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Research Article

THE IMPACT OF PHYTOCHROME B SILENCING USING RNAI ON SOME AGRONOMIC CHARACTERS IN *ARABIDOPSIS THALIANA*

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RNA interference (RNAi) is a powerful reverse genetic tool to study gene function by the interference with the gene activity. *PHYB* is the dominant phytochrome in light-grown plants, which plays an important role in plant development by the absorption of red (R) and far-red (FR) light and the transduction of intracellular signals during plant development. To construct transformation vectors that produce RNAs capable of duplex formation, gene-specific sequences (*PHYB* gene) in the sense and antisense orientations were linked and placed under the control of a strong viral promoter (35S). When introduced into the genome of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*-mediated floral dipping transformation, double-stranded RNA expressing constructs corresponding to *PHYB* gene, caused specific and heritable genetic interference (efficiently knocked-down). It is shown that *PHYB*-RNAi transformed lines have significantly taller hypocotyls and larger leaf area compared with the NO.O wild plants. Reverse transcriptase-PCR (RT-PCR) revealed a correlation between the new *PHYB*-RNAi phenotypes and declining of normal *PHYB*-mRNA accumulation, suggesting that endogenous mRNA is the target of double-stranded RNA-mediated genetic interference. The ability to generate stably heritable RNAi and the resultant specific phenotypes allows to selectively reducing *PHYB* gene function in *A. thaliana.*

INTRODUCTION

Environment has a great effect on plant development. Environmental factors such as light, perhaps has the most determinative influence on the development of a plant. Signals from the light circumstances are involved in the regulation of seed germination, formation of seedlings, determination of growth pattern, and the transition to flowering. Plants have developed wide collection of photoreceptors to recognize information about their light circumstances. Red (R) and far-red (FR) lights are essential environmental signals for the regulation of plant development, with major roles in seedling deetiolation, neighbor detection and photoperiodism (Smith, 1994; Franklin and Whitelam, 2005).

Phytochromes are the plant R/FR primary photoreceptors, they are soluble chromoproteins with a 120-130kD apoprotein and a linear tetrapyrrole chromophore. Phytochrome apoproteins are encoded by a smallgene family (*PHY*), that absorb R and FR lights and transducer the intracellular signals in the course of light-regulated plant development (Sharrock and Clack, 2002).

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Early physiological and biochemical studies showed that higher plants have at least two distinct forms of phytochrome (Hillman, 1967; Abe *et al.*, 1985; Shimazaki and Pratt, 1985; Tokuhisa *et al.*, 1985). These forms aretype I or "light-labile" phytochrome, which dominate in etiolated tissue, type II or "light-stable" phytochrome, which dominate in light-grown tissue (Furuya, 1993; Pratt *et al.*, 1995). *PHY* gene families were first identified in *Arabidopsis* and subsequently in many other plant species (Mathews *et al.*, 1995; Mathews and Sharrock, 1997; Alba *et al.*, 2000).

All examined higher plants contain multiple and distinct phytochrome forms that are the products of a divergent gene family (Mathews and Sharrock, 1997; Franklin and Whitelam, 2005). In *Arabidopsis*, which is the subject for the most extensive studies, there are five types of phytochromes; they are PhyA through PhyE (Sharrock and Ted, 2002). PhyA, the product of the *PHYA* gene, is light labile, which accumulates to comparatively high levelsin etiolated seedlings. PhyB and PhyC are light stable forms.PhyB predominates in light-grown tissues (Somers *et al.*, 1991). However, a previous studyrevealed that the *Arabidopsis* model may not entirely represent some flowering plant groups, sinceother*PHY* loci linked to *PHYA* and *PHYB* of *Arabidopsis*seemingly have evolved independently some times in dicots and monocot

flowering plants may lack orthologs of *PHYD* and *PHYE* of *Arabidopsis* (Mathews and Sharrock, 1996).

PhyB can be photoconverted between two relatively stable forms: the R light-absorbing inactive Pr form and the FR light-absorbing active Pfr form (BurgieandVierstra, 2014).

Etiolated seedlings of the *phyB* mutant revealed deficient responses to red light (Koomneef *et al.*, 1980; Reed *et al.*, 1993). Light-grown *phyB*mutant seedlings have a lengthened, early flowering characteristic just like the shade-avoidance syndrome of wild-type seedlings grown under a low R/FR or at end of day (EOD) far-red-lighttreatments. Seedlings of*phyB*mutant show attenuated responses to a low R/FR or to EOD far-red light, leading to the suggestion that *PHYB*plays a vital role in the shade-avoidance response (Nagatani *et al.*, 1991; Whitelam and Smith, 1991). Moreover, Hirschfeld *et al.* (1998) showed that the PhyCapoprotein level is significantly reduced in *phyB*mutants of *Arabidopsis*, proposing that the phenotypes associated with *phyB*mutant genes may lead to the attenuation of PhyC signaling. Although some shade-avoidance responses of the *phyB*mutant to a low R/FR or to EOD far-red light such as petiole elongation, are severely attenuated (Nagatani *et al.*, 1991), others, such as reduction in leaf area and acceleration of flowering, are clearly retained (Robson *et al.*, 1993; Halliday *et al.*, 1994). This implicates theaction of phytochromes rather than PhyBin the control of shade avoidance. Devlin *et al.* (1996) confirmed that *phyA-phyB* double mutants respond to EOD far-red light by the quickening of flowering and by theelevationof elongated internodes between rosette leaves (Devlin *et al.*, 1996). ,By subsequent treatment with red light, these responsesare reversible, representing that one or more of *PHYC*, *PHYD*, or *PHYE* controls flowering time and internode elongation (Devlin *et al.*, 1996).

To study the function of certain genesin plants, several reverse genetic techniques are being used, such as targeted induced local lesions in genomes (WLLING), tagged mutants, co-suppression andantisense suppression. A previous study have reported that the formation of double stranded RNA(dsRNA) can lead to effective and sequence-specific degradation of homologous mRNA in a post-transcriptional manner (Tavernarakis *et al.*, 2000). It has become clear that this manner is ancient. RNA interference (RNAi), the oldest and most ubiquitous antiviral system, which appeared before the divergence of plants and animals. Evidence for the participation of dsRNA in facilitating gene silencing was first discovered in *Caenorhabditiselegans*, and was termed RNA interference (Fire *et al.*, 1998). Since then, it has become clear that dsRNA can efficiently defeat gene expression in a wide range of organisms including plants, nematodes, insects and mammals (Elbashir *et al.*, 2001). RNAiis a biological mechanism by which double-stranded RNA (dsRNA) induces gene silencing by degrading the complementary mRNA, developing a way to study gene functions. Scientists can quickly and easily reduce the expression of a particular gene, for the first time, often by 90% or greater, to analyze the effect that gene might has in cellular function without affecting the expression of other genes. The ease of the method, for conducting RNAi experiments, has driven its amazingly rapid adoption by the research community (Chuang and Meyerowitz, 2000; Younis*et al.,* 2014; Heigwer *et al.,* 2018).

Agrobacterium-mediated transformation ofgene constructs delivered into plants are stably integrated into the genome of the host cells. Therefore, RNA expression from these constructs in transgenic plants can be persistent and heritable. In this study, we introduced dsRNA-expressing constructs of *PHYB* gene (*PHYB* RNAi constracts) into *Arabidopsis* plants using *Agrobacterium transformation* system to investigate the *PHYB* gene role on some yield components such as, leave area and plant length.

MATERIALS AND METHODS

Plant materials

All studies were carried out on *Arabidopsis thaliana*, ecotype NO.O previously described source (Aukerman *et al.*, 1997).

Bacterial strains

Agrobacterium tumefaciens strain GV3101 containing the pGRNAi-*PHYB*plasmid was used to transform Arabidopsis. The binary vectors were introduced intoGV3101 strain by electroporation (Shaw, 1995) and grown for 2 days at 28°C underkanamycin selection (50 µg/ml±1). Integrity of inverted repeat constructs was checkedby restriction digestion with *EcoRV*and then the positive clones were sequenced.

Cloning of PHYB-RNAi

According to Sharrock *et al.*, (2003) the pGENT123was constructed using pBI123 by replacing the kanamycin-resistance gene with a gentamycin-resistance gene. The pGRNAi was constructed by ligating a 326bp *BamHI/Smal* fragment to form the loop, then to create the pGRNAi-*PHYB* a 183bp *BamHI/Xbal* in the antisense direction fragment from the 3' end of the ecotype NO.O *PHYB* gene into the *BamHI/Xbal* site of pGRNAi the same fragment with *Kpnl/Sacl* sites was inserted in the sense direction. This resulted in *PHYB* antisense and sense orientations, which linked with a 326bp fragment and controlled by the 35S promoter as shown in Fig. (1).

The targeted regions were first amplified using PCR and subsequently cloned as BamHI/Xbal for the antisense using 5’-acaggatcc-ggcgtgtccaggtgaagg-3’ and 5’-acatctaga-gggagttccagaatgatgagg-3’ primers. The sense fragment was amplified as *Kpnl/Sacl* using 5’-acaggtacc-ggcgtgtccaggtgaagg-3’ and 5’-acagagctc-gggagttccagaatgatgagg-3’ primers.

Agrobacterium tumefaciens-mediated transformation

Floral dipping technique was used to transfect *Arabidopsis* plant with the desired construct according to Clough and Bent (1998).

The transgenic seeds were screened using MS-kanamycin plates (1x Murashige & Skoogsalts, pH was adjusted to 5.7 with 1M KOH, 0.7% Difco agar, autoclaved, cooled, and antibiotic; kanamycin of 50 µg/ml.

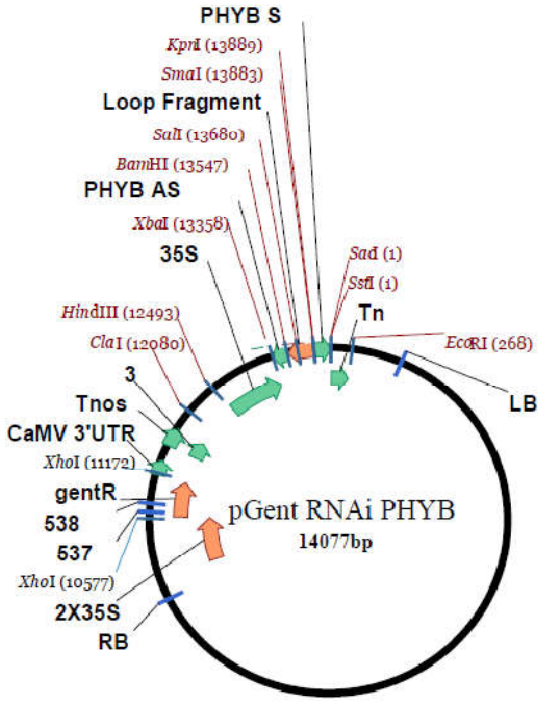

Fig 1 A map of the pGent-PHYB-RNAi construct.

Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from 4-week-old *Arabidopsis* shoot tissue using RN easy kit (Promega) followed by DNAsel (Promega) treatment. cDNA was made from1 mg total RNA using the Taqman Gold RT-PCR kit (Perkin-Elmer Applied Biosystems) in a total volume of 50 µl using the supplied hexamer primers. PCR reactions were performed using 1 µl cDNA as a template. The following PCR-program was used (40cycles of 94°C for 2min, 94°C tor 20 sec, 56°C for 20 sec, 72°C for 30 sec), then 4°C(infinite).

RESULTS AND DISCUSSION

Cloning of PHYB-RNAi

Arabidopsis PHYB genomic and cDNA sequences from GenBank database, which have L.09262 and NM_127435 numbers, respectively were used to design primers to amplify a 184bp fragment from exon number 4 of *PHYB*gene. The 184bp fragment was cloned in the sense and antiesense direction to the pGRNAi vector to construct the pGRNAi-*PHYB*. The pGRNAi-*PHYB* construction was proved using *EcoRV*restriction enzyme (Fig. 2), then one clone was sequenced (Fig. 3) and that clone was used for transforming *Agrobacterium* strain GV3101.

Agrobacterium tumefaciens-mediated transformation

Agrobacterium tumefaciens-mediated transformation technique is a fast and effective way to generate adventitious, genetically transformed seeds. In order to select transformed seeds; a binary vector was developed that enables a kanamycin resistance selection. Transformed seeds can be discriminated by growing them on MS-kanamycinplates (Zhou *et al.*, 2005). The identification of transformed seeds provided the opportunity to examine the extent of systemic spread of the silencing signal in the composite of *Arabidopsis*. Floral dipping technique was used to transform *Arabidopsis* plants with the *PHYB*-RNAi fragment.

Twelve *PHYB*-RNAi transformed T_1 seedlings were obtained, they were selected using MS kanamycin (50 µg/ml) plates.

Six outof the Twelve *PHYB*-RNAi transgenic seedlings and collected T_1 seeds were randomly chosen. By using genomic DNA from T_1 plants as templates, we were able to check for the presence of 220bp PCR product from the *PHYB*-RNAi fragment. The results revealed that all the transgenic plants have that product, so that all of them have the *PHYB*-RNAi fragment integrated in their genomes (Fig. 4).

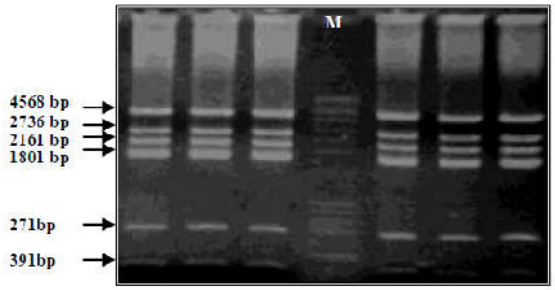

Fig 2 Restrection analysis for six colonies to check for the right pattern for the pGENT-RNAi-PHYB cut with *EcoRV*.

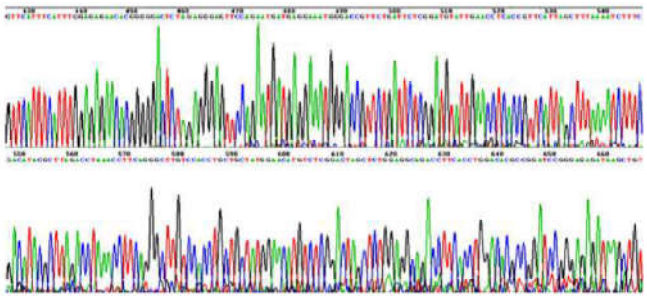

Fig 3 A Chromatogram of the sequencing results for the pGENT-PHYB-RNAi construct

Agronomic performance of PHYB-RNAi lines

Hypocotyls length and leaf area for the T_1 and T_2*PHYB*-RNAi plants compared to the NO.O wild type plants were determined using Image J software. The statistics have been made using SAS statistics package.

The same six T_1 plants were used for further studies. After seven days of germination, we found that all of the six T_1 seedlings have significantly taller hypocotyls than the NO.O wild-type seedlings. For the T_2*PHYB*-RNAi plants, three lines of *PHYB*-RNAi were used to measure the length of the hypocotyls, all of them showed significant differences between their hypocotyls length and the NO.O hypocotyls length (Fig. 5). The severityof the new phenotypes varied between transgenic lines. The mean length of hypocotyls for the NO.O plants was 0.192 ± 0.01, while the mean length of the hypocotyls in the three *PHYB*-RNAi lines was0.862±0.02,0.863±0.02 and 0.863±0.02. Analysis of variance showed ahighly significant difference between their means, while Duncan test showed asignificant difference between the NO.O plants and all *PHYB*-RNAi lines, while there was no difference between the first two lines of the *PHYB*-RNAi. The same observation was described in the null *ArabidopsisphyB* mutant by Somers *et al.* (1991).

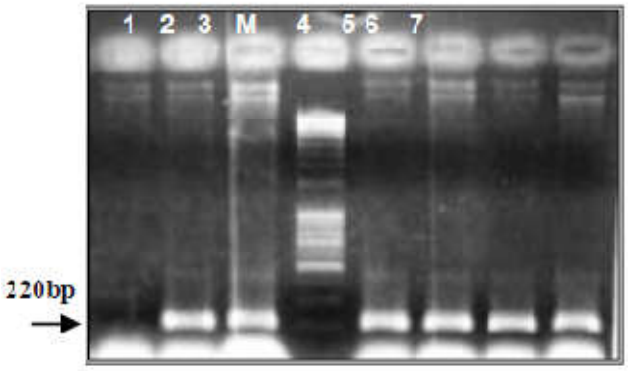

Fig 4 PCR screening results to check for the present of the PHYB-RNAi in six transgenic plants lane 1 = Negative control, lanes 2, 3, 5, 6 and 7 = six transformed plants with PHYB-RNAi and lane M = exACTGene DNA Ladders 1kb Plus.

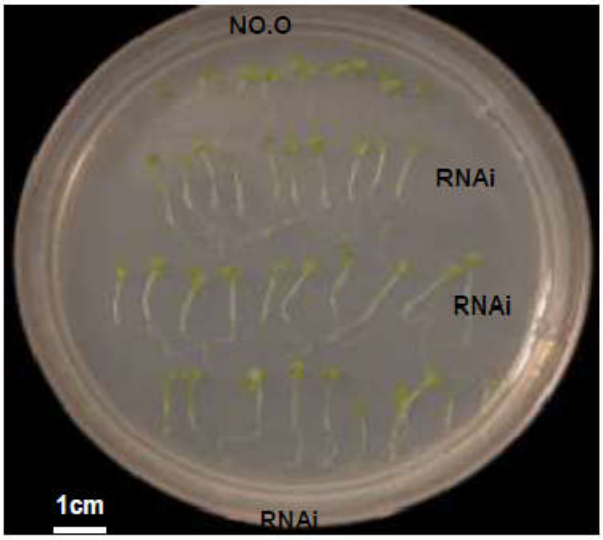

Fig 5 The differences in hypocotyl length between T_2*PHYB*-RNAi transgenic seedlings and the NO.O wild type plants after 7 days of germination.

The three-week old T_1*PHYB*-RNAi transformed plants showed taller leaves with smaller leaf area than the NO.O wild plants (Fig. 6). The mean for ten leaves obtained from the six T_1*PHYB*-RNAi transgenic plants was 7.543±0.1953, while the mean for the leaf area for the ten NO.O wild type leaves was 5.732±0.1754. T-test showed a highly significant difference in the leaf area between the two means, ($P<0.01$). Null *ArabidopsisphyB* mutant showed the same observation (Robson *et al.*, 1993; Halliday *et al.*, 1994). On the other hand, instead of the smaller area that was observed in the three-weekold plants, the mature plants (six-week-old) showed larger leaf area for the *PHYB*-RNAi transformed plants than the NO.O wild plants (Fig. 7). T_2*PHYB*-RNAi transformed lines showed the same observation for the leaf area (Fig. 8).

Fig 6 Shows the differences in leaf area between *PHYB*-RNAi transgenic plants (a) and the NO.O wild type plants (b) after threeweeks of germination.

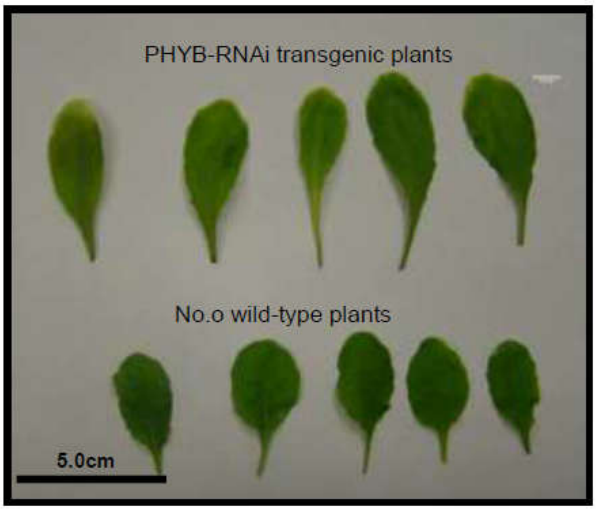

Fig 7 The differences in leaf area between T_1*PHYB*-RNAi transgenic plants (up) and the NO.O wild type plants (down) after six weeks of germination.

Fig 8 The differences in leaf area between T_2*PHYB*-RNAi transgenic plants and the NO.O wild type plants after 6 weeks of germination.

PHYB-RNAi lines show reduced levels of PHYB-mRNA

To verify knock-down of *PHYB* in the *PHYB*-RNAi transformed lines, *PHYB* mRNA levels were determined by reverse transcriptase PCR (RT-PCR). Total RNA was extracted from *PHYB*-RNAi transgenic lines and the NO.O wild type. These RNAs were used as templates to make cDNA. To amplify 570bp fragment from the *PHYB*-mRNA, we used cDNA from *PHYB*-RNAi transgenic lines and the NO.O wild type as templates, to check the levels of expression of the *PHYB* gene in both of them. Agarose gel electrophoresis was used to check these PCR products. The results show that *PHYB* mRNA is substantially reduced in the *PHYB*-RNAi transgenic lines compared with NO.Owild type as shown in Fig. (9), suggesting that endogenous mRNA is the target of double-stranded RNA-mediated genetic interference.

In conclusion, *PHYB*gene is the dominant phytochrome gene in light-growndicots and monocot plants. *PHYB* has been shown to control stem and petiole elongation, chloroplast development by the absorption of red (R) and far-red (FR) light and the transduction of intracellular signals during plant development. Reducing the level of expression of the *PHYB* gene using the RNAi technology can lead to a several changes in some characters in the *Arabidopsis* plants like increasing leaf area and elongated hypocotyls. The results revealed a great deal about the silencing of *PHYB* gene using RNAi technology, which can be used to improve some other economic crops to increase the leaf area, which can lead to higher yield components.

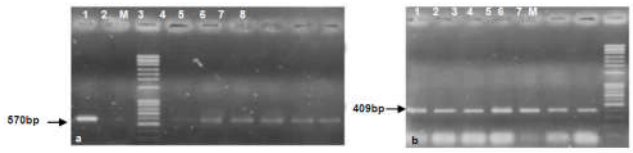

Fig 9 RT-PCR results to check for the level of expression for the *phyB* gene in 6 transgenic plants; lane 1 = NO.O wild type, lane 2 = negative control lane M =exACTGene DNA Ladders 1kb Plus,lanes 3-8 = sixtransgenic *PHYB*-RNAi plants(a) and RT-PCR results for the CBP20 housekeeping gene for the same six transgenic plants, 1 = NO.O wild type, lane 2-7 = six transgenic *PHYB*-RNAi plants.

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