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Second Cycle Degree (MSc)

In Biotechnologies for food sciences

"Elucidating the role of a basic helix loop helix (bHLH) transcription factor (TF) during bud break in hybrid aspen"

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Contents

Riassunto

Le piante sviluppano meccanismi che le mantengono sincronizzate con l'ambiente e si adattano ai cambiamenti ambientali durante l'anno. Negli alberi perenni, l'adattamento si manifesta nel ciclo di crescita e dormienza degli alberi; eventi come l'arresto della crescita, l'allegagione e l'uscita dalla dormienza delle gemme sono processi che avvengono a seconda delle stagioni. Gli spunti che innescano le risposte genetiche e i processi che si occuperanno dell'adattamento sono la temperatura e il fotoperiodo, soprattutto l'ultimo, per la sua affidabilità rispetto ai continui cambiamenti di temperatura ogni anno. Per questo motivo, i cambiamenti di fotoperiodo guidano la fenologia del pioppo, in particolare i fattori di trascrizione che controllano il tempo di fioritura, come la famiglia di proteine basic helix loop helix (bHLH), in particolare il gene CIB1, che in Arabidopsis ha dimostrato di svolgere un ruolo importante nel promuovere la trascrizione dell'iniziazione floreale dipendente da CRY2. Il modo in cui questi fattori abiotici controllano la rete di segnalazioni molecolari è stato ampiamente studiato in piante modello come l'Arabidopsis, mentre in specie come il Pioppo è poco conosciuto.

In questo progetto di ricerca l'obiettivo è stato quello di chiarire il fattore di trascrizione CIB nell'ibrido T89 (T89CIB) di Aspen (Populus tremula x Populus tremuloides). Lo scopo principale è stato definire la localizzazione subcellulare della proteina nella cellula, la regolazione della proteina in condizioni di luce blu e la generazione di linee di complementazione di Arabidopsis per lo studio dell'interazione delle proteine con T89CIB. Per raggiungere questi obiettivi, sono stati generati due costrutti pGWB6 (35S::GFP T89 CIB, (B) 35S::GFPAtCIB1), che sono stati utilizzati per le applicazioni a valle. La localizzazione subcellulare della proteina T89 CIB è stata ottenuta mediante trasformazione con Agrobacterium e trasformazione transiente di N. Benthamiana mediante Agro-infiltrazione , seguita da analisi in microscopia confocale che hanno evidenziato la localizzazione nucleare di T89CIB. Inoltre, per comprendere la regolazione di T89CIB in condizioni di luce blu, è stata eseguita un'analisi SDS PAGE e Western blot su foglie di N. Benthamiana infiltrate con i costrutti e poste in diverse condizioni di luce (buio e 20 µmol.m^{-2}. s^{-2} for per 20 minuti). Questo saggio ha mostrato la regolazione positiva della proteina alla luce blu e la sua assenza al buio. Inoltre, per avere una prima visione dell'interazione tra il fattore di trascrizione T89CIB e il criptocromo 2, è stata eseguita una trasformazione mediata da Agrobacterium di T89CIB per la complementazione di linee di cry2 e sovraespressione in Col-o in Arabidopsis.

Abstract

Plants develop mechanisms to keep them synchronized to the environment and adapt to the environmental changes throughout the year. In perennial trees, adaptation is shown in the cycle of growth and dormancy of the trees, events such as growth cessation, bud set and bud break are processes happening depending on the seasons. The cues that trigger the genetic trigger and processes which will deal with the adaptation are temperature and photoperiods, especially the latest one, due to its reliability compared to the continue changes in Temperature every year. For this reason, photoperiods changes drive the phenology in poplar, especially transcription factors controlling flowering time like the protein family basic helix loop helix (bHLH) especially CIB1 gene which has been show in Arabidopsis to play an important role in promoting CRY2-dependent floral initiation transcription. How these abiotic factors control the network of molecular signalings have been widely study in model plants such Arabidopsis, while in species such as Poplar is poorly understood.

In this research project the aim was to elucidate the basic helix loop helix transcription factor CIB in T89 hybrid Aspen. The main purpose was to subcellular localized the protein in the cell, the regulation of the protein under blue light and the generation of Arabidopsis complementation lines for the study of interaction of proteins with T89CIB. To achieve these objectives, two pGWB6 contructs (35S::GFP T89 CIB, (B) 35S::GFPAtCIB1) were clone, which were used for downstream applications. The subcellular localization of the protein T89 CIB was achieved by Agrobacterium transformation and Transient Transformation of *N. Benthamiana* by Agro-Infiltration followed by analysis in Scanning confocal microscopy which result in the nuclear localization of T89CIB. Furthermore, to understand the regulation of T89CIB under blue light a SDS PAGE and Western blot analysis was performed to *N. Benthamiana* leaves that were Agro infiltrated with the constructs, plants infiltrated were put under different light conditions (darkness and 20 µmol.m^{-2}. s^{-2} for 20 minutes) with a Photon System Instruments SL 3500-R-D, this assay showed the positive regulation of the protein to blue light and the absence under darkness. Moreover, to have the first insight into the interaction between the Transcription factor T89CIB and the Cryptochrome 2, T89CIB Agrobacterium-mediated transformation on cry2 and Col-o complementation lines in Arabidopsis were performed.

1. Introduction

1.1 Ecology of Poplar

Poplar (genus: *Populus sp*.) is part of the willow family (*Salicaceae*), divided into three groups: cottonwoods, balsam poplars, and aspens. They are distributed throughout the northern temperate and boreal regions, extending from Euroasia through North America and northern Africa (Brunner et al., 2004). There are 35 species of poplar, classified into six sections. Poplars are dioecious, with wind-pollinated flowers. The flowers bloom in drooping catkins, and seeds mature as leaves develop; this characteristic facilitates wind pollination (Strobawa, 2014). Moreover, its flowers appear before its leaves (heart-shaped with toothed margins) do.

1.2 Common Species

Two of the most well-known species are the European aspen or common aspen (*Populus tremula*) and the trembling aspen (*Populus tremuloides*); both share similarities, such as a height of about 30 meters and narrow trunks (Pakull et al., 2009). We can differentiate them due to their physiology: there are marked differences in their leaf shapes. For instance, in *P. tremuloides*, one can find small teeth along the margins of its leaves, light-colored bark, and flattened petioles (Jelinski & Cheliak, 1992). On the other hand, the leaf edges of *P. tremula* have fewer and bigger teeth, making the leaf shape different (Luquez et al., 2008).

The aforementioned specie *P. tremula* is mainly used for their wood but over the past few years have been a matter of study due to its fundamental role in bioenergy production. This species has been chosen for being a fast-growing temperate tree and for its resources, like the whole genome sequence which facilitates research studies on the plant(An et al., 2021).

1.3 Transgenic Poplar

T89 hybrid aspen is a well-known species used for research due to its characteristics, such as height, leaf development, bud burst, diameter, and volume growth; for example, it is estimated that its annual growth rate in height is 4.2cm per 7 days, while the rate in wild-type *P. tremula* is 2.4 cm (Q. Yu et al., 2001)

The main species used in the transformation facility at Umea Plant Science Center (UPSC) is called T89. This clone is a hybrid aspen between *P. tremula x P. tremuloides*, originating in the Czech Republic (UPSC, 2022). The method used for plant transformation is mediated by Agrobacterium infection of leaf explants, petioles, and stem segments. It takes around eight to ten months for the trees to be transferred to the greenhouse. The pipeline starts with the transformation and continues by selecting plates, growing the callus, growing the shoots, and selecting the shoots to get the individual lines (UPSC, 2022).

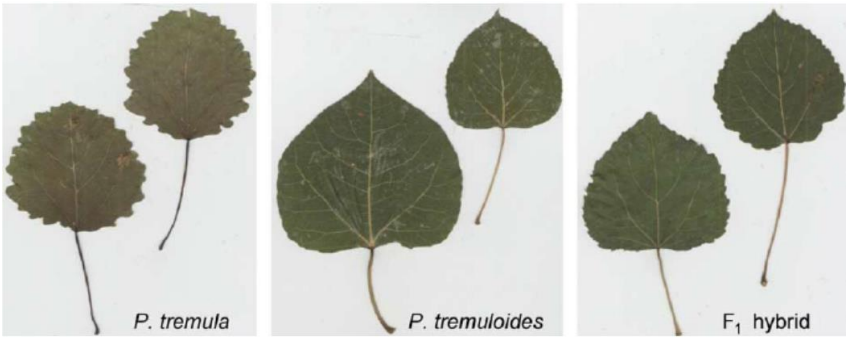

Figure 1. Leaves shapes of different species of *Populus*. From left to right: *P. tremula*, *P. tremuloides*, and the F_1 hybrid (T89 hybrid aspen) (Tullus et al., 2012).

1.4 Environment Drives in Poplar Phenology

Aspen trees growing in taiga forests experience annual shifts in temperatures ranging from -60°C in the winter to as high as 40°C in the summer; these trees have developed mechanisms to coordinate the growth patterns with the different weather and day-length conditions throughout the year (van Cleve et al., 1991).

Due to their geographic location, the environmental cues that determine the tree's flowering phenology and developmental processes are temperature and photoperiods(Huang et al., 2020; Wang et al., 2020). In the boreal regions, due to the Earth's rotation around the sun, the photoperiod varies throughout the year, which means an increase in light during summer and reduced hours of sunlight in winter(Wareing, 1956). This cue is regular from year to year, turning it into a perfect signal for the tree to sense and adapt to a variation in the climate (Wang et al., 2020).

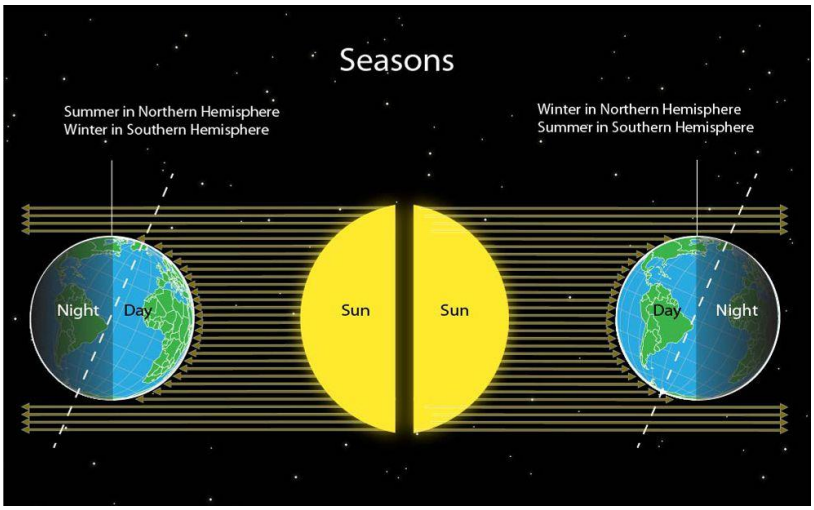

Figure 2. Photoperiod throughout the different seasons of the year in the two hemispheres. Image source: nationalgeographic.org

Plants develop processes that keep them synchronized to the environment and sense climate changes; studying these processes is essential due to the changing timing of recurring biological events caused by climate warming(Huang et al., 2020). The seasonal cycle of growth and dormancy in aspen is an example of this adaptation; events such as growth cessation, bud set, bud dormancy, bud flush, and development happen during environmental adaptation(Azeez & Sane, 2015).

The genetic network and processes for the adaptation in poplar start when the photoperiod falls below the critical day length; it begins with growth cessation, builds up until bud set, and the acquiring of cold hardiness. At this stage of dormancy switch, the circadian clock, light signaling, and orthologs of *Arabidopsis* flowering time regulators connect gene expression with environmental signals(Downs & Borthwick, 1956).

1.5 Adaptation Stages of Poplar to Daylight

The stages the poplar tree goes through to adapt when the day length is reduced are as follows (Figure 3):

- Bud set
- Cold disruption of the circadian clock
- Dormancy establishment
- Dormancy release
- Bud burst

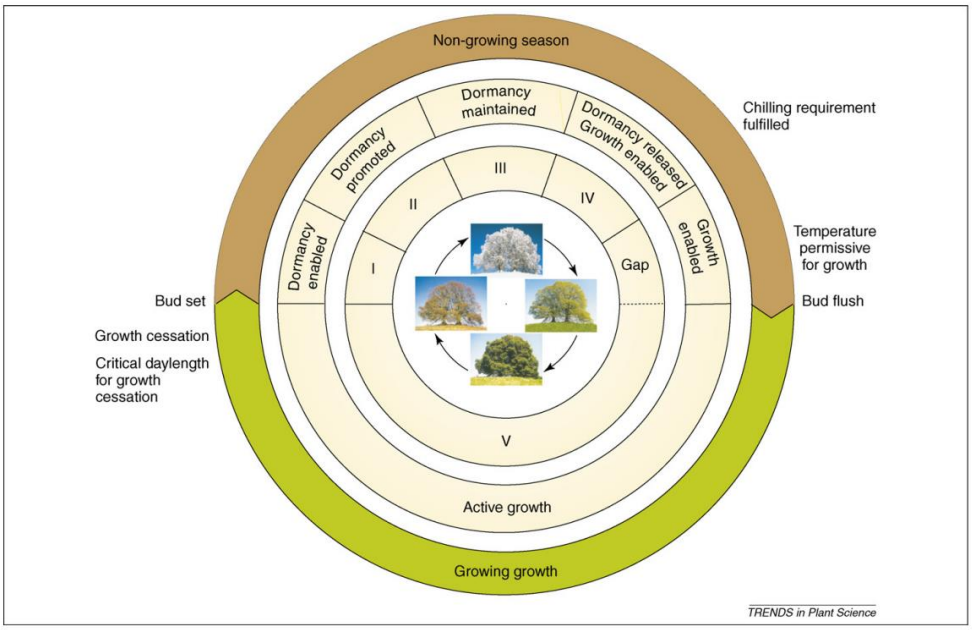

Figure 3. Plant dormancy is affected by day length in the perennial context (Rohde & Bhalerao, 2007)

Dormancy is defined as "the inability to initiate growth from meristems and other organs and cells with the capacity to resume growth under favorable conditions" (Samish, 1954). This proposed definition considers four aspects. First, dormancy is only established in meristems, shoot, or cambial apical but not physical dormancy. Second, it encompasses dormant axillary buds due to an inhibiting organ covering them. The third aspect is the ability of the tree to avoid growth even if the growth capacity is restored in the later stages of winter. Last, the definition considers dormancy as quantitative, in which molecular and physiological changes occur in the structures formed during the dormancy stage.

The stages that the plant goes through from growth cessation to dormancy release are as follows:

- **Growth cessation**
  The first step in establishing dormancy is aroused by environmental cues such as temperatures or day length. The first signs of changes in these cues emit a signal to the leaves, which will transmit to the apex to evoke inactivity of growth(Petterle et al., 2013).

- **Dormancy establishment**
  Once growth ceases, the dormancy establishment begins progressively until it reaches the incapacity of the meristem cells to activate and respond to growth promoters. Between these

two processes, the bud is formed essentially for survival in harsh conditions (Petterle et al., 2013).

- **Dormancy release**
  The release of this state requires long-term exposure to chilling temperatures; this condition restores the ability to grow and must not promote it. This process takes place after the end of cell division; no cell division can be found in the apical meristem of buds (Petterle et al., 2013).

1.6 Photoperiod and temperature mediate phenology control in trees

The control of developmental transitions that occur in trees has been an essential research topic. To understand the molecular basis of developmental processes, it is better to go back to the basics of the model plant *Arabidopsis*, since this plant has been widely studied and most of the molecular pathways have been unveiled. In *Arabidopsis,* the flowering process is controlled mainly by the photoperiodic machinery known as the circadian clock; this endogenous regulator is entrained by 24h clock and photoperiodic cues(Kinmonth-Schultz et al., 2021). This "clock" controls endogenous rhythmic processes, such as perfectly timing gene expression and regulating responses when there are photoperiodic signals. The principal components of the central oscillators in *Arabidopsis* are *PPR1/Timing of cab2 expression 1 (TOC1), Circadian clock associated1 (CCA1), Late elongated hypocotyl (LHY)*, as well as photoreceptors such as the blue light receptor cryptochrome *(CRY)* and red-light photoreceptor phytochrome A *(PHYA)* (Cervela-Cardona et al., 2021; Ponnu & Hoecker, 2022). Even if their primary purpose is providing energy via photosynthesis, they are also vital in growth and plant development (Ponnu & Hoecker, 2022). For example, the mechanism by which *PHY* controls gene expression in response to light is mediated by encoding an apoprotein post-transcriptionally converted to the holoprotein by a covalent attachment of a linear tetrapyrrole pigment (Su et al., 2015). Phytochrome (inactive Pr) is transformed into a Pfr state via photoisomerization; this state acts in the cytosol and translocates to the nucleus, which opens the cytosolic calcium channels, this, in turn, releases cyclic guanosine monophosphate (cGMP), which is in charge of activating the transcription factors, which will control the gene expression (Su et al., 2015). Another example is cryptochrome; this photoreceptor has holoproteins containing chromophores (FAD and MTFH) to perceive light that responds primarily to a blue wavelength (400–500 nm), although some studies have shown it responding to UVA light (315–400 nm) and being sensitive to green light (520–560 nm)(Ponnu & Hoecker, 2022). Due to their importance in de-etiolation, plant development, and flowering, both photoreceptors have been widely studied in *Arabidopsis.* Meanwhile, poplar studies have arisen showing phy and cry targets of Late elongated hypocotyl 1 (LHY1) and 2 (LHY2), responsible for encoding putative transcription factors involved in circadian rhythm (Mizoguchi et al., 2002).

Besides the two photoreceptors mentioned above, the ZEITLUPE/ADAGIO (ZTL/ADO1) family responds to blue and UVA light; their structure consists of a light-oxygen-voltage-sending domain (LOV) used to sense environmental conditions. Inside the domain, a prosthetic group is found containing oxidized FMN. This family of proteins' role is to regulate the speed of the circadian oscillator, sense photoperiods, and de-etiolation processes (Kim et al., 2007).

Many studies (Franklin, 2003; Sullivan & Deng, 2003a; Swift et al., 2022) have been performed on *Arabidopsis,* unraveling the molecular mechanisms used by the plant to sense environmental conditions. The goal in the near future is to elucidate these pathways in tree plants, such as poplar.

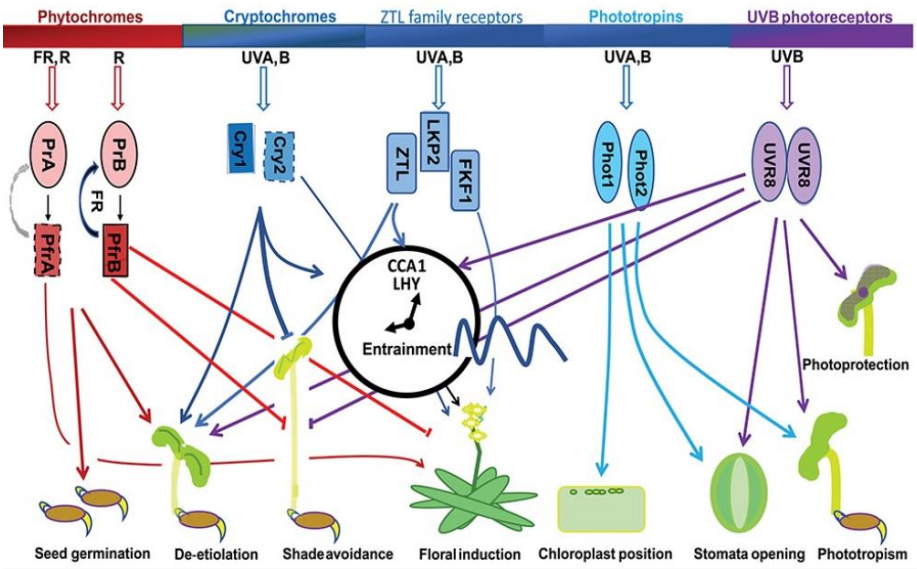

Figure 4. The role of photoreceptors in *Arabidopsis* development (Sullivan & Deng, 2003).

The photoperiodic changes in the environment drive the phenology in poplar; exposure to short days (SDs) before the winter is a crucial step for the tree to survive; it works as an acclimation period in which adaptative pathways are activated (Triozzi et al., 2018). This was proven by working with poplar overexpression lines of photoperiod-insensitive oat (*PHYA)*. Further evidence proved the alteration of the transcription of specific genes when exposed to a reduction in light hours, circadian clock, and light signaling-regulated genes (Maurya & Bhalerao, 2017). An example of induced genes in such conditions are *Dehydrin (DHN), Cold regulated genes (COR),* and *Late embryogenesis-abundant (LEA).* Furthermore, as mentioned before, thanks to the studies Click or tap here to enter text.performed in annuals (Triozzi et al., 2018), particularly in *Arabidopsis,* some orthologs in trees have been found to play similar and critical roles in photoperiodic control in poplar. Besides *PHYA*, the poplar ortholog of *Arabidopsis* Flowering Locus T (*FT*) has played an essential role in understanding the influence of photoperiod in growth cessation. SD signals are perceived and targeted by *FT2, and* most importantly, it has been shown (Hsu et al., 2012; Li et al., 2020)that *FT* in hybrid aspen is regulated upstream in a similar way to *Arabidopsis* by Constans (*CO*), which is highly expressed during LDs due to its expression occurring mainly in the dark. For this reason, when there is a variation in the day length from more to fewer hours of light reaching the critical day length, *CO* protein cannot maintain *FT*, causing inhibition of growth (Hsu et al., 2012).

Regulation following exposure to SDs is regulated upstream by CO. As mentioned before, this is not the only regulation component; the *CO/FT* module is also regulated downstream by genes downregulated during SDs, targeting *FT2* (Böhlenius et al., 2006). The genes involved in this network are *Aintegumenta-like (AIL1),* whose action is not directed to *FT2;* it is necessary for the intervention of the *Apetala1 (AP1)* as a linker between *AIL1* and *FT* to target *FT2,* inducing growth cessation (Krizek, 2011). As we can observe in the mechanisms, the transcriptional network for the photoperiodic control of growth in poplar is being elucidated; *Arabidopsis* and model plants are fundamental due to the orthologous genes and evolution of pathways by controlling the same processes in plants and trees (Krizek, 2011).

Daylength variations do not only trigger an increase in gene expression. Still, it also stimulates important phytohormones such as Ethylene and Abscisic acid (ABA) involved in the regulation of bud set development, and it plays an essential role in the adaptive responses of the plant (D. Yu et al., 2019).

Photoperiod has drawn attention as the primary environmental cue affecting growth cessation and bud set. Therefore, it has been intensively studied at the molecular level. In the past years, another variable appeared as a modulator, correlating with the timing of growth cessation and bud set; the first approach to test whether there is a relationship was through a study (Osnato et al., 2022) that compared the timing in trees sampled in 12 locations in Sweden along a latitudinal cline of 55.9-66.0 N, in which the sample was put in a growth chamber with a constant temperature of 20°C and hours of light that were reduced by 1 hour per week. The results obtained indicated a non-effect of temperature (T) in bud set phenology. This might suggest that temperature has little to no effect, but other studies (Rohde et al., 2011) have shown the impact of a low T in bud formation; they indicate the influence that it has upon modulating the response to photoperiodical growth cessation and the rate at which bud set is formed.

Temperature is an essential modulator of the bud set; it is a thermosensitive process in the tree. The climate affects when this process happens; it has been shown that poplar is highly sensitive to small changes in temperature; the lower the temperature, the more delay to the bud set. It has been discovered that 134 genes are associated with diurnal transcription patterns in control conditions (Day 25°C/ Night 12°C); in this pool, an ortholog of *Arabidopsis* genes involved in responses to decreasing temperatures, freezing tolerances, phospholipid metabolism, and stomatal closure was expressed (Rohde et al., 2011). Examples of these genes are *Phospholipase D delta (PLDDELTA)* (Q. Liu et al., 2010)*, C-repeat-binding factor 4 (CBF4)* (Tian et al., 2017)*, and Circadian rhythm and RNA binding 1 (CCR1)* (Tamasloukht et al., 2011). These examples indicate the correlation between nighttime temperature decrease and the bud set.

1.7 Dormancy Release and Bud Break

Once dormancy is established, all the growth-promotive signals are shut down by the plants. As soon as the environmental conditions start to change, the dormancy state must be released; this process is carried out by prolonged exposure to low temperatures during the last weeks of winter(Liao et al., 2021). This release of dormancy enables the reactivation of growth in the plant, manifested by bud break(Busov, 2019).

The molecular mechanisms involved in both processes still need to be elucidated, although dormancy release and bud break are intimately linked(Lloret et al., 2018). To understand the regulation of these pathways, studies (Rohde et al., 2007; Zawaski & Busov, 2014) based on transcriptomics and physiology have been performed. For example in Rohde et al., 2007, it has been shown that gibberellic acid (GA) promotes bud break, while the contrary occurs with ABA. The endogenous mechanism in which these hormonal pathways are involved in bud break regulation occurs because they are downstream targets of the *short vegetative phase-like (SVL)* transcription factor. The *SVL* MADS-box transcription factor has been shown to participate in bud break control; it was discovered by screening hybrid aspen lines overexpressing transcription factor genes involved in bud break; poplar *SVL* had a significant effect on bud break delay, confirming the negative regulatory role played by *SVL* on bud break. Lines reducing the expression of *SVL* were generated, resulting in an early bud break phenotype, further confirming the overexpression data. Furthermore, the expression of the gene is regulated by temperature signals, especially under low temperatures where it is downregulated. Therefore, as mentioned earlier, ABA induces *SVL* expression, acting as a positive regulator of *SVL* expression. Interestingly, thanks to the study of transcriptional regulation of ABA biosynthesis and response machinery during bud break in different lines, it was discovered that ABA and *SVL* work in a sort of feedback loop since *SVL* positively regulates ABA biosynthesis and signaling-related genes, while enhancing GA metabolism, therefore, blocking growth (Busov, 2019; Maurya et al., 2018).

Moreover, to understand the regulatory mechanisms, a gene that regulates the timing of bud break has been identified and characterized: *Early Bud Break 1 (EBB1)* (Yordanov et al., 2014) was discovered and isolated from mutant poplar (*ebb1D*), showing accelerated bud break. To reiterate the effect of *EBB1* in bud break, overexpression lines (*EBB1*-oe) were formed, and the results obtained showed no visible phenotype in growth cessation and bud set; instead, a precocious bud break flushed faster than the wild-type trees (Yordanov et al., 2014). Furthermore, changes in the anatomy of the transformed lines were observed. First, embryonic leaves, as well as bud scales, seemed to be thicker at the base, while the bud adopted a more oval figure, leaving the meristem in a more exposed shape. *EBB1* encodes a transcription factor of the APETALA2/Ethylene responsive transcription factor family, which suggests a protagonist role as a positive regulator of bud break and in the reactivation of meristem activity after dormancy, due to the role APETALA 2 transcription factor has in the plant. (Yordanov et al., 2014).

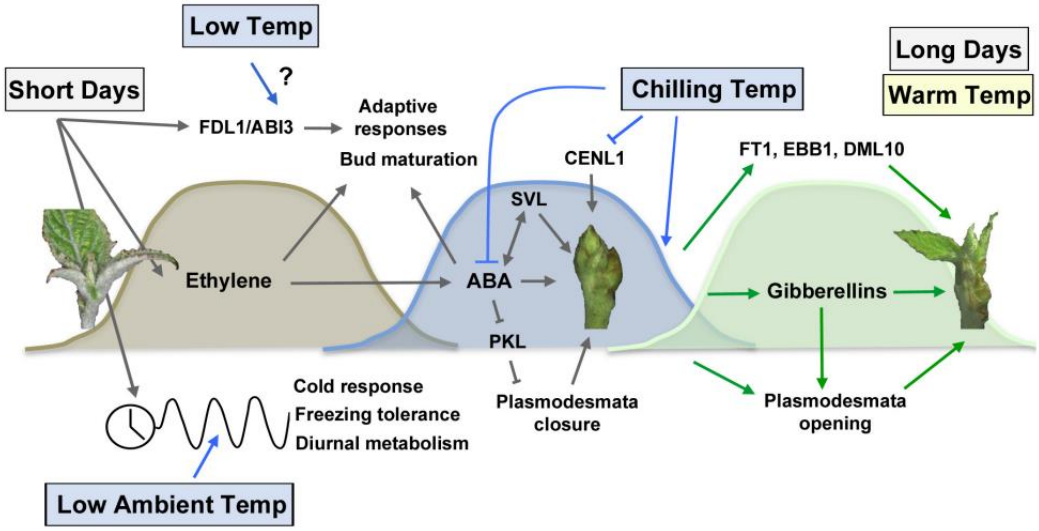

Figure 5. Photoperiodic and temperature control of bud set, dormancy, and bud break (Maurya et al., 2018)**.**

Several transcription factors control flowering time; the protein family basic helix-loop-helix (bHLH) is highly conserved throughout eukaryotic organisms, particularly the amino acid motif (Y. Liu et al., 2013a) The domain of these proteins is composed of two conserved motifs: a helix-loop-helix and a primary region; the first one is necessary for dimerization to change the expression of genes relevant to signaling pathways, such as flowering, while the second one is involved in DNA binding to the G box (CACGTG) or E box (Enhancer box) both act as DNA regulatory elements. This superfamily has been characterized in *Arabidopsis* due to the vital part its members play in survival mechanisms to deal with the environment, including light signal transduction, stress, flowering, and plant growth(Castelain et al., 2012a; Hao et al., 2021a; Jang et al., 2008).The pathways in which this protein is involved include seed germination, flowering, cell fate, plant mineral nutrition, abiotic stress, stress response, light signaling, hormone signaling (JA, IAA, ABA), and cross-talk between light and phytohormones (Hao et al., 2021b).

We will focus our attention on the bHLH proteins involved in the flowering pathway; genes such as CIB1, FBH1, NFL, MYC2, MYC3, MYC4, BEE1, PIF4, and SPT have been characterized. Understanding

how abiotic factors control this network of molecular signalings is essential for the plant's survival. CIB1 was highlighted due to its function in promoting CRY2-dependent floral initiation (FT) transcription (Liu et al., 2018).

Light is an important signal that induces plants to start different processes regarding growth and development. Plants perceive light conditions through the photoreceptors and three types are known: phytochromes, cryptochromes, and phototropin (Casal, 2007). The former is known to be responsible for the absorption of red and far-red light, and the second and latter for the blue region and blue light respectively. The main ones known are blue light photolyase-like receptors cryptochromes: CRY PTROCHROME 1 (CRY1) and CRY PTROCHROME 2 (CRY2) (Castelain et al., 2012). The first photoreceptor is involved in blue light-dependent de-etiolation responses, while the second is in photoperiodic regulation of floral initiation (Y. Liu et al., 2013a)

CRY2 blue light-specific interactions are with CIB1 in *Arabidopsis* and yeast cells; this, plus the interaction with CIB1-related proteins, triggers floral initiation (Hongtao et al., 2008). Moreover, it is worth emphasizing that CRY is the only photoreceptor found in bacteria, plants, and animals. The role it plays in the first two is well understood, while its role in mammals continues to be a subject of study (Y. Liu et al., 2018). As mentioned before, this photoreceptor is involved in FT mRNA expression; the mechanisms by which this happens are being elucidated. So far, three are known: direct modulation of FT transcription, suppression of CO protein degradation, and regulation of light entrainment of the circadian clock (Hongtao et al., 2008; Y. Liu et al., 2018). We will focus our attention on the latter.

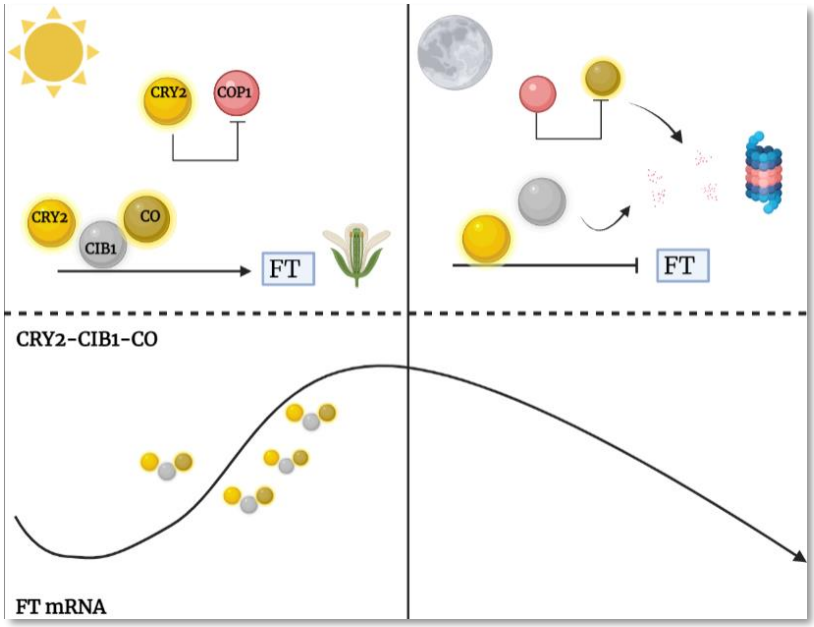

Figure 6. With interaction between the CRY2-CO and CRY-CIB1 pathways, they cooperate to regulate the FT mRNA and flowering. Based on Y. Liu et al. (2018). Figure created with BioRender.com

The photoperiodic control of floral initiation by the regulation produced between the interaction of CRY and bHLH proteins is now more relevant than ever in understanding how this mechanism works. The proteins that most affect flowering are CIB1, CIB4, CIB5, CIL1, and HBI1(Y. Liu et al., 2018). CIB1 is localized in the nucleus. It is part of the 17-member bHLH subfamily 18 and was the first blue light-dependent CRY2-interacting protein discovered in plants, with positive dependent regulation of CRY2 for floral initiation(Hongtao et al., 2008; Y. Liu et al., 2018). The response to light regulates mRNA expression of CIB1; this responseis for only a specific spectrum of light (380 to 500nm, i.e. blue light.

Assays (Hongtao et al., 2008), in which yeast cells were irradiated with blue light (15 µmol and 100 µmol m^{-2} s^{-1}) for 60min and red light (18 µmol m^{-2} s^{-1}) for 360 min, determined that the exposition of a higher fluence rate of blue lamplight generates a more significant expression of the interaction between CRY2 and CIB1. Red light does not create any interaction between CIB1 and the cryptochrome. Furthermore, the interaction between CIB1 and CO (Y. Liu et al., 2018) acts co-dependently with CO to promote flowering, while CO works independently. It is worth mentioning that both are co-expressed and regulated by photoperiods, which means that the peak of protein expression is in the early morning hours and again at nightfall on LDs, provoking *FT* transcription.

The Rishi Bhalerao group (Maurya et al., 2018; Singh et al., 2018, 2019; Yordanov et al., 2014) identified players involved in the bud break process; however, the molecular mechanism is still unknown. As a starting point for the elucidation of these mechanisms, gene expression profiles were reviewed for the identification of the bHLH Transcription Factor. Experiments were carried out (Bhalerao, unpublished) to assess the involvement of Transcription Factor 97 (TF97) during bud break. The results (Figure 7) obtained showed a reduction in expression as temperature and daylight decreased, while the opposite happened when daylight increased, preparing the trees for spring-inducible bud break.

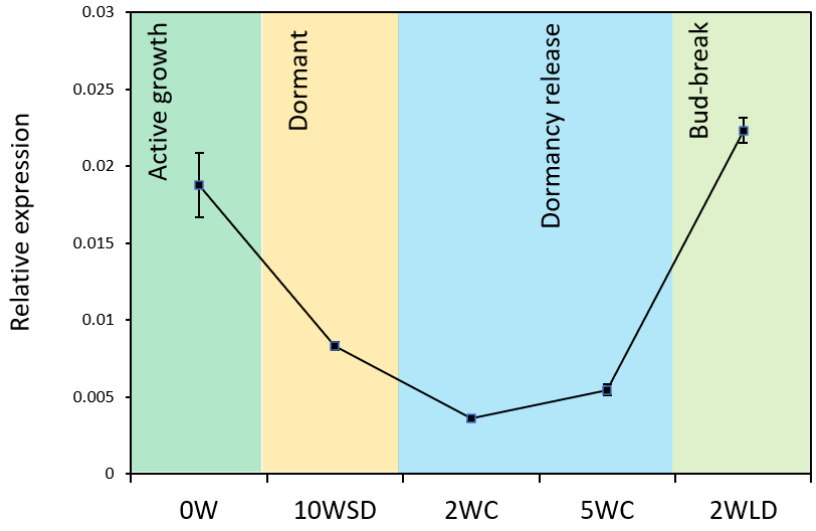

Figure 7. Expression pattern of TF97. Short day represses the expression from (0W-10WSD) it was reduced from 0.02 to around 0.01, which remains low during dormancy release and then is induced upon transfer to bud break inducing warm long days(2WLD) coinciding with time of bud break (Bhalerao, unpublished).

To sustain this hypothesis Bhalerao (unpublished) created overexpression lines of bHLH, in which the bud break rate and percentage were quantified. All the lines showed a reduction in time taken for bud break to come about compared to the wild type hybrid T89 (Figures 8 and 9).

Figure 8. Overexpression results TF97 in early bud break. T89 (Wild type) vs bHlH overexpression lines, plants were analyzed after 10weeks of short day, 5 weeks of cold and 4 weeks of long days. During the transfer from 5 weeks cold to long conditions, overexpressed lines open the bud faster compared to the wild type. In the picture it can be observed three overexpressed lines (1A, 3A and 2B) , (1A) showed the line which took less time to bud break, followed by (2B), leaving the less efficient to (3A) line (Bhalerao, unpublished).

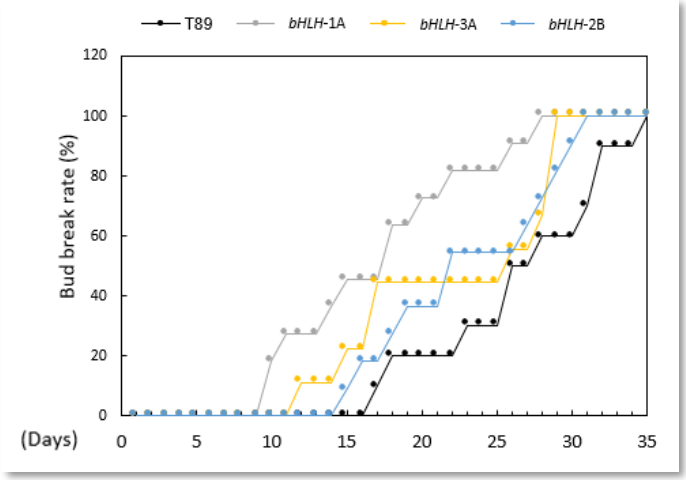

Figure 9. Bud break rate (%) in overexpression lines of a bHLH compared to T89 (WT). As observed in the graph overexpressed lines showed a higher bud percentage rate, starting the bud break in less days compared to T89, line bHLH-1A showed the best bud break percentage among the other two overexpressed lines (Bhalerao, unpublished).

With the evidence obtained it can be suggested the involvement of the Transcription Factor (TF97) in bud break, however it is still necessary to elucidate the pathways and mechanisms involving it in *populus spp.*

2. Aim of thesis

The aim of this thesis work was to identify and functionally characterise the transcription factor (TF) CIB of hyrid aspen T89 (*Populus tremula x Populus tremuloides*) by analyzing the role of blue light in the regulation of the expression of the TF and. For this purpose, Gateway cloning vectors carrying T89CIB and AtCIB were used for the overexpression of the gene, for the generation of *Arabidopsis thaliana* complementation lines, and for its subcellular localization experiments.

3. Materials and Methods

3.1 PCR amplification

T89 ID. Potrx055869g18047A provided by the Bhalerao team, was used as plasmid DNA. The primers used were: forward (5'- CACCATGGAAAGAGATAAGTTGTTTGTGAGCG - 3') and reverse (5'- TTATAGCTCAATTTTCATGTGAGTTGAGGGC -3'); primers used for the amplification of the T89CIB gene were synthesized by Eurofins Genomics. Primers were dissolved in ultrapure water (90µL miliQ-H20 + 10 µL primer). For PCR, the following reagents were mixed and filled up with water to a total volume of 20 µl: 2 µL plasmid DNA, 1 µL of each primer, 0.4 µL dNTP, 4 µL 5X Buffer, 0.6 µL DMSO, and 0.2 µL Taq DNA polymerase (5U/ µL, Thermo Scientific).

PCR was performed using a T100 thermocycler (Bio-Rad) at 95˚C for 3 minutes; 34 cycles at 95˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 1 minute and 30 seconds; and 72˚C for 5 minutes. The agarose gel was prepared with 1% agarose gel (100ml) and 8ul Midori Green; the solution was poured into a gel casting tray.

DNA was mixed with 5 µL loading dye (6X) (Thermo Scientific) and loaded on the agarose gel using 120 V for 25 minutes in TAE buffer. The separated DNA fragments were visualized using a UV transilluminator (365 nm) and cut. DNA was extracted from the gel slice using QI Aquick ® Gel extraction kit, QIAGEN. The concentration of DNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific).

The same procedure was done with *Arabidopsis*, sequence AT4G34530.1 as a plasmid DNA template. The primers used were (5'- GCGACAAGATCACAGGCAAA - 3') and reverse (5'- CCGGCCTTGGATTCACAATT-3').

3.2 TOPO TA Cloning

The T89 CIB PCR product (1.5 µL) was cloned into the pENTR™/D-TOPO™ Vector, as outlined by the manufacturer´s instructions using the pENTR™/D-TOPO™ Cloning kit, with One Shot™ TOP 10 Chemically Competent *E.coli* (Invitrogen, Cat. #K2400-20).After the TOPO Cloning reaction, the T89 CIB pENTR™ TOPO® construct was transformed into competent *E.coli*. One shot® TOP10 Chemically Competent *E.coli*, following the manufacturer´s instruction using pENTR™ Directional TOPO® Cloning Kits (Invitrogen, Cat.#K2400-20).

Transformed competent *E.coli* was spread in a selective plate Luria Broth (LB) + Kanamycin 50 µg/mL and incubated overnight at 37˚C. The Analysis of the transformants was made by colony PCR and Sequencing. For colony PCR, the following reagents were mixed and filled up with water to a total volume of 13µL; picked colony from the selective plate, 0.75 µL of T89 CIB reverse and forward primer, and 7.5 µL of GoTaq® DNA Polymerase (5U/ µL, Promega). PCR was performed using a T100 thermocycler (Bio-Rad) with the same setting mentioned earlier. DNA was extracted following the manufacturer's instructions using QIAprep ® Spin Miniprep kit (QIAGEN) from overnight cultures for sequencing validation. The concentration of DNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Sequencing samples were filled with water up to a total volume of 15 µL for a quantity of 1500 ng of DNA in a volume of 15 µL and M13 Reverse primer (5´-CAGGAAACAGCTATGAC – 3´). Eurofins Genomics, Ebersberg – Germany performed sequencing.

3.3 Gateway cloning and transformation of DH5α

Positive entry constructs were used for LR recombination reaction following the manufacturer's instructions using Gateway LR Clonase® II enzyme mix, Invitrogen. The binary Gateway vector used was pGWB6 (35S promoter, N-sGFP) (--35S promoter-sGFP-R1-CmR-ccdB-R2—) (Nakagawa,2002). The vector pGWB6 was chosen due to its capability to fuse with GFP, the N-terminal end was selected as

a place to fusion due to the signal peptide sequence it possesses which helped the subcellular localization of T89CIB in the organelle where it is expressed.

Figure 10. Illustration of the PGWB6 vector with CaMV35S::HPT-NosT marker containing a GFP reporter at the N-terminal (Image obtained from Nakagawa, 2002).

In a 1.5 mL microcentrifuge tube at room temperature, 2 µl of T89 CIB TOPO, 2 µl of pGWB6, 4 µL of 5x LR Clonase reaction buffer, and 8 µl of TE Buffer, pH 8 were mixed. To the mixed components, 4ul was added of LR Clonase enzyme and vortex. The reaction was incubated at 25C overnight; the next day, 2 ul of 2 µg/µL Proteinase K solution was added and incubated at 37C for 10 minutes.

Transformed competent DH5α™ *E.coli* was spread in a selective plate (LA + 50 µg/mL Kanamycin (KM25) + 50 µg/mL Hygromycin) and incubated overnight at 37˚C. The Analysis of the transformants was made by colony PCR and sequencing, the same procedure mentioned earlier.

3.4 Agrobacterium transformation and Transient Transformation of *N. Benthamiana* by Agro-Infiltration

The binary vectors pGWB6 GFP T89 CIB and pGWB6 GFP AtCIB1 were transferred into *A. tumefaciens* GV3101 by the freeze-thaw method (Weigel & Glazebrook, 2006). Transformed Agrobacterium cells were plated on selection plates (kanamycin 50 ug/ml, tetracycline 10 µg/mL, rifampicin 50µg/mL, and gentamicin 25 µg/mL) and incubated upside down for two days.

Positive colonies were selected and grown in liquid culture containing the same antibiotics used in the selection plates overnight at 28˚C and 200rpm in a test tube of 5mL. *N.Benthamiana* was removed from the 25 ˚C chamber, watered, and let stand on the bench for 5 hours for easier infiltration. Transformed *Agros* were harvested by centrifugation in 50mL Falcon tubes at 4000 rpm for 20 min, followed by the resuspension of the pellet in infiltration buffer (1omM MgCl2, 150 mM Acetosyringone) by shaking for 3 hours, the same volume used for the overnight culture was used. After incubation, the OD600 was measured from 5mL culture by measuring the resuspended pellet. The final OD was adjusted to 1, with the infiltration buffer; the p19 strain was included as an *Agros* helper for a better expression of the transgene. Leaves were infiltrated using a needleless syringe, prioritizing young leaves. Plants were put back on the bench top, covered with a black blanket for one day, and the remaining two days under 12h light/ 12 h photoperiod; leaves were harvested for downstream applications after three days.

Nine plantlets in total were infiltrated (three with T89CIB, three with AtCIB1, and three with the empty pGWB6 vector). The plants were divided into two groups; one was used for the subcellular localization of the protein by confocal laser scanning microscopy, while the other was used for protein expression study under blue light. Plants used for the protein expression study were kept in darkness for three days after the infiltration and then exposed for 20 minutes under blue light (20 µmol.m^{-2}. s^{-2}.) with a Photon System Instruments SL 3500-R-D (Czech Replublic).

3.5 Confocal laser scanning microscopy

Ziess LSM 780 confocal laser scanning microscope was used for confocal laser-scanning microscopy. The detection of transiently expressed tagged T89CIB and AtCIB1 proteins with GFP (pGWB6 GFP T89CIB and pGWB6 GFP AtCIB1) was studied. Leaves were taken after three days of infiltration. A thin layer from the epidermis of the leaf was gently peeled off using forceps and put on a microscope slide with the abaxial side up, with Mili-Q water on top covered with coverslips and adjusted in the microscope stage. The filter viewing selected was GFP, the fluorescence protein- tagged was excited at 488 nm. GFP fluorescence emissions were detected in the 493 – 510 nm window. Images were captured and processed by using a GaAsP detector and ZEN software (Zeiss – Germany).

3.6 SDS PAGE and WESTERN BLOT

Samples of leaves were taken from the infiltrated plants (T89CIB and AtCIB1 darkness for three days and 2o min blue light) and immediately placed in liquid nitrogen.

3.6.1 Lysis of infiltrated leaves

Sample lysis was performed by dissecting the infiltrated leaf with a sterile forcep and scalpel on liquid nitrogen (LN2). Leaves were ground with LN2, pestle, and mortar until a fine powder was obtained. Each sample was placed in Eppendorf tubes with 500µL of SII Buffer (150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 5mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), TritonX100, 10 mM sodium fluoride (NaF), bartezomid, 10 mM N-Ethylmaleimide (NEM) and protease inhibitor cocktail (pic)) – all chemicals used were from Sigma Aldrich (United States of America).

3.6.2 Determination of extracts protein concentration

Protein quantification assay was done by using 1000mg of fine powder of the infiltrated leaves; The Pierce™ BCA Protein Assay kit was used. For the determination of protein concentration, the first preparation of diluted bovine serum albumin (BSA) standards were made, and the final BSA concentrations (µg/mL) were 0, 2.5, 5, 10, 20, 40, 60, 80 and secondly a preparation of the BCA working reagent was prepared for quantification by mixing 50 parts of BCA reagent "A" and 1 part of BCA reagent "B". To calculate the protein concentration, in a fresh Eppendorf tube 2 µl sample, 98 µL SII Buffer and 1.9 mL of BCA were added, while for the blank 100 µl of SII Buffer and 1.9 mL of BCA were mixed. All the samples were transferred to a water bath for 10 min at 38°C.

An LKB Novaspec model 4049 spectrophotometer (Viena, Austria) was set to 562 nm. To set the zero baseline a cuvette filled with the blank (SII Buffer) was used. The absorbance from all the samples was measured. A standard curve was prepared by plotting the average blank corrected at 562 nm for BSA standard vs concentration in µg/mL. The standard curve was used to determine the protein concentration of the samples.

3.6.3 Western blotting

Protein samples and BenchMark™ Prestain protein ladder (Novex by life technologies, California US) were loaded in equal amounts of protein into the wells of a 7.5 % SDS PAGE gel. The gel was run at 100 V until samples migrated through the stacking gel, then the voltage was increased to 120 V until finished, 1 hour and 30 minutes approximately.

The gel was transferred to the stack containing a polyvinylidene difluoride membrane, the membrane was activated with methanol for 1 min and rinsed with transfer buffer (25mM tris base, 39 mM glycine, 20% ethanol) before preparing the stack. The stack was left overnight at 4° C and 20 V.

After the night, the membrane was blocked with 5 % milk powder with TBS-T (tris-buffered saline and Tween 20) for 1 hour. The membrane was incubated with 1:8000 Goat Anti-Mouse IgG (H+L) – HRP conjugate #1706516 in a blocking buffer for two hours. After this time the membrane was washed in three washes of TBS-T, 10 min each. A second incubation was made with 1:8000 Anti-GFP antibody in blocking buffer for 1 hour. Finished this time the membrane was washed three times with TBS-T 10 min each.

For signal development, enhanced chemiluminescent (ECL) Thermo Scientific™ Super Signal™ West Pico PLUS Chemiluminescent Substrate Prod #1863098 was used, 300 µL of each reagent was mixed, and poured into the membrane, then the membrane was covered in transparent plastic wrap. Protein signals in the membrane were scanned and digitized using the AZURE c600 Imaging system by Azure Biosystems.

3.7 *Agrobacterium-mediated* transformation of *Arabidopsis thaliana* using the floral dip method

The A*grobacterium* strain GV3101 is C58 (rif R) Ti pMP90 (PTiC58DT-DNA) (gent R) Nopaline, that harbored T89CIB was prepared by inoculating a single *Agrobacterium* colony into 5 ml Luria-Bertami (LB) medium containing antibiotics (50 µg/mL kanamycin, 50 µg/mL hygromycin, 50 µg/mL gentamicin, 25 µg/mL rifampicin). The culture was incubated at 28C° for two days. After this time, the culture was used as a starter to inoculate 500 mL liquid LB with the same concentration of antibiotics (Zhang et al., 2006). The culture was grown at 28C° for 16-24 h until the cells reached the stationary phase at an OD of 1- 1.5. The cells were collected by centrifugation at 4° C, 4000 rpm x 30 min, and were resuspended in 1 volume of 5% (wt/vol) sucrose solution. Before the dipping, Silwet L-77 was added to a concentration of 0.02% (vol/vol). Plants were inverted for the dipping, and the aerial parts from *Arabidopsis* were dipped in the *Agrobacterium* suspension for 10 s approx. After being soaked, the submerged plants were drained for around 5 s, and transformed plants were put on a tray and covered with a dome to maintain their humidity. The dome was removed after three days; treated plants were placed back in the Greenhouse where they grew for 1 month, and watering was stopped when siliques turned brown; at this moment drying plants and loose bolts were wrapped with wax paper. Finally, dry seeds were collected using a sieve mesh.

The screening of the transformants was done by pouring square Petri dishes (120 x 120 x 17mm) containing (hygromycin, 50 µg/mL)D in a 1/2 MS agar. The seeds that were collected previously were sterilized by treating them with 50X volume of 70% of ethanol for 1.30min and mixing the suspension from top to bottom, followed by treating them with 50x volume of 50X volume of 10% sodium hypoclorate containing 8% chlorine for 10 minutes mixing the suspension thoroughly. To conclude the sterilization process, the seeds were rinsed three times with sterile water and put to dry under a laminar flow hood. The sterilized seed was spread in the square Petri dishes containing the appropriate antibiotics and ½ MS agar. Once the plates dried and the seeds became stable in the square petri dish, the plates were covered in aluminum foil and placed at 4°C for 3d. After this period of time, the Petri dishes were moved to a growth chamber under long-day conditions (25° C, 16 hours of light / 8 hours of dark). After 7 days, seedlings with green cotyledons, true leaves, and roots were moved directly to pots with soil and transferred to the greenhouse:

	Day	**Night**
Light source	LED (Fionia FL300 Sunlight) plus sunlight	
Irradiance	150-250 µmol m^{-2} s^{-1}	
Temperature	22° C	18° C
Light	18 hrs	6 hrs

Humidity	60%	60%

Table 1. Greenhouse room settings for *Arabidopsis Thaliana*.

After 15 days of growth, true leaves were taken from the transformed plants. DNA extract was prepared by freezing the leaves in liquid nitrogen and grinding it in a tissue lyser (Retsch MM301) for 1 min at 20 Hz using acid-washed stainless-steel beads (diameter: 5mm). Following, the bead was removed, and the obtained powder was mixed with Edward buffer (200mM Tris (pH 7.5), 250 mM NaCl, 25mM EDTA, 0,5% SDS) in a 2µL Eppendorf tube. The samples were centrifuged at 16,000 rpm for 2 min. The supernatant corresponding to the DNA was transferred to a new 1.5 µL tube, in which 300 µL of Isopropanol was added; this was followed by another spin at 16,000 rpm for 5 min. The pellet was washed with 70% EtOH and let it dry at room temperature. Once dried the pellet was resuspended in 200 µl H20 and used for PCR reaction.

4. Results and Discussion

4.1 T89 CIB of hybrid aspen shares sequence similarity to AtCIB1

A blast protein alignment between Arabidopsis (AT4G34530) and T89 Hybrid Aspen was carried out to search for shared sequences that could lead us to discover proteins in Hybrid Aspen that may act in the same way as in *Arabidopsis*. Both sequences matched in the region of the basic helix-loop-helix (bHLH) transcription factor of *Arabidopsis;* this result suggests that in T89 Hybrid Aspen, there must be a protein that acts in the same way as the CIB1 protein in *Arabidopsis.* From this result, the characterization of what I called T89CIB was performed.

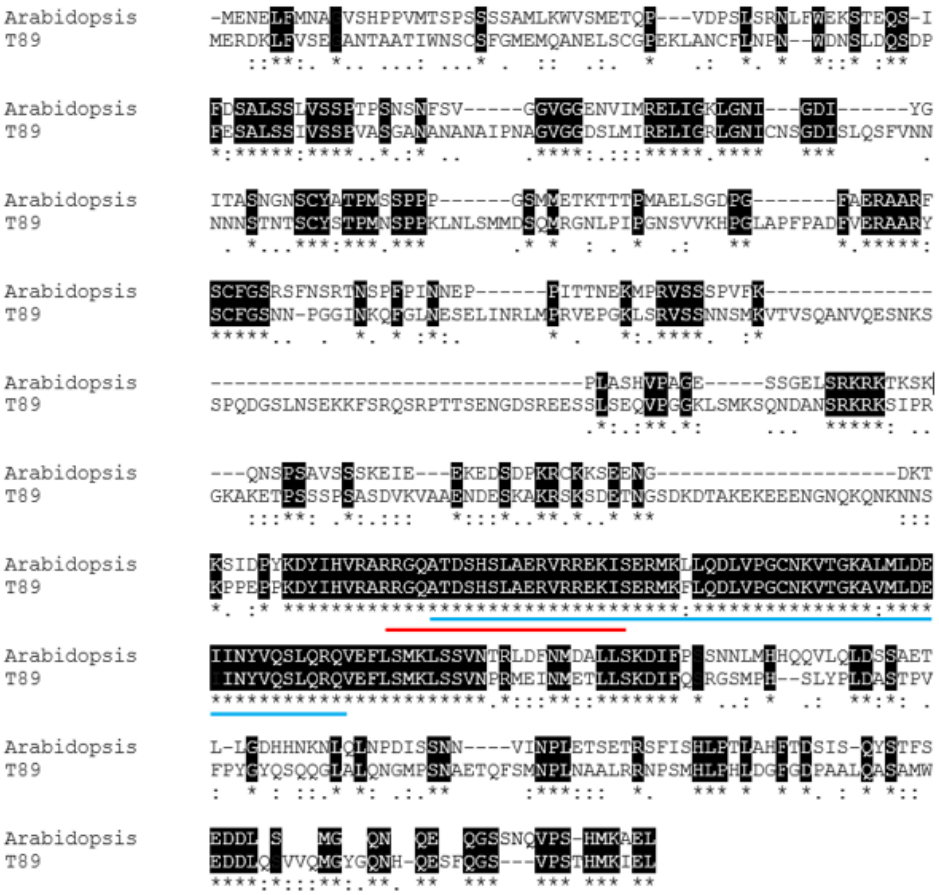

Figure 11. BLAST report protein alignment between Arabidopsis (AT4G34530) and T89 Hybrid Aspen CIB1 proteins. The dark highlighting represents identical amino acids. The region underlined in blue was where most of the identical proteins were found in the basic helix-loop-helix region. The area underlined in red shows both sequences' nuclear localization signal (NLS).

4.2 Plasmid construction and cloning

To construct the pGWB6 T89CIB/AtCIB1 plasmid Gateway® binary vector used for transformation in *A. tumefaciens*, the T89 CIB/At CIB was amplified and cloned into the attR1-(CmR-ccdB)-attR2 sites of pGWB6; the genes of interest replaced ccdB to generate an expression clone, used for downstream applications.

In figure 12, we can observe a representation of the pGWB6 with the oriented cloning of T89CIB or AtCIB1. The plasmid map contains elements that are important for the experiments performed; it includes a GFP reporter marker for protein localization and for the visualization of the expression of the protein under light conditions. The antibiotic resistance genes Kanamycin and Hygromycin allow bacteria to grow with the insertion of the plasmid; the promoter CaMV 35S is a constitutive promoter for the constitutive transcription of T89 CIB and AtCIB1, essential for the study of the gene. Finally, in the *att*R sites is the insert (T89 CIB and AtCIB1). The final two plasmids were pGWB6 GFP T89 CIB and pGWB6 GFP AtCIB1.

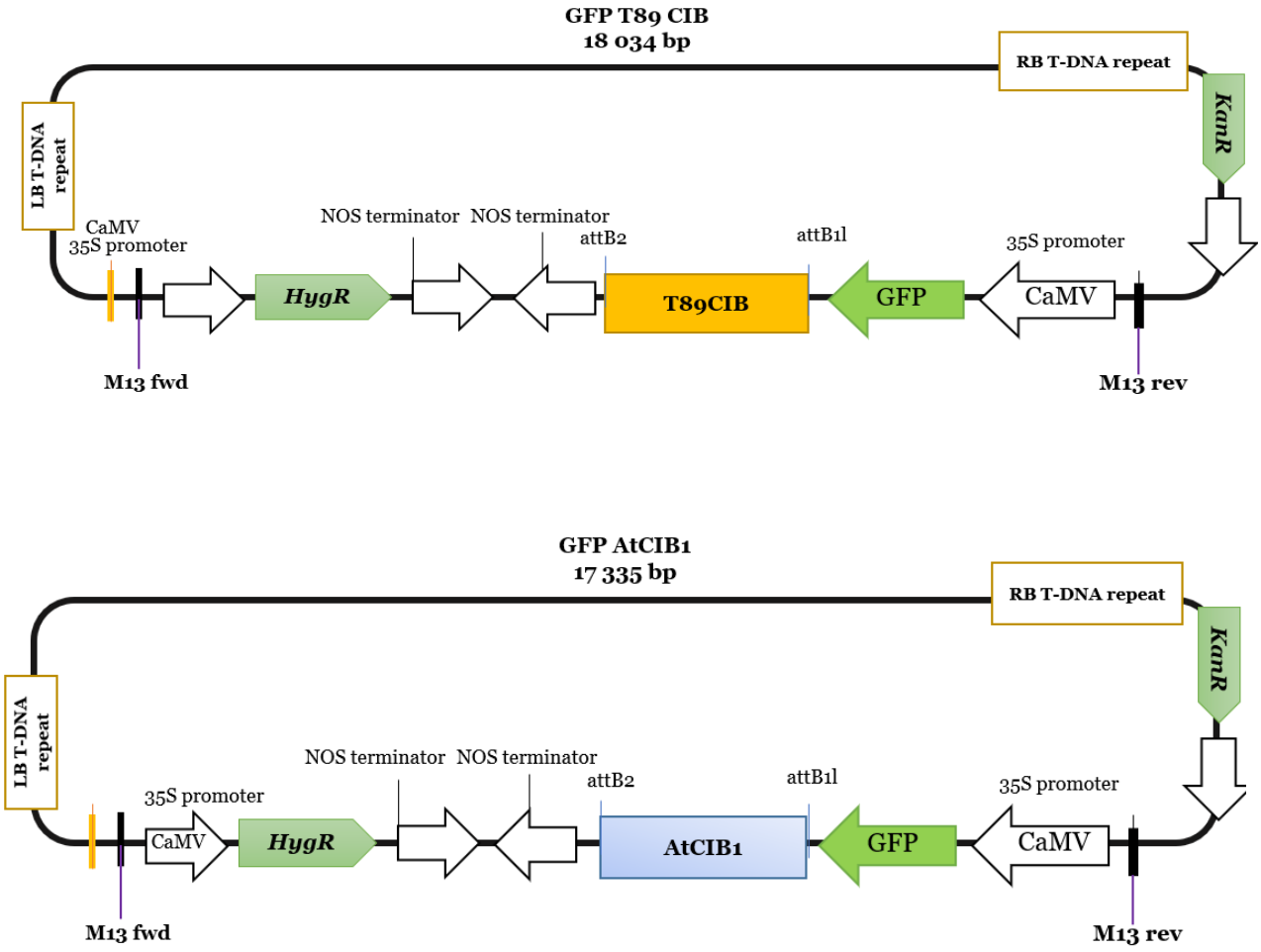

Figure 12. (upper panel) and *AtCIB1* (lower panel) genes inserted in the pGWB6 plasmid, the sizes are 18,034 bp and 17,335 bp, respectively. Antibiotic resistance genes are Kanamycin (KanR) and Hygromycin (HygR), and the constitutive promoter is the cauliflower mosaic virus (CaMV) 35S; the visual marker for both plasmids is Green Fluorescent protein (GFP).

Figures 13 and 14 display the two methods performed to verify the presence and orientation of the clone in the plasmid. As shown in Figure 13, we observed the presence of bands with the size expected with the clones after colony PCR. Furthermore, the same colonies were sent for sequencing to

determine the best colonies with the correct orientation and best alignment. These two analyses of the transformants helped me choose the best colonies to work with for the next steps of my research.

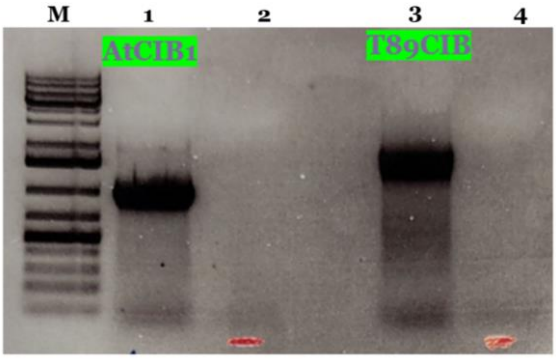

Figure 13. Gel analysis of transformants using colony PCR. Using AtCIB1 and T89CIB primers, colony PCR was conducted, producing products of the expected size (~1,200 bp and ~1,700 bp respectively) with the presence and absence of the clone insert. Column M: Marker; Column 1: *AtCIB1* insert; Column 2: no insert; Column 3: *T89CIB* insert; Column 4: no insert.

1

2

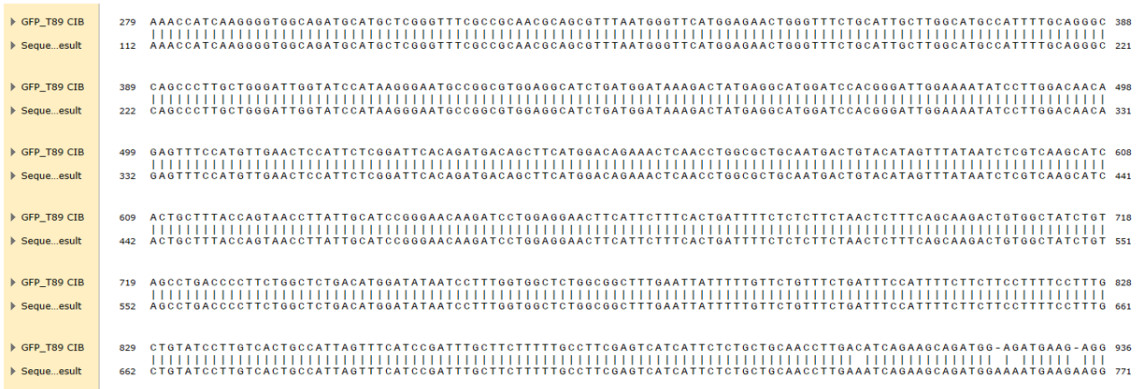

Figure 14. Sequencing analysis of transformants. To confirm the transformants' presence of the genes, *T89CIB* and *AtCIB1* colonies were sent for sequencing. A blast was performed between the sequencing result and the sequence with the clone. In panel 1, the blast between GFP AtCIB1 and the colony sequencing is shown, confirming the presence and correct orientation of the clone; in panel 2, the alignment of GFP T89CIB and the colony sent for sequencing is shown. In both cases, we know that cloning was performed successfully.

4.3 Subcellular Localisation of GFP T89 CIB

T89CIB is a putative orthologue of the Arabidopsis CIB1 gene, as shown previously. To test this hypothesis, T89CIB was transiently expressed in *N.benthamiana* lines. It was generated an N-terminal tagged T89CIB and AtCIB1 – GFP fusions, generating transgenic tobacco lines, and assayed the reporter fluorescence in epidermal cells. This analysis showed that both T89CIB and AtCIB1 were subcellularly localized in the nucleus; the result in *Arabidopsis* was confirmed, as previously demonstrated by (Hao et al., 2021). A pGWB6 binary vector, without the gene of interest, was used as a control. As expected, the protein T89CIB was expressed in the nucleus, suggesting its function as a DNA-binding transcription factor involved in the regulation of DNA-templated transcription in T89 hybrid Aspen, which is similar to AtCIB1 in *Arabidopsis* (Y. Liu et al., 2013).

Confocal microscopy revealed the subcellular localization of fluorescence in cells expressing GFP in tobacco. The fluorescence was accumulated in the cytoplasm, plasma membrane, and nucleus in the leaf tissues, expressing untargeted GFP (pGWB6-GFP) (Figure 15 – C). In comparison, targeted GFP was accumulated in the epidermal tobacco cells' nuclei (Figure 15, A–B).

Transient expression assay was chosen for subcellular localization due to its established conductions of the reporter gene, favoring the gene delivery into the host cells. Although subcellular localization is possible, a problem arises when one's aim is to decrypt the protein's function; this happened because many multigene families occur in plants. Therefore, with the nuclear subcellular localization of T89CIB, it can be inferred that the protein's function might take place within the cell due to the sequence similarities being shared with AtCIB1. Therefore, it is necessary to compare an individual member of the multigene families encoding T89CIB to comprehend the function in hybrid aspen.

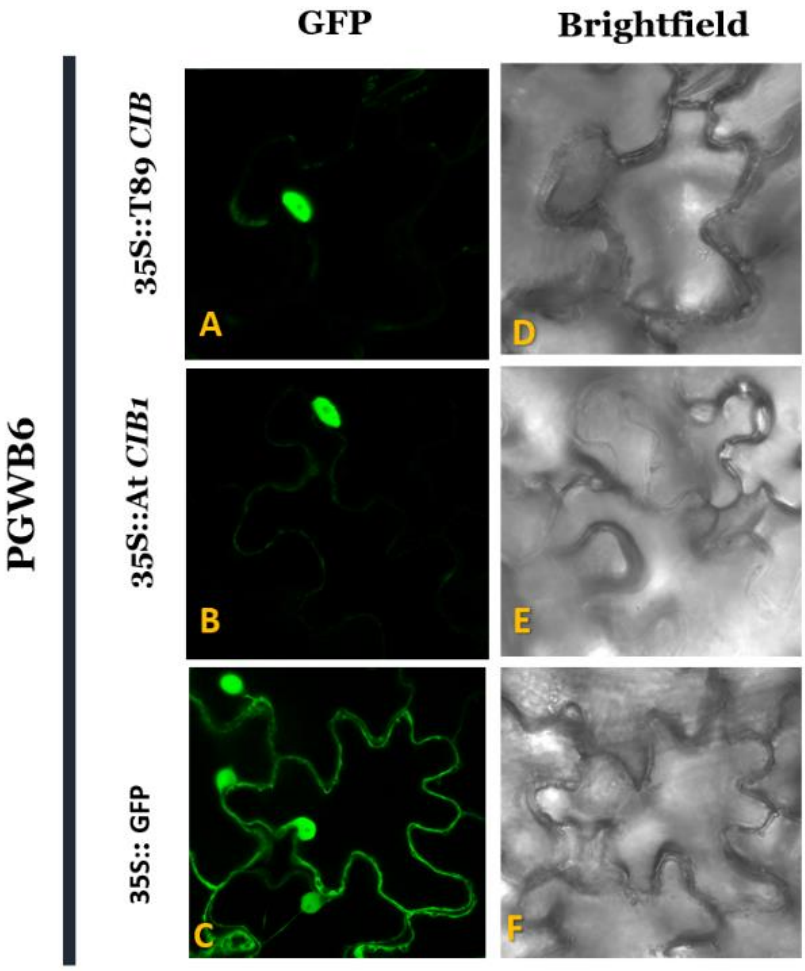

Figure 15. Confocal images of *Nicotiana benthamiana* leaf epidermal cells showing transient expression of (A) 35S::GFP T89 CIB, (B) 35S::GFPAtCIB1, and (C) 35S::GFP. (D), (E), and (F) show the Brightfield imaging. Panels (A) and (B) show the subcellular localization in the nucleus of GFP T89CIB and GFP AtCIB1, respectively. Panel (C) shows the fluorescence of GFP in the nucleus and membrane in an unspecific localization.

4.4 T89 CIB protein expression in the absence and presence of blue light

To examine the stabilization of T89CIB protein expression under blue light, the Pierce BCA (Bicinchoninic acid) Protein Assay was realized for the determination of protein concentration. In Figures 16 and 17, we can see the preparation of the standards, the dilution of the albumin standard (BSA), and their absorbances. With these values, it was possible to plot the standard curve, which helped to calculate the concentration of the proteins T89CIB DARK and T89 CIB BLUE 20min. The concentration obtained in Figure 18 was based on the gel's protein load for the SDS page. In the gel, it was loaded with 15.26 µl of T89 CIB DARK, 10.73 µl T89 CIB BLUE 20 min (1), and 11.89 µl T89 CIB BLUE 20 min (2). The remainder was filled with the buffer for a total volume of 25 µl.

4.4.1 Bicinchoninic acid (BCA) protein assay

BSA (µg/µl)	OD 562 nm
0	0
2.5	0.054
5	0.083
10	0.136
20	0.238
40	0.45
60	0.637
80	0.813

Table 16. Bovine serum albumin (BSA) dilutions to generate a standard curve for the BCA protein concentration assay. BSA ampule (2mg/mL) was diluted, with the following concentrations 0, 2.5, 5, 10, 20, 40, 60, and 80 µg/µl. The absorbance obtained with each concentration (OD 562nm) was used for the plot of the standard curve.

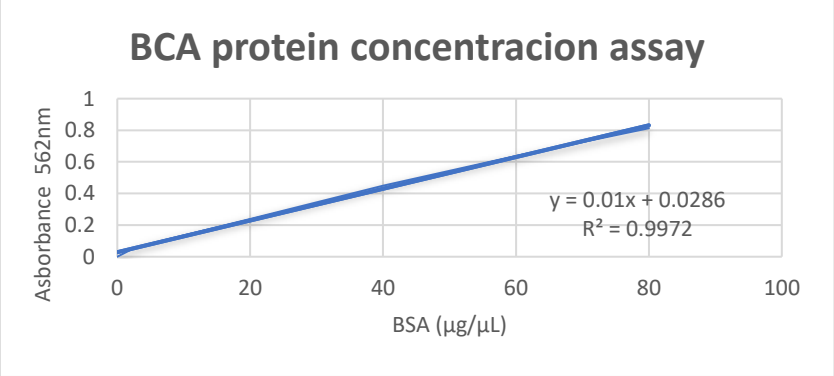

Figure 17. Standard curve for T89CIB protein quantification assay. Albumin standard (BSA) in (2mg/mL) was used to generate the standard curve for the BCA T89CIB concentration assay. The concentration of protein (µg/µL) was determined using the equation of the line y=0.01x+0.0286 with a coefficient of determination (R^2) of 0.0997, where y is the absorbance and x is the concentration. The standard curve is needed for the calculation of the protein concentration.

	OD 562		µg/µl (x)	for 50 µg	for 25 µl
T89 CIB DARK	0.081	0.0524	5.188119	9.637405	15.3626
T89 CIB BLUE 20 min - 1	0.064	0.0354	3.50495	14.26554	10.73446
T89 CIB BLUE 20 min-2	0.067	0.0384	3.80198	13.15104	11.84896

Table 18. Protein concentration of T89 CIB DARK and T89CIB BLUE 20 min -1 and -2. The protein concentration of each sample was calculated with the equation of the line y=0.01x+0.0286 which led to the following protein concentrations (µg/µL): 5.18, 3.50, and 3.80 µg/µL, respectively. The quantity load into the gel of each sample in a 25 µL total volume were the following: 9.63, 14.26, and 13.15 µL.

4.5 T89CIB is positively regulated by blue light

To better understand the role of T89CIB in the light regulation of plant development, it was necessary to investigate if blue light affects protein expression. To achieve this, tobacco transient expressing epitope-tagged T89CIB (35S::GFP-T89CIB) was used to analyze the expression of the protein. In the proposed experiment, I transformed tobacco plants by *A. tumefaciens* to transiently express 35S::GFP-T89CIB; after the infiltration, they were transferred to darkness for two days, and then the plants were exposed to blue light for 20 min under 20 µmol.m^{-2}. s^{-2} and the level of T89 protein was established. The result of this experiment showed that little or no T89CIB expression was detected on plants that were treated only in darkness; on the contrary, the levels rise significantly within 20 min of blue light treatment (Fig #). The Ponceau S stain was used for protein transfer verification.

The result obtained establishes, the level of T89CIB protein expression is positively regulated by blue light, while in darkness it is negatively regulated.

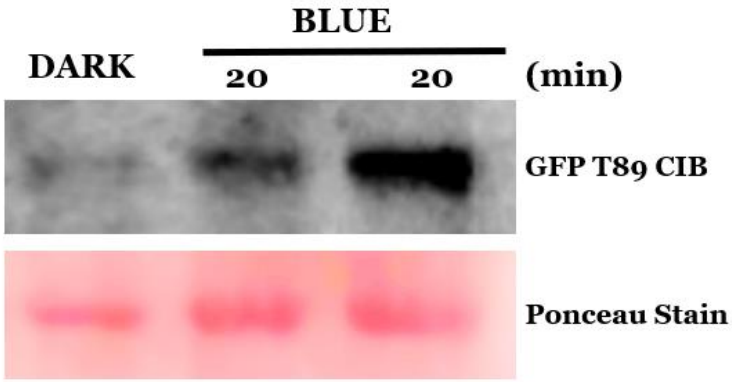

Figure 19. T89 CIB is degraded in the absence of blue light**.** Immunoblots show the expression of T89 CIB protein in tobacco plants expressing GFP T89 CIB. Samples were fractionated by 7.5% SDS PAGE, blotted, probed with anti-goat mouse IgG, stripped, and reproved with a second antibody: the anti-GFP antibody. The plant from the first column was transferred to darkness and the remaining plants to blue light (20 µmol.m^{-2}. s^{-2}) for 20 min. One replicate was made for the plants exposed to blue light. The protein transfer verification was carried out by Ponceau Staining.

4.6 T89CIB *Agrobacterium-mediated* transformation on cry2 and Col-o complementation lines in *Arabidopsis thaliana*

To examine how T89CIB affects the flowering time in *Arabidopsis*, two lines were used; the first one was Columbia (COL-o), and the second was with *cry2* mutant background. Floral dip

agrotransformation generated two independent lines expressing the 35S::89CIB transgene in the wild-type and in *cry2* mutant background. After one month, T1 seeds were collected to screen the transformants. As shown in Figure #, a screening of primary transformants was performed in a ½ MS agar medium with Hygromycin; one can observe positive germination of transgenic lines expressing 35S::T89CIB transgene, while the wild-type (COL-o) showed no germination. In Hygromycin, selection plates non-transformants also have a high rate of germination, and their cotyledons can remain green for more than one month (Zhang, 2006). Nonetheless, seedlings were selected to transfer into the soil based on three criteria: survival, size of cotyledons, and normal root development. Selected seedlings were transferred into the soil, where they grew for three weeks (Fig. #). The plants chosen for downstream analyses were based on the phenotype (flowering time) shown after the mentioned period; the leaf tissues were excised, and the integration of the 35S::T89CIB transgene was examined by PCR. As shown in Fig. #, the two lines (wild-type (COL-o) and *cry2* mutant) did not show any band with the transgene. However, in 2/3 of the 35S::T89CIB/COL-o lines, there was a positive insertion of the gene, showing the presence of a band of around 2,500 bp. This was also the case with the 35S::T89CIB/*cry2* lines, where all the plants showed a band with the transgene. These results further confirm the positive insertion of the gene T89CIB in the two *Arabidopsis* lines (COL-o and *cry2*)*;* these new lines will help the scientific community study the interaction between *cry2* and T89CIB and their codependence at flowering time.

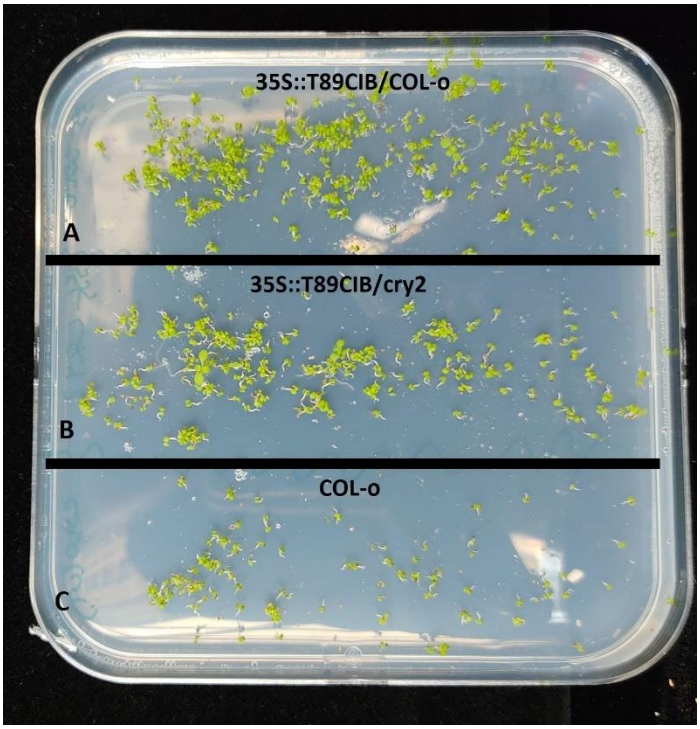

Figure 20. Screening of primary transformants (T1). Square selection plate of ½ MS plus Hygromycin (50 µg/ml) containing sterilized seeds of A) 35S::T89CIB/COL-o, B) presence of the 35S::T89CIB*/cry2,* and C) COL-o. A) and B) show germinated seedlings, showing a positive resistance to the antibiotic (Hyg +), while in C), the rate of germination of the seedlings was low due to the Hygromycin selection plates.

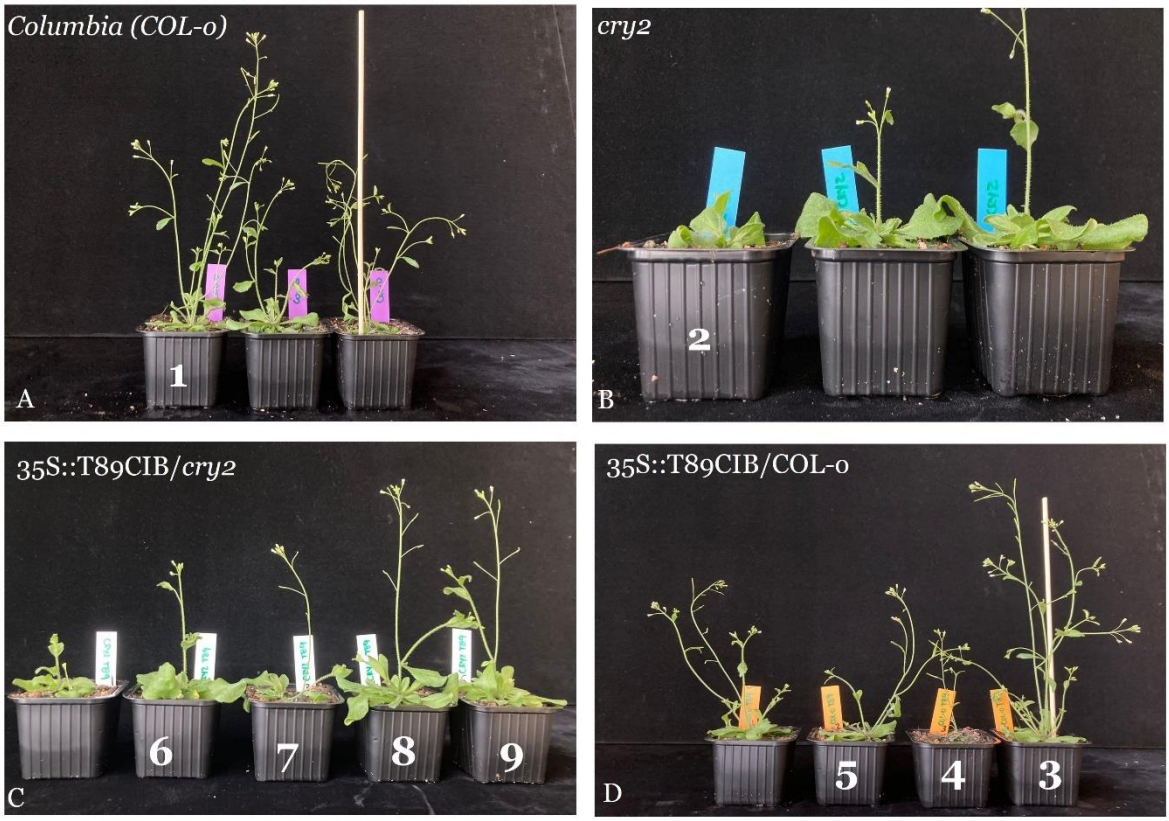

Figure 21. Transgenic plantlets and controls transferred to soil. (A–B):

Images of control lines Columbia (COL-o) and *cry2* mutant background; (C–D): Images of transgenic plants (T1) expressing the gene T89CIB. Plants were grown in Long Day photoperiods (18 hrs light / 6 hrs dark) for three weeks. There is a phenotype in the transgenic lines, as opposed to the lines without T89CIB, suggesting a positive regulation by the transgene during early flowering (C–D).

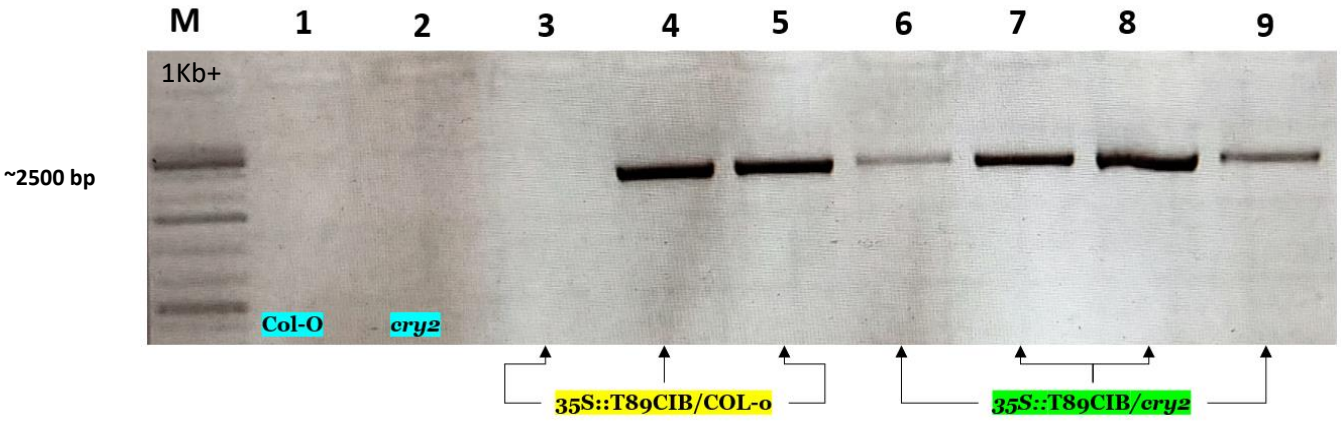

Figure 22. Transformant selection using PCR. Using T89 CIB primers, PCR was conducted on *Arabidopsis thaliana* plants germinating on MS plates with the antibiotics (Kanamycin 50 µg/mL, Hygromycin 50 µg/mL, and Gentamicin, 25 µg/mL). Positive transformants were analyzed via PCR.

Column M: Marker; Column 1: Col-o control; Column 2: cry control; Column 3: 35S::T89CIB/COL-o gene was not present; Column 4: presence of 35S::T89CIB/COL-o; and Column 5: presence of 35S::T89CIB/COL-o. Columns 6–9: presence of the 35S::T89CIB*/cry2*.

5. DISCUSSION

This study reports the control of bud break, a role for CIB transcription factors in hybrid aspen. The subcellular localization data and *in vivo* assays helped in the nuclear localization of the hybrid aspen transcription factor T89 CIB. Furthermore, the role of blue light in the T89 CIB was analyzed, indicating this light wavelength's ability to positively regulate the protein's expression. In order to better understand the interaction between photoreceptor CRY2-signaling proteins like T89 CIB, *Arabidopsis thaliana* complementation lines were generated. *Arabidopsis thaliana* has a cryptochrome-interacting basic helix-loop-helix CIB protein, which is a nuclear-localized protein (H. Liu et al., 2008) and is blue-light dependent (H. Liu et al., 2013). It physically interacts with photoreceptor CRY2, which helps the regulation of the function and works together to promote photoperiodic flowering(H. Liu et al., 2008).This research has shown that T89CIB is a nuclear-localized protein. The accumulation of the T89CIB protein in blue light was further demonstrated, whereas no expression is observed in the dark. Furthermore, two transgenic lines in *Arabidopsis* (COL-o and *cry2*) were constructed, inserting T89CIB as complementation lines to study the effect of the protein in flowering. These results support the hypothesis stated at the beginning of the study due to the shared sequence similarities between T89 hybrid aspen and *Arabidopsis* in the region of the genome where CIB1 is located; therefore, the "unknown protein" in T89 sharing the same bases might have a similar biological process, cellular localization, and molecular function to Arabidopsis CIB1.

This study has shown the regulation of the protein T89CIB in a blue-light-specific manner and the degradation of the protein in the absence of light. The mechanism and receptors mediate the blue-light suppression for the degradation of T89 CIB. Although, as discussed previously, the discovered protein in T89 is acting as Arabidopsis CIB1. A hint can be obtained using the same analysis to understand the mechanism by which T89CIB is degraded in darkness. Interestingly, the mechanism in *Arabidopsis* is more elucidated: as shown in (Casal, 2007; Sullivan & Deng, 2003), there exist photoreceptors – such as light-oxygen-voltage (LOV)-domain F-box proteins FLAVIN-BINDING KELCH REPEAT 1(FKF1), ZEITLUPE (ZTL), and LOV KELCH PROTEIN 2 (LKP2) – which regulate the expression of genes as a photoperiod changes response, having a direct influence on the flowering time in *Arabidopsis*. In addition, those authors studied the light-dependent and 26-S proteasome proteolysis, as this is a well-studied mechanism of regulation in signaling proteins. As expected, they suggested that the absence of the protein in darkness is attributable to the proteolysis of CIB1 by the 26-S proteasome. Furthermore, they investigated the role of LOV-domain F-box proteins in CIB1 under the exposition of blue light; interestingly, they found a protective effect of the ubiquitin ligase that targets the degradation of the protein, interrupting the action of the 26-S proteasome. Since the main aim was to elucidate the principal characteristics of the transcription factor (TF) T89 CIB, such as subcellular localization and light-dependent regulation, this study did not include a deep analysis of the mechanisms involved during the light regulation nor of proteins involved in the expression and inhibition of the T89 CIB, although the results obtained during this project may suggest that the regulation and action of the protein might occur in a similar way to hybrid aspen. Now that the essential aspects (localization and regulation) have been confirmed, it is necessary to study where this TF fits in the pathway during flowering and establish if it acts upstream or downstream of FT (Flowering time). This will elucidate the paths that lead to bud break in hybrid aspen.

As reported previously, CIB1 in *Arabidopsis* is a blue-light-dependent, *cry2*-interacting protein promoting FT transcription (H. Liu et al., 2008). In addition, as reported in (H. Liu et al., 2013), *cry2* and CIB1 are colocalized in the nucleus, and when exposed to blue light, the accumulation of the complex *cry2*-CIB1 is enhanced, affecting the modulation of FT transcription. Given all the information explaining the relationship between the cryptochrome and the bHLH TF, it is hypothesized that T89 CIB might need the interaction with the *cry2* photoreceptor to regulate transcription and bud break

in hybrid aspen. Due to the time of growth (one needs at least 11 weeks to research hybrid-aspen-clone T89 plants) (Q. Yu et al., 2001), complementation lines in *Arabidopsis* were constructed due to their convenient time of growth and the well-known elucidated pathways. To test the interaction between T89 CIB and endogenous Arabidopsis *cry2*, the transgene T89 CIB was inserted in the wild-type (COL-o), and the mutant *cry2*. As shown in Fig. #, a positive insertion was made in some of the lines that were agroinfiltrated by floral dip. As the main goal was to test the positive insertion of the T89 CIB in the *Arabidopsis* line by PCR and protein expression, a complete phenotype study that suggests a phenotype when T89 CIB is expressed without *cry2* cannot be assessed, although as observed in Fig. #, the plants with the transgene have a different phenotype compared to the controls. It can be observed that plants 35S::T89CIB*/cry2* started to flower before the line with the mutation *cry2*; in the same way, 35S::T89CIB*/*COL-o showed early flowering compared to the wild-type, as can be observed in the difference in height between them: COL-o started flowering when the shoots were taller. This raised questions such as: is T89CRY2 required for T89 CIB functionality? As happens in *Arabidopsis*, it is necessary to test both interactions. Further studies are needed to elucidate the function of T89 CIB. This would be necessary to generate CRISPR lines, analyze the phenotype (bud burst and growth cessation), and validate if T89 functions upstream of FT.

6. Conclusion

The discovery of bHLH transcription factor T89CIB in hybrid aspen is an important step in the elucidation of the genetic mechanisms regulating bud break. The study of this new TF in hybrid aspen opens new insights into the control of dormancy release in perennial plants. This study hereby reports the identification and characterization of the hybrid aspen bHLH T89 CIB protein. T89 CIB is nuclear localized and is stabilized in a blue-light-dependent manner. These results are consistent with the hypothesis that hybrid aspen T89 CIB acts similarly to atCIB1 due to the sequence similarities that they share. Furthermore, the effects of atCRY2 (Cryptochrome) interacting with T89CIB on the flowering time cannot be explained in a single essay with complementation lines of *Arabidopsis*. Although some *cry2* mutants and T89CIB in COL-o showed an accelerated flowering phenotype, suggesting how the interaction between atCRY2 and T89CIB regulates flowering time. It is necessary to explore this further with experiments to test the T89CIB-T89CRY2 interaction and T89CRY2 CRISPR/T89CIBoe to have a better understanding of T89CIB functionality. Further studies are needed to elucidate T89CIB interaction in the network involved in bud break. New discoveries in T89 CIB will provide a genetic understanding of plant regulation under environmental cues.

7. Future perspectives

The characterization of T89CIB in hybrid Aspen gives an insight of the role Transcription Factors play in the adaptation of the tree towards the environmental cues each season throughout the year. The discoveries such as localization, light regulation of T89CIB help in elucidating the pathway in which bHLH transcription factor is involved in bud break. Following the present work there are some experiments in the pipeline such as he generation of T89 CRISPR lines to observe the phenotype (bud burst and growth cessation); in addition, as mentioned before it has been shown the interaction in Arabidopsis between CIB1 and CRY2 to promote FT2 expression, therefore it would be interesting to explore this interaction T89 hybrid aspen. For this the generation of plants with the deletion of *cry2* have been made; it is necessary to do assays such as Bimolecular fluorescence complementation (BiFc). Soon, the understanding of the genetic network of bud break in hybrid Aspen will be fundamental to overcome he environmental challenges due to the climate change.

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