# Role of splice variant of CONSTANS and Carbon Nanoparticles in flowering time control in *Arabidopsis thaliana*

**By**

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Abhishek Kumar

# DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Abhishek Kumar

# LIST OF PUBLICATIONS PERTAINING TO THESIS

## Published

1. **Abhishek Kumar**, Anamika Singh, Madhusmita Panigrahy, Pratap Kumar Sahoo, Kishore C.S Panigrahi. (2018).Carbon nanoparticle influences photomorphogeneis and flowering time in *Arabidopsis thaliana.* Plant Cell Report.https://doi.org/10.1007/s00299-018-2277-6.

2. **Abhishek Kumar**, Madhusmita Panigrahy, Kishore CS Panigrahi. (2018).Optimization of soil parameters and cost effective way of growing *Arabidopsis thaliana* from an Indian perspective. International Journal of Basic and Applied Agricultural Research.16.1.

3. **Abhishek Kumar**, Aaram A. Kumar, Aditya P.Nayak, Priyanka Mishra, Madhusmita Panigrahy, Pratap K. Sahoo, Kishore CS.Panigrahi.(2019).Carbohydrates and polyphenolics of extracts from genetically altered plant acts as catalyst for *in-vitro* synthesis of silver nanoparticle. Journal of Biosciences 44:6 DOI: 10.1007/s12038-018-9826-6.

## Manuscript under preparation

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## Conferences/Symposiums/workshops

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1. **Abhishek Kumar**, Anamika Singh Madusmita Panigrahy and Kishore C.S Panigrahi. (2016). Carbon nanoparticles accelerate the flowering time in *Arabidopsis thaliana.* 2nd International Conference on Translational Research, 2016 held from 15th -17th December, 2016.

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## Conferences attained

4. XXXV All India Cell Biology Conference, 16th -18th Dec 2011, NISER Bhubaneswar.

5. 99th Indian Science Congress, 2012, KIIT, Bhubaneswar.

6. Indian Immunological Society Odisha chapter, 2012, Bhubaneswar.

7. National Seminar on Science and Technology for Indigenous Development in India, 2015.

8.National Seminar on Forestry& Agriculture for Sustainable Future, 2016.

9. International Interdisciplinary Conference on Humanitarian Technology, 2016, KIIT University, Bhubaneswar.

9. 2nd International Conference on Translational Research, 2016 held from 15th -17th December, 2016.

10. 2nd Immunological Society Odisha chapter, 2017 NISER, Bhubaneswar.

11. Nextgen Genomic, Biology, Bioinformatics and Technology (NGBT) Conference, held from 2nd -4th October, 2017.

12.International Conference on Plant Developmental Biology and 3rd National *Arabidopsis* Meeting 12th-16th December 2017, NISER, Bhubaneswar.

13. 42th National Conference on Biotechnology Interventions for Enviornmental Stress Management in Plant and Microbes, 20th-21th January 2018, Utkal University, Bhubaneswar.

14. Biotechnological Initiatives for Crop Improvement (BICI-2018) 08th-09th December 2018 Bihar Agricultural University, Sabour, (Bhagalpur) Bihar.

15. National Workshop on Horizon of Bioinformatics in Environmental Management & Biodiversity Conservation, University Centre of Bioinformatics, TMBU, Bhagalpur,7th-9thJanuary, 2019.

## Others

16. Received Young scientist award by Orissa Botanical Society in 42th National conference on Biotechnology Interventions for Environmental stress Management in plant and Microbes, 20th-21th January 2018, Utkal University, Bhubaneswar.

17. First prize in research presentation in National conferences in Biotechnological Initiatives for Crop Improvement (BICI-2018) 08th-09th December, 2018.

Abhishek Kumar

# DEDICATION

Dedicated to my parents and family

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Abhi

Abhishek Kumar

# CONTENTS

# Homi Bhabha National Institute

# Ph. D. PROGRAMME

# SYNOPSIS

1. **Name of the Student:** ABHISHEK KUMAR
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## INTRODUCTION

*Arabidopsis thaliana (At)* is a facultative long day (LD) plant (Johansson M et al., 2014). Flowering in *At* is influenced by the duration of light and dark period, called photoperiod (Garner etal., 1920). It flowers earlier in summer long days typically, with cycles of 16h light followed by 8h dark (LD) in a 24h diurnal rhythm. The flowering is normally delayed in winter short days (SDs, of about 16h dark and 8h light cycles. The photoperiodic regulation of flowering time is controlled by circadian clock. In both LDs and SDs, a plant specific nuclear protein GIGANTEA, transcriptionally up regulate the master regulator B-box zinc finger protein CONSTANS (CO) (Truck et al., 2006). The post-translational stability of CO protein accumulation is dependent on the synergistic activity of far-red light photoreceptor PHYA, blue light photoreceptors CRY1 and CRY2. The coincidence of external light stabilizes the PHYA, CRY1 and CRY2 proteins there by, increasing CO protein as has been proposed in the external coincidence model of photoperiodic

flowering time control (Valverde et al., 2004). The accumulation of CO activates the transcription of *flowering locus T* (*FT*). CONSTANS (COα) strongly induces *FT* expression by directly binding to *FT* promoter (Song et al., 2015). Mutation in either *CO or FT genes* delays floral transition in LDs and SDs confirming the pivotal role of these two components in photoperiodic flowering-time control (Jang et al., 2008; Yoo et al., 2005). Over-expression of *CO* has been shown to abolish the photoperiod discrimination (Goto et al., 2003) and therefore leads to flowering roughly at similar time frame under both LDs and SDs.

In current study first we optimised the all essential parameters such as temperature, light intensity, relativity humidity and soil composition i.e. required to evaluate the flowering in *At.* We have tested five different soil types available and also formulated a new mixed soil (Soilrite, Red soil and Garden soil in proportion of 5:4:1) for optimum growth of *At* in controlled environmental conditions. We have standardized and fixed the soil and nutrient requirement for *At* growth in growth chambers in local conditions of Bhubaneswar, Odisha (Kumar et al., 2018). This will provide necessary guidelines for Indian researchers and base for obtaining optimal observations of flowering time under mixed soil composition.

In silico data suggested another possible splice form of *CONSTANS* (*COβ*). However, how the improper splicing form of transcript (*COβ)* is formed is not known. In current study we demonstrated that in the absence of GIGANTEA improper splicing of *CONSTANS* occur and that delays flowering. However early flowering after treatment of carbon nanoparticles to the COβ over-expression plant suggests photoperiod independent flowering control. The extract of these transgenic plants also enhanced the silver nanoparticle synthesis in vitro.

The objectives of the present study are

1. Optimization of soil composition for flowering time evaluation in *Arabidopsis thaliana*

2. Generation of transgenic lines and evaluation of flowering time with phenotypic characterization of COβ in different genetic background (Col-0, *co-10* and *phyb-9*)

3.To Study the of expression pattern of *COβ* transcript at various diurnal and developmental stages

4. To understand the role of Carbon nanoparticles (CNPs) in flowering time in COβ and Col-0 background in *Arabidopsis thaliana*

5. To understand the role of COβ in silver nanoparticles synthesis

This thesis is divided into six chapters

1. Introduction

2. Materials and methods

For our studies we optimised all essential protocol and used appropriate materials for the work, to full fill the specific objectives.

The result section is divided into four chapters:

3. Chapter: Mixed soil grown plants behave standard flowering time in *Arabidopsis thaliana*

4. Chapter:.Over-expressed lines of COβ delay flowering time and it is diurnally and developmentally regulated in *Arabidopsis thaliana*

5. Chapter: Carbon nanoparticles (CNPs) accelerate flowering in over-expressed line of COβ and Col-0 in *Arabidopsis thaliana*

6. Chapter: Over-expression line of COβ enhances the Silver nanoparticles synthesis

7. Conclusion and future prospective

8. Bibliography

## 3.0 Chapter: Mixed soil grown plants behave standard flowering time in *Arabidopsis thaliana*

### 3.1 Introduction

*Arabidopsis* is a model plant used for the understanding of biochemical pathways and molecular genetics, because of short life cycle, relatively short genome size and mutation can be easily induced. It requires specific soil, nutrient and watering conditions for optimal growth that is recommended by *Arabidopsis* biological resource centre (ABRC, UK). However, the growth conditions of *At* in Indian conditions is not optimal due to non-availability of similar soil mix. We tested different soil types and formulated a new soil mix proportion for optimum growth and flowering time evaluation of*At* from an Indian perspective.

### 3.2 Result

*Arabidopsis* plant growth was retarded on clay and red soil and finally died after 15 days, where as it could reach the bolting stage on garden soil, soilrite and mixed soil. Leaf growth and area is a positive indicator of plant growth (Weraduwage et al., 2015).The plants on the garden soil showed less healthy which was accompanied by obvious small leaf size, thin stem, less number of leaves and anthocyanin accumulation on leaves. Plants grown on soilrite were comparatively equally healthy as those on mixed soil, however plant having pale leaves. The plants on mixed soil showed best plant growth in terms of leaf number, greenness, leaf length and optimal number of leaves at bolting stage .To access the plant growth and healthy status, root length was measured from the plants grown on five different soil conditions. Root length was maximum in mixed soil and minimum in red soil with high iron content.

In present study, leaf area and perimeter was accessed in the plants grown on garden soil, soilrite and mixed soil. In all the 3 soil conditions tested, the leaf area and perimeter is higher under SD conditions than under LD conditions. Leaves from SD conditions had longer petioles. Higher

vegetative growth under SD conditions is known in *Arabidopsis* (ABRC) as compare to long days plant.

To access the photosynthetic potential and stress parameters, total chlorophyll, carotenoid and anthocyanin was quantified in the leaves from the plants grown in 5 different soil conditions .Under both LDs and SDs, total chlorophyll was highest in the plants grown on mixed soil indicating better plant growth than other soil conditions tested. Carotenoids and anthocyanin are considered in this study as indicators of abiotic stress, since these are known to accumulate in different kind of abiotic stress conditions including nutrient deficiencies, toxicity and disease (Ramakrishna et al., 2011). Carotenoid and anthocyanin under both LDs and SDs were highest in the plants grown on garden soil and least in mixed soil indicating that the plants grown on mixed soil reflect a healthy and almost stress-free growth environment.

Rosette leaf number at bolting stage represents the reproductive status of the plant (Fornara et al., 2009). Presently, the leaf number at bolting stage was optimal which are about 13 leaves in plants grown on mixed soil. Whereas leaf number was less in case of plants on garden soil (9) and soilrite (11).The plants were on clay and red soil died at 2 leaf stages. Seed pod length, weight and number of seeds per pod were significantly highest in the plants grown on mixed soil followed by soilrite and then garden soil being the least.

The results from nutrient and soil parameter analysis presented following data. Clay soil sowed lowest pH indicating the most acidic soil. The pH of garden soil and mixed soil was same at neutral pH. Optimum pH of soil for healthy plant growth ranges from 5.5 to 7.5 (Hanlon et al., 1993). Electrical conductivity (EC) is a measure of salinity and each plant species has a specific threshold of soil EC for obtaining best yield (Hanlon et al., 1993). *At* root hair cells are affected by changes in the electrical properties of the plasma membrane (Lew. 2004). Mixed soil in present study showed highest EC indicative of highest movement of ions into plant.

### 3.3 Conclusion

We tested the mixed soil to be best for the *Arabidopsis* growth with respect to reproductive potential of the plants as the plants grown on mixed soil had highest biomass and seeds per pod. Mixed soil grown plants showed least accumulation of carotenoids and anthocyanin indicating the better plant growth conditions. Mixed soil could be due to favourable pH, high electric conductivity (EC), high N, P and K content. Though the organic content (OC) of the mixed soil was low, it could be efficiently taken up by the plant due to high EC.

## 4.0 Chapter: Over-expressed lines of COβ delay flowering time and it is diurnally and developmentally regulated in *Arabidopsis thaliana*

### 4.1 Introduction

Flowering in *Arabidopsis* is influenced by the duration of light and dark period, called photoperiod (Garner et al., 1920). The photoperiodic regulation of flowering time is controlled by circadian clock. The accumulation of CONSTANS (CO) activates the transcription of *flowering locus T* (*FT*). In silco data suggested the presence of an improper splicing form of CONSTANS (COβ) (Mater thesis of Alexander Ternois MPIPZ, 2008). In current study we demonstrated that in the absence of GIGANTEA, improper splicing of *CONSTANS* occurs to a higher level and that delays flowering in a photoperiodic dependent manner in *At*.

### 4.2 Result

After optimization of all essential parameters to evaluate flowering time, then we verified the occurrence of improper splice form *CONSTANS* (COβ) in vivo by a set of gene specific primer.COβ was detected, amplified and cloned in gateway compatible vectors containing CFP and YFP N-terminus tag. The expression a contract containing the insert of COβ was transformed into the *Agrobacterium tumefaciens* strain GV3101 using electroporation method.The correct insertion of the construct in *Agrobacterium* was confirmed by colony PCR. Transformation of *At*

with these constract was done by floral dip method. The transformed seeds were grown in controlled environmental conditions (as described in objective 1) and positive plants were selected using BASTA (herbicide selection) followed by PCR with gene and vector specific primers. Homozygous stable transgenic lines expressing COβ were generated after rigorous selection by both BASTA and PCR in each single plant up to F3 generation. The various over-expressing transgenic lines of COβ *were* generated in Col-0, *co-10* and *phyb-9* in backgrounds as the follow

| | | |
|---|---|---|
| 1. 35S::COβCol-0 | 2. 35S::CFP:COβCol-0 | 3. 35S::YFP:COβCol-0 |
| 4. 35S::COβ*co-10* | 5. 35S::CFP:COβ*co-10* | 6. 35S::YFP:COβ*co-10* |
| 7. 35S::CFP:COβ *phyb-9* | 8. pSUC2::COβCol-0 | 9. pKNAT1::COβCol-0 |

In order to evaluate the flowering behaviour total number of leaves (both rosettes leaves and cauline leaves) were recorded at the time of bolting. We observed a delay in flowering in the over-expressed lines of COβ as compare to controls Col-0, *co-10* and *phyb-9* under LDs and SDs however significance difference was minimum under SDs as compare to LDs. Since the plants were more greenish in the over-expressed line of COβ, therefore chlorophyll content was also examined. Both under LDs and SDs the total chlorophyll was significantly higher in over-expressed lines of COβ as compared to Col-0, suggesting the COβ alter the chlorophyll synthesis pathway.

Our result showed higher percentage of carbohydrate accumulation and more numbers of seeds per pod clearly suggested the higher yield performance in COβ over-expressing lines than the controls. In order to understand the COβ can be influence by the abundance of *GIGANTEA* quantative, RT-PCR was performed. A significant higher level of *COβ* transcript in *gi-100* mutant lines were observed that clearly suggested the absence of GIGANTEA may lead to improper splicing of *CONSTANS* forming COβ and that delays flowering in *At* in a photoperiod dependent manner.

### 4.3 Conclusion

Our result showed delay in flowering time in the over-expressed lines of COβ in different genetic background as compare to controls Col-0, *co-10* and *phyb-9.*This result also suggested an involvement of a common mechanism irrespective of genotypes. We also observed significantly high level of transcript of *COβ* in *gi-100* mutant lines. From this result we can conclude that GIGANTEA it required for proper splicing of *CO* and that delays flowering in *At* in a photoperiodic dependent manner.

## 5.0 Chapter: Carbon nanoparticles accelerate flowering in over-expressed line of COβ and Col-0 in *Arabidopsis thaliana*

### 5.1 Introduction

Application of carbon nano-tubes in agriculture and plant biology research is an emerging area of plant nano-biotechnology. However, the mechanisms of carbon nanoparticles (CNPs) in plant systems are poorly understood (Husen et al., 2014). In the present study, we tried to understand the importance of CNPs in some crucial physiological processes in *Arabidopsis thaliana* (*At*) and over-expressed line of COβ.

### 5.2 Result

Effect of CNPs on *At* and over-expressed line of COβ were tested by growing the seedling in petriplates and in planton boxes. A higher percentage of bolting correlated with the increased concentration of CNPs from 10, 100 and 500 µg/ml was observed. Subsequent experiments were conducted using 500 µg/ml of CNPs concentration. Bolting of Col-0 was observed at about 12 (±1) rosette leaves and in between 24 to 28 d whereas the CNPs treated plants flowered at around 8 (±1) rosette leaves and in about 20 LDs cycles. However under LDs bolting of 35S::COβ lines were (delayed flowering phenotype) observed in about 21 rosette leaves in the CNPs treated plants

compared to about 30 rosette leaves in untreated plants. In order to ascertain that the early flowering phenotype was specific to CNPs and not due to carbon per say, Col-0 plants were grown in presence of 500 μg/ml Charcoal under LDs. Charcoal-treated plants showed larger yellowish,leaf and flowering were delayed as compared to CNPs treated plants.

To determine whether CNPs are taken up systemically through the plant roots, CNPs accumulation was investigated in the homogenate of 20 days old plant root, stem leaf and flower using Raman Spectroscopy. The amount of CNPs accumulation was found to be highest in the leaf extracts and least in the extracts of flower.

Significant ($p \leq 0.01$) increase in hypocotyls length under white light (WL) or even in red light under LDs after CNPs treatment was observed.To determine whether the hypocotyls growth was a developmental defect, seedlings were grown for 8 days in complete darkness after germination induction with or without CNPs at 22. Both the CNPs treated and untreated seedlings showed elongated hypocotyls and closed pale-yellow cotyledons suggesting the enhanced hypocotyls elongation growth in presence of CNPs is light dependent.

To further analyse the red-light specific increment of hypocotyls length is due to the involvement of phytochrome B (PHYB) was investigated in 10 days old seedlings with or without CNPs treatment under LDs. The expression of *PHYB* transcript was analysed using reverse transcription PCR (Cao et al., 2005) at zeitgeber (Zt) Zt 8 and Zt 12, as compared to the transcript level of *ACTIN* at respective Zt. *PHYB* transcript levels were higher at all the time points in light phase in the untreated seedling than their respective CNPs treated seedlings. These results suggested that the expression level of *PHYB* was down regulated upon CNPs treatment. We further tested the involvement of the flowering time master regulator gene CONSTANS (CO). Real time PCR was performed in CNPs treated plants for evaluating the expression of *CONSTANS* (*CO*) grown under LDs. The transcript levels of *CO* at Zt 8, and 12 in compared with the transcript level of *ACTIN* was analysed and found that there were no significant differences.

Furthermore dramatic up regulation of *GIGANTEA* and *LFY* transcript were also observed in CNPs treated plants explaining the early flowering in the presence of CNPs.q-RT- PCR result also suggested that the transcript level of *PIF4* in CNPs treated plant was higher under LDs. This could explain the longer hypocotyls and smaller cotyledon angle of CNPs treated seedlings under the LDs. This novel molecular understanding of CNPs accelerating flowering may have broad implications and the application in plant biotechnology and agriculture.

### 5.3 Conclusion

Here we conclude that carbon nano-particles are taken up by *Arabidopsis* and induce early flowering with longer hypocotyls length in presence of CNPs. Other pathways such as PIF4 dependent pathways might play a significant role in these CNPs treated plants transcript is normally high at around Zt 16 under LDs. This could explain the longer hypocotyl of CNPs treated seedlings compared to their control under white light. In q-RT PCR experiments, dramatic down regulation of *PHYB* transcript at Zt 4, 8 and 12 by CNPs treatment was observed. Such understanding may have broad implications in plant biotechnology and agriculture.

## 6.0 Chapter: Over-expressed line of COβ enhances the silver nanoparticles synthesis

### 6.1 Introduction

The synthesis of nanoparticles (NPs) fromplants extracts is now exciting area because of their adaptability and reduced use of unsafe solvents (Abdel et al., 2011). It has been shown that the plant extract containing phenolic, alkaloids, terpenoids and flavonoids are responsible for the reduction of ionic metal into metallic nanoparticles (Chung et al., 2016). However, the molecular mechanisms of silver nanoparticles (AgNPs) synthesis are poorly understood. In this study, we tried to understand the in vitro synthesis of AgNPs in *At* and genetically altered *At* plants.

## 6.2 Result

The in vitro synthesis of AgNPs was performed using plant extracts of different transgenic plants of *Arabidopsis thaliana* (*At*) a genetic model plant and from the Chilika lagoon. The intensity of the brown colour was directly proportional to the increase in particle size of AgNPs. The UV-Vis spectra of the plant extract solution containing $AgNO_3$ after the reaction showed a peak in 400-700 nm windows indicating the abundance of AgNPs. The amount of AgNPs synthesis was also found to be dependent on the individual extracts. However, the morphology of the particles was similar for AgNPs synthesized using different plant extracts as evident from SEM and also from TEM images. Results from SEM showed that NPs were spherical or rhomboidal in shape having a poly-dispersive size distribution with a size of about 10 - 20 nm in diameter.

To understand the mechanism of AgNPs synthesis, we chose *Arabidopsis thaliana,* plant, for its capability to synthesize AgNPs. The UV-Vis absorption spectrum of the suspension obtained after incubation of silver nitrate with the *Arabidopsis* leaf extracts showed absorption maxima between 450 - 470 nm. We also observed the size distribution of the NPs that was obtained by reaction of silver nitrate with the *Arabidopsis* leaf extract. This demonstrated that whole plant extracts of *Arabidopsis* is capable of synthesizing AgNPs. In order to identify the chemical nature of the substances that facilitate AgNPs synthesis, depletion experiments were performed with extracts of *Arabidopsis,Scendesmus, Enteromorpha, Potamogeton* and followed by analysis using NMR spectroscopy. The results revealed chemical shifts in the range of 3-4 ppm as an indication of carbohydrates. These experimental evidences suggested that oligosaccharides most likely acted as reducing as well as stabilizing agents for synthesis of NPs. Notably, in case of *Arabidopsis* and *Potamogeton*, NMR peaks were obtained in the aromatic region around 6.8 suggestive of presence of the phenolic.

In order to understand the nature of the compound that is required and sufficient for AgNPs synthesis, we performed depletion experiments. The depletion of DNA, RNA and proteins did not alter the AgNPs yield significantly, while the removal of carbohydrate fraction lead to a sharp decline in the synthesis of AgNPs. GI acts antagonistic to SPINDLY (SPY). An improper splice form of CONSTANS (COβ) was also used in this study which showed higher amount of sugar accumulation. The effect of *spy-5, gi-100* mutation, in plants constitutively producing GI and COβ under the control of viral (35S) promoter for AgNPs biosynthesis was investigated. Consistent with our hypothesis, the *spy-5* and 35S::GI extracts yielded lesser amount of AgNPs, whereas *gi-100* and COβ over-expresser yielded nearly two and three-fold higher as compared to Col-0 plant extracts respectively. Results of the SEM analysis for the AgNPs from the plant extracts of Col-0, *gi-100*, 35S::GI and could demonstrate that the AgNPs synthesized had variable sizes with 35S::GI extracts containing the smallest and *gi-100* and 35S::COβ being the largest. These results indeed confirmed that the amount of the sugar accumulated in the different genotypes positively correlated with the AgNPs yield.

### 6.3 Conclusion

Here we conclude that the plant extracts of over-expresses lines of COβ with other genetic backgrounds in *Arabidopsis thaliana* and as well as from weed species of Chilika lagoon could be used for synthesis of AgNPs in an eco-friendly manner. The synthesis of AgNPs in different plant species is varied in their intensity might be because of the carbohydrate and polyphenols differ in different plant species. However, the similar properties of AgNPs from different extracts raised a possibility of a common mechanism in their synthesis. From our studied, we also conclude that carbohydrate and the polyphenols in the extracts strongly influence the AgNPs synthesis.

## 7.0 Chapter: Conclusion and future prospective

Our overall observations as below

The mixed soil proved to be best for *Arabidopsis* growth and development with better health since the accumulation of anthocyanin is least. This optimised mixed soil may substitute for the growth of *Arabidopsis* plant for flowering time evaluation from Indian prospective. We further evaluated the flowering time in over-expressed lines of COβ in different genetic background. Our result showed a delay in flowering as compare to controls Col-0, *co-10* and *phyb-9* that suggested an involvement of a common mechanism. Our result with more chlorophyll and carbohydrate accumulation consistence with pod weight supported that the yield parameter in COβ lines is higher than the controls. We further observed a significant high level of COβ transcript in *gi-100* mutant lines that clearly suggested the absence of GIGANTEA may lead to improper splicing of *CONSTANS* (COβ) and that delays flowering in *At* in a photoperiod dependent manner. In continuation with other role of COβ we also observed the early flowering of these lines by CNPs treatments. In this study, we tried to understand the importance of CNPs in over-expressed line of COβ in *At.* From our results we conclude that CNPs could taken-up by the plant and accumulate in leaf tissues and can induce early flowering in PHYB, FT dependent and CO independent manner. In summary, we conclude that, CNPs serve as important agent to induce early flowering. Exploring the importance of CNPs in crop plants may open up a novel non transgenic approach for crop improvement.

Since the carbohydrate accumulation is high in COβ lines we tried to further evaluate the role of COβ in the AgNPs synthesis. In this regard plant extract of COβ and other genetic background as well as from Chilika lagoons were used for synthesis of AgNPs. We observed the synthesis of AgNPs is varied and dependent upon genetic background. The plant extracts enriched in carbohydrates consistently showed higher amount of AgNPs synthesis. Our result also suggested the sugar conjugated proteins are most likely important component over free-proteins for AgNPs synthesis irrespective of the sugar concentration of the extracts that need to further evaluate in future. We conclude that the ability of the plant species from Chilika lagoons to synthesize AgNPs

sparked hope for its use in industry. Production of AgNPs using *Arabidopsis* extract offered its usage in dissecting out the biochemical pathway that is involve in its synthesis through molecular genetics.

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**Publications**

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3. **Abhishek Kumar**, Aaram A. Kumar, Aditya P.Nayak, Priyanka Mishra, Madhusmita Panigrahy, PratapK. Sahoo, Kishore CS. Panigrahi.(2019) Carbohydrates and polyphenolics of extracts from genetically altered plant acts as catalyst for *in-vitro* synthesis of silver nanoparticle.Journal of Bioscience.

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1. Role of splice variant of CONSTANS (COβ) in flowering time control in *Arabidopsis thaliana.*

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**Poster presented**

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| | |
|---|---|
| ABRC | Arabidopsis Biological Resource Center |
| AGL20 | AGAMOUS-LIKE 20 |
| AgNPs | Silver Nanoparticles |
| *At* | *Arabidopsis thaliana* |
| B | Blue Light |
| CCA | Circadian Clock Associated |
| CCT | CONSTANS, CONSTANS-like and Time of CAB |
| °C | Degree celcius |
| Col-0 | Columbia ecotype |
| COP | Constitutive Photomorphogenesis |
| CRY | Cryptochrome |
| CFP | Cyan Fluoresces Protein |
| CNPs | Carbon Nanoparticles |
| CO | CONSTANS |
| COα | CONSTANSα |
| COβ | CONSTANSβ |
| D | Days |
| DOF | DNA-binding One Zinc Finger |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| Dnase | Deoxyribonuclease |
| dNTPs | deoxy Nucleotide Tri Phosphate |
| EC | Electric Conductivity |
| EDTA | Ethylenediaminetetra acetic acid |
| ELF4 | Early Flowering 4 |
| EtBr | Ethidium Bromide |
| FCA1 | Flowering time control protein A1 |
| FKF1 | Flavin-binding, Kelch repeat, F-box 1 |
| FLC l | Flowering Locus C1 |
| FW | Fresh weight |
| FR | Far red light |
| FT | Flowering locus T |
| FTRI | Fourier-transform infrared spectroscopy |
| GA | Gibrellic Acid |
| GI | GIGANTEA |
| h | Hour |
| HOS1 | High Expression of Osmotically Responsive Genes 1 |
| IDD | Indeterminate Domain |
| IDD14 | Indeterminate Domain14 |
| JAZ | Jasmonate ZIM-domain |
| K | Potash |
| Kb | Kilo Base |
| KV | Kilo volt |
| LD | Long Day |
| LFY | LEAFY |
| LHY | Late Elongated hypocotyls |
| µg/ µl | Microgram per micro liter |

| | |
|---|---|
| mg | Milli-gram |
| mm | Milli-meter |
| ml | Millilitre |
| µF……. | Micro Farad |
| µg $mg^{-1}$ | Microgram per milli gram |
| µm | Microgram meter |
| MiRNA | micro RNA |
| Min | Minute |
| MPIPZ | Max Planck Institute for Plant Breeding Research |
| MS | Murashige and Skoog |
| MWNT | Multi-walled carbon nanotubes |
| N | Nitrogen |
| nm | Nano meter |
| NMR | Nuclear Magnetic Resonance |
| NPs | Nanoparticles |
| OC | Organic Content |
| OD | Optical Density |
| PAT | Phosphinothricin acetyltransferase |
| P | Phosphorus |
| PCR | Polymerase Chain Reaction |
| pH | Potential of hydrogen |
| PHY…….. | Phytochrome |
| PHYB | Phytochrome B |
| PIF4 | Phytochrome Interacting Factor 4 |
| ppm | part per million |
| PVPP | Polyvinyl pyrrolidine |
| R | Red Light |
| RNA | ribo nucleic acid |
| RNase A | Ribonuclease A |
| Rpm | Revolution per minute |
| RPS4 | Ribosomal protein S4 |
| RT | Room temperature |
| RT-PCR | Reverse-Transcriptase PCR |
| SD | Short-day |
| SEM | Scanning Electron Microscopy |
| SOC1 | Suppressor of Overexpression of Constans |
| *spy-5* | *spindly*-5 |
| SWNT | Single walled carbon nanotubes |
| SL | Silique Length |
| SRR1 | Sensitivity to red light reduced |
| SW | Seed Weight |
| TAE | Tris-acetate Buffer |
| TE | Tris- EDTA |
| TOC1 | Time of CAB1 |
| TPC | Total Phenolic compounds |
| V | Volt |
| VRN1 | VARNALIZATION1 |
| WL | White Light |
| WT | Wild Type |
| YFP | Yellow Fusion Protein |

| | |
|---|---|
| W/v | Weight per volume |
| Zt | Zeitgeber |

# CHAPTER: 1

# INTRODUCTION AND REVIEW OF LITERATURE

# 1. Introduction

### 1.1 *Arabidopsis thaliana*

*Arabidopsis thaliana (At)* is a facultative long day (LD) plant (Johansson M et al., 2014). It flowers earlier under summer long days typically, with cycles of 16h light followed by 8h dark (LD) in a 24h diurnal rhythm (Karlsson et al., 1993; Redei 1962; Johansson M et al., 2014)). The flowering is normally delayed under winter short days (SDs) of about 16h dark and 8h light cycles (Garner et al., 1933). The timing to flowering of *At* is determined by the combinitorial interaction of developmental competence and environmental cues that signal the favourable conditions for reproductive success (Simpson G et al., 1999). Flowering is influenced by the duration of light and dark period, called photoperiod (Garner et al., 1920). The seasonal change in photoperiod is one of the very important environmental factors that affect flowering time (Kobayashi and Weigel, 2007; Amasino, 2010). It is widely distributed on earth from 10° N to 60°N (Li et al., 1998, Minorksy, 2001). *Arabidopsis thaliana* plant growth and its variation in morphology (Lacey, 1986; Potvin, 1986; Reinartz, 1984), phenology (Zhang et al., 1994), physiology (Minorksy, 2001) and growth rate (Li et al., 1998) have been well documented according to ecotype variations and belong to Brassicaceae family.

### 1.2 *Arabidopsisthaliana* a genetic model plant

*Arabidopsis* is an herbaceous, monocarpic, annual plant, a commonly growing road side weed, which bears an erect plant form. It is widely used for understanding of biological mechanisms in diverse range of researches in different parts of the world. Its wide acceptability and preferred use is imparted to its short life span of 90-120 days, simple genomic organization and genetic regulations. The plant growth varies according to environmental condition, 22-24°C temperature being the best growth condition for the plant. *Arabidopsis* is a long day plant and under natural conditions it grows up to 2 feet height, flowers at a minimum of 12-14 leaves. Unlike natural growth conditions which incorporate milder fluctuations in diurnal light, temperature and humidity, growth conditions in research laboratories maintain constant temperature, humidity

and light cycles. Under controlled growth conditions *Arabidopsis* grows up to 12 leaves stage under LD and 25 leaves under SD conditions after which it flowers (ABRC).

### 1.3 Soil composition for *Arabidopsis* growth

*Arabidopsis* requires certain kind of soil, nutrient and watering conditions because of which *Arabidopsis* biological resource centre (ABRC) has prescribed standard procedures for healthy growth of *Arabidopsis* in growth chambers. Perdue Agriculture standards describe "PRO-MIX BX" soil mix (Perdue plant growth facility) and Osmocote fertilizer mix for healthy *Arabidopsis* growth (ABRC). However, growth conditions of *At* in Indian conditions deviate significantly to the recommendations of ABRC standards due to non-availability of optimal similar soil mix. This introduces problems to attain standard growth of *At* in Indian soil conditions, which is the basic pre-requisite to perform experiments and obtain reproducible results. Here we have examined five different soil types available and also formulated a new soil mix proportion for optimum growth of *Arabidopsis* in growth chambers under controlled conditions. This will provide necessary guidelines for Indian researchers and set a base for obtaining reproducible results under controlled conditions.

### 1.4 Regulation of flowering time in *Arabidopsis thaliana*

Flowering is regulated by both positive and negative regulators that feedback each other in different flowering pathways such as autonomous, vernalization, geberrelic acid and photoperiodic pathways. The photoperiodic regulation of flowering time is controlled by circadian clock (Suarez-Lo et al., 2001). Under both LDs and SDs, a plant specific nuclear protein GIGANTEA is regulated by circadian clock, transcriptionally up regulate the master regulator B-box zinc finger protein CONSTANS (COα) (Truck et. al., 2008; Suarez-Lopez et al., 2001). The post-translational stability of COα protein accumulation is dependent on the synergistic activity of far-red light red-far red photoreceptor PHYA and blue light photoreceptors (CRY1 and CRY2.), COα protein is stabilized by the ubiquitin action and

degraded in a proteasome-dependent manner (Valverde et al., 2004). Among these, the distinct pattern of diurnal COα accumulation, which peaks in late afternoon under long days (LDs), is critical for induction of flowering (Valverde et al., 2004).The protein HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) triggers the degradation of COα in the early morning (Lazaro et al., 2012). Meanwhile, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) directs COα degradation at night (Jang et al., 2008; Liu et al., 2008).Notably, flavin-binding, Kelch repeat, F-box 1 (FKF1) works synergistically with blue light signals in the late afternoon to stabilize CO under LDs (Song et al., 2012b).

### 1.5 CONSTANS (CO*α*) a master regulator of flowering time

The accumulation of COα a transcription factor plays a key role in photoperiodic flowering by regulating the expression of the floral integrator *flowering locus* T (FT) (Corbesier et al., 2007; Andres and Coupland, 2012; Song et al., 2013). COα strongly induces *FT* expression by directly binding to *FT* promoter (Song et al., 2015). Mutation in either *COα or FT* genes delays floral transition under both LDs and SDs confirming the pivotal role of these two components in photoperiodic flowering-time control (Jang et al., 2008, Yoo et al., 2005). Over-expression of COα has been shown to abolish the photoperiod discrimination (Goto et al., 2003) and therefore leads to flowering roughly at similar time under both LDs and SDs. However, despite the constitutive over-expression of full-length of *CONSTANS* from a viral promoter (35S), *Arabidopsis* normally flowers only after about fifteen LD cycle. Despite recent advances in our understanding of the molecular mechanisms underlying the diurnal COα accumulation, it is currently unclear whether COα plays a some what active role in its own accumulation dynamics during developmental transition.

### 1.6 Importance of splicing in *Arabidopsis*

In the recent years, advancement of high-throughput sequencing-based analysis helped the study of alternative splicing and its dynamic interactions between the different proteins isoforms

became apparent to understand the regulative mechanism that regulates transcription activity in *Arabidopsis* (Seo et al., 2011, 2012; James et al., 2012; Staiger and Brown, 2013). It has been also suggested through the plant transcriptomes data that about the 60% of plant gene loci encode alternatively spliced mature transcripts (Sergei F et al 2015). These studies have also revealed that alternative splicing in plants can be regulated by cell type, developmental stage, the environment and the circadian clock.This is also high lights coupled to RNA surveillance and processing mechanisms, including nonsense mediated decay (Sergei F et al., 2015).Recently, non-protein-coding transcripts have also been shown to undergo alternative splicing (Sergei F et al., 2015).

It has been shown that the gene like *Circadian clock associated 1* (*CCA1*), an important clock component, undergo alternative splicing, that produces two protein isoforms such as CCA1a and CCA1b (James et al., 2012; Seo et al., 2012). The inhibitious activity of CCA1a through the CCA1b without DNA binding important in the physiological processes like the freezing tolerance (Seo et al., 2012). Similarly, the *indeterminate domain 14 (IDD14)* also showing alternatively splicing isoforms such as *IDD14a*, *IDD14b* and IDD14b the latter negatively regulate the IDD14a activity by forming heterodimers (Seo et al., 2011). The JASMONATE ZIM-domain (JAZ) a proteins act as repressors of jasmonate (JA) signalling pathway also have splice variant (JAZ10.4 and JAZ10 that lack the Jas domain and, as a consequence, is highly resistant to JA-induced degradation (Hoo SH et al., 2009). Recent studies in the tobacco N and the *Arabidopsis* RPS4 genes, both encode Toll-like plant R proteins, showed that intron-deprived genes have reduced or no activity, suggesting that alternative splicing plays a crucial component in these signalling pathways (Tina J et al., 2002). Similar alternative splicing mechanisms also have been observed in intrinsic region of miR400 (intron 306 bp) that down regulated the host gene At1g32583 when it was co-transcribed because of alternative splicing specifically induced by heat stress (Kang Y et al., 2012).

In silco data suggested the there are two splice form of CONSTANS (Mater thesis of Alexander Ternois MPIPZ, 2008) one is proper splice form of *CONSTANS* (COα) and another is an improper splicing form of *CONSTANS* (*COβ*).The correctly splice form of CONSTANS (COα) is about 373 amino acid long containing B-box domain in N-terminal half and CCT (CONSTANS, CONSTANS-like and Timing of CAB) domain in C-terminal part. It has been shown that B-BOX motifs involved in protein - protein interaction (Tripathi et al., 2016). The CCT domain within the second half of the CCT motif has been shown to be involved in nuclear localization and in light signal transduction (Strayer et al., 2000).

The other improper splice form i.e. the truncated form of CONSTANS (COβ) which is about 274 amino acid long if transcribedwould be contains B-box domain only.The transcript of (COβ) that if encoded, would produce a truncated protein devoid of C- terminal half. In current study we demonstrated that in the absence of GIGANTEA, improper splicing of *CONSTANS* occurs and that delays flowering in a photoperiodic dependent manner in *At.*The extract of these transgenic plants also enhance the silver nanoparticle synthesis.

**1.7 Role of nanoparticles and carbon nanoparticles in plants**

Nanomaterial based on carbon has been used to understand plant development and productivity (Zaytseva et al., 2016; Sera et al., 2015; Sekhon, 2014; Husenand Siddiqi 2014; Ditta, 2012). Carbon nanomaterials fall into several categories such as nanotubes, fullerenes, nanoparticles, Nano horns, Nano beads, dots, Nano fibres and Nano diamonds (Salata 2004; Nowack and Bucheli 2007;Sharon and Sharon 2010;Hossain et al., 2015; Mukherjee et al., 2016; Wang et al., 2016). Among these, single walled carbon nanotubes (SWNT), multi-walled carbon nanotubes (MWNT) and carbon nanoparticles (CNPs) attracted attention and have been studied in plants (Mukherjee et al., 2016; Garcia-Sanchez et al. 2015). They can have both positive and negative effects on plant development (Mukherjee et al., 2016). SWNT and MWNT have been shown to be beneficial in tobacco (Samaj et al., 2004) and in rice (Lin et al., 2009). These agents penetrate

and are internalized into plant roots serving as nano-transporters that probably facilitate nutrient uptake. Carbon nanotubes can increase tomato production up to two-fold in tomato, and fullerenes can lead to significant increase in the medicinal compounds in bitter melon seeds (Husen and Siddiqi 2014). However, exposure to SWNT and MWNT showed deleterious effect in *Arabidopsis thaliana* (*At*) resulting in cell aggregation, chromatin condensation, $H_2O_2$ accumulation, decrease in cell dry weight, cell viability, chlorophyll content and superoxide dismutase activities (Zaytseva et al., 2016). Induction of programmed cell death was observed in *At* and rice due to SWNT (Shen et al. 2010). Despite these studies, the effect and mechanism of CNPs on various physiological processes in different plant systems are not well studied.

Here, we investigated the role of CNPs on the seedling development and flowering in *At*. Seedling development in *At* starts after germination and includes several photomorphogenic events. These processes include hypocotyls growth inhibition, opening of apical hook, cotyledon opening.Cotyledon expansion, synthesis of chlorophyll and root growth. All of these are controlled by various photoreceptors; among which phytochrome is extensively studied as the Red (R) and Far-red (FR) light receptors. Upon activation by light, the inactive Pr form (Pr indicates the red-light absorbing form) is converted into the active Pfr form (Pfr indicates the far-red light absorbing forms). The Pfr form of the phytochrome further interact with several down-stream signalling components including transcription factors, activators or repressors to elicit the plethora of photomorphogenic responses. The developmental plasticity is thus fine-tuned according to various environmental stimuli. In *At*, the phytochrome gene family consists of five members (PHYA-E). Out of these, PHYB is detected as the major light stable phytochrome for regulating most of the photomorphogenic responses. PHYB has also been shown to be the most important photoreceptor for sensing and responding to shade. A drastic reduction of R to FR ratio in the incident light between 0.05 - 0.7 is defined as shade, which naturally occurs underneath the canopies (Franklin et al., 2005). Shade signals cause

distinguishable changes in the plant form including elongation of hypocotyl, petiole, stem, increased height, increased chlorophyll and accelerated flowering times which are collectively known as shade avoidance syndrome (Casal, 2012). Under shade, increased abundance of Phytochrome Interacting Factor 4 (PIF4), PIF5, degradation of DELLA proteins, increased synthesis and redirection of auxin are the major events among the molecular processes that arise down stream of PHYB (Casal, 2012). PHYB also plays a major role in regulation of flowering time in a photoperiod dependent manner. It acts antagonistically with Phytochrome A to activate GIGANTEA (GI) and stabilize CONSTANTS (CO), which in turn positively regulate FLOWERING LOCUS T (FT) under summer long-days (Song et al., 2015; Valverde, 2004).

### 1.8 Importance of carbon nanoparticles in flowering time

Floral transition between the vegetative and reproductive phases of plant development is an important event not only to manipulate yield but also as a mechanism to escape abiotic stress (Song et al., 2015). Flowering is regulated by a complex network of various environmental factors which quantitatively modulate the flowering-time integrators (Kumar et al.,2012; Fornara et al., 2010;Mouradov et al., 2002) such as FT, LEAFY (LFY) and SUPPRESSOR OF OVEREXPRESSIONOFCONSTANS (SOC1), also known as AGAMOUS-LIKE 20 (AGL20) (Samach et al., 2000). Molecular and genetic studies have shown that at least four distinct mechanisms leading to floral transition are possible: (i) Photoperiod (duration of light), (ii) Low temperature (vernalization), (iii) Gibberellic acid (GA) and (iv) Autonomous pathway (Boss et al., 2004). Photoperiod pathway stimulates flowering under inductive long-days (LDs). Signals perceived by the photoreceptors together with the nuclear proteins such as ELF3 and GI entrain the circadian oscillators, which consists of LHY, CCA, TOC1 and ELF4 (de Montaigu et al. 2010). Changes in the day length is detected by the circadian clock and transduced to the photoperiod components by initiating cyclic expressions of COα. Under short-days (SDs), COα expression peaks at the night phase, whereas under the LDs its expression overlaps with the end-

of-the-day light phase and this expression pattern leads to the stability of COα that mirrors the FT accumulation. Under LDs, toward the middle of the day the accumulation of GI along with FKF1 forms a complex that degrades the DOF repressors which allows transcription of *COα*, thereby inducing expression of the floral promoter FT (Fornara et al., 2009; Sawa et al., 2007; Imaizumi et al., 2003). By contrast under SDs, FKF1 accumulatesCOα ~3h after GI a peak that does not allow the formation of the GI-FKF complex, thereby leading to a low abundance of *COα* transcripts, hence delays timing to flower. In vernalization pathway, signal from prolonged cold is perceived by the plant which strongly down-regulates the FLC level that stimulates flowering. This mechanism is controlled by the combinatorial effect of *VRN1* and *VRN2* gene products. The plant growth regulator GA can also promote flowering in a photoperiod independent manner (Khan et al., 2014). It has been shown that mutation in the repressor of GA signalling like SPINDLY can also induce flowering most likely antagonizing the effect of GI. The genetic interaction of GI and SPINDLY has also been documented and reviewed (Mishra et al., 2015). In the autonomous pathway, components involved in RNA processing and epigenetic regulation such as FCA and FLC have been characterized that modulate the flowering-time integrators at the shoot apical meristem (Khan et al., 2014). Genes of the autonomous pathway constitute a subgroup which target FLC expression and function to reduce its accumulation (Sheldon et al., 1999; 2000).

In the present study, we have investigated the uptake, accumulation and effect of CNPs on development of *Arabidopsis thaliana* and evaluated seed germination rate, chlorophyll content, photomorphogenesis and flowering time. Our data showed that CNPs induces the shade avoidance response in seedlings and also accelerates flowering in PHYB-dependent and CO-independent manner.

### 1.9 In vitro synthesis of silver nanoparticles by plant extracts

Nanoparticles synthesis using plant extracts with different phytochemical agents have drawn more attention than the existing physical, chemical and hybrid methods due to their adaptability in surgical, pharmaceutical purposes and reduced use of unsafe solvents that they offer (Abdel-Halim et al., 2011). “Green nanotechnology” can replace the need of chemical synthesis and significantly reduce the amount of hazardous waste products (Singaravelu et al., 2007). The list of biological agents used in “green” synthesis of nanoparticles (NPs) includes different organisms such as plants, algae, fungi, yeast, bacteria and their bioactive components (Chung et al., 2016, Kumar et al., 2012, Sastry et al., 2003). The use of nanomaterial’sthemselves as therapeutic and immune-modulatory agents is prevalent as seen in the application of silver NPs at surgical or ectopic infection sites as antibacterial agents (Morones et al., 2005; Tripathy et al., 2009). Silver as nanoparticle have got special attraction due to their striking characteristics such as catalytic, antibacterial activity and chemical stability (Sharma et al., 2009) To this end green synthesis has been successfully accomplished from different plants, algae, yeast, bacteria, microorganisms, biopolymers (Sharma et al., 2009) Plant extracts from about 41 species specifically from leaves have been extensively exploited for AgNPs synthesis (Shankar et al., 2003; Chandran et al., 2006; Chung et al., 2016).In most of the cases, AgNPs were synthesized from leaf extracts, while in few cases other plant parts such as stem, petals, rhizome, root, fruit, seed, latex, bark, whole plant and see-weeds were also used. The shapes of synthesized AgNPs ranged from circular, cubical, rectangle, and optical, crystalline, poly-dispersed, triangular, cuboidal, while spherical being the most usual shape. Size of the AgNPs synthesized ranged from 2 nm in the extracts of *Euphorbia helioscopia* to 250 nm in the extracts of *Carica papaya* (Chung et al., 2016).

### 1.10 Important of secondary metabolites for silver nanoparticles synthesis

It has already been shown that the secondary metabolites of the plant extract such as phenolic, alkaloids, terpenoids and flavonoids are responsible for the reduction of ionic metal into

metallic nanoparticles (Chung et al., 2016). It has also been documented that the first bio-reduction of silver occurs by trapping of the silver ions on the surface of the proteins due to electrostatic interactions, leading to changes in the secondary structure of the metal ion and formation of silver nuclei (Li et al., 2007). Further, the aldehyde, amide and polypeptides are known for the subsequent reduction of silver ions at the silver nuclei leading to metallic AgNPs. The functional group –C=O and −C≡N of the amides could efficiently cap the ionic substances into metallic nanoparticles. Unique pH of different plant extracts can regulate the size and shape of silver nanoparticles, with lower pH (potential of hydrogen) is most often being favourable for larger nanoparticles being synthesized (Mock et al., 2002).

### 1.11 Understanding the molecular mechanism of silver nanoparticles synthesis

However, the exact mechanisms of involvement of the different phytochemicals and pH in controlling the variable shape and sizes of AgNPs are poorly understood (Shameli et al., 2012).Properly controlled biological systems could work as efficient "Nano-scale production factories" to yield nanomaterial of desired properties. However, this would require a greater understanding of the precise mechanisms to regulate its synthesis. Despite all these excellent discoveries, there has been no model plant optimized for in vitro synthesis of AgNPs under controlled conditions. In this study, we have experimented to establish *Arabidopsis thaliana* (*At*) as the model system for understanding the in vitro synthesis of AgNPs. Moreover, for the first time using genetically altered *At* plants to show the biochemical basis of AgNPs synthesis as a proof of principle.

In current study first we optimized the all essential parameters such as temperature, light intensity, relativity humidity and soil composition i.e. required to evaluate the flowering time in *At.* We have tested five different soil types available and also formulated a new mixed soil (Soilrite, Red soil and Garden soil in proportion of 5:4:1) for optimum growth of *At* in controlled environmental conditions. We have standardized and fixed the soil and nutrient requirement for

*At* growth in growth chambers in local conditions of Bhubaneswar, Odisha. We also showed that in the absence of GIGANTEA improper splicing of *CONSTANS* occurs and that delays flowering in *At*. However,the early flowering after treatment of carbon nanoparticles to the COβ over-expression plant or even in Col-0 suggests the involement of common mechnaisms. The extract of this transgenic plants also enhances the silver nanoparticle synthesis.

The objectives of the present study are

1. Optimization of soil composition for flowering time evaluation in *Arabidopsis thaliana*

2. Generation of transgenic lines and evaluation of flowering time with phenotypic characterization of COβ in different genetic background (Col-0, *co-10* and *phyb-9*)

3.To Study the of expression pattern of *COβ* transcript at various diurnal and developmental stages

4. To understand the role of Carbon nanoparticles (CNPs) in flowering time in COβ and Col-0 background in *Arabidopsis thaliana*

5. To understand the role of COβ in silver nanoparticles synthesis

# CHAPTER: 2

# MATERIALS AND METHODS

# 2.0 Materials and Methods

## 2.1 Materials used

### 2.1.1 Soil types

Clay soil local available soil outside NISER campus from local rice fiel, Jatni. Red soil local available soil at NISER campus Jatni. Garden soil local soil available nursery at Bhubaneswar. Soilrite was obtained from Keltech Energies limited, Bangalore, which was a mixture of horticulture grade expanded perlite, iris peat moss and exfoliated vermiculite in equal ratio.The new mixed soil components were designed by us which is a mixture of soilrite mix: red soil: garden soil proportion of 5:4:1. All five kinds of soil were initially autoclaved to avoid contamination and cooled before use.

### 2.1.2 Chemicals used

$NH_4NO_3$, $HNa_2PO_4$, $AgNO_3$, NaOH, Tris-Cl, EDTA, Glacial Acetic Acid, Potassium Acetate, Agarose, and KCL.

### 2.1.3 Plant materials

The plant species *Potamogeton pectinatus, Enteromorpha intestinalis, Gracilaria verrucosa* and *Scenedesmus acuminatus* were collected from the back waters of Chilika Lake, Odisha, India. Seeds of *Arabidopsis thaliana* Columbia ecotype (Col-0), *gigantia-100* (*gi-100), spindly*-5 (spy-5) 35S::GI and 35S::CONSTANSβ (35S::COβ) were used.

### 2.1.4 Plasmid vector

| | |
|---|---|
| Entry vector | pDNOR201 |
| Source/Vendor | Invitrogen, |
| Plasmid Type | Gateway Donor vector |
| Plasmid Size | 4470bp, |
| Bacterial Resistance | Kanamycin. |

### 2.1.5 Expression vector

We used different Gateway destination vectors such as 35S::GW, 35S::YFP:GW and 35S::CFP:GW.

### 2.1.6 *Escherichia coli* strain

DB3.1, DH5α, as mentioned by the Gatewaycloning method.

### 2.1.7 *Agrobacterium tumefacience* strain GV3101 ((pMP90)

Agrobacterium tumefaciens strain GV3101(pMP90) (Koncz and Schell, carrying the binary plasmid pBINm-gfp5-ER (Haseloff et al., 1997) was used in this experiment.

### 2.1.8 Carbon Nanoparticles

We purchased from Sisco Research Laboratories Pvt. Ltd. India as Graphite Nano powder, Type-1, APS: 400 nm.

### 2.1.9 MS medium

From Himedia (Catalogue # PT100-1L).

### 2.1.10 RNeasy plant mini kit

**(**Cat No: 74904, Qiagen**)**

### 2.1.11 DNeasy Plant mini Kit

**(**Cat No: 69104, Qiagen**)**

### 2.1.12 YEBS media

| | |
|---|---|
| Yeast extract | 1.0 g/L |
| Beef extract | 5.0 g/ L |
| Bcto-peptone | 5.0 g/L |
| Sucrose | 50.0 g/L |
| Magnesium Sulphate | 0.5 g/L |
| pH (adjusted to) | 7.0 |

### 2.1.13 LB media

| | |
|---|---|
| Bcto-peptone | 10.0 g/L |
| Yeast Yeast | 5.0 g/L |
| Sodium Chloride | 5.0 g/L |

### 2.1.14 TAE Buffer

For one liter 50 x (stock Solution), 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA pH 8.0. Working solution is 1x.

### 2.1.15 Miniprep solutions

Solution I: 50 mM Glucose, 25 mM Tris-Cl pH 8.0 and 10 mM EDTA pH 8.0

Solution II: 0.2 N NaOH (freshly prepared), 1% SDS (freshly prepared)

Solution III: For 100 ml: 60.0 ml,5 M Potassium acetate,11.5 ml glacial acetic acid and 28.5 ml distilled water.

### 2.1.16 Hard-shell 384-well PCR plates

(Supplied by Bio-Rad, Cat #: HSP3805)

### 2.1.17 Equipments used

**1. Gel DOC**

BIO-RAD, Molecular imager Gel DOCCTMXR, Imaging System**,** Model No: Universal Hood, Serial no: 721BR01263, Input: 100/115/230V, Maximum rated Power: 200VA, Frequency: 50/60 Hz System

**2. Merck Milli Pore System Water** Purification System

Cat no: ZLXE010WW, Serial no: F4DA38148B, Mfg.dates: 30-APR 2014

**3. Spectrophotometer A23616**

Back Man Coulter, DU$^{R}$ 720, and general-purpose UV/Vis spectrophotometer**,** Frequency: 50/60 Hz

4**. Electroporation**

BIO-RAD**,** Model No: Gene PulserXCell$^{TM}$ with Bio-Rad**,** Module: PC and CE module, Serial no: 617BR107392, Input: 100-120/220-240 V, Maximum Current: 2A, Frequency: 50/60 Hz

**5. Thermo-Cycler**

Master Cycler Pro S Vapo Protect, Model No: eppendorf AG (6325), Serial no: 6325YL202561, Frequency: 50/60 Hz

**6. Q-RT: Bio-Rad CFX384TM Real Time PCR**

**7. Centrifuge**

Model No: eppendorf AG (6325)**,** Serial no: 5426YN131059, 5426YN231063**,** Maximum Current: 1-4 A, Frequency: 50/60 Hz

**8. Thermo- mixture: Comfort, made in Germany**

Model No: eppendorf AG (2331), Serial no: 53552694603, Input: 100-240 V, Maximum Current:1-6 A, Frequency: 50/60 Hz

**9. Laminar air flow, NUNRE**

Model No: NU-201-430E, Serial no: 134054111709**,** Input: 230V, Maximum Current: 4A, Frequency: 50/60 Hz

**10. Rotospin:**

TARSON**,** Cat No: 3090XSerial no: 1501RMM051 Input: 240V, Maximum Current: 4A,

Frequency: 50 Hz

**11. Heating Block**

TARSON**,** Cat No: 6040, Model No: Spigot digital module MC02, Serial no: D1601009, Input: 230V, Maximum Current: 4.9A, Frequency: 50 Hz

**12. Shaker LABNET**

Model No: S2030-RC-220, Serial no: 0982020, Input: 230V, Maximum Current: 0.25A, Frequency: 50/60 Hz

**13. Spectrophotometer JASCO** made in JAPAN

Model No: V-730, Serial no: A037361798, Input: 240V, Frequency: 50/60 Hz

**14. Microscope: Olympus Jpto system India, Pvt.ltd** were visualized by Olympus microscope (Model: 1X73/m. PS.O Camera and S/W) at 100x magnification

Model No: CH201B1MSerial no: 16C3565, Input: 220- 240V, Maximum Current: A, Frequency: 50/60 Hz

**15. Electronic Balance**

Sartorius, quintix**,** Serial no: 0032905891, Maximum weight: 220g

**16. Digital Dry bath Labnet**

Model No: D1200-230VSerial no: Se2A13337, Input: 230V, Maximum Current: 1A, Frequency: 60 Hz

**17. Vortex mixer**

Labnet Model No: S0200-230V-EU**,** Serial no: 25020039, Input: 230V, Maximum Current: 0.3A, Frequency: 60 Hz

**18. Tissue Lyser 11** Manufacture by Retsch, Qiagen

Model No: 20-74-0001, Serial no: 129030717, Input: 100-240V, Maximum Current: 4.0 A, Frequency: 50/60 Hz

**19. 525 nm Grunlichtleuchte**

Albert-Ludwigs universities, Freiburg, Green light, Serial no: 2008-0003-10, Input: 230V AC, Maximum Current: 4.0A

**20. Percival Growth Chamber**

Model No: CU36L6, Serial no: 14743.08.10L, 14743.08.10L Input: 230V, Maximum Current: 10 A, Frequency: 50 Hz

**21. Incubator Shaker series**

New Brunswick, Model No: Innova 42, Serial no: A037361798, Input: 230V, Frequency: 60 Hz

**22. Incubator Shaker series**

New Brunswick Model No: Innova 44 R. Serial no: 110154384, 110254886, Input: 230V,

Frequency: 50 Hz

**23**. **Spectrophotometer**

JASCO made in JAPAN Model No: V-730, Serial no: A037361798, Input: 240V, Frequency: 50/60 Hz

**24. Confocal microscope**

Leica microsystem (ILS, Bhubaneswar)

**25.Epson L220 scanner**

**26. Raman spectroscopy**

EzRaman Reader V5.8.5 (EzRaman S/N 158270, E.McDurmottste A-1 Irvine CA 29614, USA)

**2.2.1 Cloning strategy**

*Gateway Cloning method*

**2.2.1a Designing attB PCR Primers for *COβ*amplification**

F1 Forward Primer COβ
5‘GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTGAAACAAGAGAGTAAC- 3'

R1 Reverse primer for COβ

5‘GGGGACCACTTTGTACAAGAAAGCTGGGTCTCA CCCTGCTGCGTTATG GGT 3'

### 2.2.1b Amplification of attB- PCR Products: PCR Conditions

For the 25.0 µl reaction mixture of PCR consist of the following composition

| | |
|---|---|
| Reaction Buffer | = 2.5µl |
| dNTPs | = 0.5 µl |
| Forwadr Primer | = 0.5 µl |
| Reverse Primer | = 0.5 µl |
| DNA template | = 1. 0 µl (50-80 ng/ µl) |
| Taq DNA Polymerase | = 0.2 µl |
| Water | = 25 µl to adjust the volume |

The PCR thermo cycler program was used as follows: 95°C: 5 min, 95°C: 30 s, 57°C: 30 s, 72°C: 1.30 min and 72°C: 5 min.

The amplified product of PCR was checked by 1.5% agarose gel electrophoresis. The size of the PCR products was determined by the comparison with a DNA ladder run on the same gel along with the amplified products.

### 2.2.2 Purifying attB-PCR Products

**By Poly ethylene Glycol (PEG) Purification method**

This method is useful for removing the DNA less than of 300 bp such as prime dimer.The 50 ul of amplified attB-PCR product was added in 1.5 ml eppendorf tube with 150 µl of TE, pH 8.0. Then 100 µl of 30% PEG 8000/30 mM $MgCl_2$ was added in same tube. Sample was vortexes thoroughly and centrifuged immediately at 12,000 x g for 15 minutes at room temperature and removed the supernatant. The settle down pellet was dissolved in 50 µl of TE buffer, pH 8.0. The quality of recovered *att*B-PCR product was checked on 1.5% agarose gel.

### 2.2.3 Creating entry clones

Gene of interest was cloned between the gateway site in entry vectors plasmid, pDNOR201 by using the BP recombination reaction**.**

### 2.2.4 BP recombination reaction

The BP recombination reaction takes places between an *att*B-flanked DNA fragment and an *att*P-containing donor vector to generate an entry clone. The BP Clonase™ enzyme mix was removed from -

20°C and thawed on ice (~ 2 minutes) and BP Clonase™ enzyme mix was vortexed briefly twice (2 seconds each time). 2 µl of BP Clonase™ enzyme was added to each sample above and mixed well by vortaxing briefly twice (2 seconds each time). The reactions were incubated at 25°C for 1 hour. Proteinase K solution was added 1.0 µl of to each reaction tube and incubated for 10 minutes at 37°C.This 1.0 µl of BP recombination reaction taken out and transformed the E.coli DB3.1 competent cell and plated on antibiotic LB agar and incubated for overnight at 37°C. Observed colonies were used for plasmid minipreparation.

| | |
|---|---|
| attB-PCR product (30 ng/ µl) | = 1.0 µl |
| PDONR201 (150ng) | = 1.0 µl |
| TE buffer | = 6.0 µl |
| BP clonase mix | = 2.0 µl |
| Total | = 10.0 µl |

### 2.2.5 Preparation of competent cell of *E coli* strain

Streaked LB plate of glycerol stock of DB3.1 and DH5α strain. Plates were left for over night at 37°C. Single colony was taken added 5ml LB broth and grown for 3-4 h at 37°C with vigorous shaking at 220 rpm. 5ml inoculated broth was added into 100 ml of LB broth. The OD (600 nm) was checked an interval of 50 to 60 minute. When OD reached about 0.5, flask was inoculated and kept inside the ice, for 30 minutes. Culture was centrifuged at 5000 rpm for 5 minutes at 4°c. The supernatant was discarded and washed the pellet with mixing 10 mL cold 0.1M $CaCl_2$ gently by pipet controller inside the ice inside the laminar hood. Similarly, culture was centrifuged at 5000 rpm for 5 minutes at 4°c. The supernatant was discarded and washed the pellet with mixing 10 mL cold 0.1M $CaCl_2$ gently by pipet controller inside the ice inside the laminar hood. The supernatant was discarded, and pellet was gently resuspended on 20 mL cold 0.1MCaCl with 15% Glycerol. 50 µl of competent cell was aliquoted in each 1.5 ml eppendorf tubes and kept at -80°C.Viability test of competent cell was carried out. These competent cells we used for transformation up to 3 months.

### 2.2.6 Transformation of competent cell

50µl of competent cell was taken from -80°C and store on ice. 1µl of entry clone was added (plasmid) in tube within 5-10 minutes and mixed by tipping. The tube was stored on ice for 30 minutes. Tube was transferred at 42°C preheated water bath for 30-40 second without shaking. The tube was transferred to ice for 3-5 minutes. 800 µl of LB was added in tube and incubate at 160 to180 rpm for 45-50 minutes at 37°C for recovering and expressing the antibiotics resistance. The culture was centrifuged at 6000 rpm for 1 minute. The supernatant was removed of about 700 µl and mixed gently remaining solution by pipetting up and down. The mixed cell of 20 µl was taken and spared on appropriate antibiotics plate by spreader. When liquid has been absorbed from plate, incubate the plate at 37°C for 12 to 16 h by inverting the plate. The transformed colonies were observed.

### 2.2.7.Minipreparation of transformed colonies

In 4 ml of LB culture (LB+ Antibiotics) single colonywas inoculated and grown at 37°C for 14 hours. 1.5 ml of culture was taken in eppendorf tube and centrifuge at 13000 rpm for 1 minute and supernatant was removed. Again 1.5 ml of culture was taken in same eppendorf tube and centrifuged at 13000 rpm for 1 minute, and supernatant was removed. The pellet was resuspended in 200µl of ice-cold solution I, and tube was vortexes vigorously so that pellet was dissolved properly. 400µl of solution II was added and inverted the tube rapidly 5 times gently and incubated at room temperature for 5 minutes. 300µl of ice-cold solution III was added and inverted the tube 20 times rapidly. Then tubes were kept inside the ice for 5 minutes and centrifuged at 13000 rpm for 12 minutes at 4°C. The supernatant was transferred in fresh eppendorf tubes and add equal volume of phenol: chloroform: isoamyl alcohols (25:24:1) solution and tubes were inverted 20 times. The tube was centrifuged at 13000 rpm for 7 minutes at 4°C. The supernatant (aqueous phase) was taken in fresh tubes and added one volume of ice chilled isopropanol and vortexes gently and kept on ice for 30 minutes and centrifuged at 13000 rpm for 10 minutes at 4°C. The supernatant was discarded and added 1ml of 70% ethanol

inverted the tubes 10 times and centrifuged at 13000 rpm for 5 minutes at 4°C. Supernatant was discarded and dried the pellet at 37°C for 30 minutes. 30µl of TE buffer was added containing 20 µg/ml. RNase A The tubes were stored at -20°C for further use.

### 2.2.8 Selection of positive clone

By PCR based selection as mention in section 2.2.1b.

### 2.2.9 Creating expression clones by LR recombination reaction

This expression clones were created inbetween the entry clone and a destination vector by using LR recombination reaction. The reaction mixture was transformed into an *E. coli* DH5α strain.

The LR Clonase™ enzyme mix was taken out from -80°C and thawed on ice for ~ 2 minutes. LR Clonase™ enzyme was vortexes briefly twice 2 seconds each time. 4 µl of LR Clonase™ enzyme mix was added to tube and mixed by overtaxing briefly twice 2 seconds each time. Reactions tube was incubated at 25°C for 1 hour. 2 µl of the Proteinase K solution was added to each tube and incubated for 10 minutes at 37°C. The 1 µl of recombinase reaction was used for transformation with *E. coli* strain DH5α.

### 2.2.10 Transformation of expression clone in *Escherichiacoli*strainDH5α

Transformation was done as mention in section 2.2.6.

### 2.2.11 Selection of positive clone

Antibiotic based selection and then PCR based selection after minipreparation of positive colonies were done as mention in section 2.2.1b. Positive expression clone was transformed to *Agrobacterium* competent cell GV3101 (pMP90) by electroporation method.

### 2.2.12 Agrobacterium competent cell preparation

LA plate (Rifampicin) was streak with glycerol stock of GV3101 (pMP90) *Agrobacterium* strain. Plate was Left for two days for growing at 28°c. The single colony was taken and added into 5 ml LB broth, grown for over-night at 28°C with vigorous shaking at 220 rpm. 5 ml of inoculated broth was taken and added into 100 ml of LB broth having Rifampicin (50µg/µl). OD

(600nm) was taken an interval of 50 to 60 minute. When OD was reached about 0.5, inoculated flask was kept inside the ice, for 25 minutes. The inoculum was centrifuged at 5000 rpm for 5 minutes at 4°c. The supernatant was discarded and washed the pellet with 40 ml of 10% glycerol 3 times. 1 ml of 10% glycerol was added in tube and aliquoted 50 µl in each eppendorf tubes and kept at -80°C.Viability test of competent cell was carried out in rifampicin LA plate. The competent cells were used up to 3 months.

**2.2.13 Electroporation method**

The optimised electroporation condition for efficient transformation was as follow

1. Capacitance = 25 µF
2. Voltage = 2.4 KV
3. Resistance = 200 Ω
4. Pulse length = 5 Second

The 1.5 ml of eppendorf tube containing 50µl competent cell of *Agrobacterium* was taken from -80°C and kept on ice inside the laminar hood. 1µl of plasmid containing insert was added and gently mixed the tube by pipetting and transferred to pre-chilled electroporation corvette. The cell was shocked by electroporation machine 2 to 3 times in above mention optimised conditions. Immediately after the electroporation 1ml of LB was added inside the laminar hood to the cuvette and transferred the bacterial suspension to 1.5 ml eppendorf tube. The cell was incubated for 8 h at 175 rpm and 28°C. The cell was collected by centrifuging at 6000 rpm for 1 minute. The supernatant was discarded about 800µl and mixed the remaining cell broth slowly with pipette. The 30µl of cell broth was taken and spreaded on the plate containing antibiotics (Rifampicin anotherconstructs selected antibiotics). Plates were wrapped with aluminium foil and incubated for 2 days at 28°C. The colonies were observed. The transformed *Agrobacterium* strains with expression clone were selected on LB plate containing with Rifampicin (40 µg/ml) and construct specific antibiotic. Colonies PCR were performed to confirm the presence of

desired insert. These positive colonies were transformed to the plants by floral dip method of transformation.

### 2.3 Floral dip method of transformation

Gene with gateway overhang in *Agrobacterium* was cultured in 100 ml YEBS media. 160µl of rifampicin (stock 25 mg/ml) and 100 µl of ampicillin (stock 100 mg/ml) was added to the YEBS media. The culture was set in a shaking incubator at 220 rpm and 28$^{o}$C and culture was grown until the O. D reached 0.8 at 600 nm wavelength. The 0.02% Silwet 77 was added to the culture as the Silwet being a surfactant ensures that the bacteria uniformly cover the entire surface. The aerial parts of the plants were dipped in the *Agrobacterium* culture for 60 seconds. Care was taken that the soil did not touched the bacterial culture. The plants were kept in dark (1 hour without cover and 14 hours wrapped in a polybag) since the T-DNA transfer is a light sensitive process. The plants were transferred to normal light conditions and again second time transformed plants were dipped in same component of fresh media after 4 days of interval. Transformed plant were grown in optimised growth conditions andseeds were harvested in polythene bags. Harvested seeds were grown in mixed soil in control growth conditions and positive plants were selected by BASTA (BASTA (Phosphinothricin) is a broad-spectrum herbicide and derivative of glufosinate. It irreversibly deactivates the enzyme glutamine synthetase, which is essential for production of glutamine and ammonia detoxification. Phosphinothricin acetyltransferase (Pat) is an enzyme which inactivates phosphinothricin by adding an additional acetyl group and thereby acts as a selectable marker. The transformed plant shall bear the gene thus can survive after the application of phosphinothricin, whereas the non-transformed plants will die in the presence of BASTA.

### 2.4 BASTA selection

Transformed plants were grown in optimised growth condition till to maturation stage and mature siliques were collected in seed collection bag. Seeds have been dried for 48 h at room

temperature. Dried mature seed were grown on mixed soil in optimised growth conditions. The 0.1% BASTA was sprayed on the 5 days old seedling. BASTA selected plant were grown in optimised growth conditions and again second time BASTA selection was performed after 4 days of interval. The resistant plants were survived while the non-resistant plants died eventually with pale yellow colour. Positive BASTA selected plants were transplanted on new soil bed and further it was confirmed by PCR with gene specific primers. The plants (called F0 generation) were similarly grown for F1 and F2 generation.

**2.5 Generation of transgenic lines**

The F2 positive plants were further grown and seeds were collected separately in collecting bags. The harvested seeds were grown in optimised growth conditions. Basta selection was performed on 5 day's old seedlings. The mature seeds were harvested. The following over-expressed and miss-expressed transgenic lines of COβ were generated in Col-0, *co-10* (CONSTANS mutant) and *phyb-9* (Phytochrome mutant) in Columbia genetic background as the follow

| | | |
|---|---|---|
| **1**. 35S::COβ Col-0 | **2.** 35S::CFP:COβ Col-0 | **3.** 35S::COβ *co-10* |
| **4.** 35S::YFP:COβCol-0 | **5.** 35S::CFP:COβ*co-10* | **6.** 35S::YFP:COβ*co-10* |
| **7.** 35S::CFP:COβ *phyb-9* | **8.** pSUC2::COβ Col-0 (Phloem specific promoter expressed transgenic line) | **9**. pKNAT1::COβCol-0 (Meristematic specific promoter expressed transgenic line) |

**2.6 Bulking of seeds**

The F3 generation of plants were similarly grown in controlled environmental conditions and mature seeds were collected for further experimental purposes.

**2.7 Disinfection of Seeds**

Some seeds were taken in setline tube inside the laminar hood. 800 µl of 70 % ethanol was added in tube and kept for 5 minutes inside hood with gentle tipping. Seeds were spin at 2500 rpm for 30 second, and alcohol was discarded from the tubes. 100 % ethanol was added in

eppendorf tube and kept for 5 minutes inside hood with gentle tipping. Tube was spin at 2500 rpm for 30 second and alcohols was removed. Again, seeds were spin at 2500 rpm for 30 second. The sterilized tubes cap was open inside hood and keep for 2 h to evaporate alcohols. Meanwhile a tube was slowly tipping inside the hood. Disinfected seeds were put on the MS media for the experimental analysis.

### 2.8 Plant growth conditions

The disinfectant seeds were stratified at 4 °C for 2 days in dark and further grown under white light for all seedling phenotypic measurements. For adult plant phenotype *Arabidopsis* seedlings were grown till the induction of bolting and rosette leaf number was counted. The white light was obtained from Philips 17-watt F17T8/TL741 USA Alto II technology tubes with 100% light intensity, which was equivalent to ~120 µmols $min^{-1}$. All the data represented in this study were obtained from plant were grown under white light in plant growth chambers (Percival, USA) set to 22°C and 70% relative humidity. The growth chambers were programmed to provide 16 hours light and 8 hours dark (LDs) or 8 hours light and 16 hours dark (SDs).

### 2.9 Plant phenotypic measurements

All data presented are average of 3 biological replicates with each data point is the mean of at least 15 measurements. Error bars refer to the SD. Pearson's correlation for significance was performed at $p < 0.01$ and indicated as * above the significant data point.

#### 2.9.1 Seedling phenotypes measurement

Seeds of *At* were surface sterilized (Kumar et al., 2018;Fornara et al., 2009) by rinsing in 70% ethanol and 100% ethanol for 5 min each followed by air drying under laminar air flow. Surface sterilized seeds were sown on 50 ml of sterile MS medium from Himedia (Catalogue # PT100-1L) with increasing concentrations of CNPs starting from 10 µg/ml, 100 µg/ml and 500 µg/ml respectively in LD growth conditions. Medium plates without CNPs and similar concentration of charcoal (Merck, Catalog # 102514, EC No. 1907/2006) served as the control. CNPs were added

to the MS medium placed in 6.4cm × 6.4cm × 10 cm planton tissue culture containers (TarsonsProduct Pvt. Ltd. India). For all phenotypic measurements of seedling, the seeds were stratified at 4°C for 3 days (d) in the dark and further grown for 6 d under white light (WL). For adult plant phenotype, *At* seedlings were grown till bolting on the MS medium and rosette leaf number was counted. The WL was obtained from Philips 17-watt F17T8/TL741 USA Alto II technology tubes with 50% light intensity, which is equivalent to ~100 µmols/$m^2$. All the seedling data represented in this study were obtained from seedlings (6-day-old) grown under white light (WL) plant growth chambers (model-AR36, Percival, USA) set to 22°C and 70% relative humidity. The growth chambers were programmed to provide cycles of 16 h light and 8 h dark (LDs) or 8 h light and 16 h dark (SDs). For monochromatic red light (RL), far-red light (FRL) and blue light (BL) irradiation, seedlings were grown in E-30 LED chambers (Percival, USA) maintained at 22°C temperature and with 70% relative humidity. The intensities of incident lights were 80 µmol/$m^2$/s for WL, 50 µmol/$m^2$/s for RL, 6 µmol/$m^2$/s for FRL and 30 µmol/$m^2$/s for BL.

### 2.9.2 Root length measurement

Root length measurements of seedlings were carried out by taping them on a black paper and before scanned images were obtained with Epson L220 scanner. Images were analysed using Image J software (Version 1.46). All data presented were average of at least 3 biological replicates, and each data point was mean of at least 20 measurements

### 2.9.3 Hypocotyl length measurement

Relative hypocotyl length measurements of seedlings were carried out by taping them on a black paper and before scanned images were obtained with Epson L220 scanner. Images were analysed using Image J software (Version 1.46). All data presented were average of at least 3 biological replicates, and each data point was mean of at least 20 measurements. Relative

hypocotyl lengths were obtained as ratios of hypocotyl length of seedlings grown in light to their respective dark control.

### 2.9.4 Cotyledon angle measurements

Seedlings cotyledon angle were measured by taping them on a black paper and before scanned images were obtained with Epson L220 scanner. Images were analysed using Image J software (Version 1.46). All data presented were average of at least 3 biological replicates, and each data point was mean of at least 20 measurements were measured from the angle between the cotyledon and the hypocotyl in seedlings without or with CNPs treatment. Data were analysed in Prism5 software using two ways ANOVA. Pearson's correlation for significance was performed at $p < 0.01$ and indicated as significant data point (indicated by ***).

### 2.9.5 Leaf area and perimeter

Leaves were taped on a black paper and scanned images were taken with Epson L220 scanner. Images were analysed using Image J software (Version 1.46).

## 2.10 Adult phenotypes

### 2.10.1 Root length

About 22 days old plants were taken and the washed the root system properly in tap water and extra water were wipe out by tissue paper. Root length was measured manually by ruler.

### 2.10.2 Pod length, Pod weight and Seeds per pod

Pod length and weight measurement were done from 35-45 days old plant samples. Pods were taped on a black paper and scanned images were taken with Epson L220 scanner. Images were analysed using Image J software (Version 1.46). Pod weight measurement was carried out using balance (from Sartorius S/N 0032905891, Germany).

### 2.10.3 Leaf number at bolting

Leaves (both cauline leaf and rosette) were counted in 22-25 days old plants. Data represented was a mean of about 15 independent measurements.

### 2.10.4 Biomass determination

Biomass was determined from 45-50 days old plants after 3 days of air drying after growth stage 6.90 flowering completed (Boyes et al., 2001).

### 2.11 Chlorophyll content and carotenoid estimation

The total chlorophyll was quantified according to (Arnonet al., 1949). Briefly, about 10 mg of leaf from 22 days old plants were taken and incubated with 1.25 ml of 80% acetone for 48 hrs at 4°C in dark. Eppendorfs were centrifuged at 13000 rpm for 5 min and absorbance of the supernatant was measured at 645 nm and 663 nm and 475 nm.

Total chlorophyll was quantified as described previously (Kumar et al., 2018; Arnon et al., 1949). Briefly, 10 mg of leaf from seedlings were isolated and incubated with 1.25 ml of 80% acetone for 48 h at 4°C in the dark. Eppendorf tubes with the acetone extract centrifuged at 13000 rpm for 5 min, followed by measurement of absorbance of the supernatant at 645 nm and 663 nm. Total chlorophyll was calculated as the formula mention below:

Total chlorophyll ($\mu g\ mg^{-1}$) = 20.2 (A645) + 8.02 (A663)

Total Carotenoids ($\mu g\ mg^{-1}$) = C x+c= (1000A470 – 1.82Ca –85.02Cb)/198

### 2.12 Estimation of anthocyanin amounts

The Anthocyanin's was estimated according to (Panigrahy, 2004). About 10 mg of leaf from 22 days old plants were taken in 1.5 ml Eppendorf tube and added 300 µl anthocyanin extraction solution. Incubated the sample at 95 °C for 5 minutes. After cooling the samples kept for 24 h at 4 °C in dark. Eppendorf tubes were centrifuged at 13000 rpm for 5 minutes, followed by taking absorbance of supernatant at 535 nm and 650 nm. The data represented is mean of at least 15 measurements. Anthocyanin's ($\mu g\ ml^{-1}$) = A535-2.2 (A650)

### 2.13 Raman spectroscopy

Various plant samples (100 mg FW) were taken, washed three times with distilled water and homogenized with mortar and pestle in 3 ml of distilled water. The, max power output = 500 mW, maxi wavelength = 785 nm, Envaveoptronicsinc18271 homogenate was collected in a

borosilicate cuvette compatible to the Raman spectrometer and the spectra were collected with EzRaman Reader V5.8.5 (EzRaman S/N 158270, E.McDurmottste A-1 Irvine CA 29614, USA) using a frequency stabilized 785 nm narrow bandwidth laser source in backscattering mode. For all the samples 400 mW of output laser power was used through a permanently aligned single fibber and the spectra were collected through a 200 µm collection fibber having numerical aperture of 0.22 and working distance of 7 mm. The spectrometer has the spectral resolution of 6 $cm^{-1}$ in the range of 250-2350 $cm^{-1}$. Highly sensitive linear CCD array having pixel size of 14 µm x 200 µm (2048 pixels) in TEC cooled (~15$^{o}$C) detector was used for collecting the data. The background was subtracted from an observed spectrum through manually (Kumar et al., 2018). For intensity comparison and good statistics, the integration time of 40 s was kept constant for all the samples. Percentage of intake of CNPs and statistical analysis were carried out using Prism5 software.

**2.14 Light microscopy**

Transverse sections of leaves were made with the help of a razor blade and were visualized under 100x magnification using Inverted microscope from Olympus (Model: 1X73/m. PS.O Camera and S/W) from DSS image tech Pvt. Ltd. Size and structure of CNPs was visualized in MS medium alone as a positive control or in the leaf mesophyll cells of the plant material grown with CNPs. The uptake of CNPs by seedlings could be visualized by the microscopic observation of CNPs fine particle aggregates in the CNPs-treated plant samples only in contrast to the control Col-0 plants

**2.15 RNA isolation and qRT-PCR**

For RNA isolation, 10 day-old seedlings grown on MS medium with or without 500 µg/ml CNPs were harvested at Zt 4, 8 and 12 under LDs. Total RNA was isolated using Qiagen Plant RNA isolation mini kit (Cat #74104 Qiagen). The cDNA was prepared from 1µg of RNA of each sample using Bio-RadiScript™ Reverse Transcription Super-mix, (Cat # 1708840). The

quantitative RT-PCR (qRT-PCR) was performed using CFX384 Touch™ Real time detection system (Bio-Rad laboratories), following the manufacturer's manual of iTaq™ universal SYBR green supermix. Gene-specific primers used for qRT-PCR were designed using Primer Quest software tool and are listed in Supplementary Table I. All reactions were carried out in a Hard-shell 384-well PCR plates (supplied by Bio-Rad, Cat #: HSP3805), with a reaction volume of 10 µl per well. The PCR mix consisted of

| | |
|---|---|
| 2x iTaq universal SYBR Green super mix | = 5 µl |
| Template | = 2 µl (~50 ng) |
| Primers (500 nM) | = 1 µl |
| H2O | = 2 µl |

The PCR thermo cycler program used was as follows: 95°C: 5 min, 95°C: 10 s, 60°C: 30 s, 60°C: 30 s and 65°C: 5 s. Transcript levels were normalized with ACTIN. Each qRT-PCR reaction was performed in at least triplicate and all data were presented as mean ± SEM.

**2.16 Plant species used for preparation of crude extracts**

The plant species *Potamogeton pectinatus, Enteromorpha intestinalis, Gracilaria verrucosa* and *Scenedesmus acuminatus* were collected from the backwaters of ChilikaLake, Odisha, India. Seeds of *Arabidopsis thaliana* Columbia ecotype (Col-0), *gigentia-100* (*gi-100),* *spindly*-5 (spy-5) 35S:GI and 35S:CONSTANS$_\beta$(35S:CO$_\beta$) were surface sterilized and grown under sterile conditions on MS media plates (Fornara et al., 2009) in the Percival plant growth chambers (Model No- CU36L6; Perry) at 22 °C, relative humidity of 70 %, $CO_2$ concentration of 400 ppm and illumination of ~135 $\mu$mol$^{-2}$ s$^{-1}$ from white fluorescent tube lights (Philips F17T8/TL741, 17 Watt).Two-week old *Arabidopsis* seedlings were grown under 16 h light and 8 h dark cycles were harvested and air dried. Nearly 1 g of dried plant tissue was powdered using mortar and pestle, re-suspended in 10 ml Milli-Q water. The resultant mixture was autoclaved at 121**°C** for 20 minute and filtered using Whatman filter paper. The filtrate was centrifuged at 10,000 x g for 5 minute and the cell-free supernatant was normalized with respect

to the standard protein concentration using Bradford analysis and diluting appropriately with Milli-Q water and used as source.

### 2.17 In vitro synthesis of AgNPs

For the synthesis of AgNPs, 5 ml of plant extract as prepared in section 2.1, was mixed with 2.5 ml of 10 mM silver nitrate aqueous solution and the volume was made up to 25 ml with Milli-Q water. The mixture was incubated at room temperature (RT) (25 °C) and formation of AgNPs was monitored as indicated by a colour change to yellowish brown. In order to obtain sugar capped NPs, the NPs' suspension was mixed with 0.01 M glucose or maltose followed by stirring overnight with an end-to-end mixer at RT.

### 2.18 UV-Vis Spectra analysis

The reduction of pure $Ag^+$ ions was monitored by measuring the UV-Vis absorbance spectrum of the suspension at different time points after the induction of NPs synthesis. The NPs containing solution was centrifuged at 10000g for 10 minutes and the pellet was suspended in Milli-Q water and the UV-Vis spectrum was measured using a Nano Drop 2000 Analyser (Fischer Scientific Ltd.)

### 2.19 Scanning electron microscopy (SEM)

For SEM analysis thin films of the sample were prepared on a carbon coated copper grid by adding a 10 µl sample on the grid. The film on the SEM grid was allowed to dry. The surface morphology of the prepared samples was examined using Field Emission Scanning Electron Microscopy (FEG–SEM; Neon 40 cross-beam system; M/S Carl Zeiss GmbH). The sample solution was drop casted on Si substrate and dried under a mercury lamp for 5 min. The dries substrates were loaded in to FESEM for measurement. Secondary electron detector was used to collect the micrograph. All the micrographs were recorded with 20 kV accelerating voltage.

### 2.20 NMR and FTIR analysis

The plant extracts were dried by evaporation on a dry bath at 60 °C and the residues were dissolved in 500 µl of $D_2O$ (Heavy water). The $^1H$ NMR of the solutions was performed using a 400 MHz NMR spectrometer (Model No: Bruker Arx 400 MHz). In order to pellet down the capped AgNPs from the suspension, the mixture was centrifuged at 10000 g for 10 min. The resulting pellet was suspended in of 1 ml Milli-Q water. The pelleted NPs were washed thrice with Milli-Q water followed with centrifugation. Thereafter, the purified pellet was dried to powder by evaporation on a dry bath at 60 °C and used for FTIR sample preparation (Kareru et al., 2007). The powder was mixed with potassium bromide and made into a pellet using a hydraulic pellet pressure. The pellet was analyzed by FTIR spectroscopy.

### 2.21 Depletion of polyphenolics

Removal of polyphenolic compounds from the extract was done according to Lodhi et al (1994). To the aqueous extracts of total plant tissue, 4 % (w/v) cross-linked polyvinyl pyrrolidine (PVPP) suspension was added in 1:1 (v/v) ratio. The mixture was incubated at 55 °C for 1 h, centrifuged at 10,000 g for 10 minutes at RT. The polyphenols compounds were precipitated along with PVPP and the supernatant fraction devoid of phenolic compounds was used for NPs synthesis.

### 2.22 Depletion of carbohydrate

Carbohydrates were depleted from the aqueous extract by acetone precipitation according toKu et al., (2003). Briefly, four volumes of acetone were added to one volume of the extract and the mixture was incubated overnight at 4 °C. It was warmed to RT followed by centrifugation for 10 minutes at 10,000g. The carbohydrate deficient supernatant was used for the synthesis of NPs.

### 2.23 Depletion of DNA, RNA and protein

DNase treatment was performed according to Guillemaut et al., (1992). The 10x DNase I buffer (0.1 volume) and 2 units of recombinant DNase I were added to the extract and mixed gently. The mixture was incubated at 37 °C for 30 min, 0.1 volumes of 50 mM EDTA was added and mixed well. After 2 minutes of incubation, the mixture was centrifuged at 10,000 g for 10 minute and the supernatant was transferred into fresh tube and used for AgNPs synthesis. RNase treatment was carried out as described in (Sambrook et al., 1989) and the RNA deficient fraction was used for AgNPs synthesis. For obtaining protein deficient fraction, 5 units of proteinase K was added in 50 mM Tris-Cl buffer containing 5 mM $CaCl_2$ and the depleted fraction was used for AgNPs synthesis after inactivation of the proteinase K by incubating the suspension at 95 °C for 10 min.

### 2.24 Estimation of polyphenols, carbohydrates, DNA, RNA and protein

The quantitative estimation of different bio-molecules was done spectrophotometrically. DNA and RNA concentrations were determined by measuring the absorbance at 260 nm using UV-Vis spectrophotometry. Protein concentrations were determined by Bradford method (Bradford 1976). The analysis of polyphenol content was performed using Folin-Ciocalteau method while carbohydrate estimation was done by Nelson's method (Miller 1952).

### 2.25 Northern blot analysis

The RNA blot was performed as described by Akbergenov et al. (2006) and Shiva Prasad et al., (2012). About 10 µg of total RNA were suspended in 8 µl loading buffer (0.10 % bromophenol blue, 0.10 % xylene cyanol in 100 % de-ionised formamide), heated at $95^0$C for 1 min and loaded on 15 % denaturing polyacrylamide gel (a 19:1 ratio of acrylamide to bis-acrylamide, 8 M urea). The gel was run at 100 V for 3 h and then transferred to a Hybond N+ membrane by electro blotting at 10 V overnight at $4^0$C. The hybridization was performed at $35^0$C for 12 h in UltraHyb-oligo buffer (Ambion). Short DNA oligos (please add oligo sequences); end-labelled with $^{32}$P-ATP (Board of Radiation and Isotope Technology, India) using polynucleotide kinase

(NEB) and purified through Micro Spin G-25 columns (GE Healthcare), were used as probes. The blot was washed twice with 2X SSC, 0.5 % SDS for 30 min at $35^0$C. The signal was detected after exposure on a phosphor imager screen using a Molecular Imager). For repeated hybridisation, the membrane was stripped and re-probed and probe in this analysis mention below.

U6-I: GGCCATGCTAATCTTCTCTGTATCGTT

U6-II: CCAATTTTATCGGATGTCCCCGAAGGGAC

miR156: GTGCTCACTCACTTCTGTCA,

**2.26 Confocal microscopy**

We used Leicamicrosystem (Leica logo) with help of institute of life sciences Bhubaneswar, for image processing. Condition used for CFP signal was excited wave length 405 nm and fluorescence was detected inbetween 454-503 nm wavelength.

**2.27 Soil nutrient analysis**

Soil nutrient analysis was performed at Orissa University of Agriculture and Technology, Bhubaneswar according to standard procedures (Singh et al. 1999).

**2.28NPK Solution**

1 $NH_4NO_3$ : 6.516 g dissolve in 1000 ml = 2000 ppm
2 $HNa_2PO_4$ : 1.824 g dissolve in 1000 ml = 400ppm
3 KCL : 7.624 g dissolve in 1000 ml = 1000ppm

N (1): P (2): K (3) =20:4:10

Take 100 ml each solution 1, 2 and 3 = 300 ml
Add auagurd water = 300 ml
Total = 600 ml

After mixing properly then solution were irrigated to each 3 days of interval with *Arabidopsis* plants.

**2.29 Statistical analysis**

Pearson's' Correlation and significance test was performed on the biomass values of each soil against each component of the soil. The significant differences were confirmed by Mann

Whitney Unpaired t-test statistical analysis for the significance level at $p < 0.01$and ANOVA Bonferroni's multiple comparisons statistical analysis for the significance level at $p < 0.01$

# CHAPTER: 3 RESULT AND DISCUSSION

## MIXED SOIL GROWN PLANT BEHAVE STANDARD FLOWERING TIME OF *ARABIDOPSIS THALIANA*

## 3.0 Result

### 3.1 Mixed soil offer better growth of *At*

*Arabidopsis* plant growth was rigorously retarded on clay and red soil, where as it could reach the bolting stage on garden soil, soilrite and mixed soil and Max Planck Institute for Plant Breeding Research Koln grown soil (taken as positive control to evaluate standard flowering time)(Figure 3.1 and Table 6). The plants on the garden soil was less healthy which was accompanied by obvious small leaf size, thin stem, less number of leaves and anthocyanin accumulation on leaves. Plants grown on soilrite were comparatively healthy as those on mixed soil, however plants were having pale leaves.

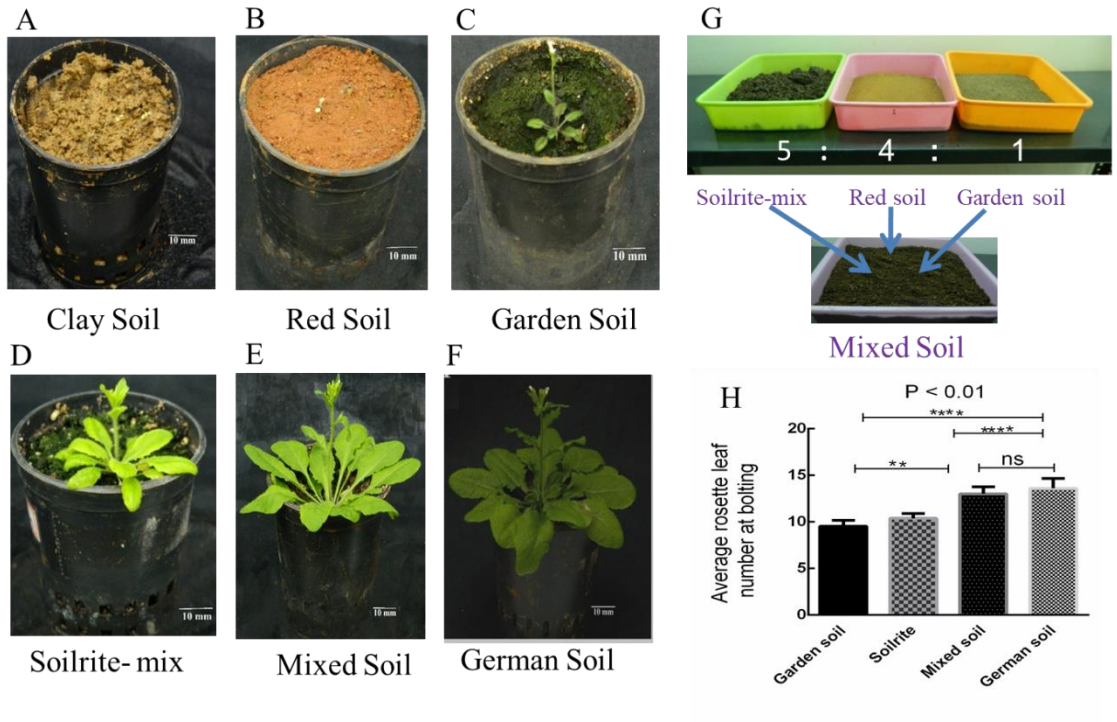



**Figure 3.1 Plant Phenotype of Col-0 grown in different soil conditions. Phenotype of *Arabidopsis* Col-0 grown on Clay soil (A), Red soil (B), Garden soil (C), Soilrite (D) Mixed soil (E), German soil (Max Plank Institute soil) (F) soil proportion of Mixed soil (G) and statistical analysis of flowering time at bolting stage of garden soil, soilrite, mixed soil and German soil (H).**

The plants on mixed soils showed best plant growth in terms of leaf number, greening, leaf length and higher number of leaves at bolting stage and it was comparable to plants gown in German soil grown soil. Flowering time at bolting stage in garden soil, soilrite, mixed soil and

German soil were evaluated (Figure 3.1H). The data showed that less number of rosste leaves both in garden soil and soilrite which is about 10, however in mixed soil grown plants showed about 13 which is comparable to standard German soil.

### 3.2 Mixed soil showed longest roots

To access the plant growth and healthy status, root length was measured from the plants grown on five different soil conditions (Figure 3.2).

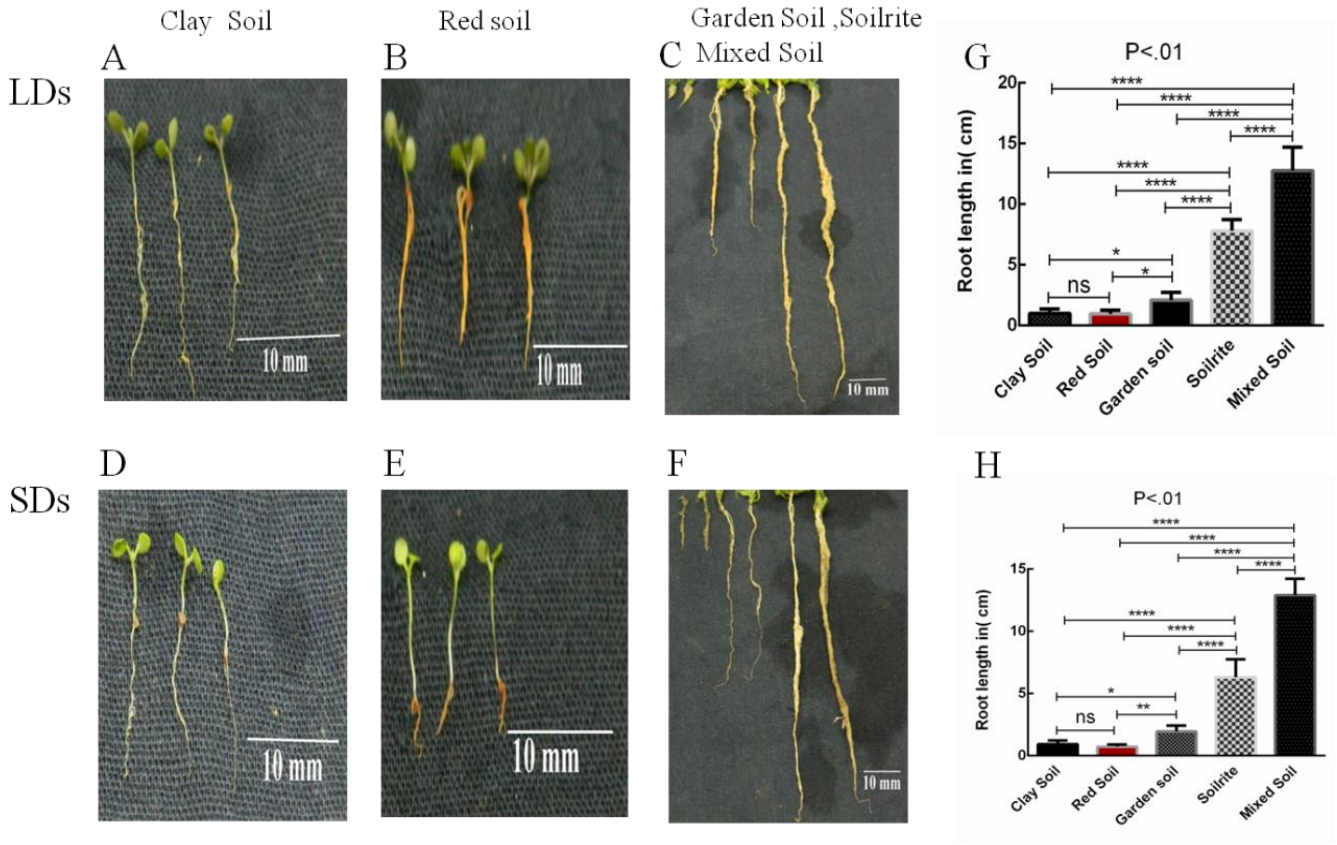



**Figure 3.2 Seedling phenotype of Col-0 grown under long days. Seedling phenotype of *Arabidopsis thaliana* Col-0 grown under Long Day (A, B, C and G ) and Short Day growth conditions (D,E, G and H) on clay soil (A, D), Red soil (B, E) and Mixed soil (C, F) respectively. Observed maximum root length under LD (G), and SD growth conditions (H) respectively. Areal parts were excised from plants grown on mixed soil for clarity of root pictures (C and F).**

Due to poor growth, root length on clay and red soil could be taken from seedlings after 15 days, whereas those from the other three soils were accessed around 25 days. Root growth was observed under both long days (LDs) and short days (SDs) to understand the photoperiod effect.

Root length was maximum in mixed soil and minimum in red soil. Secondary roots and root hairs were higher in red soil under LD conditions.

### 3.3 Leaf area and perimeter are highest in mixed soil grown plants

Leaf area is a positive indicator of plant growth (Weraduwage et al., 2015). In present study, leaf area and perimeter were accessed in the plants grown on garden soil, soilrite and mixed soil (Figure 3.3).

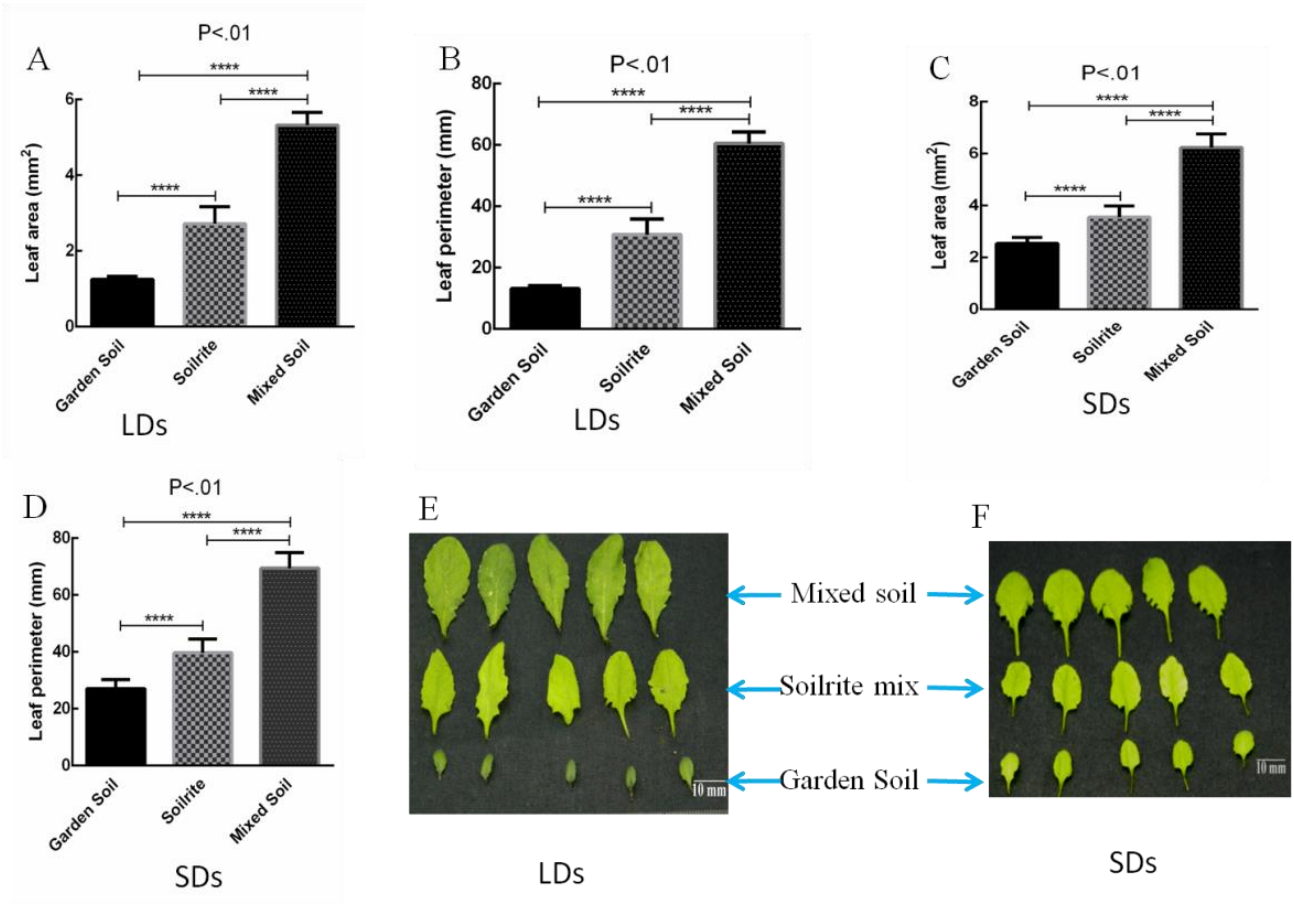



**Figure 3.3 Leaf shape and area of Col-0 grown in different soil conditions.Leaf shape of *Arabidopsis thaliana* Col-0 (E, F) plants grown in mixed soil (E and F: 1st row), soilrite (E and F: 2nd row), garden soil (E and F: 3rd row) under Long Day and Short Day growth conditions respectively.). Leaf area (A, C) and Leaf perimeter (B, D) in garden soil, soilrite and mixed soil under LD and SD growth condition respectively.**

As the seedlings on clay and red soil died after15 days only at the cotyledon leaf stage, their leaf area and perimeter could not be included in this data. In all the 3 soil conditions tested, the leaf area and perimeter was higher under SD condition than under LD condition. Leaves under SD

conditions had longer petioles. Higher vegetative growth under SD conditions is known in *Arabidopsis* (ABRC) as it is a long day plant.

### 3.4 Chlorophyll content was higher however carotenoid and anthocyanin amount were less in the mixed soil grown plants

To access the photosynthetic potential and stress parameters, total chlorophyll, carotenoid and anthocyanin was quantified in the leaves from the plants grown in 5 different soil conditions (Figure 4).

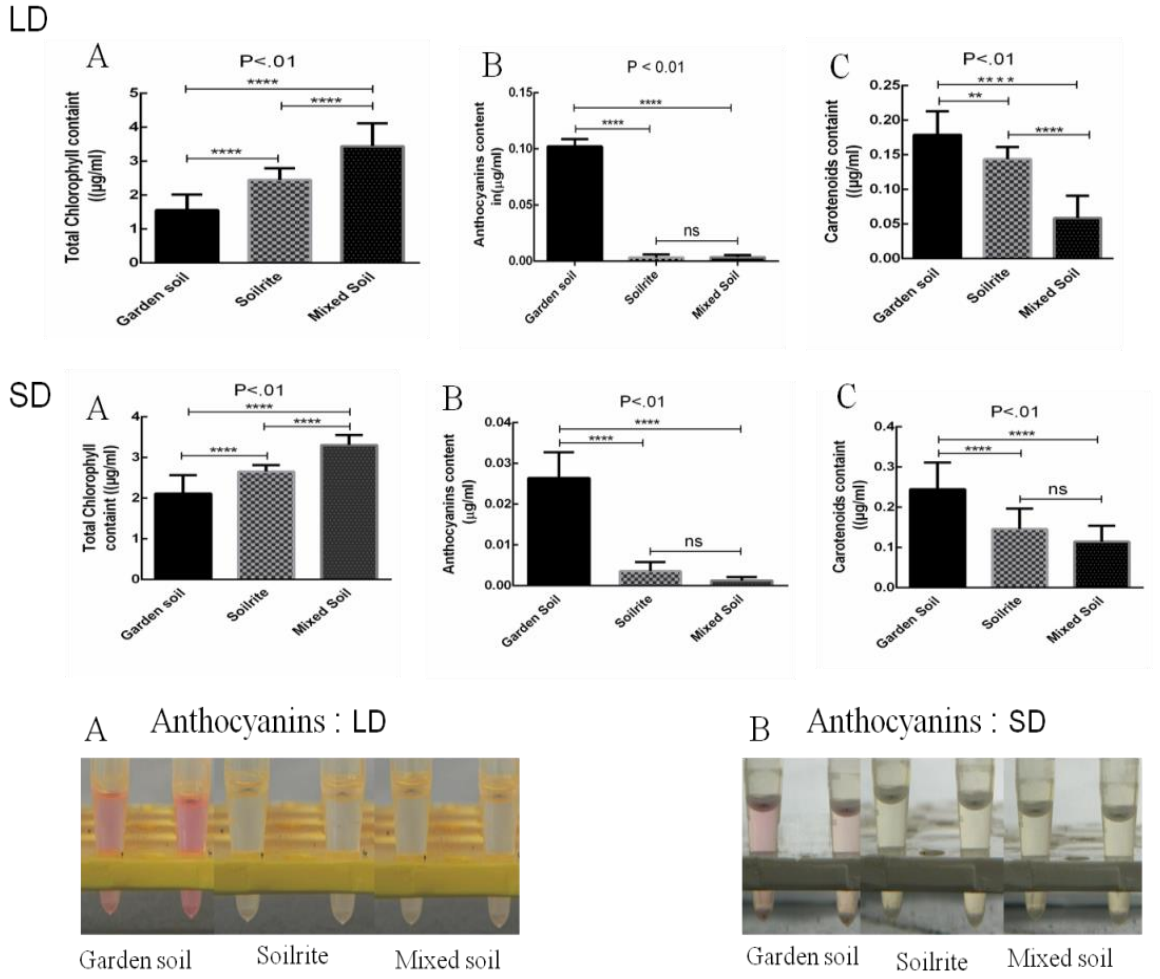



**Figure 3.4 Chlorophyll, Carotenoid and Anthocyanin of Col-0 grown under long day and short-day conditions. Chlorophyll (A, D), Carotenoid (B, E) and Anthocyanin (C, F) of Col-0 from leaf samples grown under Long Day (A, B and C) and Short Day (D, E and F) growth condition respectively.**

Under both LDs and SDs, total chlorophyll was highest in the plants grown on soil mix indicating better plant growth than other soil conditions tested. Carotenoids and anthocyanin are

considered in this study as indicators of abiotic stress, since these are known to accumulate in different kind of abiotic stress conditions including nutrient deficiencies, toxicity and disease (Ramakrishna et al., 2011). Carotenoid and anthocyanin under both LDs and SDs were highest in the plants grown on garden soil and least in soil mix indicating that the plants grown on soil mix reflect a healthier and stress-free growth environment.

### 3.5 Mixed soil grown plants exhibited a higher reproductive potential

Leaf number at bolting represents the reproductive status of the plant (Fornara et al., 2009).

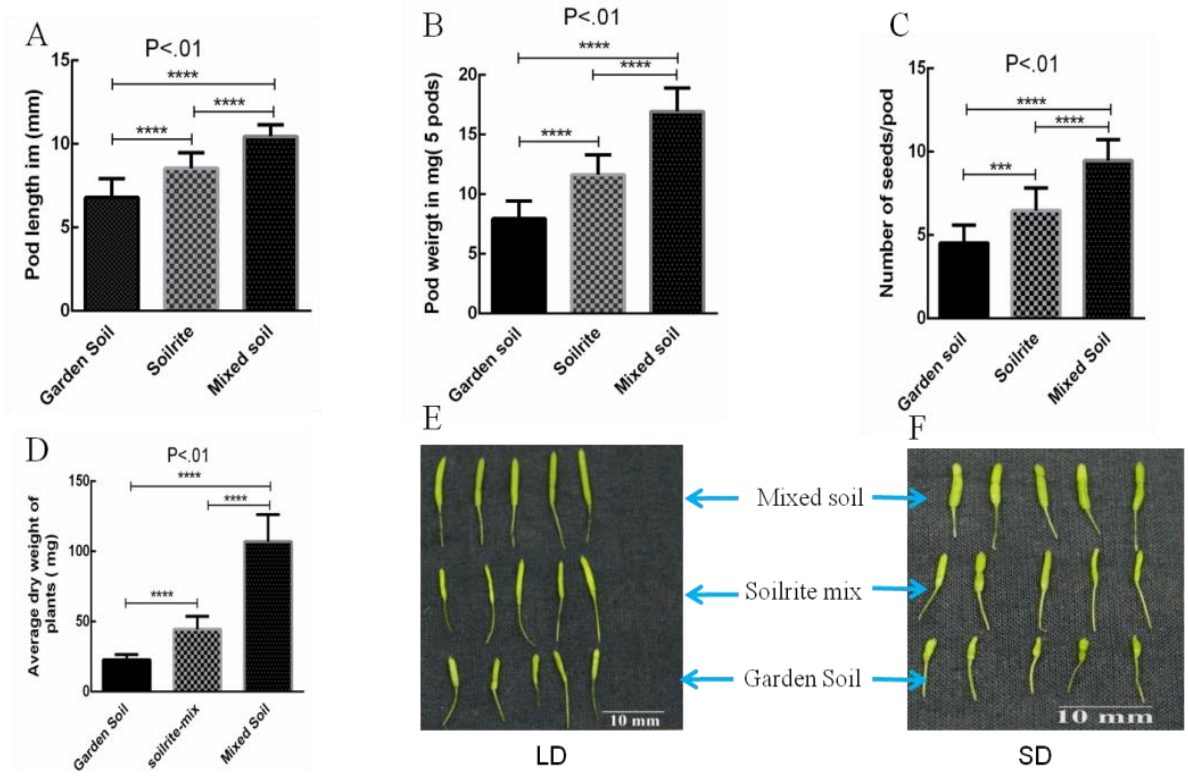



**Figure 3.5 Plant Reproductive feature in different soil conditions.Plant reproductive characteristics in different soil conditions. (A) Pod length (B) Pod weight, (C) Number of seeds per pod, (D) Average dry weight (E) representative pod pictures under LD growth conditions and (F) SD growth conations of *Arabidopsis thaliana* Col-0. Pod length of plants grown in mixed soil (E&F: 1st row), soilrite (E&F: 2nd row), garden soil (E&F: 3rd row) under Long Day (LD) growth conditions and short-day condition respectively.**

Presently, leaf number at bolting was highest at 15 leaves in plants grown on soil mix (Figure 5.5). Whereas leaf number was similar in case of plants on garden soil and soilrite, plants on clay and red soil died at 2 leaf stages. Seed pod length, weight and number of seeds per pod was

significantly highest in the plants grown on soil mix followed by soilrite and that from garden soil being the least

### 3.6 Soil nutrients status, pH and electric conductivity in different soil conditions

The results from nutrient and soil parameter analysis as mention in table (1.1.) presented following data. Clay soil sowed lowest pH indicating the most acidic soil. The pH of garden soil and soil mix was same at neutral pH. Optimum pH of soil for healthy plant growth ranges from 5.5 to 7.5 (Hanlon, 1993), whereas ABRC standard (ABRC) procedures recommend pH 5.7 for healthy *At* growth, which is close to the pH of red soil in present case. Electrical conductivity is a measure of salinity and each plant species has a specific threshold of soil EC for obtaining best yield (Hanlon, 1993). Richards (1954) defined four different ranges of EC and categorized different crops such as bean, barley, corn and wheat into sensitive, moderate and tolerant crops. Moreover, *At* root hair cells are affected by changes in the electrical properties of the plasma membrane (Lew, 2004). Mixed soil in present study showed highest EC indicative of highest movement of ions into plant. Among the five types of soil tested, organic content was highest in soilrite followed by clay, red soil, and garden soil respectively and was least in the soil mix. ABRC standards recommended 14%: 14%: 14% for the N: P: K amounts in the soil mix (ABRC). Nitrogen content was highest in garden soil followed by soil mix. Phosphate content was found to be least in all soil, whereas the entire N, P and K content was least in soilrite.

| S.N0 | Parameters | Red soil | Clay soil | Garden Soil | Soil rite | Mixed Soil | German soil mix |
|---|---|---|---|---|---|---|---|
| 1 | pH | 5.40 | 4.62 | 7.22 | 6.48 | 6.2 | 6.03 |
| 2 | EC (dSm-1) | 0.006 | 0.055 | 0.260 | 0.028 | 0.408 | 0.907 |
| 3 | OC (g/ kg soil) | 4.72 | 7.98 | 3.45 | 11.7 | 7.75 | 11.4 |
| 4 | Avl. N ( Kg/ha) | 189.0 | 164.0 | 428.0 | 0.30 | 208 | 1023.0 |
| 5 | Avl. P ( Kg/ha) | 5.85 | 5.28 | 147.0 | 0.04 | 57.4 | 321.0 |
| 6 | Avl. K ( Kg/ha) | 143 | 180.0 | 176.0 | 0.03 | 295 | 1015.0 |
| 7 | Avl. Ca ( Kg/ha) | 1.5 | 8.04 | 13.9 | 0.49 | 3.18 | 32.9 |
| 8 | Avl. Mg (meq/100 gm) | 0.49 | 3.53 | 5.10 | 0.18 | 3.1 | 7.0 |
| 9 | Avl. Fe ( mg/kg) | 97.4 | 7.4 | 6.44 | 0.09 | 13.20 | 8.36 |
| 10 | Avl. Mn ( mg/kg) | 93.0 | 134.8 | 1.4 | 0.002 | 6.32 | 6.98 |
| 11 | Avl. Cu ( mg/kg) | 0.99 | 3.08 | 9.19 | 1.9 | 3.18 | 3.75 |
| 12 | Avl. Zn ( mg/kg) | 1.08 | 5.58 | 25.2 | 5.5 | 5.0 | 10.24 |
| 13 | Avl. B ( mg/kg) | 4.3 | 2.6 | 10.2 | 0.01 | 0.50 | 0.82 |

**Table 1.1 Nutrient content and soil parameters in five different soil conditions.**

K content was found highest in the soil mix. Soil EC correlates different soil properties including soil texture, exchangeable Calcium and magnesium ions, drainage conditions, organic matter and subsoil characteristics and water content (Kadam, 2016). Calcium and magnesium ions are comparable among the red soil and soil mix, whereas highest in the garden soil. Soil properties has been highly influenced by micronutrient status which ultimately affects the plant health (Mandal et al., 2015) Iron content has attained highest value in red soil, whereas the other micronutrients such as Zn, Cu and B content was highest in the soilrite among the 5 soils tested. The dry biomass and seeds / pod showed positive correlation with pH, EC, and K, Fe and Mn content.

## 3.7 Discussion

The soil mix designed in the present study at ratio of soilrite, red soil and garden soil in 5:4:1 proportion proved best for *Arabidopsis* plant growth with respect to vegetative growth and reproductive potential of the plants, as the plants grown on soil mix had highest biomass, seeds per pod. Soil mix grown plants showed least accumulation of secondary metabolites such as carotenoid and anthocyanin indicating better plant health conditions. Plant growth on red soil was not possible after about 15 days possibly due to high iron content. The better plant growth on soil mix could be due to favourable pH, high EC, high N, P and K content. Though the OC content of the soil mix was low, it could be efficiently taken up by the plant due to high EC content. The soil mix designed in this study could be useful for Indian researchers for healthy *Arabidopsis* growth in plant growth chambers.

# CHAPTER 4: RESULTS & DISCUSSION

## OVER-EXPRESSED LINES OF COβ DELAY FLOWERING TIME AND IS DIURNALLY AND DEVELOPMENTALLY REGULATED IN *ARABIDOPSIS THALIANA*

## 4.0 Result

### 4.1 Splice variant of CONSTANS

The in silico data suggested the presence of two forms of CONSTANS, one is full length of CONSTANS (COα) which is correctly spliced (Figure 1A.) while another is improper splice form of CONSTANS (COβ) (Figure 4.1B).The COα is 373 amino acid long containing B-box domain in N-terminal and CCT (CONSTANS, CONSTANS-like and Time of CAB) domain in C-terminal. It has been shown that B-BOX motifs is involved in protein–protein interaction (Tripathi et al., 2016).The CCT domain within the second half of it has been shown to be involved in nuclear localization and in the light signal transduction (Strayer et al., 2000).

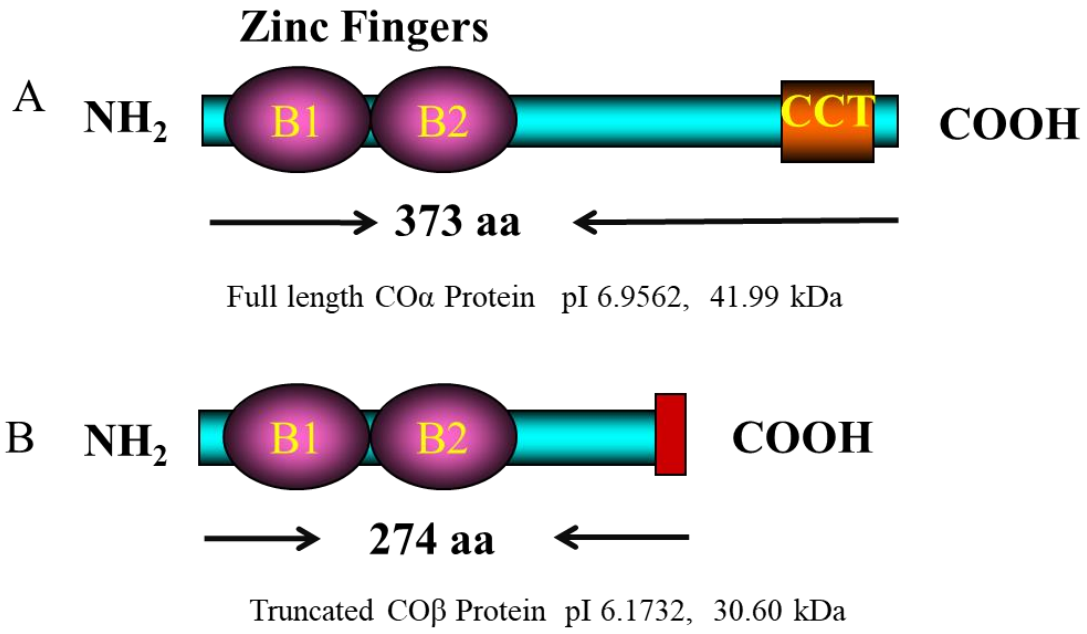



**Figure 4.1 Splice forms of (A) CONSTANS. Proper splice form of CONSTANS (COα) is full length of CONSTANS. It contain B-BOX domain in N-terminus and CCT domain in C-terminus, (B) improper splice form (truncated splice form) of CONSTANS (COβ) contain only the B-BOX domain.**

The improper splice form i.e. the truncated form of COβ, which might encode putative protein of 274 amino acid long (Figure 4.1B) with a 20 amino acid from the intron region of the C-terminus.

### 4.2 Confirmation of the presence of splice variants of CONSTANS

The in silco analysis suggested the two forms of CONSTANS in *At* (Figure 4.2A). Further the varification for the presence of the both form of transcript by sequence specific primers was performed by q-RT-PCR and identified COα and COβ (Figure 4.2B).The COα was 180 bp and COβ was 250 bp.

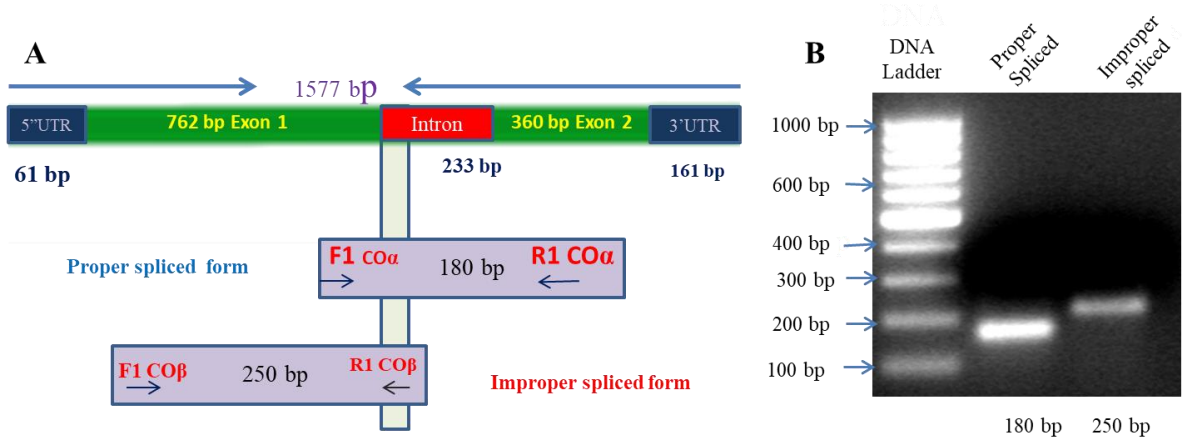



**Figure 4.2 Experimental confirmation of splices forms of CONSTANS *in vivo*. Diagrammatic representation of position of forward primer (F1COα) and reverse primer (R1COα) for COα and forward (F1COβ) and reverse primer (R1COβ) for (COβ) (A) and cDNA amplified products of COα is 180 bp and COβ is 250 bp (B).**

### 4.3 Generation of transgenic lines of COβ

After in vivo confirmation of COβ, was amplified and cloned in gateway compatible vectors containing CFP and YFP N-terminus tag (Figure 4.3A) by a set of gene specific primers.The expression constructs containing the COβ was transformed into the *Agrobacterium tumefaciens* strain GV3031 using electroporation method. The correct insertion of the construct in *Agrobacterium* was confirmed by colony PCR (Figure 4.3B). Transformation of Col-0 (wild type) *co-10* (CONSTANS mutant) and *phyb-9* (Phytochrome B mutant) with these constructs was done by floral dip method (Figure 4.4A).Transformed seeds were harvested in paper bag separately (Figure 4.4B). The transformed seeds were grown in controlled environmental conditions (Figure 4.5A) and positive plants were selected using BASTA (Figure 4.5B) followed by PCR with gene and vector specific primers (Figure 4.5C).

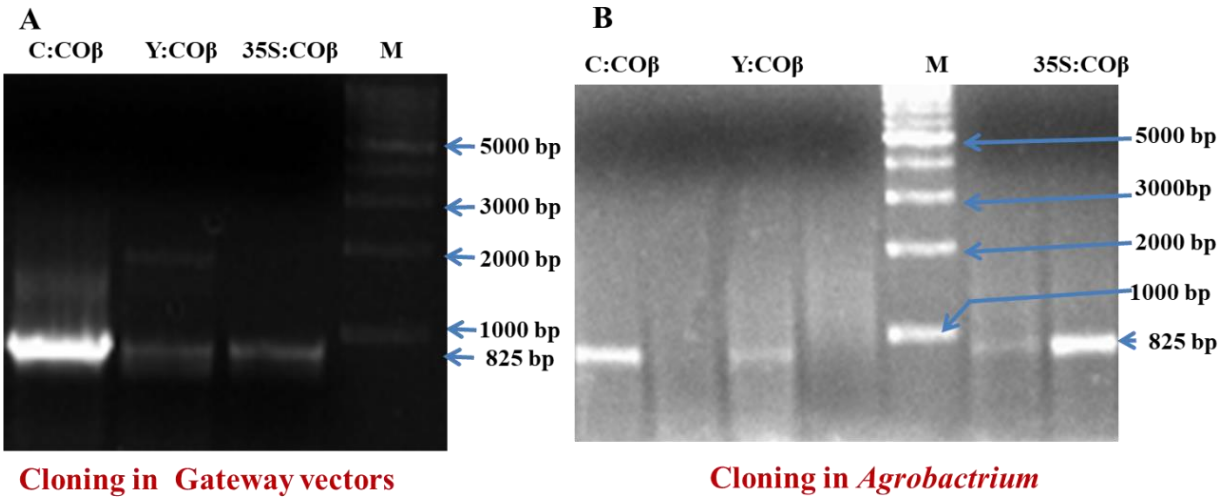



**Figure 4.3 Cloning of COβ in the Gateway vector and transformation into the *Agrobacterium tumefaciens*. Cloning of COβ was done in Gateway compatible vectors containing CFP and YFP as N-terminal tag and confirmation by colony PCR. Size of amplified product is 825 bp (A.). Evaluation of positive transforments of COβ into the *Agrobacterium* by colony PCR (B).Symbolic representation of C:COβ;35S::CFP:COβ destination vector,Y:COβ;35S::YFP:COβ destination vector and 35S::COβ; 35S::COβ destination vector.**

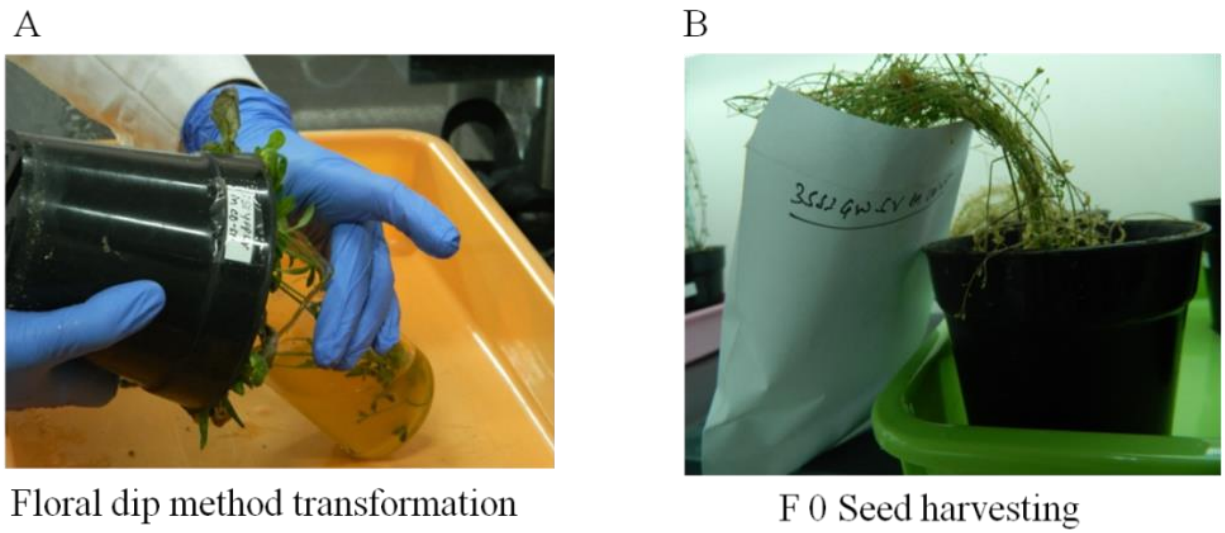



**Figure 4.4 Floral dip method of transformation of *Arabidopsis thaliana* and harvesting of seeds.The COβ gene containing construct was transformed by floral dip method (A.) and seeds were harvested in a paper bag (B.)**

Homozygous stable transgenic lines expressing COβ were generated after continous selection by both BASTA and PCR for each single plant up to F3 generation. The various over-expressed

transgenic lines of COβ were generated in Col-0, *co-10* and *phyb-9* genetic background as the follow

1. 35S::COβCol-0 (Figure 4.6A)
2. 35S::CFP:COβCol-0 (Figure 4.6B
3. 35S::YFP:COβCol-0 (Figure 4.6C)
4. pSUC2::COβCol-0 (Phloem specific promoter,Figure 4.6D)
5. 35S::CFP:COβ*phyb-9* (Figure 4.7D)
6. 35S::COβ*co-10* (Figure 4.7A)
7. 35S::CFP:COβ*co-10* (Figure 4.7B)
8. 35S::YFP:COβ *co-10*(Figure 4.7C)
9. pKNAT1::COβCol-0 (Meristem specific promoter, Figure 4.6E)

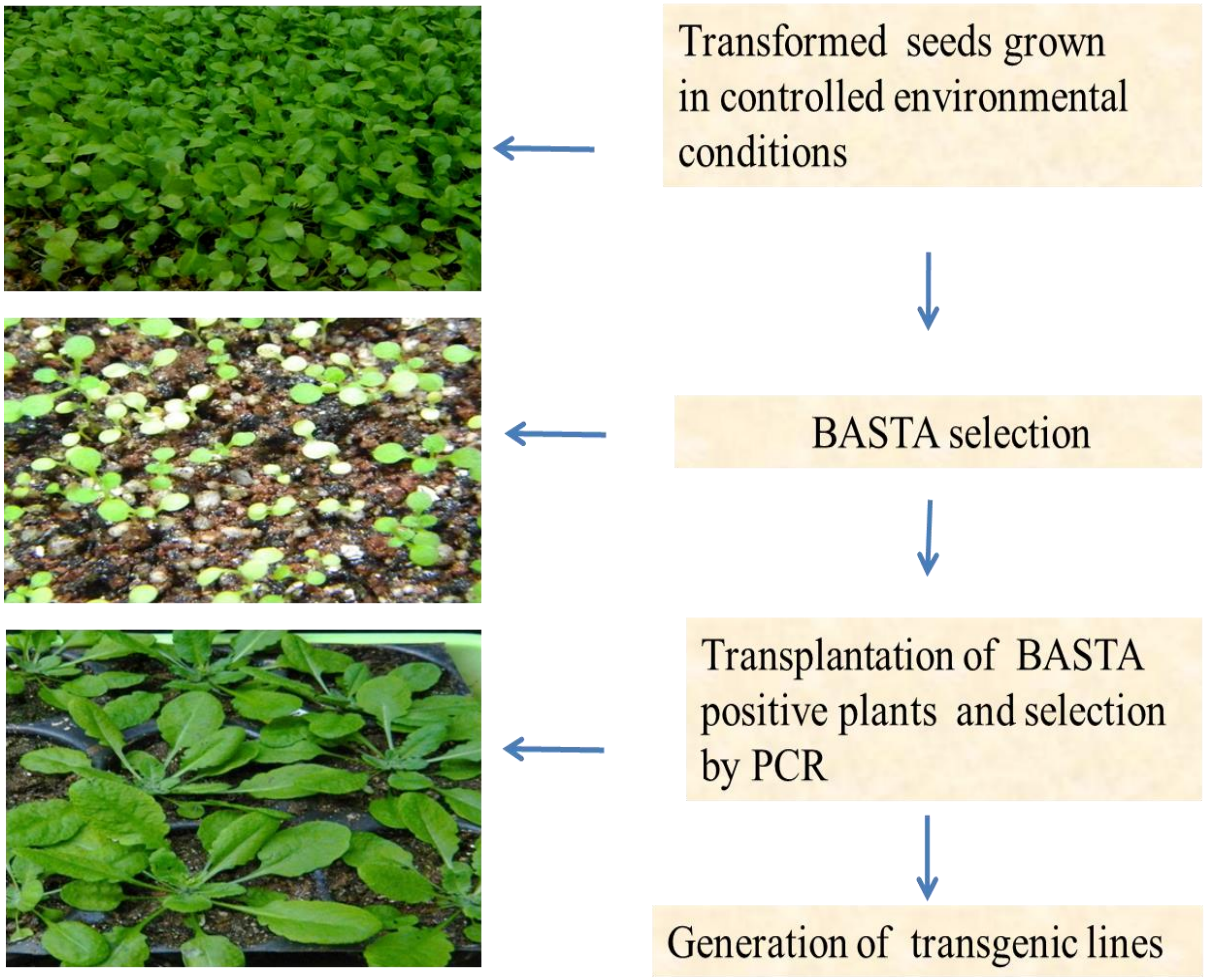



**Figure 4.5 Selection of transformed plants by BASTA followed by PCR. Seeds from the transformed plants were grown under control environmental conditions (A), followed by BASTA selection (B), BASTA positive plants were re-evaluated by PCR with gene and vector specific primers (C) followed by generation of transgenic lines of COβ.**

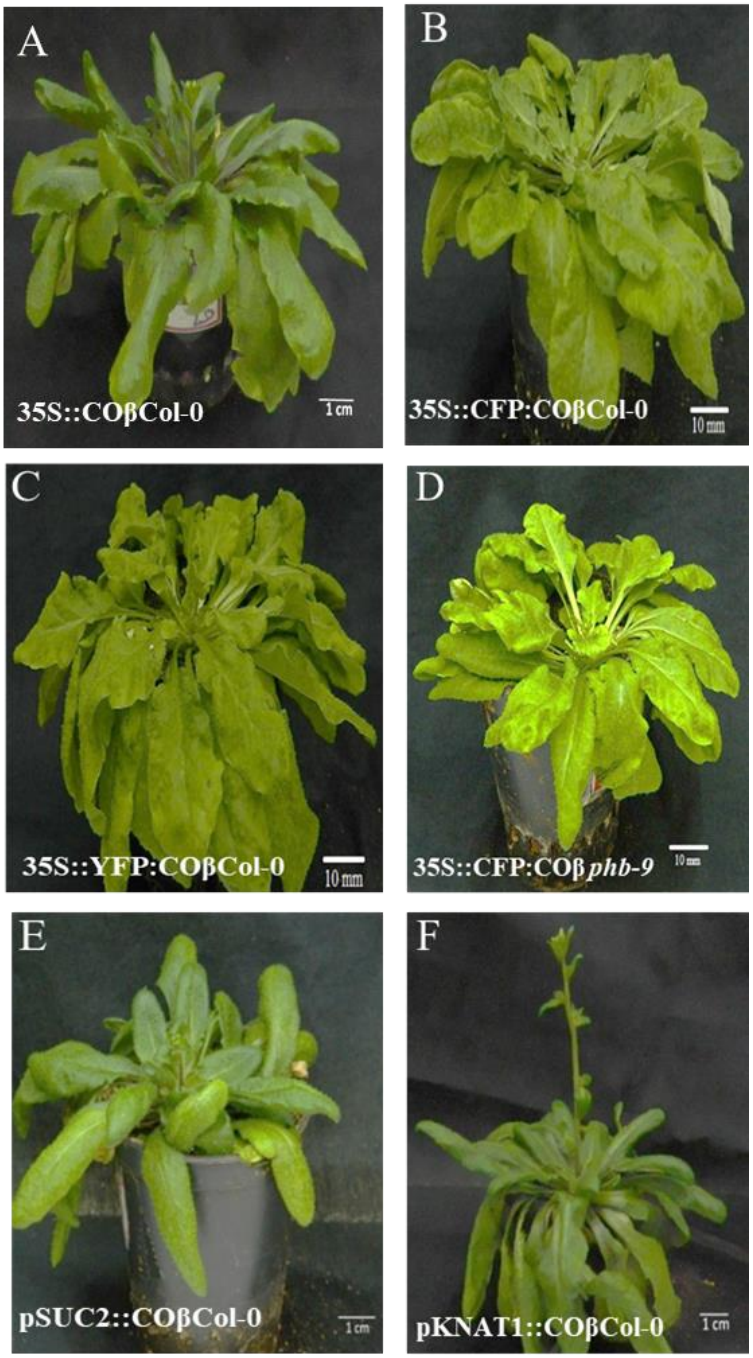



**Figure 4.6 Representative images of over-expressed transgenic lines of COβ in Col-0 and *phyb-9* background. Constitutive over-expressed lines of COβ with 35S viral promoter as 35S::COβCol-0 (A), with CFP N-terminal fusion as 35S::CFP:COβCol-0 (B), with YFP N-treminal fusion as 35S::YFP:COβCol-0 (C) and with CFP N-terminal fusion as 35S::CFP:COβ *phyb-9* (D).Phloem tissue driven expression of COβ pSUC2::COβCol-0 (E), meristem tissue driven expression of COβ pKNAT1::COβCol-0 (E).**

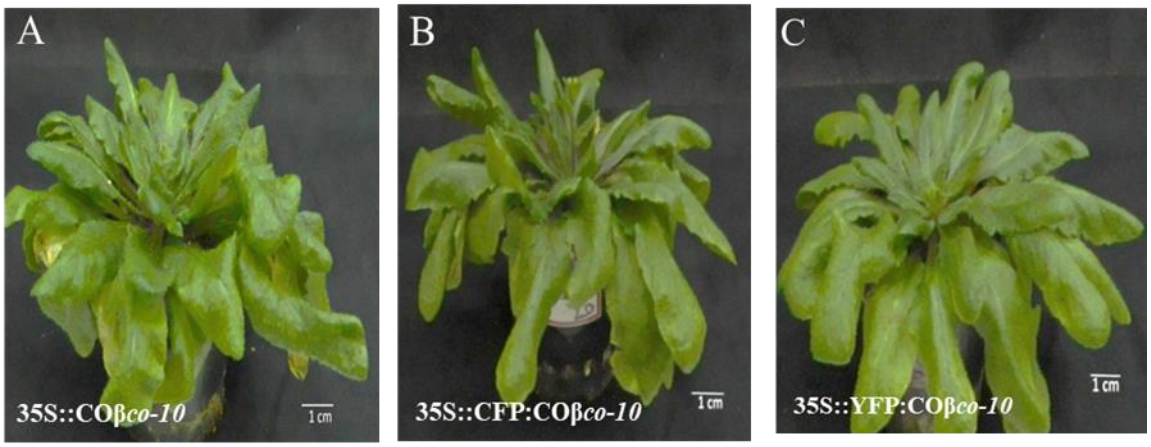



**Figure 4.7. Representative immages of over-expressed transgenic lines of COβ in *co-10* background.Constitutive over-expressed lines of COβ with 35S viral promoterin *co-10* background as 35S::COβ*co-10* (A), with CFP N-terminal fusion as 35S::CFP:COβ*co-10* (B) and with YFP N-treminal fusion as 35S::YFP:COβ *co-10* (C).**

### 4.4 Over-expression of COβ delay flowering under long day conditions

In order to evaluate the flowering time of the transgenic plants total leaves (both rosettes and cauline leaves) were counted as soon as the first bolting stem emerged. A delayed flowering was noticed which took about 44 long day ( 50 total leaves) in the over-expressed lines of COβ (Figure 4.10A and Table 4.1) while compared with Col-0 (Figure 4.10A) that flowered in about 23 LD cycle (13 leaves, Table 4.1).

| Genotypes | No of rosette leaves | No of total leaves | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 9 ± 2 | 13 ± 1.06 | 23 ± 1.3 | 20 |
| 35S:COβCol-0 | 45 ± 2.7 | 50 ± 3.41 | 44 ± 3.2 | 20 |
| *co-10* | 46 ± 3.58 | 56 ± 3.46 | 49 ± 3.5 | 20 |
| 35S:COβ*co-10* | 54 ± 4.21 | 61 ± 4.51 | 54 ± 4.7 | 20 |
| *Phyb-9* | 9 ± 1.27 | 12 ± 1.51 | 27 ± 1.4 | 20 |
| 35S::CFP:COβ*phyb-9* | 52 ± 4.03 | 57 ± 4.84 | 54 ± 3.4 | 20 |

**Table 4.1 Flowering time evaluation under long day conditions in Col-0, *co-10* and *phyb-9* genetic backgrounds**

However, in co-*10* (T-DNA insertions mutant of CONSTANS) background, over-expressed

lines of COβ flowered at about 61 leaves with about 54 LDs cycle (Table 4.1 and Figure 4.10A ) but *co-10* flowered with 56 leaves about 47 long days cycle (Figure 4.10A and Table 4.1). This result suggested that the delay in flowering of COβ lines in *co-10* background as compared to *co-10* is CONSTANS independent.

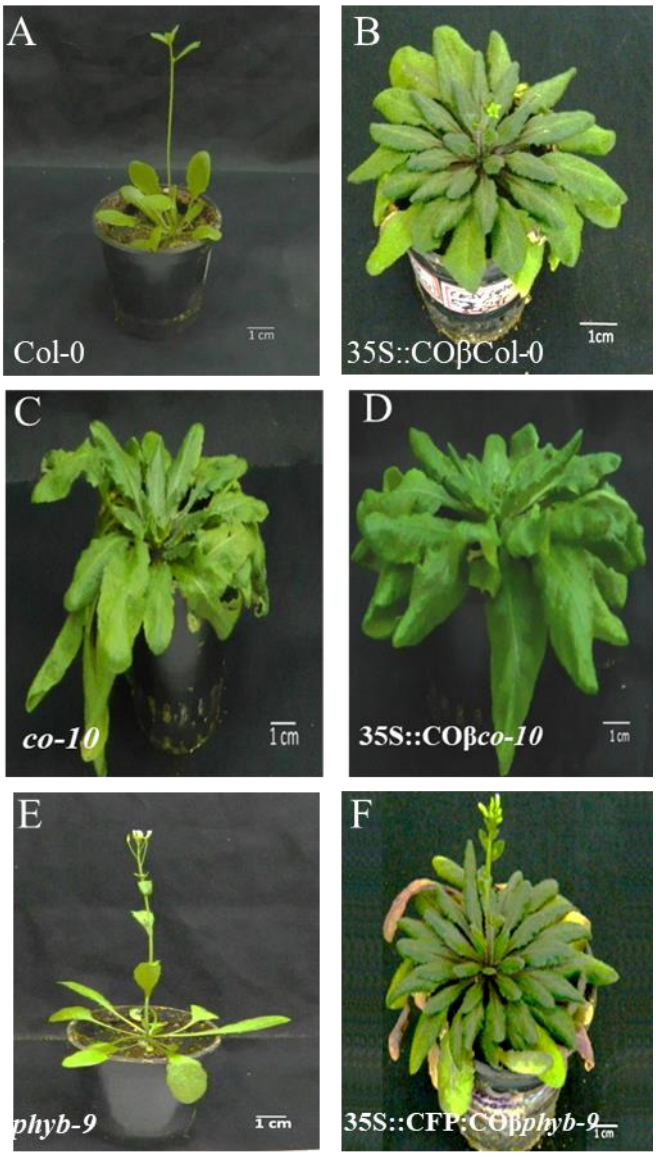



**Figure 4.8 Pictorial representation of transgenic lines of COβ with their contols at the time of bolting grown under LD cycles. In a Col-0 (A), over-expressed line of COβ in Col-0 (B), *co-10* (C), over-expressed line of COβ in *co-10* (D), *phyb-9* (E), and over-expressed line of COβ in *phy-9* (F).**

The over-expressed lines of COβ also delayed flowering which is in 54 long days (Table 4.1) with 56 leaves (Figure 4.10A and Figure 4.8F) with respect to control *phyb-9* background

(Figure 4.8E) flowering in 27 long days with 12 leaves stage, (Table 4.1,Figure 4.10A). This delay in flowering even in *phyb-9* background suggested a PHYB independent mechanism. The significant statistical analysis with significant result of total number of leaves is represented in (Figure 4.10A).

| Genotypes | No of total leaves | No of total leaf | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 9.3 ± 1.35 | 12.6 ± 1.84 | 24 ± 1.24 | 20 |
| pSUC2::COβCol-0 | 52.5 ± 3.28 | 58 ± 4.11 | 50 ± 4.55 | 20 |
| pKNAT1::CO□Col-0 | 52 ± 3.25 | 57 ± 3.51 | 56 ± 4.3 | 20 |

**Table 4.2 Flowering time evaluation of COβ over-expression by tissue specific promoters in Col-0 background grown under LDs.**

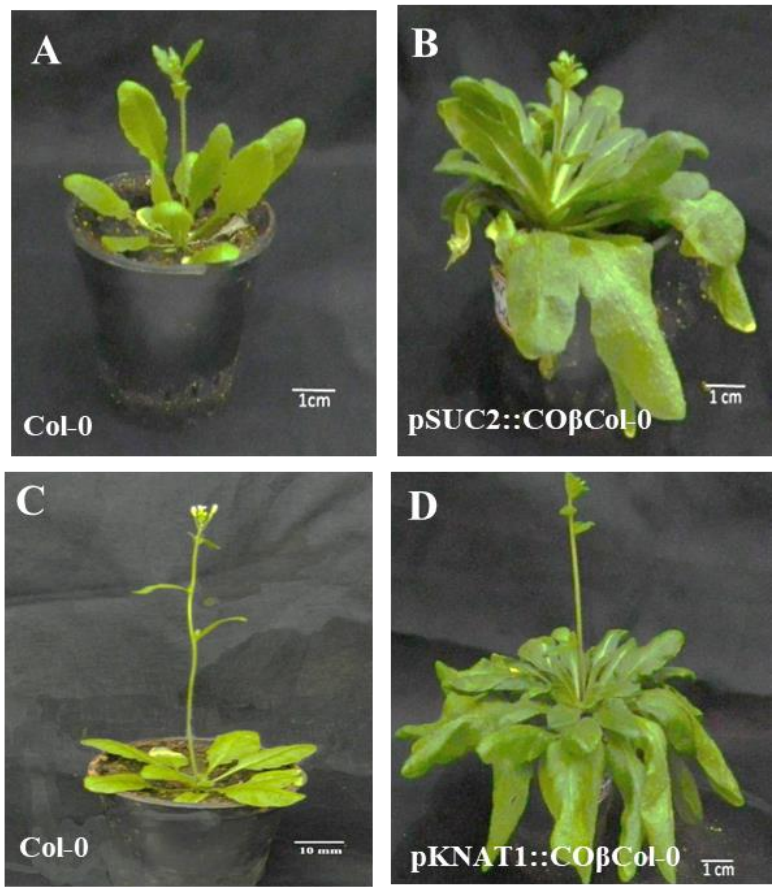



**Figure 4.9 Pictorial representation of transgenic lines of COβ with their contols at the time of bolting grown under LD cycles. Flowering time evaluation using tissue specific promoter under long days conditions. In Col-0 (A), with phloem tissue specific promoter**

**(pSUC2::Col-0) (B), Col-0 with meristem specific promoter lines of COβ), (D) in Col-0 background.**

It was also observed delayed flowering with the two tissue specific promoters tested such as phloem tissue specific (pSUC2::COβ) and meristem tissue specific (pKNAT::COβ) in Col-0 background.

With phloem tissue specific promoter plants were bolted with 53 leaves (Figure 4.10B) at about 52 long days (Figure 4.9B and Table 4.2) as compared to control Col-0 which bolted at about 13 leaves ( Figure 4.9A and Figure 4.10B ) at about 24 long days cycles ( Table 4.2 ). Similarly in meristematic tissue specific promoter driven lines of COβ plant was bolted at about 57 leaves ( Figure 4.10B and Figure 4.9D ) with 54 long days cycle ( Table 4.2 ) as compared to control Col-0 which was bolted at about 12.5 leaf (Figure 4.10B and Figure 4.9B ) at about 24 long days cycle ( Table 4.2 ).This result suggested that the delayed flowering phenotype noticed by the tissue specific expression of COβ.

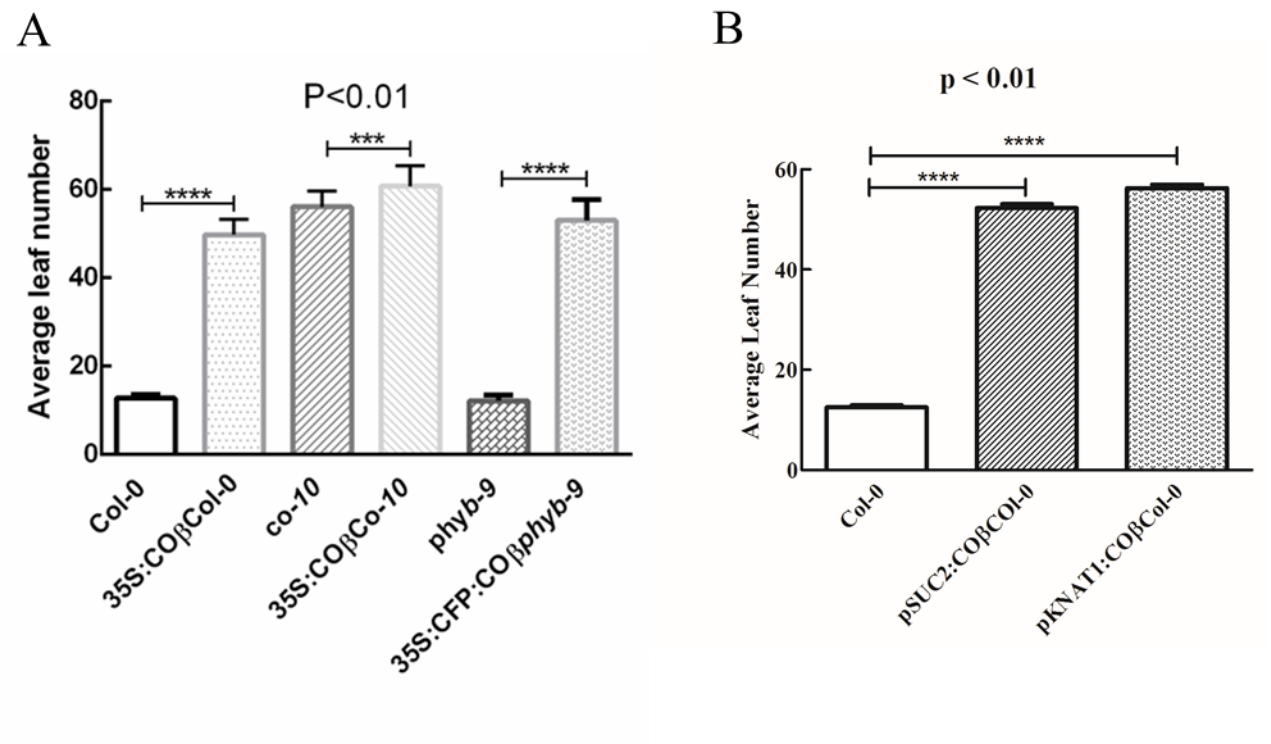



**Figure 4.10 Statistical analysis of flowering time under long day conditions in Col-0, *co-10* and *phyb-9* background. In constitutive over-expression of COβ (A) and tissue specific**

**(phloem specific,pSUC2::COβ and meristematic specific, pKNAT1::COβ) promoter in (B). The data presented is at least the mean of 20 individual plants.**

### 4.5 Over-expressed lines of COβ also delayed flowering under short day conditions

We did observe a delay in flowering under SD conditions in over-expressed lines of COβ in all the three genetic backgrounds such as Col-0 (Figure 4.11B), *co-10* (Figure 4.11D), and *phyb-9* (Figure 4.11F) as compared to their respective control. However, the significance difference was less in SD as compared to LD conditions of respective backgrounds.

| Genotypes | No of rosette leaves | No of total leaves | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 50 ± 3.7 | 57 ± 2.86 | 49 ± 3.1 | 20 |
| 35S::COβCol-0 | 54 ± 3.6 | 61 ± 3.41 | 54 ± 4.7 | 20 |
| *co-10* | 52 ± 5.07 | 58 ± 3.58 | 53 ± 3.5 | 20 |
| 35S::COβ*co-10* | 55 ± 4.21 | 62 ± 3.8 | 58 ± 4.7 | 20 |
| *Phyb-9* | 46 ± 4.01 | 52 ± 4.07 | 44 ± 1.7 | 20 |
| 35S::CFP:COβ *phyb-9* | 57 ± 4.84 | 61 ± 4.03 | 54 ± 3.4 | 20 |

**Table 4.3 Flowering time evaluation under short day conditions in Col-0, *co-10* and *phyb-9* genetic backgrounds.**

It was also observed delay flowering even under short days (SDs) in tissue specific promoters such as phloem tissue specific promoter ( Table 4.4 ) and meristematic tissue specific promoter ( Table 4.4 ) in Col-0 background.

| Genotypes | No of rosette leaves | No of total leaves | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 52 ± 3.24 | 58 ± 3.45 | 51 ± 3.77 | 20 |
| pSUC2::COβCol-0 | 57 ± 3.06 | 64 ± 3.3 | 55 ± 3.78 | 20 |
| pKNATI::COβCol-0 | 60.65 ± 4.36 | 65.5 ± 5.15 | 54 ± 4.45 | 20 |

**Table 4.4 Flowering time evaluation under short day conditions with tissue specific promoters in Col-0 background.**

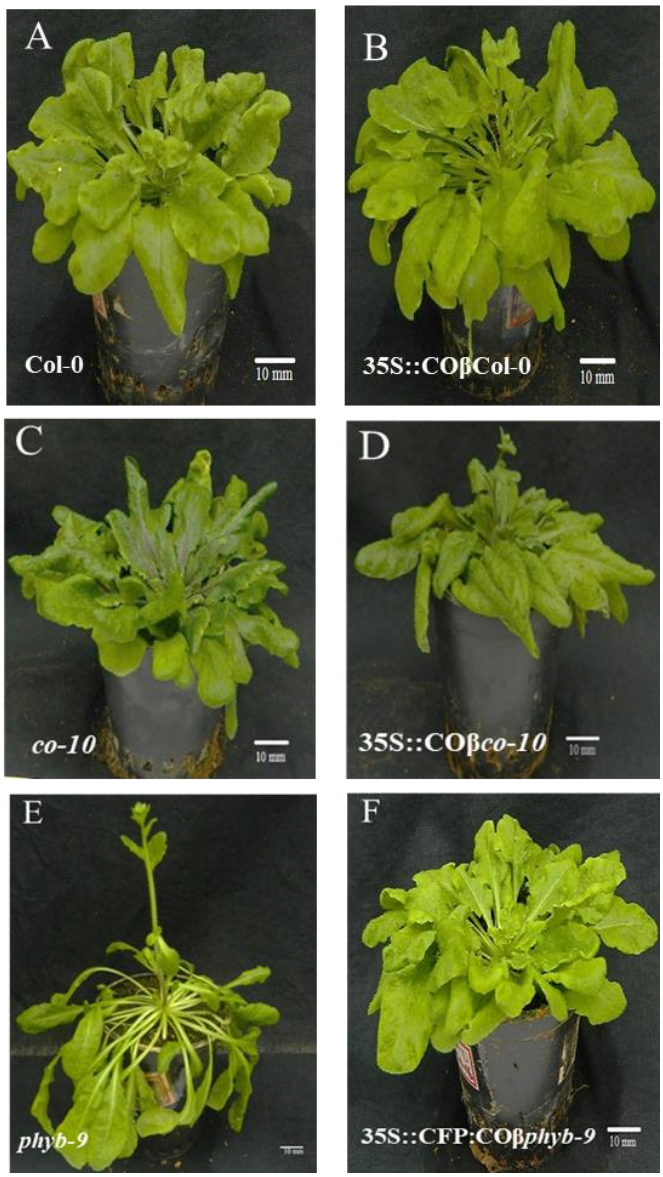



**Figure 4.11 Pictorial representation of transgenic lines of COβ with their contols at the time of bolting grown under SD cycles. In Col-0 (A), over-expressed line of COβ in Col-0 (B), *co-10* (C) , over-expressed line of COβ in *co-10* (D) and *phyb-9* (E), over-expressed line of COβ in *phyb-9* (F).**

In phloem tissue specific promoter driven plants were bolted at about 64 leaf (Figure 4.13B) at about 55 long days (Figure 4.12B and Table 4.4) stage as compared to control Col-0 which was bolted at about 58 leaves (Figure 4.12A and Table 4.4 ) in 51 long days (Figure 4.13B).Similarly in meristematic tissue driven lines of COβ plant was bolted at about 66 leaves (Figure 4.12D and Table 4.4) (Figure 4.12D and Table 4.4) with 54 long days cycle (Figure 4.13B) as compared to

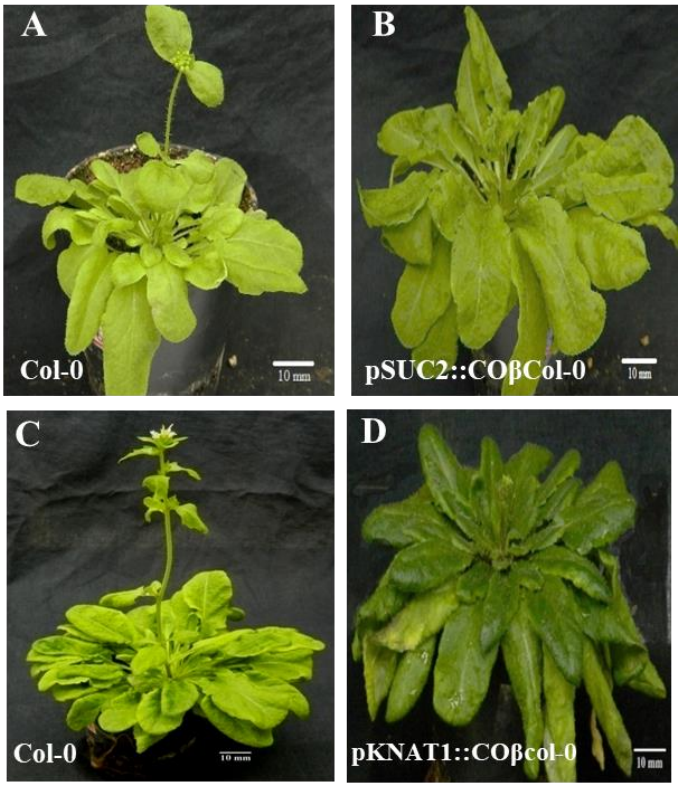



**Figure 4.12 Flowering time evaluation in tissue specific promoter under short days conditions. In phloem tissue specific promoter (pSUC2) (A) ( Col-0 and B.pSUC2::COβ), and meristematic specific promoter (C) ( Col-0 and D. pKNAT1::COβ) in Col-0 background.**

**.**

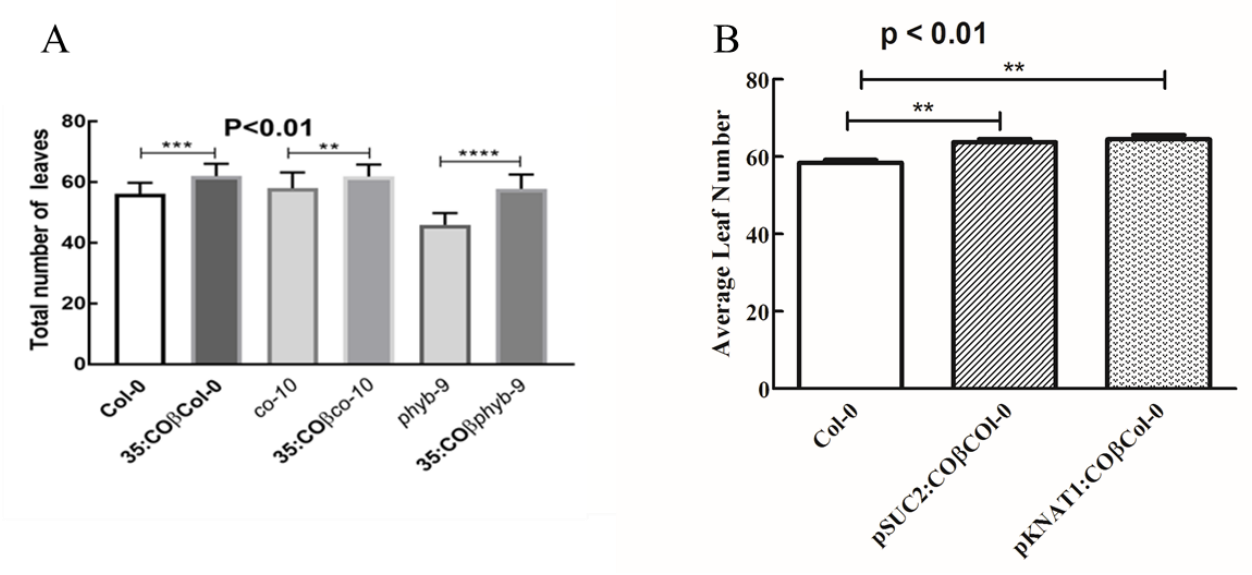



**Figure 4.13 Statistical analysis of flowering time under short day conditions. By constitutive over-expression of COβ (A) and by tissue driven promoter (phloem specific,pSUC2::COβ and meristem specific, pKNAT1::COβ) promoter in (B).The data presented is the mean of 20 individual plants.**

control Col-0 which was bolted at about 52 leaves (Figure 4.13B) with 48 long days cycle (Figure 4.12C). However, the significance difference was less under SDs as compared to LDs conditions of their respective control.The significant statistical analysis of delayed flowering represented in (Figure 4.13B). This result of delay flowering under short days suggested that the delay flowering in over-expressed line of COβ is also photoperiodic dependent.

### 4.6 COβ over-expression causes accumulation of chlorophyll

Since the 35S::COβ plants were greener bigger in size as compared to Col-0 control, therefore total chlorophyll content was quantified.

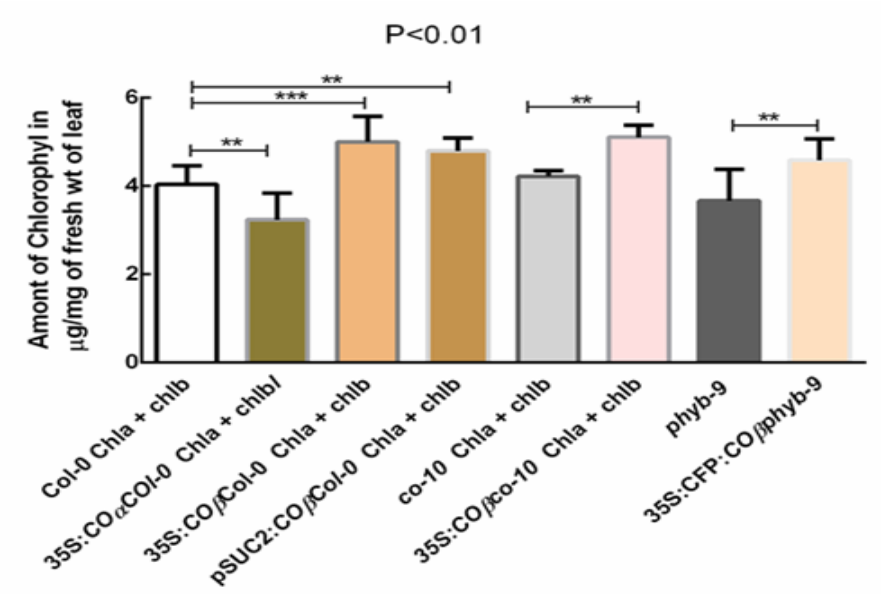



**Figure 4.14 Chlorophyll estimation form over-expressed line of COβ under long days in different genetic backgrounds. Here white histogram represent col-0, olive histogram 35S::CO*α*, marigold histogram 35S::COβ, golden histogram pSUC2::COβ, grey histogram *co-10*, pink histogram 35S::COβ*co-10*,black histogram *phyb-9*, frute pink histogram in 35S::CFP:COβ*phyb-9*.The data presented is the mean of 20 individual plants.**

The total chlorophyll content was found to be significantly higher (Figure 4.14) in all the constitutive over-expressed lines of COβ and those driven by pSUC2 (Phloem specific promoterin Col-0 background) as compared to col-0,*co-10* and *phyb-9* genetic backgrounds under LD conditions. However the total chlorophyll content was less in over-expressed line of COα (35S:COα) as compared to Col-0 and over-expressed lines of COβ under LD conditions.

### 4.7 Hypocotyls length was shorter whereas root length, cotyledon angle and cotyledon area were longer in over-expressed lines of COβ

In order to understand its effect on other phenotype such as hypocotyls length, root length cotyledon angle and cotyledon area of over-expressed lines of COβ, we have grown the seedling of over-expressed lines of COβ and Col-0 in MS media till 8 days under different light conditions and recorded each phenotype. The hypocotyl length was shoter in over-expressed lines of COβ (Figure 4.15A) in different light qualities such as white light, red light, far red light and blue light as compared to control. To

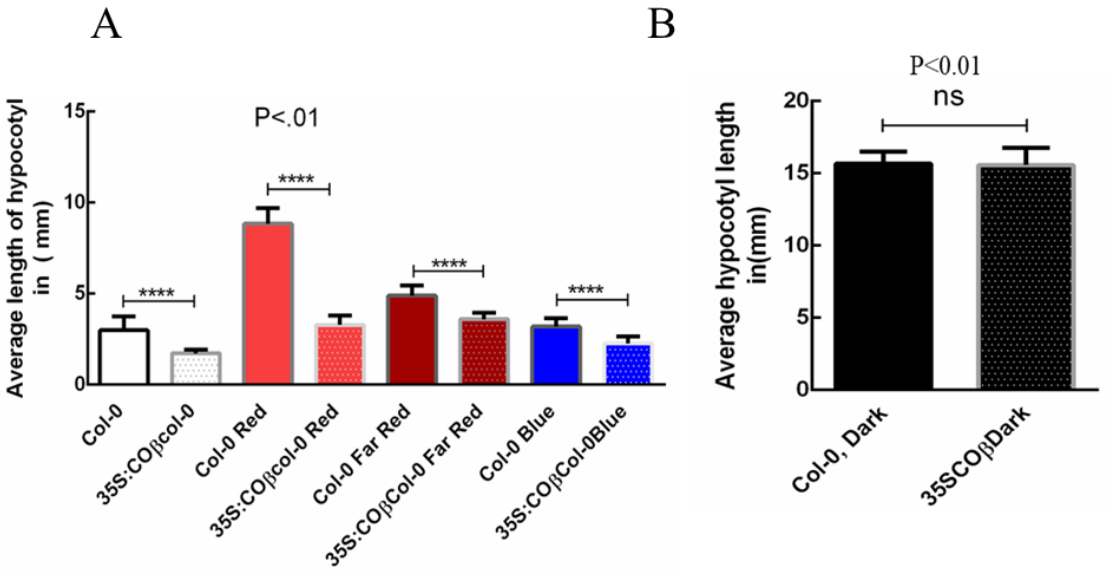



**Figure 4.15 Measurements of hypocotyl length in over-expressed lines of COβ under different light qualities. In different light qualities (A) and dark grown seedling (B).This non-significant differences in dark grown seedling was confirmed by Mann Whitney Unpaired t-test statistical analysis for the significance level at p <0.01.The data presented is the mean of 20 individual plants. White histogram represents white light, red histogram represents red Light, wine colour histogram represents far-red Light and blue histogram represent blue Light.**

confirm this observed phenotype is not because of developmental defect, ssedlings were also grown in dark conditios and seedling hypocotyl length were similar both in Col-0 and 35S::COβ lines (Figure 4.15B) respectively. However the root length was longer in over-expressed lines of COβ (Figure 4.16A) as compared to their respective control and it is independent of light quality such as red light (R) and far red light (FR).This observed phenotype is not because of developmental defect as it was shown in the dark grown seedling with similar root length both in Col-0 and COβ lines (Figure 4.16B).

As the leaf growth and area is the positive indicator of plant growth (Weraduwage et al., 2015), in present study, we also measured the cotyledon area, perimeter and cotyledon angle in over-expressed lines of COβ as compared to control (Figure 4.17).

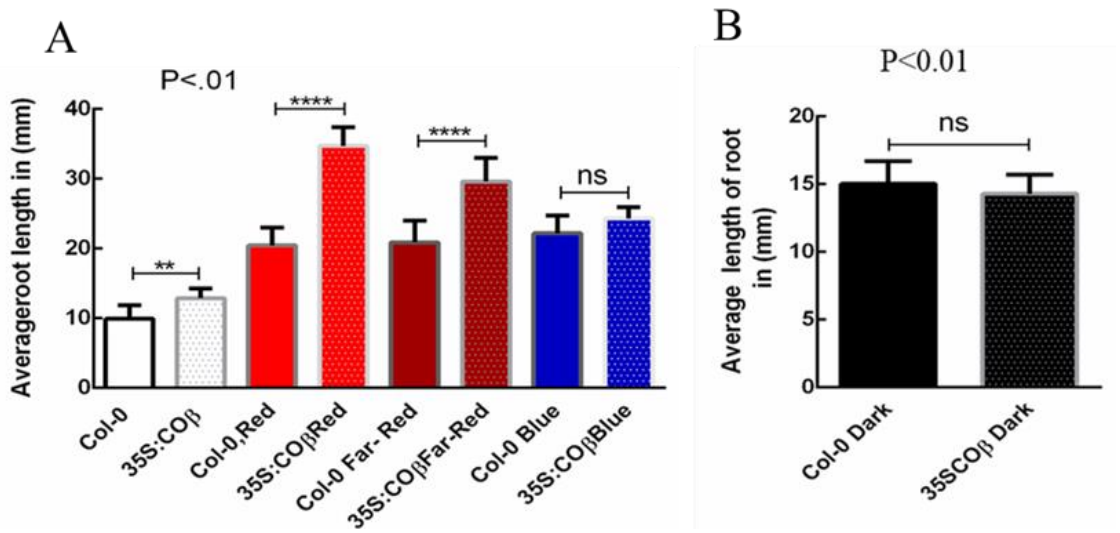



**Figure 4.16 Measurements of root length in over-expressed lines of COβ in different light qualities. In different light quality (A) and dark grown seedling (B).This non-significant differences in dark grown seedling was confirmed was confirmed by Mann Whitney Unpaired t-test statistical analysis for the significance level at p <0.01.White histogram represent white light, red histogram represent red Light, wine colour histogram represent far-red Light and blue histogram represent blue Light.**

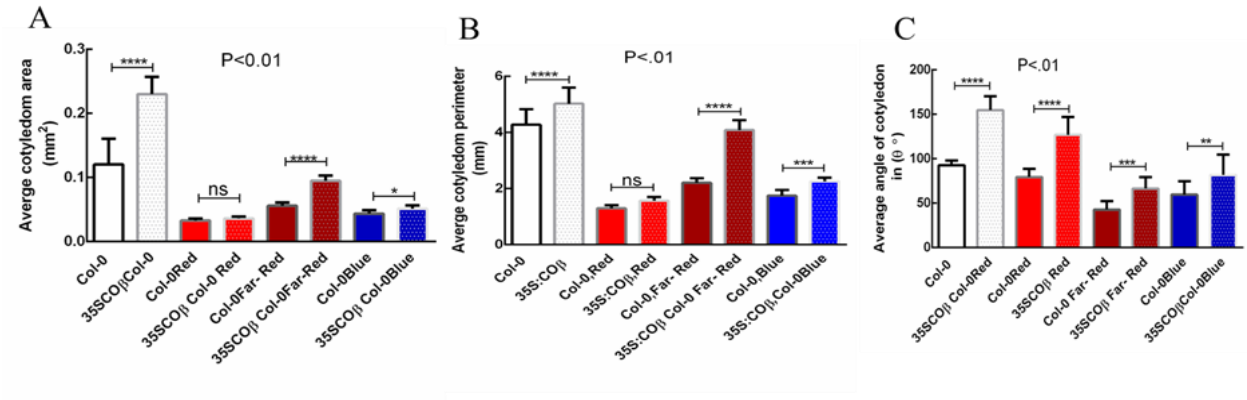



**Figure 4.17 Measurement of cotyledon area, cotyledon perimeter and cotyledon angle in over-expressed lines of COβ under different light qualities.Here (A) represent cotyledon area,(B) cotyledon perimeter and (C) angle of cotyledon.White histogram represent white light, red histogram represent red Light, wine colour histogram represent far-red Light and blue histogram represent blue Light. The data presented is the mean of 20 individual plants.**

We also observed more cotyledon area (Figure 4.17A), cotyledon perimeter (Figure 4.17B) and

cotyledon angle (Figure 4.17C) in over-expressed lines of COβ as compared to the respective control and this observed phenotypes are independent of light quality such as red light, blue light and far red light. However the cotyledon area and perimeter changed in FR and Blue suggests the of role of PHYA and also here a weak blue light functions

### 4.8 Diurnal and developmental expression pattern of COβ in wild type and *gi-100* mutant

It was also studying the diurnal and developmental expression pattern of the *COβ* transcript in Col-0 and *gi-100* (mutant of GIGANTEA, Figure 4.18) and developmental expression pattern (Figure 4.19) of *COβ* transcript in Col-0 background. We observed the transcript level of*COβ* at different Zt (Zt-0, Zt-4, Zt-8, Zt-12, Zt-16 and Zt-20) was significantly higher in *gi-100* as compared to Col-0. Highest transcript level of *COβ* was observed at Zt-8 in *gi-100* and Zt-12 in Col-0.

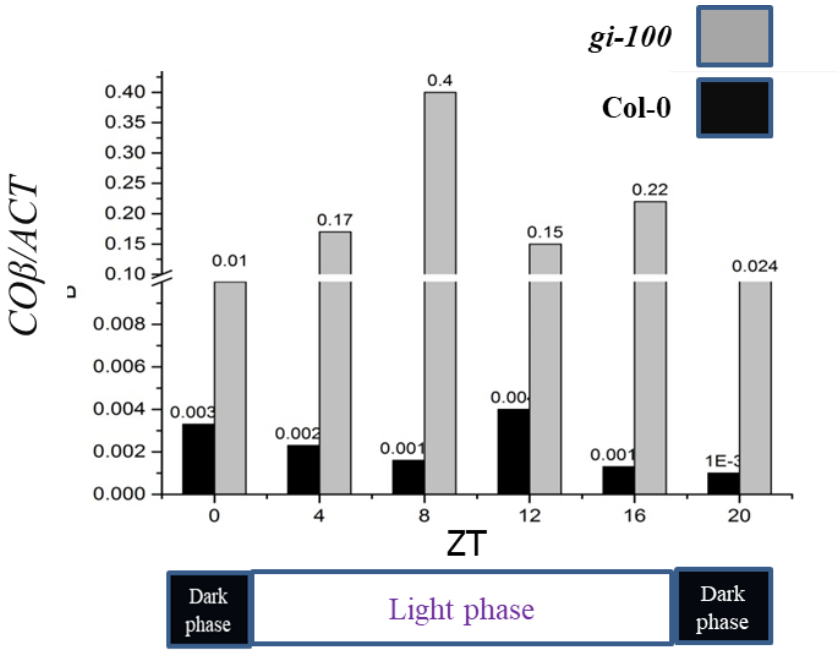



**Figure 4.18 Quantitative RT-PCR analysis of diurnal expression pattern of *COβ* transcript at different Zts. At Zt-0, Zt-4, Zt-8, Zt-12, Zt-16 and Zt-20 in Col-0 (black histogram) and *gi-100* (faint white histogram) under long day conditions. Highest transcript of *COβ* observed at Zt-8 in *gi-100*.**

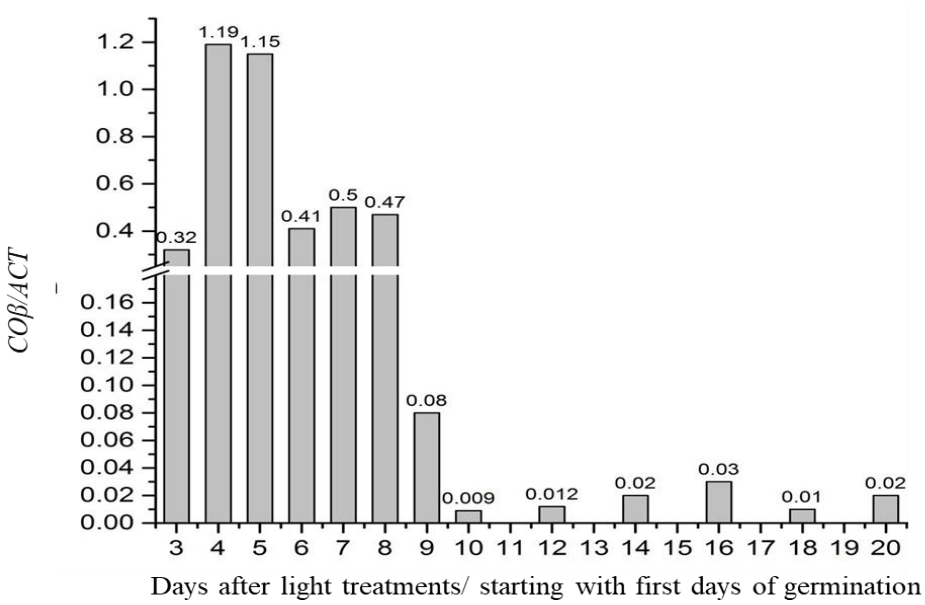



**Figure 4.19 Quantitative RT-PCR analysis of developmental expression of *COβ* transcript in Col-0 background at Zt-12 under long day conditions. *ACTIN* was taken as control.**

However,in the developmental regulation of *COβ* transcript expression was the significantly high till 9 days after light treatments under long day conditions in col-0, after that the transcript of *COβ* goes down till 20 days.

### 4.9 Sub cellular localization of COβ in leaf tissue

In order to understand the localization pattern of COβ we generated the transgenic lines of COβ with N-terminal CFP fusion protein (35S::CFP:COβ).

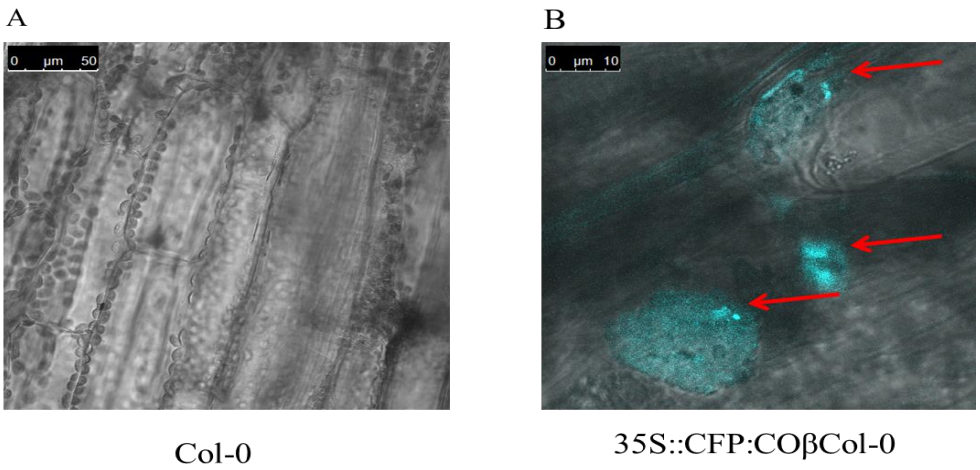



**Figure 4.20 Cellular localization pattern of 35S::CFP:COβ fusion in leaf mesophyll Cell The CFP signal of COβ is marked by red arrow head (B) and no signal observed in Col-0 (A).**

We observed the localised pattern of COβ in the form of CFP signal in leaf mesophyll cell (Figure 4.20B) with respect to control Col-0 (Figure 4.20A). Since it was the leaf tissue, we can also expect autoflorence signal of chlorophyll.

### 4.10 Adaxial leaf trichome number is higher in over expressed lines of COβ

It was also counted number of trichomes at adaxial surface of leaves in over-expressed lines of COβ (Figure 4.21B) were founded about 18 as compared to Col-0 (Figure 4.21A) which was about 4.The statistical significant has been tested byMann Whitney Unpaired t-test statistical analysis for the significance level at p <0.01(Figure 4.22C).

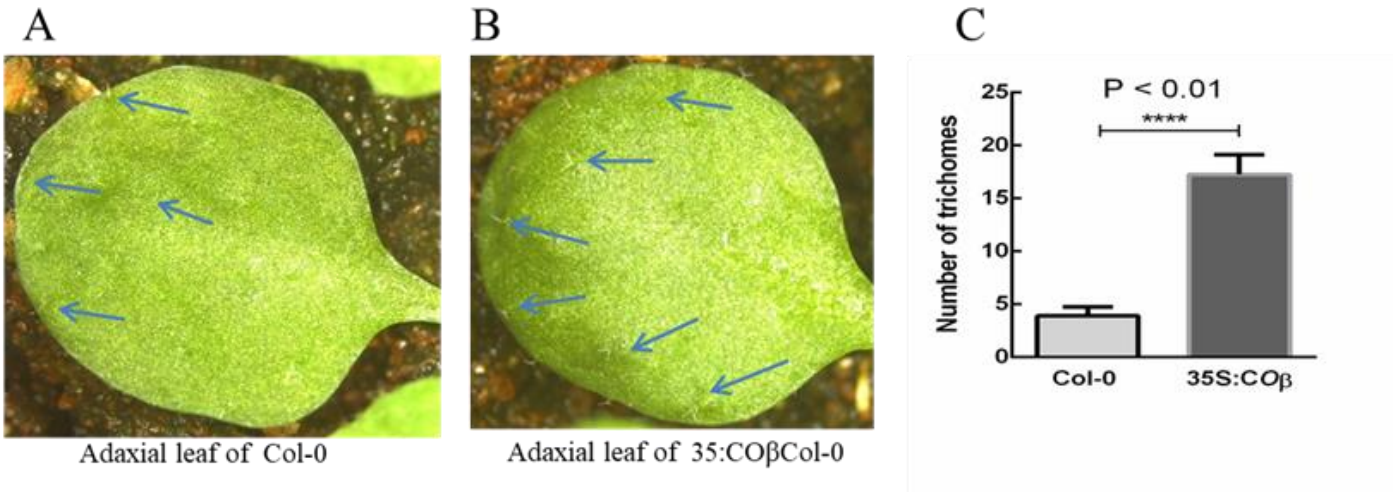



**Figure 4.21 Counting of trichome number in adaxial leaf surface of over-expressed lines of COβ.Trichome number was calculated in 35S::COβ in (B), and Col-0 (A) .The comparative statistical analysis of trichomes number in (C).This significant differences was confirmed by Mann Whitney Unpaired t-test statistical analysis for the significance level at p <0.01.**

### 4.11 COβ over-expression causes regulation of of miR156

Since miR156 is required for trichome formation (Gang et al., 2009) therefore its abudance was investigatedas described by Shiva P et al., (2012) in over-expressed lines of COβ it was found that miR156 was relatively high in 35S::COβ lines as compared to Col-0, after the 12$^{th}$ day and 16$^{th}$ day of light treatments (Figure 4.22).

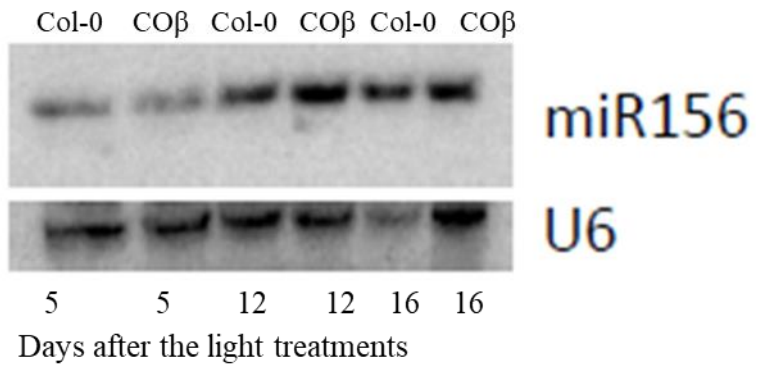



**Figure 4.22 Expression pattern of miR156 in COβ lines under long days cycles. Here we have taken U6 as an expression control. Samples were harvested after the 5 days of light treatments till 16 days. Here COβ top on figure represents the over-expressed lines of COβ and Col-0 as a control.**

### 4.12 COβ over-expression causes higher seed yield

It was shown that the yield performance is correlated to pod length (Silique length) and pod weight (silique weight) (Yuhua et al., 2016, and Ying et al., 2015), therefore we verified the yield performance in different transgenic lines of COβ (35S::COβ) by mesurering the average pod length (Figure 4.23) and pod weight (Figure 4.24). It was found to be significantly higher in over-expressed lines of COβ irrespective of genetic backgrounds such as Col-0, *co-10* and *phyb-9* compared to their respective control.

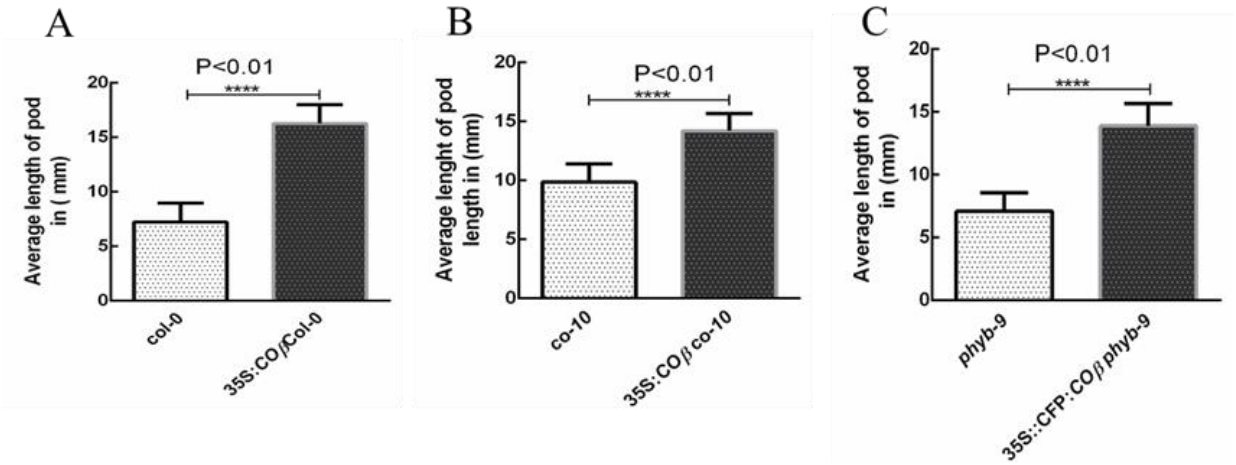



**Figure 4.23 Measurements of pod length in over-expressed lines of COβ, in different genetic backgrounds. In col-0 (A), *co-10* (B) and *phyb-9* (C) genetic background. This significant difference was confirmed by Mann Whitney Unpaired t-test statistical analysis for the significance level at p <0.01. Here white histogram represent Col-0 and black histogram represent 35S::COβ.**

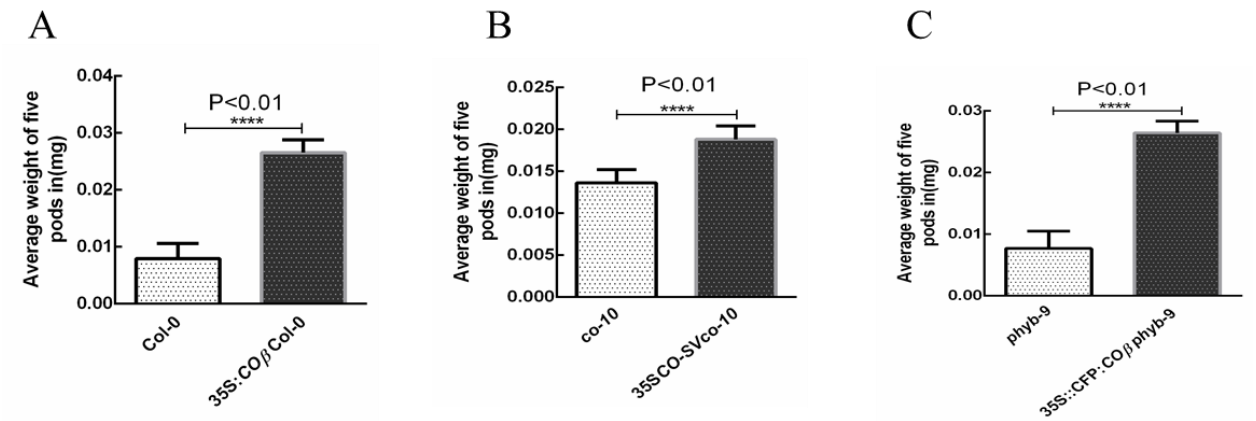



**Figure 4.24 Measurements of pod weights in over-expressed lines of COβ, in different genetic backgrounds. In col-0 (A), *co-10* (B) and *phyb-9* (C) genetic background. This significant difference was confirmed by Mann Whitney Unpaired t-test statistical analysis for the significance level at p <0.01.**

## 4.13 Discussion

In the current study, it was demonstrated that over-expression lines of COβ delayed flowering irrespective of genetic background (Col-0, *co-10* and *phyb-9*) tested irrespective of day length periods. However, the delayes found to be less significance under SDs. This result suggested an involvement of a common mechanism. As the the over-expressed lines of COβ responded to the difference day-length differently in terms of their flowering time, theseare photoperiod sensitive. The differences in rosette leaf number at the time of bolting between control and the COβ lines were significantly higher under LDs than compared to under SDs, suggesting that the delayed flowering phenotype in the over-expressed lines of COβ plants were marginally photoperiod dependent. Delayed in flowering in the over-expressed lines of COβ plants even in *co-10* background, suggested a COα independent mechanism by passing the classical (CO-FT) photoperiod module.

Our results also showed the delay in flowering time by over-expressing of COβ in specific tissues such as phloem and meristematic tissue in similar way both under LD and SD conditions. The level of significance was found to be less under SDs indicating that this phenotype is not due to the tissue specific expression of COβ. It was also observed more vegetative growth in the over-expressed lines of COβ plant with respect to controls suggesting higher potential of these

plants for biomass production. The higher chlorophyll content in the over-expressed lines of COβ also suggested the involvement in chlorophyll homeostatic.

It was also observed shorter hypocotyl in the over-expressed lines of COβ in white light (WL), red light (R), far red light (FR) and blue light (B) compared to Col-0.The shortest hypocotyl length was observed in red light, which was similar to over-expressed phenotype of phytochrome (PHYB) (Wagner al 1995).These results indicated that COβ over-expression might lead to the up regulation the PHYB. It is correlated with the delayed flowering in the over-expressed lines of COβ.This observation was already published supported by the physical interactions of PHYB with CONSTANS that resulting delay in flowering (Endowed et al., 2013). Since the over-expressed lines of COβ increase the cotyledon angle, cotyledon area cotyledon perimeter and root length under different light qualities (White Light, red light far red light (FR) and blue light (B) suggesting the regulation of COβ by the combinatorial effect.

In this study it was demonstrated that significantly higher level of transcript of *COβ* in *gi-100* genetic background compared to Col-0 under LDs. From this result suggested that GIGANTEA may be required for proper splicing of *CONSTANS.* However, when analysis for the developmental regulation of *COβ* transcript was performed a significantly high in the *COβ* transcript till 9 days was observed then goes down till 20 days after light treatments. This suggested, that COβ could be the one of factor that coincides the juvenile phase in *Arabidopsis thaliana*. It was also observed that the localised pattern of COβ with CFP fusion protein in leaf tissue, suggested that putative truncated protein of COβ is functional.

In the current study also observed significantly higher number of trichomes at adaxial leaf surface in over-expressed lines of COβ (Figure 21B) ascompared to Col-0,as trichomes are known to be developed form a specialized cell by differentiation processes (John C et al., 1996). It is already shown that (Gang et al., 2009) in over-expressed line of miR156 have a greater number of trichomes.We could find remarkably higher transcript level of miR156 in 35S:COβ

lines as compared to Col-0. This suggested that over-expression of COβ notably increases the trichomes numbers by increasing the miR156 transcript expression.

Our result showed the seed yield in over-expressed lines of COβ remarkably higher in Col-0, *co-10* and *phyb-9* transgenic lines. This result directly signifying that photosynthetic performances of over-expressed lines of COβ are high therefore the dry matter of pod (silique) is more (Yuhua et al., 2016). Moreover, the silique also serves to coordinate the seed filling and protects seeds against biotic and abiotic stresses. The silique length (SL) and silique weight (SW) is significantly more in COβ plant, positively correlated with more seed weight one of the components of plant grain yield (Ying et al., 2015). Being siliqueis a desirable trait in over-expressed lines of COβ, it can be useful for convential breeding programs.

# Chapter 5: RESULTS &DISCUSSION

## CARBON NANOPARTICLES ACCELERATE FLOWERING IN OVER-EXPRESSEDLINE OF COβ AND COL-0 IN *ARABIDOPSIS THALIANA*

# 5. Result

## 5.1 CNPs treatment induces early flowering in dosage dependent manner in *Arabidopsis thaliana*

Increasing concentration of CNPs resulted in dosage dependent earlier flowering in *At* (Figure 1a-d and Supplementary Figure 5.2A-D). Under LDs, the untreated Col-0 plants showed bolting at around the 11 ± 1 rosette leaf stage. A significant reduction in leaf number was observed following the exposure of increasing concentration of CNPs from (10-500 µg/ml), with the plants flowering on an average 2 leaves earlier (Figure5.1B, C and E).

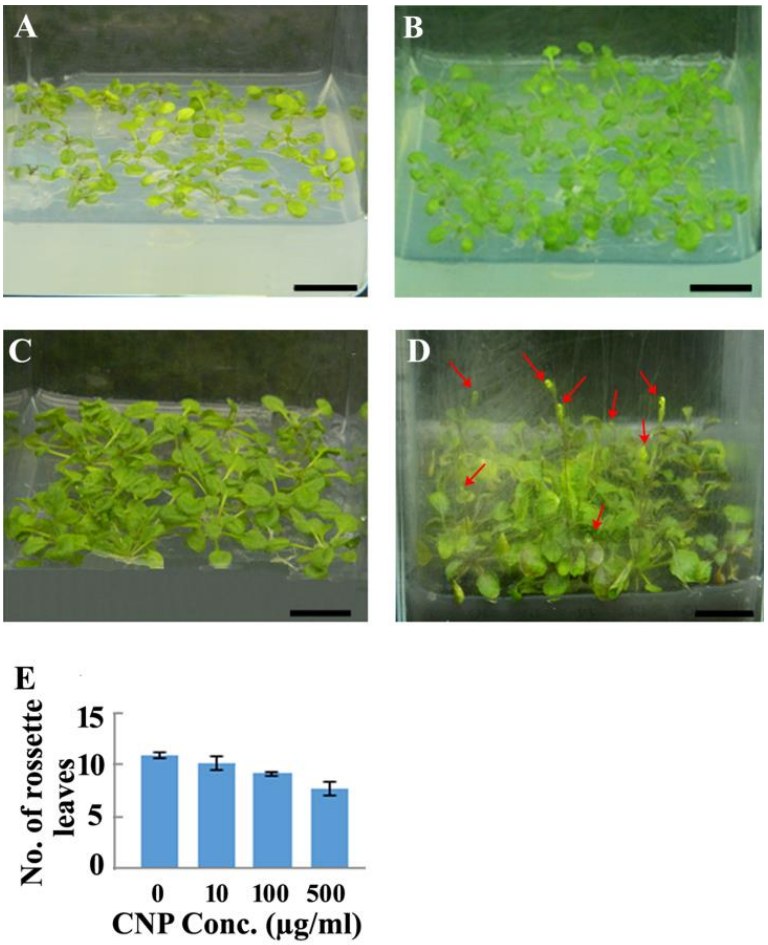



**Figure 5.1 Dose dependent effects of carbon nanoparticles in flowering time A, Without CNPs or B, 10 µg/ml C, 100 µg/ml and D 500 µg/ml of CNPs. Plants showed bolting were marked with red arrows. E, Evaluation of flowering time of Col-0 plant grown with different concentration of CNPs in MS media. Rosette leaf number was considering as an indicator of flowering time which was counted at the time of bolting. The data presented was the mean of 20 individual plants. Col-0 plants normally bolted in 22 d on the soil however on MS media bolting was delayed by ~3 d in all biological replicate.**

Further increasing CNPs concentration (500 µg/ml) resulted bolting even earlier at 3 ± 0.5 leaves as compared with the untreated plants (Figure 5.1D-E and Supplementary Figure5.2D). Therefore, all subsequent experiments were conducted using a CNPs concentration of 500 µg/ml in a medium.

### 5.2 CNPs induce early flowering in *Arabidopsis thaliana*

To confirm that the early induce flowering phenotype by CNPs was not a result due to crowding; individual seedlings were grown in single plantons in the presence of CNPs in a similar method as described above (Figure 5. 2A and 5.2B).

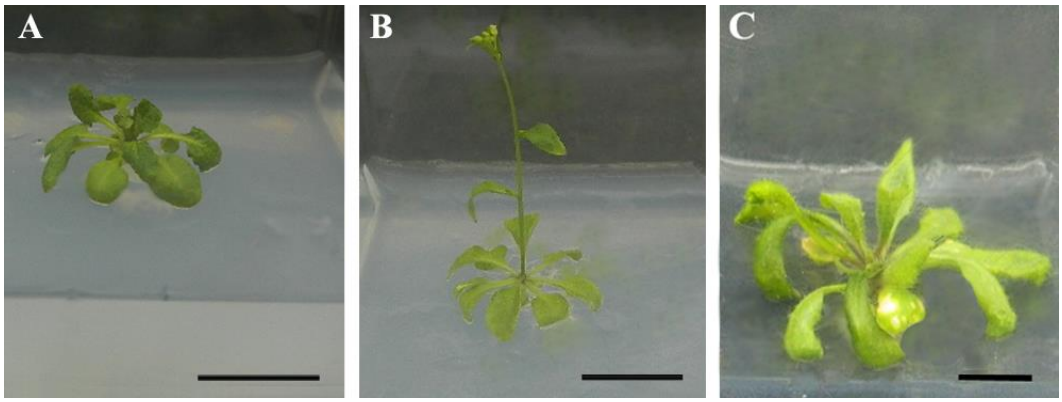



**Figure 5.2 Effect of carbon nanoparticle treatment on individual *Arabidopsis* plants.Effect of CNPs treatments on the phenotype of individual plant on MS medium under LD (16 h light / 8 h dark cycles) growth conditions. A, Without or B with CNPs of 500 µg/ml till bolting stage. C, Representative Picture of a plant grown in the presence of 500 µg/ml charcoal as an additional control. Plants were photographed on 20-21 d after transferring to light. Distinct earlier flowering was observed only in plants with CNPs in the specified growth conditions, while larger leaf size and pale greenish plants were documented in all biological replicates in presence of charcoal. Scale bar: 10 mm.**

In order to ascertain that the early flowering phenotype was specific to CNPs and not due to the higher amount of carbon *per se*, Col-0 plants were grown in presence of 500 µg/ml charcoal under LDs (Figure5.2C). Charcoal-treated plants showed very different rosette morphology (Figure5.2C).Average rosette leaf size was larger and longer. These plants were less green and showing some sign of localized chlorosis or senescence as yellow patches but eventually flowered 5 rosette leaves or more compared to its untreated control under LDs.

### 5.3 CNPs accumulated to highest in leaf tissues

To determine whether CNPs were taken up systemically through plant roots, CNPs accumulation

Was investigated in root, stem and leaf extracts made from in 20d old plants as described in

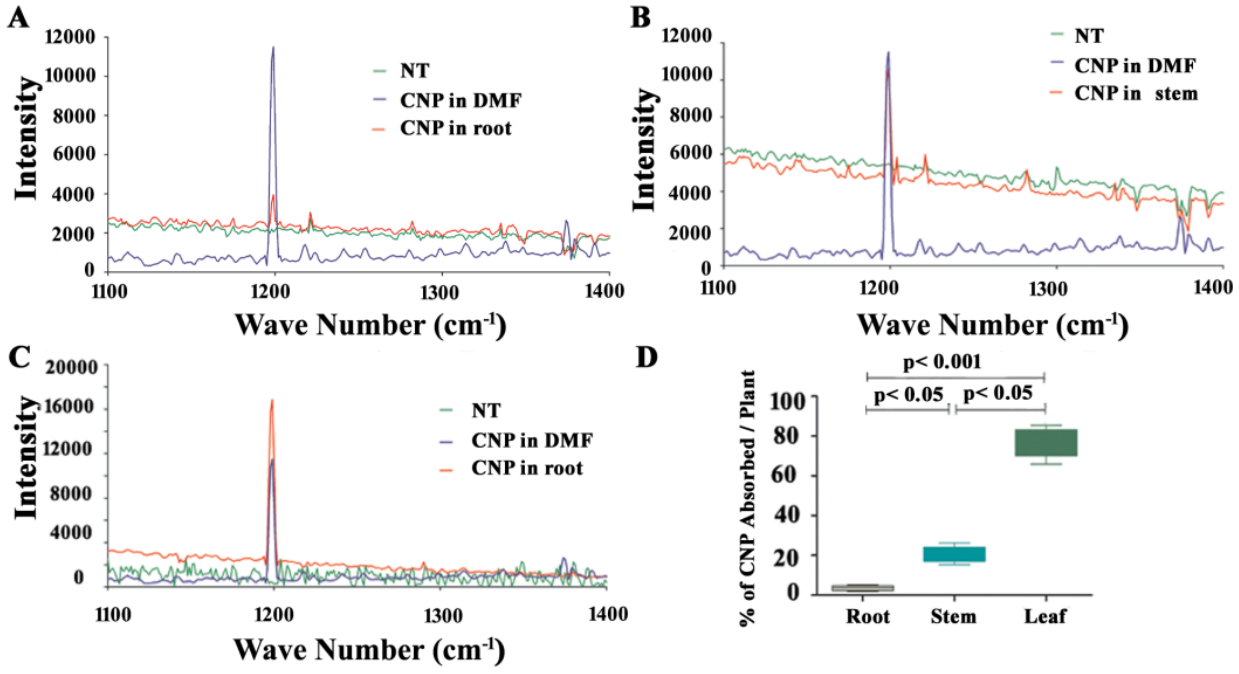



**Figure 5.3 Accumulation of carbon nanoparticles assayed using Raman Spectroscopy.Col-0 plants Raman Spectra obtained from root A, stem B or leaf C showed a peak at wave number 1200 cm-1 (red) indicative of CNPs (as shown by arrows), as the CNPs control alone in di-methyl formamide (DMF) (blue) overlap with the same wave number. D, Quantitative estimation of CNPs was done from the area of the peaks using Prism 5 software and presented. Percentage of CNPs accumulation in root (shown in grey), stem (shown in blue) or leaf (shown in green) indicates significant difference of CNPs accumulation (P < 0.001).**

Materials and methods and were subjected to Raman spectroscopy (Figure 5.3). As a positive control, CNPs at 500 µg/ml in di-methyl formamide (DMF) solvent showed sharp peak at wave number 1200 $cm^{-1}$. All the tissue extracts tested showed accumulation of CNPs at wave number comparable with that of DMF solvent. However, the amplitude of accumulation was highest in the leaf extracts and least in the extracts of root. The percentage of CNPs accumulation was quantified as ~75% in leaf, ~20% in stem and ~5% in root extracts. These results indicated that

the accumulation of CNPs in Col-0 plants showed tissue specificity and was predominately enriched in the leaf. Tissue specificity is an interesting point to understand the interaction of nanomaterial's with plants, also documented in *At* (Bao et al., 2016) and in tomato (Dan et al., 2015).

### 5.4 CNPs exposure affected different physiological traits at various growth stages of *Arabidopsis thaliana*

We first examined whether the CNPs-induced earlier flowering response is due to higher germination rate. CNPs treatment showed a marginally enhanced effect on the percentage of seed germination in the Col-0 seeds as scored after 3 days of irradiation under WL in LDs(LDs: 16 h light / 8 h dark). While Col-0 seeds showed 83% germination on MS medium, CNPs treated seeds showed nearly 5% higher 88.7% germination.CNPs treatment affected the hypocotyl length significantly ($p \leq 0.01$) under WL both under LDs and SDs(8 h light / 16 h dark) (Figure 5.4A and 5.4C, Supplementary Figure 5.3A and Supplementary Figure5.5). To determine whether the hypocotyl growth inhibition was not a developmental defect, seedlings were grown for 8 days in darkness after germination induction light treatment without or with CNPs. Both the CNPs treated andun-treated seedlings showed distinct etiolated phenotype with elongated hypocotyls and closed pale-yellow cotyledons (Figure5.4B) suggesting the hypocotyl elongation growth in presence of CNPs was light dependent. Compared to the respective controls in LDs and SDs, CNPs treated seedlings under WL consistently showed about 60.6% (Figure5.4C) and 30% (Supplementary Figure5.3A) increase in hypocotyl length, respectively. To determine if the increase in length of hypocotyl was light quality-dependent, seedlings were grown under monochromatic R, FR, or blue lights.Compared to controls, a dramatic increase of 2-fold in hypocotyl length was observed when the seedlings were grown under red light.Root length in seedlings grown under white light was also significantly affected due to CNPs (Figure 5.4D, Supplementary Figure 5.3B). Compared to WL controls, the CNPs treatedseedlings showed significantly ($P<0.01$) longer primary roots in LDs and SDs. Under differentqualities of

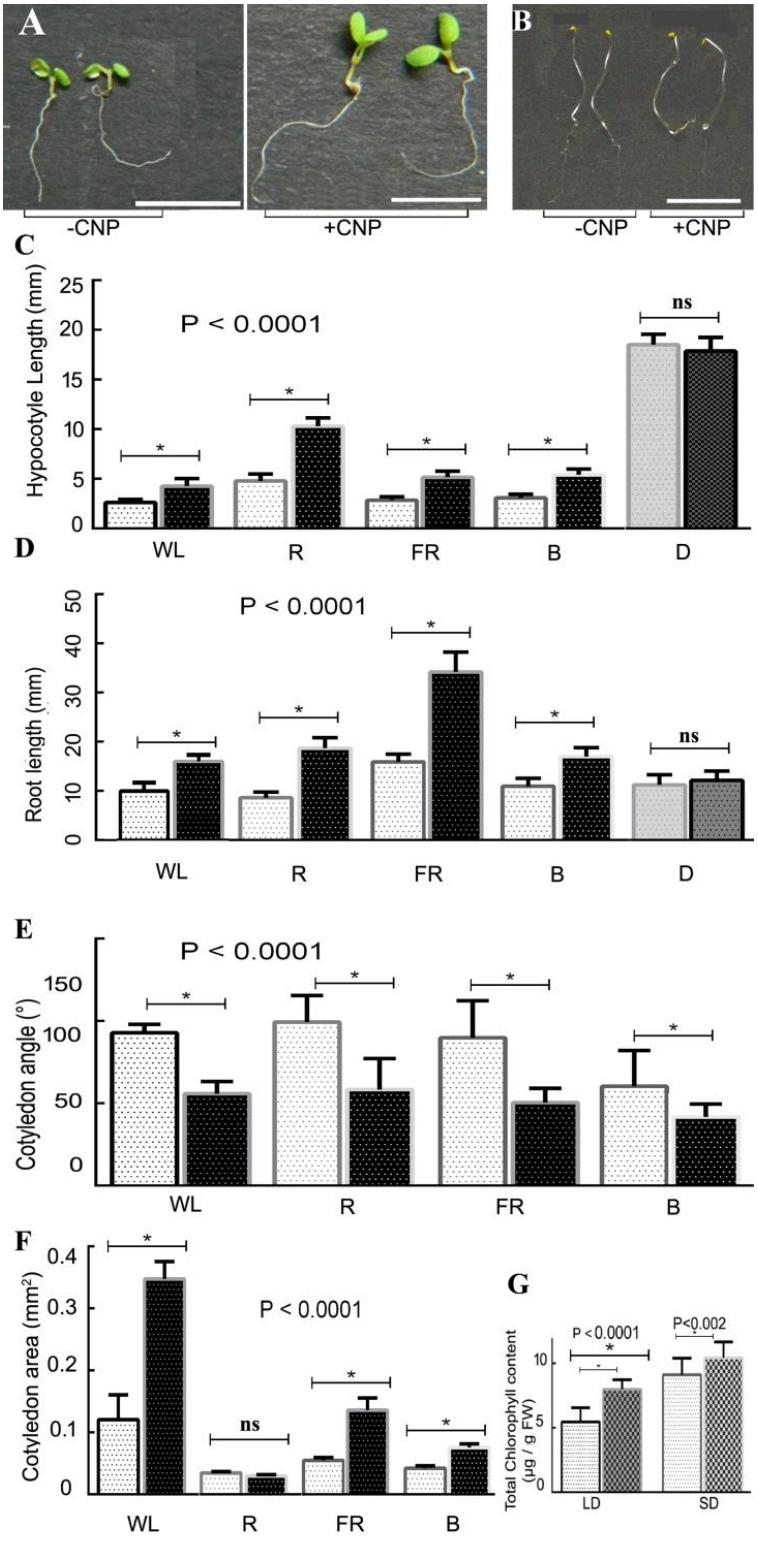



**Figure 5.4 Effect of carbon nanoparticles treatment on seedling phenotype. Pictures of two representative seedlings grown under white light A, etiolated seedlings without (-CNPs) or with (+CNPs) B. C-F Average hypocotyl length, root length, cotyledon angle and cotyledon area of seedlings grown under White (WL), Red (R), Far-red (FR), Blue (B) lights or Dark (D) respectively. White dotted histograms indicate – CNPs while black dotted histograms indicate + CNPs. g Seedlings were grown for 8 d either in Long days (LDs) or Short days (SDs) and chlorophyll content estimation was done as described in materials and methods.**

monochromatic light, although the root length of CNPs treated seedlings wasincreased as observed in WLthe increase was highest and significant under the FR with more increased as

than double than that of the untreated seedlings.CNPs treatment also affected cotyledon opening of seedlings (Figure 5.4e, Supplementary Figure5.3C). Cotyledon angle in 8 d old Col-0 seedlings was ~93°, whereas in the case of CNPs-treated seedlings it was ~56° (Figure5.4E). The cotyledon angle of seedlings grown in SDs also followed the LDs pattern (Supplementary Figure5.3C). Cotyledon angle also showed similar pattern as the LDs under different qualities of monochromatic lights R, FR and Blue (Figure 5.4E). The cotyledon area of the CNPs treated seedlings was significantly higher than that of the untreated seedlings in both LDs and SDs (Figure 5.4F, Supplementary Figure5.3D). CNPs treatment resulted in enhanced chlorophyll accumulation. Under LDs, total chlorophyll content increased from 4.8 µg/mg in control seedlings to 7.5 µg/mg in the CNPs-treated seedlings (Figure 5.4G).

**5.5 Early flowering induced by CNPs treatment is photoperiod dependent**

Bolting, as indication of flowering time, in Col-0 was observed at 11 (±1) rosette leaves and in between 25 - 28 d, whereas the CNPs-treated plants flowered at around 8 (±1) rosette leaves and took about 20 LD cycles.CNPs treatment in the SDs growth conditions also resulted in earlier flowering. CNPs-treated plants bolted at about 5 d earlier (with 28-30 SD cycles), while Col-0 plants bolted at 34-36 SD cycles post-germination. Since the photoperiod is perceived at the leaf (Song et al., 2015), and the mechanism operates from phloem companion cells, it was further asked if the early flowering phenotype due to CNPs treatment was photoperiod dependent.The leaf number at bolting stage in LDs was counted in the CNPs treated and untreated plants (Figure5.5A and 5.5B).Average rosette leaf number in the Col-0 plants with and without CNPs treatment showed a significance difference ($p < 0.01$).

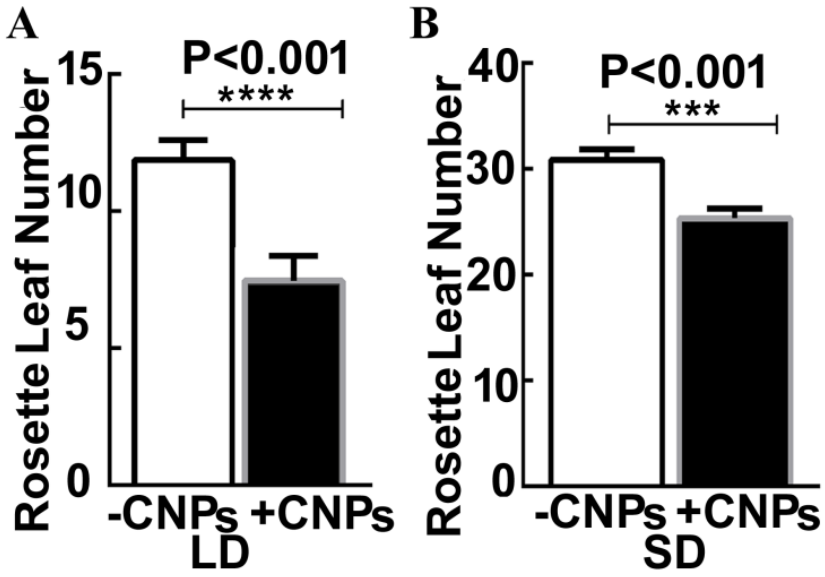



**Figure 5.5 Leaf number at bolting stage. Flowering time of Col-0 under long days and Short days. Plants were grown in MS medium without or with CNPs (500 µg/ml) treatment. Col-0 plants grown for 20 d in LDs A, or in SDs B, for 30 d. White histograms represent without CNPs and black histograms represent with CNPs. Rosette leaf number data presented is a mean of at least 3 individual experiments.**

Further, the leaf number was counted in plants grown under SDs (Supplementary Figure 5.4).

The control plants flowered at 31.8 ± 0.7 leaves, whereas the CNPs treated plants flowered earlier with 30.33 ± 0.57 leaves in 10 µg/ml, 28.7 ± 0.2 leaves in 100 µg/ml and 25.7 ± 0.7

leaves in 500 µg/ml of CNPs respectively. Hence, it indicated that the early flowering phenotype due to CNPs is partially photoperiod dependent, the responses being pronounced in LDs.

**5.6 CNPs treated samples have less *PHYB* transcript compared to the untreated seedlings**

Involvement of Phytochrome B (PHYB) was investigated to understand the red-light specific increment of hypocotyl length, in 10 d old seedlings with or without CNPs treatment under LDs (Figure 5.6A). The expression of *PHYB* transcript was analysed using qRT-PCR at Zeitgeber (ZT) 8, 12 and 20, relative to the transcript level of *ACTIN*.*PHYB* transcript levels were higher at all the time points of light phase tested in the untreated seedlings than their respective CNPs

treated seedlings. The most contrasting decrease in the *PHYB* expression was found to be at ZT 12 in the CNPs treated seedlings. These results suggested that the expression level of *PHYB* was down-regulated upon CNPs treatment.

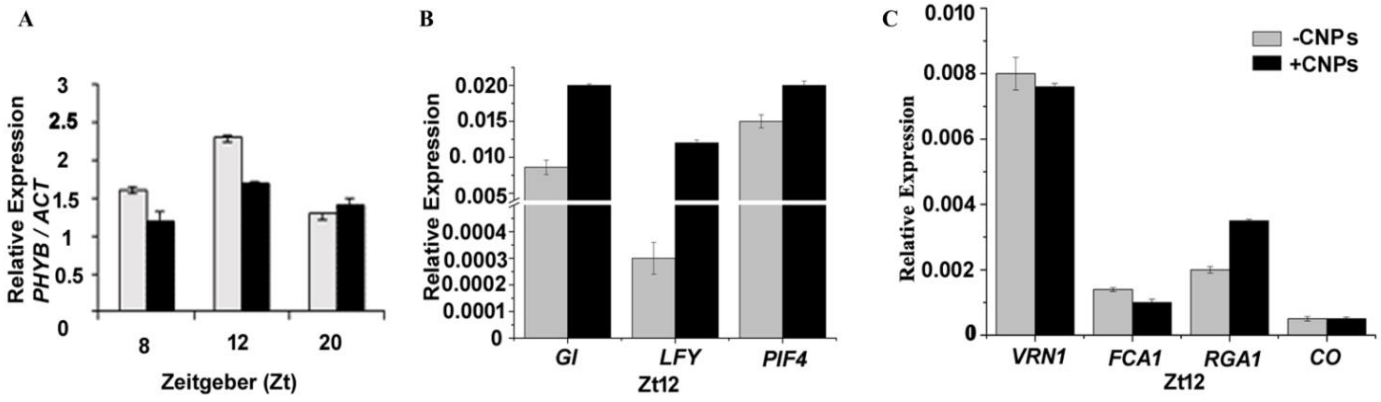



**Figure 5. 5 Effect of carbon nanoparticles treatment on transcript levels of representative genes of different flowering pathways.Quantitative RT-PCR analyses of representative transcripts from flowering pathways. Relative transcript level of *PHYB* using qRT-PCR at ZT 8, ZT 12 and ZT 20 A, and normalized with the expression of *ACTIN* (*ACT*). Relative transcript level of *GIGANTEA (GI), LEAFY (LFY)* and *Phytochrome Interacting Factor 4 (PIF4)* and c*VRN1, FCA1, RGA1* and *CONSTANS (CO)* at ZT12 B. Gary histograms represent without CNPs and black histograms represent with CNPs.**

### 5.7 CNPs treated 35S:PHYBGFPseedlings have less PHYB protein as compared to untreated plant

Involvement of Phytochrome B (PHYB) functional protein was investigated in 35SPHYB::GFP seedlings with or without CNPs treatment under LDs (Figure5.7D, E). We observed less protein

Of PHYB in CNPs treated plants (Figure 5.7E) as compare to un-treated plant (Figure 5.7D).Tested in the untreated seedlings than their respective CNPs-treated seedlings. However, in *phyb-9*

CNPs treated (Figure 5.7B) and un treated plant (Figure 5.7A) showed no difference in flowering time that was confirmed by statistical analysis (Figure 5.7 C), suggesting that CNPs treated plants has PHYB dependent flowering behaviour.

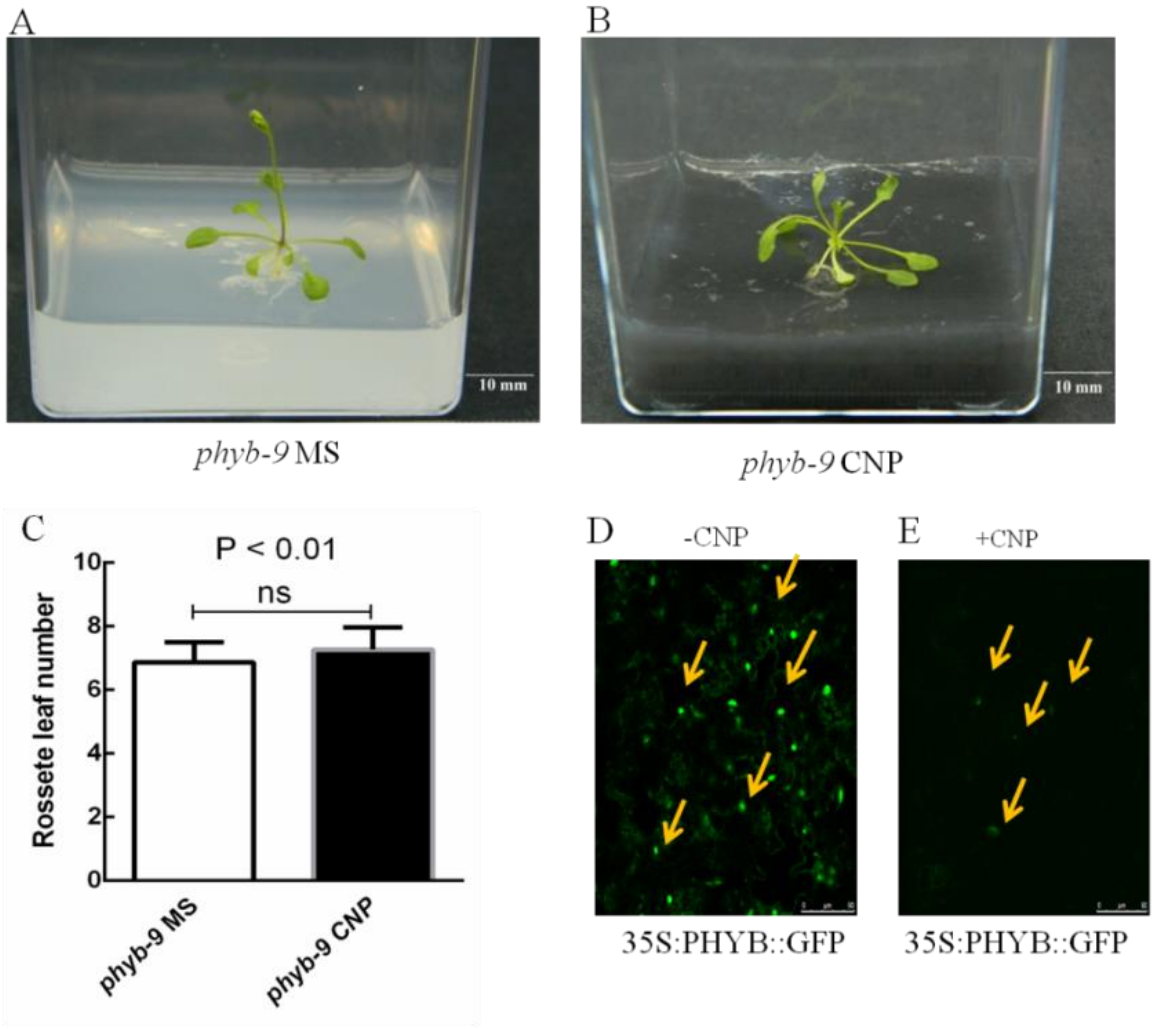



**Figure 5.6 Figure 7.Effect of CNPs treatment on phyb-9 plants phenotypes under long days. Pictures of two plants s grown under white light without (-CNPs) A, or with (+CNPs) B. Non-significant flowering time analysis without (-CNPs) and with (+CNPs), C.The reduce expression of PHYB protein in CNPstreated seedling in E, as compare to un-treaded CNPs seedling in D, respectively. White dotted histograms indicate – CNPs while black dotted histograms indicate + CNPs. Plants were grown till bolting stage inlong days (LDs).**

### 5.8 Early flowering due to CNPs is regulated by photoperiodic flowering time pathway components

Since CNPs induced the down regulation of *PHYB*, we further analysed the expression level of a selected component of the photoperiod pathway, the GIGANTEA (GI), at ZT12 in CNPs-treated seedlings. GI is a nuclear protein, regulated by circadian clock, and can directly activate the

expression of FT to regulate flowering time under LDs (Sawa et al., 2011). *GI* transcript levels in CNPs treated seedlings were nearly 2.1-fold higher than the untreated seedlings at ZT12 (Figure5.6B). The CONSTANS (*CO*) transcript levels in the CNPs-treated seedlings remained unaffected (Figure 5.6c) indicating a low probability of CO involvement and possible direct interaction of GI with FT to elicit the early flowering phenotype. This direct interaction of GI and FT was also documented previously (Sawa et al., 2011).To confirm that the early flowering was caused due to possible altered expression of other components of photoperiod pathway, transcript levels of Phytochrome Interacting Factor (*PIF4*) and *LEAFY* (*LFY*) were estimated in the CNPs treated seedlings. It was shown that interactions involving GI, FT and LFY influence the flowering and floral development (Blázquezet al., 1997; Nilsson et al., 1998). *At* ZT12, *LFY* transcript levels in the CNPs treated seedlings were highly induced with nearly 40-fold up-regulation compared to the untreated seedlings (Figure 5.6B). *PIF4*, which has been shown to negatively interact with PHYB (Huqet al., 2002), showed ~1.25-fold up-regulation showed ~1.25-fold up-regulation in the CNPs-treated seedlings at ZT12 compared to the untreated controls (Figure5.6B). These results further confirmed the involvement of photoperiod pathway for causing the early flowering phenotype.

To further analysis the CONSTANS is a master regulator in photoperiod pathway. Therefore, we checked CNPs treated phenotypes in CONSTANS mutant*co-10* background (T-DNA insertion mutant). The CNPs treated *co-10* plantshowed early flowering (Figure 5.8B) as compare to un-treated plant (Figure 5.8A) this results indicates the CNPs induce early flowering is CONSTANS independent. Statistical analysis of flowering time represented by (Figure 5.8C) showed significant further confirmed the involvement of for causing the early flowering phenotype.

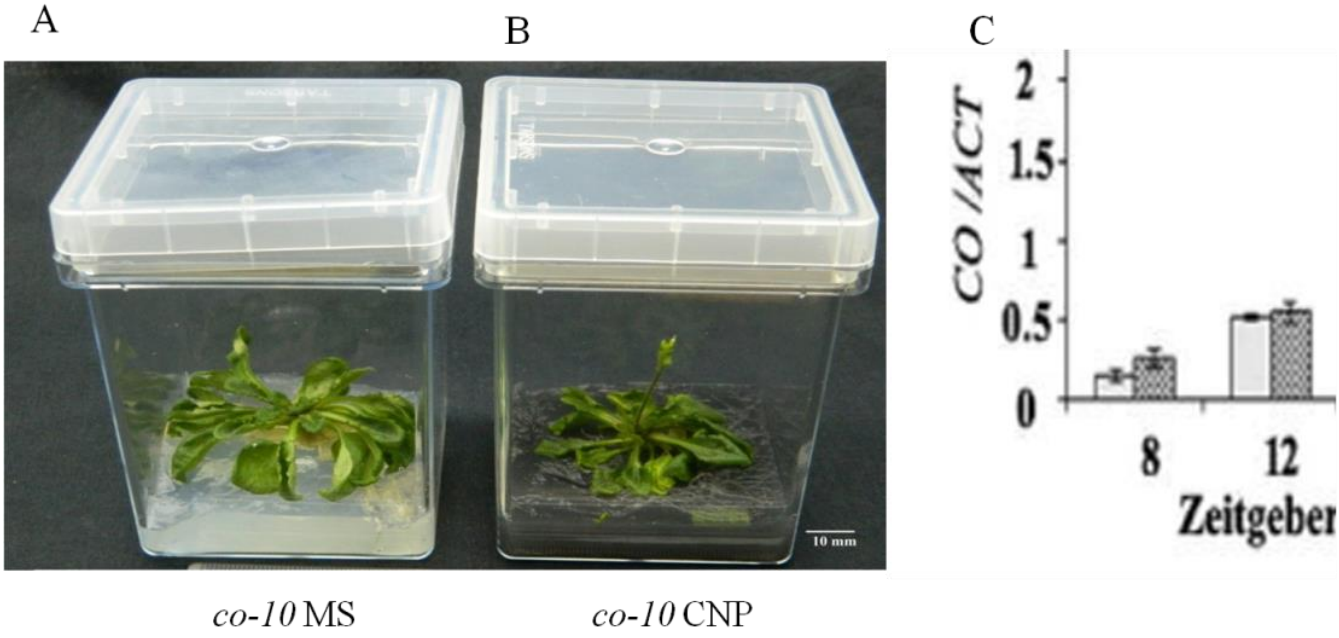



**Figure 5.7 Effect of CNPs treatment on *co-10* plants phenotypes. Pictures of two plants grown under white light without (-CNPs) (A) or with (+CNPs) (B). Non-significant result of q-RT analysis without (-CNPs, white histogram) and with (+CNPs, faint cross histogram) (C) at ZT-8 and ZT-12.Plants were grown till bolting stage in Long days (LDs).**

### 5.9 CNPs-induced early flowering is independent of GA, Vernalization and Autonomous pathways

To further dissect the involvement of other pathways for the early flowering phenotype due to CNPs treatment, one major component from each pathway (*FCA* for autonomous pathway, *RGA* for GA pathway and *VRN1* for vernalization pathway) was selected for expression analysis at ZT12 in the CNPs treated seedlings. The transcript levels of *VRN1* and *FCA1* in the CNPs-treated seedlings were nearly similar as compared with their controls (Figure5.6C). However the *RGA1* transcript levels showed about 1.7 fold up-regulations in the CNPs treated seedlings.

### 5.10 CNPs-induced early flowering in over-expressed line of COβ

CNPs also induced early flowering in in over-expressed line of COβ (Figure5.9B) as compare to untreated line of COβ (Figure 5.9A) in MS media under LDs.

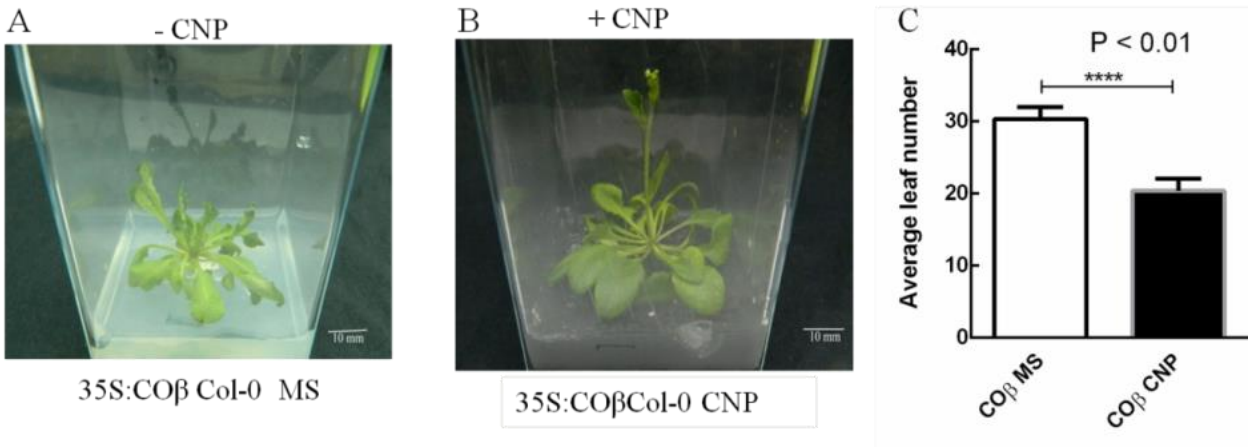



**Figure 5.8 Effect of CNPs treatment on 35S::COβ plants phenotypes. Pictures of two plants grown under white light without (-CNPs) A, or with (+CNPs) B. Significant difference of flowering time analysis without (-CNPs, White histograms) and withCNPs (black histogramsindicate) C. Plants were grown till bolting stage in Long days (LD) conditions.**

CNPs treated plant of over-expressed line of COβ showed bolting at around leaf stage 21 ± 1 while untreated plants at around leaf 32 ± 2rosette leaf stage. Statistical analysis of flowering time presented in (Figure5.9C).

### 5.11 A proposed model to explain flowering behaviour after CNPs treatments

A possible model explaining the early flowering caused due to CNPs treatment is drawn in. An

expression level of LFY determines the identity of primordia that arises at the shoot apical meristem and positively correlate the transition to flowering (Blázquez et al.,1997 It has been shown that mutation in flowering genes like *GI* causes reduction in *LFY*expression, hence, represses flowering (Nilsson et al., 1998). Also, PHYB, which causes late flowering, act as a negative regulator of the LFY (Nilsson et al., 1998) and delays flowering in WT. Here, we observed that after CNPs treatment expression of *PHYB* is down-regulated which results in the up-regulation of *LFY* expression also, *GI* transcript levels were enhanced.These combined effects lead to, hence resulting in early flowering. It has also been shown thatPHYB acts as a

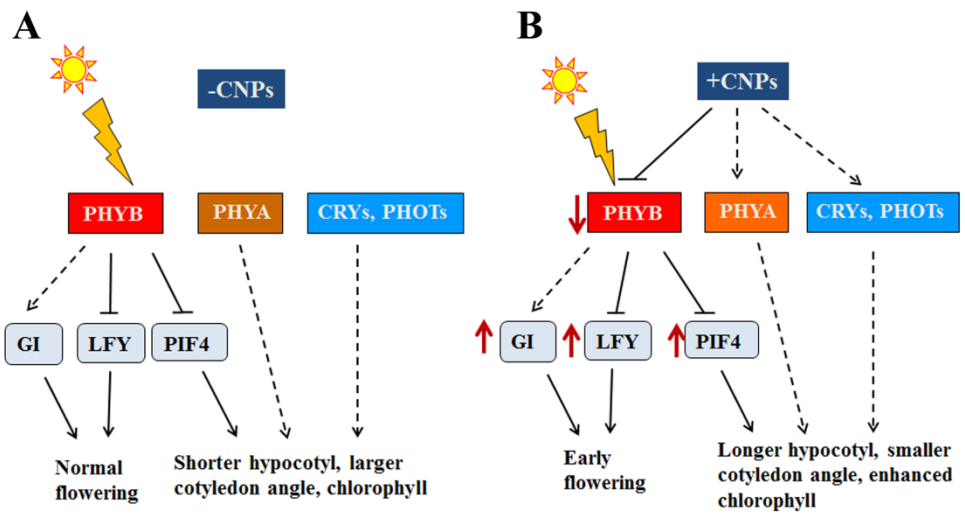


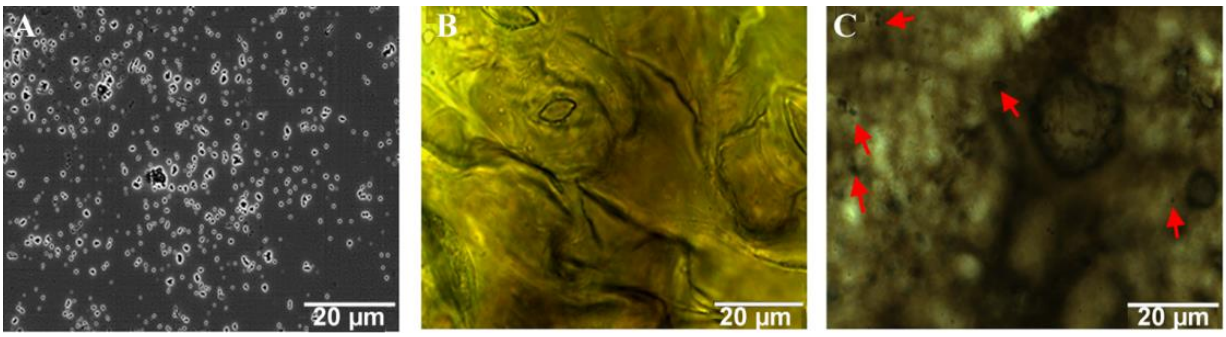

**Figure 5.9 Proposed model explaining possible involvement of CNPs in photomorphogenesis and flowering. In the absence of CNPs under white lighthigher transcripts of *PHYB* lead to the degradation of *PIF4* A. Ultimately -CNPs lower transcript levels of *PIF4* results in shorter hypocotyl and normal flowering. +CNPs treatment leads to down regulation of *PHYB* and ultimately accumulation of transcripts of *PIF4* B, since PHYB acts as a repressor of PIF4. *GI* transcript level is enhanced which leads to the up-regulation of *LFY* expression. Dotted lines between PHYB and GI indicate the involvement of multiple clock components leading and output to GI, Such as PHYA: Phytochrome A, CRY: Cryptochromes, PHOT: Phototropins.**

repressor for *PIF4* gene which causes short hypocotyl and large cotyledon angle in WT (Huq et al., 2002). Since after CNPs treatment *PHYB* transcript level was down-regulated, we found higher level of *PIF4* transcript which resulted in longer hypocotyl and smaller cotyledon angle compared to WT. In this study, we also observed enhancement in *RGA1* transcripts, which is a repressor of GA pathway. To further confirm that the reduced *PHYB* transcript caused early flowering in a GA-independent manner, we measured hypocotyl length of seedlings grown in the dark. The strongly etiolated phenotype and the differences in hypocotyl length in the dark after CNPs treatment supported the view that the process is GA-independent.

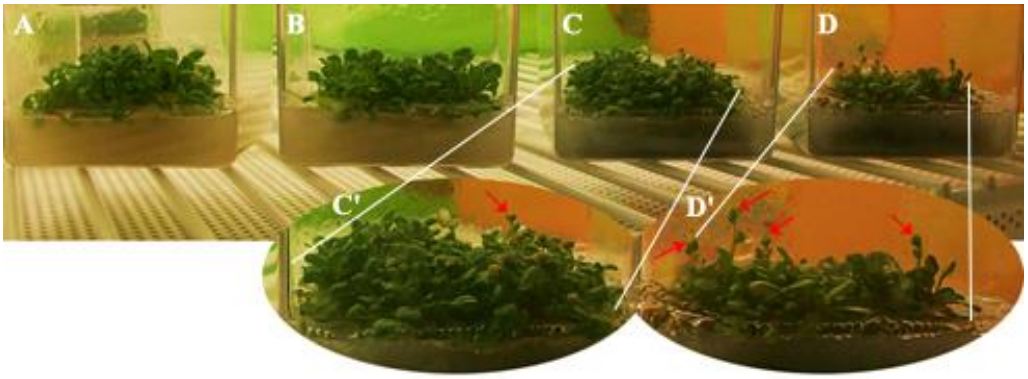
Smaller cotyledon angle in WL and under different qualities of light suggested the down-regulation of multiple photoreceptor functions and possible involvement of a common signalling pathway. Larger cotyledon area in all the light qualities tested suggested a complex combinatorial output of interaction of different signalling pathways. However, the higher

chlorophyll content in WL suggested significant down-regulation of PHYA and PHYB dependent pathways that mimic a *phyAphyB*double mutant plant, which also flowers relatively earlier compared to its WT control (Franklin et al., 2010; Neff et al., 1998). Since PIF4 is epistatic to PHYB and acts antagonistic to it, the *phyB* mutant accumulates PIF4 that result in longer hypocotyl and larger cotyledon area explaining the observation in the current study (Huq et al., 2002). Earlier flowering by CNPs treatment without the involvement of CO suggests an alternate mechanism that by passes the classical (CO-FT) photoperiod module possibly by direct interaction of GI on *FT* promoter.

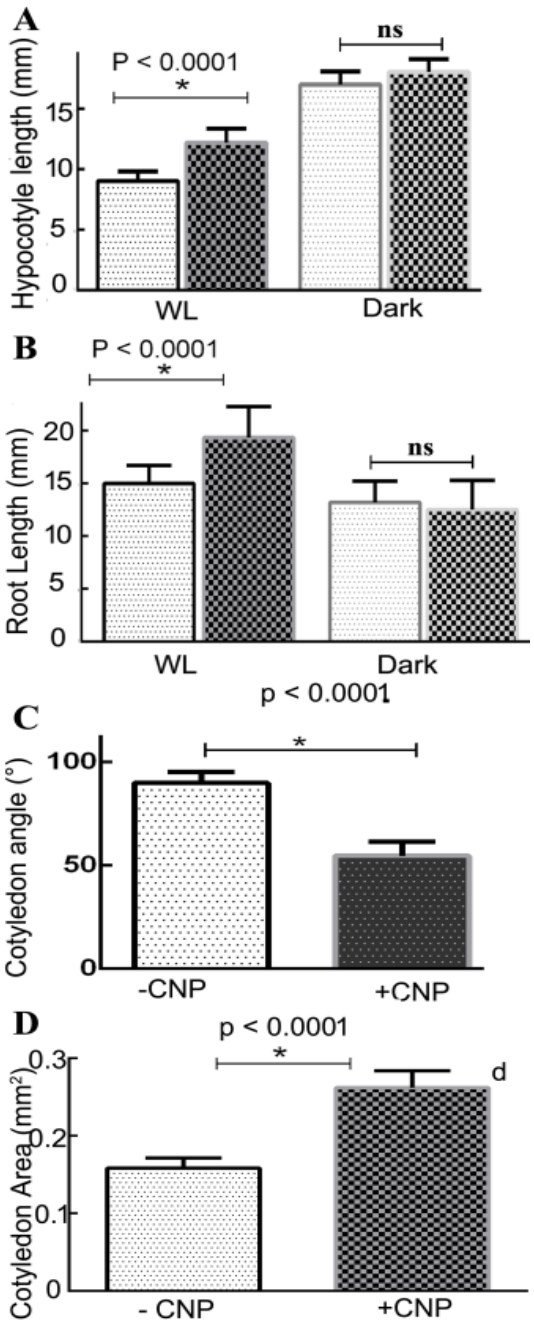
## SUPPLEMENTARY FIGURES



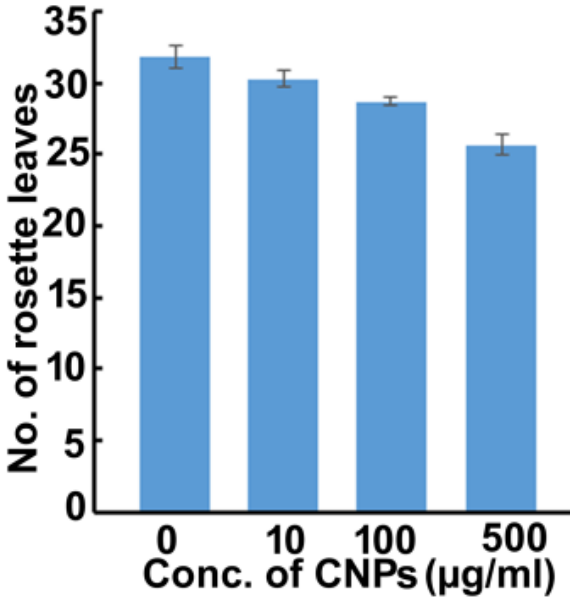

**Supplementary Figure 5.1 Visualization of carbon nanoparticles particle aggregates using light microscopy. CNPs at 500ug/ml was solubilizedin MS media and observed under light microscope (A) Leaf mesophyll cells of20 days old plants untreated (B) and treated (C) CNPs fine particle aggregates are marked with red arrows. Scale bar: 20 µm.**



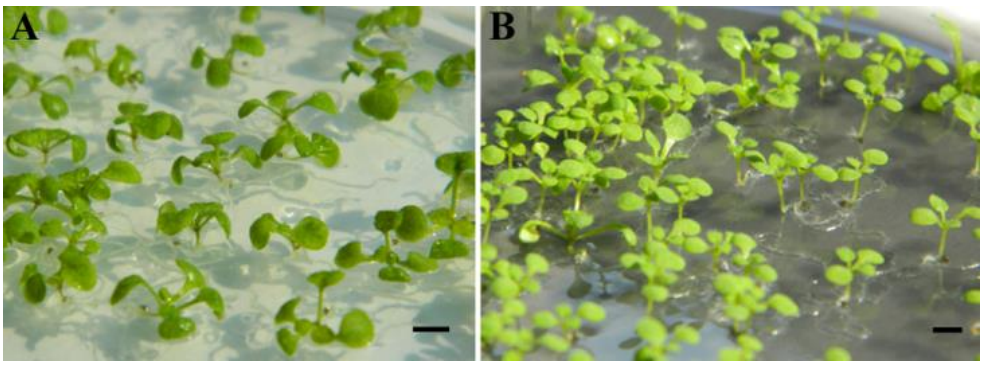

**Supplementary Figure 5.2 Dose dependent effect of carbon nanoparticles on induction of bolting. Col-0 seeds were surface sterilized and grown in planton boxes containing 50 ml of MS medium as described in materials and methods either A without or B with CNPs of concentrations 50 µg/ml, C 250 µg/ml or D 500 µg/ml for an average of 20 d. C' and D' are the zoomed inset of C and D respectively showing bolting marked with red arrows.**




**Supplementary Figure 5.3 Effect of carbon nanoparticles treatment on seedling phenotype in short days. Seedlings of Col-0 ecotype were grown in the presences or absence of CNPs under short days (8 h light / 16 h dark) as described in materials and methods. A Hypocotyl length, B root length of 8 D dark grown seedlings (Dark) or subjected to 8 d white light SD cycles (WL) post sowing were estimated from at least 20 seedlings of each and plotted. White histograms were data from untreated while black histograms were data from treated seedlings. Furthermore, C cotyledon angle, D cotyledon area were estimated from 20 seedlings each and statistical significance was analyzed using prism 5 and plotted. All experiments were carried out at least thrice. Significantdifferences at p < 0.0001 or ns (non-significance) as confirmed through unpaired student t-test were indicated.**




**Supplementary Figure 5.4 Dosage dependent effects of carbon nanoparticles on flowering time in short days. Col-0 plants were grown in plantons containing 50 ml of MS medium without (0) or with CNPs of concentrations 10, 100 or 500 µg/ml for up to 30 days. Rosette leave numbers, indicative of flowering time was evaluated from 20 plants each per treatment and the mean of three independent experiment was presented.**




**Supplementary Figure 5.5 Effect of carbon nanoparticles treatment on seedling hypocotyls length. Seedlings of Col-0 ecotype were grown on MS medium under white light (WL) in long day with A 10 µg/ml or B 500 µg/ml CNPs. longer hypocotyl was observed in case of seedlings grown in presence of CNPs. Scale bar: 5 cm.**

## 5.13 Discussion

In the present study, an attempt has been made to understand the importance of carbon nanoparticles in some of the crucial physiological processes in the important plant model system, *Arabidopsis thaliana*. The results demonstrated that CNPs may serve as important modulator to induce early flowering. Using a series of experiments, our results suggested that this effect is PHYB, photoperiod-dependent and CO independent. To our knowledge, this is the first report

demonstrating the direct application of CNPs as flowering time regulator in plants which may also be employed as a strategy to escape abiotic stress.

In this study, we have demonstrated that CNPs were taken up by *At* seedlings. Plants flowered earlier, when seeds were germinated and grown on the medium containing CNPs. This suggested that the CNPs were taken up systemically by the root system. Most likely the transport of CNPs did not use the plasmodesmata route, but it must have occurred by the endocytosis pathway. Similar uptake and distribution of C60 and C70 fullerenes was demonstrated in rice (Lin et al., 2009), tomato (Khodakovskaya et al., 2009) and *Arabidopsis* (Garcia-Sánchez et al., 2015).CNPs aggregates have been shown to be taken up in both simplistic and apoplastic route (Tripathi et al., 2017). However, most likely they did not aggregate before being taken up by the plant. This observation was strengthened by the fact that such aggregates are not detected in the vasculature. CNPs were observed in the stem tissue though in lesser quantity indicating that xylem could be the possible player for its translocation. In the present study, CNPs uptake didn't show any adverse effect in terms of growth of *At*. To understand, if the dosage dependent early flowering phenotype was result of combinatorial presence of CNPs and shading due to over-crowding of plants in a single pot, experiments were performed in single pots to minimize the shade avoidance response primarily regulated by PHY photoreceptors.CNPs may not necessarily inhibit only PHYB response but also might alter other biochemical pathways including auxin, cytokinin and brassinosteroids.

The early flowering phenotype and the longer hypocotyl length observed in all experimental plants exposed to CNPs prompted us to look into the photoperiod and light signalling regulatory components at the molecular level. The photoperiod is sensed at the leaves (Song et al., 2015) and in our studies the CNPs were predominantly accumulated in the leaf tissues. Since, the CNPs accumulation was highest in leaf tissues and was at un-detectable level in the isolated leaf veins (data not shown); our work suggested that the photoperiodic control

may have been altered. Accordingly, the plants responded to the day-length differently in terms of their flowering in the presence of CNPs under both LDs and SDs although with different number of rosette leaves upon treatment with CNPs on MS medium. These indicated that the CNPs-treated plants are photoperiod sensitive. The differences in rosette leaf number were significantly lower and are about less than half under LDs than under SDs suggesting that the early flowering phenotype in the CNPs-treated seedlings was partially photoperiod sensitive.

A hypothetical model explaining the early flowering caused due to CNPs treatment is drawn in Figure 7. Expression level of *LFY* determines the identity of primordia that arises at the shoot apical meristem and positively correlate the transition to flowering (Blázquez et al., 1997). It has been shown that mutation in flowering genes like *GI* causes reduction in *LFY*expression, hence, represses flowering (Nilsson et al., 1998). The PHYB, also which causes late flowering, act as a negative regulator of the LFY (Nilsson et al., 1998) and delays flowering in WT. Here, we observed that after CNPs treatment expression of *PHYB* is down regulated, whereas, *GI* transcript levels were enhanced. These combined effects lead to the up-regulation of *LFY* expression, hence resulting in early flowering. It has also been shown that PHYB acts as a repressor for *PIF4* gene which causes short hypocotyl and large cotyledon angle in WT (Huq et al., 2002). Since after CNPs treatment *PHYB* transcript level was down-regulated, we found higher level of *PIF4* transcript which resulted in longer hypocotyl and smaller cotyledon angle compared to WT. In this study, we also observed enhancement in *RGA1* transcripts, which is a repressor of GA pathway. To further confirm that the reduced *PHYB* transcript caused early flowering in a GA-independent manner, we measured hypocotyl length of seedlings grown in the dark. The strongly etiolated phenotype and the differences in hypocotyl length in the dark after CNPs treatment supported the view that the process is GA-independent.

Smaller cotyledon angle in WL and under different qualities of light suggested the down-regulation of multiple photoreceptor functions. Larger cotyledon area in all the light qualities

tested suggested a complex combinatorial output of interaction of different pathways. However, the higher chlorophyll content in WL suggested significant down-regulation of PHYA and PHYB dependent pathways that mimic a *phyAphyB* double mutant plant, which also flowers relatively earlier compared to its WT control (Franklin et al., 2010; Neff et al., 1998). Since *PIF4* is epistatic to *PHYB* and acts antagonistic to it, the *phyB* mutant accumulates PIF4 that result in longer hypocotyl and larger cotyledon area explaining the observation in the current study (Huq et al., 2002). Earlier flowering by CNPs treatment without the involvement of CO suggests an alternate mechanism that by passes the classical (CO-FT) photoperiod module possibly by direct interaction of GI on *FT* promoter.

# CHAPTER 6: RESULT AND DISCUSSION

## OVER-EXPRESSION LINE OF COβ ENHANCES THE SILVER NANOPARTICLES SYNTHESIS

# 6. Results

## 6.1 *In vitro* synthesis of AgNPs using plant extract

The *in vitro* synthesis of AgNPs was performed using plant extracts of *Enteromorpha intestinalis*, *Gracilaria verrucosa*and, *Scendesmus acuminatus*, and *Potamogeton pectinatus* from the Chilika lagoon and the model plant *Arabidopsis thaliana* as described in materials and methods section.

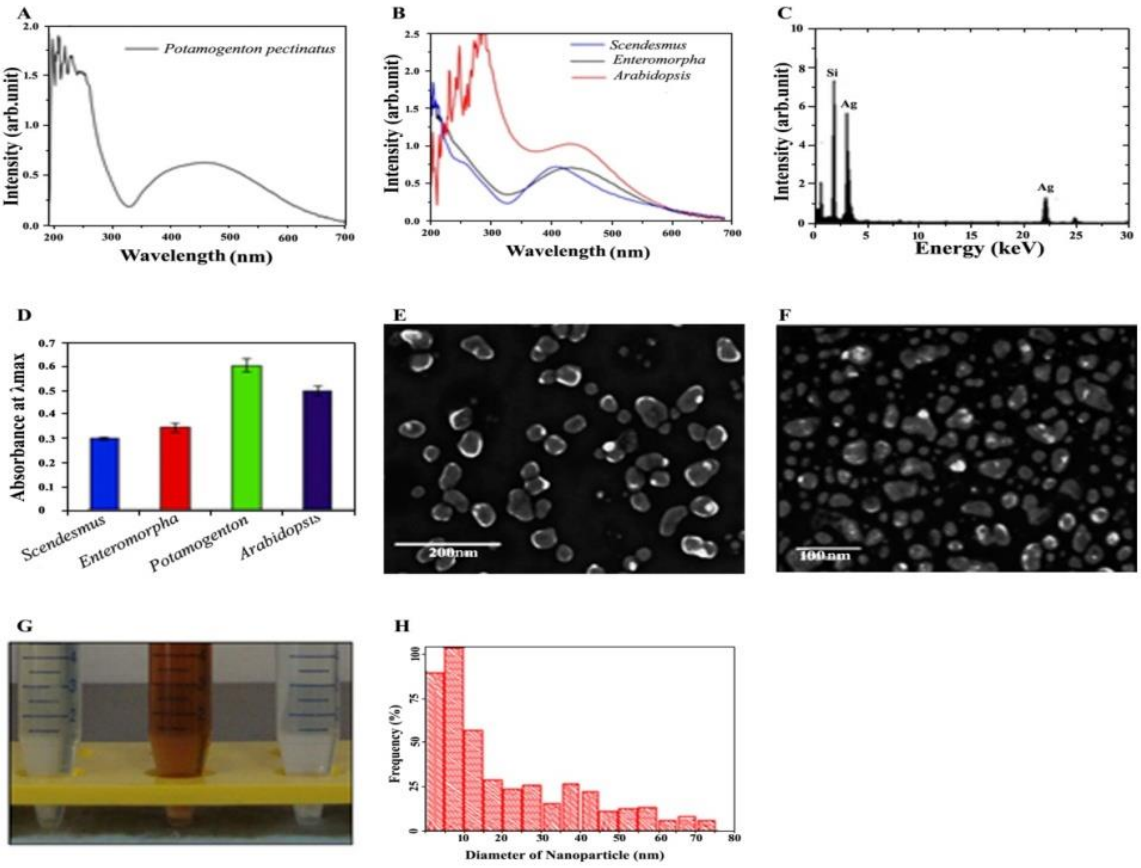



**Figure 6.1 Synthesis of silver nanoparticles from plant extracts using silver nitrate AgNO3 solution.(A) UV-Vis absorption spectrum of silver nanoparticles synthesized from 10 mM $AgNO_3$ using *Potamogeton pectinatus* extract., (B) UV-Vis spectra showing similar λmax for AgNPs produced by *Scenedesmus*, *Enteromorpha*, *Arabidopsis* extracts, (A and B) Intensity is presented in arbitrary unit, (C) Energy dispersive spectrum (EDS) for bio-synthesized AgNPs coated on Si plates, (D) Nanoparticle yield for different plant extracts. Nanoparticle yield is represented as the function of absorbance at λmax, (E and F) SEM images of AgNPs produced using *Arabidopsis* and *Potamogeton* extracts respectively, scale bar: 200 nm and 100 nm respectively (G) Digital images of (from left): *Potamogeton* aqueous extract, 10 mM $AgNO_3$ with *Potamogeton* extract after 1h of incubation, and 10 mM $AgNO_3$ without *Potamogeton* extract after 1h of incubation, (H) Particle size distribution of silver nanoparticles synthesized using *Arabidopsis* extracts.**

The UV-Vis spectra of the plant extract solution containing $AgNO_3$ after the reaction showed a peak in 400-700 nm windows indicating the abundance of AgNPs (Figure 6.1A). The AgNPs synthesized with different extracts although varied in their relative intensities, fall into the range of similar absorption maxima ($\lambda_{max}$) as visualized by UV-Vis spectroscopy (Figure 6.1B). The presence of elemental silver was confirmed from energy dispersive spectroscopy (EDS) of AgNPs (Figure6.1C). The amount of AgNPs synthesis was also found to be dependent on the individual extracts, *Potamogeton* being the best among the four species tested (Figure 6.1D).

However, the morphology of the particles was similar for AgNPs synthesized using different extracts as evident from SEM images. Results from SEM showed that NPs were spherical or rhomboidal in shape having a poly-dispersive s distribution with a size of about 10 nm in diameter (Figure 6.1E, 6.1F).

However, the morphology of the particles was similar for AgNPs synthesized using different extracts as evident from SEM images. Results from SEM showed that NPs were spherical or rhomboidal in shape having a poly-dispersive size distribution with a size of about 10 nm in diameter (Figure6.1E, 6.1F). The biosynthesis of AgNPs using extracts of *Potamogeton* could be easily visualized and correlated with a change of color of the solution from yellowish to brown (Figure 6.1G). The intensity of the brown colour was directly proportional to the increase in particle size of AgNPs.

**6.2 Understand the mechanism of silver nanoparticles synthesis**

To understand the mechanism of AgNPs synthesis, we chose *Arabidopsis thaliana,* an excellent model dicot plant, for its capability to synthesize AgNPs. The UV-Vis absorption spectrum of the suspension obtained after incubation of silver nitrate with the *Arabidopsis* leaf extracts showed absorption maxima between 450 – 470 nm (Figure 6.2A). The size distribution of the

NPs that was obtained by reaction of silver nitrate with the *Arabidopsis* leaf extract is shown in the Figure 6.1H. This demonstrated that whole plant extracts of *Arabidopsis* are capable of synthesizing AgNPs. The amount and size of AgNPs produced was dependent on the initial concentration of $AgNO_3$ (Figure 6.2A). *At* higher concentration of about of 5 mM $AgNO_3$, aggregation started to occur as observed by the extra Quadra polar resonance peak (marked with an arrow) appearing at lower wavelengths in UV–Vis spectra. The increase in average size of NPs was also evident from Raman spectra of AgNPs with different $AgNO_3$ concentrations (Supplementary Figure 6.S1).

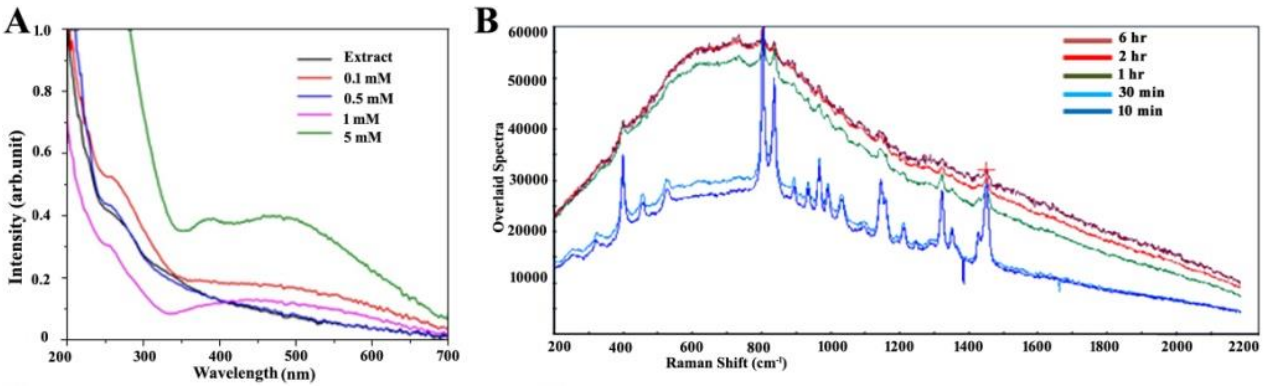



**Figure 6.2 Raman spectra spectra of silver nanoparticles.Raman spectra of silver nanoparticles (A) Raman spectra of silver nanoparticles (AgNPs) synthesized using different concentrations of $AgNO_3$. (B) Raman spectra of AgNPs synthesized during different time periods.**

The increase in average size of NPs was also evident from Raman spectra of AgNPs with different $AgNO_3$concentrations (Supplementary Figure 6.S1). AgNPs synthesis was found to be saturated around 1hr post incubation reaching marginally highest after around 2 hrs as evident from Raman spectra of the AgNPs at different time periods (Figgure6.2B).

### 6.3 Carbohydrates and polyphenolsin plant extracts facilitate AgNPs synthesis

In order to identify the chemical nature of the substances that facilitate AgNPs synthesis, depletion experiments were performed with extracts of *Scendesmus, Enteromorpha*, *Potamogeton* and *Arabidopsis* followed by analysis using NMR spectroscopy. The results

revealed chemical shifts in the range of3-4 ppm (encircled in Figure 6.3 C-F) as an indication of carbohydrates. The intensity patterns of the peaks in these regions correlated with the ability of the extracts to synthesize agnostically $^{1}$H NMR chemical shifts of carbohydrate ring protons which are 3-6 ppm (Figure 6.3C-F). Established Oligosaccharide $^{1}$H NMR spectral data has been shown to be in the region of the spectra from 3–4 ppm (Jansson et al., 2006). These experimental evidences suggested that oligosaccharides most likely acted as reducing as well as stabilizing agents for synthesis of NPs.

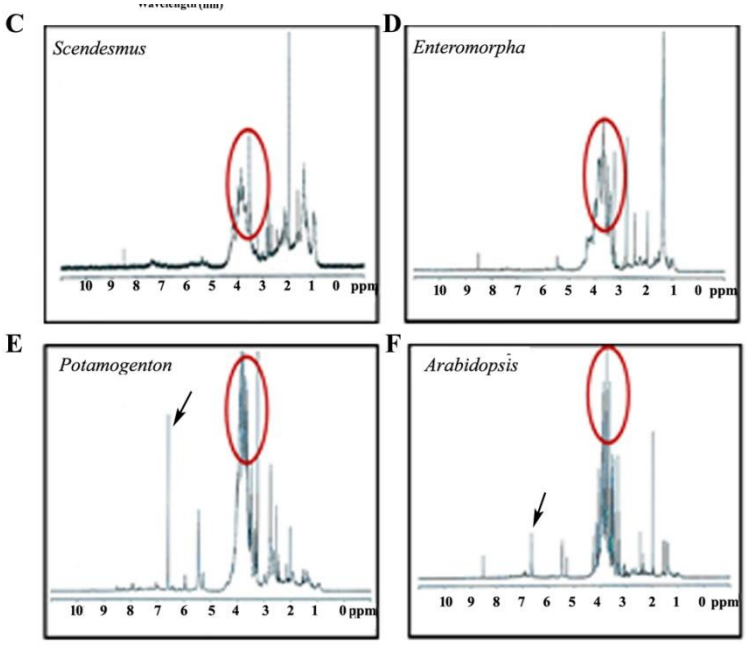



**Figure 6.3 NMR spectra of the synthesized AgNPs enhance the signal from the plant. Plant extract, (C-F) 1H NMR spectra of various plant extracts used for synthesizing silver nanoparticles. Peaks of Plant extracts from Scendesmus, Enteromorpha, Potamogeton and *Arabidopsis* from left to right. Chemical shifts are shown increasing from right to left (0-10 ppm). Similar chemical shifts can be observed in all the extracts in the range of 3-4 ppm suggesting presence of oligosaccharides**.

They are most likely in varying concentrations in different species as evident from the intensity of the peaks which might be the reason for variable sizes and amounts of NPs synthesized by different extracts. Notably, in case of *Arabidopsis* and *Potamogeton*, NMR peaks were obtained in the aromatic region around 6.8 suggestive of presence of the phenolic (Figure 6.3 E, 6.3F, marked with arrow).

### 6.4 Understand the nature of the compound required for silver nanoparticles synthesis

In order to understand the nature of the compound that is required and sufficient for AgNPs synthesis, we performed depletion experiments. The depletion of the biochemicals in the *Arabidopsis* plant extracts were performed as described in method section and verified (Supplementary Figure 6.S2). The depletion of DNA, RNA and proteins did not alter the AgNPs yield significantly, while the removal of carbohydrate fraction lead to a sharp decline in the synthesis of AgNPs (Figure 6.4A, 6.4B).

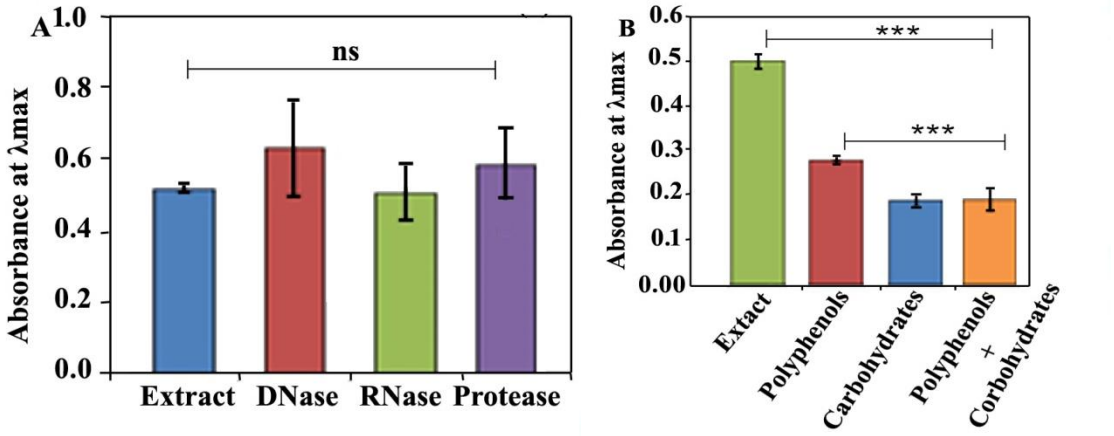



**Figure 6.4 Potential roles of different biomolecules in nanoparticle synthesis in *Arabidopsis*. A, Absorbance of plant extracts of *Arabidopsis* treated with DNase, RNase and protease was measured at 450 nm for detection of AgNPs. 'ns': non-significant difference; B, Absorbance of plant extract of *Arabidopsis* depleted with polyphenols, carbohydrates or for both was measured at 450 nm. ***: significant difference at $p < 0.0001$.**

*Arabidopsis* extracts showed nearly 80% decreased synthesis of AgNPs up on carbohydrate depletion (Supplementary Figure 6.S3). In *Arabidopsis* and *Potamogeton*, removal of polyphenols also leads to decline in AgNPs biosynthesis suggesting a role of polyphenols in reduction of $Ag^{+}$ into the zero-valent NPs (Supplementary Figure 6.S3). However, it should be noted that in *Arabidopsis*, depletion of carbohydrates by acetone precipitation not only induced a decrease in intensity of peaks in the 3-4 ppm region but also caused the peak in the polyphenol region 7.27 ppm to disappear (Figure 6.5). Notwithstanding, we noted that the

total phenolic and carbohydrate content in the plant species tested were different and their abundance correlated with the extent of NPs' synthesis (Supplementary Figure6. S4).

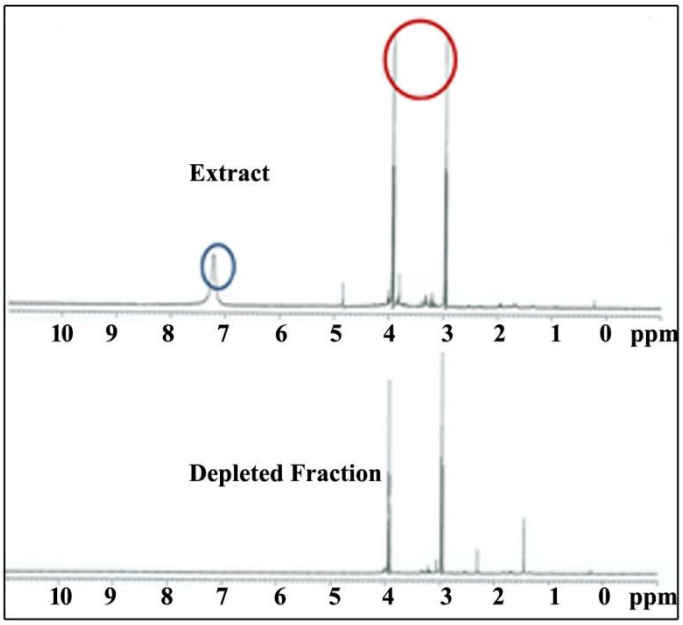



**Figure 6.5 Potential roles of different biomolecules in nanoparticle synthesis in *Arabidopsis*.NMR results of *Arabidopsis* extract before depletion and after depletion of carbohydrate. $^{1}$H NMR spectra of aqueous extracts of *Arabidopsis* (upper panel) and fraction of the same extract depleted of carbohydrates using acetone precipitation method (lower panel). The depleted fraction has decreased intensities in characteristic carbohydrate regions (3-4 ppm, encircled red) but lacks the polyphenol peak (encircled blue).**

### 6.5 *Arabidopsis* mutants altered in sugar accumulation show differential degree of AgNPs biosynthesis

To validate our hypothesis that carbohydrates and conjugated sugars play a crucial role in the reduction of ionic silver to metallic zero-valent AgNPs, we studied the *in vitro* synthesis of AgNPs in the *Arabidopsis* Col-0 mutants and over-expresser, aberrant in sugar homeostasis and conjugation. The mutants of GIGANTEA gene (*gi-100)* accumulate different types and complex carbohydrates and also starch (Mishra et al., 2015). GI acts antagonistic to SPINDLY (SPY) (Mishra et al., 2015). A splice form of CONSTANS (COβ) was also used in this study which showed higher amount of sugar accumulation (Gil et al., 2017). The free sugar level in

all these genotypes was evaluated (Supplementary Figure 6.S5). The free sugar level in all these genotypes was evaluated (Supplementary Figure 6.S5). The effect of *spy-5*, *gi-100* mutation, in plants constitutively producing GI and COβ under the control AgNPs biosynthesis,

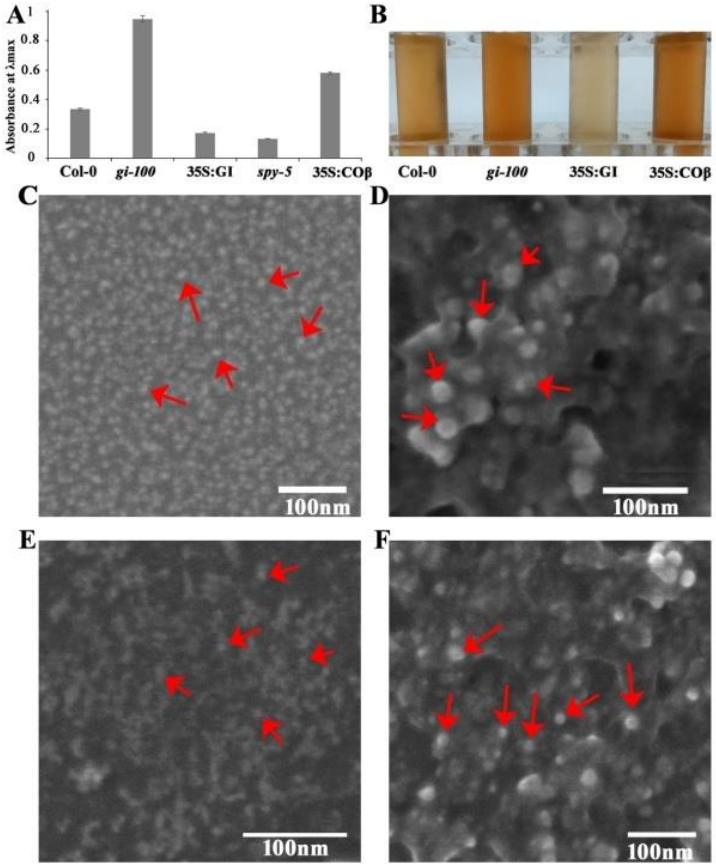



**Figure 6.6 Silver nanoparticle synthesis in different mutants and over-expressor lines of *At* altered in sugar homeostasis.**(A) **Differential nanoparticle yield for Col-0, *gi-100* mutant, 35S::GI lines, 35S::COβ lines and *spy-5* mutant of *Arabidopsis thaliana*. AgNPs yield was estimated by measuring absorbance of the NPs solution at $\lambda_{max}$. (B) images of plant extracts showing variable colour intensity with 10 mM $AgNO_3$ after 1 hrs of incubation (from left): Col-0, *gi-100*, 35S::GI and 35S::COβ. (C-F) SEM images AgNPs synthesized in plant extracts of Col-0 (D), *gi-100* (E), 35S::GI (F) and 35S::COβ (G); AgNPs are marked with red arrows; scale bar: 100nm.**

consistent with our hypothesis, the *spy-5* and 35S::GI extracts yielded lesser amount of AgNPs, whereas *gi-100* and COβ over-expressor yielded nearly two and three-fold higher as compared to Col-0 plant extracts respectively (Figure 6.6A). Here again, the colour intensity of the AgNPs formed in the plant extracts of different genotypes correlated with the AgNP yield (*gi-100* being highest and *spy-5* being lowest, Figure 6.6B). Results of the SEM analysis for the

AgNPs from the plant extracts of Col-0 (Figure 6.6C), *gi-100* (Figure 6.6D), 35S::GI (Figure 6.6E) and 35S::COβ (Figure 6.6F) could demonstrate that the AgNPs synthesized had variable sizes with 35S::GI extracts containing the smallest and *gi-100* being the largest.

We estimated the carbohydrate content of the different mutant and transgenic lines of *Arabidopsis* mentioned above and found that, *gi-100* as described earlier and 35S::COβ plants reported in this work contained highest amount of sugars compared to its WT (Supplementary Figure6.S5).

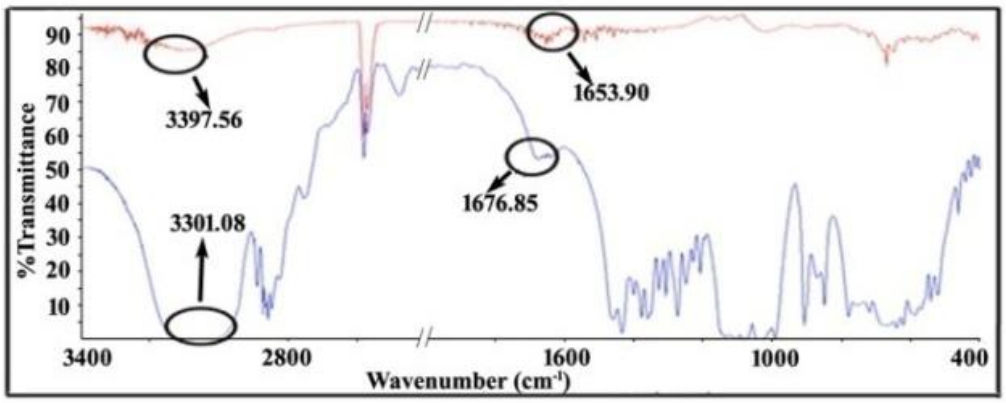
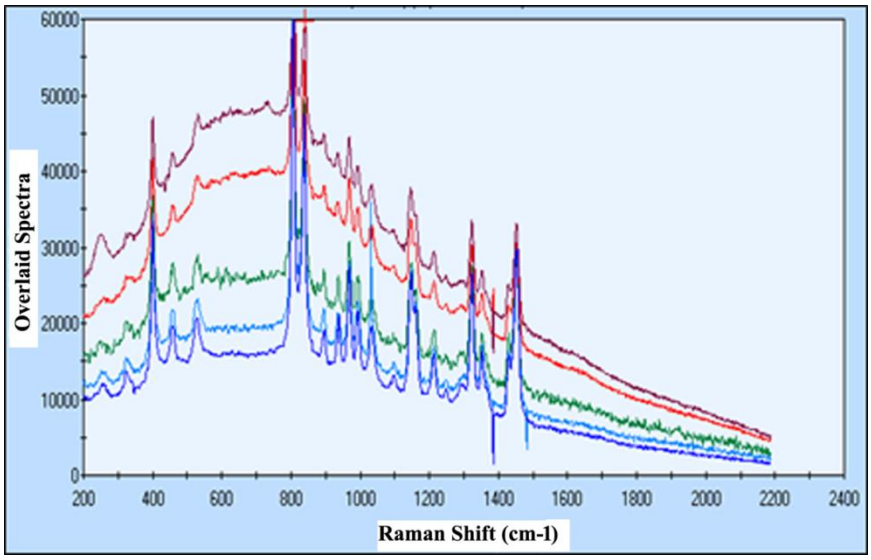



**Figure 6.7 FTIR spectra of maltose-capped silver nanoparticles. FTIR spectra of maltose-capped silver nanoparticles (red color) and pure maltose (blue color). The shift in peaks of potential co-ordinate bond formation sites – OH (circles on the left side) and – C=O bonds (circles on the right) of maltose indicate the capping of nanoparticles by maltose. The band seen at 1675 $cm^{-1}$ corresponds to the C=O stretching frequency. In pure maltose, maltose capped silver nanoparticle this band is shifted to 1653 $cm^{-1}$ indicating conjugation of C=O bond with silver nanoparticles. Similarly, the band observed at 3302.08 $cm^{-1}$ corresponds to the –OH stretching frequency and in maltose capped silver nanoparticles, increased transmittance to 3397.56 suggested decrease in free –OH group indicating that the hydroxyl group is involved in capping of nanoparticles.**

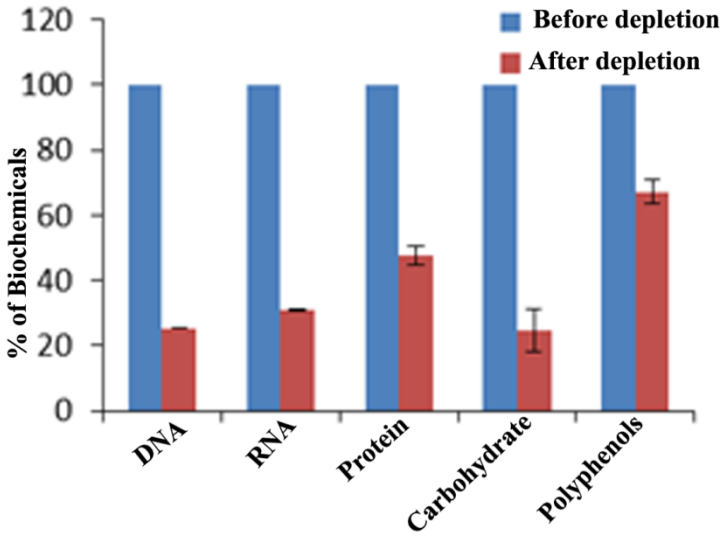
These results indeed confirmed that the amount of the sugar accumulated in the different genotypes positively correlated with the AgNPs yield. Again, it confirmed that the colour of the AgNPs formed was due to the size of the AgNPs and not due to the concentration. In order to strengthen the observation of involvement of oligosaccharides in the formation AgNPs synthesis, maltose capping experiment was performed with the *Arabidopsis* plant extract and

evaluated using FTIR spectroscopy. The –OH and C=O bond stretching in maltose in FTIR spectra (Figure 7) indicated.The successful legend capping of nanoparticles. With the successful ligand capping modifications possible, we showed here that silver nanoparticles obtained through biosynthesis using different plants extracts could be stabilized.

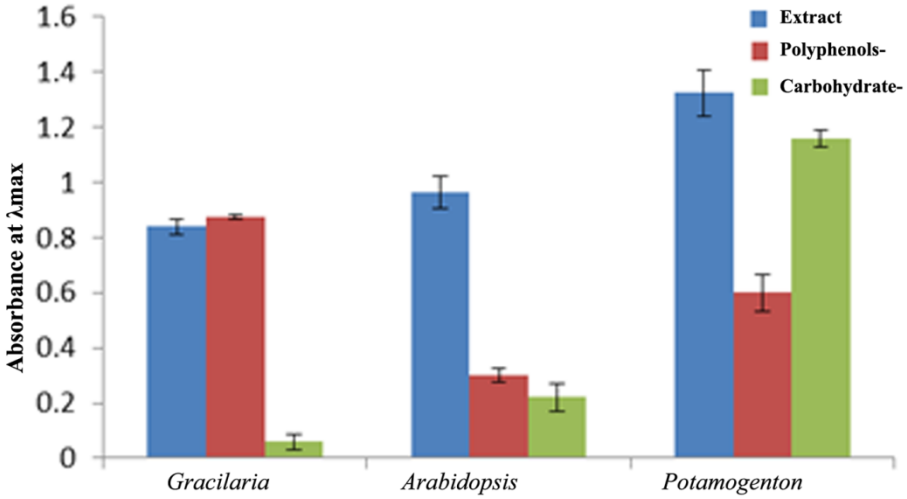
## SUPPLEMENTARY FIGURES



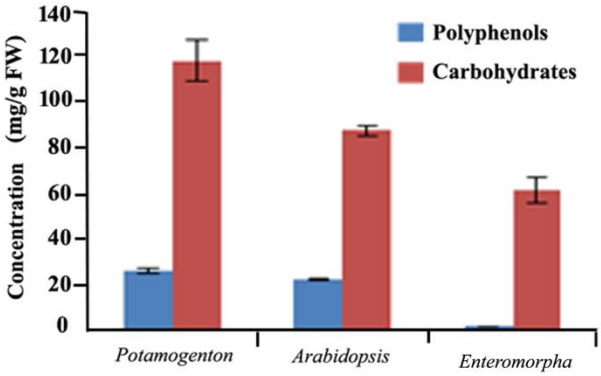

**Supplementary Figure 6.1 Raman spectra of silver nanoparticles synthesized using different concentrations of $AgNO_3$. The synthesized AgNPs enhance the signal from the plant extract of *Arabidopsis*.**



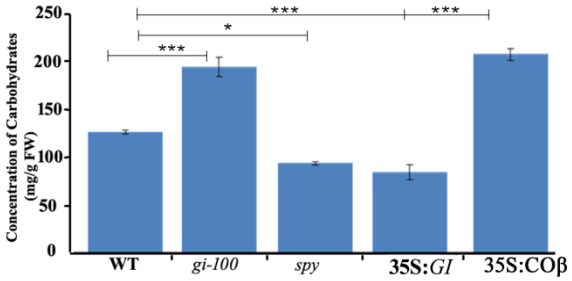

**Supplementary Figure 6.2 Percentage depletion of different biochemical in plant extracts. Percentage depletion of DNA, RNA, protein, carbohydrate and polyphenols in plant extracts.Before depletion in blue histogram and after depletion in red histogram.**




**Supplementary Figure 6.3 Depletion of carbohydrates or polyphenols influence the AgNPs yield is different plants extracts.*Gracilaria, Arabidopsis* and *Potamogeton.* Extract: Crude Plant Extract, in blue histogram; Polyphenols: Polyphenols depleted fraction, in red histogram; Carbohydrate: Carbohydrate depleted fraction in green histogram.**




**Supplementary Figure 6.4 Phenolic and carbohydrate content vary significantly among plant species and correlates with their ability to synthesize silver nanoparticles.Total phenolic in blue histogram and carbohydrate red histogram content vary significantly among plant species such as *Potamogeton, Arabidopsis* and *Enteromorpha* correlates with their ability to synthesize silver nanoparticles.**




**Supplementary Figure 6.5 Total carbohydrate content varies in different genetic background of *Arabidopsis thaliana* such as Wild type (Col-0), *gi-100*, and 35S::GI and 35S::COβ. *: significant difference at p < 0.01 and ***: significant difference at p < 0.0001.**

### 6.6 DISCUSSION

Here we showed that plant extracts could be used for synthesis of AgNPs in an eco-friendly manner. AgNPs were synthesized using aqueous extracts of weed species from Chilika lagoon, namely *Potamogeton pectinatus*, *Enteromorpha intestinalis*, *Gracilaria verrucosa* and *Scendesmus acuminates* as well as from *Arabidopsis thaliana*. Synthesis of AgNPs from Enteromorpha flexuosa and its antibacterial properties was reported by Yousefzadi et al., (2014). The AgNPs synthesized in the different species in our study varied in their intensity, however the amount synthesized was highest from the *Potamogeton* extracts. Similarity of the AgNPs properties from different extracts raised a possibility of a common mechanism in their synthesis. The UV-Vis spectra of the solution containing AgNPs showed peak in between 400-700 nm.

Plant extract in absence of silver showed absorption peaks in the range of 200-350 nm and not in the spectral range of 400-700 nm supported by the previous observation (Kumar et al., 2012).Synthesis of AgNPs is favoured by increasing pH as documented in many reports earlier (Alqadi et al., 2009; Singh et al., 2009). However, presently the synthesis of AgNPs did not require any such pH requirement, which is an advantage over the earlier reports, as milder pH is safer to handle.The *Potamogeton* and *Arabidopsis* extracts fell in to a different category having enriched with both aromatic compounds as well as carbohydrates, consistently showed higher amount of AgNPs synthesis. This indicated that these groups of compounds might influence AgNPs synthesis. Possibly, the decline in AgNPs synthesis upon carbohydrate removal by acetone is caused in part by the simultaneous depletion of polyphenol compounds. The total carbohydrate and polyphenols differ in different plant species and thus might be the reason of difference in the amount and size of the AgNPs synthesized. Since autoclaving the extract takes away most of the protein activity and targeted protein depletion using proteinase K did not affect the NPs synthesis in our experiments, we conclude minimal role of protein in the synthesis process. The supernatant of autoclaved or boiled plant extracts is known to be enriched in hydrophilic proteins, LEA proteins and glyco-proteins (Panigrahi 1998, PhD thesis). Also, the acetone precipitation has an ability to precipitate proteins non-specifically along with carbohydrates therefore; acetone precipitation may have precipitated sugar conjugated proteins or glycoprotein those might be the important component of the autoclaved extracts that induces AgNP synthesis.

We wished to test this hypothesis by using molecular genetics tool. The GI gene of *Arabidopsis* has been shown to play a role in regulating sugar homoeostasis (Tseng et al., 2004). SPINDLY (SPY) encodes an O-linked β-N-acetyl glucosamine transferee involved in glycosylation of substrates including proteins and was shown to genetically interact with GI that functions in an antagonistic manner (Tseng et al., 2004). Since the absence of GI lead to an increase in sugar

content in *Arabidopsis* and the conjugating activity of sugars by SPY are high in this mutant line, it was expected that the extract of GI mutant would show a higher level and size of AgNPs.This is exactly what we observed in our experiment. Similarly, spy mutant or plants over-expressing of GI lead to the least accumulation of AgNPs by a down-regulation of SPY function in both (Figure 6.4A). We further wanted to validate this observation by taking another genetically modified line of *Arabidopsis*, published recently that interact with photo-periodic pathway, biological clock and therefore most possibly with GI. Both GI mutants and the over-expresser of COβ form shows similar late flowering phenotype, alters diurnal rhythm and therefore might share the same function (Gil et al., 2017).In the Over-expressing line of COβ we observed a higher AgNPs synthesis consistent with our hypothesis.

In our study, we conclude that carbohydrate and polyphenol compounds in the extracts strongly influence the effectiveness of AgNPs synthesis. The results from NMR analysis, biochemical depletion and mutant studies that carbohydrates and polyphenols might be agents responsible for NPs biosynthesis is an encouraging step towards unravelling the mechanisms of AgNPs synthesis. While the ability of the plant species from Chilika lagoon to synthesize AgNPs sparked hope for its use in industry, production of AgNPs using *Arabidopsis* extract offered its usage to dissect out the pathways that control its synthesis using molecular genetics.

# CHAPTER: 7

# CONCLUSION AND FUTURE PROSPECTIVES

## 7. Conclusion and Future prospectives

In summary we conclude that the mixed soil tested to be the best suited for the *Arabidopsis* growth with respect to reproductive potential of the plants as the plants grown on mixed soil had highest biomass and seeds per pod. Mixed soil grown plants showed least accumulation of carotenoids indicative of less stress and better for plant growth.From our result we conclude that optimised mixed soil may substitute for the growth of *Arabidopsis* plant for the evaluation of flowering time from Indian prospective.

Our result with the delay flowering in over-expressed lines of COβ in different genetic background such as Col-0, *co-10* and *phyb-9* not only consistent with constitutive over-expressed with 35S promoter but also with the phloem specific promoter (pSUC2) and meristematic specific promoter (pKNAT1) in Col-0 background.From this result we conclude that there is involvement of a common mechanism for delay flowering. Our result with more chlorophyll, carbohydrate accumulation with more leaf size and area in over-expressed lines of COβ irrespective of genetic backgrounds. From this result we conclude that over-expressed lines of COβ have the high photosynthetic performance consistence with increased pod length, pod weight and seed per pod co-relating the high yield performance.

Our result over-expressed lines of COβ induced decrease in hypocotyl length under white light (WL) red (R), far-red (FR) and blue light (B) and the lowest in the red light due to predominantly by the hypersensivity of phytochrome B signalling and also sensitive effects of all other light signalling pathways such as phytochrome A and cryptochromes. From this result we conclude that, the decrees hypocotyl length is most probably due to the down regulation of light signalling components. The longer root system, higher chlorophyll content and delay flowering observed in over-expressed lines of COβ plants are beneficial agronomic traits from the perspective of abiotic stress tolerance and can be exploited for sustainable optimal yield even under stress conditions.

In this study it was also observed a significant high level of *COβ* transcript in *gi-100* mutant under long day conditions irrespective of Zt, from this result we conclude that in the absence of GIGANTEA might lead to improper splicing of *CONSTANS* (COβ) and transcript of *COβ* is diurnally regulated. That delays flowering in *At* in a photoperiod dependent manner. We further observed high transcript level of COβ is till 9 days of light treatment under long day conditions and drastically decrees after 9 days from this result we conclude that the transcript of *COβ* is developmentally regulated and might the one of the reasion to maitain juvinility in *Arabidopsis thalian*. Therefore, form both above study we conclude that transcript of *COβ* both diurnally and developmentally regulated.

As we also visualised the functional protein of COβ result by the confocal imaging of 35S::CFP:COβ in seedling concluding the functional protein of COβ is there and it is correlating our result with high transcript of COβ in seedling .Our result with high transcript of miR156 in over-expressed lines of COβ after 10 days of light treatments under long day conditions concluding that the transcript of miR156 is developmentally regulated by COβ. In continuation with other role of COβ we also observed the early flowering of these lines by CNPs treatments as compare with the without CNPs treatments. From this result we conclude that the importance of COβ in crop plants under control conditions may open up a novel transgenic approach for crop to escape from abiotic stress.

Use of nano-materials in biotechnology and agriculture have also been attempted to treat plants with nanoparticles. Here we have demonstrated that carbon nano-particles are taken up by *Arabidopsis* and induce early flowering with longer hypocotyls length in presence of CNPs. Other pathways such as PIF4 dependent pathways might play a significant role in these CNP treated plants transcript is normally high at around Zt 6 under LDs. This could explain the longer hypocotyl of CNPs treated seedlings compared to their control under white light. In q-

RT PCR experiments, dramatic down regulation of *PHYB* transcript at Zt 4, 8 and 12 by CNP treatment was observed.

From this study of carbon nanoparticles, we conclude that CNPs could taken-up by the plant and accumulate in leaf tissues and can induce early flowering in PHYB, FT dependent and CO independent manner. In summary, we conclude that, CNPs serve as important agent to induce early flowering. Such understanding may have broad implications in plant biotechnology and agriculture.

It was also showed that the plant extracts of over-expresses lines of COβ with other genetic background in *Arabidopsis thaliana* and as well as from weed species of Chilika lagoon could be used for synthesis of AgNPs in an eco-friendly manner. The synthesis of AgNPs in different plant species is varied in their intensity might be because of the carbohydrate and polyphenols differ endogenouslyin different plant species. However, the similar properties of AgNPs from different extracts raised a possibility of a common mechanism in their synthesis. From our studied, we conclude that carbohydrate and the polyphenols in the extracts strongly influence the AgNPs synthesis.

Since the carbohydrate accumulation is high in over-expressed lines of COβ lines we tried to further evaluate the role of splice variant of CONSTANS in the AgNPs synthesis. In this regard plant extract of over-expressed lines of COβ and other genetic background as well as from Chilika lagoons were used for synthesis of AgNPs. We observed the synthesis of AgNPs is varied and dependent upon genetic background. The plant extracts enriched in carbohydrates consistently showed higher amount of AgNPs synthesis. Our result also suggested the sugar conjugated proteins are most likely important component over free-proteins for AgNPs synthesis irrespective of the sugar concentration of the extracts that need to further evaluate in future.We conclude that the ability of the plant species from Chilika lagoons to synthesize AgNPs sparked

hope for its use in industry. Production of AgNPs using *Arabidopsis* extract offered its usage in dissecting out the biochemical pathway that is involve in its synthesis through molecular genetics.

# CHAPTER: 8

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# ANNEXURE

## LIST OF TABLES

### Table 1 The primer sequences used in this study

| Gene Name | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| *ACTIN* | *GGTAACATTGTGCTCAGTGGTGG* | *AACGACCTTAATCTTCATGCTGC* |
| *PHYTOCHROME B* | CAGAACCGTGTCCGAATGATAG | GTAGAACCAACCAAGCACATAGA |
| *GIGANTEA* | GCTTCTCGAGGATCTGGTAAAC | GTCAGCGTAGCAGTCTCATATC |
| *CONSTANS* | CCATCAGCGAGTTCCAATTCTA | CTTCTCTGGATCGGTCATTGTT |
| *PIF4* | CCTCCAGATGAAGACCCATTC | GACTTAGGCTTAACCGTCTCTG |
| *LFY* | CAGCAGAGACGGAGAAAGAAA | CTACCTCCGTTGCCGTTATC |
| *RGA* | CGCAGATTGGTGGAGTCATAG | CTTGCGAGTCAACCAGGATAA |
| *VRN1* | CTGAGGGTCCCAGATAAGTTTG | GTCAGCTTTCCTTAGTCCTACAC |
| *FCA* | TTTCGTCCAATGGGTCCTAAC | TGGACTCTCTGAAGGAGGATAC |
| Gateway sequenes | GGGGACAAGTTTGTACAAAAAAGCAGGCTTC | GGGGACCACTTTGTACAAGAAAGCTGGGTCA |

### Table 4.1 Flowering time evaluation under long day conditions in Col-0, co-10 and *phyb-9* genetic backgrounds

| Genotypes | No of rosette leaves | No of total leaves | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 9 ± 2 | 13 ± 1.06 | 23 ± 1.3 | 20 |
| 35S:COβCol-0 | 45 ± 2.7 | 50 ± 3.41 | 44 ± 3.2 | 20 |
| *co-10* | 46 ± 3.58 | 56 ± 3.46 | 49 ± 3.5 | 20 |
| 35S:COβ*co-10* | 54 ± 4.21 | 61 ± 4.51 | 54 ± 4.7 | 20 |
| *Phyb-9* | 9 ± 1.27 | 12 ± 1.51 | 27 ± 1.4 | 20 |
| 35S::CFP:COβ*phyb-9* | 52 ± 4.03 | 57 ± 4.84 | 54 ± 3.4 | 20 |

**Table 4.2 Flowering time evaluation under long day conditions with tissue specific promoters**

| Genotypes | No of total leaves | No of total leaf | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 9.3 ± 1.35 | 12.6 ± 1.84 | 24 ± 1.24 | 20 |
| pSUC2::COβCol-0 | 52.5 ± 3.28 | 58 ± 4.11 | 50 ± 4.55 | 20 |
| pKNAT1::CO□Col-0 | 52 ± 3.25 | 57 ± 3.51 | 56 ± 4.3 | 20 |

**Table 2.3 Flowering time evaluation under short day conditions in Col-0, co-10 and phyb-9 genetic backgrounds**

| Genotypes | No of rosette leaves | No of total leaves | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 50 ± 3.7 | 57 ± 2.86 | 49 ± 3.1 | 20 |
| 35S::COβCol-0 | 54 ± 3.6 | 61 ± 3.41 | 54 ± 4.7 | 20 |
| *co-10* | 52 ± 5.07 | 58 ± 3.58 | 53 ± 3.5 | 20 |
| 35S::COβ*co-10* | 55 ± 4.21 | 62 ± 3.8 | 58 ± 4.7 | 20 |
| *Phyb-9* | 46 ± 4.01 | 52 ± 4.07 | 44 ± 1.7 | 20 |
| 35S::CFP:COβ *phyb-9* | 57 ± 4.84 | 61 ± 4.03 | 54 ± 3.4 | 20 |

**Table 4.4 Flowering time evaluation under short day conditions with tissue specific promoters**

| Genotypes | No of rosette leaves | No of total leaves | Days to bolting | No of Plants |
|---|---|---|---|---|
| Col-0 | 52 ± 3.24 | 58 ± 3.45 | 51 ± 3.77 | 20 |
| pSUC2::COβCol-0 | 57 ± 3.06 | 64 ± 3.3 | 55 ± 3.78 | 20 |
| pKNATI::COβCol-0 | 60.65 ± 4.36 | 65.5 ± 5.15 | 54 ± 4.45 | 20 |

**Table 3.1 Soil nutrients analysis in different soil**

| S.N0 | Parameters | Red soil | Clay soil | Garden Soil | Soil rite | Mixed Soil | German soil mix |
|---|---|---|---|---|---|---|---|
| 1 | pH | 5.40 | 4.62 | 7.22 | 6.48 | 6.2 | 6.03 |
| 2 | EC (dSm-1) | 0.006 | 0.055 | 0.260 | 0.028 | 0.408 | 0.907 |
| 3 | OC (g/ kg soil) | 4.72 | 7.98 | 3.45 | 11.7 | 7.75 | 11.4 |
| 4 | Avl. N ( Kg/ha) | 189.0 | 164.0 | 428.0 | 0.30 | 208 | 1023.0 |
| 5 | Avl. P ( Kg/ha) | 5.85 | 5.28 | 147.0 | 0.04 | 57.4 | 321.0 |
| 6 | Avl. K ( Kg/ha) | 143 | 180.0 | 176.0 | 0.03 | 295 | 1015.0 |
| 7 | Avl. Ca ( Kg/ha) | 1.5 | 8.04 | 13.9 | 0.49 | 3.18 | 32.9 |
| 8 | Avl. Mg (meq/100 gm) | 0.49 | 3.53 | 5.10 | 0.18 | 3.1 | 7.0 |
| 9 | Avl. Fe ( mg/kg) | 97.4 | 7.4 | 6.44 | 0.09 | 13.20 | 8.36 |
| 10 | Avl. Mn ( mg/kg) | 93.0 | 134.8 | 1.4 | 0.002 | 6.32 | 6.98 |
| 11 | Avl. Cu ( mg/kg) | 0.99 | 3.08 | 9.19 | 1.9 | 3.18 | 3.75 |
| 12 | Avl. Zn ( mg/kg) | 1.08 | 5.58 | 25.2 | 5.5 | 5.0 | 10.24 |
| 13 | Avl. B ( mg/kg) | 4.3 | 2.6 | 10.2 | 0.01 | 0.50 | 0.82 |

# PUBLICATIONS

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ORIGINAL ARTICLE

# Carbon nanoparticles influence photomorphogenesis and flowering time in *Arabidopsis thaliana*

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**Abstract**

***Key message*** **Inclusion of carbon nanoparticles in growth medium accelerates timing to flower by down-regulating phytochrome B in a CONSTANS-independent but photoperiod-dependent manner in *Arabidopsis thaliana*.**

**Abstract** Despite the recognized importance of nanoparticles in plant development over the last decade, the effect of carbon nanoparticles (CNPs) on plant processes such as photomorphogenesis and flowering time control is poorly understood. We explored the uptake, accumulation and effect of CNPs on seedling development and flowering time control in *Arabidopsis thaliana* (*At*). CNPs uptake was demonstrated using Raman spectroscopy and light microscopy that affected *At* seedling growth and flowering time in a dose-dependent manner. The highest accumulation of CNPs was observed in leaves followed by stem and root tissues. CNPs treatment enhanced seed germination, showed elongated hypocotyl, larger cotyledon area and increased chlorophyll content in *At* seedlings. CNPs treatment induced early flowering in both long-day and short-day growth conditions indicating a photoperiod-dependent effect. CNPs-treated seedlings showed a drastic reduction in the relative abundance of *phytochrome B* (*PHYB*) transcript. Further, we analyzed the transcript abundance of at least one major component involved in various pathways that regulate flowering such as (1) photoperiod, (2) gibberellic acid (GA), (3) vernalization and (4) autonomous. An up-regulation of transcript levels of *PHYTOCHROME INTERACTING FACTOR 4 (PIF4), GIGANTEA (GI), REPRESSOR OF GIBBERELLIC ACID 1 (RGA1)* and *LEAFY (LFY)* were observed, however, there were no changes in the transcript levels of *CONSTANS (CO), VERNALIZATION 1* (*VRN1)* and *FLOWERING CONTROL LOCUS A (FCA)*. Despite the up-regulation of *RGA1*, we conclude that the earlier flowering is most likely GA-independent. Here, we demonstrated that the early flowering in CNPs-treated seedlings was *PHYB* and photoperiod-dependent.

**Keywords** Carbon Nanoparticles · *Arabidopsis* · Phytochrome B · CONSTANS · Flowering · Photoperiod

**Abbreviations**

| | |
|---|---|
| CNPs | Carbon nanoparticles |
| PHY | Phytochrome |
| PHYB | Phytochrome B |
| LD | Long-day |
| SD | Short-day |
| ZT | Zeitgeber time |
| FW | Fresh weight |
| WL | White light |

## Introduction

Nanomaterial based on carbon has been used to understand plant development and productivity (Zaytseva and Neumann 2016; Serag et al. 2015; Sekhon 2014; Husen and Siddiqi 2014; Ditta 2012). Carbon nanomaterials fall into several categories such as nanotubes, fullerenes, nanoparticles,

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nanohorns, nanobeads, dots, nanofibres and nanodiamonds (Salata 2004; Nowack and Bucheli 2007; Sharon and Sharon 2010; Hossain et al. 2015; Mukherjee et al. 2016; Wang et al. 2016). Among these, single walled carbon nanotubes (SWNT), multi-walled carbon nanotubes (MWNT) and carbon nanoparticles (CNPs) attracted attention and have been studied in plants (Mukherjee et al. 2016; García-Sánchez et al. 2015). They can have both positive and negative effects on plant development (Mukherjee et al. 2016). SWNT and MWNT have been shown to be beneficial in tobacco (Samaj et al. 2004) and in rice (Lin et al. 2009). These agents penetrate and are internalized into plant roots serving as nanotransporters that probably facilitate nutrient uptake. Carbon nanotubes can increase tomato production up to two-fold in tomato, and fullerenes can lead to significant increase in the medicinal compounds in bitter melon seeds (Husen and Siddiqi 2014). However, exposure to SWNT and MWNT showed deleterious effect in *Arabidopsis thaliana* (*At*) resulting in cell aggregation, chromatin condensation, $H_2O_2$ accumulation, decrease in cell dry weight, cell viability, chlorophyll content and superoxide dismutase activities (Zaytseva and Neumann 2016). Induction of programmed cell death was observed in *At* and rice due to SWNT (Shen et al. 2010). Despite these studies, the effect and mechanism of CNPs on various physiological processes in different plant systems are not well studied.

Here, we investigated the role of CNPs on the seedling development and flowering in *At*. Seedling development in *At* starts after germination and includes several photomorphogenic events. These processes include hypocotyl growth inhibition, opening of apical hook, cotyledon opening, expansion, synthesis of chlorophyll and root growth. All of these are controlled by various photoreceptors, among which phytochromes (PHY) are extensively studied as the red (R) and far-red (FR) light receptors. Upon activation by red light, the inactive Pr form (with absorption maxima of 660 nm) is converted into the active Pfr form (with absorption maxima of 730 nm of far-red light). The Pfr form of the phytochromes further interact with several down-stream signaling components including transcription factors, activators or repressors to elicit the plethora of photomorphogenic responses. The developmental plasticity is thus fine-tuned according to various environmental stimuli. In *At*, the phytochrome gene family consists of five members (PHYA-E). Out of these, PHYB is detected as the major light stable phytochrome for regulating most of the photomorphogenic responses. PHYB has also been shown to be the most important photoreceptor for sensing and responding to shade. A drastic reduction of R to FR ratio in the incident light between 0.05 and 0.7 is defined as shade, which naturally occurs underneath the canopies (Franklin and Whitelam 2005). Shade signals cause distinguishable changes in the plant form including elongation of hypocotyl, petiole, stem, increased height, and increased chlorophyll and accelerated flowering time which is collectively known as shade avoidance syndrome (Casal 2012). Under shade, increased abundance of PHYTOCHROME INTERACTING FACTOR (PIF) PIF 4 and PIF5, degradation of DELLA proteins, increased synthesis and redirection of auxin are the major events among the molecular processes that arise downstream of PHYB (Casal 2012). PHYB also plays a major role in the regulation of flowering time in a photoperiod-dependent manner. It acts antagonistically with PHYA to activate GIGANTEA (GI) and stabilize CONSTANTS (CO), which in turn positively regulate FLOWERING LOCUS T (FT) under summer long-days (Song et al. 2015; Valverde et al. 2004).

Floral transition between the vegetative and reproductive phases of plant development is an important event not only to manipulate yield but also as a mechanism to escape abiotic stress (Song et al. 2015). Flowering is regulated by a complex network of various environmental factors which quantitatively modulate the flowering-time integrators (Kumar et al. 2012; Fornara et al. 2010; Mouradov et al. 2002) such as FT, LEAFY (LFY) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), also known as AGAMOUS-LIKE 20 (AGL20) (Samach et al. 2000). Molecular and genetic studies have shown that at least four different mechanisms leading to floral transition are possible: (1) photoperiod (duration of light), (2) low temperature (vernalization), (3) gibberellic acid (GA) and (4) autonomous pathways (Boss et al. 2004). Photoperiod pathway stimulates flowering under inductive long-days (LDs). Signals perceived by the photoreceptors together with the nuclear proteins such as EARLY FLOWERING 3 (ELF3) and GI entrain the circadian oscillators, which consists of LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), TIMING OF CAB EXPRESSION 1 (TOC1) and EARLY FLOWERING 4 (ELF4) (de Montaigu et al. 2010). Changes in the day length is detected by the circadian clock and transduced to the photoperiod components by initiating a cyclic expression of *CO*. In short-days (SDs), *CO* expression peaks at the night phase, whereas in the LDs its expression overlaps with the end-of-the-day light phase and this expression pattern leads to the stability of CO that mirrors the FT accumulation. In LDs, toward the middle-of-the-day the accumulation of GI along with FLAVIN-BINDING, KELCH REPEAT, F BOX 1 (FKF1) forms a complex that degrades the DNA-BINDING-WITH-ONE-FINGER (DOF) repressors which allows transcription of *CO*, thereby inducing the expression of the floral promoter FT (Fornara et al. 2009; Sawa et al. 2007; Imaizumi et al. 2003). By contrast in SDs, FKF1 accumulates ~ 3 h after GI peaks that does not allow the formation of the GI-FKF complex, thereby leading to a low abundance of *CO* transcripts, hence delays flowering

time. In vernalization pathway, signal from prolonged cold is perceived by the plant which strongly down-regulates the FLOWERING LOCUS C (FLC) level that stimulates flowering. This mechanism is controlled by the combinatorial effect of two vernalization genes, *VRN1* and *VRN2* gene products. The plant growth regulator GA can also promote flowering in a photoperiod-independent manner (Khan et al. 2014). It has been shown that mutation in the repressor of GA signaling such as SPINDLY (SPY) can also induce flowering most likely antagonizing the effect of GI. The genetic interaction of GI and SPY has also been documented and reviewed (Mishra and Panigrahi 2015). In the autonomous pathway, components involved in RNA processing and epigenetic regulation such as FLOWERING CONTROL LOCUS A (FCA) and FLC have been characterized that modulate the flowering-time integrators at the shoot apical meristem (Khan et al. 2014). Genes of the autonomous pathway constitute a subgroup which target *FLC* expression and function to reduce its accumulation (Sheldon et al. 1999, 2000).

In the present study, we have investigated the uptake, accumulation and effect of CNPs on development of *At* and evaluated seed germination rate, chlorophyll content, photomorphogenesis and flowering time. Our data showed that CNPs induces the shade avoidance response in seedlings and also accelerates flowering in PHYB-dependent and CO-independent manner.

## Materials and methods

CNPs were obtained from graphite nanopowder, type-1, APS: 400 nm (Sisco Research Laboratories Pvt. Ltd. India).

### Plant growth conditions and phenotypic measurement

Seeds of *At* were surface sterilized (Kumar et al. 2017a; Fornara et al. 2009) by rinsing in 70% ethanol and 100% ethanol for 5 min each followed by air drying under laminar air flow. Surface sterilized seeds were sown on 50 ml of sterile MS medium from Himedia (Catalogue # PT100-1L) with increasing concentrations of CNPs starting from 10, 100 and 500 µg/ml, respectively, in LD growth conditions. Media plates without CNPs and similar concentration of charcoal (Merck, Catalog # 102514, EC No. 1907/2006) served as the control. CNPs were added to the MS medium placed in 6.4 cm × 6.4 cm × 10 cm planton tissue culture containers (Tarsons Product Pvt. Ltd. India). For all phenotypic measurements of seedling, the seeds were stratified at 4 °C for 3 days in the dark and further grown for 8 days under white light (WL). For adult plant phenotype, *At* seedlings were grown till bolting on the MS medium and rosette leaf number was counted, as the rosette leaf number at the time of bolting is an indicator of flowering time. The data presented were the mean of 20 individual plants. The pattern of the result presented was similar in at least three biological replicates. Col-0 plants normally bolted in 22 days on the soil, however, on MS media bolting was delayed by ~ 3 days in all biological replicates. The WL was obtained from Philips 17 W F17T8/TL741 USA Alto II technology tubes with 50% light intensity, which is equivalent to ~ 100 µmol/$m^2$. All seedling data represented in this study were obtained from seedlings (8-day-old) grown under WL plant growth chambers (model-AR36, Percival, USA) set to 22 °C and 70% relative humidity. The growth chambers were programmed to provide cycles of 16 h light and 8 h dark (LDs) or 8 h light and 16 h dark (SDs). For monochromatic red (R), far-red (FR) and blue (B) light irradiation, seedlings were grown in E-30 LED chambers (Percival, USA) maintained at 22 °C temperature and with 70% relative humidity. The intensities of incident lights were 80 µmol/$m^2$/s for WL, 50 µmol/$m^2$/s for R, 6 µmol/$m^2$/s for FR and 30 µmol/$m^2$/s for B.

### Seedlings phenotypes

Hypocotyl and root length measurements of seedlings were carried out by taping them on a black paper and scanned images were obtained with Epson L220 scanner. Images were analyzed using Image J software (Version 1.46). All data presented were average of at least 3 biological replicates, and each data point was a mean of at least 20 measurements. Relative hypocotyl lengths were obtained as ratios of hypocotyl length of seedlings grown in light to their respective dark controls. Average cotyledon angles were measured from the angle between the cotyledon and the hypocotyl in seedlings without or with CNPs treatment. Data were analyzed in Prism5 software using two-way ANOVA. Pearson's correlation for significance was performed at $p < 0.01$ and indicated as significant data point (indicated by ***).

### Chlorophyll content

Total chlorophyll was quantified as described previously (Kumar et al. 2017b; Arnon 1949). Briefly, 10 mg of leaf from seedlings were isolated and incubated with 1.25 ml of 80% acetone for 48 h at 4 °C in the dark. Eppendorf tubes with the acetone extract centrifuged at 13,000 rpm for 5 min, followed by the measurement of absorbance of the supernatant at 645 and 663 nm. Total chlorophyll was calculated using the formula: 20.2 (*A*645) + 8.02 (*A*663).

### Raman spectroscopy

Various plant samples (100 mg FW) were taken, washed three times with distilled water and homogenized with

mortar and pestle in 3 ml of distilled water. The homogenate was collected in a borosilicate cuvette compatible to the Raman spectrometer and the spectra were collected with EzRaman Reader V5.8.5 (EzRaman *S/N* 158,270, max power output = 500 mW, maxi wavelength = 785 nm, Envaveoptronicsinc18271, E.McDurmottste A-1 Irvine, CA, 29614, USA) using a frequency stabilized 785 nm narrow band width laser source in back scattering mode. For all the samples 400 mW of output laser power was used through a permanently aligned single fiber and the spectra were collected through a 200 µm collection fiber having numerical aperture of 0.22 and working distance of 7 mm. The spectrometer has the spectral resolution of 6 $cm^{-1}$ in the range of 250–2350 $cm^{-1}$. High sensitive linear CCD array having pixel size of 14 µm × 200 µm (2048 pixels) in TEC cooled (~ 15 °C) detector was used for collecting the data. The background was subtracted from a dark spectrum automatically through the in-build Enwave Raman reader software program (Kumar et al. 2017a). For intensity comparison and good statistics the integration time of 40 s was kept constant for all the samples. Percentage of intake of CNPs and statistical analysis were carried out using Prism5 software.

### Light microscopy

Transverse sections of leaves were made with the help of a razor blade and were visualized under 100× magnification using inverted microscope from Olympus (Model: 1 × 73/m. PS.O Camera and S/W) from DSS image tech Pvt. Ltd. Size and structure of CNPs was visualized in MS medium alone as a positive control or in the leaf mesophyll cells of the plant material grown with CNPs. The uptake of CNPs by seedlings could be visualized by the microscopic observation of CNPs fine particle aggregates in the CNPs-treated plant samples only in contrast to the control Col-0 plants (Supplementary Fig. 1).

### RNA isolation and qRT-PCR

For RNA isolation, 10-day-old seedlings grown on MS medium without or with 500 µg/ml CNPs were harvested at ZT 8, 12 and 20 under LDs. Total RNA was isolated using Qiagen Plant RNeasy mini kit (Cat #74104). The cDNA was prepared from 1 µg of RNA of each sample using Bio-RadiScript™ Reverse Transcription Super-mix, (Cat # 1708840). The quantitative RT-PCR (qRT-PCR) was performed using CFX384 Touch™ Real time detection system (Bio-Rad laboratories), following the manufacturer's manual of iTaq™ universal SYBR green supermix. Gene-specific primers used for qRT-PCR were designed using Primer Quest software tool (Integrated DNA Technologies, Inc., USA) and are listed in Supplementary Table 1. All reactions were carried out in a Hard-shell 384-well PCR plates (supplied by Bio-Rad, Cat #: HSP3805), with a reaction volume of 10 µl per well. The PCR mix consisted of

2× iTaq universal SYBR Green super mix: 5 µl.
$H_2O$: 2 µl.
Template: 2 µl (~ 50 ng).
Primers (500 nM): 1 µl.

The PCR thermocycler program used was as follows: 95 °C: 5 min, 95 °C: 10 s, 60 °C: 30 s, 60 °C: 30 s and 65 °C: 5 s. Transcript levels were normalized with *ACTIN*. Each qRT-PCR reaction was performed in at least triplicate and all data were presented as mean ± SEM.

## Results

### CNPs treatment induces early flowering in *Arabidopsis thaliana*

Increasing concentration of CNPs resulted in dosage-dependent earlier flowering in *At* (Fig. 1a–d and Supplementary Fig. 2a–d). Under LDs, the untreated Col-0 plants showed bolting at around 11 ± 1 rosette leaf stage. A significant reduction in leaf number was observed following exposure to increasing concentration of CNPs from (10–100 µg/ml), with the plants flowering on an average two leaves earlier (Fig. 1b, c, e). Further increasing CNPs concentration (500 µg/ml) resulted bolting even earlier at 3 ± 0.5 leaves as compared with the untreated plants (Fig. 1d, e and Supplementary Fig. 2d). Therefore, all subsequent experiments were conducted using CNPs concentration of 500 µg/ml in medium.

To confirm that the early flowering phenotype was not a result of crowding, individual seedlings were grown in single plantons in the presence of CNPs in a similar method (Fig. 2a, b) as described above. To ascertain that the early flowering phenotype was specific to CNPs and not due to the higher amount of carbon *per se*, Col-0 plants were grown in presence of 500 µg/ml charcoal under LDs (Fig. 2c). Charcoal-treated plants showed very different rosette morphology (Fig. 2c). Average rosette leaf size was larger and longer. These plants were less green and showing some sign of localized chlorosis or senescence as yellow patches but eventually flowered later (after ~ 5 rosette leaves or more) compared to its untreated control under LDs.

### CNPs accumulated to highest in leaf tissues

To determine whether CNPs were taken up systemically through plant roots, CNPs accumulation was investigated in root, stem and leaf extracts made from in 20-day-old plants as described in materials and methods and were

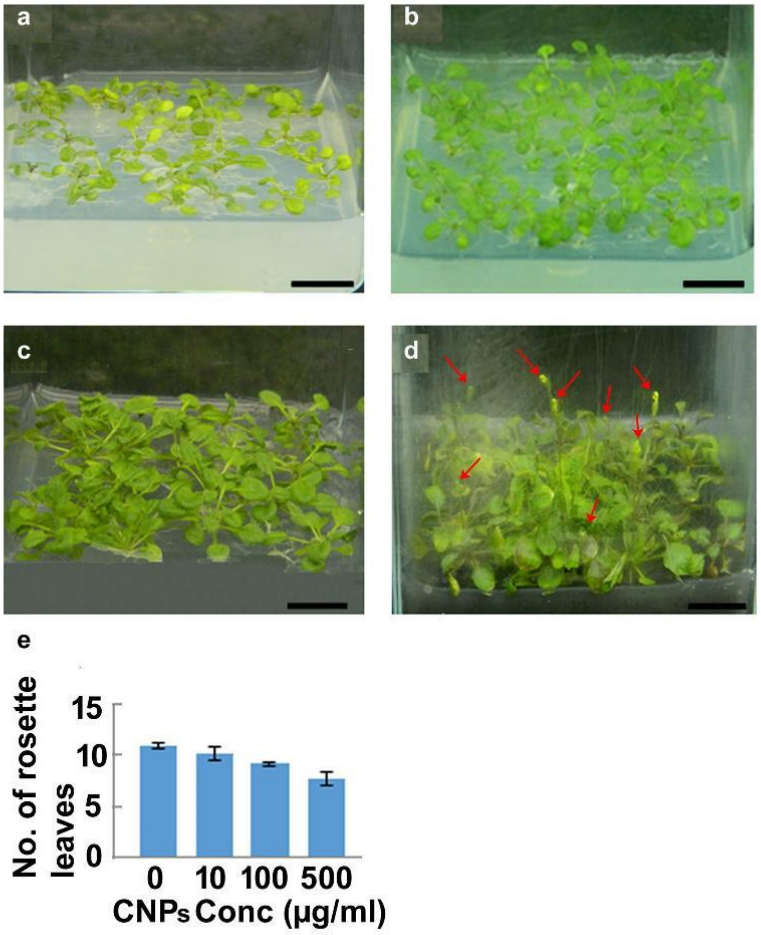


**Fig. 1** Dosage dependent effect of CNPs on the induction of bolting. Col-0 seeds (Col-0 about 20/pot) were grown in planton boxes as described in materials and methods. **a** Without CNPs or **b** 10 µg/ml **c** 100 µg/ml and **d** 500 µg/ml of CNPs. Plants showed indications of bolting were marked with red arrows. **e** Evaluation of flowering time of Col-0 plant grown with different concentration of CNPs in MS media

subjected to Raman spectroscopy (Fig. 3). As a positive control, CNPs at 500 µg/ml in di-methyl formamide (DMF) solvent showed a sharp peak at wave number 1200 $cm^{-1}$. All the tissue extracts tested showed the accumulation of CNPs at wave number comparable with that of the DMF solvent. However, the amplitude of accumulation was highest in the leaf extracts and least in the extracts of root. The percentage of CNPs accumulation was quantified as ~ 75% in leaf, ~ 20% in stem and ~ 5% in root extracts. These results indicated that the accumulation of CNPs in Col-0 plants showed tissue specificity and was predominately enriched in the leaf. Tissue specificity is an interesting point to understand the interaction of nanomaterial's with plants, also documented in *At* (Bao et al. 2016) and in tomato (Dan et al. 2015).

## CNPs exposure affected different physiological traits at various growth stages of *A. thaliana*

We first examined whether the CNPs-induced earlier flowering response is due to higher germination rate. CNPs treatment showed a marginally enhanced effect on the percentage of seed germination in the Col-0 seeds as scored after 3 days of irradiation under WL in LDs. While Col-0 seeds showed 83% germination on MS medium, CNPs-treated seeds showed nearly 5% higher (88.7%) germination (data not shown).

CNPs treatment affected the hypocotyl length significantly ($p \leq 0.01$) under WL both under LDs and SDs (Fig. 4a, c, Supplementary Fig. 3a and Supplementary Fig. 5). To determine whether the hypocotyl growth

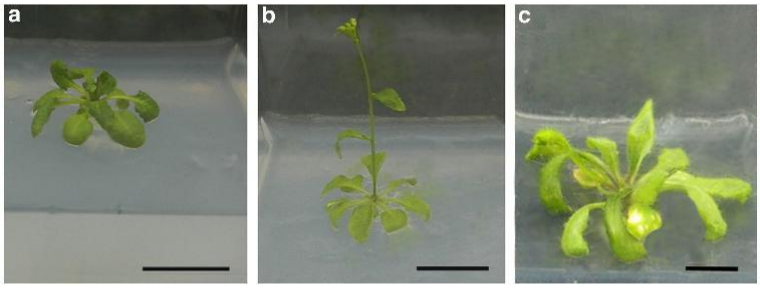


**Fig. 2** Effect of CNPs treatment on the phenotype of individual *Arabidopsis* plant. Col-0 seeds were grown individually under LD (16 h light/8 h dark cycles) growth conditions on MS medium as described in materials and methods. **a** Without or **b** with CNPs of 500 µg/ml till bolting stage. **c** Representative picture of a plant grown in the presence of 500 µg/ml charcoal as an additional control. Plants were photographed on 20–21 days after transferring to light. Distinct earlier flowering was observed only in plants with CNPs in the specified growth conditions, while larger leaf size and pale greenish plants were documented in all biological replicates in presence of charcoal. Scale bar: 10 mm. (Color figure online)

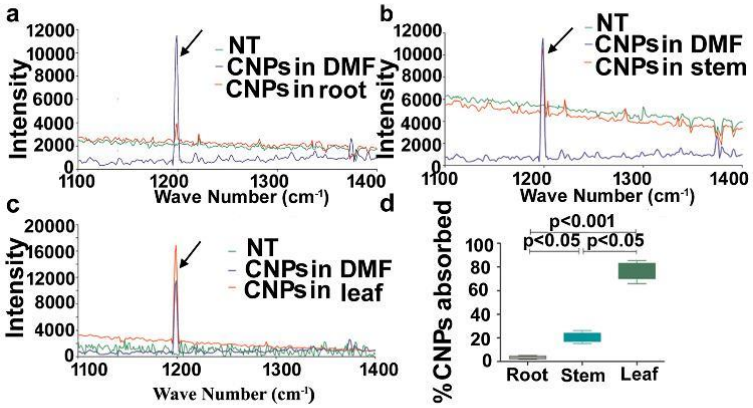


**Fig. 3** Raman Spectroscopic analysis of the accumulation of CNPs. Col-0 plants were grown for 20 days in plantons containing MS media without or with CNPs (500 µg/ml) and processed for Raman spectroscopy as described in materials and methods. Spectra obtained from **a** root, **b** stem or **c** leaf showed a peak at wave number 1200 $cm^{-1}$ (red) indicative of CNPs (as shown by arrows), as the CNPs control alone in di-methyl formamide (DMF) (blue) overlap with the same wave number. **d** Quantitative estimation of CNPs was done from the area of the peaks using Prism 5 software and presented. Percentage of CNPs accumulation in root (shown in grey), stem (shown in blue) or leaf (shown in green) indicates significant difference of CNPs accumulation ($p < 0.001$). The data represented were reproduced thrice in independent experiments. (Color figure online)

inhibition was not a developmental defect, seedlings were grown for 8 days in darkness after germination induction without or with CNPs. Both the CNPs-treated and untreated seedlings showed distinct etiolated phenotype with elongated hypocotyls and closed pale yellow cotyledons suggesting the hypocotyl elongation growth in the presence of CNPs was light-dependent (Fig. 4b). Compared to the respective controls in LDs and SDs, CNPs-treated seedlings under WL consistently showed about 60.6% (Fig. 4c) and 30% (Supplementary Fig. 3a) increase in hypocotyl length, respectively. To determine if the increase in length of hypocotyl was light quality-dependent, seedlings were grown under monochromatic R, FR, or B lights. Compared to controls, a dramatic increase of twofold in hypocotyl length was observed when the seedlings were grown under R light.

Root length in seedlings grown under WL was also significantly affected due to CNPs (Fig. 4d, Supplementary Fig. 3b). Compared to WL controls, the CNPs-treated seedlings showed significantly ($p < 0.01$) longer primary roots in LDs and SDs. Under different qualities of monochromatic

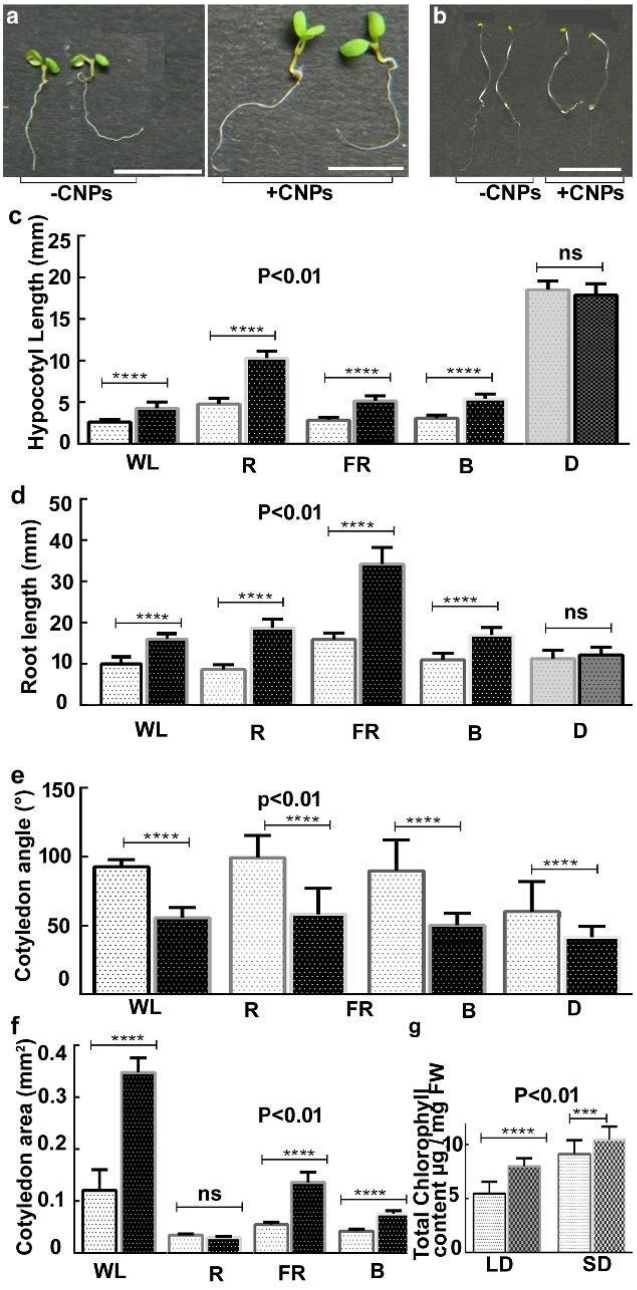


**Fig. 4** Effect of CNPs treatment on seedling phenotypes. Eight-days-old seedlings of Col-0 ecotype without or with CNPs were obtained as described in materials and methods. **a** Pictures of two representative seedlings grown under white light, **b** etiolated seedlings without (−CNPs) or with (+CNPs). **c–f** Average hypocotyl length, root length, cotyledon angle and cotyledon area of seedlings grown under white (WL), red (R), far-red (FR), blue (B) lights or dark (D), respectively. White dotted histograms indicate −CNPs while black dotted histograms indicate +CNPs. **g** Seedlings were grown for 8 days either in long days (LDs: 16 h light / 8 h dark) or Short days (SDs: 8 h light/16 h dark) and chlorophyll content estimation was done as described in materials and methods. The data represented is mean of 20 seedlings reproduced thrice. $p < 0.01$ indicates significant difference (**** or ***) confirmed through unpaired student *t* test. *ns* non-significance. The *p* value with higher number of stars is indicative of higher significance by Prism 5. Scale bar: 10 mm

light, although the root length of CNPs-treated seedlings was increased as observed in WL, the increase was highest and significant under the FR with more than double than that of the untreated seedlings.

CNPs treatment also affected cotyledon opening of seedlings (Fig. 4e, Supplementary Fig. 3c). Cotyledon angle in 8-day-old Col-0 seedlings was ~ 93°, whereas in the case of CNPs-treated seedlings it was ~ 56° (Fig. 4e). The cotyledon angle of seedlings grown in SDs (Supplementary Fig. 3c) and under different qualities of monochromatic lights R, FR and B (Fig. 4e) followed the similar pattern as the LDs.

The cotyledon area of the CNPs-treated seedlings was significantly higher than that of the untreated seedlings in both LDs and SDs (Fig. 4f, Supplementary Fig. 3d). CNPs treatment resulted in enhanced chlorophyll accumulation. Under LDs, total chlorophyll content increased from 4.8 µg/mg in control seedlings to 7.5 µg/mg in the CNPs-treated seedlings (Fig. 4g).

## Early flowering induced by CNPs treatment is photoperiod-dependent

Bolting, as indication of flowering time, in Col-0 was observed at 11 (± 1) rosette leaves and in between 25 and 28 days, whereas the CNPs-treated plants flowered at around 8 (± 1) rosette leaves and took about 20 LD cycles. CNPs treatment in the SDs growth conditions also resulted in earlier flowering. CNPs-treated plants bolted at about 5 days earlier (with 28–30 SD cycles), while Col-0 plants bolted at 34–36 SD cycles post-germination.

Since the photoperiod is perceived at the leaf (Song et al. 2015), and the mechanism operates from phloem companion cells, it was further asked if the early flowering phenotype due to CNPs treatment was photoperiod-dependent. The leaf number at bolting stage in LDs was counted in the CNPs-treated and untreated plants (Fig. 5a, b). Average rosette leaf number in the Col-0 plants without or with CNPs treatment showed a significance difference ($p < 0.01$). Further, the leaf number was counted in plants grown under SDs (Supplementary Fig. 4). The control plants flowered at 31.8 ± 0.7 leaves, whereas the CNPs-treated plants flowered earlier with 30.33 ± 0.57 leaves in 10 µg/ml, 28.7 ± 0.2 leaves in 100 µg/ml and 25.7 ± 0.7 leaves in 500 µg/ml of CNPs, respectively. Hence, it indicated that the early flowering phenotype due to CNPs is partially photoperiod-dependent, the responses being pronounced in LDs.

## CNPs-treated samples have less *PHYB* transcript compared to the untreated seedlings

Involvement of PHYB was investigated to understand the red light specific increment of hypocotyl length, in 10-day-old seedlings without or with CNPs treatment under LDs

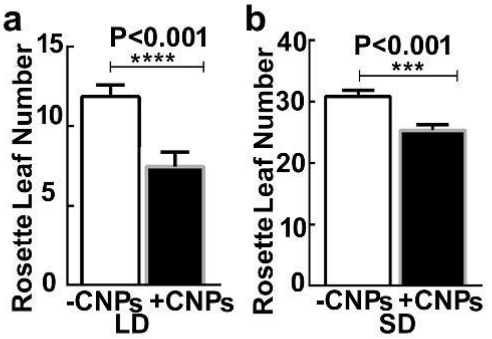


**Fig. 5** Flowering time of Col-0 under Long days and Short days. Plants were grown in MS medium without or with CNPs (500 µg/ml) treatment. **a** Col-0 plants grown for 20 days in LDs or **b** in SDs for 30 days. White histograms represent without CNPs and black histograms represent with CNPs. Rosette leaf number data presented is a mean of at least three individual experiments. Significant difference at $p < 0.001$ without or with CNPs treatment was evaluated by Prism 5

(Fig. 6a). The expression of *PHYB* transcript was analyzed using qRT-PCR at Zeitgeber (ZT) 8, 12 and 20, relative to the transcript level of *ACTIN*. *PHYB* transcript levels were higher at all the time points of light phase tested in the untreated seedlings than their respective CNPs-treated seedlings. The most contrasting decrease in the *PHYB* expression was found to be at ZT 12 in the CNPs-treated seedlings. These results suggested that the expression level of *PHYB* was down-regulated upon CNPs treatment.

## Early flowering due to CNPs is regulated by photoperiodic flowering time pathway components

Since, CNPs-induced the down regulation of *PHYB*, we further analyzed the expression level of a selected component of the photoperiod pathway, the *GI*, at ZT12 in CNPs-treated seedlings. GI is a nuclear protein, regulated by circadian clock, and can directly activate the expression of *FT* to regulate flowering time under LDs (Sawa and Kay 2011). *GI* transcript levels in CNPs-treated seedlings were nearly 2.1-fold higher than the untreated seedlings at ZT12 (Fig. 6b). The *CO* transcript level in the CNPs-treated seedlings remained unaffected (Fig. 6c) indicating a low probability of CO involvement and possible direct interaction of GI with FT to elicit the early flowering phenotype. This direct interaction of GI and FT was also documented previously (Sawa and Kay 2011).

To confirm that the early flowering was caused due to possible altered expression of other components of photoperiod pathway, transcript levels of *PIF4* and *LFY* were estimated

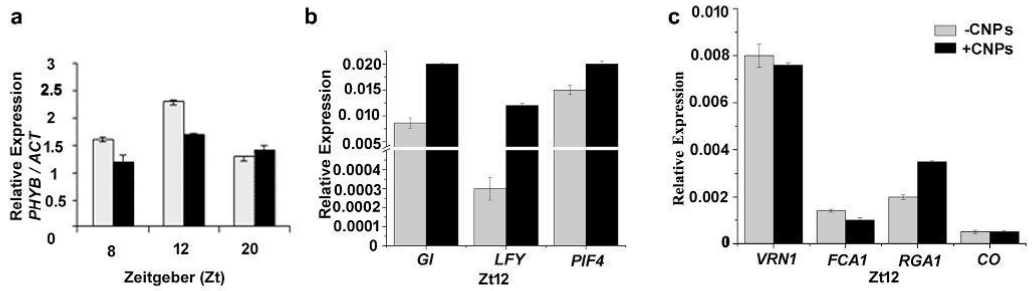


**Fig. 6** Quantitative RT-PCR analysis of representative transcripts from flowering time pathways. RNA was extracted from various samples grown under white light at different Zeitgeber (ZT) as described in materials and methods. **a** Relative transcript level of *PHYB* using qRT-PCR at ZT 8, ZT 12 and ZT 20 and normalized with the expression of *ACTIN* (*ACT*). **b** Relative transcript level of *GIGANTEA (GI), LEAFY (LFY)* and *PHYTOCHROME INTERACTING FACTOR 4 (PIF4)* and **c** *VERNALIZATION1 (VRN1)*, and *FLOWERING CONTROL LOCUS A (FCA), REPRESSOR OF GIBBERELLIC ACID 1 (RGA1)* and *CONSTANS (CO)* at ZT12. Gray histograms represent without CNPs and black histograms represent with CNPs

in the CNPs-treated seedlings. It was shown that interactions involving GI, FT and LFY influence the flowering and floral development (Blázquez et al. 1997; Nilsson et al. 1998). At ZT12, *LFY* transcript levels in the CNPs-treated seedlings were highly induced with nearly 40-fold up-regulation compared to the untreated seedlings (Fig. 6b). Since PHYB negatively regulates PIF4 (Gangappa et al. 2017), we hypothesized that the phenotypes shown after CNPs treatment could be due to enhanced PIF4 function. Interestingly, CNPs-treated seedlings showed ~ 1.25-fold up-regulation in the *PIF4* level at ZT12 compared to the untreated controls (Fig. 6b). These results further confirmed the involvement of photoperiod pathway for causing the early flowering phenotype.

### CNPs-induced early flowering is independent of GA, vernalization and autonomous pathways

To further dissect the involvement of other pathways for the early flowering phenotype due to CNPs treatment, one major component from each pathway (*FCA* for autonomous pathway, *RGA1* for GA pathway and *VRN1* for vernalization pathway) was selected for expression analysis at ZT12 in the CNPs-treated seedlings. The transcript levels of *VRN1* and *FCA* in the CNPs-treated seedlings were nearly similar as compared with their controls (Fig. 6c). However, the *RGA1* transcript levels showed about 1.7-fold up-regulation in the CNPs-treated seedlings.

## Discussion

In the present study, an attempt has been made to understand the importance of carbon nanoparticles in some of the crucial physiological processes in the important plant model system, *At*. The results demonstrated that CNPs may serve as important modulator to induce early flowering. Using a series of experiments, our results suggested that this effect is PHYB, photoperiod-dependent and CO-independent. To our knowledge, this is the first report demonstrating the direct application of CNPs as flowering time regulator in plants which may also be employed as a strategy to escape abiotic stress.

In this study, we have demonstrated that CNPs were taken up by *At* seedlings. Plants flowered earlier, when seeds were germinated and grown on the medium containing CNPs. This suggested that the CNPs were taken up systemically by the root system. Most likely the transport of CNPs did not use the plasmodesmata route, but it must have occurred by the endocytosis pathway. Similar uptake and distribution of C60 and C70 fullerenes was demonstrated in rice (Lin et al. 2009), tomato (Khodakovskaya et al. 2009) and *Arabidopsis* (García-Sánchez et al. 2015). CNPs aggregates have been shown to be taken up in both symplastic and apoplastic route (Tripathi et al. 2017). However, most likely they did not aggregate before being taken up by the plant. This observation was strengthened by the fact that such aggregates are not detected in the vasculature. CNPs were observed in the stem tissue though in lesser quantity indicating that xylem could be the possible player for its translocation. In the present study, CNPs uptake did not show any adverse effect in terms of growth of *At*. To understand, if the dosage dependent early flowering phenotype was result of combinatorial presence of CNPs and shading due to over-crowding of plants in a single pot, experiments were performed in single pots to minimize the shade avoidance response primarily regulated by PHYB photoreceptors. CNPs may not necessarily inhibit only PHYB response but also might alter other biochemical pathways including auxin, cytokinin and brassinosteroids.

The early flowering phenotype and the longer hypocotyl length observed in all experimental plants exposed to CNPs prompted us to look into the photoperiod and light signaling regulatory components at the molecular level. The photoperiod is sensed at the leaves (Song et al. 2015) and in our studies the CNPs were predominantly accumulated in the leaf tissues. Since, the CNPs accumulation was highest in leaf tissues and was at un-detectable level in the isolated leaf veins (data not shown), our work suggested that the photoperiodic control may have been altered. Accordingly, the plants responded to the day-length differently in terms of their flowering in the presence of CNPs in both LDs and SDs although with different number of rosette leaves upon treatment with CNPs on MS medium. These indicated that the CNPs-treated plants are photoperiod sensitive. The differences in rosette leaf number were significantly lower and are about less than half under LDs than under SDs suggesting that the early flowering phenotype in the CNPs-treated seedlings was partially photoperiod sensitive.

A possible model explaining the early flowering caused due to CNPs treatment is drawn in Fig. 7. Expression level of *LFY* determines the identity of primordia that arises at the shoot apical meristem and positively correlates the transition to flowering (Blázquez et al. 1997). It has been shown that mutation in flowering genes such as *GI* causes reduction in *LFY* expression hence, represses flowering (Nilsson et al. 1998). Also, PHYB which causes late flowering, act as a negative regulator of the LFY (Nilsson et al. 1998) and delays flowering in wild type (WT). Here, we observed that after CNPs treatment, expression of *PHYB* is down-regulated which results in the up-regulation of *LFY* expression also, *GI* transcript levels were enhanced. These combined effects lead to early flowering. It has also been shown that PHYB acts as a repressor for PIF4 which causes short hypocotyl and large cotyledon angle in WT (Huq and Quail 2002). Since, after CNPs treatment *PHYB* transcript level was down-regulated, we found higher level of *PIF4* transcript which resulted in longer hypocotyl and smaller cotyledon angle compared to WT. In this study, we also observed enhancement in *RGA1* transcripts, which is a repressor of GA pathway. To further confirm that the reduced *PHYB* transcript caused early flowering in a GA-independent manner, we measured hypocotyl length of seedlings grown in the dark. The strongly etiolated phenotype and no difference in hypocotyl length in the dark after CNPs treatment supported the view that the process is GA-independent.

Smaller cotyledon angle in WL and under different qualities of light suggested the down-regulation of multiple photoreceptor functions and possible involvement of a common signaling pathway. Larger cotyledon area in all the light qualities tested suggested a complex combinatorial output of interaction of different signaling pathways. However, the higher chlorophyll content in WL suggested significant down-regulation of PHYA and PHYB-dependent pathways that mimic a *phyA phyB* double mutant plant, which also flowers relatively earlier compared to its WT control (Franklin and Quail 2010; Neff and Chory 1998). Since PIF4 is epistatic to PHYB and acts antagonistic to

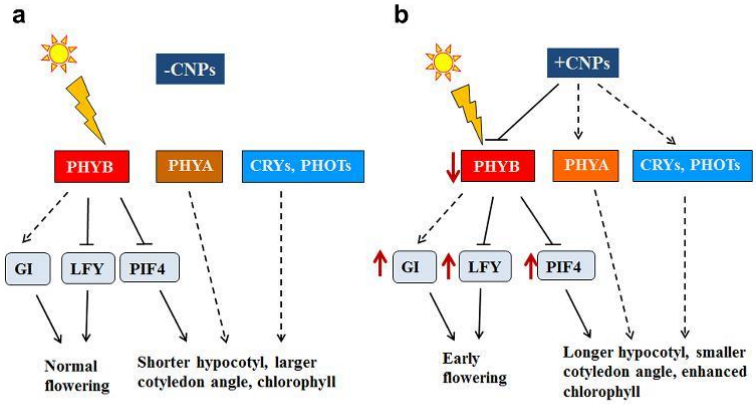


**Fig. 7** Proposed model explaining possible involvement of CNPs in photomorphogenesis and flowering. **a** In the absence of CNPs under white light, higher transcripts of *PHYB* lead to the degradation of *PIF4*. Ultimately a lower transcript levels of *PIF4* results in shorter hypocotyl and normal flowering. **b** CNPs treatment leads to the down regulation of *PHYB* and ultimately accumulation of transcripts of *PIF4*, since PHYB acts as a repressor of PIF4. *GI* transcript level is enhanced which leads to the up-regulation of *LFY* expression. Dotted lines between PHYB and GI indicate the involvement of multiple clock components leading and output to GI. *PHYA* phytochrome A, *CRYs* cryptochromes, *PHOTs* phototropins. Dotted arrows between +CNPs and PHYA, CRYs, PHOTs indicates proposed regulation from the results of this study

it, the *phyB* mutant accumulates PIF4 that result in longer hypocotyl and larger cotyledon area explaining the observation in the current study (Huq and Quail 2002). Earlier flowering by CNPs treatment without the involvement of CO suggests an alternate mechanism that by passes the classical (CO-FT) photoperiod module possibly by direct interaction of GI on FT promoter.

## Conclusion

In summary, we conclude that, CNPs is taken up by the plant, accumulates in leaf tissues and can induce earlier flowering by altering both PHYB and photoperiod-dependent pathway. CNPs induced increase in hypocotyl length under red, far-red and blue light due to the hyposensitive effects of all the three major photoreceptor signaling pathways by a yet unknown mechanism. Consequently, the early flowering phenotype is most probably due to the down regulation of light signaling components. The longer root system, higher chlorophyll content and early flowering observed in CNPs-treated plants are beneficial agronomic traits from the perspective of abiotic stress tolerance and can be exploited for sustainable yield under stress. Exploring the importance of CNPs in crop plants under control conditions and soil may open up a novel non-transgenic approach for crop improvement.

**Author contribution statement** AK and AS have performed the experiments and contributed equally. KCP, MP and PKS have designed the experiments and written the MS. MP has analyzed the data, written the MS and made the figures. All authors have read and approved the MS.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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# Optimization of soil parameters and cost effective way of growing *Arabidopsis thaliana* from an Indian perspective

ABHISHEK KUMAR[1,2], MADHUSMITA PANIGRAHY[1,2] and KISHORE CS PANIGRAHI[1,2]

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**ABSTRACT :** Arabidopsis Biological Resource Centre (ABRC) has prescribed standard procedure for healthy growth of *Arabidopsis* in growth chambers and green house. However, standard growth of *Arabidopsis* thaliana in local conditions of Bhubaneswar, Odisha is found to be dependent on the type of soil mixture used. In the present study, we have tested 5 different types of soil combinations available (i.e. clay, red soil, garden soil, soilrite and mixed soil) and recorded different plant growth parameters such as root length, leaf area, chlorophyll, carotenoid, anthocyanin content, number of leaves at bolting, pod length, pod weight, seeds / pod and biomass to access healthy plant growth condition. We could observe the best plant growth on mixed soil (soil mixture of soilrite, red soil and garden soil) determined by us. Mixed soil grown plants show optimal vegetative growth as well as reproductive potential. Finally, we have fixed the soil mixture composition as soilrite, red soiland garden soil in 5:4:1 proportion for optimum plant growth of *Arabidopsis* in growth chambers. This proportion of mixed soil could be taken as the bench mark for the researchers in Indian conditions for *Arabidopsis* research.

**Key words:** *Austempered* ductile iron, soilbin, rotavator, power and torque

*Arabidopsis thaliana* is the model dicot plant, belonging to Brassicaceae family, most widely used as the platform for understanding plethora of biological mechanisms in diverse range of researches in different parts of the world. Its wide acceptability and preferred use is imparted to its short life span of 90-120days, simple genomic organization and genetic regulations. Arabidopsis is an herbaceous, monocarpic, annual plant, a commonly growing road side weed, which bears anerect plant form. It is widely distributed on earth from 10° N to 60°N (Li *et al.*, 1998, Minorksy (2001). *Arabidopsis thaliana*plant growth and its variation in morphology (Lacey, 1986; Potvin, 1986; Reinartz 1984), phenology (Zhang *et al.*, 1994), physiology Minorksy (2001) and growth rate (Li *et al.*, 1998) have been well documented according to ecotypic variations. The plant growth varies according to environmental condition,22-24°C temperature being the best growth condition for the plant. Arabidopsis is a long day plant and under natural conditions it grows up to 2 feet height, flowers at a minimum of 12-14 leaves. Unlike natural growth conditions which incorporate milder fluctuations of daily light, temperature and humidity, growth conditions in research laboratories maintain constant temperature, humidity and light cycles. Under controlled growth conditions *Arabidopsis* grows up to 12 leaves stage in long day and 25 leaves in short day conditions after which it flowers (ABRC). *Arabidopsis* requires certain kind of soil, nutrient requirements and watering conditions because of which *Arabidopsis* biological resource centre (ABRC) has prescribed standard procedures for healthy growth of *Arabidopsis* in growth chambers. Perdue Agriculture standards describe "PRO-MIX BX" soil mix (Perdue plant growth facility) and Osmocote fertilizer mix for healthy Arabidopsis growth (ABRC). However, growth conditions of *Arabidopsis* in Indian conditions vary significantly to the recommendations of ABRC standards due to non-availability of similar soil mix. This introduces problems to attain standard growth of *Arabidopsis* in Indian soil conditions, which is the basic pre-requisite to perform experiments and obtain standard results. We have tested five different soil types available and also a new mixed soilproportion for optimum growth of *Arabidopsis* in growth chambersunder controlled conditions. In this paper we have standardized and fixed the soil and nutrient requirement for *Arabidopsis* growth in growth chambers in local conditions of Bhubaneswar, Odisha. This will provide necessary guidelines for Indian researchers and base for obtaining true observations under experimental conditions.

## MATERIALS AND METHODS

### *Soil proportions*

Clay soil: local available soil outside NISER campus, Bhubaneswar. Red soil: local available soil at NISER campus, Bhubaneswar. Garden soil: Local soil available nursery at Bhubaneswar. Soilrite was obtained from Keltech Energies limited, Bangalore, which a mixture of horticulture grade expanded perlite, irishpeat moss and exfoliated vermiculite in equal ratio. Mixed soil compositions were designed by us is thus a noble mixture of soilrite mix: red soil: garden soil proportion of 5:4:1. All five kinds of soil was initially autoclaved to avoid contamination and cooled before use.

### *Plant growth conditions*

The seeds were stratified at 4 °C for 2 days in dark and further grown for under white light for all seedling phenotypic measurements. For adult plant phenotype *Arabidopsis* seedlings were grown till the induction bolting and rosette leaf number was counted. The white light was obtained from Philips 17 watt F17T8/TL741 USA Alto II technology tubes with 100% light intensity, which was equivalent to ~120µmols $min^{-1}$. All the data represented in this study were obtained from plant were grown under white light in plant growth chambers (Percival, USA) set to 22°C and 70% relative humidity. The growth chambers were programmed to provide 16 hours light and 8 hours dark (LD) or 8 hours light and 16 hours dark (SD).

### *Plant phenotypic measurements*

All data presented are average of 3 biological replicates with each data point is the mean of at least 15 measurements. Error bars refer to the SD. Pearson's correlation for significance was performed at $p < 0.0001$ and indicated as * above the significant data point.

### *Root length*

About 22-days-old plants were taken and the washed the root system properly in tap water and extra water were wiping out by tissue paper. Root length was measured manually by ruler.

### *Leaf area and perimeter*

Leaves were taped on a black paper and scanned images were taken with Epson L220 scanner. Images were analyzed using Image J software (Version 1.46).

### *Pod length, Pod weight and Seeds per pod*

Pod length and weight measurement were done from 35-45 days old plant samples. Pods were taped on a black paper and scanned images were taken with Epson L220 scanner. Images were analyzed using Image J software (Version 1.46). Pod weight measurements were carried out using balance (from Sartorius S/N 0032905891, Germany).

### *Leaf number at bolting*

Leaves (both cauline leaf and rosette) were counted in 22-25-days-old plants. Data represented was a mean of about 15 independent measurements.

### *Biomass*

Biomass was determined from 45-50 days-old plants after 3 days of air drying after growth stage 6.90 flowering completed (Boyes *et al.*, 2001).

### *Chlorophyll and carotenoid estimation*

The total chlorophyll was quantified according to ( Kumar *et al.*, 2018). Briefly, about 10 mg of leaf from 22-days-old plants were taken and incubated with 1.25 ml of 80% acetone for 48 hrs at 4 °C in dark. Eppendorfs were centrifuged at 13000 rpm for 5 min and absorbance of the supernatant was measured at 645 nm and 663 nm and 480 nm.

Total chlorophyll (µg $mg^{-1}$)= 20.2 (A645) + 8.02 (A663)

Total Carotenoids (µg $mg^{-1}$)= C x+c= (1000A470 – 1.82Ca –85.02Cb) 198

### *Estimation of anthocyanin amounts*

The Anthocyanins was quantified according to Panigrahy ( 2004). About 10 mg of leaf from 22 days old plants were taken in 1.5 ml Eppendorf tube and added 300 µl anthocyanin extraction solution. Incubated the sample at 95 °C for 5 minutes. After cooling the samples kept for 24 h at 4 °C in dark. Eppendorf tubes were centrifuged at 13000 rpm for 5 min, followed by taking absorbance of supernatant at 535 nm and 650 nm. The data represented is mean of at least 15 measurements.

Anthocyanins(µg $ml^{-1}$)=A535-2.2 (A650)

***Soil nutrient analysis***

Soil nutrient analysis was performed at Orissa University of Agriculture and Technology, Bhubaneswar according to standard procedures (Singh *et al.*, 1999).

***Statistical analysis***

Pearson's' Correlation and significance test was performed on the Biomass values of each soil against each component of the soil analysis table using two-tailed t-test.

## RESULTS AND DISCUSSION

*Arabidopsis* plant growth was rigorously retarded on clay and red soil, where as it could reach the bolting stage on garden soil, soilrite and mixed soil figure 1. The plants on the garden soil showed less healthy which was accompanied by obvious small leaf size, thin stem, less number of leaves and anthocyanin accumulation on leaves. Plants grown on soilrite were comparatively equally healthy as those on mixed soil, however were having pale leaves. The plants on mixed soil showed best plant growth in terms of leaf number, greenness, leaf length and higher number of leaves at bolting stage.

To access the plant growth and healthy status, root length was measured from the plants grown on five different soil conditions Figure 2. Due to poor health condition, root length on clay and red soil could be taken from seedlings of maximum 15 days, whereas those from the other three soils were accessed at 25 days. Root growth was studied in both long day (LD) and short day (SD) to perform a thorough analysis in varied photoperiods. Root length was maximum in soil mix and minimum in red soil. Secondary roots and root hairs were higher in red soil in LD condition.

Leaf growth and area is a positive indicator of plant growth (Weraduwage *et al.*, 2015). In present study, leaf area and perimeter was accessed in the plants grown on garden soil, soilrite and mixed soil only Figure 3. As the seedlings on clay and red soil died after15 days, their leaf area and perimeter could not be included in this data. In all the 3 soil conditions tested, the leaf area and perimeter is higher in SD condition than in LD condition. Leaves from SD condition had longer petioles. Higher vegetative growth in SD condition is known in Arabidopsis (ABRC) as it is a long day plant.

**Table 1: Nutrient content and soil parameters in five different soil conditions**

| S.No. | Parameters | Red soil | Clay soil | Garden Soil | Soil rite | Mixed Soil |
|---|---|---|---|---|---|---|
| 1 | pH | 5.40 | 4.76 | 7.22 | 6.48 | 7.2 |
| 2 | EC (dSm-1) | 0.006 | 0.055 | 0.260 | 0.028 | 0.408 |
| 3 | OC (g/kg soil) | 4.72 | 7.98 | 3.45 | 11.7 | 2.24 |
| 4 | Avl. N (Kg/ha) | 189.0 | 164.0 | 428.0 | 0.30 | 208 |
| 5 | Avl. P (Kg/ha) | 5.85 | 5.28 | 147.0 | 0.04 | 57.4 |
| 6 | Avl. K (Kg/ha) | 143 | 180.0 | 176.0 | 0.03 | 295 |
| 7 | Avl. Ca (Kg/ha) | 1.5 | 8.04 | 13.9 | 0.49 | 3.18 |
| 8 | Avl. Mg (mg/kg) | 0.49 | 3.53 | 5.10 | 0.18 | 3.1 |
| 9 | Avl. Fe (mg/kg) | 7.76 | 97.4 | 6.44 | 0.09 | 13.20 |
| 10 | Avl. Mn (mg./kg) | 93.0 | 134.8 | 1.4 | 0.002 | 6.32 |
| 11 | Avl. Cu (mg./kg) | 0.99 | 3.08 | 9.19 | 1.9 | 3.18 |
| 12 | Avl. Zn (mg./kg) | 1.08 | 5.58 | 25.2 | 5.5 | 5.0 |
| 13 | Avl. B (mg/kg) | 4.3 | 2.6 | 10.2 | 0.01 | 0.50 |

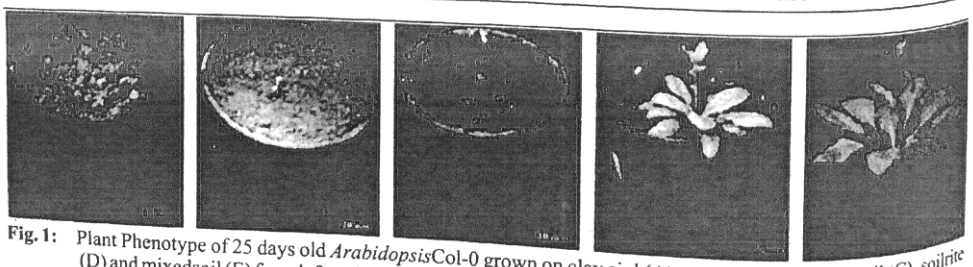

**Fig. 1:** Plant Phenotype of 25 days old *Arabidopsis*Col-0 grown on clay siol (A), red soil (B), garden soil (C), soilrite (D) and mixedsoil (E) from left to right respectively.

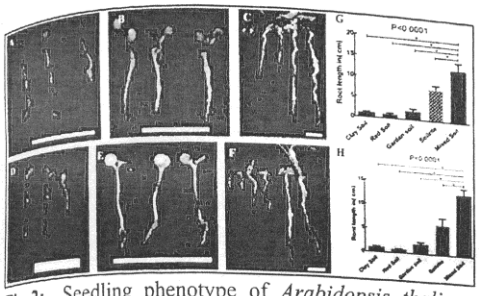

Fig. 2: Seedling phenotype of *Arabidopsis thaliana* Col-0 grown under Long Day (LD) (A-C and G) and Short Day (SD) growth conditions (D-G and H) on clay soil (A, D); Red soil (B, E) and Mixed soil (C, F) respectively. Observed maximum root length under LD (G), and SD growth conditions (H) respectively. Areal parts were excised from plants grown on mixed soil for clear root pictures (C and F).

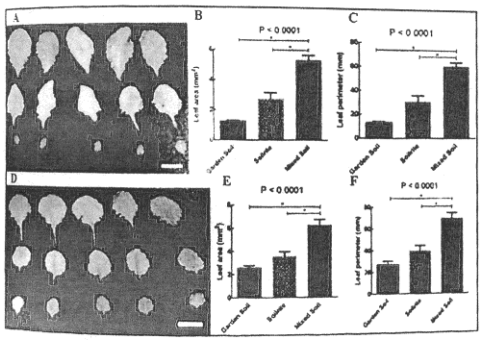

Fig. 3: Leaf shape of *Arabidopsis thaliana* Col-0 (A,D) plants grown in mixed soil (A and D: 1st row), soilrite (Aand D: 2nd row), garden soil (Aand D: 3rd row) in Long Day (LD) and Short Day (SD) growth conditions respectively.). Leaf area (B,E) and Leaf perimeter (C, F) in garden soil,soilrite and mixed soil underLong Day (LD) and Short Day (SD) growth condition respectively.

To access the photosynthetic potential and stress parameters, total chlorophyll, carotenoid and anthocyanin was quantified in the leaves from the plants grown in 5 different soil conditions Figure 4. In both LD and SD, total chlorophyll was highest in the plants grown on soil mix indicating better plant growth than other soil conditions tested. Carotenoids and anthocyanin are considered in this study as indicators of abiotic stress, since these are known to accumulate in different kind of abiotic stress conditions including nutrient deficiencies,

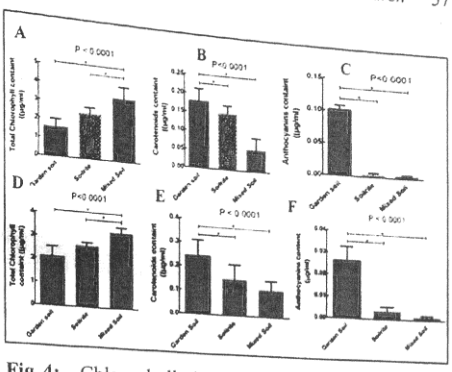

Fig. 4: Chlorophyll (A, D), Carotenoid (B,E) and Anthocyanin (C,F) of *Arabidopsis thaliana* Col-0 from leaf samples grown under Long Day (LD) (A, B and C) and Short Day (SD) (D, E and F) growth condition respectively.

toxicity and disease Ramakrishna *et al.* (2011). Carotenoid and anthocyanin in both LD and SD were highest in the plants grown on garden soil and least in mixed soil indicating that the plants grown on soil mix reflect a healthy and stress-free growth environment.

Leaf number at bolting represents the reproductive status of the plant Fornara *et al.*, (2009). Presently, leaf number at bolting was highest at 15 leaves in plants grown on mixed soil Figure 5. Whereas leaf number was similar in case of plants on garden soil and soilrite, plants

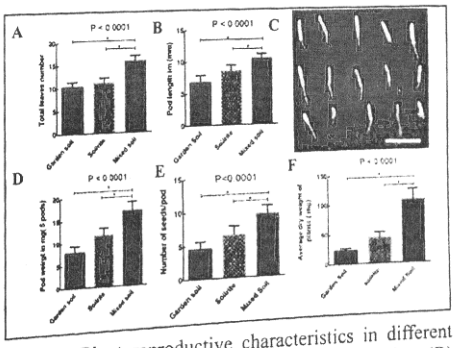

Fig. 5: Plant reproductive characteristics in different soil condition. (A) Total Leaves at bolting, (B) Pod length, (C) Pod weight, (D) Number of seeds per pod and (E) representative pod pictures in LD growth condition of *Arabidopsis thaliana* Col-0. Pod length of plants grown in mixed soil (C: 1st row), soilrite (C: 2nd row), garden soil (C: 3rd row)in Long Day (LD) growth conditions.

on clay and red soil died at 2 leaf stage. Seed pod length, weight and number of seeds per pod was significantly highest in the plants grown on mixed soil followed by soilrite and that from garden soil being the least.

The results from nutrient and soil parameter analysis presented following data. Clay soil sowed lowest pH indicating the most acidic soil. The pH of garden soil and soil mix was same at neutral pH. Optimum pH of soil for healthy plant growth ranges from 5.5 to 7.5 Hanlon (1993), whereas ABRC standard (ABRC) procedures recommend pH 5.7 for healthy *At* growth, which is close to the pH of red soil in present case. Electrical conductivity (EC) is a measure of salinity and each plant species has a specific threshold of soil EC for obtaining best yield Hanlon (1993). Richards (1954) defined four different ranges of EC and categorized different crops such as bean, barley, corn and wheat into sensitive, moderate and tolerant crops. Moreover, *At* root hair cells are affected by changes in the electrical properties of the plasma membrane Lew ( 2004). Mixed soil in present study showed highest EC indicative of highest movement of ions into plant. Among the five types of soil tested, organic content was highest in soilrite followed by clay, red soil, garden soil respectively and was least in the mixed soil. ABRC standards recommended 14%: 14%: 14% for the nitrogen (N): phoshorus (P): potash (K) amounts in the soil mix (ABRC). Nitrogen content was highest in garden soil followed by mixed soil. Phosphate content was found to be least in all soil, whereas all the N, P and K content was least in soilrite. K content was found highest in the soil mix. Soil EC correlates different soil properties including soil texture, exchangeable Calcium and magnesium ions, drainage conditions, organic matter and subsoil characteristics and water content Kadam (2016). Calcium and magnesium ions are comparable among the red soil and mixed soil, whereas highest in the garden soil. Soil properties has been highly influenced by micronutrient status which ultimately affects the plant health (Mandal *et al.*, 2015) Iron content has attained highest value in red soil, whereas the other micronutrients such as zinc, copper and boron content was highest in the soilrite among the 5 soils tested. The dry biomass and seeds / pod showed positive correlation with pH, EC, and K, iron and manganese content.

## CONCLUSION

The mixed soil designed in the present study at ratio of soilrite, red soil and garden soil in 5:4:1 proportion proved best for *Arabidopsis* plant growth with respect to vegetative growth and reproductive potential of the plants, as the plants grown on mixed soil had highest biomass, seeds per pod. Mixed soil grown plants showed least accumulation of secondary metabolites such as carotenoids and anthocyanin indicating better plant health condition. Plant growth on red soil was not possible after about 15 days possibly due to high iron content. The better plant growth on mixed soil could be due to favourable pH, high electric couductivity, high nitrogen, phoshorous and potash content. Though the OC content of the mixed soil was low, it could be efficiently taken up by the plant due to high EC content. The mixed soil designed in this study could be useful for Indian researchers for healthy *Arabidopsis* growth in plant growth chambers.

## ACKNOWLEDGEMENTS

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DOI: 10.1007/s12038-018-9826-6



# Carbohydrates and polyphenolics of extracts from genetically altered plant acts as catalysts for *in vitro* synthesis of silver nanoparticle

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Eco-friendly biosynthetic approach for silver nanoparticles production using plant extracts is an exciting advancement in bio-nanotechnology and has been successfully attempted in nearly 41 plant species. However, an established model plant system for systematically unraveling the biochemical components required for silver nanoparticles production is lacking. Here we used *Arabidopsis thaliana* as the model plant for silver nanoparticles biosynthesis *in vitro*. Employing biochemical, spectroscopic methods, selected mutants and over-expressor plants of *Arabidopsis* involved in pleotropic functions and sugar homeostasis, we show that carbohydrates, polyphenolics and glyco-proteins are essential components which stimulated silver nanoparticles synthesis. Using molecular genetics as a tool, our data enforces the requirement of sugar conjugated proteins as essentials for AgNPs synthesis over protein alone. Additionally, a comparative analysis of AgNPs synthesis using the aqueous extracts of some of the plant species found in a brackish water ecosystem (*Gracilaria, Potamogeton, Enteromorpha* and *Scendesmus*) were explored. Plant extract of *Potamogeton* showed the highest potential of nanoparticles production comparable to that of *Arabidopsis* among the species tested. Silver nanoparticles production in the model plant *Arabidopsis* not only opens up a possibility of using molecular genetics tool to understand the biochemical pathways and components in detail for its synthesis.

**Keywords.** *Arabidopsis*; carbohydrates; *Gracilaria*; *Potamogeton*; *Scendesmus*; silver nanoparticles

## 1. Introduction

Nanoparticles synthesis using plant extracts containing different phytochemical agents have drawn more attention than the existing physical, chemical and hybrid methods due to their adaptability in surgical, pharmaceutical purposes and reduced use of unsafe solvents that they offer (Abdel-Halim *et al*. 2011). 'Green nanotechnology' can replace the need of chemical synthesis and significantly reduce the amount of hazardous waste products (Singaravelu *et al*. 2007). The list of biological agents used in 'green' synthesis of nanoparticles (NPs) includes different organisms such as plants, algae, fungi, yeast, bacteria and their bioactive components (Sastry *et al*. 2003; Sharma *et al*. 2009; Kumar *et al*. 2012; Chung *et al*. 2016). The use of nanomaterial themselves as therapeutic and immune-modulatory agents is prevalent as seen in the application of silver NPs at surgical or ectopic infection sites as antibacterial agents (Morones *et al*. 2005; Tripathy *et al*. 2009). Silver as nanoparticles have received special attention due to their striking characteristics such as catalytic, anti-bacterial activity and chemical stability (Sharma *et al*. 2009). Plant extracts from more than about 41 species specifically from leaves have been extensively exploited for AgNPs synthesis (Shankar *et al*. 2003; Chandran *et al*. 2006; Chung *et al*. 2016). In most of the cases, AgNPs were synthesized from leaf extracts, while in few cases other plant parts such as stem, petals, rhizome, root, fruit, seed, latex, bark, whole plant and see-weeds were also used. The shapes of synthesized AgNPs ranged from circular, cubical, rectangle, optical, crystalline, poly-dispersed,

*Electronic supplementary material: The online version of this article (https://doi.org/10.1007/s12038-018-9826-6) contains supplementary material, which is available to authorized users.*

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## Ph.D. Thesis Evaluation Report

1. **Name of the Constituent Institution:** National Institute of Science Education and Research
2. **Name of the Student:** Abhishek Kumar
3. **Enrolment No.:** Life 11201004012
4. **Title of the Thesis:** Role of Splice Variant of CONSTANS and Carbon Nanoparticle in flowering time control in Arabidopsis thaliana
5. **Name of the Board of Studies:** Life Science

### Recommendations

Tick one of the following:

1. The thesis in its present form is commended for the award of the Ph.D. Degree. ☑
2. The thesis is commended for the award of the Ph.D. degree. However, my suggestions for improving the thesis may be considered at the time of the viva voce examination and if the viva voce board deems it appropriate, the same may be incorporated in the thesis based on the discussions during the viva voce examination. The revised thesis need not be sent to me. ☐
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Detailed report enclosed.

1. **Name of the Student: Abhishek Kumar**

2. **Title of the Thesis:** Role of splice variant of CONSTANS and Carbon Nanoparticles in flowering time control in *Arabidopsis thaliana*

## DETAILED REPORT

This thesis presents a piece of impressive work that advances the knowledge on the photoperiodic flowering time control using *Arabidopsis thaliana* as a model plant. Abhishek's work convincingly demonstrates the splice variant of CONSTANS (CO) play role in regulating flowering time and that carbon nano-particles accelerate flowering time. The work also demonstrates possible mechanism of their regulations and throws light on their diurnal and developmental regulation. Interestingly, the findings indicate a possible yet unidentified feed-back mechanism that might regulate the expression of clock-controlled component GIGANTEA by splice variant of CONSTANS (COβ). Moreover, the role of COβ in delaying flowering seems to have a photoperiod independent regulation on and above the photoperiodic control. At the same time the work uncovers that the action of COSV is light dependent but light quality independent. It also proposes a Phytochrome B independent control of flowering time, by passing the central CO-FT module. Overall, the thesis is well written, results are presented and discussed in organized manner, and hypothesis is logically explained.

Despite it is an excellent piece of work, I have few minor concerns on the thesis as follows :

Scientific:

1. Page 52 – Result section =Table 4.1: It seems there is a typographical error in case of leaf numbers (total and in phyB9 plant); same in page 54 –Result Section- Table 4.2P – leaf number of pSUC2::COβCol0 plant and control.
2. Page 62 - Although not in the purview of this work one can find out the 'gi' transcript level in COβ over expressing plants?

Non-scientific:

3. Minor mistakes for example –
   Page 4 – under 1.5 – 'In over expression of.....in the dark': rewrite the sentence,
   Page 8- fist line – delete and before increased, 3rd line - underline P, I and F (PIF 4),
   Page 11 – last line – 'Thus will provide.....soil composition': looks like repetition,
   Page 14 – under 2.1.3 –gigantea-100 : spelling,
   Page 16 – under 2.1.17 –heading – Equipment used,
   Page 18 – under 2.2.1a – .....primers for COSV amplification may be indicated,
   Page 21 – first sentence – Single colony was .... Rewrite
   Page 27- 2.9.3, 2.9.3 and in 2.9.4: delete extra S in Measurement, Hypocotyl and angle
   Page 37 – heading of Chapter 3 0: 'Exhibit' would be better than 'behave'.
   Page 38 – under 3.1 –Heading change to: 'Mixed soil offers better growth of Arabidopsis'
   Page 73- Figure 5.1 – first line – put a , after A.

The corrections of error are minor and therefore, may also be taken care while publishing the work.

The candidate may be asked following questions during viva voce -

(1) COβ over expression lead to more greening- so is it possible that cytokinin production or signaling is altered?

(2) Can CNP be used for changing flowering time in crops?

Name of Examiner: Dr Ananda K. Sarkar

Signature and Date: 12/03/2019

Dr. Ananda K. Sarkar
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5. **Name of the Board of Studies:** Life Sciences

## Recommendations

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3. The thesis should be revised as per the suggestions enclosed. I would like to see the revised thesis incorporating my suggestions before I give further recommendations. ☐
4. The thesis is not acceptable for the award of the Ph.D. degree. ☐

Date: 10th March

(Signature):

Name of Examiner: Prof. RP Sharma
And affiliation Repository of Tomato Genomics Resources
Dept of Plant Sciences
University of Hyderabad
Hyderabad-500 046
India

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Version approved during the meeting of Deans held during 29-30 Nov 2013

1. **Name of the Student:** Abhishek Kumar

2. **Title of the Thesis:** Role of splice variant of CONSTANS and CarbonNanoparticles in flowering time control in Arabidopsis thaliana

## DETAILED REPORT

The signed detailed report is attached at next page.

Name of Examiner: Prof RP Sharma

Signature and Date:

10th March 2019

Version approved during the meeting of Deans held during 29-30 Nov 2013

**PhD Thesis Report**

**Name of the Candidate:** Abhishek Kumar
NISER

**Title of the thesis:** Role of splice variant of CONSTANS and Carbon Nanoparticles in flowering time control in Arabidopsis thaliana

The flowering is the most fascinating aspect of plant development. The molecular mechanism underlying the flowering have evolved from initial observations of discovery of photoperiodism in plants, prediction of a flowering regulating mobile element- florigen and final discovery of molecule that is potential candidate for florigen- FT protein in Arabidopsis. However, the formation of FT protein in Arabidopsis is modulated by a complex regulation, wherein among many component CONSTANS (CO) plays a major role. In current thesis, the candidate has attempted to decipher the role of its splice variant and also influence of carbon nanoparticles on flowering time.

The first chapter though unusual but reports standardization of a soil formulation for Arabidopsis growth. Several parameters were examined and evaluated along with phenotype changes in plants. The candidate finally achieved a formulation that appeared to be promising for the reproductive growth of the plants. However, the underlying fact is that the flowering behavior of Arabidopsis in nature including molecular regulation is very different than in the controlled conditions in the laboratory. Therefore, there is still a wide gap in understanding flowering behaviors of plants in nature compared to the growth rooms.

The candidate next examined the role of splice variant of CONSTANS (COβ) by generating a COβ overexpression line. This line had different phenotype such as more seeds. Since the candidate observed higher level of transcript of COβ in a *gigantea* mutant, it appears that GIGANTEA is needed for the proper splicing of COβ in Arabidopsis, which influences its photoperiodic behavior.

The candidate then followed his studies with influence of carbon nanoparticles (CNPs) in COβ over-expressing and in Col-0 ecotype. Interestingly, a concentration dependent increase in bolting was observed with CNPs. The treatment advanced bolting in both Col-0 ecotype and 35S:Coβ over-expressing lines. The treatment of CNPs also influenced the seedling phenotype with longer hypocotyls in light. In seedlings while expression of PHYB was down-regulated by CNPs, it up-regulated GIGANTEA and LFY transcripts in adult plants. I have reservation about usage of 500 µg/ml charcoal as control. The observed phenotype in charcoal-treated plants could be due to strong sequestration of minerals and other molecules in the medium.

The final chapter deals with the relationship between over-expression of COβ and silver nanoparticles synthesis. Several plants were examined for nanoparticle synthesis. It is hard to pinpoint a mechanism how plants do that, but irrespective of mechanism, it is an interesting area of research for future.

Though the research work presented by Abhishek Kumar is interesting and advances information about flowering. The overall presentation of the work in thesis is rather poor with several mistakes in the language and the presentation of figures and graphs. I recommend that the candidate revise the thesis and get it certified from supervisor for clarity and consistency in the language and the presentation before holding the viva voce examination.

I recommend the award of Ph.D. Degree to Abhishek Kumar after satisfactory completion of *Viva-Voce*.

Prof. RP Sharma
Repository of Tomato Genomics Resources
Dept of Plant Sciences
University of Hyderabad

# Homi Bhabha National Institute

## Ph.D. Thesis Evaluation Report

1. **Name of the Constituent Institution:** National Institute of Science Education and Research Bhubaneswar
2. **Name of the Student:** ABHISHEK KUMAR
3. **Enrolment No.:** LIFE11201004012
4. **Title of the Thesis:** Role of splice variant of CONSTANS and Carbon nanoparticles in flowering time control in Arabidopsis thaliana
5. **Name of the Board of Studies:** LIFE SCIENCES

### Recommendations

Tick one of the following:

1. The thesis in its present form is commended for the award of the Ph.D. Degree. ☑
2. The thesis is commended for the award of the Ph.D. degree. However, my suggestions for improving the thesis may be considered at the time of the viva voce examination and if the viva voce board deems it appropriate, the same may be incorporated in the thesis based on the discussions during the viva voce examination. The revised thesis need not be sent to me. ☐
3. The thesis should be revised as per the suggestions enclosed. I would like to see the revised thesis incorporating my suggestions before I give further recommendations. ☐
4. The thesis is not acceptable for the award of the Ph.D. degree. ☐

Date: 2.4.19,

(Signature):

Name of Examiner:
And affiliation

**Please give your detailed report in the attached sheet. You may use additional sheets, if required.**

Version approved during the meeting of Deans held during 29-30 Nov 2013

1. **Name of the Student:** ABHISHEK KUMAR

2. **Title of the Thesis:** Role of splice variant of CONSTANS and Carbon nanoparticles in flowering time control in Arabidopsis thaliana.

**DETAILED REPORT**

The thesis in its present form can be excepted for the award after all corrections are incorporated.

2.10.19.

Name of Examiner:

Signature and Date:

Version approved during the meeting of Deans held during 29-30 Nov 2013

# Homi Bhabha National Institute

## Evaluation Report[1] of Ph.D. Viva-Voce

## Board of Studies in LIFE Sciences

**1.Name of the Constituent Institution:** NATIONAL INSTITUTE OF SCIENCE EDUCATION AND RESEARCH, BHUBANESWAR

**2. Name of the Student: ABHISHEK KUMAR**

**3. Enrolment Number: LIFE 11201004012**

**4. Date of Enrolment in HBNI: 26-12-2010**

**5. Date of Submission of Thesis: 18-05-2018**

**6. Title of the Thesis: ROLE OF SPLICE VARIANT OF CONSTANS AND CARBON NANOPARTICLES IN FLOWERING TIME CONTROL IN ARABIDOPSIS THALIANA**

**7.Number of Doctoral Committee Meetings held with respective dates:**

| Review Period | Date | Review Period | Date |
|---|---|---|---|
| 1. 2011-2012 | 20-12-2012 | 2. 2012-2013 | 23-12-2013 |
| 3. 2013-2014 | 19-12-2012 | 4. 2014-2015 | 21-12-2013 |
| 5. 2015-2016 | 22-03-2016 | 6. 2016-2017 | 21-04-2017 |

**8. Name and Affiliation of the Examiner 1:**
Dr.Ananda Sarkar
Staff Scientist IV
National Institute of Plant Genome Research

**Recommendations of the Examiner 1 (Thesis Evaluation) (i) accepted, (ii) accepted after revisions, or (iii) rejected:** ✓

**9. Name and Affiliation of the Examiner 2:**
Prof. R. P Sharma
Repository of Tomato Genomics Resources
Dept. of Plant Sciences, University of Hyderabad

**Recommendations of the Examiner 2 (Thesis Evaluation) (i) accepted, (ii) accepted after revisions, or (iii) rejected:** ✓

[1] This is to be submitted by Dean-Academic to Central office and is not to be included in the thesis.

## Recommendations of the Viva-Voce Board

1. **Date of Viva Voce Examination: 29.03.19**

2. **Recommendations for the award of the Ph.D. degree: Recommended / Not Recommended**

   (If Recommended, give summary of main findings and overall quality of thesis)
   (If Not Recommended, give reasons for not recommending and guidelines to be communicated by Convener of the Doctoral committee to the student for further work)

   (1) The thesis describes a new component that possibly work post-transcriptionally to control photoperiodic flowering.

   (2) one another major finding is to assign a role of Carbon nano particles in inducing early flowering.

   (3) overall quality of the thesis is satisfactory.

**In case, Not Recommended, another date will be fixed by the Dean-Academic, CI, which shall not be earlier than a month after and not later than six months from the date of first viva.**

3. **Name and Signature of the Viva Voce Board (Doctoral Committee & External Examiner):**

| Sr No | Composition | Name | Signature with date |
|---|---|---|---|
| a. | **Chairman** | **Dr.Chandan Goswami** | Chandan Goswami 29/3/19 |
| **b.** | **Convener** (Guide) | **Dr.Kishore C S Panigrahi** | 29/3/19 |
| c. | **External Examiner** | **Dr.Ananda Sarkar** | 29/03/19 |
| d. | **Member** | **Prof.A.B.Das** | 29/03/2019 |
| f. | **Member** | **Dr.Praful Singru** | 29.3.19 |
| g. | **Member** | **Dr.Pratap Sahoo** | 29/03/2019 |
| | | | |

**Dean-Academic, CI**