ATFL1 (Anti-TFL1), a novel orthologue induces flowering in the masting alpine snow tussock, Chionochloa pallens (Poaceae)

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Abstract

Masting, the synchronous highly variable flowering across years by a population of perennial plants, has been shown to be precipitated by many factors including nitrogen levels, drought conditions, spring and summer temperatures. However, the molecular mechanism leading to the initiation of flowering in masting plants in particular years remains largely unknown, despite the potential impact of climate change on masting phenology. We studied genes controlling flowering in Chionochloa pallens, a strongly masting perennial grass. We used a range of in situ and manipulated plants to obtain leaf samples from tillers (shoots) which subsequently remained vegetative or flowered. Here, we show that a novel orthologue of TERMINAL FLOWER 1 (TFL1; normally a repressor of flowering in other species) promotes the induction of flowering in C. pallens (hence Anti-TFL1), a conclusion supported by structural, functional and expression analyses. Global transcriptomic analysis indicated differential expression of CpTPS1, CpGA20ox1, CpREF6 and CpHDA6, emphasising the role of endogenous cues and epigenetic regulation in terms of responsiveness of plants to initiate flowering. Our molecular-based study has provided insights into the cellular mechanism of flowering in masting plants and will supplement ecological and statistical models to predict how masting will respond to global climate change.

Introduction

Mast seeding (or masting) is synchronous highly variable seed production among years by a population of perennial plants (Kelly, 1994; Kelly, Turnbull, Pharis, & Sarfati, 2008; Schauber et al., 2002). This results in irregular heavy flowering and seeding events, which occur in a range of taxa globally, including in various woody and herbaceous endemic species in the New Zealand flora (Webb & Kelly, 1993; Schauber et al., 2002; Kelly et al., 2008). A key question is what external and internal factors allow the plants to synchronously trigger heavy reproduction in only some years. Better understanding of those factors, to allow prediction of changes under global climate change (Kelly et al., 2013; McKone, Kelly, & Lee, 1998; Rees, Kelly, & Bjornstad, 2002) requires clarification of the underlying genetic mechanisms which control masting (Samarth, Kelly, Turnbull, & Jameson, 2020).

Although masting imposes costs, such as missed opportunities for reproduction, it is selectively favoured in plants which gain benefits from one or more Economies of Scale (EOS) (Kelly, 1994; Kelly & Sork, 2002). The two most common EOSs are predator satiation (where seed predators are not able to consume all the seed production, ensuring higher survival of the offspring) or more efficient wind pollination (Kelly & Sork, 2002). In order for masting to occur, plants need some synchronising factor, typically a weather cue. Samarth et al. (2020) have suggested that a likely cue for masting comes from seasonal changes in summer temperature, so it has been speculated that increases in global temperatures may alter masting behaviour, although the

nature of this effect is uncertain (Kelly et al., 2013; Monks, Monks, & Tanentzap, 2016). Changes in masting would affect the wider community, potentially impacting on food availability for indigenous seed predators and the rest of the food chain. Molecular studies, such as Kobayashi et al. (2013), have the potential to improve mechanistic understanding that underpins forecasting of changes in mast flowering behaviour. That in turn can help show how changes in natural conditions may lead to evolution of flowering-time genes and associated regulatory mechanisms. However, there is currently very little molecular evidence on the mechanisms for temperature-driven mast flowering in plants.

Information from model plant species provides useful background to the special case of mast seeding species. Molecular and genetic approaches have revealed that various external cues interact with the developmental processes to regulate the floral transition in perennial plants (Khan, Ai, & Zhang, 2014; Kobayashi et al., 2013). Genetic pathways controlling flowering time in model crops and temperate grasses, including Arabidopsis (*Arabidopsis thaliana*), tomato, apple, rice, barley, wheat and *Brachypodium distachyon* (purple false brome), show a high degree of conservation between dicot and monocot species (Shrestha, Gomez-Ariza, Brambilla, & Fornara, 2014). Both dicots and monocots share common floral integrator genes including homologues of florigen, the universal flowering hormone. Florigen, or FLOWERING LOCUS T (FT) is a 175 amino acid long protein belonging to the**p** hosphatidyl **e** thanolamine **b** inding**p** rotein (PEBP) family, an evolutionarily conserved protein family found in all taxa of organisms from bacteria to animals and plants (Karlgren et al., 2011). Phylogenetic analysis of different homologues of the PEBP gene sequences across the plant kingdom has revealed three sub-families. These are MOTHER OF FT AND TFL (MFT), FT and TERMINAL FLOWER 1 (TFL1) (Karlgren et al., 2011). FT and TFL1 protein sequences share 60% homology with highly conserved amino acid sequences across diverse species. These genes, however, act antagonistically to each other: FT promotes flowering whereas TFL1 represses it (Liu, Yang, Wei, & Wang, 2016).

In the current study, molecular tools were used to investigate the regulation of flowering of the alpine snow tussock, *Chionochloa pallens* (Poaceae). This species is one of the most strongly masting plant species globally (Kelly et al., 2000), which gives the plant selective benefits through predator satiation (Rees et al., 2002). The possible impacts of global warming on masting in this species have been discussed in the literature (Kelly et al., 2013; McKone et al., 1998; Monks et al., 2016; Rees et al., 2002), but more information on its flowering mechanisms is required. From various plants, including some manipulated to induce or prevent flowering, we took leaf samples from tillers (shoots), some of which subsequently flowered and some remained vegetative. We later identified each leaf as coming from a plant that subsequently flowered or one from a plant that remained vegetative (Appendix S1). We then used ecological transcriptomics (Samarth, Lee, Song, Macknight, & Jameson, 2019; Todd, Black, & Gemmell, 2016) to identify the potential homologues of PEBP sequences involved in the onset of flowering. Subsequent structural, functional and expression analysis of the PEBP sequences led to the identification of an orthologous *TFL1* gene with a novel function. In addition, global transcriptomic analysis revealed crucial transcription factors including thermosensory and floral epigenetic genes involved in the initiation of flowering in *C. pallens* .

Methods

Study species

Chionochloa pallens (midribbed snow tussock) is an endemic alpine New Zealand perennial grass. Plants grow as tussocks (bunchgrasses) around 0.5-1.5 m tall and 15-50 cm in basal diameter. These are very long-lived plants ($>$ 100 years) with discrete individuals. Each individual plant typically comprises hundreds of tillers, each of which grows for four to five years in the field prior to attaining reproductive maturity (Mark, 1965; Rees et al., 2002) . Tillers may then wait some years before switching from vegetative to reproductive, producing a flower stalk (or culm) and typically at least one daughter tiller, before dying. Activation of the inflorescence and floral development in *C. pallens* occurs a year before flowering (Mark, 1965, and see Appendix S1 in the supplementary information), so we sampled leaves for genetic analysis from marked tillers and stored the samples at -80 °C until the fate of that tiller could be determined up to a year later. Informative samples were then selected for RNA analysis. The levels of flowering in *C. pallens* vary markedly

between years (Kelly et al., 2013; Kelly et al., 2000). Previous studies, including transplant experiments to different altitudes, have shown that flowering in *Chionochloa* is heavier after warm summer temperatures in the year preceding flowering (Kelly et al., 2013; Samarth et al., 2020).

Study site and transplantation experiments

In low-flowering years few or no tillers flower in the field, and even in high-flowering years only a minority (<20%) of tillers on each plant flower (Rees et al., 2002). In order to improve our chances of having both flowering and vegetative samples, we took leaf samples from unmanipulated plants in the field, and from manipulated plants that were transplanted to lower-elevation (warmer) or higher-elevation (colder) sites to make flowering respectively more or less likely (Table 1). In 2016, unmanipulated *Chionochloa pallens* plants at the main Mt Hutt site at 1070 m elevation (43032' S, 1710 33' E) were selected with leaf samples taken from three marked tillers on each of 10 marked plants, with another 30 tillers selected and sampled the following two years. None of the tagged plants at the control plot flowered in 2017 and 2018, but they did in 2019.

Several transplantation experiments to higher or lower altitudes were carried out to manipulate flowering in *C. pallens* . Each group of transplanted tussocks was identified by the year of transplantation and the treatment provided. Ten separate plants from the vicinity of the control plot were moved to near sea-level at the University of Canterbury (UC, Christchurch; 43° 31' S, 172° 35' E, 15 m a.s.l.) for the inductive summer period in December 2016 to March 2017 (17Hot transplants) and then transplanted back to the 1070 m site. Leaf samples from tagged tillers (both transplants and control plants) were collected at different time points through the year (January 2017, March 2017, October 2017 and January 2018; between 11:00 am and 2:00 pm) and were used for the gene expression studies. Leaf samples collected during the inductive summer period (January 2017) were also used for the transcriptomic analysis as described below. Two other sets of 10 plants each were permanently transplanted in December 2015 from the control site at 1070 m to different altitudes: to UC (16Hot) and to 1520 m on Mt Hutt (16Cool). Leaf samples were collected at different seasonal time-points (between 11:00 am and 2:00 pm) including summer (January 2016), autumn (March 2016), spring (October 2016) and autumn (post flowering; March 2017).

In all the experiments, all leaf samples were collected and put directly into dry ice within 20 seconds and stored at -80 ^{o}C until further analysis. The tagged tillers were labelled and subsequent behaviour (flowering or not) was recorded in the following year. The stored leaf samples could then be identified as being from tillers that subsequently flowered or remained vegetative, and suitable samples were selected for downstream analysis.

Phylogenetic, structural and functional analysis of the PEBP-sequences in *C. pallens*

The reference '*C. pallens'* transcriptome assembly published in Samarth et al. (2019) was used to identify the potential homologues of the PEBP protein family (Table 2). The identified homologous PEBP sequences were further clustered into their respective sub-families using a phylogenetic approach. The PEBP-phylogenetic tree consisting of 49 protein sequences (Table S1) was reconstructed using the maximum likelihood method with Jukes Cantor substitution matrix and 1000 bootstrap replication using the PhyML package. Homologous PEBP protein sequences were also modelled using a homology modelling approach at the SWISS-PROT server and visualised using Pymol (student version). For functional analysis, homologous PEBP-gene sequences (*CpFT1, CpFT2, CpFT3, CpFT4, CpFT5* and *CpTFL1*) were transformed into*Arabidopsis thaliana* ecotype Landsberg erecta *ft-1* mutant plants using the pB2GW7 vector. Wild-type Landsberg plants flower after producing ˜ 8-10 leaves whereas *ft-1* is a late-flowering mutant, flowering after ˜20 leaves (Martinezzapater & Somerville, 1990).

RNA-extraction

Three separate biological replicates for RNA sequencing and two separate biological replicates for expression studies, each consisting of three individual leaf samples each from three different plants, were ground under liquid nitrogen using mortar and pestle. Each sample was dissolved in 1 ml of Trizol reagent and heated at

65 ºC for 5 min. The samples were then centrifuged at 13000 g for 5 min at 4 ºC. RNA was isolated using the Qiagen Plant RNA extraction kit following the manufacturer's instructions. The extracted RNA was further purified using a DNase I digestion kit (Qiagen DNaseI kit) according to the product manual. Extracted RNAs with 260/280 values of 2.0-2.2 and 260/230 valuse of 1.8-2.0 were selected for downstream analysis. Illumina TruSeq kit 2.0 was used to prepare cDNA libraries for paired-end sequencing on an Illumina HiSeq 2500 platform. The sequencing was carried out at Macrogen Inc. (South Korea).

Transcriptomic analysis

Transcriptomic analysis was performed comparing the tillers that flowered in the next season (17Hot) and tillers that remained vegetative (17Control) as described in Samarth *et al* . (2019). 150 bp generated paired-end reads were assembled into a single reference transcriptome using the Trinity pipeline (Haas et al., 2013). Reads from each of the samples were aligned to the reference assembly to generate a count matrix using the Bowtie2.0 (Langmead & Salzberg, 2012) followed by the differential expression analysis using the DESeq2 package in R. The significantly differentially expressed genes (adj. p-value<0.05) were annotated based on orthologous proteins identified in *B. distachyon* using blastp with an E-value of 10^{-5} and 50% protein identity. Gene ontology and pathway enrichment analysis was performed based on the PANTHER server (Mi et al., 2016). Validation of the RNA-seq results was performed using RT-qPCR with a random selection of four genes from the transcriptomic data (Appendix S2).

Gene expression analysis

Expression analysis was performed as described in Samarth *et al* . (2019). The primer sequences used are listed in Table S2. In addition to*CpFT1, CpFT2, CpFT3, CpFT4, CpFT5* and *CpTFL1* , homologues of other floral-promoting genes known to regulate the expression of*FT*- like genes (Song *et al.,* 2013) including *CpGI* ,*CpHd1* , *CpEhd3* , *CpMADS1* , *CpTPS1, CpMADS50* and a temperature regulator of flowering in temperate grasses (*CpVRN1*) were also included in the expression studies. Despite much effort, the expression of *CpFT1* could not be detected in the leaf samples.

Statistical analysis

Seasonal flowering expression of genes was analysed by two-way ANOVA using the prism software with the following predictors: time-point, site, and their interaction. The detailed statistical analysis is summarised in Table S5. The comparative data between leaves from plants that subsequently flowered and those from plants that remained vegetative were analysed using two-way student's t-test.

Results

Flowering in the tillers with leaf samples collected

Control tagged plants at 1070 m did not flower in 2017 and 2018, but did in 2019, a masting year. Since activation of floral development initiates a year before anthesis (Appendix S1), leaf samples from these control plants (18Control; January 2018) were included in the study. Some tillers collected from the plants that had been moved to other altitudes (16Hot, 16Cool, and 17Hot) flowered in the next season i.e. in 2017, 2017 and 2018, respectively. Flowering was recorded for each of the tagged tillers and all the leaf samples were then correspondingly identified as being from tillers that subsequently flowered or not (Table 1).

Orthologous PEBP-genes in *C. pallens*

Seven distinct homologues of PEBP-like gene family members were identified from the draft transcriptome of *C. pallens* (SRA accession number: GHUI00000000) (Samarth et al., 2019). The GIHRV-domain and LREY/HLHWIV-domain, both characteristic features of PEBP-family proteins were found to be conserved in the homologous sequences from*C. pallens* (Fig. 1a).

The general topology of the reconstituted PEBP-protein family tree was consistent with the previous findings of Karlgren et al. (2011) and Liu et al. (2016), indicating that the PEBP phylogenetic tree can be sub-divided into three major clades. These involve MOTHER OF FT and TFL1 (MFT)-like, FLOWERING LOCUS T

(FT)-like, and TERMINAL FLOWER1 (TFL1)-like (Fig. 1b). The PEBP-protein family tree was consistent with the species tree. Separate clades for homologous PEBP sequences belonging to monocots and dicots can be seen in the phylogenetic tree. All the *C. pallens* sequences were found to clade with PEBP protein sequences from species belonging to the Poaceae family. Of the seven contigs identified, one belongs to the MFT cluster, one belongs to the TFL1 group and five belong to the FT group (Fig. 1b). All sequences grouped with functionally categorised members of their respective families with high bootstrap values.

In *C. pallens,* five of the seven contigs which clustered within the FT group showed the presence of Tyr 85 residue, a critical amino acid for flower promoting activity. These contigs were named CpFT1-CpFT5, respectively (Fig. 1a). The external loop of FT (128-145 amino acid residues), critical for its florigenic activity (Ahn et al., 2006), was conserved in all the *C. pallens* FT sequences. All the FTs from *C. pallens* also had conserved characteristic features of an FT protein including His87, Glu109, Arg139, and Gln140 near the ligand-binding pocket. Structural modelling results indicate that Tyr85, His87, and Glu109 can form hydrogen bonds and interact with the Arg139 residue (Fig. S1). However, the Gln140 residue usually found in FT-like sequences has changed to His in CpFT3 and CpFT5, and to Pro in CpFT4 (Fig. 1a). The presence of proline at position 140 in CpFT4 may disturb the ligand-binding wall and may render the protein ineffective.

One of the *C. pallens* PEBP-like sequences clustering with the TFL1 group has both critical amino acid indicators of a TFL1-like sequence, a His88 and Asp140 (Fig. 1a). Segment B of exon 4 was also seen to be highly variable in *C. pallens* . During the genetic complementation test in the *ft-1* mutant, *CpFT1, CpFT2, CpFT3, CpFT4, CpFT5* and *CpTFL1* were all found to accelerate flowering. All the *FT/TFL1* -like genes significantly shortened the number of days for the complemented *ft-1* plants to flower under inductive conditions relative to the mutant (Fig. 1c). This suggests that, in contrast to AtTFL1, which is a known floral repressor (Ahn et al., 2006), the *C. pallens TFL1* -like gene promotes the floral transition. Therefore, from here on *CpTFL1* is referred as the *ANTI-TFL1* (*CpATFL1*). It is important to point out that genetic complementation assays have been widely used by the researchers to test the functionality of genes isolated from highly diverged species (such as gymnosperms) into model plants including Arabidopsis (Karlgren et al., 2011).

Expression of *ATFL1* correlates with the induction of flowering in *C. pallens*

Leaf samples from the control plants at 1070 m and transplants that flowered in the next season collected during and after the transplantation period were analysed for the expression of selected flowering-time genes (Table 1).

Leaf samples collected from both the 17Hot transplants that flowered in 2018 and control plants (17control) that had remained vegetative showed a seasonal expression pattern ($P < 0.05$; Table S5; Fig. S2). The seasonal expression patterns for *CpFT3, CpFT4, CpFT5, CpATFL1, CpVRN1, CpMADS50, CpMADS1* and *CpHd1* (Fig. S2) were similar between the control plants at 1070 m, all of which had remained vegetative, and the transplants that flowered in the next season (Table S5). Greater expression of *FT-like* genes was observed in the spring season (October samples) aligning with studies in model plant species (Nagano et al., 2019). Expression of *FT-like* genes was similar during the spring season in both leaf sample sets, whether from tillers that flowered in the next season or remained vegetative. However, *CpFT1* transcript was not detected in any of the samples due to its expression either being below the limit of detection or because it was not expressed in the leaves of the plant during the time when leaf samples were collected. Expression analysis of *CpVRN1*(Fig. S2), another floral promoter, correlated with its known seasonal expression pattern in temperate cereal species (Woods, Ream, & Amasino, 2014).

When the expression pattern is compared between the two sets of plants during the inductive summer conditions (January 2017), *CpATFL1*had a significantly greater expression in the leaf samples of the 17Hot transplants that flowered in the next season compared to the control plants at 1070 m that remained vegetative. Along with *CpATFL1,*other floral promoters including *CpTPS1, CpMADS1, CpMADS50,* and*CpVRN1* were also highly expressed in the leaf samples associated with flowering tillers (Fig. 2a, Fig S3).

The expression of flowering-time genes in the leaf samples from the transplanted plants that had flowered in 2017 (16Hot transplants and 16Cool transplants) was also compared to the control plants at 1070 m (16Control), none of which flowered. All the gene(s) showed a seasonally variable expression pattern ($P < 0.001$; Table S5) (Fig. S4). The expression pattern of *CpFT2* , *CpFT3* , *CpFT4* ,*CpATFL1* , *CpMADS1* , *CpEhd3* and *CpTPS1* in the leaves of tillers that flowered in the next season at both the sites, UC and 1520 m (16Hot transplants and 16Cool transplants, respectively) showed a similar expression pattern to that observed in the leaf samples associated with flowering in 17Hot transplants (comparing Fig. S3 and Fig. S4). Even though *CpFT5* had a seasonal expression pattern, plants at UC showed greater expression during autumn. All the genes showed a significant differential expression between tillers which subsequently flowered vs tillers that remained vegetative during the induction summer period ($P < 0.001$), except for *CpFT4* in the 16Cool transplants (Table S5).

Similar to the 17Hot transplants, *CpATFL1* had a significantly greater expression in the tillers that flowered at both the sites (UC and 1520 m; 16Hot transplants and 16Cool transplants, respectively) during the inductive summer period (January-2016) (Fig. 2a). Expression analysis of *CpVRN1* also correlated with its known seasonal expression pattern with a higher peak in expression post winter (Shimada et al., 2009). The expression of *CpVRN1* was greater in the tillers at both the sites (UC and 1520 m) that flowered in the next season compared to the plants at the control site that had remained vegetative (Fig. S3). Expression of *CpHd1* , *CpGI* ,*CpEhd3* , *CpMADS50* and *CpMADS1* were all greater in the leaf samples of the transplanted plants which subsequently flowered compared to the control site plants that remained vegetative (Fig. S3).

Leaf samples associated with tillers that remained vegetative in both the transplants, (16Hot transplant and 16Cool transplants) were found to have significantly lower expression of *CpATFL1* during the inductive summer temperature (January 2016) compared to the leaf samples from the same groups of transplants that flowered in the next season (Fig. 2b). In addition to the transplants, leaf samples collected from the control plants during January 2018 (18 Control) that flowered in the masting year 2019, also showed a significantly greater expression of*CpATFL1* compared to the leaf samples from plants that remained vegetative in the year 2019 (Fig. 2b). In summary, expression studies suggest that an elevated expression of *CpATFL1* during the inductive summer temperature period is associated with the induction of flowering in *C. pallens* .

Transcriptomic analysis identifies potential regulators of*CpATFL1*

To study the transcriptional changes associated with the induction of flowering in more depth, differential expression (DE) profiling, using RNA-seq, was performed. Leaf samples (January 2017) from 17Hot transplants located at UC which flowered heavily the next season were compared to the control plants (17Control; January 2017) that remained vegetative in the next season which was a non-masting year.

High-throughput 150 bp paired-end sequencing yielded 32 GB of raw data with 120 million average read counts for each replicate. Reads were assembled into a reference transcriptomic assembly using the Trinity pipeline. The generated *de novo* assembly yielded 140,826 transcripts, comprised of 383,092 contigs with an average length of 1428.92 bp and an N50 length of 2414 bp (Table S3). A total of 29,566 contigs (adj $P < 0.01$) were significantly differentially expressed (DE) with 14,514 and 15,052 transcripts significantly up and downregulated, respectively (Fig. 3).

Gene Ontology analysis: To gain insights into the function of the genes that were DE, contigs were functionally categorised on the basis of putative biological processes, molecular function, and cellular localisation. Out of 29,566 DE contigs, 15,974 (54.09 %) were annotated against the *B. distachyon* protein database with an E-value of 10^{-5}. The DE genes were further categorised based on gene ontology using a hypergeometric test with a significance threshold of 0.05 to identify key correlations between the internal cellular activity and the phenotypic differences.

Upregulated genes in the leaf samples associated with tillers that flowered in the next season were significantly enriched in the cellular components belonging to the cytoplasm, organelle, and intracellular organelle as the top three categories. Proteins encoded by the upregulated genes were further clustered into separate functional categories belonging to protein binding, transferase activity and anion binding. These genes were

found to be involved in biological processes enriched in response to stimuli, oxidation-reduction processes and cellular response to stimulus (Fig. 3d). Similarly, gene ontology analysis was also carried out for the proteins encoded by the downregulated transcripts. The downregulated transcripts were significantly enriched in the cellular components assigned to the cytoplasm, cell periphery and plasma membrane. These transcripts were then further clustered into distinct molecular functions with most of them belonging to transferase activity, anion binding, and small molecule binding. Finally, these proteins were assigned to the category of biological processes involved in organo-nitrogen compound metabolic process, protein metabolic process, and biological regulation as the top three classes (Fig. 3d).

2,786 downregulated transcripts when mapped to the KEGG database were enriched in biosynthesis of secondary metabolites, metabolic pathways and circadian rhythms with a false discovery rate of less than 0.05 (Fig. S5). About 3,788 (12.9%) transcripts of the upregulated genes were found to be associated with the KEGG pathways. The genes were significantly enriched in metabolic pathways (44.1%), biosynthesis of secondary metabolites (26.1%) and protein processing in endoplasmic reticulum (4.75%) as the top three categories.

Differentially expressed orthologues of floral genes in *C. pallens*: Out of the 29,567 DE contigs, 200 homologous floral protein sequences (from *A. thaliana* and *B. distachyon*) were significantly differentially expressed in the leaves of the tillers that flowered in the next season compared with tillers that remained vegetative (Table S4). Floral integrator genes, including *CpMADS1* and *CpATFL1* , were highly expressed in the tillers that flowered in the next season which also aligns with the quantitative PCR analysis performed earlier (Fig. 3c; Appendix S2). FRIGIDA (*CpFRI*), a known floral repressor in *A. thaliana* (Choi et al., 2011) and *B. distachyon* and other *CpFRI* -interacting genes were downregulated in the tillers that flowered in the next season (Table S4).

Leaf samples from 17Hot transplants that flowered in the next season also showed an increase in the expression of thermosensory genes including *CpPIF4* , *CpPIF5* and *CpbHLH80* relative to plants at the control site. These genes also act as floral promoters in response to high temperatures (Kumar et al., 2012). *CpSPL15* a known floral promoter in perennial plants (Hyun et al., 2019), was also upregulated in the 17Hot transplants compared to the control plants. *CpVRN2* , another temperature regulated floral repressor (Yan et al., 2004) was also downregulated in the tillers during the inductive summer period that subsequently flowered (Fig. 3c).

Two gibberellin catabolism gene family members (*CpGA2ox1* and *CpGA2ox8*) were also downregulated, while genes involved in gibberellin synthesis, including *CpKS* (kaurene synthase) and *CpGA20ox2* were upregulated in the tillers that subsequently flowered. Gibberellins have been shown to promote flowering in plants either by the activation of *FT* through SPL-family proteins or by activation of *SOC1* , independent of FT (Yu et al., 2012).

Several epigenetic editor genes, known to deposit active methylation marks to activate the expression of flowering promoting genes, including *CpREF6* , *CpMSI1* , *CpFLD* , and *CpEBS* (He, 2012), were upregulated in the tillers that flowered in the next season compared to the tillers from the plants at the control site that had remained vegetative (Table S4). Additionally, genes involved in the epigenetic repression of floral repressors such as *CpFLK* ,*CpFY* , *CpFPA* and *CpVEL1* (Qüesta, Song, Geraldo, An, & Dean, 2016) were also upregulated in the tillers that subsequently flowered (Table S4).

Discussion

C. pallens is a non-model monocot species with a large genome (2n = 42) (Connor, 1991). Due to highly variable flowering in *C. pallens* growing in natural conditions, it would have been difficult to track the induction of the flowering process using only unmanipulated plants. Transplanting plants to a lower altitude (15 m, University of Canterbury) and warmer temperature conditions induced heavy flowering in *C. pallens* , as previously reported for *C. rigida* (Mark, 1965), allowing sample collection during the inductive summer period and enabling us to study the molecular regulators of flowering. With recent advances in sequencing technologies, transcriptomic profiling of non-model plant species has been demonstrated to be advantageous

and effective when studying ecological phenomena (Todd et al., 2016). In the present study, use of ecological transcriptomics resulted in the identification of key orthologous flowering-time gene(s) in *C. pallens* responsible for regulating the floral transition. Transcriptomic profiling of different orthologous floral protein sequences showed that many components of the flowering pathways identified in *A. thaliana* and *B. distachyon* were also conserved in *C. pallens* . For example, *VRN* genes, which are known to regulate the floral transition in response to vernalisation and temperature in temperate grasses (Ream et al., 2014), were also identified successfully using the global transcriptomic approach.

Phylogenetic characterisation followed by expression profiling of the *PEBP* -genes resulted in characterisation of the floral promoting genes. Like maize, which has 26 homologous PEBP sequences (Liu et. al., 2016), *C. pallens* has multiple copies of PEBP gene sequences. This may be attributed to the polyploid nature of the plant, along with gene duplication events. The identified PEBP-like sequences from *C. pallens* were categorised into their subfamilies. Five of the sequences were shown to be FT-like sequences. Most of the sequences grouped with sequences which have already been functionally characterised, which aided in predicting function. All of the *CpFT-like* genes showed similar seasonal expression patterns (except for *CpFT1*), with their greatest expression during the spring season. This pattern of expression is similar to the expression of *FTs* characterised in other plant species (Nagano et al., 2019) but none of them could be correlated with the induction of the floral transition in the tillers that flowered in the next season. This could be due to the fact that the activation of the floral transition in *C. pallens* occurs during summer rather than in spring when the expression of the *FT-like* genes was at its peak.

CpATFL1 acts as a promoter of flowering in *C. pallens*

The TFL1 protein is generally a floral repressor in annual and perennial plants, acting antagonistically to FT (Liu et al., 2016). *CpATFL1,* clustering with the TFL1 clade in the *PEBP* -gene family tree, also had the key conserved amino acid signatures of the TFL1 protein including His88, Asp144, and a variable segment B of exon 4 (Fig. 1a). However, functional analysis involving genetic complementation of the *ft-1* mutant of *A. thaliana* (Ler) with *CpATFL1* indicated that *CpATFL1* was acting as a floral promoter: the *ft-1* mutants complemented with the *CpATFL1* gene flowered significantly earlier than the wild type *A. thaliana* plants. This response prompted a computational biology investigation of *CpATFL1* . Sequence comparison of *CpATFL1* with close relatives of *FT* in other Poaceae members including *ZCN7* and *ZCN8* (Lazakis, Coneva, & Colasanti, 2011) suggested that there is a conserved residue of Gln150 between two different gene sequences. Ho & Weigel (2014) showed that position 150-152 is conserved in *FT* -like sequences and is crucial for floral promoting activities (Fig. 4**a, b**), whereas these positions are much more variable in *TFL1* -like sequences. Position 150 in *TFL1* -like sequences is generally occupied by either Glu or Asp as observed in *ZCN1* and *OsRCN4* , homologues of *TFL1* in maize and rice respectively (Fig. 4b) (Ho & Weigel, 2014).

The change in Glu 150 to Gln 150 in *CpATFL1* is caused by a single base-pair change from GAA to CAA. Glu is a negatively charged amino acid while Gln is a positively charged amino acid (Fig. 4c). Ho & Weigel (2014) showed that altering the surface charge of *FT* and *TFL1* -like sequences can alter their corresponding activities. When analysed for the surface charge potential compared to ZCN8 (an FT homologue in maize) and ZCN1 (a TFL1 homologue in maize), CpATFL1 showed a similar charge distribution to ZCN8. The surface surrounding position 150 was strongly positive in ZCN8 and CpATFL1, whereas the surface around that position in ZCN1 was found to be highly negative (Fig. 4d). This change in the surface charge could be the reason for the flower promoting activity of CpATFL1, in contrast to the usual TFL1 activity.

Our gene expression studies also support the hypothesis that *CpATFL1* is associated with the induction of flowering in *C. pallens* (Fig. 2). Elevated expression of *CpATFL1* was observed during the inductive summer condition in the leaf samples from the plants that subsequently flowered in all the transplants and plants growing at the control site (1070 m) in natural conditions. It is interesting to point out that generally expression of *TFL1* -like proteins has been restricted to apical meristems (Liu et al.,2016). However, recent studies have shown that homologous *TFL1* -like proteins are expressed in the leaves of a number of perennial plants including *Arabis alpina, Rosa chinensis, Fragaria vesca, Jatropha curcus, Chrysanthemum morifolium* and *Manihot esculenta* (Adeyemo, Hyde, & Setter, 2019; Gao et al., 2019; Iwata et al., 2012; Li et al., 2017;

Wang et al., 2011). The specifics of the regulation of *TFL1* in perennial plant species is still relatively unknown, except for *A. alpina* .

Potential regulators of *CpATFL1* initiate the floral transition in *C. pallens*

Global expression analysis of the leaves collected during the summer inductive conditions from tillers that subsequently flowered shows upregulation of crucial genes including *CpFTIP1* and *CpFDP*that are involved in the transport of florigen-like molecules from the leaves to the apex to subsequently activate the floral meristem gene(s) through formation of the florigen activation complex (FAC) (Kaneko-Suzuki et al., 2018). Calcium is also required to catalyse the formation of the florigen activation complex (Kawamoto, Sasabe, Endo, Machida, & Araki, 2015).The expression of *CpCPK6* , a kinase required for calcium signalling, was also upregulated in the tillers that flowered in the next season. It is important to acknowledge that formation of FAC occurs at the shoot apical meristem. Even though the differential expression of these genes was observed in the leaves, it may suggest that CpATFL1 may form the FAC through the activity of CpFTIP1 and CpFDP, catalysed by CpCPK6 at the shoot apical meristem. Further investigation involving a yeast two hybrid assay or bimolecular fluorescence assay would reveal whether CpATFL1 and CpFTIP1 do indeed interact and could, therefore, transport ATFL1 to the apex.

Vernalisation responses to mediate flowering-time control in temperate monocots are controlled by the *VERNALISATION (VRN)* loci (Reams et al*.,* 2014). The *VRN2* locus encodes a CCT-domain protein that blocks the floral transition. Exposure to cold temperatures increases the expression of *VRN1* , a repressor of *VRN2* (Yan et al*.,* 2004) and maintains the repressive state of *VRN2*even after vernalisation. Repression of *VRN2* leads to activation of *VRN3* , a homologue of *FT* and *Hd3a,* after plants are exposed to warm spring temperatures (Preston & Kellogg, 2008; Shimada et al., 2009; Woods, et al., 2016). Transcriptomic analysis showed an elevated expression of *CpVRN1* in the tillers that flowered during the increase in summer temperature, which is also observed in other temperate cereals during the process of floral transition (Trevaskis, 2010). On the other hand, expression of*CpVRN2* , the repressor of *FT* -like genes (Alexandre & Hennig, 2007), was downregulated in the same samples. Greater expression of *CpVRN1* during the spring season and which remained elevated through the summer may have blocked repressive signals from*CpVRN2* . This repression was potentially maintained until the next summer resulting in the activated transcription of *CpATFL1* to induce flowering.

Transcriptomic analysis also revealed an increase in the expression of thermosensory genes, including *CpPIF4* and *CpPIF5* in the tillers associated with flowering in the next season. *PIF* genes are known to regulate responses to high temperatures (Choi & Oh, 2016) and are involved in the activation of the flowering process along with similar bHLH floral promoting proteins such as bHLH76 and bHLH80 (Ito et al., 2012). In addition to *PIF* -family genes, homologues of*bHLH76* and *bHLH80* in *C. pallens* , were also upregulated in the above samples, which may interact with CpPIF4 to activate *ATFL1* . Transient assays are further required to confirm this interaction. The summer temperature cue may also have blocked the expression of several floral repressors including homologues of*AP2-LIKE* genes and *SVP* (Capovilla, Schmid, & Pose, 2015; Mateos et al., 2015).

Emphasis was also placed on the role of epigenetic modifiers known to regulate the reproductive transition in plants. Ambient temperatures have been shown to regulate the expression of floral repressors via epigenetic modification including either through deposition of repressive histone marks (to suppress gene expression) such as H3K27me3 or by removing acetyl groups from the histone tails at the gene loci (Bratzel & Turck, 2015; He, 2012). In contrast, high temperatures have been shown to lead to deposition of active histone marks (H3K4me3 or H3K36me3) at the loci of floral promoters (Avramova, 2015).*CpREF6* and *CpFLD* , homologues of *REF6* and *FLD*which are reported to promote flowering (He, 2012; Lu, Cui, Zhang, Jenuwein, & Cao, 2011), were upregulated in the leaves of plants that flowered in the next season. CpFLD may then interact with CpHDA6, a histone deacetylase complex, activating the floral promoting genes, a process well established in Arabidopsis (Yu, Chang, & Wu, 2016). Homologues of *CpFLK* , *CpFVE* , and *CpFY* , genes known to repress the expression of floral repressors epigenetically (Cho, Yoon, & An, 2017; He & Amasino, 2005), were upregulated in the tillers that subsequently flowered. Interestingly, homologues of epigenetic

editors, including *CpVEL1* and *CpMSI1* , which are known to be involved in the activation of *VRN1* and *SOC1* , respectively through deposition of active histone marks (Higgins, Bailey, & Laurie, 2010; Oliver & Finnegan, 2011) were also upregulated in the tillers that flowered in the next season. This may suggest that an external signal such as summer temperatures may lead to certain epigenetic changes enabling the transcription of *CpATFL1* to promote flowering.

Endogenous signals are also necessary to initiate flowering during inductive conditions

Transcriptomic analysis also showed an increased expression of genes involved in gibberellin synthesis in the leaf samples associated with flowering tillers. Previous studies have already shown that gibberellin is able to initiate and accelerate flowering in *Chionochloa* sp.in the glasshouse (Martin, Jameson, Mark, Yeung, & Pharis, 1993) and the field (Turnbull et al., 2012). Gibberellin has also been shown to upregulate the expression of *FT* and *SOC1* independently of the photoperiodic pathway in Arabidopsis and several temperate cereals as well (Yu et al., 2012). Greater expression of gibberellin synthesis gene(s) may have promoted flowering in *C. pallens* by escalating the expression of *CpATFL1* and *CpMADS1* as observed in the transcriptomic studies. Along with gibberellin, expression of the sugar signalling genes, *CpUGT87A2* , *CpTPS1* and *CpHXK1* , was found to be significantly greater in the leaves of the tillers that flowered in the next season. Appropriate sugar levels are required as an internal standard for a plant to respond to environmental signals to induce the floral transition (Yang, Xu, Koo, He, & Poethig, 2013).

These data emphasise that, along with the external signals, *C. pallens* may require sufficient internal signals to respond and initiate the floral transition. This may explain why certain tillers in the experimental transplants did not undergo flowering even following the perception of an external cue such as warm summer temperatures. These vegetative tillers may be either too young or may be still in process of accumulating enough resources.

Hypothesised molecular model of flowering in *C. pallens*

This study showed a strong conservation of the activity of PEBP-like gene family members as floral promoters in *C. pallens* . Transcriptomic analysis further suggested that *C. pallens*undergoes flowering through the interaction of external and internal signals which can be integrated at the *CpATFL1* loci to initiate flowering.

We suggest that flowering in the masting plant *C. pallens* is a coordinated two-step process (Fig. 5). In the first stage, winters may suppress the activity of *CpVRN2* by increasing the expression of*CpVRN1* , thereby elevating the expression of *FT* -like genes in the spring, each year. However, an increase in the FTs does not necessarily correlate with flowering in plants in the next season. In the second stage, only plants with sufficient internal cues and consequently a greater expression of *CpATFL1* may be able to respond to the floral transition and, subsequently, flower. This two-stage process almost certainly requires additional epigenetic factors acting on *CpATFL1* to control the reproductive transition. Further analysis based on CHiP-seq may allow the identification of the specific epigenetic genes and the corresponding methylation changes in the histones to activate the flowering process in response to the environmental cues.

Because of the potential impacts of global climate change on masting phenology (Monks et al., 2016), and the potential down-stream impacts on introduced and native fauna (Griffiths & Barron, 2016), it is becoming increasingly important to accurately predict masting years. Such predictions are strongest when they are based on a detailed mechanistic understanding of the underlying control mechanisms. The current study has identified significant molecular regulators of flowering in *C. pallens* which can be used to explore changes in flowering gene expression under altered climates. This will allow the design of new masting models with greater confidence and to understand how mast flowering may change in the face of climate change.

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Performance of the research- Samarth and RL

Data analysis- Samarth, RL, RM and DK

Collection, or interpretation- Samarth, RL, RM, AMP, MT, DK and PEJ

Writing the manuscript- Samarth, DK, PEJ, MT and RM

TABLES

Table 1. *Chionochloa pallens* plants used for leaf sample collection in this study. All plants were initially growing at 1070 m on Mt Hutt. For each row, we took leaf samples from 3 tagged tillers. from each of 10 tagged plants

Name	Site	Treatment	Year collected	Fate of sampled tillers the n
16Control	Hutt 1070 m	unmanipulated	2016	vegetative only
17Control	Hutt 1070 m	unmanipulated	2017	vegetative only
18Control	Hutt 1070 m	unmanipulated	2018	flowering and vegetative
17Hot	UC 15 m	moved to 15 m for summer then back to 1070 m	2017	flowering and vegetative
16Hot	UC 15 m	moved to 15 m	2016	flowering and vegetative
16Cool	Hutt 1520 m	moved to 1520 m	2016	flowering and vegetative

Table 2: List of flowering pathway genes selected for expression analysis in *C. pallens*

Gene	*A. thaliana* homologue	*A. thaliana* locus ID	Rice homologue	Rice protein ID	% Identity	E-value
Cp GI	*GIGANTEA*	*AT1G22770*	*OsGI*	CAB56058.1	95.24	0
Cp Ehd3			*OsEhd3*	BAI77463.1	78.35	3.27E-52
Cp MADS50		*AT2G45660*	*OsMADS50*	Q9XJ60.1	78.39	0.00
CpHd1	*CO*	*AT5G15840*	*OsHd1*	BAB17628.1	73.06	1.00E-93
CpTPS1	*TPS1*	*AT1G78580*	*OsTPS1*	BAG89812.1	86.09	0.00E+0
CpMADS1	*SOC1*	*AT2G45660*	*ZmMADS1*	NP_001105152.1	78.8	8.50E-99
CpFT2	*FT*	*AT1G65480*	*OsHd3a*	BAB61030.1	85	3.90E-63
CpFT3	*FT*	*AT1G65480*	*OsHd3a*	BAB61030.1	62.75	1.26E-68
CpFT4	*FT*	*AT1G65480*	*OsHd3a*	BAB61030.1	72.9	4.09E-57
CpFT5	*FT*	*AT1G65480*	*OsHd3a*	BAB61030.1	66.06	6.13E-51
CpVRN1	*VRN1*	*AT3G18990*	*BdVRN1*	XP_010237091.1	86.08	3.57E-84
CpATFL1	*TFL1*	*AT5G03840*	*ZCN1*	ABW96224.1	82	0

Figures

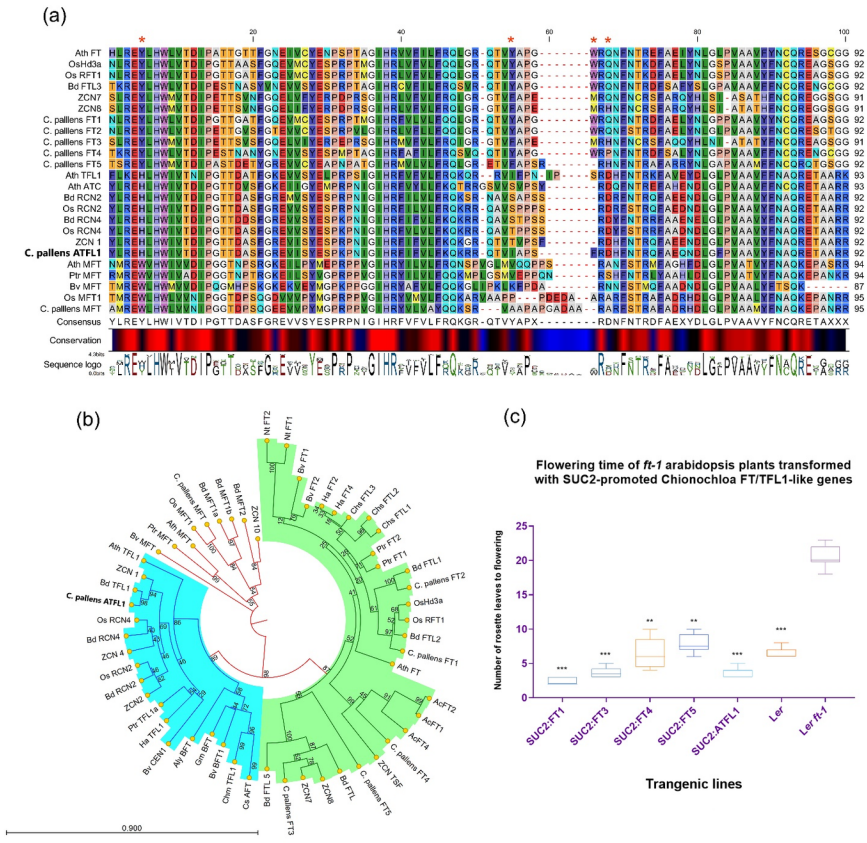

Fig. 1: **(a) Multiple sequence alignment of PEBP-like protein sequences.** Sequences were aligned using the MUSCLE alignment tool. The red asterisks indicate the conserved and key amino acid residues required for the corresponding activity of FT and TFL1-like sequences. The band at the bottom of the alignment represents the percentage of conservation of the amino acids at a position. Red indicates stronger conservation while blue represents variable sequences. **(b) Phylogenetic reconstruction of the PEBP-gene family with homologous *C. pallens* sequences** . The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Aly: *Arabidopsis lyrata* subsp. lyrata, Ath: *Arabidopsis thaliana* , Ac: *Allium cepa,* Bv:*Beta vulgaris,* Bd: *Brachypodium distachyon,* Cs:*Chrysanthemum seticuspe* , Chm: *Chrysanthemum X morifolium,*Gm: *Glycine max,* Ha: *Helianthus annuus,* Nt:*Nicotiana tabacum* , Osa: *Oryza sativa,* Ptr:*Populus trichocarpa,* ZCN: *Zea mays* and *C. pallens* . Branches in red colour represent the MFT clade, blue represent theTFL1 clade and green represent the FT clade. **(c) Functional characterisation of the *C. pallens* PEBP- like sequences.** The number of rosette leaves of the transgenic and the wild-type plants at flowering (mean ± SD, n = 10). (Significance of transgenic and control plants compared to Ler *ft-1* , Student's t-test *** P < 0.001, ** P <0.01).

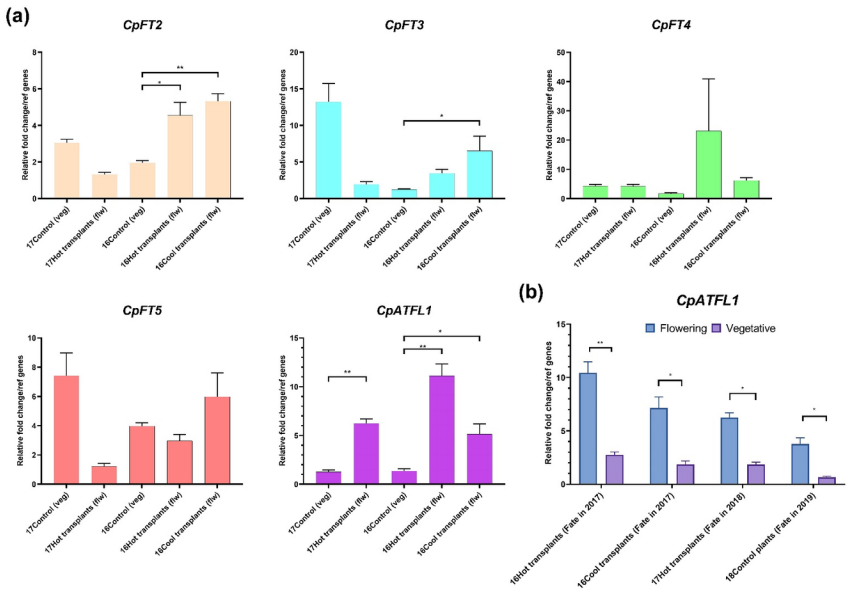

Fig. 2: **Gene expression analysis** of the orthologous PEBP-sequences in *C. pallens* during the inductive summer period in the plants (a) control plants at 1070 m in 2016 and 2017 (16 control and 17 control), plants translocated to UC for summer (December 2016-March 2017) (17Hot transplants) that flowered in 2018, plants transplanted to UC (16Hot transplants) and 1520 m Mt Hutt (16Cool) that flowered in 2017 (b) expression of *CpATFL1* in the leaves of the tillers that either flowered or remained vegetative at distinct altitudes including UC (15 m), 1520 m Mt Hutt and control plot (1070 m Mt Hutt). The data are represented by the mean ± S.D. of two biological replicates each with three technical replicates. The x-axis indicates the transplanted site from where leaf samples were collected and the fate of the plant either remaining vegetative (veg) or flowering (flw) the next season. The data were calculated using relative fold changes against two internal reference genes, *CpExP* and *CpTHP* . (two-way ANOVA, ** $P < 0.01$, * $P < 0.05$; Table S5).

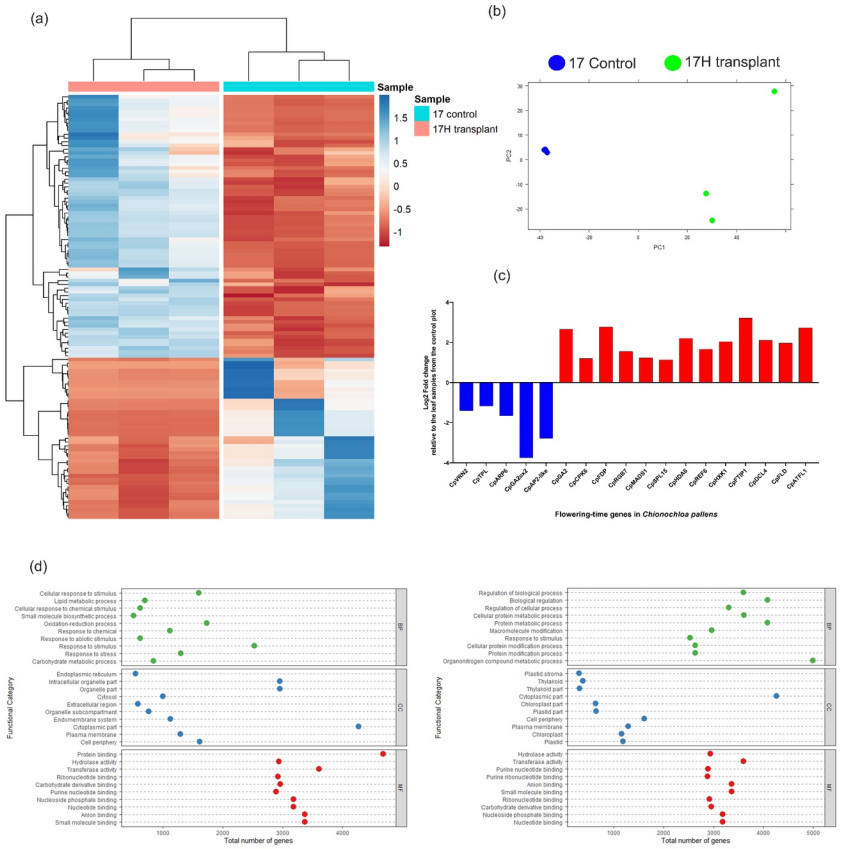

Fig. 3: (a) **Heat-map of 50 most significantly differentially expressed genes in the leaves of 17Control and 17Hot transplants (17H)**collected during the inductive summer period (January-2017). The heatmap was constructed using the R packages *DESeq2* and *heatmap.2* . Hierarchical clustering is based on the Spearman's coefficient calculated from the log-transformed RNA-seq data. (b) **PCA plot**of the transcriptomic data from three separate biological replicates, each for 17 control and 17Hot transplants. (c) **Differentially expressed flowering-pathway genes in *C. pallens*.** Logarithmic ($\log_2$) fold change in the orthologous floral genes found to be significantly differentially expressed in the leaves of the 17Hot transplants that flowered in the next season relative to the leaf samples from the control plot plants that remained vegetative. (d)**Gene Ontology analysis.** GO classification of the upregulated genes (left-hand side) and downregulated genes (right-hand side) in the leaves of the 17Hot transplants that flowered compared to the leaves from the plants at the control plot with a significance level of 0.05 after the Benjamini and Hochberg FDR correlation. (BP: Biological Process, CC: Cellular Component, MF: Molecular Functions)

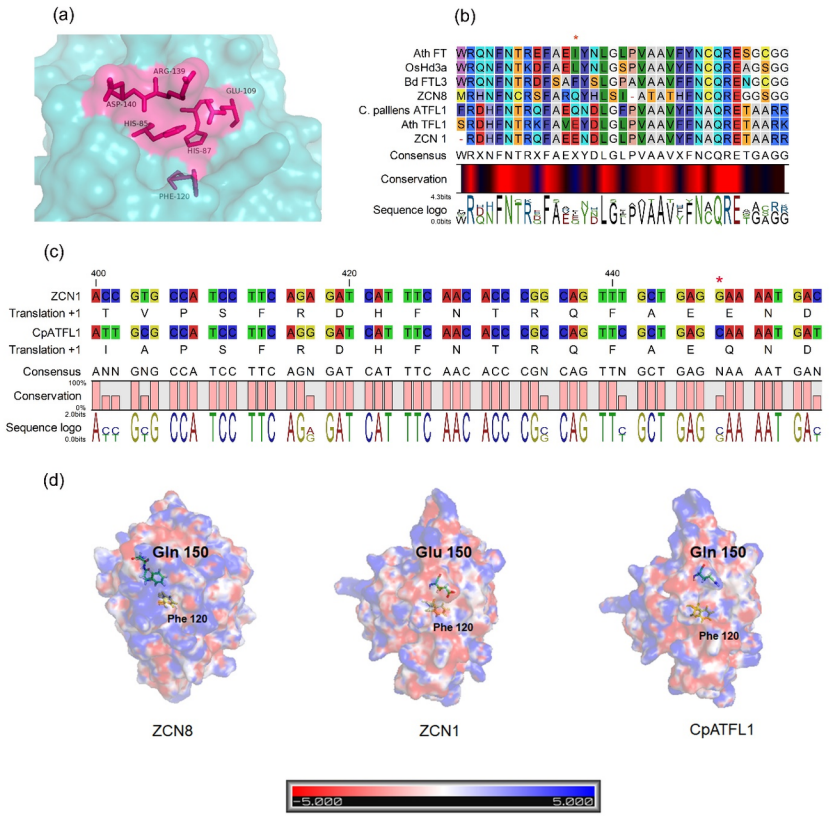

Fig. 4: (a) **Molecular structure of CpATFL1.** The protein structure was built based on the *ab initio* approach from the QUARK server. The highlighted pink region represents the conserved critical amino acids for the activity of TFL1 as a repressor with a reference amino acid, Phe at the position 120, situated towards the bottom region. (b) **Multiple sequence alignment of FT and TFL1 homologues in *A. thaliana* (ATH), *Zea mays* (ZCN), *O. sativa* (Os), *B. distachyon* and *C. pallens*.** Sequences were aligned using the MUSCLE alignment tool. The level of conservation of amino acids is represented by the colour graph at the bottom of the alignment. Red represents stronger conservation while black represents variable amino acid sites. The red asterisk represents the critical amino acid change (Glu-> Gln) in CpATFL1 which may be responsible for its floral-promoting activity. (c) **Codon usage between ZCN1, a TFL1 homologue in *Z. mays*, and CpATFL1.** * represents the single nucleotide change coding for amino acid at position 150. (d) **Electrostatic surface charge for ZCN8, ZCN1 and CpATFL1** . Electrostatic surface potential for each sequence was calculated from the CHARMM server. Protein structures were viewed using the pymol represented by the amino acid at position 150 and a reference amino acid, Phe, at position 120 to orient the structures.

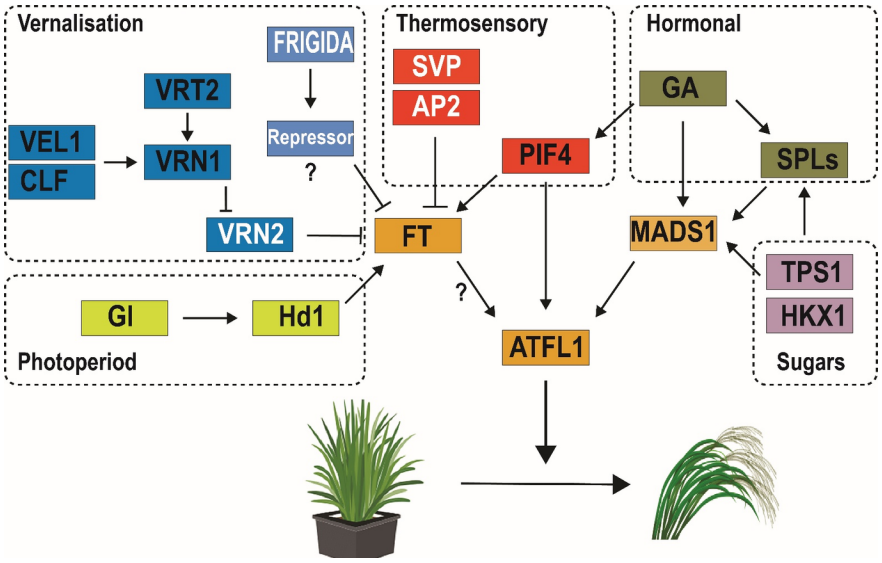

Fig. 5: **Hypothesised flowering network in *C. pallens*** . In *C. pallens* , *ATFL1* acts as the key floral integrator gene. During non-inductive conditions, homologues of floral repressors including FRI-like, AP2 and SVP can block the process of floral transition. Photoperiodic (GI, CO) and vernalisation (VRN1, VRN2) pathways may interact to activate the expression of *FT* -like genes during inductive conditions every year. However, only plants with sufficient internal cues (e.g. sucrose content (*TPS1*), maturity (*SPLs*) and gibberellin (GA)) are able to respond to the external signals such as summer temperatures (PIF4,5) which can then elevate the expression of *CpATFL1* to induce flowering in the following season. The yellow boxes indicate homologues of the floral integrator genes identified in *C. pallens*. The solid lines indicate co-expression of genes based on the transcriptomic data and known literature, while question mark indicates a hypothetical co-regulation between two genes which may exist in model species but have not been discovered yet. Genes represented with the black and the white ink, respectively represent the up- and down-regulation in the expression of corresponding genes observed in the tillers that flowered in the next season compared to tillers that remained vegetative.