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Full Length Research Paper

Phytochrome B mRNA expression enhances biomass yield and physiology of cotton plants

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A wide variety of physiological responses, including most light responses, also are modulated by photoreceptor gene such as PHYB. Phytochrome B (PHYB) expression patterns may be significant in the daily regulation of plant physiology and indicate an unexpectedly intimate relationship between the components of the input pathway. The present study shows successful transformation and mRNA expression in Phytochrome B transformed CIM 482 cotton plants. Transgenic cotton plants expressing Phytochrome B mRNA have showed more than two times increase in relative leaf growth rate (RLGR) and photosynthetic rate, more than one time increase in Root Weight Ratio (RWR), nearly half time increase in Stem Weight Ratio (SWR). Higher Relative leaf Growth Rate led to improved physiology of cotton plants, which have ultimate effects in fruit size and number. From this study we come to conclusion that Phytochrome B plants have showed significant increase in number of bolls but significant decrease in height as compared to control cotton plants.

Key words: Phytochrome B, mRNA, cotton physiology.

INTRODUCTION

A plants ability to maximize its photosynthetic productivity depends on its capacity to sense, evaluate and respond to light quality, quantity and direction (Briggs and Olney, 2001). Likewise the timing of developmental phenomenon such as flowering or entrance into dormancy depends on a system of measuring and responding to change in wavelength. Fluctuations in light can be crucial to competition and survival. Plants have evolved highly complex sensory mechanisms to monitor their surroundings and adapt their growth and development to the prevailing environmental conditions (Franklin et al., 2004). Light signals, perceived via the Phytochrome, Cryptochrome and phototropin photoreceptor families, are especially important environmental signals (Franklin et al., 2005). Phytochromes in plants are light receptors that are responsible for releasing chemicals that trigger processes in cells like germination or flowering. They work when exposed to red light with a specific wavelength. Two different Phytochrome forms exist: one is active and one inactive. Switching between them depends on the lights wavelength.

The primary photoreceptors involved in regulating the red/farred light-induced responses are the Phytochrome pigments. In *Arabidopsis*, the Phytochrome family has five members (PHYA–E) that can be separated based on amino acid sequence similarity into two main subfamilies, PHYA/C and PHYB/D/E (Clack et al., 1994; Matthews and Sharrock, 1997). Phytochrome A is a light-liable Phytochrome that predominates in dark-grown tissues whereas PHYB is light stable and predominates in light-grown tissue. The other Phytochromes (PHYC, PHYD and PHYE) are also light stable and have complex over-lapping and differential roles relative to PHYA and PHYB (Franklin et al., 2003; Monte et al., 2003). Phytochrome B (PHYB) is a major photoreceptors that control flowering. Leaves are the major sites for the perception of light. It has been discovered recently that PHY B expression in mesophyll but not in vascular bundles suppresses the expression of a key flowering regulator, flowering Locus T (FT), in vascular bundles (Endo et al., 2005). Said et al. (2007) transformed tomato with Phytochrome A and B and observed high anthocyanin pigments with high

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photosynthetic rate.

Phytochromes play key roles in vegetative plant development, including the regulation of stem elongation, leaf development and chlorophyll accumulation. Hormones have been implicated in the control of these processes in de-etiolating seedlings. However, the mechanisms by which the phytochromes regulate vegetative development in more mature plants are less well understood (Eloise et al., 2006)

Jiao et al. (2007) have determined that plants have evolved complex and sophisticated transcriptional networks that mediate developmental changes in response to light. These light-regulated processes include seedling photomorphogenesis, seed germination and the shade-avoidance and photoperiod responses. Understanding the components and hierarchical structure of the transcriptional networks that are activated during these processes has long been of great interest to plant scientists. Traditional genetic and molecular approaches have proved powerful in identifying key regulatory factors and their positions within these networks. Recent genomic studies have further revealed that light induces massive reprogramming of the plant transcriptome and that the early light-responsive genes are enriched in transcription factors. These combined approaches provide new insights into light-regulated transcriptional networks (Jiao et al., 2007).

Light conditions have direct influence on yield and quality of plant products that exist during plant growth and development (Figure 2). Responses to photosynthetic photon flux, red (R), far-red (FR) and blue (B) light responses have been studied extensively under controlled environments by Monteith (1965) and Kasperbauer (1988). These wavebands can be influenced in the field because of competitive absorption by and reflection from nearby growing plants (Monteith, 1965; Kasperbauer, 1988).

Conventional growth analysis has been used to study the response of plants to varying nutrient conditions (Lambers et al., 1989) and to develop ecological theory (Grime and Hunt, 1975). It has proven to be very valuable for ecological studies because of its clear connections with physiology and morphology, which provides a high level of understanding of growth rate and its related components in experiments with varying environmental factors. However, because classical growth analysis theory is limited to the phase of exponential plant growth, most physiological studies only consider allocation during vegetative plant growth and stop when reproductive structures are formed. By contrast, ecologists and evolutionary biologists are mainly interested in the final reproductive output of an individual (that is, its fitness) and less interested in the variety of physiological processes leading to this output. Since the fitness of plants depends on the vitality of the adult plant and its seed output, more information on changes in relative growth rate (RGR) and allocation during the whole life-cycle are needed to get a better insight in how plants regulate their vegetative and reproductive growth (Garnier and Freijsen, 1994). In present studies expression of exogenous Phytochrome in cotton in the form of RLGR, LAR, SWR, Fresh biomass and dry biomass was calculated in differently expressing PHYB transformed plants.

MATERIALS AND METHODS

Screening of cotton variety

Fifteen local cotton varieties namely CIM 497, CIM 482, CIM446, CIM499, CIM473, CIM 443, BH 118, BH 75, BH 79, BH 95, BH 557, MNH 93, NIAB 78, FH 672 and FH 673 were screened for transformation through tissue culture as done by (Rao et al., 2006) Cotton variety CIM 482 was selected for transformation on the basis of tissue culture response.

Plant transformation

The mature embryos of cotton variety CIM 482 were transformed with PHYB gene through *Agrobacterium* LBA-4404 according to the procedure described by Majeed et al. (2000).

Polymerase Chain Reaction (PCR)

Genomic DNA isolated from transgenic as well as control cotton plants leaves was analyzed by PCR for detection of PHYB by amplifying internal fragments of PHYB genes by a modification of the method by Saiki et al. (1988). The sequence of PHYB forward primer was 5' TAGGGCTCCTCATGGTTGTC 3' and the sequence of reverse primer was 5' TCGCAGTGTGAGATCGAAAC 3'.

The PCR was carried out with the following primers to amplify 646 bp fragment. DNA extracted from untransformed plants was used as negative control and that of plasmid *pBinPhyb* as positive control. The PCR was performed at 93ºC for 3 min 94ºC for 5 minutes 52ºC for 5 and 72ºC for 5 min followed by 35 times. The amplified PCR fragments were resolved on 1% agarose gel and observed under UV light.

Total RNA extraction

Total RNA extraction from cotton leaves was performed as described by (Jaakola et al., 2001). First of all samples of cotton leaves were grinded with the help of pestle and mortal in liquid nitrogen. Then extraction buffer was added for each 0.1 g, 1.2 ml buffer was added. Samples in extraction buffer were placed at 70°C for 20 min and three times vortex was done during this time. After this 4°C centrifugation was done at 5K for five minutes. Aliquot was put in 1.5 ml tube and spin for 20 min. Equal volume of Chloroform isoamylalcohol was added with vigorous vortex and spin at room temperature for 15 min. This step was repeated again and supernatant was taken. 60% LiCl was added and incubation was done at 4°C. Centrifugation was done at 4°C for 20 min. Pellet was washed with 70% ethanol (0.5 ml). Pellet was dried. 100 µl SSTE buffer was added at 65 - 70°C. Samples were pooled and equal volume of phenol chloroform isoamylalcohol was added with vigorous vortex (pH 4.5). Supernatant was taken and 2 volume of ice cold ethanol was added and incubation was done at -20°C for over night. In the very next day centrifugation was done for 20 min at full

speed and washing was done with 70% ethanol. After this pellet was dried and resuspended in water and volume was adjusted according to size of the pellet and stored at -20°C.

Northern blotting

Preparation of poly (A+) RNA from leaves and subsequent northern analysis were performed as described by (Heyer and Gatz, 1992a). 1.2% formaldehyde gel was prepared. RNA samples of both control and PHYB plants were loaded in the gel. The gel was run overnight at 15 V. Gel was transferred to nitrocellulose membrane (Hybond N). BCIP/NBT was used as substrate for color detection.

Quantitative real-time RT–PCR

Primer used for real time PCR are Forward Primer: CTCCTGGC TGAGTTTCTGCT and Reverse Primer: GCTTGTCCACCTGC TGCTAT. Real-time PCR reactions were carried out in an iQ5 cycler (BIO-RAD) with a 96-well plate (Bio-Rad) and using the IQTM SYBR_ Green Super mix (Bio-Rad). Primers used were same as for RT-PCR analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize the data. 50 ng of cDNA was used in each reaction. The reaction conditions were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s and final elongation step at 72°C for 10 min. A melting curve analysis was carried out by continuously monitoring fluorescence between 60 and 95°C with 0.5°C increments every 30s.

Statistical analysis of the real-time results was performed using iQ5 software (Bio-Rad) version 1.0 on the basis of CT values of the gene in different samples converted to their linear form normalized with GAPDH gene. Analysis of variance (ANOVA) was performed to analyze significant difference in transcript expression in leaves of control and stressed plants.

Physiological studies

Photosynthetic activity

Photosynthetic activity of transformed as well as control cotton plants was determined by estimating the CO_2 uptake per leaf area using IR spectroscopy with a transportable gas-exchange porometer (ADC, Hoddesdon, UK, model LCA3). The terminal leaflets of leaves 6 to 8 (leaf 1 was the first leaf larger than 1 cm) of 32 to 37 day old plants were used. Before measurement, the leaflet was fixed in the chamber and exposed to 50 to 500 mmol m^{-2} s^{-1} white light provided by a 150-lux lamp (Flexilux, Scholly Fiberoptik, Denzlingen, Germany) at 22 to 25°C until CO_2 assimilation reached a maximum steady-state level (10 – 15 min).

Measurement of growth factors

Relative leaf growth rate

The increase in plant leaf per unit of leaf present per unit of time was calculated by using the formula as described: RLGR = (log W2-logW1)/(t2-t1).

Root weight ratio

It was determined as quotient of total plant dry weight to root weight and determined by formula.

RWR = Root weight/Total plant weight

Stem weight ratio

The increase in plant stem weight per unit of stem present per unit time was calculated as

SWR = Stem weight/Total plant weight

Fresh biomass

The plants both control as well as transgenic which are taken from the pots were weighed by using an electric balance of model Sartorius BP 4100.

Dry biomass

After the measurement of fresh weight of the plants the fresh material after labeling was kept in an oven at 80°C for 48 h. After 48 h the plant material was removed from the oven and then re-weighed. This gave the dry weight of the plant.

RESULTS

Agrobacterium-mediated transformation of cotton variety CIM-482

The cotton variety CIM-482 was studied for its response towards *Agrobacterium* mediated transformation procedures. Plasmid vector construct pBin harboring PHYB gene was transformed in cotton variety CIM-482 by using *Agrobacterium tumefaciens* strain LB4404. Results on selection medium shows presence of required gene in the plant.

Detection of phytochrome B gene in cotton plants

The presence of PHYB gene in cotton plants were confirmed by PCR. The 646 bp fragment was amplified with internal gene specific primers. Total of 8 plants were detected positive out of 100 Putative transgenic cotton plants. No amplification was detected in negative control plant Figure 1. Lane 12 in Figure 1 determined the 1 kb ladder, Lane 11 is positive control lane 2 is negative control while all others are transgenic plants. It is clear from Figure 1 that only lane 7 out of nine experimental plants shows no amplification.

PhyB mRNA expression

Northern blotting

Northern blot analysis of PHYB transformed plants and non transformed cotton plants was conducted to check PHYB mRNA expression. The PCR amplified DIG labeled fragment of PHYB gene was used as Probe. The

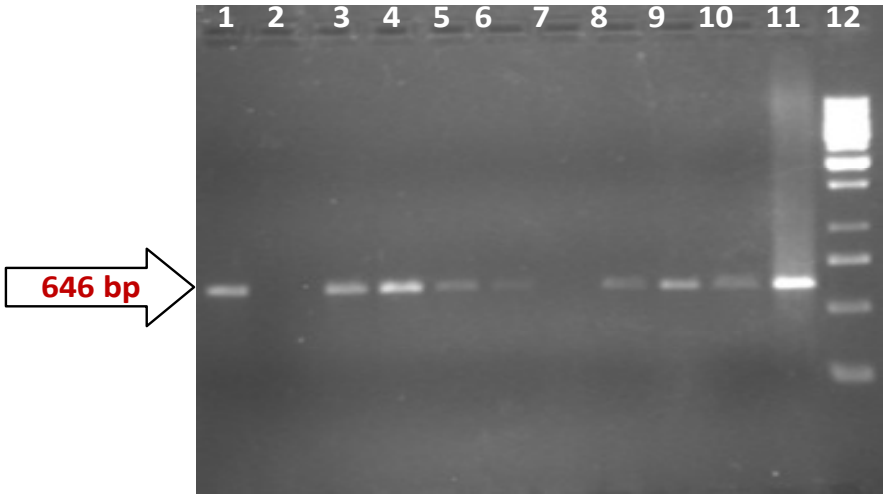


Figure 1. PCR amplification of *Phy B* plants. Lane 1-10: Plant samples. Lane 2: Negative control. Lane 11: Positive control. Lane 12: 1KB Ladder.

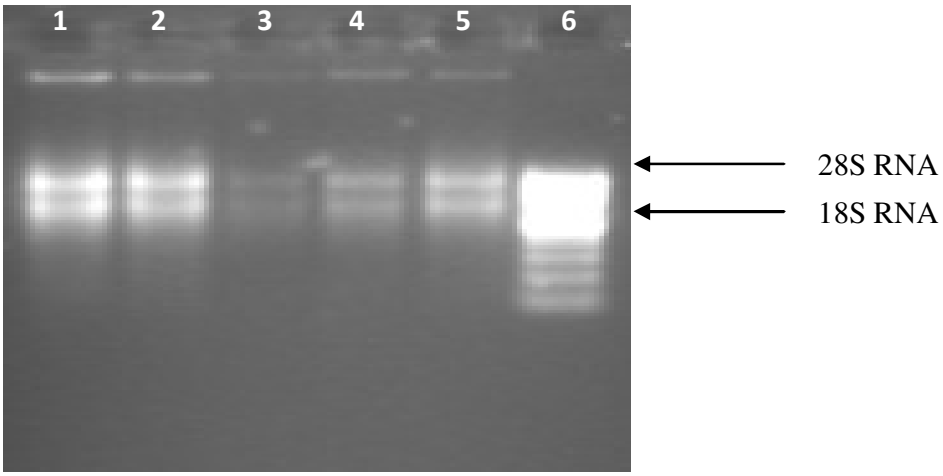


Figure 2. Total RNA extraction from cotton Leaves.

Kpn1 digested gel eluted 3.8 kb fragment of PHYB was used as positive control and non transformed cotton plant was used as negative control. In Figure 3 lane one is negative control lane 6 determines positive control while all others are PHYB plants RNA. Its is clear from the Figure that expression of PHYB RNA in QCC11 is much higher as compared to the other PHYB plants same is the case with photosynthetic rate of that plant.

Quantification of phytochrome B gene mRNA in transgenic cotton plants

Currently the gold standard for mRNA analysis offering best sensitivity dynamic range and reproducibility of any standard technique is quantitative RT PCR. In qRT mRNA transcripts were first reverse transcribed into cDNA using oligo (dt) random oligomer. The cDNA of PHYB were then exponently amplified by PCR using gene specific PCR. The concentration of amplicon in reaction is monitored with cybergreen dye. cDNA of eight plants were used for quantification by real time PCR. From Figure 3B, it is clear that plant line QCC2, QCC7, QCC11 and QCC10 are showing maximum expression as compared to other lines (Figure 3B) of PHYB plants (Table 1).

Physiological study

Photosynthetic rate

Figure 4 shows the photosynthetic activity of mature control and transgenic leaves. Young leaves from non-

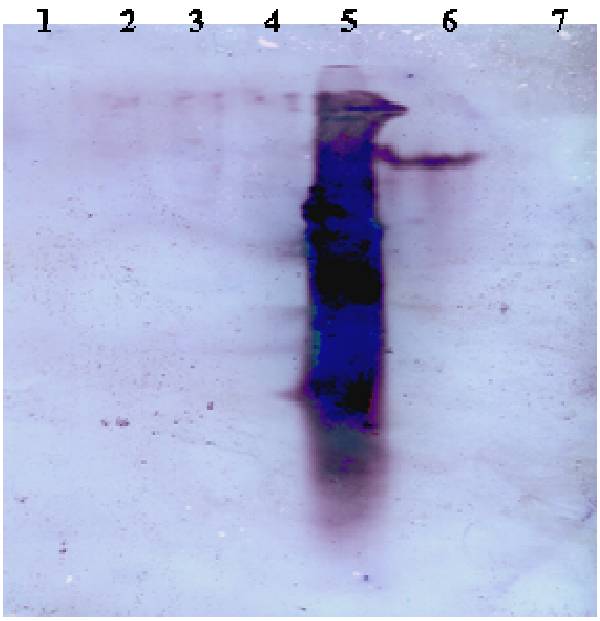


Figure 3A. mRNA expression in PHYB plants. Lane 1-Negative control cotton plant. Lane 2-5 PHYB Transgenic Southern Positive Plants. Lane 6 Kpn1 Digested gel eluted 3.8 kb fragment of PHYB DNA. Lane 7 λ/HindIII Marker.

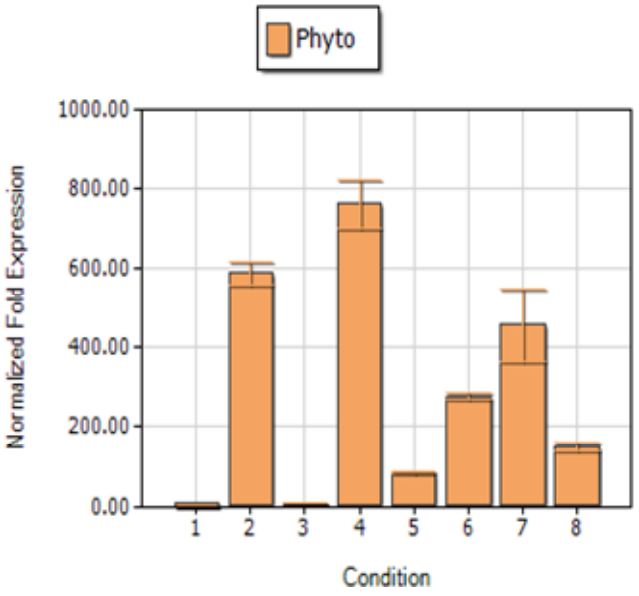


Figure 3B. qRT PCR of PHYB plants. Lane 1: QCC15. Lane 2: QCC10. Lane 3: QCC25. Lane 4: QCC11. Lane 5: QCC13. Lane 6: QCC 7. Lane 7: QCC 2. Lane 8: QCC14.

senescent plants (32-37 d old,) were used. Photon fluxes of more than 250 mmol m22 s21 in the transgenic plants showed higher rates of leaf-area photosynthesis than in the control plants. The increased rates per unit leaf area can thus be attributed to thicker leaf cross-sections and

Table 1. RNA Quantification by Nanodrop.

Samples	Ratio A260/A280	Yield (ng/µl)
QCC2	1.64	1085.88
QCC7	1.71	948.66
QCC10	1.56	692.64
QCC11	1.84	1506.46
Negative Control	1.59	1041.19

elevated chlorophyll contents. Because the total amount of leaf area was unaffected, increases in photosynthetic rates per individual plant from (0.54 mm/m^2/sec to 1.28 mm/m^2/sec.) was observed (Figure 4).

Growth analysis

Relative leaf growth rate (RLGR)

The physiological efficiency of cotton plants expressed in terms of relative leaf growth rate (RLGR) revealed the significant increase in case of PHYB transformation as compared to control. It was found that with the passage of time RLGR of PHYB plants increased significantly. 75% increase in RLGR was observed in PHYB transformed plants after 1st ten days of growth comparison as compared to control, while after twenty and thirty days 54 and 55% respectively increase was observed in PHYB plants as compared to control (Figure 5).

Stem weight ratio (SWR)

A stem is an above ground axis of the plant which acts as pillar for whole weight of the plant. So physiologically healthy plants should have greater weight ratio. Figure 6 shows significant increase in stem weight ratio of PHYB plants as compared to control. Figure 6 determines that after first ten days there is maximum (52%) increase of SWR in PHYB as compared to control but there is insignificant increase in SWR (8.3%) after twenty days in PHYB transformed plant as compared to control while PHYB plants shows significant increase in SWR (19%) in PHYB plant as compared to control (Figure 6).

Fresh biomass and dry biomass

Biomass is generally reported as grams of dry plant material per square meter. Biomass production is energetically understood as a long-term storage of hydrogen. Endogeneously, the hierarchy of energy storage begins with the electric and proton gradient across the thylakoid membrane. A significant increase of fresh and dry biomass

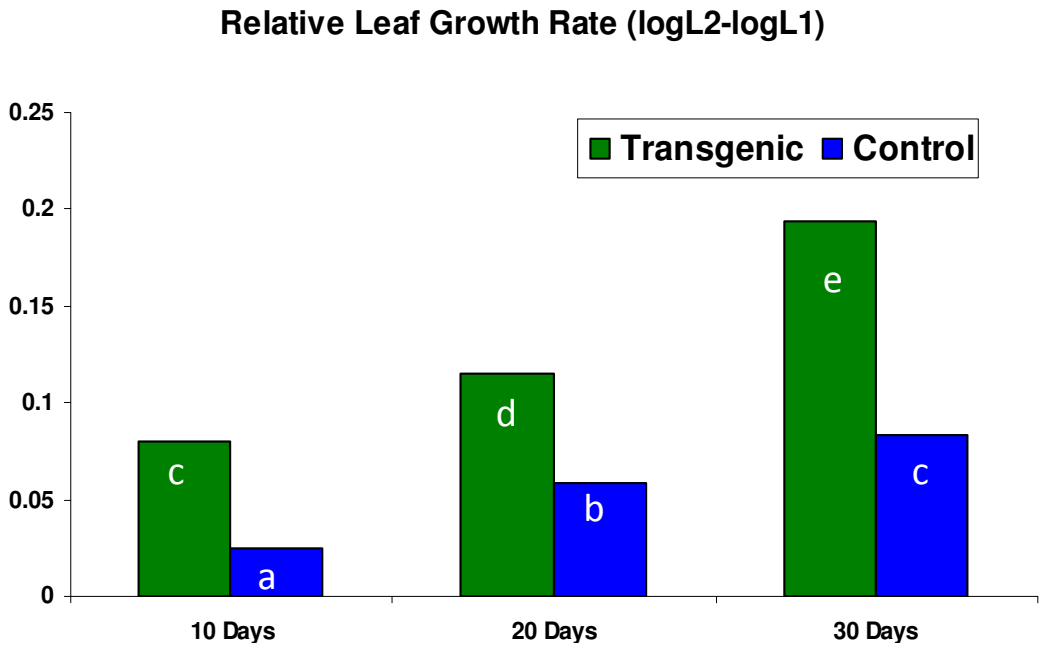
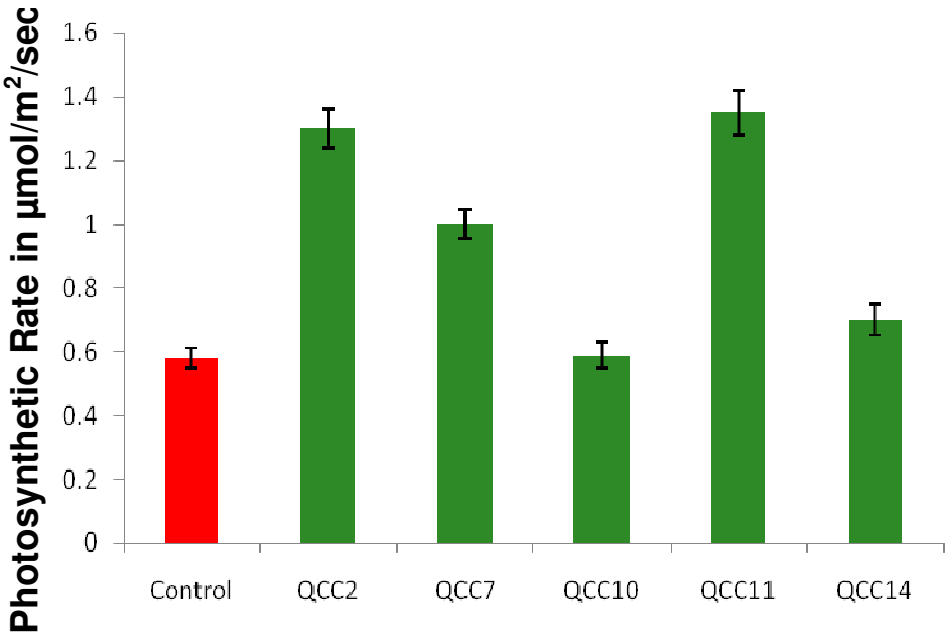


Figure 4. Comparison of Photosynthetic Rate of PHYB cotton plants with control.



Figurer 5. Comparison of Relative Leaf Growth Rate of PHYB cotton plants with control.

of cotton plant expressing Arabidopsis PHYB was attributable to higher photosynthetic rate of these plants as compared to control. Figures 6 and 7 clearly determines that Fresh and dry biomass increases much efficiently after 10 days in PHYB plants as compared to control (77%) similar was the case after thirty days (87%) but a little bit slower increase in SWR was observed during 10 to 20 days (75%) (Figures 6 and 7).

DISCUSSION

Phytochromes are known to play roles in cotton plant architecture, floral initiation and fiber elongation. The Phytochrome gene family has not been characterized in cotton (*Gossypium species*) (Ibrokhim et al., 2006). Being particularly interested in the transgenic approach, we generated cotton plants over expressing PHYB.

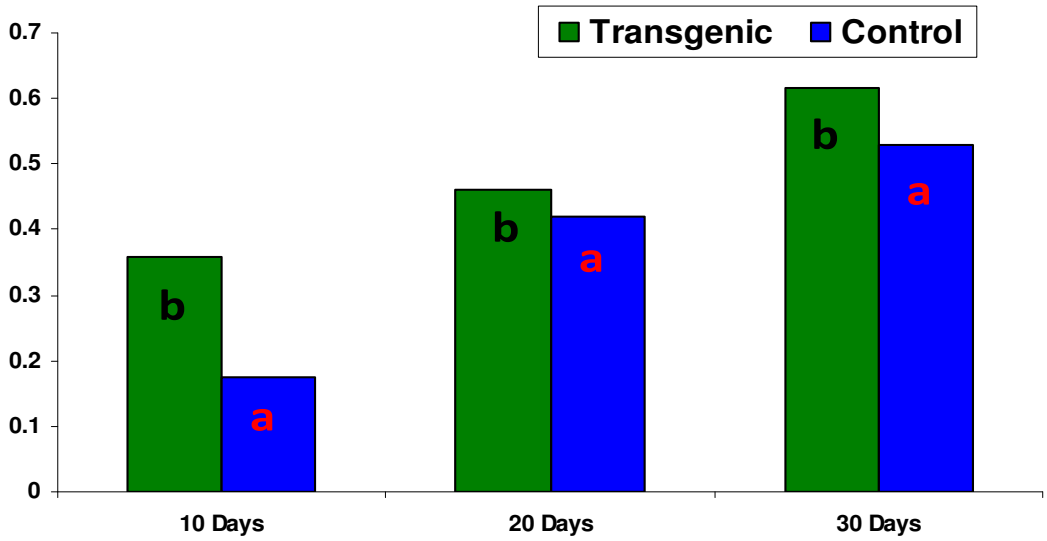
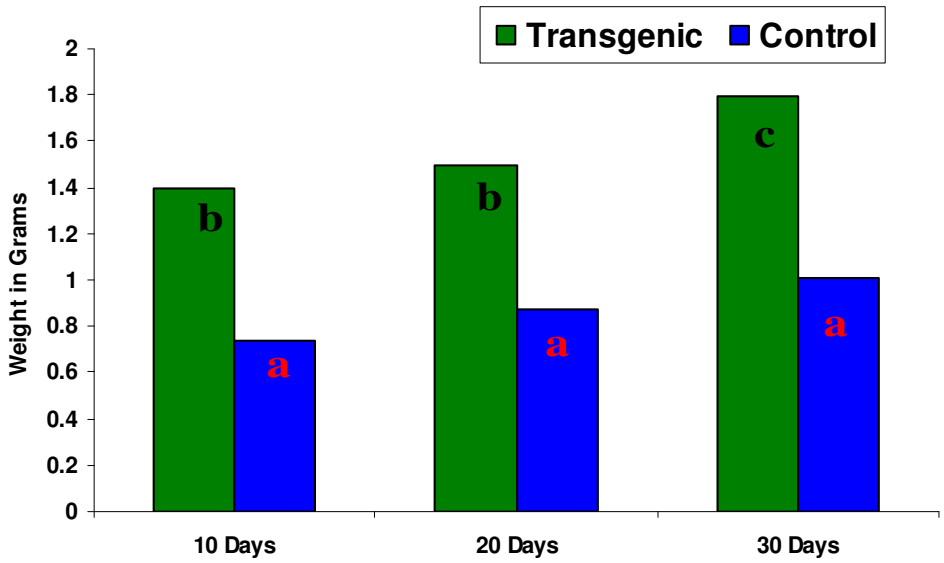


Figure 6. Comparison of Stem Weight Ratio of PHYB cotton plants with control.



Figyre 7. Comparison of total Fresh biomass yield of PHY B cotton plants with control.

Data of Molecular analysis indicated that 8 out of total 100 putative transgenic cotton plants were PCR positive (Figure 1), while 4 were expressing PHYB at mRNA level as indicated by Nouthern blot analysis (Figure 3). It is also clear from the Figure that expression level of mRNA in QCC11 which is lane 4 is much higher as compared other PHYB plants. The results of qRT real time PCR have determined that QCC11 exhibit higher level of mRNA expression as compared to other plants where mRNA expression is quite low. The results clearly determined that expression level is variable among different lines of PHYB, Expression of QCC2, QCC10 and QCC11 was much higher as compared to other lines (Figure 3B). These results are similar to Dong and Li (2007) in which they determined the variation in expression level of transgenic plants, which may be due to the nucleotide sequence of the gene, promotor and the insertion point of the gene in the DNA of the transgenic variety, transgene copy number, the internal cell environment, as well as several external factors in the environment (Rao, 2005, Warren, 2007).

Transgenic cotton plants transformed with the Arabidopsis PHYB cDNA under the control of the CaMV 35S promoter supported the notion that phytochrome-over expressing plants may have agricultural importance. We discuss the data presented here in the context of

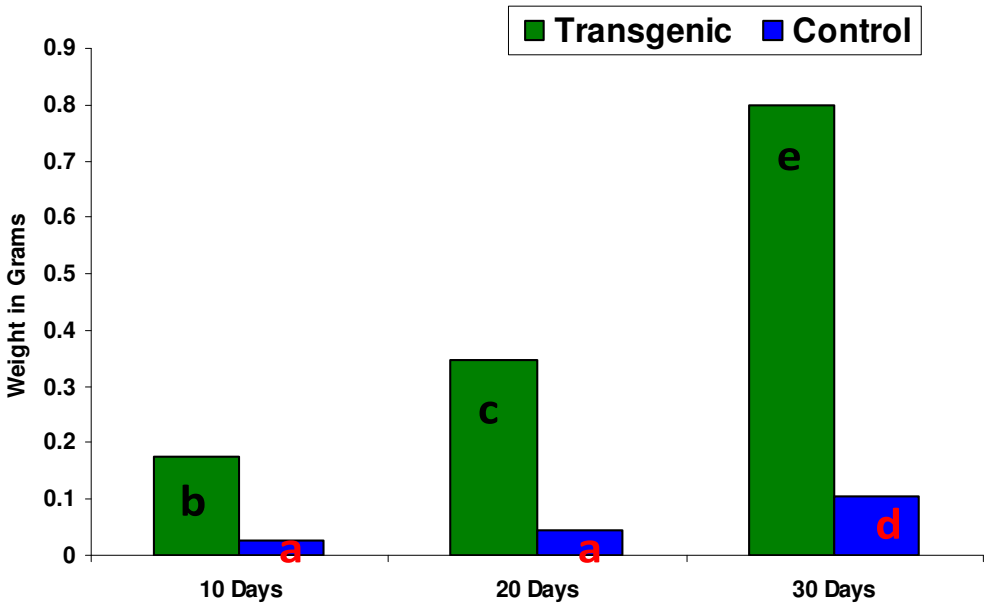


Figure 8. Comparison of total dry biomass yield of PHYB cotton plants with control.

results previously obtained with other PHYB or PHYA over expressing plants. Transgenic cotton plants over expressing Arabidopsis PHYB exhibited a small phenoltype and thicker stems, increased apical dominance, smaller but thicker leaves, higher rates of photosynthesis, prolonged time span for photosynthesis, this small phenotype of cotton plant in field is same as the Phytochrome expression in potato (Thiele et al., 1999). Phytochromes therefore appear to be involved in mediating seasonal germination timing, a trait of great ecological importance and one that is under strong natural selection (Donohue et al., 2007). The morphological data indicate that morphology of plant is directly related to its physiology with increasing photosynthetic rate. This was consistent with the result transgenic tobacco plants over expressing PHYA (Robson et al., 1996).

The PHYB plants displayed higher photosynthesis rates (Figure 4). This effect may have been proportional to the increased chlorophyll levels resulting in lower excitation of pigments compared with control leaves (Thiele et al., 1999). Phytochrome (PHY)-mediated responses of plants to their light environment is an important goal in providing an overall understanding of light-regulated growth and development (Quail, 2007). The enhanced responsiveness of the transgenics to white light led to increase thickening of leaves and thus to higher photosynthetic performance, higher biomass production of the aerial parts of the plants, but also to a preferential allocation of assimilates to the leaves at the expense of the stems. Moreover it was found that in plant QCC11 showing overexpression of PHYB results greater photosynthetic rate as compared to other PHYB transformed plants and no expression in control Figure 4.

RLGR and Photosynthetic rate of PHYB plants were much more than control, this is because simple correlation exists between RLGR and photosynthetic rate (Najafi et al., 2007). RLGR is strongly and positively correlated with Photosynthetic rate as depicted from Figure 5. Ultimately with more photosynthetic rate and growth rate PHYB plants have shown 50% more fresh and dry biomass ratio as compared to control this is same as (Thiele et al., 1999) in which PHYB potato showing more yield as compared to control. Similarly, over expression of gene in PHYB plants lead to more Biomass allocation (Figures and 7 and 8).

Conclusion

The results of this study support the idea that higher photosynthetic rates led to more Relative Leaf Growth Rate and yield biomass of cotton plants. It is clear from the results that transgenic plants are potentially more productive, especially in areas with high irradiation; therefore, reduced photo inhibition becomes advantageous. As plants were able to assimilate CO_2 for a longer time because of decelerated chlorophyll breakdown, they might be valuable in areas with long growing seasons.

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