transformation. (b) Southern blot analysis of transgenic lines. Genomic DNA (15 $\mu$g) from each transgenic plant was digested with either *Hin*dIII or *Eco*RI and then probed with the *BAR* gene. Numbers in lanes represent independent transgenic lines selected for analysis, and asterisks indicate the *BAR* bands. (c) Northern blot analysis of transgenic lines. Total RNA was isolated from the leaves of transgenic plants and the *PHYB* gene was used as a probe. Total RNA was shown as a loading control.

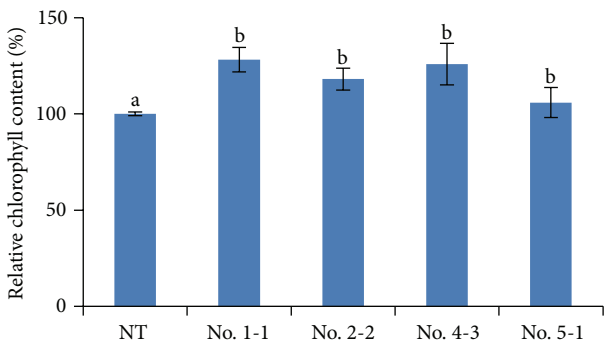


Figure 4: Relative chlorophyll content of transgenic *M. sinensis* plants with *PHYB*. The third leaf of the tillers was used for the extraction of chlorophyll (chl). Relative chlorophyll content was calculated by setting the chlorophyll content of nontransformed control plant (NT) to 100%. Error bars indicate standard errors ($n$ = 5). Means with different letters are significantly different at $P < 0.05$, using Duncan.

(Figures 5(a) and 5(b)). These results are consistent with the previous reports that overexpression of *Arabidopsis* phyB shows phenotypes of semidwarfism with decreased apical dominance in potato and cotton [37, 38]. In addition, the transgenic plants displayed significant delays in both heading and flowering (Figure 5(c)). On average, approximately 2 week delay in flowering was observed in the transgenic plants compared with the control plant. Again, transgenic lines with higher *PHYB* expression showed more delay in heading and flowering. In rice, phyB has been reported to suppress the expression of *Hd3a*, an ortholog of Arabidopsis *FT* (*FLOWERING LOCUS T*) that induces flowering in plants [41]. This result indicates that overexpression of phyB might suppress flowering in the monocot. Thus, delayed flowering in the transgenic *M. sinensis* plants overexpressing *PHYB* is consistent with this previous result. Collectively, the results suggest that overexpression of *Arabidopsis* phyB increases chlorophyll biosynthesis, delays flowering with reduced plant height in *M. sinensis*.

## 4. Conclusions

In this work, we have successfully produced transgenic *M. sinensis* plants overexpressing *Arabidopsis PHYB* gene. To our knowledge, this might be the first report to obtain genetically engineered *M. sinensis* plants with a useful gene other than

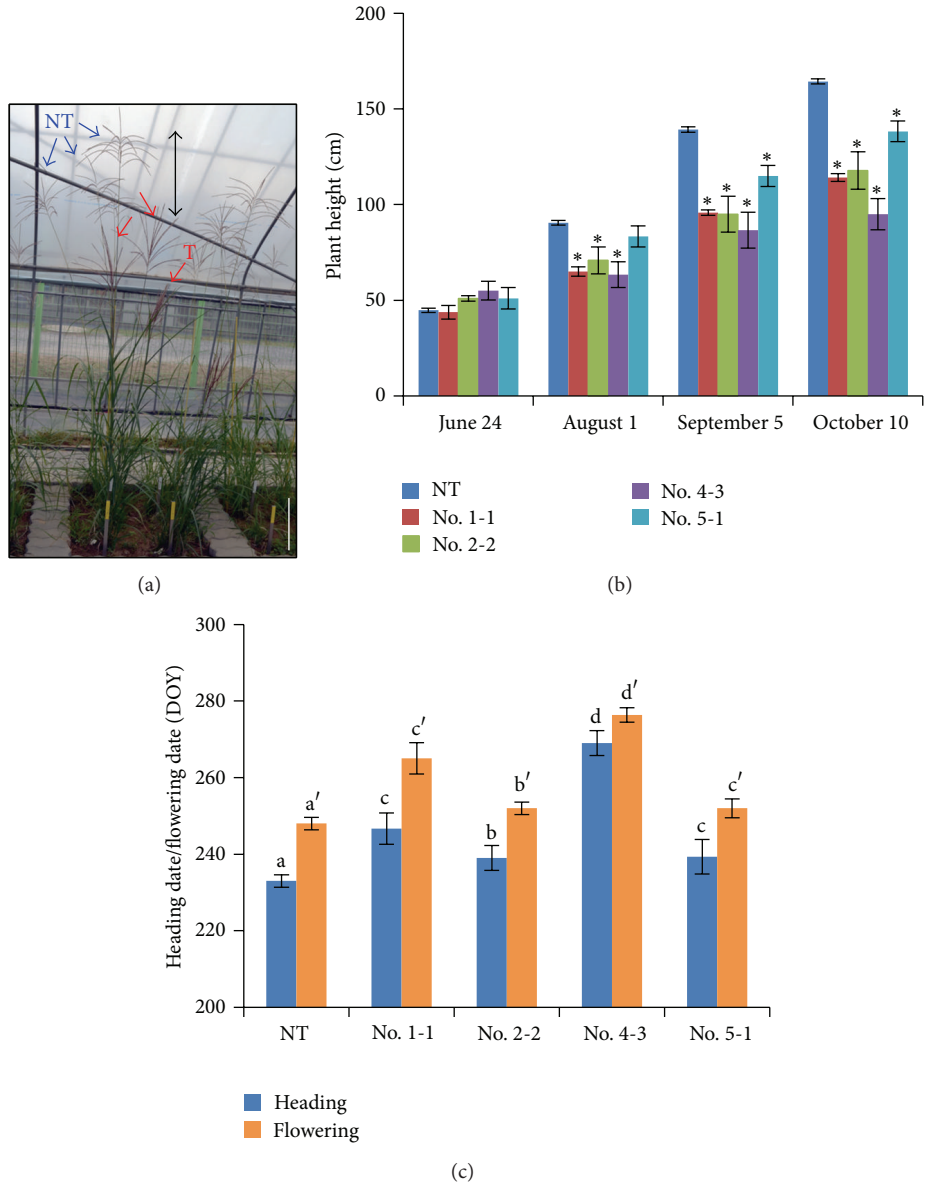


FIGURE 5: Phenotypic analyses of transgenic *M. sinensis* plants overexpressing *PHYB*. (a) Representative picture of nontransformed control plant (NT) and transgenic *M. sinensis* plants (T). Blue and red arrows represent the control and transgenic plants, respectively. The pictures were taken on DOY (day of year) 273, and the black arrow indicates the difference of plant heights between the control and transgenic plants. Bar: 20 cm. (b) Measurements of plant heights. Error bars indicate standard deviations ($n = 3$). Statistically significant changes compared with NT are indicated by * when $P < 0.05$, using LSD. (c) Measurements of heading and flowering time. Heading and flowering were observed and measured on date (DOY). Error bars indicate standard deviations ($n = 3$). Means with different letters are significantly different at $P < 0.05$, using Duncan.

selectable marker genes using an *Agrobacterium*-mediated transformation method. Furthermore, phenotypic analysis of the transgenic plants demonstrated that overexpression of dicot phyB from *Arabidopsis thaliana* shows pleiotropic effects in the monocot crop *M. sinensis*, which includes increased chlorophyll content, reduced plant height, and delayed flowering. The observed phenotypes are consistent with previous results with the overexpression of phyB in other plants such as potato and cotton. Assuming that increased chlorophyll content induces efficient photosynthetic performance and delayed flowering increases biomass productivity, the transgenic *M. sinensis* plants developed in this work might be considered as a high-value added cultivar of energy crops. In addition, the genetically engineered *M. sinensis* plants obtained in this work contained the *BAR* gene that confers herbicide-resistance to the plants. Therefore, the *M. sinensis* plants with increased phyB function and herbicide resistance reported herein offer economic

and environmental advantages over wild-type *Miscanthus* plants.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] M. M. Neff, C. Fankhauser, and J. Chory, "Light: an indicator of time and place," *Genes & Development*, vol. 14, no. 3, pp. 257–271, 2000.

[2] J. Chory, "Light signal transduction: an infinite spectrum of possibilities," *Plant Journal*, vol. 61, no. 6, pp. 982–991, 2010.

[3] N. C. Rockwell, Y. S. Su, and J. C. Lagarias, "Phytochrome structure and signaling mechanisms," *Annual Review of Plant Biology*, vol. 57, pp. 837–858, 2006.

[4] P. H. Quail, M. T. Boylan, B. M. Parks, T. W. Short, Y. Xu, and D. Wagner, "Phytochromes: photosensory perception and signal transduction," *Science*, vol. 268, no. 5211, pp. 675–680, 1995.

[5] G. Bae and G. Choi, "Decoding of light signals by plant phytochromes and their interacting proteins," *Annual Review of Plant Biology*, vol. 59, pp. 281–311, 2008.

[6] P. H. Quail, "Phytochrome photosensory signalling networks," *Nature Reviews Molecular Cell Biology*, vol. 3, no. 2, pp. 85–93, 2002.

[7] M. Chen, J. Chory, and C. Fankhauser, "Light signal transduction in higher plants," *Annual Review of Genetics*, vol. 38, pp. 87–117, 2004.

[8] Y.-J. Han, P.-S. Song, and J.-I. Kim, "Phytochrome-mediated photomorphogenesis in plants," *Journal of Plant Biology*, vol. 50, no. 3, pp. 230–240, 2007.

[9] J. A. Sullivan and X. W. Deng, "From seed to seed: the role of photoreceptors in *Arabidopsis* development," *Developmental Biology*, vol. 260, no. 2, pp. 289–297, 2003.

[10] P. R. H. Robson and H. Smith, "Fundamental and biotechnological applications of phytochrome transgenes," *Plant Cell and Environment*, vol. 20, no. 6, pp. 831–839, 1997.

[11] P. R. H. Robson, A. C. McCormac, A. S. Irvine, and H. Smith, "Genetic engineering of harvest index in tobacco through overexpression of a phytochrome gene," *Nature Biotechnology*, vol. 14, no. 8, pp. 995–998, 1996.

[12] K. A. Franklin, "Shade avoidance," *New Phytologist*, vol. 179, no. 4, pp. 930–944, 2008.

[13] T. H. Kebrom and T. P. Brutnell, "The molecular analysis of the shade avoidance syndrome in the grasses has begun," *Journal of Experimental Botany*, vol. 58, no. 12, pp. 3079–3089, 2007.

[14] A. J. Ragauskas, C. K. Williams, B. H. Davison et al., "The path forward for biofuels and biomaterials," *Science*, vol. 311, no. 5760, pp. 484–489, 2006.

[15] E. A. Heaton, F. G. Dohleman, and S. P. Long, "Meeting US biofuel goals with less land: the potential of Miscanthus," *Global Change Biology*, vol. 14, no. 9, pp. 2000–2014, 2008.

[16] E. A. Heaton, F. G. Dohleman, A. F. Miguez et al., "Miscanthus: a promising biomass crop," *Advances in Botanical Research*, vol. 56, pp. 76–137, 2010.

[17] T. van der Weijde, C. L. Alvim Kamei, A. F. Torres et al., "The potential of C4 grasses for cellulosic biofuel production," *Frontiers in Plant Science*, vol. 4, article 107, 2013.

[18] I. Lewandowski, J. M. O. Scurlock, E. I. Lindvall, and M. Christou, "The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe," *Biomass & Bioenergy*, vol. 25, no. 4, pp. 335–361, 2003.

[19] H. W. Zub and M. Brancourt-Hulmel, "Agronomic and physiological performances of different species of *Miscanthus*, a major energy crop. A review," *Agronomy for Sustainable Development*, vol. 30, no. 2, pp. 201–214, 2010.

[20] D. G. Christian, N. E. Yates, and A. B. Riche, "Establishing *Miscanthus sinensis* from seed using conventional sowing methods," *Industrial Crops and Products*, vol. 21, no. 1, pp. 109–111, 2005.

[21] K. Jakob, F. Zhou, and A. H. Paterson, "Genetic improvement of C4 grasses as cellulosic biofuel feedstocks," *In Vitro Cellular & Developmental Biology-Plant*, vol. 45, no. 3, pp. 291–305, 2009.

[22] X. Wang, T. Yamada, F.-J. Kong et al., "Establishment of an efficient in vitro culture and particle bombardment-mediated transformation systems in *Miscanthus sinensis* Anderss, a potential bioenergy crop," *Global Change Biology Bioenergy*, vol. 3, no. 4, pp. 322–332, 2011.

[23] O.-J. Hwang, M.-A. Cho, Y.-J. Han et al., "*Agrobacterium*-mediated genetic transformation of *Miscanthus sinensis*," *Plant Cell Tissue and Organ Culture*, vol. 117, pp. 51–63, 2014.

[24] Y.-J. Han, H.-S. Kim, Y.-M. Kim et al., "Functional characterization of phytochrome autophosphorylation in plant light signaling," *Plant and Cell Physiology*, vol. 51, no. 4, pp. 596–609, 2010.

[25] J.-I. Kim, Y. Shen, Y.-J. Han et al., "Phytochrome phosphorylation modulates light signaling by influencing the protein-protein interaction," *Plant Cell*, vol. 16, no. 10, pp. 2629–2640, 2004.

[26] H. Chen, R. S. Nelson, and J. L. Sherwood, "Enhanced recovery of transformants of *Agrobacterium* tumefaciens after freeze-thaw transformation and drug selection," *Biotechniques*, vol. 16, no. 4, pp. 664–670, 1994.

[27] Y.-J. Han, K.-C. Cho, O.-J. Hwang et al., "Overexpression of an *Arabidopsis* $\beta$-glucosidase gene enhances drought resistance with dwarf phenotype in creeping bentgrass," *Plant Cell Reports*, vol. 31, no. 9, pp. 1677–1686, 2012.

[28] Y.-J. Han, Y.-M. Kim, J.-Y. Lee et al., "Production of purple-colored creeping bentgrass using maize transcription factor genes *Pl* and *Lc* through *Agrobacterium*-mediated transformation," *Plant Cell Reports*, vol. 28, no. 3, pp. 397–406, 2009.

[29] S. Mathews, "Evolutionary studies illuminate the structural-functional model of plant phytochromes," *Plant Cell*, vol. 22, no. 1, pp. 4–16, 2010.

[30] J. Li, G. Li, H. Wang, and X. W. Deng, "Phytochrome signaling mechanisms," *The Arabidopsis Book/American Society of Plant Biologists*, vol. 9, Article ID e0148, 2011.

[31] J. W. Reed, P. Nagpal, D. S. Poole, M. Furuya, and J. Chory, "Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development," *Plant Cell*, vol. 5, no. 2, pp. 147–157, 1993.

[32] T. D. Elich and J. Chory, "Biochemical characterization of *Arabidopsis* wild-type and mutant phytochrome B holoproteins," *Plant Cell*, vol. 9, no. 12, pp. 2271–2180, 1997.

[33] C. de Wilde, H. van Houdt, S. de Buck, G. Angenon, G. de Jaeger, and A. Depicker, "Plants as bioreactors for protein production: avoiding the problem of transgene silencing," *Plant Molecular Biology*, vol. 43, no. 2-3, pp. 347–359, 2000.

[34] H. Vaucheret, C. Beclin, T. Elmayan et al., "Transgene-induced gene silencing in plants," *Plant Journal*, vol. 16, no. 6, pp. 651–659, 1998.

[35] D. Wagner, J. M. Tepperman, and P. H. Quail, "Overexpression of phytochrome B induces a short hypocotyl phenotype in transgenic *Arabidopsis*," *Plant Cell*, vol. 3, no. 12, pp. 1275–1288, 1991.

[36] K. J. Halliday, B. Thomas, and G. C. Whitelam, "Expression of heterologous phytochromes A, B or C in transgenic tobacco plants alters vegetative development and flowering time," *Plant Journal*, vol. 12, no. 5, pp. 1079–1090, 1997.

[37] A. Thiele, M. Herold, I. Lenk, P. H. Quail, and C. Gatz, "Heterologous expression of *Arabidopsis* phytochrome B in transgenic potato influences photosynthetic performance and tuber development," *Plant Physiology*, vol. 120, no. 1, pp. 73–82, 1999.

[38] A. Q. Rao, M. Irfan, Z. Saleem, I. A. Nasir, S. Riazuddin, and T. Husnain, "Overexpression of the phytochrome B gene from *Arabidopsis thaliana* increases plant growth and yield of cotton *(Gossypium hirsutum)*," *Journal of Zhejiang University: Science B*, vol. 12, no. 4, pp. 326–334, 2011.

[39] M. T. Boylan and P. H. Quail, "Phytochrome a overexpression inhibits hypocotyl elongation in transgenic *Arabidopsis*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 23, pp. 10806–10810, 1991.

[40] D. Wagner and P. H. Quail, "Mutational analysis of phytochrome B identifies a small COOH-terminal-domain region critical for regulatory activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 19, pp. 8596–8600, 1995.

[41] R. Ishikawa, M. Aoki, K. Kurotani et al., "Phytochrome B regulates *Heading date* 1 *(Hd1)*-mediated expression of rice florigen *Hd3a* and critical day length in rice," *Molecular Genetics and Genomics*, vol. 285, no. 6, pp. 461–470, 2011.