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UNDERSTANDING THE REGULATION OF FLOWERING TIME THROUGH *PHOTOPERIOD-1* (*Ppd-1*) and *FLOWERING LOCUS T* (*FT*) GENES IN BREAD WHEAT

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Abstract

Wheat plants have evolved ingenious and controlled ways to initiate flowering to maximise reproductive success. These avoid the extremes of high and low temperatures associated with deep summer and winter to successfully produce viable offspring in the form of fertile seed. One of these systems of control is known as the photoperiod response and involves the sensing of daylength which when extended over 12 hours, means it is suitable for *Triticum aestivum* (bread wheat) to initiate flowering. The main gene responsible for the detection of the daylength extension is known as *PHOTOPERIOD 1* (*Ppd-1*). Ppd-1 initiates flowering through the activation of *FLOWERING LOCUS T* 1 (*FT1*), which trans-locates from the leaf to the apex and begins the apical transition from being vegetative to reproductive. Previous work has also identified and characterised *FT2* and *FT3* function, relating it to the commencement of the transition to terminal spikelet from lemma primordia. However, despite other *FT*s being identified (*FT4-12*), their functions remain unknown in hexaploid wheat.

My research shows that expression of the higher *FT*s (*FT4-12*) is present in various of the apical meristem stages in wheat and suggests potential Ppd-1 regulation of *FT9* and *FT10*. Furthermore, direct interaction of Ppd-1 with the *FT1* promoter has never been observed and speculation of this interaction comes from expression studies of these two genes in *Ppd-1* Near Isogenic Lines (NILs). In this thesis, I present evidence of this interaction through a yeast-1-hybrid study and show, to a degree of 500 base-pairs, where *Ppd-1* interacts. Previous studies have focused on flowering time and spikelet number when phenotyping the NILs associated with *Ppd-1*. I confirm this and also show that *Ppd-1* also has an effect on other traits such as internode length and ear length.

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Chapter 1: Introduction

1.0 The role of photoperiod in regulating flowering time in bread wheat

The story of human farming and domestication of crops begins in the fertile crescent (modern day Middle East) around 9000 BCE with the very first cultivation of plants for food and the ancestor to the wheat we farm today (Kilian, et al., 2010). Over 11,000 years of trial and error, humans have carefully selected desirable traits that we observed within wheat today. There are two main wheat species being the tetraploid durum/pasta (*Triticum durum*) and the hexaploid bread (*Triticum aestivum*) wheat (Nesbitt, 2001). Wheat has the third highest global gross production value in terms of agricultural products and provides us with a stable supply of carbohydrates and protein in the form of commodities such as bread and pasta (Shiferaw, et al., 2013). It is consequently very important that we gain knowledge of an important process in terms of securing a high yield and better food security – flowering time. Armed with a greater understanding of this, we can accurately predict when plants will flower depending on their variety within the degree of a few days. We could also manipulate the genes to create new strains that will flower reliably according to the local growing environment, maximising its efficiency in the chosen environment.

It is only now that through modern genetic analysis we can see the precise effects of such breeding and how we can improve on it to further develop the wheat crop. One way in which we have started to do this is by selecting for plants that are either sensitive or insensitive to photoperiod length (Royo, et al., 2020). The photoperiod is the period of daylength, or light, that is received within one day. Many organism's, including wheat, utilize this duration, in the form of monitoring the 24 hour daily or circadian cycle to co-ordinate growth and development with the environment and so improve survival rates (Worland, et al., 1998) (Fjellheim S, 2014). Wheat that is sensitive to the photoperiod is responsive to the changing daylengths, and so can interpret different seasons. From an evolutionary perspective, this improves the chances of flowering at a time when the weather is optimal, and the plant therefore has a better chance of not receiving damage to its reproductive organs. Photoperiod insensitivity is when the plant initiates floral development irrespective of the day-length (Royo, et al., 2020). Conscientious selection for insensitivity to photoperiod occurred during the green revolution and was part of the key to the adaptation improvements in wheat which enabled it to be grown for higher yields around the globe (Fjellheim, et al., 2014). Both photoperiod insensitivity and sensitivity have several advantages depending on the climate in which they are grown. For example, it would be preferable to grow photoperiod insensitive wheat in a country like Spain due to the short growing season where the plants can flower regardless of the photoperiod. Whereas in countries with a much longer growing season like the U.K photoperiod insensitive wheat is advantageous and produces a higher yield than photoperiod insensitive wheat grown under the same conditions (Royo, et al., 2020)

There are similarities between the genetic mechanisms in the monocot *T. aestivum* and the model dicot *Arabidopsis thaliana* in terms of flowering, but complexities develop due to evolutionary divergence and as wheat has a hexaploid genome rather than the more usual diploid genome found in *Arabidopsis* (Peng, et al., 2015). Wheat is an allohexaploid and so has three distinct diploid genomes, the A, B and D genomes, and

so, due to the highly syntenic nature of these genomes, it is usual that three copies of any gene exists as opposed to one found in diploid. This means understanding the wheat plant becomes more complex. To address some of the complexity researchers have used the relationship between photoperiod and flowering in other closely related grasses such as rice, barley and maize has provided further insight into how photoperiod and flowering genes interact with each other (Peng, et al., 2015).

1.1 Co-ordinating flowering with seasons

The timing of flowering in *Arabidopsis* is closely regulated through the circadian clock and genes associated with this. Over a 24-hour period the plant uses different genes associated with being more highly expressed according to the stage in the diurnal cycle. This ensures that the plant is initiating different processes over the course of a day at the optimal times (Boden & Gauley, 2020). In photoperiod sensitive varieties of *Arabidopsis*, the day/night cycles are monitored across the seasons, initiating flowering only once the photoperiod is of the appropriate length. This regulation ensures that the apical transition from vegetative to reproductive only occurs in conditions that are favourable to floral survival, i.e. not too cold or too hot (Danyluk, et al., 2003) (Trevaskis, et al., 2003) (Bentley, et al., 2013) (Kitagawa, et al., 2012). Flowering genes were first studied in *Arabidopsis* and related back to wheat due to both plants possessing photoperiod dependant flowering. When the photoperiod length is adequate for *Arabidopsis* to begin flowering, *FLOWERING LOCUS T* (*FT*) is expressed. This gene is expressed in the form of a mobile florigen protein signal that travels from the leaf, through the phloem, to the apical meristem (Corbesier, et al., 2007). Here, it forms a complex with *FLOWERING LOCUS D* (*FD*) which is a bZIP transcription factor and 14-3-3 protein (Li, et al., 2015). This then interacts with floral identity genes that transform the apex to being in a reproductive state.

The photoperiod response comes in two main forms: sensitivity and insensitivity and these stem from one gene in wheat – *PHOTOPERIOD 1* (*Ppd-1*) (Bentley, et al., 2013). In countries with long growing seasons, varieties that are Ppd sensitive are sown in autumn where the plant will germinate and breach the surface (Bentley, et al., 2013). However, once they reach around the four-leaf stage above the ground, they will remain dormant and stay in a vegetative state. This may seem counter-intuitive, but evolutionarily speaking, there is a calculated reason for it. The winter months that succeed autumn are harsh and cold and usually threaten to physically damage the florets that the wheat would produce if they were to flower in this time. Vernalization, the requirement for a prolonged period of cold before flowering will occur, is also involved for this tightly timed flowering, and works in conjunction with *Ppd-1* to facilitate this. The vernalization response is controlled by *VERNALIZATION 1* (*Vrn-1*) and *VERNALIZATION 2* (*Vrn-2*) genes that are activated in response to the change of temperatures detected by the wheat plant as the seasons become warmer or colder (Chen & Dubcovsky, 2012) (Dubcovsky, 2006). As the temperature increases towards the end of winter and the cold period requirement has been met, *Vrn-1* acts to repress *Vrn-2*, which was repressing the *FLOWERING LOCUS T* gene (*FT1*) prior to and during the cold period (Dubcovsky, 2006)

(Trevaskis, et al., 2006). In addition, the area where wheat first evolved also had extreme high temperatures in the summer to contend with, so these varieties evolved to flower late enough to avoid the cold and early enough to avoid drought (Fjellheim, et al., 2014).

Over thousands of years of evolution, grasses that grew in polarised seasons had to finely tune their reproductive cycles to avoid sterility and death caused by weather extremities and therefore go on to pass on their genes when the conditions were milder and more suited for them to do so (Fjellheim, et al., 2014). It is therefore vital that they can detect the temperature and day-length of their environment, so they do not flower too quickly or too late. Vernalization and photoperiod pathways manage the response to these stimuli. Once the vernalization requirement is met, *Ppd-1* further activates the expression of *FT1* which was being repressed by *Vrn-2* prior to vernalization. FT1 is believed to trans-locate from the leaf to the apical meristem where it triggers the transition through the apical reproductive stages (Corbesier, et al., 2007) (Li, et al., 2015). In *Ppd-1* sensitive lines, *Ppd-1* expression follows a diurnal cycle where its expression peaks in the morning but has low expression at dawn. *Ppd-1* insensitive varieties have high expression, even when there is no light and do not conform to this diurnal pattern. This, in turn, means there are high levels of FT1 causing the vegetative to reproductive transition to occur earlier (Jones, et al., 2016).

As *T. aestivum* is a hexaploid, there are three copies of the *Ppd-1* gene, one on each of the three genomes, with each gene contributing differently to the photoperiod response. The strength of insensitivity to the photoperiod is dependent on whether there is either a deletion of specific promoter motifs or an increase in copy number in the A, B and D genomes. A bread wheat plant can have different combinations of these allelic variations, and this is what gives rise to these varying degrees of insensitivity. The cause of photoperiod insensitivity to the photoperiod in the D genome is a 2069 base pair deletion (Figure 3) in the promoter region of the *Ppd-1* exon and it is thought that the nucleotides that have been removed are responsible for coding regulatory motifs (Beales, et al., 2007). Deletion of this region therefore leads to less repression of *Ppd-1* expression causing the loss of usual daily gene expression pattern that we observe. By using a PCR assay, Beales et al (2007) detected this 2kb deletion in a number of varieties of bread wheat (Beales, et al., 2007). These varieties were then grown until ear emergence under 9-hour light, 15-hour dark photoperiods to see if the deletion correlated with the emergence time. It was found that known sensitive lines such as Hunter and Club did not carry the deletion and insensitive varieties such as Texel and Talent did. This confirmed that the sequence that has been removed in insensitive wheat must be responsible for the regulation of *Ppd-D1* expression and once gone, this control is reduced. This deletion in the regulatory region is thought to be similar in the A genome but as the transcript is not prominent in insensitive lines it has less of an effect compared with *Ppd-D1* insensitive varieties (Beales, et al., 2007)

In the B genome, an increase in copy number causes photoperiod insensitivity. Copy number variation was determined using a Taqman® assay to detect the specific *Ppd-B1* copies in different varieties (Diaz, et al., 2012). In the varieties that are known to have a photoperiod-insensitive phenotype such as Chinese Spring, extra copy numbers were detected. Whereas in varieties known to be photoperiod sensitive, none were found.

This genotypic variation of extra copy number confers a similar mis-expression seen in insensitive *Ppd-A1* and *Ppd-D1* wheat which are instead caused by a promoter mutation (Diaz, et al., 2012) (Seki, et al., 2013). A photoperiod sensitive *Ppd-B1* and a photoperiod insensitive *Ppd-B1* winter variety were treated with the same vernalization period. The photoperiod sensitive variety flowered 143 days after germination and the photoperiod insensitive variety flowered 181 days. This difference of 38 days displays the impact that an increase in copy number at the *Ppd-B1* allele can have on theflowering time phenotype. The group also used Taqman® assays to see if there were any copy number variants (CNVs) in *Ppd-A1* and *Ppd-D1* but none were found. It is unclear as to why the *Ppd-B1* insensitivity is different from the other alleles in the A and D genomes, but the most probable theory is that it is due to chance (Diaz, et al., 2012).

A study into the link between photoperiod sensitivity/insensitivity and dry weight produced at harvest was conducted by Perez-Gianmarco et al (2019). Plants were grown in controlled conditions in glasshouses and 4 different genotypes of *Ppd-1* sensitivity were used: Three variants of single insensitive; *Ppd-A1a*, *Ppd-B1a*, *Ppd-D1a* and triple insensitive. A wild-type Paragon variety was used as a control and the plants with varying sensitivities to the photoperiod were near-isogenic lines of this (Perez-Gianmarco, et al., 2019). The photoperiod was either 12 hours of light and 12 hours of dark or 16 hours of day and 8 hours of dark. At anthesis the plants were then dissected to count the number of fertile florets per meristem and were considered to be fertile if they displayed yellow anthers or were given a floret score of over 9.5. After the counting had taken place, the spikelets were then dried out in the oven at 65^{o}C to determine the dry weight at anthesis (Perez-Gianmarco, et al., 2019). The findings showed that triple insensitive varieties have lowered spike weight at anthesis compared to the wild-type Paragon (*Ppd-1* sensitive) control. However, the triple effect of the combined alleles in the triple insensitive line appeared to reach a maximum that fell close to single insensitive Ppd-D1 on its own. This would back up the theory that the *Ppd-D1* allele provides the majority of the transcript in insensitive varieties.

It seems to be the deletion of the regulatory region in the D genome that has the biggest effect on insensitivity. *Ppd-1* is part of a class of genes known as *PSEUDO RESPONSE REGULATORS* (*PRR*'s) and these are responsible for the timing of events in the plants circadian clock. Due to *Ppd-1* being homologous to the *Arabidopsis PRR7 and PRR3* genes it was assumed that it may functioned similarly. Despite this homology, it is not the case as mutations to *Ppd-1* do not affect the function of other circadian related genes, e.g., *PRR73*, *TOC1*, *GI* or *CDF* in wheat (Shaw, et al., 2012).

1.2 *Ppd-1* Loss of function in bread wheat

The role of Ppd-1 has been further studied through loss of function mutants, in mutants developed in the Spring cultivar, Paragon. A study by Shaw et al (2013) covered many aspects of *Ppd-1* and flowering and one of the main components was to observe what happened when the *Ppd-1* gene was removed altogether. Mutants were developed in Paragon where they had a single loss-of-function deletion on the A, B or D genome, a double loss-of-function on the A and B, A and D, or B and D or the triple loss-of-function

deletion on the A, B and D genomes (Shaw, et al., 2013). The initial mutations were developed through gamma irradiation to identify *Ppd-B1* and these plants were then crossed with lines lacking either *Ppd-D1* and *A1* genes to create the genotype combinations. The wheat plants were then grown over 18-hour day, 6-hour night cycle at 16^{o}C where samples were taken every 3 hours. These samples were then analysed for *TaFT* and *TaCO* expression and some interesting results were found (Shaw, et al., 2013).

In the wild-type Paragon varieties there are no deletions of *Ppd-1* and high expression of *FT1* which peaks in the morning at the 3-hour point after dawn, decreases throughout the day, and then peaks again between 15-18 hours.. The other genotypes which include *Ppd-D1a* (D insensitive) and *Ppd-B1a* (B insensitive) followed a similar pattern however there is a notably large difference in *FT* expression with Paragon with a deletion of *Ppd-1* in all three of the genomes, A, B and D. When the *TaCO* expression was analysed, the expression pattern that was observed with *TaFT* was completely flipped with all genotypes showing a peaked expression in at 3 hours and then again at 15-21 hours. The Paragon Wild-type had the lowest *TaCO1* expression whilst the Paragon lacking Ppd-1 in all three genomes had the highest. This supports the hypothesis that there is a feedback loop between *FT1* and *CO1*. Flowering still occurred in the triple-knockout wheat; however, it was around 28-30 days later than the Paragon (Shaw, et al., 2013).

CONSTANS 1 and *2* (*CO1 and CO2*) genes are regulated in the plant circadian clock and help modulate the flowering response to external environmental changes in daylight length. As wheat are long day plants, the genes work in unison with *Ppd-1* to promote flowering when the days become longer than 12 hours (Chen, et al., 2014) (Shaw, et al., 2013).

The deletion of the *Ppd-B1* allele on its own had the highest single effect on flowering time, with a 10-15 day later flowering compared to the control. Also, the *Ppd-A1* deletion had a 1-5 day later flowering effect and there was no difference observed between the *Ppd-D1* deletion and the Paragon. The fact that the *Ppd-B1* deletion had the greatest effect suggests that the dominant transcript is present on the B genome. On the other hand, the authors of this paper suggest this dominance may only exist in Paragon varieties and more studies need to be done on other lines to confirm if this phenomenon is universal (Shaw, et al., 2013). They also suggest that criticism of this theory could come in the form of adjacent gene removal causing the phenotype observed. However, the direct effect of *Ppd-B1* is favoured by increased lateness of alleles with *Ppd-1* mutational permeations (Shaw, et al., 2013).

1.3 The *Ppd-1* flowering response target - *FT1*

Flowering in bread wheat is an extremely controlled process that requires multiple activator and repressor genes to work in tandem to maximise reproductive success. One family of genes which appears to be particularly important in photoperiod mediated flowering control are the *FLOWERING LOCUS T* (*FT*) genes. Members of this family are known to act as both activators and repressors of the flowering response. Genetic and genomic studies have allowed to us identify a large family of *FT* genes in the monocots

(Bennett. & Dixon, 2021) (Halliwell, et al., 2016). In bread wheat there are 12 *FT* genes, with copies on the A, B and D genomes and additional duplications making 12 in total; however, we only know the approximate function of 2 of the 12 found. The first of the family to be characterised is *FT1* which has been identified through QTL studies relating to the vernalization and photoperiod response (Chen, et al., 2014) The relationship between *Ppd-1* and *FT1* has recently been established as key to photoperiod regulated flowering development. In photoperiod sensitive varieties, once the daylight is of the appropriate length (>12 hours), *Ppd-1* will become expressed, and the protein is believed to subsequently bind *FT1* in the leaf and activate its expression. This FT protein is thought to travel to the apex which is believed to cause it to transition from vegetative to reproductive and is observed in *Arabidopsis FT* (Tiwari, et al., 2010). When a wheat plant is triple insensitive to photoperiod, there is a deletion of the promoter region in the A and D and increase of copy number in the B (Beales, et al., 2007) (Diaz, et al., 2012). This gives wheat the greatest insensitivity to photoperiod, and increased Ppd-1 expression levels which means *FT1* levels are therefore constantly elevated (Shaw, et al., 2012).

Studies from vernalization experiments had identified that the *FT1* gene on the B-genome was the dominant allele in the flowering response (Yan, et al., 2006). Expression studies also confirmed it to be the major target for *Ppd-1* regulation (Shaw, et al., 2012). Overexpression via *FT1* promoter mis regulation from the Hope-1 allele of *FT1* (Nitcher, et al., 2014) caused the plant to ignore floral repression signals and initiate flowering at a very early stage (Dixon, et al., 2018). This allele originated from the Hope variety of wheat which is known to exhibit and early flowering phenotype. In plants lacking *FT-B1* extremely late flowering was observed flowering approximately thirty days later in 18^{o}C and 10-15 days later in 24^{o}C. Flowering still occurred which could be as *FT-A1* and *FT-D1* were still activated by the vernalization and photoperiod pathways but as *FT-B1* is dominant, this happened at a later stage (Dixon, et al., 2018).

The wheat apexes were dissected at different timepoints to see how their development rate was affected by the lack of *FT-B1* expression and the results are what you might expect. There was a substantial delay between when the *FT-B1* null apexes transitioned to the double ridged stage compared with the parental lines and this delay was also observed for the transition to terminal spikelet. Interestingly, however, there was a noticeable increase in the number of spikelets produced in the *FT-B1* null wheat. This indicates that *FT-B1* may also have a role in accelerating the plant through all of the apical developmental stages and not just to initiate the process (Dixon, et al., 2018). More tillers were also seen to have grown in the *FT* null line. Dixon *et al* suggest this is due to tillers stemming from vegetative nodes and as *FT-B1* accelerates the development of plants to reach maturity, less grow out when the FT1 signal is strong. This experiment was conducted in relation to temperature and how this affects floral development. I would be very interesting to investigate how the plants reacts when the prominent *FT1* allele is deleted with respect to photoperiod signalling via Ppd-1 (Dixon, et al., 2018).

As previously discussed, when the photoperiod is of more than 12 hours, Ppd-1 activates *FT1* in the leaf (Chen, et al., 2014). A florigen signal, largely believed to be formed of FT

protein, is sent from the leaf phloem to the apical meristem where it binds and initiates flowering through expression of the floral development genes (Dixon, et al., 2018). The meristem then transitions through the reproductive stages from double ridged through to terminal spikelet. Studies in rice have shown that *FT* genes *Heading Date 3a* (*Hd3a)*, *Rice flowering locus 1* (*RFT1*), bZIP transcription factors and Gf14 proteins combine into either florigen activator or repressor complexes which determine whether the meristem flowers depending on external environmental stimuli (Li, et al., 2015). It is thought that these interactions may also happen in other grasses species such as barley or wheat. There are parallels that can be made despite rice and barley being diploid and *Triticum aestivum* being a hexaploid. All are grass species that have very similar flowering pathways and if these flowering repressor and activator complexes are present in rice and barley it is very likely that they are in wheat also (Li, et al., 2015).

To investigate this, yeast 2-hybrids were generated to study protein interactions between integrated flowering gene products and 14-3-3 proteins (Li, et al., 2015). Different 14-3-3 proteins were used as bait and variations of *HvFDL* and *TaFDL* were used as prey. When these proteins interacted with each other, they activate reporter genes which enable growth on selective agar. It was found that there were complexes formed between *TaFT* and most of the 14-3-3 proteins tested. They also found that 14-3-3C formed a complex with barley *FT3*, *FT4* and *FT5*. This may therefore also occur between wheat *FT3-5* and 14-3-3C (Li, et al., 2015).

1.4 The *Ppd-1* flowering response target - *FT2*

Recently, a role for *FT2* has been identified in wheat (Boden & Gauley, 2020). Through taking meristem samples at different timepoints during the development of the apical meristem: vegetative, double-ridged, lemma primordia and terminal spikelet, the expression of *FT2* could be traced in different Ppd-1 allelic backgrounds (Boden & Gauley, 2020). This type of analysis is incredibly valuable as they are studying the mRNA and therefore seeing exactly what the cells are expressing. The analysis identified that when there were higher levels of *FT2* expressed it correlated with a decrease in the number of spikelets produced. Furthermore, it was observed that as there was higher expression in *Ppd-1* insensitive varieties compared to the wild-type Paragon, it was therefore concluded that these patterns might be linked (Boden & Gauley, 2020). *FT2* expression begins to rise at the lemma primordia stage and peaks at the terminal spikelet stage of the apex. The results indicate that increased Ppd-1 leads to increased *FT2*, as observed for FT1 expression, and this causes a quicker transition between these two stages which results in fewer spikelets being produced (Boden & Gauley, 2020). To further test this theory, they grew mutants of the *FT-B2* allele which encodes a non-functional protein. These mutants had roughly 12 more spikelets than the wild-type control with a functional *FT-B2* gene (Boden & Gauley, 2020). The *FT-B2* mutants still progressed to the terminal spikelet stage which suggests, similar again to *FT-B1* regulation, that a single deletion can be compensated by other gene copies. The plants in the experiment still had active *FT-A2* and *FT-D2* alleles which would still be functioning to generate FT2 proteins. Alternatively, other *FT* genes may act as failsafe's that kick in

when the plant detects that the transition has not occurred. These findings were also echoed in *Brachypodium* where overexpression of *FT2* led to fewer spikelets being produced and *FT2* expression was seen later in plant development as seen in wheat (Shaw, et al., 2019). One prospect regarding these findings could be altering *FT2* expression in order to obtain more spikelets without a penalty to the heading time. As we find out more about how the other *FT*s interact with each other, we could design plants that have this trait.

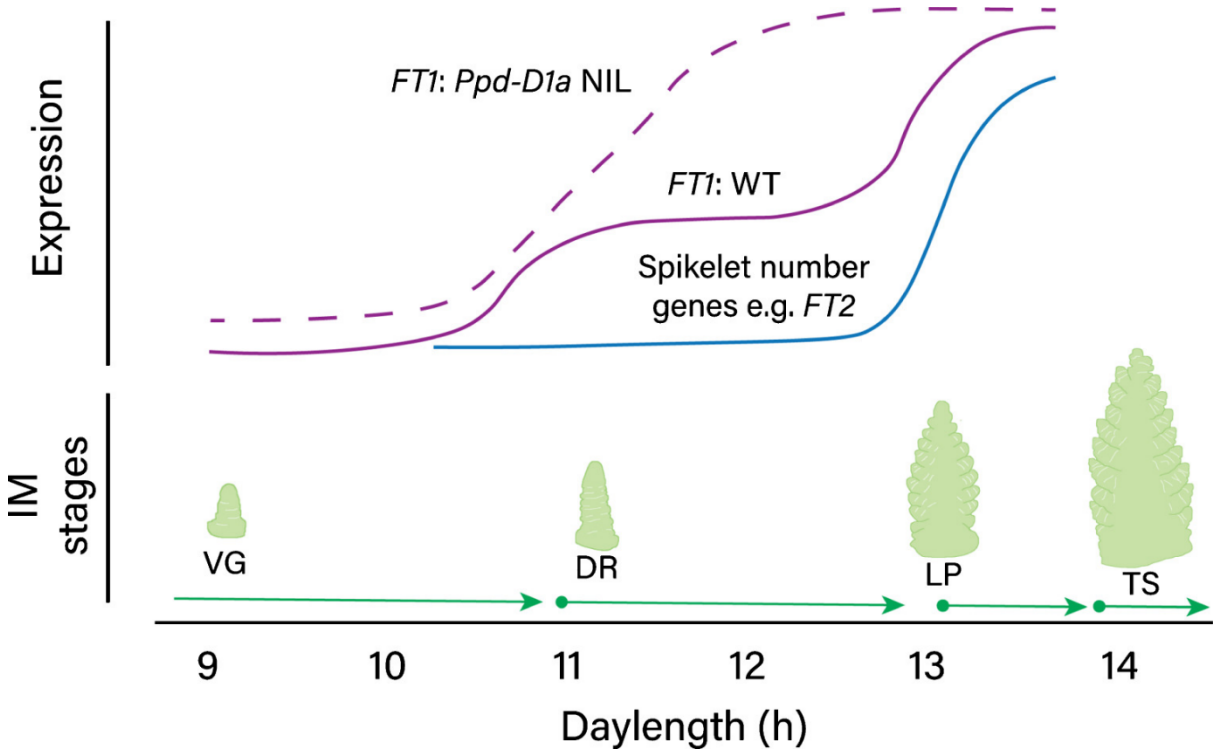

Figure 1.2: Investigation into *FT1* and *FT2* expression in WT Paragon and single insensitive *Ppd-D1a* NILs (SI) across different stages of apical development. Apical stages are represented as: VG (vegetative), DR (double ridge), LP (lemma primordia), TS (terminal spikelet). *FT1* shown by purple line with the dashed line representing *Ppd-D1a* NILs and solid line representing WT Paragon. *FT2* expression is represented by a solid blue line. Graph taken from (Boden & Gauley, 2020).

The described relationship between *Ppd-1* and spikelet production at the level of different *Ppd-1* alleles (discussed in section 1.1 (Perez-Gianmarco, et al., 2019)) echo the findings in (Boden & Gauley, 2020) as the increased *FT2* expression in the insensitive lines would mean that the dry weight and spikelet number would be decreased (see Figure 1.3) (Boden & Gauley, 2020). As previously stated, this could be because of *FT2* expression in the transitionary stage between lemma primordia and terminal spikelet, speeding up the change. This, in turn, will limit the number of spikelets produced and therefore affect the dry weight.

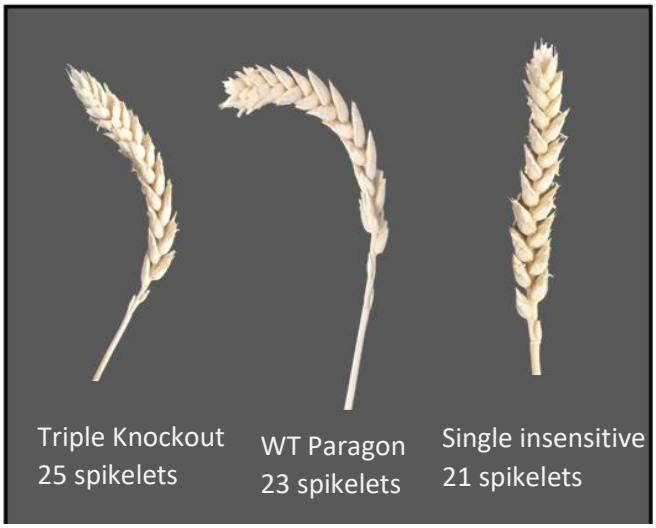

Figure 1.3: Differences in spikelet number between Ppd-1 NILs; single insensitive (SI) and triple knockout (TK) and WT Paragon. Wheat ears showing differences in spikelet number between the three genotypes.

1.5 *FT* genes 3-12 have been identified

Until now only two of the *FT* genes in *T. aestivum* have been properly identified and their functions characterised in any detail – *TaFT1* and *TaFT2*. Through further analysis of the genome, nine more *FT* genes have been identified that we believe have a role in the flowering process of wheat and functions of these genes have been hypothesised in subsequent reviews of the topic (Bennett. & Dixon, 2021). A similar degree of FT family expansion has also been identified in barley (Halliwell, 2016; Pieper 2020; Bennett, 2021). As barley is a diploid, it is easier to conduct genetic analysis in, but the results are often extremely applicable to wheat due to their shared evolutionary history and similar developmental programmes. Two additional FT's have been characterised in barley and to a lesser extent in wheat. FT3 is also referred to as Ppd-2 as it was identified as a minor effect photoperiod QTL.

Some investigation into *HvFT3* has been conducted in barley where they discovered a potential function through *HvFT3* overexpression and then the subsequent monitoring of this expression. They found that the overexpression of *HvFT3* led to the process of spikelet formation being accelerated but did not have any significant effect on flowering time (Muhammed, et al., 2018). This was evident in the transgenic winter varieties of *Hordeum vulgare* where stronger *HvFT3* signals did not initiate flowering in short days like you would expect when *FT1* is overexpressed (Dixon, et al., 2018). This shows that *HvFT1* and *HvFT3* are functionally different. However, the overexpression of *HvFT3* was dominant over its repression by *Vrn-2* in spring and winter varieties in short-days and therefore spikelet formation was initiated earlier (Muhammed, et al., 2018). These findings were further confirmed through expression of *HvFT3* in barley where they found

that the vernalization gene *Vrn-H1* was upregulated by *HvFT3* (Mulki, et al., 2018). One could postulate that these mechanisms of *HvFT3* would be the same in *T.aestivum*. This comparison between *T. aestivum and H. vulgare FT3* genes have been made where it was found through qPCR and phylogenetic analysis that 95-96% of the coding region and 96-97% of the amino acid sequence are identical (Halliwell, et al., 2016). Furthermore, when recessive alleles of *HvFT3* were studied it was found that this initiated flowering in short day (SD) conditions (Halliwell, et al., 2016). This implies *FT3s* role as a short-day inducer of flowering and this phenotype may protect the barley from early flowering and therefore colder temperatures associated with short photoperiods.

With regards to *HvFT4*, overexpression conferred a later flowering time in long days (Pieper, et al., 2021). Overexpression also decreased the grain mass harvested by reducing grain size, number of tillers and spikelet number. This indicates *HvFT4* potentially having a repressive role in barley floral development and as *HvFT3* share protein and gene homology with *TaFT3*, this could also be the case with *FT4*. For *FT5-12*, there has been little to no research into their function in grass species such as in wheat. We can only make educated guesses on their function based on their amino acid sequences that may indicate if they are activators or repressive (Bennett. & Dixon, 2021). These educated guesses came in the form of amino acid homology between members of the *FT* family (*FT1-12*) and attempt to predict if these genes are regulated through *Ppd-1*, like seen with *FT1* and *FT2*, and if they play repressive or activator functions. To do this, the *FT*s were split into three clades based on amino acid sequence: clade 1 containing *FT1* and *FT2*, clade 2 containing *FT3-5* and clade 3 containing *FT6-12*. It was found that conserved regions associated with interaction with 14-3-3 protein were found in *FT1-FT5* and therefore it is predicted that these genes may form a complex with 14-3-3 and therefore be regulated through *Ppd-1* like seen in *FT1* (Corbesier, et al., 2007). To assess an *FT*'s potential of being repressive or active, another conserved amino acid sequence was identified between *FT1* and *FT2*, which are both activators. *FT3*, *FT4* and *FT5* were all found to not contain these amino acids and are therefore predicted to be repressive, whereas in *FT6-12*, they are present and are therefore predicted to be active.

CHAPTER 2: The role of *Ppd-1* in regulating plant architecture

2.0 Introduction

Flowering time is an important agronomic trait, particularly in the context of wheat (*T. aestivum*) as the heat of the summer months and cold of the winter months can be detrimental to the amount of grain harvested and even the plants survival. This has led to farmers and breeders producing and growing varieties that are suited to the local growing seasons (Royo, et al., 2020). *PHOTOPERIOD 1* (*Ppd-1*) has a profound effect on flowering time and is essential in facilitating the photoperiod response in cereals where the gene is functional (Bentley, et al., 2013). In plants carrying a photoperiod insensitive *Ppd-1* allele flowering time is accelerated irrespective of photoperiod and flowering time is slowed when the gene is non-functional, such as in varieties where *Ppd-1* exons are deleted (Shaw, et al., 2013). The production of insensitive varieties have been very useful in contributing to the increase of global wheat yields as they have led to wheat being able to flower when the growing season is much shorter. For examples, countries such as Spain where the extreme heat in summer causes sterility means that it is advantageous for farmers to grow wheat that can transition to reproductive development in short days (Royo, et al., 2020). Studying the response of wheat to photoperiod and the associated phenotypes surrounding genetic variation of *Ppd-1* is essential as it may unlock faster and slower ways of growing wheat in seasons that are continuously altering due to the actions of climate change (Shiferaw, et al., 2013). Improving the adaptation of wheat to its local environment is vital to maximising the yield that we can produce.

Once wheat is mature enough to harvest, the parts of the plant that are removed and consumed are the grain, housed within the spikelets. Previous studies have shown that when plants make the vegetative to floral transition in short-day conditions, plants that are photoperiod sensitive produce more spikelets than their photoperiod insensitive near-isogenic in the region of *Ppd-1* counterparts (Boden, et al., 2015). This lower number of spikelets correlates to lower numbers of grain which in turn lowers the dry weight harvested (Perez-Gianmarco, et al., 2019). This is interesting as it implies that *Ppd-1* has a direct/indirect impact on agronomic traits separate to just flowering time.

Studies understanding flowering time regulation and spikelet number have demonstrated that *Ppd-1* is an important gene within these processes (Perez-Gianmarco, et al., 2019) (Dixon, et al., 2018). However, flowering time regulation in cereals involves many other traits including the timing of the vegetative to floral transition, regulation of each of the inflorescence meristem stages, internode elongation, spike architecture (spike length, spikelet arrangement, spikelet number) and the floral regulation of the tillers. To study how *Ppd-1* regulates these phenotypes, I set up experiments in long day (LD) and field (F)conditions. These conditions were selected as the reflect growing environments experienced during stem elongation stages of areas with short growing seasons (LD). This was complemented with phenotyping from the field (UK) to assess if additional developmental phenotypes occurred outside. The near isogenic lines (NILs) used had previously been developed and used in a number of studies and was developed in *Triticum aestivum* cv. in Paragon background which is a spring variety (Bentley, et al., 2013) (Shaw, et al., 2012) (Shaw, et al., 2013). The common genetic background of Paragon enabled direct comparison of the *Ppd-1* allelic effects as

well as having the advantage of not having a vernalization requirement. This means that plants would transition to floral development in higher temperature, long-day glasshouse conditions. Four different genotypes with variations of the *Ppd-1* allele were used; Triple-Knockout (where *Ppd-1* is knocked out in all three genomes), Wild-Type Paragon (photoperiod sensitive), Single Insensitive (deletion in the promoter region in D genome) and Triple insensitive (deletion in promoter region in A and D genomes and increase in copy number in the B genome).

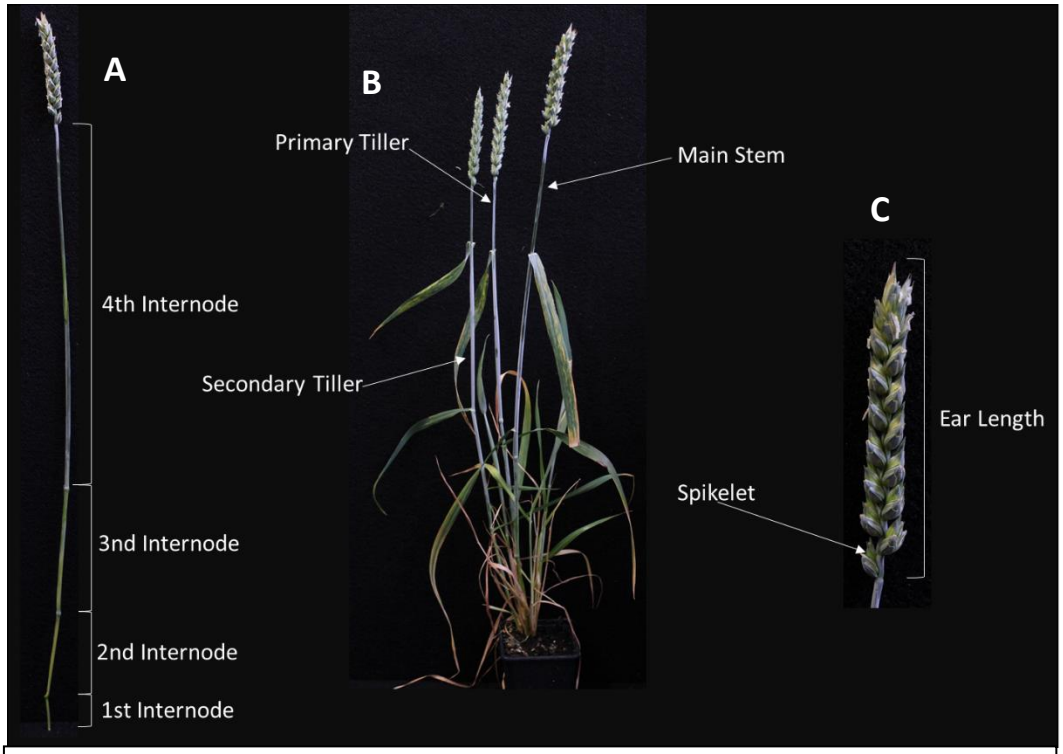

Figure 2.1: Morphological features of a wheat plant. (A) The internode lengths, internodes 1-4. (B) The whole wheat plant, highlighting the main stem and tillers. (C) The spike highlighting the spikelets and ear length.

Using the *Ppd-1* Paragon NILs, I wanted to understand how *Ppd-1* affected plant growth, not just flowering time. I was particularly interested in identifying if different *Ppd-1* alleles affected all the floral meristem's growth, not just the main stem. In addition, the lines were grown in the field to enable a comparison of the phenotypes observed in the glasshouse with those under natural, variable conditions. Characteristics I wanted to study were internode lengths, ear length and spikelet number which are all key traits when determining either flowering time and/or final yields. Previous studies have mainly focused on flowering time and spikelet number in the context of *Ppd-1* and have

not looked in detail at other phenotypic traits that may also be affected by it such as internode lengths.

In addition to assessing development under LD and field conditions plants were also grown under short and long-day conditions at 20ºC. This comparison was conducted to firstly confirm the photoperiod insensitivity of the single and triple insensitive Paragon NILs as well as further understand tiller development. For each genotype, meristems were dissected and imaged at each leaf stage, starting from 4th leaf. I started with the 4-leaf stages as this is when the apex is known to begin the transition from a vegetative state.

2.1 Materials and Methods

2.1.1 Germplasm:

All plants used in the phenotyping experiments were in a *Triticum aestivum* cv. Paragon (WT) background which is a photoperiod sensitive spring hexaploid variety. The photoperiod sensitivity had then been altered through varying *Ppd-1* alleles. The triple knockout (TK) variety was developed using germplasm by Shaw et al (2013) with Norstar providing the *Ppd-A1* and *Ppd-D1* deletions and then being backcrossed 4 times into Paragon. The *Ppd-B1* deletion was induced through seeds being exposed to gamma radiation, screened for the deletion, and then backcrossed into Paragon. For production of the insensitive varieties, *Ppd-D1a* allele was donated from Sonora64 into Paragon background, which was then backcrossed to produce the Paragon single insensitive (SI) plants. *Ppd-A1a* and *Ppd-B1a* alleles were donated from GS-100 and Sonora64 respectively and backcrossed with the *Ppd-D1a* allele to give triple insensitive (TI) plants (Shaw, et al., 2012).

2.1.2. Long-day Glasshouse Phenotyping:

Wheat plants of the hexaploid wild-type Paragon (WT), Triple knockout (TK), single insensitive (SI) and triple insensitive (TI) were grown in cereal mix developed by the John Innes Centre which consisted of 40% medium grade peat, 40% sterilised soil, 20% horticultural grit, 1.3 kg/m^3 PG mix 14-16-18 + Te base fertiliser, 1 kg/m^3 osmocote mini 16-8-11 2 mg + Te 0.02% B, Wetting agent, 3 kg/m3 maglime, 300g/m^3 exemptor. Plants were grown in 30 mm x 34 mm x 6 cm cells and then potted up to 9cm x 9cm x 9.5cm pots once at the 3-leaf stage. Phenotypic traits flowering time, internodal lengths (0-1, 1-2, 2-3, 3-4), ear length and spikelet number were recorded when the wheat flowered and subsequent grain ripening in long-day conditions (16h light 8h dark) at 22ºC occurred. The plants were grown under Attis 7 3W Epistar LED's that emitted an average of 2.13 μ mol/w/m2 delivered at crop level.Once flowered, the traits flowering time internode length, spikelet number and ear length were recorded. This was done for the main stem, primary and secondary tillers.

2.1.3. Field Phenotyping:

Wheat plants of wild-type Paragon (WT), Triple Knockout (TK), single insensitive (SI) and triple insensitive (TI) in the Paragon background were sown in March 2021 at the University of Leeds farm, Tadcaster (CIEL Spen Farm), grid reference SE 43294, 40829. Once flowered, flowering time, internode length (0-1, 1-2, 2-3, 3-4), spikelet number and ear length were recorded for main stem, primary tillers, and secondary tillers.

2.1.4. Short and Long-day experiment:

18 plants of each genotype; triple knockout, wild-type Paragon, single insensitive and triple insensitive were placed in a 23cm x 36cm x 6cm tray with 30mm x 34mm x 6mm cells. One of these trays was placed in short-day conditions (10h light 14h dark) and the other was placed in long-day conditions (16h light 8h dark). Both conditions were controlled by Sanyo Phytotron walk-in controlled environment rooms. The plants were then dissected at each leaf stage starting from when the wheat produces a fourth leaf

and was then stopped once one of the genotypes had reached terminal spikelet. Once the apex had been dissected, they were placed onto a petri dish and imaged. Three samples were taken at each stage and the length of the apex was measured using ImageJ.

Results 2.2

2.2.1 The role of *Ppd-1* in regulating internode length

To assess the role of *Ppd-1* in regulating internode length and plant height in *Ppd-1* NILs in the background Paragon, along with Paragon, were grown under glasshouse conditions of long-day (LD: 16 h light: 8 h dark) photoperiods and constant 20°C. All plants phenotyped had fully flowered and dried before phenotyping, ensuring that full stem elongation had occurred. Plant height in LD glasshouse conditions show at least one of the tillers to be shorter in length than the main stem as shown in (Figure 2.2A). Significant differences were observed between the main stem length and primary/secondary tiller length in all four genotypes (TK, WT, SI, TI). For the plants phenotyped there were 4 internode lengths stemming from the base of the plant to the rachis. The first internodal length showed different developmental responses between the genotypes with only TK internode length being significantly different to any of the other genotypes, SI and TI. There was no significant difference between (WT, SI and TI) indicating the first internode length could be key to contributing to taller overall plant length as seen in TK. In the second internodal space (Figure 2.2 C), the genotypes seem

to conform with what is seen in the first (Figure 2.2B) with TK being significantly longer in size to the other genotypes and WT, SI, TI having no significant difference between each-other. For the third internode length, there was no significant difference between any of the genotypes observed (Figure 2.2D). Finally, in the fourth internode space, a significant length difference was observed between TK and WT and TK and TI (Figure 2.2E). In this case, the TK samples were shorter than WT and TI. However, there was no difference seen in the lengths of the WT, SI and TI. My results indicate that functioning *Ppd-1* alleles as seen in the WT, SI and TI display similar internodal phenotypes in LD glasshouse conditions, whereas in TK, they seem to have longer internode lengths in the first and second internodes and shorter in the fourth. Overall, this contributes to taller plants in these conditions.

In field conditions (Tadcaster, West Yorkshire), TK also displays a taller overall plant height (Figure 2.3A) as seen in the LD glasshouse conditions (Figure 2.2A). For the other genotypes, WT, SI and TI, there was no significant difference in stem length observed. This suggests that indicate *Ppd-1* is influencing node elongation in stable temperatures at 23°C and variable temperatures in the field. There was a large increase in overall plant height observed for all genotypes (TK, WT, SI, TI) in glasshouse LD conditions when compared with the field (Figures 2.3B-2.3E). This may suggest that constant high temperatures of 23°C positively influence wheat plant stem length regardless of phenotype as opposed to variable, inconsistent temperatures and photoperiod that is seen in the field.

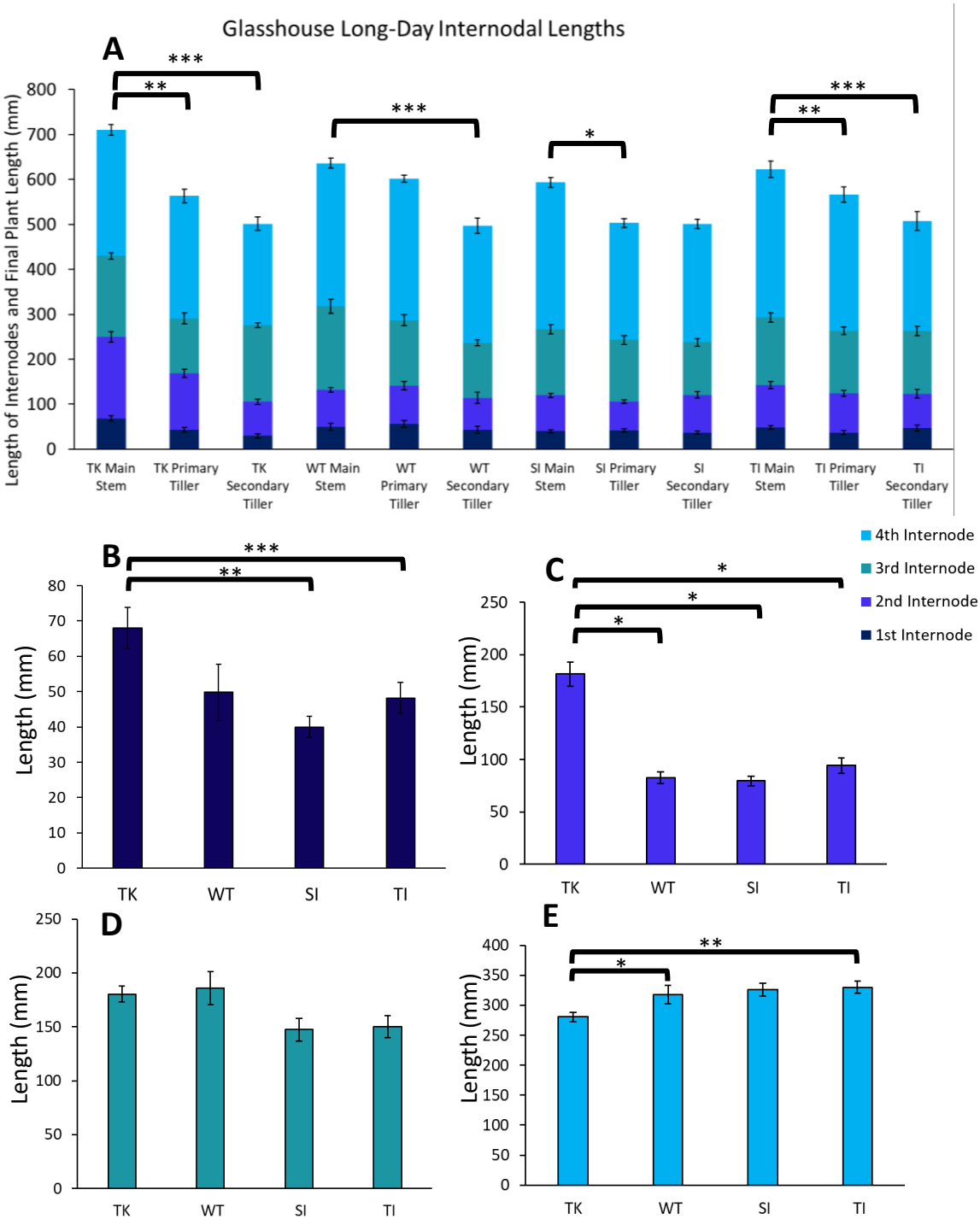

Figure 2.2: Internodal length of *Triticum aestivum* grown under long-day glasshouse conditions with varying *Ppd-a* alleles. (A) Combined-internodal lengths 0-1, 1-2, 2-3, 3-4 for Paragon (WT), *Ppd-D1* triple knockout (TK), *Ppd-D1* insensitive (SI), *Ppd-D1* triple insensitive (TI) where dark blue is 1st internode, mid-blue is 2nd internode, green is 3rd internode and sky blue is 4th internode. Comparisons made between overall plant height in (A). The internode lengths are shown separately in (B) 1st internode, (C) 2nd internode, (D) 3rd internode and (E) 4th internode in LD glasshouse conditions. Significance is shown by two-tailed-T test where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. N = 10 and error bars are standard error of the mean.

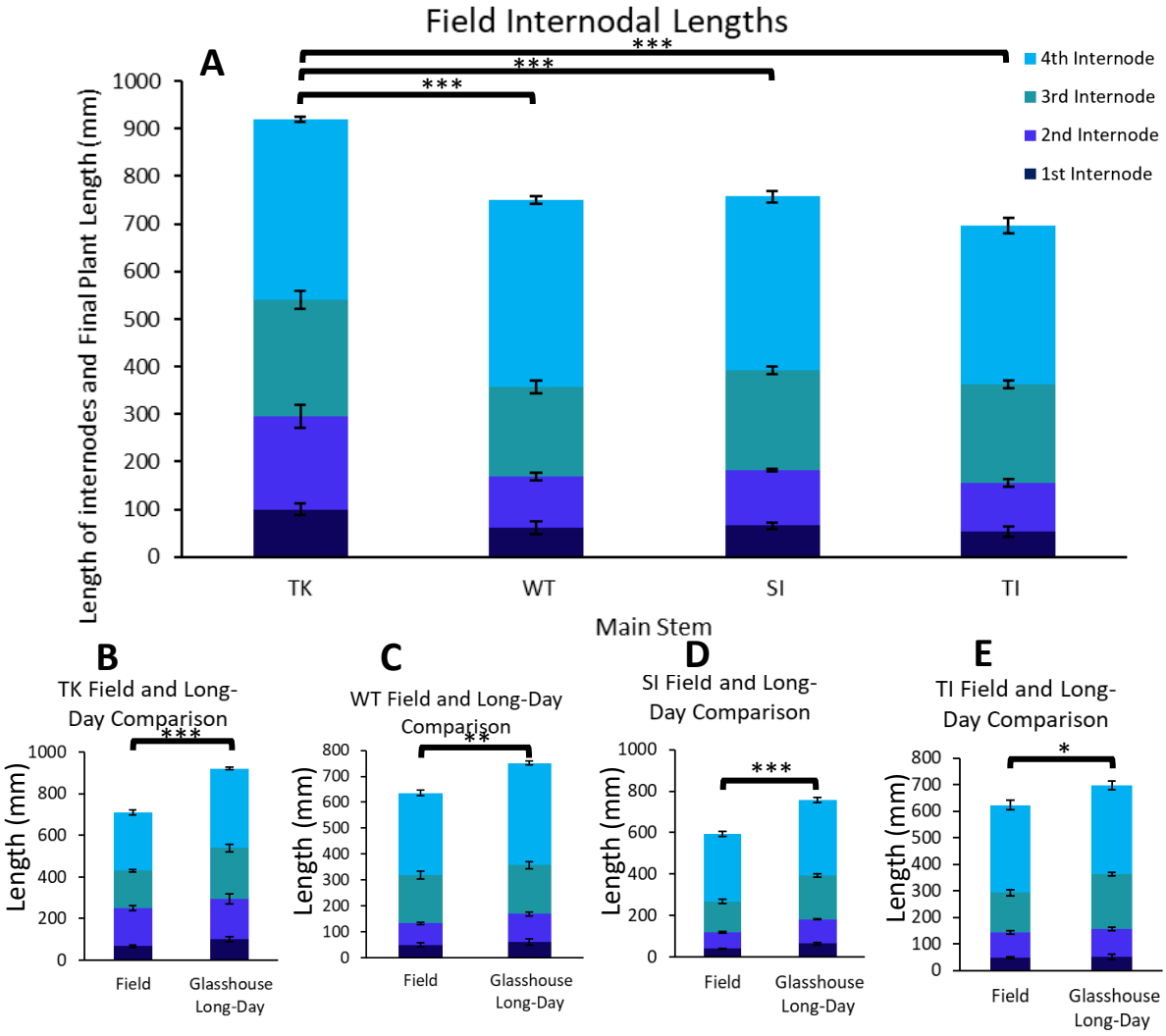

Figure 2.3: Internodal length of *Triticum aestivum* grown under field conditions with varying *Ppd-a* alleles. (A) Combined-internodal lengths 0-1, 1-2, 2-3, 3-4 for Paragon (WT), *Ppd-D1* triple knockout (TK), *Ppd-D1* insensitive (SI), *Ppd-D1* triple insensitive (TI) where dark blue is 1st internode, mid-blue is 2nd internode, green is 3rd internode and sky blue is 4th internode. Comparisons were made between overall plant heights in (A). (B-E) show the comparison between the overall plant height between wheat plants grown in field conditions and LD glass house conditions. Significance is shown by two-tailed-T test where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. N = 10 and error bars are standard error of the

2.2.2. The role of *Ppd-1* in regulating ear lengths:

To assess the role of *Ppd-1* in regulating ear length *Ppd-1* NILs in the background Paragon, along with Paragon, were grown under glasshouse conditions of long-day (LD: 16 h light: 8 h dark) photoperiods and constant 20^{o}C. All plants phenotyped had fully flowered and dried before phenotyping, ensuring that full ear development had occurred. As wheat ears are the part of the plant that hold the spikelet containing the grain, it is an important agronomic trait to study. I observed that ear lengths generally show an increase in size when comparing the main stem to the tillers across the genotypes TK, WT and TI in LD glasshouse conditions. In TK, the ear lengths of the main stem were significantly larger than the primary and secondary tiller (Figure 2.4A). Similarly in the WT, and TI, there was a significant difference between the main stem and secondary tiller. However, for SI, there was no significant difference in size between any of the tillers (Figure 2.4A). The length of the ear for the main stems were significantly larger in the TK than in both photoperiod insensitive (SI and TI) plants but not when compared with WT (Figure 2.4B). This implies that the polymorphism in *Ppd-1* promoter region which is used in both insensitivity NILs has a direct/indirect role in wheat ear elongation in the main stem. There was no significant difference observed between any of the genotypes in the primary and secondary main stems in LD greenhouse conditions (Figures 2.4C, 2.4D).

Field main stem ear lengths were also longest in TK where they were observed to be significantly larger compared to WT and SI (2.5A). Data was not recorded for TI due to deer consuming the wheat ears that were grown in the field, presumably because they were the first cultivar in the field to mature. WT wheat ear lengths were also seen to be significantly longer when compared with the SI. This reinforces the theory that increased levels of *Ppd-1* could play a role in shortening the ear length. When comparing ear length data in the wheat in genotypes TK, WT, SI in glasshouse LD conditions with those grown in the field, they all were observed to be significantly shorter in the field (2.5B-D). This could indicate a consistent temperature at 23^{o}C, or consistent LD photoperiod could influence increasing the size of the wheat ears.

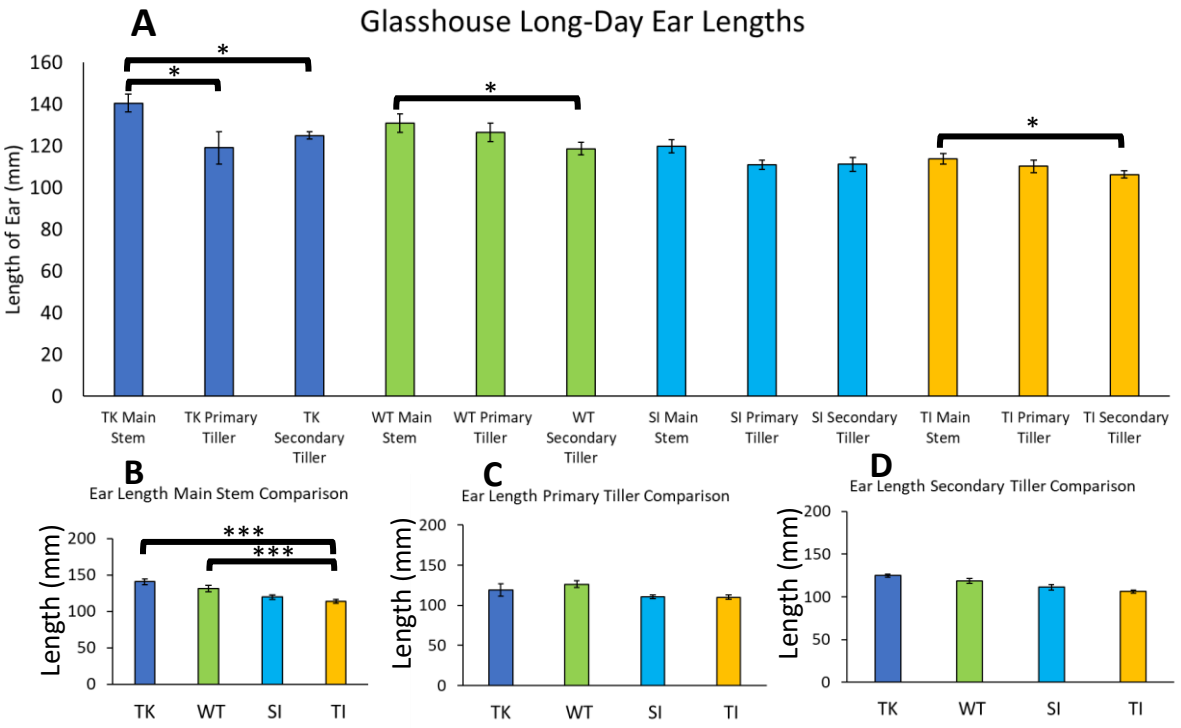

Figure 2.4: Ear length of *Triticum aestivum* grown in LD glasshouse conditions with varying *Ppd-a* alleles: (A) shows the comparison between 4 for Paragon (WT) (lime green), *Ppd-D1* triple knockout (TK) (dark blue), *Ppd-D1* insensitive (SI) (turquoise), *Ppd-D1* triple insensitive (TI) (orange) main stems in field conditions, (B-D) compare the ear lengths of the main stems, primary stems and secondary stems between the genotypes in LD glasshouse conditions. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. N = 10 and error bars are standard error of the mean.

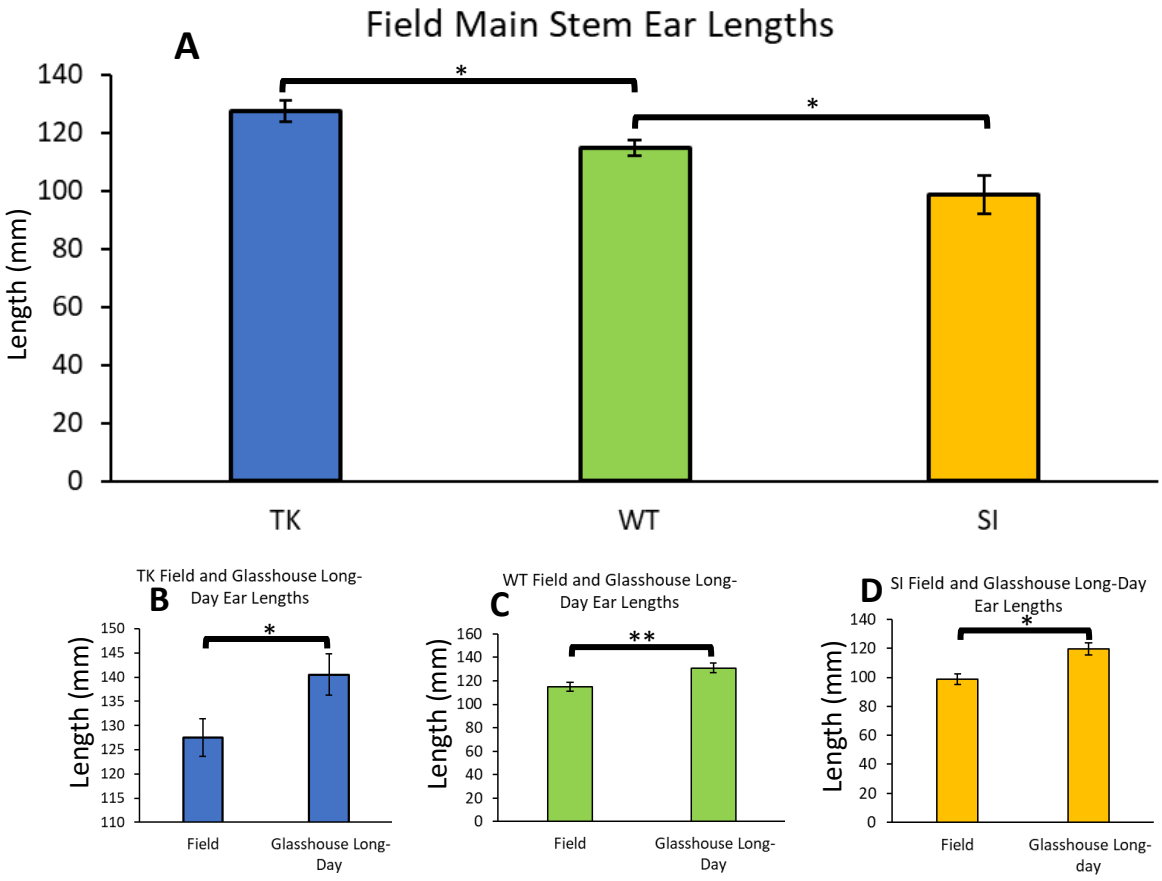

Figure 2.5: Ear length of *Triticum aestivum* grown in field conditions with varying *Ppd-a* alleles. (A) shows the comparison between 4 for Paragon (WT), *Ppd-D1* triple knockout (TK), *Ppd-D1* insensitive (SI) main stems in field conditions, (B-D) compare the main stem ear lengths in the field with main stem ear length of LD glasshouse conditions. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. N = 10 and error bars are standard error of the mean.

2.2.3. The role of *Ppd-1* in regulating spikelet number:

To assess the role of *Ppd-1* in regulating spikelet number *Ppd-1* NILs in the background Paragon, along with Paragon, were grown under glasshouse conditions of long-day (LD: 16 h light: 8 h dark) photoperiods and constant 20^{o}C. All plants phenotyped had fully flowered and dried before phenotyping, ensuring that spikelet number was accurately recorded. Grain within the spikelets is the part of the wheat plant that is harvested and therefore spikelet number directly influences the yield that is gained and for this reason, it was important to study this. For glasshouse LD conditions, spikelet number was much higher in the main stems across all four genotypes (TK, WT, SI, TI) when compared with the secondary tillers (Figure 2.6A). There was no significant difference between the main stem and the primary tiller in terms of spikelet number. In all cases in this study, WT were observed to have the most spikelets when comparing the four genotypes. TK is usually expected to produce more spikelets than WT, SI and TI but this may not occur in LD glasshouse conditions (Shaw, et al., 2013). In the main stem, primary tiller and secondary tiller, WT had significantly more spikelets in contrast to TK, SI, TI (Figures 2.6B-D). There were no significant differences observed in the number of spikelets between the TK, SI and TI genotypes for the three tillers examined. This indicates that polymorphisms around the *Ppd-1* allele may play a role in spikelet formation.

In field conditions, TK was observed to have significantly more spikelets than WT and SI (Figure 2.7A). TI spikelet number was not recorded due to deer consuming the wheat heads. For the WT wheat used in this study, there were significantly more spikelets produced when compared to the SI. TK having the most spikelets could be due to lack of *Ppd-1* interacting with floral transition causing a delay in key apical developmental stages such as spikelet and floret formation, meaning that more are generated. When comparing the field and glasshouse LD conditions between the genotypes TK, WT and SI in the context of spikelet number, considerably more spikelets seem to be produced in the latter for WT and SI (2.7B-D). There was no significant difference recorded for TK between the field and glasshouse LD conditions (Figure 2.7B).

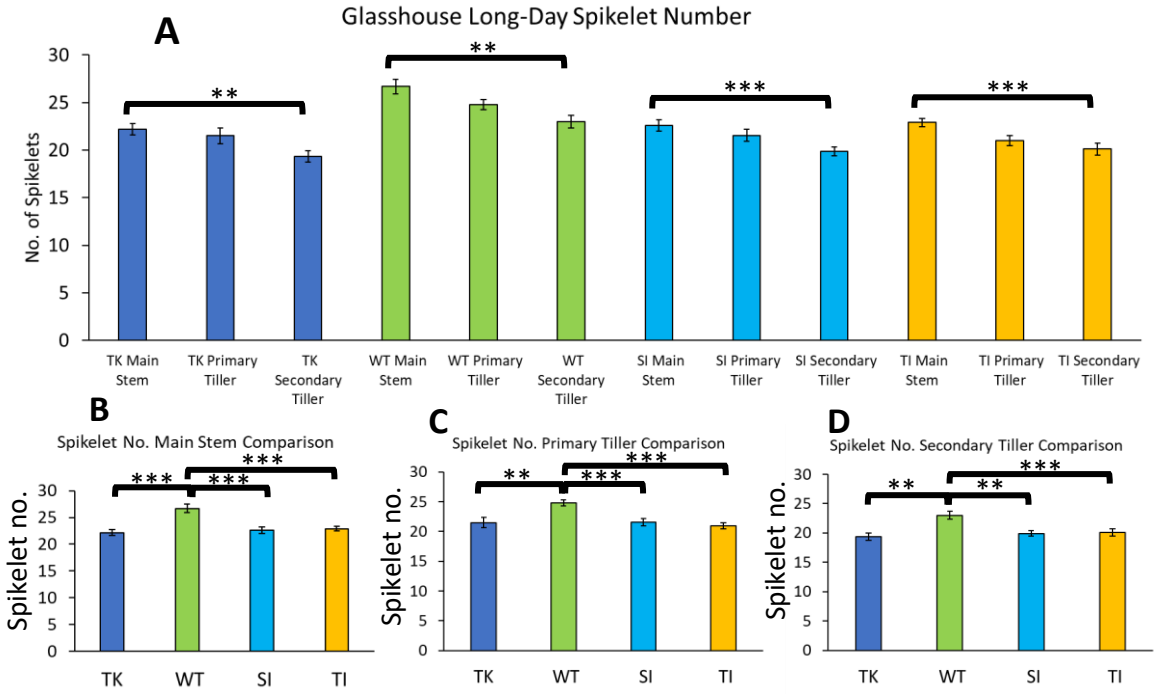

Figure 2.6: Spikelet number of *Triticum aestivum* grown in LD glasshouse conditions with varying *Ppd-a* alleles. (A) shows comparisons between the main stem, primary and secondary tillers of the same genotype which include 4 for Paragon (WT) (lime green), *Ppd-D1* triple knockout (TK)(dark blue), *Ppd-D1* insensitive (SI)(turquoise), *Ppd-D1* triple insensitive (TI)(orange). Analysis of if there are significances between their lengths in LD glasshouse conditions. (B-D) show comparisons of spikelet number of the main stem, primary tiller, and secondary tiller between the genotypes in LD glasshouse conditions. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. N = 10 and error bars are standard error of the mean.

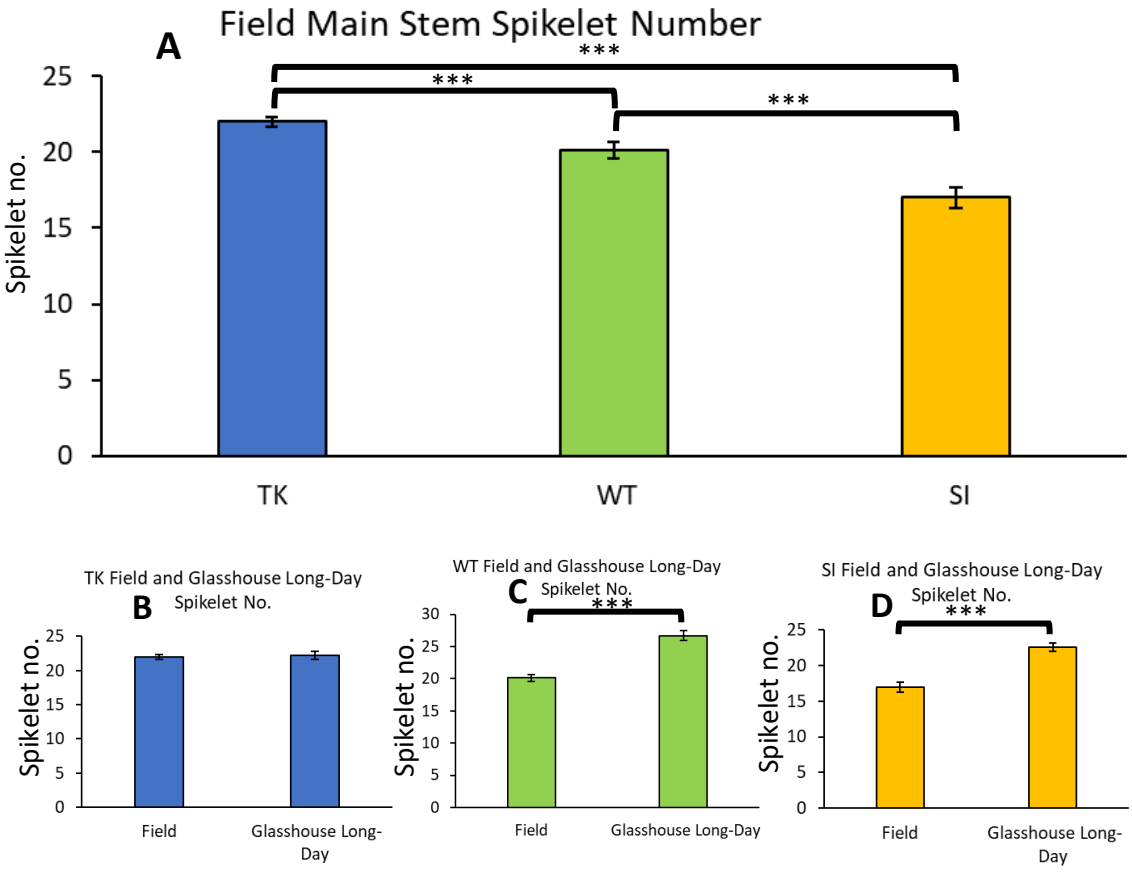

Figure 2.7: Spikelet number of *Triticum aestivum* grown in field conditions with varying *Ppd-a* alleles. (A) shows the comparison between Paragon (WT)(lime green), *Ppd-D1* triple knockout (TK)(dark blue), *Ppd-D1* insensitive (SI)(orange) main stems in field conditions. (B-D) compare the main stem spikelet number in the field with main stem spikelet number of LD glasshouse conditions. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. N = 10 and error bars are standard error of the mean.

2.2.4 Short and Long-day Apex length experiment:

The effects that the *Ppd-1* alleles have on apical development and their relation to phenotypes displayed was a vital component to understanding this project. The short (SD) and long-day (LD) experiments were designed to study how the different photoperiods determined floral maturation, quantify the differences between the genotypes and determine if there is significance. In SD's, there were no significant differences between the apex lengths at the 4-leaf stage and only one genotypic comparison (TK:SI) resulted in a significant difference at the 5-leaf stage (Figure 2.8B, Table 2.1). The 6-leaf stage is where the differences between the apices become significant with there being strong significant differences between all genotypic comparison combinations besides SI:TI (Figures 2.8A). These differences are to be expected, however, with WT apices only developing quicker than TK and both insensitive varieties being significantly faster in development than WT and TK. This is due to SI and TI constantly expressing *Ppd-1* and therefore *FT1* regardless of the environmental photoperiod, initiating flowering at the earliest possible point which is not the case with WT and TK (Shaw, et al., 2012).

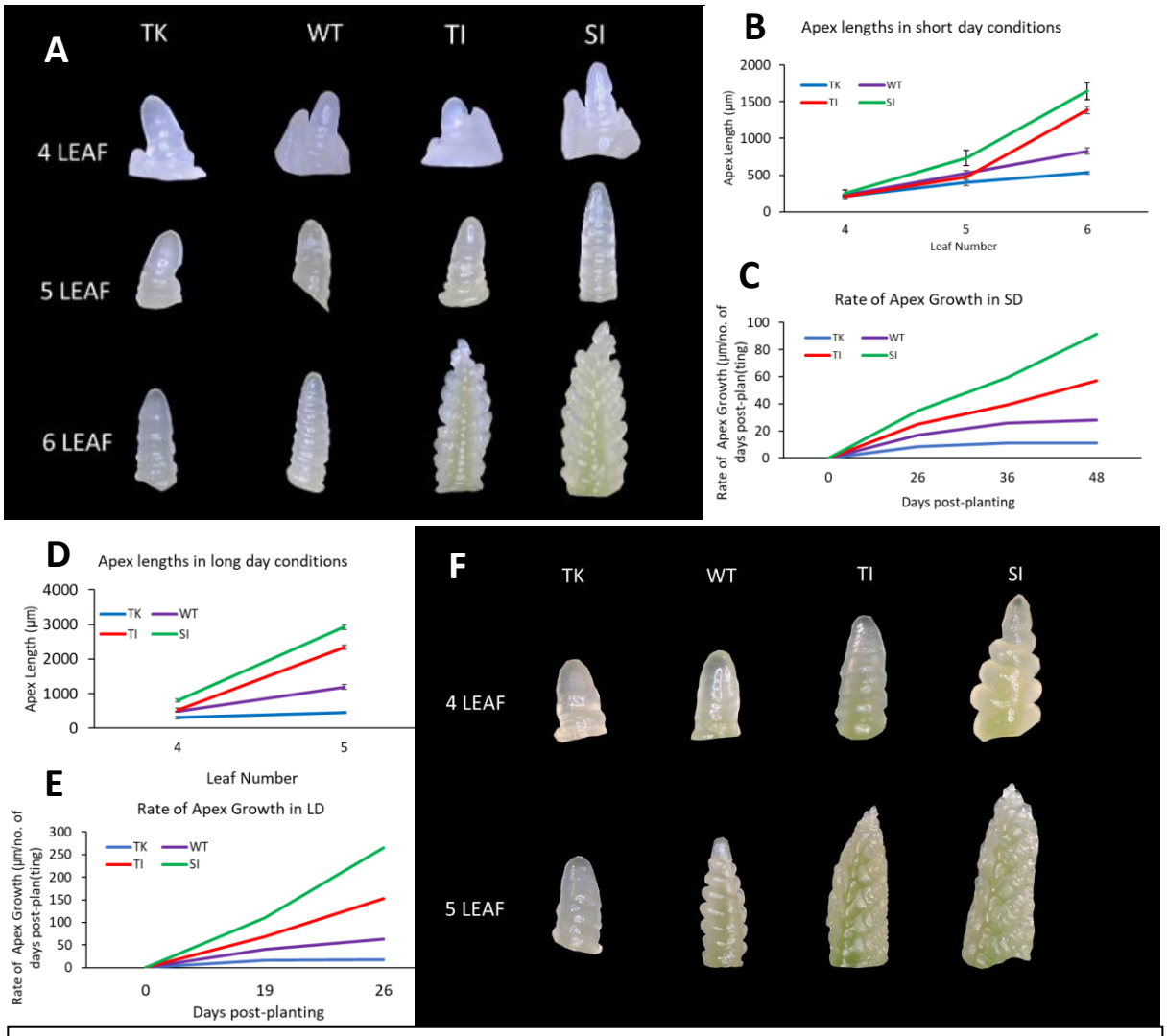

Figure 2.8 Short and Long day *Triticum aestivum* apex length tracking in short and long-day conditions with varying *Ppd-a* alleles: (A) shows image representation of the apical stages of each genotype: 4 for Paragon (WT)(purple), *Ppd-D1* triple knockout (TK)(blue), *Ppd-D1* insensitive (SI)(green), *Ppd-D1* triple insensitive (TI)(red) at each leaf stage in SD (10h light 14h). Graphs (B,C) also show the average apex lengths across each genotype at the 4th, 5th and 6th leaf stage and rate of growth of each genotype. This was calculated by average apex length/days since planted. (D) shows apex length data of TK, WT, SI, TI but in LD (16h light 8h dark) conditions. Images showing apex length across different leaf stages also feature (F) with rate of growth graph(E). N = 3 and error bars are standard error of the mean.

SHORT DAY: A

4 LEAF	TK:WT	TK:SI	TK: TI	WT:SI	WT:TI	SI:TI
P Value	0.33861	0.458424	0.780692	0.633389	0.43365	0.424554
Significance	n/a	n/a	n/a	n/a	n/a	n/a
5 LEAF	TK:WT	TK:SI	TK: TI	WT:SI	WT:TI	SI:TI
P Value	0.08241	0.040636	0.249491	0.137936	0.413568	0.08425
Significance	n/a	*	n/a	n/a	n/a	n/a
6 LEAF	TK:WT	TK:SI	TK: TI	WT:SI	WT:TI	SI:TI
P Value	0.002535	0.000657	0.00074	0.002622	0.000964	0.108012
Significance:	**	***	***	**	***	n/a

LONG DAY: B

4 LEAF	TK:WT	TK:SI	TK: TI	WT:SI	WT:TI	SI:TI
P Value	0.066913	0.009626	0.09383	0.012252	0.411851	0.071269
Significance	n/a	**	n/a	*	n/a	n/a
5 LEAF	TK:WT	TK:SI	TK: TI	WT:SI	WT:TI	SI:TI
P Value	0.00036	<0.00001	0.000011	0.000045	0.00024	0.003288
Significance	***	***	***	***	***	**

Table 2.1 Short and Long day *Triticum aestivum* apex length tracking statistics table between varying alleles of *Ppd-a.* Tables for significance values of apex lengths between all of the four genotypes, WT, TK, SI, TI, shown. (A) = Short Day and (B) = Long Day. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2.3 Discussion

2.3.1 *Ppd-1* allelic effect on floral development

The effect that *Ppd-1* has on aspects of floral development such as spikelet number and flowering time is a well-studied area (Jones, et al., 2016) (Shaw, et al., 2012) (Perez-Gianmarco, et al., 2019) and my findings in this chapter seem to reinforce the consensus. Across all of the genotypes tested, there seemed to be a higher number of spikelets produced in glasshouse LD conditions when compared to the field (Figures 2.7,2.8). This may be due to consistently higher temperatures and longer photoperiods providing conditions that are less stressful for the wheat grown in them. This result was echoed in ear lengths where they were consistently longer in glasshouse conditions (Figures 2.5, 2.6). There is a clear relationship between higher number of spikelets and longer ear lengths and the reason for this is probably due to the need for a longer ear to hold more spikes. Longer ear lengths and higher spikelet number were also found be the case when comparing the main stem to the tillers, especially when comparing the main stem to secondary tillers. One reason for this might be the main stem having a longer amount of time to develop therefore resulting in this difference. Another could be differences in gene expression between the main stem and tillers. For example, repressive genes in the *FLOWERING LOCUS T* (*FT*) gene family could be more highly expressed in tillers which gives the main stem a head start and therefore a longer time to develop. Levels of genes such as *FT2*, which is responsible for spikelet termination, could be higher in tillers resulting in fewer spikelets (Boden & Gauley, 2020). Other genes could similarly repress ear elongation resulting in the phenotypes seen.

Spikelet number was seen to be the highest in WT in the glasshouse LD conditions whereas TK produced the most in the field (Figures 2.6B, 2.7A). However, when comparing ear length, TK was seen to have the longest in both field and glasshouse LD conditions. This could contradict what was previously stated regarding the positive relationship between ear length and spikelet number. A further observation of note is that the photoperiod insensitive NILs (SI and TI) produce far less spikelets compared to the WT in both field and glasshouse LD setting which provides more evidence for *Ppd-1* influencing spikelet number. Elevated levels of *Ppd-1* translate to more *FT1* being expressed which would indicate why photoperiod insensitive wheat plants develop faster. However, this does not explain why they would have less spikelets and shorter ear lengths as *FT1*'s known function is to initiate the vegetative to reproductive floral transition and does not have a known role in spikelet formation/ termination. This implies that *Ppd-1* influences the expression of genes that have a role in ear elongation and spikelet formation, some of which may belong to the *FT* gene family. This has already been shown to be the case with *FT2* which has been shown to have a role in spikelet termination. *FT2* expression has also been shown to be positively affected by *Ppd-1* and therefore there is a shorter gap between lemma primordia and terminal spikelets in plants with elevated levels of *Ppd-1* such as in SI and TI when compared to WT. This interaction between *Ppd-1* and *FT2* could also be the case with other genes in the *FT* family that may play a role in determining ear length.

Results from the short and long-day experiment show that *Ppd-1* has a clear effect on early floral development when comparing *Ppd-1* NILs in Paragon background. In both cases, the photoperiod insensitive plants developed quicker than the WT by the end of the experiment. This is indicative of elevated levels of *Ppd-1* and therefore *FT1* causing floral reproductive initiation occurring earlier in photoperiod insensitive plants. One result that is surprising, is in LD conditions SI seemed to develop quicker than TI at the 5-leaf stage with apices being longer on average in comparison (Figure 2.8D). One reason for this difference could be from higher *Ppd-1* expression coming from three genomes as opposed to just one, causing increased expression of repressive genes that may slow apex development up until this stage. For *T. aestivum* that are photoperiod insensitive through polymorphisms around the *Ppd-1* promoter, it is understood that *Ppd-1* is constantly expressed due to its mis-regulation, which in turn, constantly expressed *FT1*. This means that there should be equal expression of *Ppd-1* and therefore *FT1* regardless of the photoperiod, however, it is clear that there is a difference in developmental rates. This implies that there are likely other genes detecting the longer photoperiod in plants in LD conditions and thus advancing development rates when compared to SD.

2.3.2 *Ppd-1* effect on stem elongation and plant height

An area that is less documented is the effect that *Ppd-1* has on internodal lengths and plant height. In the first two internodal lengths, TK plants are considerably longer than the other 3 genotypes used in this study in glasshouse LD conditions, with no differences in internode length in any of the other internodes compared (Figure 2.2). This implies that in these first two internodal elongation stages, *Ppd-1* has a role in repressing length here. Alternatively, as TK plants have a floral development that is slower than in WT, the plant may just keep growing until the booting stage, causing the plants to be overall taller as booting occurs later. Furthermore, the fact that tillers grew to be overall shorter than the main stem could also indicate this as they arise at a later stage and therefore have less time to grow. In the field and glasshouse LD conditions, TK are the tallest plants overall, which could indicate that *Ppd-1* influences genes controlling plant height (Figures 2.2, 2.3). However, this could also be explained by slower floral development in TK plants. The glasshouse plants grew to be much taller in all four genotypes studied when compared to the field. This echoes the findings in ear length and spikelet number where LD glasshouse conditions appeared to be more favourable for longer ear length and increased number of spikelets. This may be due to conditions that convey a more consistent photoperiod and temperature when compared to the field.

2.3.3 Future experiments regarding phenotyping

Phenotyping is an informative way of observing whether a change to a gene has an ffect on traits within a whole plant system. However, in the context of studying *Ppd-1* alleles, there are limitations with this way of study. One of these would be that it does not tell you if *Ppd-1* has a direct effect on traits such as spikelet number or if it is regulating other gene pathways that indirectly influence it. To investigate the effect that *Ppd-1* is

having on the traits discussed in this chapter further, overexpressed *Ppd-1* NILs could be produced. These NILs could supplement the growing data that *Ppd-1* has a role in influencing flowering phenotypes in wheat. Furthermore, dissecting the apex at earlier stages and analysing gene expression through qPCR in the context of comparison between the *Ppd-1* NILs, may give more insight into the genes involved in regulating spikelet number, ear length and internode length and whether or not they are *Ppd-1* regulated.

CHAPTER 3: Investigating whether *Ppd-1* acts as a transcription factor

3.0 Introduction

A core theory regarding the floral initiation pathway in wheat is that Ppd-1 initiates *FT1* expression in the leaf through interaction with *FT1*'s promoter. The *Ppd-1* gene is expressed in response to changes in photoperiod in sensitive *T. aestivum* varieties and it is believed that the Ppd-1 protein then binds to a specific motif in the promoter of *FT1*, thereby regulating its expression and acting as a transcriptional factor (Corbesier, et al., 2007). *FT1* is a mobile floral activator and is considered to be part of the florigen signal that can move from the leaf, down the phloem and to the vegetative meristem. Here, FT1 forms a complex with FD-like protein and 14-3-3 and this complex interacts with floral identity genes that kickstart floral development in wheat (Li, et al., 2015). In spring photoperiod sensitive hexaploid wheat, this whole process is regulated through the plants' monitoring of the photoperiod of its environment and is therefore important that we understand the specifics of this interaction and even if there is a direct interaction at all. If it were found to be the case that *Ppd-1* acts as a transcriptional factor for *FT1* it could have wider implications for wheat breeding as it would imply that this one gene has a massive effect on flowering time and potentially other traits related to flowering. These other traits such as spikelet number could be controlled through other members of the *FT* family which also may be regulated through *Ppd-1*. It is therefore essential that we discover the complex workings and interactions of these genes to create varieties that are more suited to the environment they are grown in, whilst also ensuring high yields.

However, this interaction has not been directly proven and is only currently speculated as the mode of action for the Ppd-1 protein. One piece of evidence supporting the theory is the decrease in *FT1* in *Ppd-1* knockout lines and then the increase in lines carrying a photoperiod insensitivity trait; showing a clear correlation between the levels of expression of the two genes (Shaw, et al., 2013). Further evidence is provided through the study of photoperiod insensitive wheat varieties. Insensitivity phenotypes arise from one of two ways; a deletion of the promoter region before the *Ppd-1* exon or an increase in copy number (Shaw, et al., 2012). It is thought that within the 2kb deletion in the promoter there are regulatory motifs that repressive proteins would usually bind to limit *Ppd-1* expression. Without these motifs, *Ppd-1* is freely expressed and therefore confers an early flowering phenotype. It is probable that Ppd-1 is regulating its own expression, which is supported through the increase in expression in *Ppd-1* TK lines where *Ppd-1* is non-functional (unpublished data from Dixon group).

Additional support for a direct interaction between *Ppd-1* and the *FT1* promoter comes from comparison with Arabidopsis. Homology studies show that wheat Ppd-1 is most similar to the *PSEUDO RESPONSE REGULATOR* (*PRR*) gene family in Arabidopsis. In Arabidopsis, *PRR* genes bind to circadian clock genes and *FT* via specific motifs in their promoters. Some of these specific motifs have been identified in Arabidopsis (Liu, et al., 2016) (Kamada & Miwa, 1992). I therefore hypothesise that it would therefore be plausible that Ppd-1, a pseudo response regulator-like gene, will bind to *FT1* through similar motifs.

Studies into *FT2* expression in hexaploid wheat have also shown that its expression correlates with levels of *Ppd-1*, which suggests that Ppd-1 may also interact with the *FT2* promoter (Boden & Gauley, 2020). *FT2* was shown to be highly expressed between the lemma primordia and terminal spikelet stages of the apex development and higher expression of *Ppd-1* in the photoperiod insensitive resulted in higher *FT2* expression. This indicates *FT2*'s possible role in spikelet allocation and formation and increased expression could suggest why insensitive *Ppd-1* NILs present a lower number of spikelets on the wheat apex (Royo, et al., 2020). If both *FT1* and *FT2* are regulated directly through Ppd-1, there may be conserved regions across the 3 genomes and both genes in their promoters that could indicate where Ppd-1 specifically binds. Finding homologous sequences that they all share could identify the precise motif sequences. Ppd-1 is also thought to bind its own promoter so if a shared motif is found, then it may be present in the *Ppd-1* promoter as-well. Furthermore, *T. aestivum* contains a vastly expanded *FT* gene family (FT1-12) on three genomes (A, B and D) and so it would be interesting to know if Ppd-1 can regulate all of these *FT* genes in a similar way. Beyond having a role in floral development initiation (*FT1*) (Corbesier, et al., 2007)and transitioning between two apical stages (*FT2*) (Boden & Gauley, 2020), functions are largely unknown for the other 10 genes in the *FT* family. Understanding the functions of these *FT*'s and whether they are regulated through photoperiod could provide new insight into specific mechanisms associated with floral development.

To investigate the possibility of Ppd-1 binding *FT1* (and potentially other *FT* gene promoters) I first looked, *in silico*, for domains in the *FT* genes promoters with similarity to the *Arabidopsis PRR* motif. Then, I looked for conserved domains between the genes which we believe to be bound by Ppd-1. In parallel I also tested for interaction between Ppd-1 and the *FT1* promoter via yeast-1-hybrid. In the event that I observed an interaction via yeast growth, it would confirm that Ppd-1 does directly interact with the promoter region of *FT1*. Using this method, the idea would be to find which 500bp fragment contains the motif and then split that into smaller and smaller fragments until the motif was found. To start with, I split the promoter into 500bp fragments. Additionally, as we think that Ppd-1 binds the *FT1*, *FT2* and its own promoter, one could assume that there may be highly conserved motifs between these 3 genes. To find this out, I used a bioinformatical approach with the aim of using identified motifs from the to guide the design of the DNA fragments tested via yeast-1-hybrid interaction.

3.1 Materials and Methods

3.1.1 Yeast-1-Hybrid:

3.1.1.1 Cloning bait fragments

The bait genomic DNA was cloned from Cadenza via PCR. Each fragment contained a 500 base pair sequence taken from the promoter region of *FT1* and represent the 1st 500 – 4th 500 base pairs before the exon i.e. covering the 2kb upstream of the *FT1* start site. The sequence used was taken from Ensembl Plants *Triticum aestivum* v2 for the *FT1* gene with the ID '*TraesCS7B02G013100.1'*. These fragments were extracted from wheat genomic DNA using PCR according to the protocol provided by Jena Bioscience (Löbstedter Str. 71, 07749 Jena, Germany) and then run on a 1% agarose gel where the bands at 500bp were extracted and purified using New England Biolabs inc. gel extraction kit. They were then sequenced using Eurofin© overnight sequencing to confirm the product was correct.

For Dof and ZF-HD domains, forward and reverse single-stranded primers were acquired (IDNA genetics) and annealed together in a PCR reaction which reagents quantities were 4µl Q5 reaction buffer, 0.2µl Q5 Taq Polymerase, 1µl F1 primer, 1µl R1 primer, 0.4µl dNTPs and 13.4µl nuclease-free water where primer-dimers were formed.

3.1.1.2 Generating the yeast-1-hybrid constructs

The *Ppd-1* gene was provided courtesy of Dr Adam Gauley in the pGAD424 plasmid. *FT1* promoter 500bp fragments were transferred into the pHIS2 plasmid which contained tryptophan and histidine selection. Restriction enzymes (all from NEB, Massachusetts, USA) Sac1 and EcoR1 were used to cut the plasmid open and the same restriction enzymes (EcoR1, Sac1) were used to add restriction sticky ends to the fragments. This was done with 1µl Sac1 and EcoR1 enzymes, 2µl cutsmart buffer, 5µl plasmid and 11µl water for the plasmid reaction and 16µl fragment for the fragment reaction. Once restriction digested, the fragment and plasmid are combined through ligation in a mixture of 5µl T4 ligation buffer (NEB), 1µl plasmid, 3µl DNA fragment and 1µl T4 ligase (NEB) where they are left at 16^{o}C overnight.

Next, the plasmids containing the promoter fragments of *FT1* were transformed into *E. coli*. Briefly, 1µl of plasmid was added to 25µl of competent E.coli cells and left on ice for 30 minutes. A heat shock treatment of 30 seconds in a water bath which was heated to 42^{o}C, enabled the plasmid to be transformed into the *E. coli* cell. Then, 950µl of *E. coli* nutrient media is added and incubated for 60 minutes at 37^{o}C at 180 RPM. 100µl of *E. coli* cells were plated onto LB agar plates containing (1/1000 dilution) kanamycin and then incubated at 37^{o}C overnight. The fragment was confirmed to be in the *E.coli* via colony PCR. Here, 16.7µl water, 2µl ruby buffer (Jena Bioscience, Germany), 0.4µl (10mM) dNTPs, 0.1µl Taq Polymerase (Jena Bioscience, Germany) and 0.4µl of the

forward and reverse primers. A sterile toothpick was then used to remove part of a colony and was inserted into the tube and mixed until the colony was suspended. The PCR product was run on a 1% agarose gel and illuminated with Biotium Nucleic Acid stain, gel red. Bands at 500bp were the fragments that I had inserted.

Colonies that were confirmed to have the plasmid with the fragment insert were cultured in 5ml LB solution with 5µl kanamycin (1/1000 dilution) in a shaker overnight set at 37^{o}C, 180RPM.From the culture, the plasmid was then isolated using the Monarch plasmid miniprep kit according to the manufacturer's instructions. The plasmid was confirmed to contain the fragment via Eurofins overnight sequencing.

3.1.1.3 Yeast Transformation

The plasmid was transformed into yeast. Salmon sperm was thawed out and boiled for 5 minutes, 15µl of this was added to an Eppendorf tube along with 5µl plasmid DNA. 100µl TM solution (19.8% sterile 2m lithium acetate, 79.4% sterile 50% PEG, 0.8% 2-beta mercaptoethanol) was added and the mixture was vortexed for 15 seconds. For the host yeast, AH109 was used. A yeast colony was taken and inserted into the tube until it was uniformly suspended in the solution. This was then incubated at 29^{o}C, 180RPM in a shaker for 45 minutes. The yeast cells were taken out of the shaker and plated onto plates with an absence of tryptophan. This agar was made with 0.74% yeast nitrogen base with no amino acids, 0.07% Formedium amino acid mix (CSM, Silsden, UK), 2.2% Agar and 2.2% sugar into 225ml dH_2O. Yeast cells that had transformed with the plasmid were able to grow on the plate as it contained the ability to synthesis tryptophan.

Ppd-1 was isolated and cloned using the same method as the *FT1* promoter fragments, however a different plasmid, pGAD424, was used. This plasmid carries a different antibiotic resistance tag to ampicillin and therefore ampicillin was used in the plates. Once the plasmid had been successfully inserted into *E. coli* and then confirmed through PCR and gel electrophoresis the plasmids were isolated using the Monarch plasmid miniprep kit as per the manufacturer's instructions. The plasmid was then transformed into yeast cells containing pHIS2 using the yeast plasmid transformation method described above. To confirm that the yeast contained both plasmids, agar was made that lacked tryptophan and leucine. Only yeast cells with both plasmids would have the ability to colonise on the plate. Finally, to test the interaction, agar that contains no leucine, tryptophan and histidine was made and the cells were streaked on. If the interaction was present, colonies would grow. Stronger interactions were filtered out using serial dilutions of the yeast colonies.

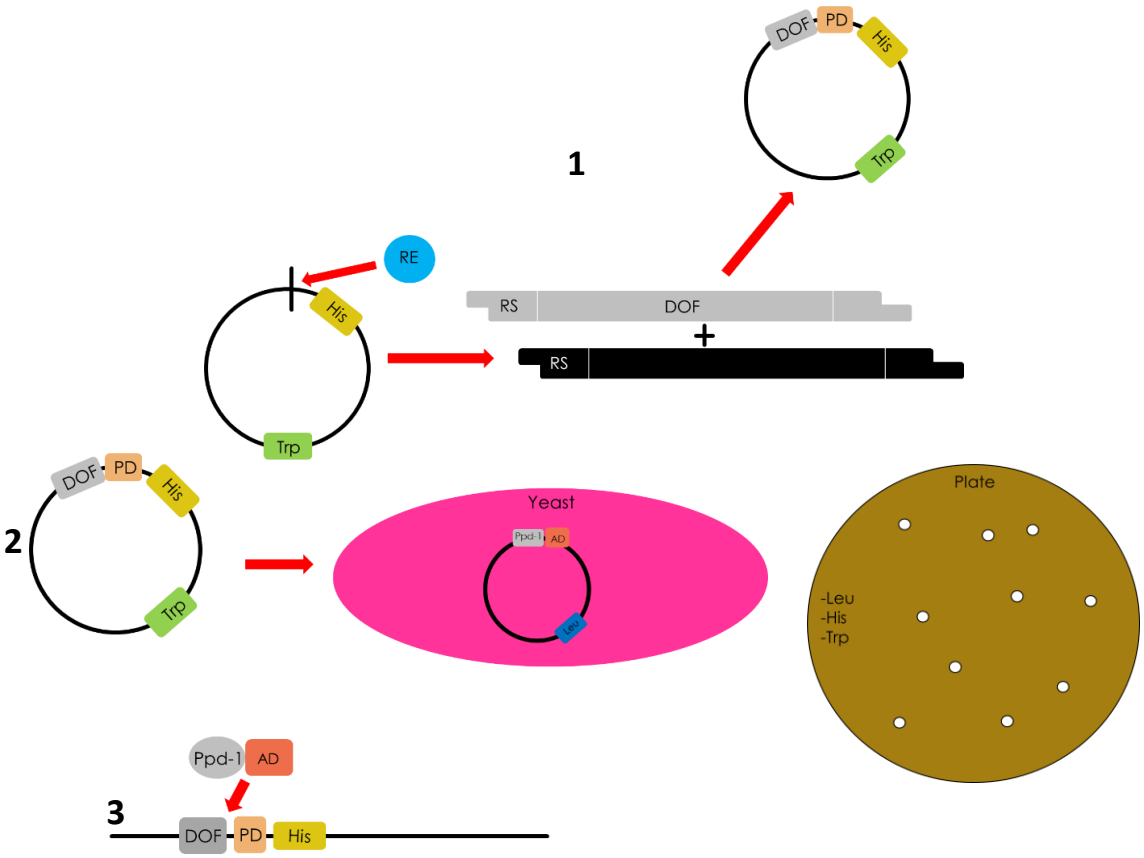

Figure 3.1: Yeast-1-Hybrid protocol: Schematic diagram showing the yeast-1-hybrid protein-gene interaction method. The promoter fragment 'Dof' domain was used as an example. Leu = Leucine, Trp = Tryptophan, His = Histidine. AD = Activation Domain, PD = Promoter Domain, RS = Restriction Site. (1) Restriction enzyme breaking open the plasmid and insertion of the fragment of interest into the plasmid. The plasmid in this case was pHIS2 and carries a histidine and tryptophan tag (2) pHIS2 plasmid transformed into yeast already containing pGAD424 with *Ppd-1* exon insert. If an interaction occurred, the yeast would form colonies, as shown. In (3), the specific interaction between Ppd-1 and the fragment in the *FT1* promoter is shown.

Primers Used:

Primer Name:	Orientation	Sequence
FT1 Promoter 1st 500bp	Forward	CCGAATTCATTCCCTGTGCCCGCTTG
FT1 Promoter 1st 500bp	Reverse	CCTATCCCTACCGGCCATTA
FT1 Promoter 2nd 500bp	Forward	CCGAATTCTGCAGCTCATACCTTTGGAA
FT1 Promoter 2nd 500bp	Reverse	CCGAGCTCCAAGCGGGCACAGGGAAT

FT1 Promoter 3rd 500bp	Forward	CCGAATTCGCATGTCGGTCGCACTTTAA
FT1 Promoter 3rd 500bp	Reverse	CCGAGCTCTTCCAAAGGTATGAGCTGCA
FT1 Promoter 4th 500bp	Forward	CCGAATTCCAATCAATGTGGGTGCTCAAAG
FT1 Promoter 4th 500bp	Reverse	CCGAGCTCTTAAAGTGCGACCGACATGC
Dof (Fragment only)	Forward	CCGAATTCGAGCTTATTACGGCAGAGAGCTCG
Dof (Fragment only)	Reverse	CGAGCTCTCTGCCGTAATAAGCTCGAATTCGG
ZF-HD (Fragment only)	Forward	CCGAATTCTATGCATGCCTTTTTCCCCTGAGCTCG
ZF-HD (Fragment only)	Reverse	CGAGCTCAGGGGAAAAAGGCATGCATAGAATTCGG
Dof (Region)	Forward	CCGAATTCGCCTTGAGCTCG
Dof (Region)	Reverse	CGAGCTCAAGGCGAATTCGG
ZF-HD (Region)	Forward	CCGAATTCATTACGAGCTCG
ZF-HD (Region)	Reverse	CGAGCTCGTAATGAATTCGG

3.1.2 Searching for Conserved regions between *Ppd-1*, *FT1* and *FT2* promoter regions:

For the identification of conserved regions, the promoter regions up to 3000 bp upstream of the genes start codon were run on a motif-searching software called PlanPAN 3.0 (http://plantpan.itps.ncku.edu.tw/promoter.php). This locates known motifs in the DNA and reports the gene family known to bind it, its position, and the hit sequence. The data was then exported to Microsoft Excel where it could be studied. To see if these highly conserved regions exist, the columns were grouped together: 'Family' and 'Position' for *FT1A*, *FT1B* and *FT1D* promoter regions and then compared them using the MATCH function. Once *FT1A* and *FT1B* had been compared, the common Family & positionswere taken from the 2 genes and compared them with *FT1D*. This result then showed the motifs in common between all three genes that were in the exact same position. This process was also applied to *FT2A*, *FT2B* and *FT2D*. Once the 3 genome copies of the *FT1* and *FT2* were compared, the same method was used to see the Family and Positions in common and this result was subsequently compared with *Ppd-D1*.

3.1.3 Finding *PSEUDO RESPONSE REGULATOR* (*PRR*) Promoter Domains in *FLOWERING LOCUS T* (*FT*) gene family promoters:

Promoter regions up to 3000 bp for each of the *FT* genes (*FT1-12*) were identified using Ensembl Plants (http://plants.ensembl.org/Triticum_aestivum/Info/Index) and pasted into a Microsoft Word document to be analysed. The promoter regions were then analysed to see if they contained motifs of interest. Motifs searched for were 'CACGTG', 'CCAAT', 'TTTGTT' and 'CCAAAAAGG'. The programme then highlighted if and where the domains were present in all the promoter regions which I then recorded the frequency of in a Microsoft Excel document. The motifs were then assessed if there were any upstream and/or downstream of the exon.

3.2 Results

3.2.1 Surveying the FT gene promoters for potential Ppd-1 binding domains

Ppd-1 is thought to directly regulate the expression of *FT1* and *FT2* via interaction with their promoter, however, the motif to which it interacts is unknown. In Arabidopsis there are motifs that certain PSEUDO RESPONSE REGULATOR (PRR) proteins interact with in order to activate floral development (Li, et al., 2015). Similar domains may be used by Ppd-1, as it has homology to *PRR* genes, to activate *FT1* and *FT2* expression. Motifs that were analysed were the G-Box, CCAAT box, 'TTTGTT' and the CCAxGG domain (Liu, et al., 2016), (Kamada & Miwa, 1992) (Tiwari, et al., 2010). These domains were searched for in FT1 and *FT2* but also in *FT3-12*. If it is discovered that Ppd-1 does regulate *FT1* and *FT2* expression directly, then we could assume that some of the other genes in the *FLOWERING LOCUS T* family may also be regulated via this pathway.

	Gene name:	No. Times G- Box present:	Upstream:	Downstream:
FT1	*TraesCS7A02G115400.1*	2	Y	N
	TraesCS7B02G013100.1	1	Y	N
	TraesCS7D02G111600.1	1	Y	N
FT2	*TraesCS3A02G143100.1*	1	Y	Y
	TraesCS3B02G162000.1	0	N	N
	TraesCS3D02G144500.1	1	Y	Y
FT3	*TraesCS1A02G338600.1*	1	Y	Y
	TraesCS1B02G351100.1	1	Y	N
	TraesCS1D02G340800.1	0	N	N
FT5	*TraesCS5A02G546900.1*	1	Y	Y
	TraesCS4B02G380600.1	0	N	N
	TraesCS4B02G379100.1	0	N	N
	TraesCSU02G130900.1	1	Y	N
	TraesCS5A02G546800.1	1	Y	N
	TraesCSU02G130800.1	2	Y	Y

	TraesCSU02G130700.1	0	N	N
FT4	*TraesCS2A02G132300.1*	2	Y	Y
	TraesCS2B02G154800.1	1	Y	N
	TraesCS2D02G134200.1	1	Y	N
	TraesCS7B02G272600.1	0	N	N
FT6	*TraesCS6A02G160200.1*	2	Y	Y
	TraesCS6B02G191200.1	2	Y	Y
	TraesCS6D02G152500.1	2	Y	Y
FT7	*TraesCS5A02G154600.1*	0	N	N
	TraesCS5B02G152800.1	2	Y	Y
	TraesCS5D02G159500.1	0	N	N
FT8	*TraesCS2A02G536700.1*	1	Y	N
	TraesCS2A02G536600.1	0	N	N
	TraesCS2A02G536900.1	0	N	N
	TraesCS2B02G567400.1	0	N	N
	TraesCS2B02G567200.1	0	N	N
	TraesCS2D02G538100.1	0	N	N
	TraesCS2D02G485100.1	2	Y	N
	TraesCS2B02G511400.1	3	Y	N
FT11	*TraesCS4B02G073800.1*	0	N	N
	TraesCS4D02G072300.1	0	N	N
FT9	*TraesCS2A02G347000.1*	1	Y	N
	TraesCS2B02G365300.1	1	Y	N
	TraesCS2D02G345700.1	1	Y	N

	TraesCS5B02G020300.1	0	N	N
FT12	*TraesCS6D02G197100.1*	1	Y	N
	TraesCS6A02G214400.1	2	Y	N
	TraesCS6B02G244400.1	1	Y	N
FT10	*TraesCS5A02G297300.1*	0	N	N
	TraesCS5B02G296600.1	1	Y	N
	TraesCS5D02G304400.1	1	Y	N

Table 3.1: G-box motifs in *FT1-12* promoter regions. Table showing *FT* genes 1-12 from all three genomes in hexaploid wheat. First and second columns show the '*FT*' name and *Triticum aestivum* names which denotes the genome position that the gene resides on. The third column shows the motif in question and then how many times it features within the promoter. Columns 4 and 5 show whether the motif was found to be upstream and/or downstream of the exon.

	Gene name:	No. Times CCAAT present:	Upstream:	Downstream:
FT1	*TraesCS7A02G115400.1*	5	Y	Y
	TraesCS7B02G013100.1	7	Y	Y
	TraesCS7D02G111600.1	5	Y	N
FT2	*TraesCS3A02G143100.1*	6	Y	Y
	TraesCS3B02G162000.1	3	Y	Y
	TraesCS3D02G144500.1	3	Y	Y
FT3	*TraesCS1A02G338600.1*	4	Y	Y
	TraesCS1B02G351100.1	5	Y	Y
	TraesCS1D02G340800.1	4	Y	Y
FT5	*TraesCS5A02G546900.1*	3	Y	Y
	TraesCS4B02G380600.1	1	N	Y
	TraesCS4B02G379100.1	2	Y	N
	TraesCSU02G130900.1	2	Y	Y
	TraesCS5A02G546800.1	4	Y	N
	TraesCSU02G130800.1	5	Y	Y
	TraesCSU02G130700.1	4	Y	Y
FT4	*TraesCS2A02G132300.1*	4	Y	Y
	TraesCS2B02G154800.1	5	Y	Y

	TraesCS2D02G134200.1	3	Y	Y
	TraesCS7B02G272600.1	4	Y	Y
FT6	*TraesCS6A02G160200.1*	4	Y	N
	TraesCS6B02G191200.1	5	Y	Y
	TraesCS6D02G152500.1	7	Y	Y
FT7	*TraesCS5A02G154600.1*	2	Y	Y
	TraesCS5B02G152800.1	3	Y	N
	TraesCS5D02G159500.1	3	Y	N
FT8	*TraesCS2A02G536700.1*	2	Y	N
	TraesCS2A02G536600.1	2	Y	N
	TraesCS2A02G536900.1	1	Y	N
	TraesCS2B02G567400.1	7	Y	Y
	TraesCS2B02G567200.1	4	Y	Y
	TraesCS2D02G538100.1	2	Y	N
	TraesCS2D02G485100.1	4	Y	N
	TraesCS2B02G511400.1	3	Y	Y
FT11	*TraesCS4B02G073800.1*	3	Y	N
	TraesCS4D02G072300.1	2	Y	N
FT9	*TraesCS2A02G347000.1*	6	Y	Y
	TraesCS2B02G365300.1	5	Y	N
	TraesCS2D02G345700.1	5	Y	N
	TraesCS5B02G020300.1	3	Y	N
FT12	*TraesCS6D02G197100.1*	5	Y	Y
	TraesCS6A02G214400.1	5	Y	Y
	TraesCS6B02G244400.1	9	Y	Y
FT10	*TraesCS5A02G297300.1*	3	Y	Y
	TraesCS5B02G296600.1	6	Y	Y
	TraesCS5D02G304400.1	4	Y	Y

Table 3.2: CCAAT motifs in *FT1-12* promoter regions. Table showing *FT* genes 1-12 from all three genomes in hexaploid wheat. First and second columns show the '*FT*' name and *Triticum aestivum* names which denotes the genome position that the gene resides on. The third column shows the motif in question and then how many times it features within the promoter. Columns 4 and 5 show whether the motif was found to be upstream and/or downstream of the exon.

	Gene name:	No. Times TTTGTT present:	Upstream:	Downstream:
FT1	*TraesCS7A02G115400.1*	4	Y	N
	TraesCS7B02G013100.1	2	Y	N
	TraesCS7D02G111600.1	1	Y	N
FT2	*TraesCS3A02G143100.1*	3	Y	Y
	TraesCS3B02G162000.1	4	Y	Y
	TraesCS3D02G144500.1	3	Y	Y
FT3	*TraesCS1A02G338600.1*	4	Y	Y
	TraesCS1B02G351100.1	2	Y	Y
	TraesCS1D02G340800.1	4	Y	Y
FT5	*TraesCS5A02G546900.1*	2	Y	N
	TraesCS4B02G380600.1	5	Y	Y
	TraesCS4B02G379100.1	3	N	Y
	TraesCSU02G130900.1	5	Y	Y
	TraesCS5A02G546800.1	3	N	Y
	TraesCSU02G130800.1	3	Y	Y
	TraesCSU02G130700.1	5	Y	Y
FT4	*TraesCS2A02G132300.1*	6	Y	Y
	TraesCS2B02G154800.1	5	Y	Y
	TraesCS2D02G134200.1	5	Y	Y
	TraesCS7B02G272600.1	5	Y	Y
FT6	*TraesCS6A02G160200.1*	3	Y	Y
	TraesCS6B02G191200.1	2	Y	Y
	TraesCS6D02G152500.1	1	N	Y

FT7	*TraesCS5A02G154600.1*	2	Y	Y
	TraesCS5B02G152800.1	2	Y	N
	TraesCS5D02G159500.1	2	Y	N
FT8	*TraesCS2A02G536700.1*	2	Y	Y
	TraesCS2A02G536600.1	1	N	Y
	TraesCS2A02G536900.1	2	N	Y
	TraesCS2B02G567400.1	6	Y	Y
	TraesCS2B02G567200.1	4	Y	Y
	TraesCS2D02G538100.1	2	Y	Y
	TraesCS2D02G485100.1	4	Y	Y
	TraesCS2B02G511400.1	2	Y	Y
FT11	*TraesCS4B02G073800.1*	4	Y	Y
	TraesCS4D02G072300.1	2	Y	Y
FT9	*TraesCS2A02G347000.1*	1	N	Y
	TraesCS2B02G365300.1	0	N	N
	TraesCS2D02G345700.1	1	Y	N
	TraesCS5B02G020300.1	3	Y	Y
FT12	*TraesCS6D02G197100.1*	2	Y	Y
	TraesCS6A02G214400.1	4	Y	Y
	TraesCS6B02G244400.1	5	Y	Y
FT10	*TraesCS5A02G297300.1*	1	N	Y
	TraesCS5B02G296600.1	3	Y	Y
	TraesCS5D02G304400.1	5	Y	Y

Table 3.3: 'TTGTTT' motifs in *FT1-12* promoter regions. Table showing *FT* genes 1-12 from all three genomes in hexaploid wheat. First and second columns show the '*FT*' name and *Triticum aestivum* names which denotes the genome position that the gene resides on. The third column shows the motif in question and then how many times it features within the promoter. Columns 4 and 5 show whether the motif was found to be upstream and/or downstream of the exon.

	Gene name:	No. Times CCAAAAAGG present:	Upstream:	Downstream:
FT1	*TraesCS7A02G115400.1*	1	Y	N
	TraesCS7B02G013100.1	0	N	N
	TraesCS7D02G111600.1	0	N	N
FT2	*TraesCS3A02G143100.1*	0	N	Y
	TraesCS3B02G162000.1	0	N	N
	TraesCS3D02G144500.1	0	N	N
FT3	*TraesCS1A02G338600.1*	0	N	N
	TraesCS1B02G351100.1	0	N	N
	TraesCS1D02G340800.1	0	N	N
FT5	*TraesCS5A02G546900.1*	0	N	N
	TraesCS4B02G380600.1	1	Y	N
	TraesCS4B02G379100.1	1	Y	N
	TraesCSU02G130900.1	0	N	N
	TraesCS5A02G546800.1	0	N	N
	TraesCSU02G130800.1	0	N	N
	TraesCSU02G130700.1	0	N	N
FT4	*TraesCS2A02G132300.1*	0	N	N
	TraesCS2B02G154800.1	0	N	N
	TraesCS2D02G134200.1	0	N	N
	TraesCS7B02G272600.1	0	N	N

FT6	*TraesCS6A02G160200.1*	0	N	N
	TraesCS6B02G191200.1	0	N	N
	TraesCS6D02G152500.1	0	N	N
FT7	*TraesCS5A02G154600.1*	0	N	N
	TraesCS5B02G152800.1	0	N	N
	TraesCS5D02G159500.1	0	N	N
FT8	*TraesCS2A02G536700.1*	0	N	N
	TraesCS2A02G536600.1	1	Y	N
	TraesCS2A02G536900.1	1	Y	N
	TraesCS2B02G567400.1	0	N	N
	TraesCS2B02G567200.1	0	N	N
	TraesCS2D02G538100.1	0	N	N
	TraesCS2D02G485100.1	1	Y	N
	TraesCS2B02G511400.1	1	Y	N
FT11	*TraesCS4B02G073800.1*	0	N	N
	TraesCS4D02G072300.1	0	N	N
FT9	*TraesCS2A02G347000.1*	0	N	N
	TraesCS2B02G365300.1	0	N	N
	TraesCS2D02G345700.1	0	N	N
	TraesCS5B02G020300.1	0	N	N
FT12	*TraesCS6D02G197100.1*	0	N	N
	TraesCS6A02G214400.1	0	N	N
	TraesCS6B02G244400.1	0	N	N
FT10	*TraesCS5A02G297300.1*	0	N	N

	TraesCS5B02G296600.1	0	N	N
	TraesCS5D02G304400.1	0	N	N

Table 3.4: 'CCAAAAAGG' motifs in *FT1-12* promoter regions. Table showing *FT* genes 1-12 from all three genomes in hexaploid wheat. First and second columns show the '*FT*' name and *Triticum aestivum* names which denotes the genome position that the gene resides on. The third column shows the motif in question and then how many times it features within the promoter. Columns 4 and 5 show whether the motif was found to be upstream and/or downstream of the exon.

The motifs displayed in (Tables 3.1, 3.2, 3.3, 3.4) (G-Box, CCAAT Box, TTTGTT, CCAxGG domain) were found across many of the promoter regions of the *FT* genes. Domains 'TTTGTT' and 'CCAAT' were found in high frequency in the majority of the *FT*s with only one, *FT9B*, that did not contain either motif (Table 3.2, 3.3) The ubiquitous nature of these domains could suggest that they may not play a role in their regulated expression as they are not specific to certain genes that we may expect to be regulated by *Ppd-1*. The 'CCAAAAAGG' motif was only found 7 of the genes analysed (Figure 3.4). Only *FT1A* was found to possess a CCAAAAAGG motif which indicates it has no role as a *Ppd-1* binding domain. If we expect Ppd-1 to bind to this motif in the promoter, we would expect to find it in the promoters of *FT1*, *FT2* and other genes in the *FT* family. The G-Box was found in low numbers in approximately half of the genes analysed (29/46) and were found in 5 out of the 6 *FT1* and *FT2* gene promoters (Table 3.1). This is interesting as the study by (Liu, et al., 2016) shows that they feature at PRR binding regions and are potentially used to regulate the genes that *PRR*s bind to. The Reference genes 'TraesCS3D02G330500' and *RL1* (Jakobus & Scholtz, 2013) were also analysed for presence of motifs of interest where most were found in abundance and sporadically throughout the promoter (Tables 3.1-3.4). This suggested that the motifs are not specific to *Ppd-1* or even *PRR* genes as the housekeeping genes are not expected to be regulated by Ppd-1 and therefore this analysis was not carried on further.

3.2.2 Identifying conserved domains within promoters known to be bound by *Ppd-1*

As the initial approach of using motifs identified in Arabidopsis did not identify promising candidates a second approach was taken. To find the conserved regions between the three genes, the promoter regions up to 3000bp were aligned and multiple comparisons were made in Microsoft Excel to establish the common motif families between them using the domain search engine software Plant Pan 3.0. Once the three genes had been compared, I found that there were two motifs in common at the same position. The first was a Zinc Finger-Homeodomain transcription factor (*ZF-HD*) at position -1540 and the other was a DNA binding with one finger (*Dof*) domain located at position -1183 before the exon (Figure 3.2). Using the Interpro Protein classification (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR006456/), *ZF-HD* genes have been shown to be expressed exclusively in floral tissue and are associated with development

of floral organs. This motif is therefore of higher interest than the Dof domain with regards to Ppd-1 regulation of FT as it has been documented to have a role in floral development. However, it is important to note that the Ppd-1-*FT1* interaction is thought to occur in the leaf and not the meristem.

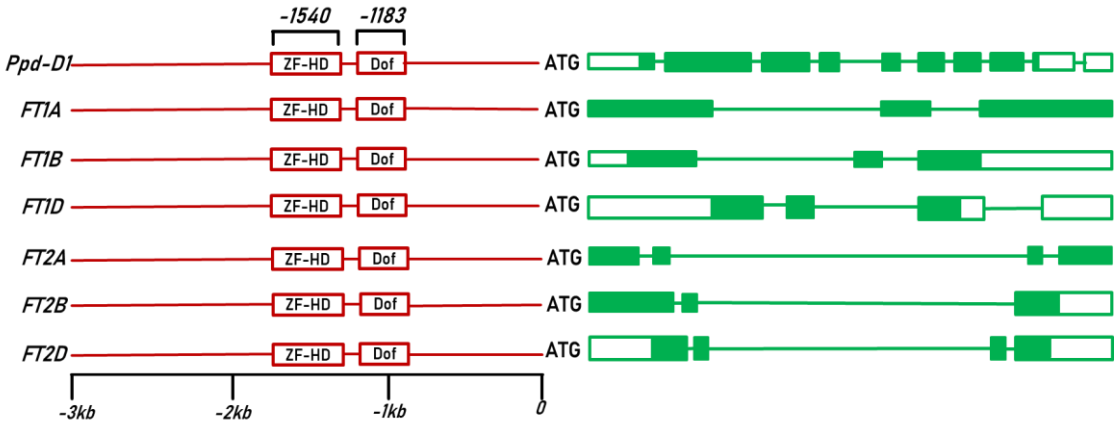

Figure 3.2: Conserved regions within the promoter regions of *Ppd-D1*, *FT1* and *FT2*. Promoter analysis for conserved regions between *Ppd-1*, *FT1A,B,D* and *FT2A,B,D*. Red areas denote the promoter region of the gene and green denotes the exon. Genes analysed for these regions are *Ppd-D1*, *FT1A*, *FT1B*, *FT1D*, *FT2A*, *FT2B*, *FT2D*. Zinc Finger Homeodomain (ZF-HD) and DNA binding with One Finger (Dof) are shown in red boxes in the promoter region. Dof domains are positioned 1183bp before the start codon of the exon (ATG) and ZF-HD domains are positioned 1540bp before the start codon.

From Dr Adam Gauley's preliminary results (presented in his doctoral thesis), he identified various genes that's expression also seemed to be correlated with *Ppd-1* expression in *T. aestivum*. These wheat genes; *CAT1, ALOG1A-D, Bzip1A-D,* that are thought to be also regulated via Ppd-1 were also analysed and whilst there was not the same level of conservation in the promoter regions, it was found that they all contained at least one Dof or ZF-HD motif (Figure 3.3).

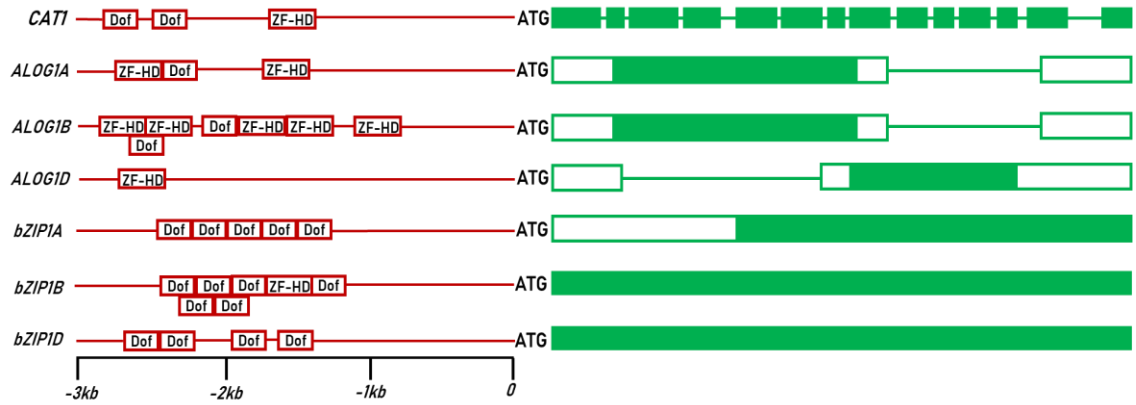

Figure 3.3: Identification of Dof and ZF-HD domains in genes thought to be regulated via *Ppd-1*. Promoter analysis for conserved regions between genes identified by Dr Adam Gauley that may also be regulated by *Ppd-1*. Red areas denote the promoter region of the gene and green denotes the exon. Genes analysed for these regions are *CAT1*, *ALOG1A,B,D, bZIP1A,B,D.* Zinc Finger Homeodomain (ZF-HD) and DNA binding with One Finger (Dof) are shown in red boxes in the promoter region.

These motifs were identified as good candidates for yeast-1-hybrid analysis. They were cloned into *E. coli* using the method referred to in the methods of this chapter, but they were not yet transformed into yeast.

3.2.3 Establishing yeast-1-hybrid system to identify Ppd-1 protein-*FT* promoter interactions

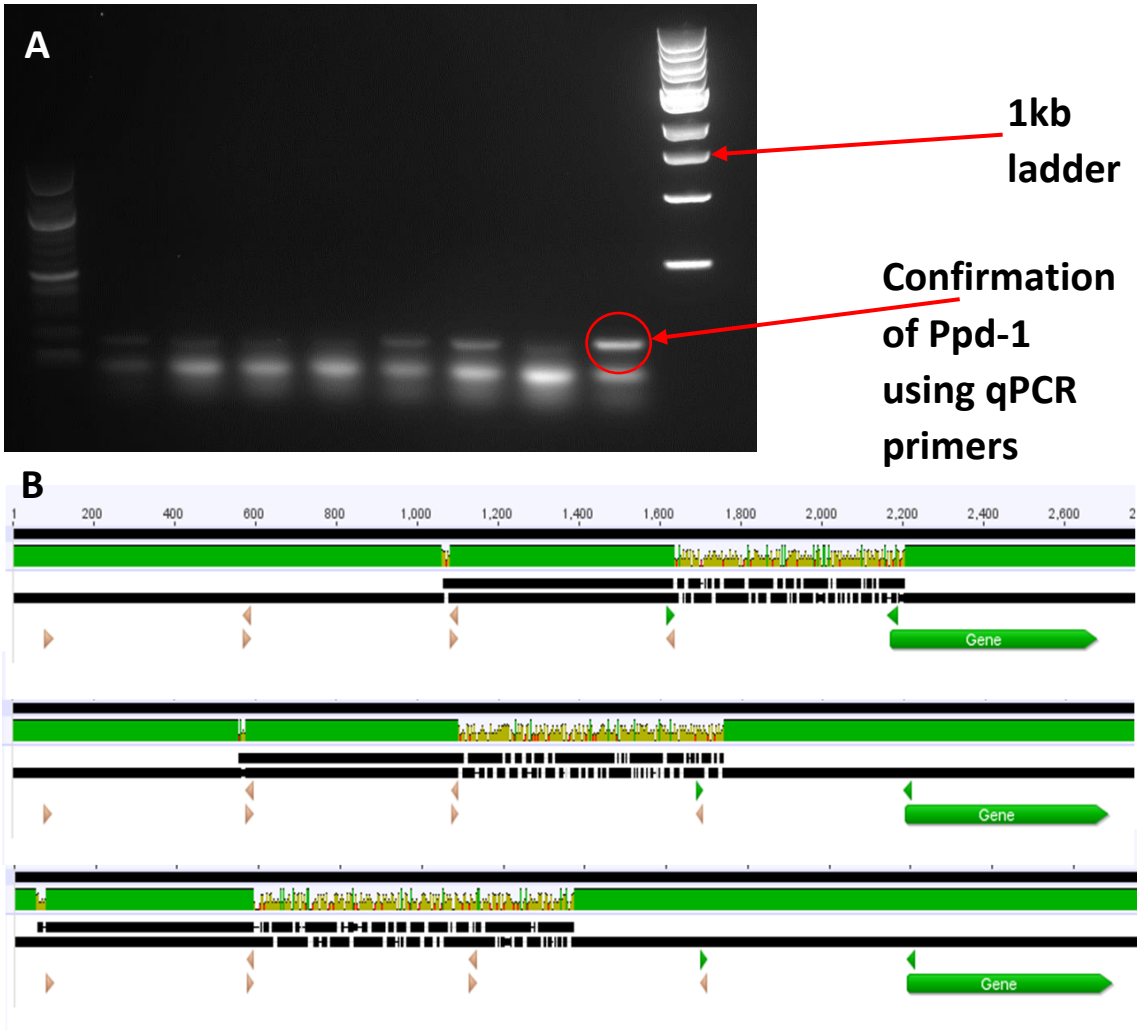

Figure 3.4: Confirmation of *Ppd-1* fragment and *FT1* promoter fragments through PCR/ gel electrophoresis and Eurofin© overnight sequencing. (A) shows confirmation of the Ppd-1 fragment successfully inserted into pGAD424 in an E.coli colony of interest of 80bp. (B) shows sequencing results of pHIS2 plasmids with inserted *FT*P2, *FT*P3, *FT*P4 (2nd 500bp, 3rd 500bp, 4th 500bp before exon).

The established expression analysis, combined with the promoter analysis presented above support the idea that Ppd-1 regulates *FT1* expression either directly or via other conserved proteins. The purpose of this experiment was to test for an interaction between Ppd-1 and the *FT1* promoter. The *FT1* promoter was split into 500bp fragments (confirmed in Figure 3.4B) and then inserted into a pHIS2 plasmid. The pGAD424 containing *Ppd-1* was also transformed into the same yeast cells and then grown on -trp, -leu, -his agar plates where colonies would form if an interaction took place. For each stage of transformation there were steps to confirm that it had been successful.

The first transformation was inserting the plasmids into *E.coli*. For pHIS2 and pGAD424, there are antibiotic resistance tags on each plasmid (Kanamycin and Ampicillin respectively), therefore any colonies that grew should be successfully transformed with the desired plasmid. As extra confirmation, plasmids were extracted (see methods) and sequenced using Eurofin© overnight sequencing (Figure 3.5B) for the promoter fragments. For Ppd-1, Part of the fragment was amplified in a PCR reaction and run on a gel (Figure 3.4A). This confirmed the presence of the pGAD424 plasmid and Ppd-1 fragment in *E. coli*; the plasmid was then extracted (see methods). The pHIS2 plasmid contains a Leucine (Leu) selection and pGAD424 contains a Tryptophan (Trp) tag. Once the plasmid had been inserted and the yeast had been transformed with pHIS2 + FT1 promoter fragments, they were spread across plates lacking Leu, so if any yeast grew, it would be confirmed that the transformation was successful. This same yeast was transformed again with pGAD424 and streaked across plates that lacked Leu and Trp as pGAD424 contains a Trp tag. Yeast that grew on this plate were therefore confirmed to have both pGAD424 and pHIS2. To study the interaction, the colonies were picked from the -Leu, -Trp plate and dropped onto a -Leu, -Trp, -His plate. Only colonies where the two plasmids had interacted could grow on the plate (Figure 3.1)(Figure 3.6A).

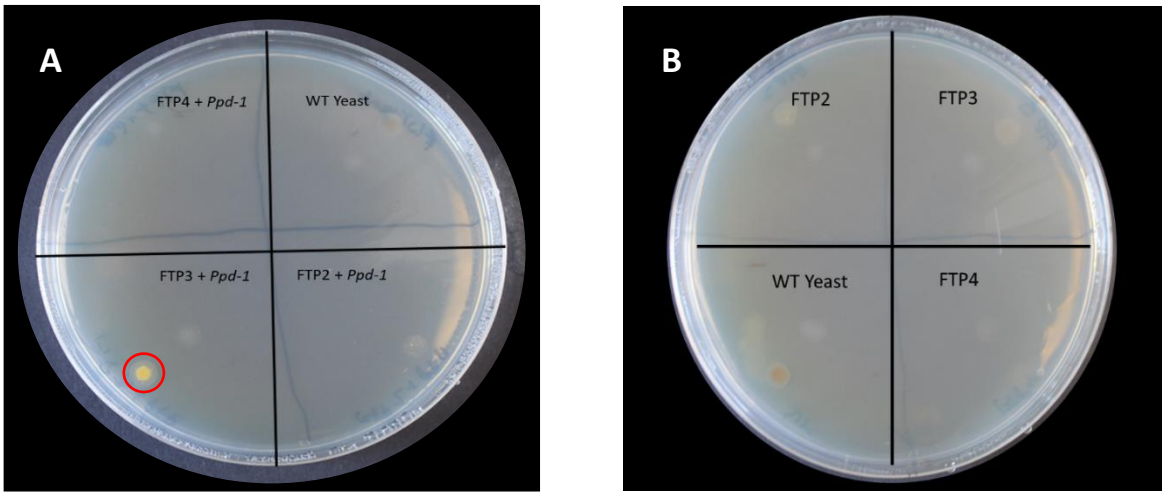

Figure 3.5: Yeast-1-Hybrid showing interaction between FT1 promoter and Ppd-1 Protein. Plates containing selective media with -Trp, -Leu, -His shown in (A) and (B). *FT*P2 represents the 2nd 500bp before the exon of *FT1*, *FT*P3 represents the 3rd 500bp and *FT*P4 represents the 4th 500bp. Yeast that were streaked onto the plate in (A) contained the pHIS2 plasmid containing the promoter region fragments and pGAD424 plasmid containing the *Ppd-1* exon. Wild-Type yeast was also used as a control. An additional control is visualised in (B) where yeast only containing the promoter region fragments were added to the plate along with some WT yeast.

Yeast-1-hybrid is an extremely powerful tool in the study of protein-gene interactions. The selective media approach is a relatively cheap and efficient way of seeing if the plasmid has been transformed into the host yeast. Additional checks can be made by extracting the plasmids and sequencing them or running them on a gel to see if bands

of the appropriate size are present. For this experiment there were many confirmation steps that were taken in order to only have colonies containing the desired plasmid, so that if there was growth on the final plate, the result is therefore as accurate as it can be. As seen in Figure 3.5A, there is growth on the plate in the '*FT*P3 + *Ppd-1'* quarter. This was confirmed to be yeast through studying it under the microscope. No colonies grew on any of the other three quarters seen in Figure 3.5, '*FT*P2 + *Ppd-1'*, '*FT*P4 + *Ppd-1*' and 'WT Yeast'. There was no growth of any of the yeast containing just the pHIS2 plasmid with *FT1* promoter fragments seen (Figure 3.5B) '*FT*P2', '*FT*P3', *FT*P4' and 'WT Yeast'. This therefore supports the hypothesis that Ppd-1 can directly bind to the *FT1* promoter and that this interaction is specific in the region *FT*P3 upstream of the *FT1* start codon.

The two conserved motifs, Zinc Finger Homeodomain (ZF-HD) and DNA binding with One Finger (Dof) are found within the 3rd 500bp and 2nd 500bp from the FT exon respectively. It is therefore interesting that the yeast that grew and confirmed an interaction between Ppd-1 and the *FT1* promoter, occurred in the 3rd 500bp before the exon where the ZF-HD motif resides. However, it is important to note that this interaction would be considered weak as only yeast from the first dilution grew on the plate.

3.3 Discussion

3.3.1 Investigating *PRR* domains in the FLOWERING LOCUS T (*FT*) family

PHOTOPERIOD 1 (*Ppd-1*) is the major gene in monocots which, through close monitoring of the photoperiod, is thought to drive the photoperiod-dependant flowering response. *Ppd-1* is thought to directly bind to the *FT1* promoter and activate its expression, which is the protein largely considered to be a major part of the mobile florigen signal. This florigen signal then moves from the leaf, down the phloem, to the vegetative meristem (apex) where it interacts with 14-3-3 protein and *FD*-like protein (a bZIP transcriptional factor) to form a complex (Corbesier, et al., 2007) (Li, et al., 2015). This complex, in turn, interacts with floral identity genes localised to the meristem that transition the plant to being in a reproductive state (Li, et al., 2015). This same system was initially discovered and has been rigorously studied in *Arabidopsis* (Notaguchi, et al., 2008).The described mechanism all stems from the plant's needs for careful detection of photoperiod changes that gives the plant an evolutionary advantage in terms of flowering success. This advantage stems from flowering occurring when temperatures are not too cold (associated with short photoperiods) and not too hot (associated with long photoperiods), where both scenarios can drastically effect plant fertility (Royo, et al., 2020). *Ppd-1* is not known by this name in *Arabidopsis* and instead its homologues are called *PRR3* and *PRR7* which are members of the *PSEUDO RESPONSE REGULATOR* (*PRR*) family of genes, and these orchestrate functions within the plant circadian clock (Nakamichi, et al., 2010). The PRR genes have been shown to bind the *FT* promoter (Hayama, et al., 2017) and so raised the question of whether the same is observed in wheat.

The first logical step in finding if Ppd-1 does indeed bind the *FT1* promoter would be to see if any known PRR domains feature in the *FLOWERING LOCUS T* (*FT*) promoter regions in wheat. As shown in Figure 3.2, it was found that many are present in the *FT* promoter region but none besides the G-box present any interesting data due to lack of motifs present or too many. The 'CCAAT' box motif seems to appear too many times in the promoter regions of most of the *FT* genes analysed to be specific for any regulatory interaction (Tiwari, et al., 2010) (Liu, et al., 2016). The CCAAT box has been shown to be involved in upregulated *FT* expression through CONSTANS (*CO*) mediated interaction and this motif was therefore of high interest. The 'CCAxGG' did not bring fruitful results, however, there are many different versions of this motif where some differ in the number of adenine present. This means that it is possible that this domain is significant but exists in a different permutation. The G-box gave interesting result when looking in the *FT* promoters with it only being present once or twice in any given promoter region and seemed to be specific to certain FT genes (Table 3.1). With G-box motifs being overrepresented in *PRR* promoter regions and being involved in transcriptional regulation of the target genes *PRR9* and *CCA1*, it makes it an interesting candidate for Ppd-1 and *FT* interaction (Liu, et al., 2016). However, when analysing the reference genes '*TraesCS3D02G330500*' and *RL1* (Jakobus & Scholtz, 2013), which are providing non-photoperiod regulated controls, all motifs analysed, including the G-box, were

found to be present in the promoter region. This implies that the motifs are not specific to *PRR* promoters and study into these were not carried further.

3.3.2 Searching for conserved regions in *Ppd-1*, *FT1* and *FT2*

Protein specificity is key to understanding how proteins such as transcriptional factors interact with gene promoter regions and regulate their expression. This idea of specificity is what drove the search for conserved regions in the *FT* promoters. However, the targeted approach of looking for specific motifs known to be regulated by the Arabidopsis *PRR* genes had not yielded obvious candidates, a second approach was taken. This approach used promoter domain searching software to identify all the known domains within specific promoter fragments. The search focused on *Ppd-1*, *FT1* and *FT2*. *Ppd-1* expression has been shown to correlate with expression of both *FT1* and *FT2* and it is thought that it may also bind its own promoter (Boden & Gauley, 2020). As the Ppd-1 protein potentially binds all three of these promoter regions it would be plausible that the motif remains the same throughout. This was found to be the case when aligning the promoter regions up to 3000bp before the first exon and gave light to the Zinc Finger Homeodomain (ZF-HD) and DNA binding with One Finger (Dof) promoter motifs (Figure 3.3). These were found to be extremely highly conserved between the promoter regions, appearing at the exact same positions in all 7 promoter regions analysed (*Ppd-D1, FT1A,B,D, FT2A,B,D*). This level of conservation could point to one of these motifs being used by Ppd-1 to interact with target promoters in order to regulate gene expression as seen in other *PRR* genes that act as transcriptional factors (Liu, et al., 2016). Equally, if not a target of the *Ppd-1* protein the high level of conservation suggests that they will be targets of important regulators for these genes. The search for conserved regions was conducted for *FT1* and *FT2* out of the 12 genes in the *FT* family and one way of predicting if an *FT* gene is photoperiod regulated would be to look for these binding motifs.

3.3.3 Testing if Ppd-1 can bind *FT1* promoter regions

It is heavily speculated that Ppd-1 interacts with *FT1* and regulates its expression, and this comes from correlated expression levels and studies with *Ppd-1* knockout and photoperiod insensitive wheat plants (Shaw, et al., 2012) (Shaw, et al., 2013). This interaction has never been directly seen to happen and in this study, yeast-1-hybird seems to suggest that is does, even if it is only a weak interaction. Yeast colonies formed in the transformation which contained the *FT* Promoter 3 (*FT*P3) which is the third 500bp in the promoter region (1000-1500bp) and Ppd-1 protein. Interestingly, this is the region where the conserved Dof motif resides. The specificity of this interaction is supported by the fact that no yeast colonies were observed for the other three *FT1* promoter fragments tested (Figure 3.5B). Whilst there are many limitations of the yeast-1-hybrid approach this is an exciting observation.

To take this forward, the Dof and ZD-HD motifs have been cloned and inserted into yeast where the interaction with Ppd-1 will be studied in a yeast-1-hybrid setting, similar to

the method used to find the *FT*P3 interaction result. If this is not successful, an alternative approach of splitting *FT*P3 into 100bp fragments and test with *Ppd-1* protein to further narrow down where the binding occurs will be conducted. Additionally, it would be interesting to understand if the interaction in the yeast system is only involving the Ppd-1 protein and *FT1* DNA. There may also be additional yeast proteins that are recruited to create a complex as seen in *FT1*, *FD* and 14-3-3 which were confirmed in yeast-2-hybrid studies (Li, et al., 2015). One way of further testing this would be through the use of surface plasmon resonance (SPR) (Douzi, 2017). This method is able to study binding affinity and protein-DNA interaction in real-time and, through inserting the Ppd-1 protein onto the chip and running the promoter region over it, interaction could be monitored. Additionally, this approach would also provide additional verification that the interaction in the yeast-1-hybrid system is specific and accurate.

Furthermore, the same region of promoter could be cloned from *FT2* and tested in a yeast-1-hybrid system to see if the area of binding for *Ppd-1* is conserved between *FT1* and *FT2*. If this is the case, accurate prediction of *Ppd-1* regulation of other members of the *FT* family (*FT3-12*) could be conducted through testing this region of the promoters against Ppd-1. The Dof domain was seen to be present in the same region of the *FT1* and *FT2* promoter regions and also resides in the region of the promoter that evidenced an interaction with Ppd-1. This could prove to be the motif used by Ppd-1 to regulate *FT1* and *FT2* transcription and, as referenced earlier, cloning this, and transforming it into yeast could bring interesting results. Furthermore, the Dof domain has been found in other genes thought to be regulated by Ppd-1 which enforces the fact that this could be the motif used (Figure 3.4). However, the Dof proteins have been shown to be mainly involved in early plant processes, such as germination so it may be unlikely that Ppd-1 uses this domain to regulate *FT* expression (Ruta, et al., 2020).

CHAPTER 4: Does *Ppd-1* regulate the expression of the wider *FT* gene family in wheat?

4.0 Introduction

In photoperiod sensitive hexaploid wheat, flowering is dependent on a tightly regulated mechanism to ensure it occurs at a time when conditions are less likely to cause damage to floral organs (Royo, et al., 2020). This mechanism occurs through regulation of the floral integrator gene *FLOWERING LOCUS T* (*FT1*) via the pseudo response regulator, *PHOTOPERIOD 1* (*Ppd-1*). These genes work in tandem to ensure precise reproductive initiation and polymorphisms around *Ppd-1* cause early and late flowering phenotypes (Shaw, et al., 2012) (Shaw, et al., 2013). The *Ppd-1-FT1* interaction occurs in the leaf where FT1 is then expressed as a florigen signal. Results from chapter 3 indicate that the interaction is likely to be directly between Ppd-1 binding *FT1* promoter. The FT1 protein is then believed to travel down the phloem to the meristem where it interacts with 14-3-3 and FD-like proteins to form a complex which in-turn interacts with floral identity genes, as observed in *Arabidopsis* (Li, et al., 2015). This then initiates reproductive development of the meristem. Another *FLOWERING LOCUS T* gene with a documented function in this process in wheat is *FT2*. Through the study of Ppd-1 NILs, it was found that *FT2* showed higher expression in *T. aestivum* that carried a photoperiod insensitive allele. This expression was increased between the apical development stages of lemma primordia and terminal spikelet and as insensitive varieties produce a lower number of spikelets when compared to their photoperiod sensitive counterparts, *FT2* has been associated with spikelet formation (Boden & Gauley, 2020) (Shaw, et al., 2019).

Recent studies into the wheat genome have discovered that there are 12 FLOWERING LOCUS T (*FT*) genes present in wheat in total (Bennett. & Dixon, 2021) (Halliwell, et al., 2016). Of these 12, we have documented a function for just two of them: *FT1* and *FT2*. This leaves 10 *FT* genes with completely unknown roles within the plant. Finding the purpose of these genes has a high importance in the context of flowering time as they are thought to play an important role in regulating this. It is also possible that they have a function outside of flowering time such as in regulating apical architecture or nutrient allocation. Some of the FTs may not be just confined to the apex and may serve as developmental signals as seen with FT *in Arabidopsis* where a florigen signal travels through the phloem from the leaf to the vegetative apical meristem, inducing a reproductive state (Corbesier, et al., 2007). Much of what we think regarding the functions of *FT3-12* are in *T. aestivum* are just educated guesses and due to the genome of wheat being sequenced only recently, very few studies have been done to try and discover them.

Studies in *H. vulgare* have identified some functions for *FT3* and *FT4*. When *FT3* was first discovered it was named *Ppd-2* as it was seen to cause variation in flowering time in barley in short-days (Laurie, et al., 1995). However, when the FTs were being identified, *FT3* was found to be in the same region of in the chromosome as *Ppd-2* and therefore the name was changed (Halliwell, et al., 2016) (Zikhali, et al., 2017). In Barley carrying recessive HvFT3 alleles, delayed flowering occurs in short day (SD) photoperiods (Zikhali, et al., 2017) (Muhammed, et al., 2018). This implies *FT3'*s role as a short-day inducer of flowering and this phenotype may protect the barley from early flowering and therefore colder temperatures associated with short photoperiods (Zikhali, et al., 2017). *TaFT3* and *HvFT3* share many similarities in terms of coding regions and predicted proteins

with a 95-96% and 96-97% similarity respectively (Halliwell, et al., 2016) and therefore *FT3* could serve the same purpose in wheat. With regards to *HvFT4*, overexpression conferred a later flowering time in long days in a study conducted in 2021 (Pieper, et al., 2021). Overexpression also decreased the grain mass harvested by reducing grain size, number of tillers and spikelet number. This indicates *HvFT4* potentially having a repressive role in barley floral development and as *HvFT3* share protein and gene homology with *TaFT3*, this could also be the case with *TaFT4*.

Amino acid sequences of the members of the *FT* family have been identified and analysed to look for sequence homology to assign either activator or repressor functions (Bennett. & Dixon, 2021). Based on this homology, the FTs have been split into clades. The first clade contains *FT1* and *FT2* and both share Arg (R), Thre (T), Pro (P), Phe (F), Arg (R) conserved sequences between them and are both thought to bind to 14-3-3 protein and from a floral complex. The second clade also has this same sequence homology, and this clade contains *FT3*, *FT4* and *FT5*. These genes therefore have also been predicted to form a complex with 14-3-3 due to the presence of these conserved binding sites. *FT1* and *FT2* both give activator phenotypes and therefore another conserved region has been associated with this; Tyr (Y) and Trp (W). In the third clade, there is *FT6*, *FT7*, *FT8*, *FT9*, *FT10*, *FT11* and *FT12* and these do not share the 14-3-3 binding sequence homology that is present in *FT1-5* (Bennett. & Dixon, 2021). However, the *FT*s in clade 3 do share the activator amino acid conservation that is found in *FT1* and *FT2* which and have therefore been predicted to be activators. *FT3*, *FT4* and *FT5* do not have the Y and W amino acid conservation and are therefore predicted to be repressors. However, evidence from studies in *FT3* seem to suggest it being a short-day inducer and therefore an activator and not repressive (Zikhali, et al., 2017). On the other hand, FT3 could be activating a gene that negatively affects flowering, thereby appearing to have a repressive role.

A well-studied and established theory surrounding *FT1* and *FT2* is that Ppd-1 regulates their expression in wheat. This begs the question, could Ppd-1 be also regulating the other *FT*s? The *FT*s have been assigned to FLOWERING LOCUS T based on the homology that they have with each other, so it is likely that some will also share their regulation through the Ppd-1 route (Bennett. & Dixon, 2021). This regulation could be direct via Ppd-1 interacting with the promoter region of the gene or indirect where *Ppd-1* would regulate a gene that in turn regulates one of the *FT*s. Seeing where these novel *FT* genes are being expressed and if they are regulated through Ppd-1 was the main focus of my project and I carried out experiments to test this.

To properly study the expression across the floral development of a wheat plant, apex samples were taken as this is where *FT* genes are deemed potentially be active. The stages of apex that were selected for dissection were vegetative (Veg), double-ridged (DR), terminal spikelet (TS), glume, florets and rachis at when the plant was booting. These stages are key points in the apex development and have great potential to host *FT* activity. The germplasm used consists of *Ppd-1* NILs and were selected to study whether *Ppd-1* expression affected FT expression besides *FT1* and *FT2*. The four NILs used were Paragon wild-type (WT), triple knockout (TK), single insensitive (SI) and triple insensitive (TI). If any of the FTs are regulated via Ppd-1, we should expect to see changes

in expression levels such as an increase in the insensitive lines and a decrease in the knockout, which in this case, would show that Ppd-1 activates its expression as seen in *FT1*.

4.1 Materials and Methods

4.1.1. Germplasm and sample collection

The gene expression studies use the same genetic material as the phenotyping described in Chapter 2: the *Ppd-1* triple knockout (TK), single insensitive (D genome) (SI), triple insensitive (TI) and wild-type Paragon (WT). These plants were planted into pots as per method in Chapter 2.1.2. The wheat was grown in controlled greenhouse conditions with 16h light/ 8h dark cycle and with the temperature set at 23^{o}C. All plants were grown in a cereal mix as detailed in Chapter 2.1.2.

Once the plant apexes were determined to be the right stage for the required sampling, they were transported to the lab and extracted with a sterile scalpel. The apexes were then placed into 1.5ml Eppendorf tubes and immediately frozen using liquid Nitrogen. These samples were cryogenically stored at -80^{o}C. For smaller samples (vegetative and double-ridged meristems), 10 apexes were collected per Eppendorf tube and for larger (at terminal spikelet), only 3 were assigned to each tube. In the case of glume and floret samples, a 1.5ml Eppendorf tube was filled to approximately quarter full and for rachis, they were cut to approximately an inch in length and placed into an Eppendorf tube.

4.1.2. RNA extraction and cDNA synthesis

The RNA was extracted from the samples using a Qiagen (Hilden, Germany) RNeasy kit as per the manufacturer's instructions and reverse transcribed them into cDNA. First, the RNA was treated with 1µl DNAse (Promega, Wisconsin, USA) and 1µl DNAse buffer (Promega) and incubated the samples at 37^{o}C for 30 minutes. Once this had finished, I added 1µl DNAse stop solution (Promega, Wisconsin, USA) to the tube and incubated this at 65^{o}C for 10 minutes. 7.5µl of this mixture was then added to a separate tube containing 0.75µl oligodTs and 0.75µl dNTPs and this was incubated at 65^{o}C for 5 minutes before being transferred to ice for 5 minutes. Next, 3µl FS buffer (Invitrogen), 0.75µl DTT (Invitrogen), 0.75µl Superscript III reverse transcriptase (Invitrogen), 0.75µl RNAse out (Invitrogen) and 0.75µl dH_20. This mixture was then subjected to a 25^{o}C (5 minutes), 50^{o}C (50 minutes), 70^{o}C (15 minutes) cycle. The cDNA was then stored at -20^{o}C.

4.1.3. Semi-qPCR

Expression profiles for each FT gene were then attained through qPCR using the Biorad C1000 Thermal Cycler and CFX96 Real-Time System. For each reaction, 2.5µl cDNA, 2.5µl of primer mix containing F1 and R1 primer (4µl of each primer was added to 92µl nuclease-free water) and 5µl Gotaq qPCR master-mix (Promega, USA). Gene expression was normalised using a housekeeping gene *Traes_5AS_019ECA143.1*. For measuring the expression of *FT3-12* I designed generic primers that would bind to all three genomes for each gene and if a gene showed expression from these, I then designed genome specific primers.

Primer design was conducted using exon sequences taken from ensembl plants (https://plants.ensembl.org/Triticum_aestivum/Info/Index) and aligning them on Bioedit©. Shared sequences between the genomes were then used for the generic primers. Below are the primers used. Analysis was conducted through the formula (2^(A-B)) with (A) being the Ct value for the housekeeping gene '*Traes_5AS_019ECA143.1'* and (B) being the average of the Ct values for each gene of interest.

Gene Name:	Common Name:	Fwd/Rvs?	Sequence
TraesCS1A02G338600, TraesCS1B02G351100, TraesCS1D02G340800	*FT3*	F	GGCAGGTGCTGAGCTAAGA
TraesCS1A02G338600, TraesCS1B02G351100, TraesCS1D02G340801	*FT3*	R	GGACACCATCCAGTGCAAG
TraesCS2A02G132300, TraesCS2B02G154800, TraesCS2D02G134200	*FT4*	F	GGTGCCTCTAACTGTGAT
TraesCS2A02G132300, TraesCS2B02G154800, TraesCS2D02G134200	*FT4*	R	CGCATCAGGATCCACCAT
TraesCS6A02G160200, TraesCS6B02G191200, TraesCS6D02G152500	*FT6*	F	GCACAGTTGATCTGACAGTG
TraesCS6A02G160200, TraesCS6B02G191200, TraesCS6D02G152500	*FT6*	R	ACAAGGGTGTATGTCACGCT
TraesCS5A02G154600, TraesCS5B02G152800, TraesCS5D02G159500	*FT7*	F	GGGAACATCGTCGGAGAAA
TraesCS5A02G154600, TraesCS5B02G152800, TraesCS5D02G159500	*FT7*	R	CCATCACCAGCGTGTATAG
TraesCS2A02G536600, TraesCS2B02G567400, TraesCS2D02G538100	*FT8*	F	AACCGCGAGATCACCAAT
TraesCS2A02G536600, TraesCS2B02G567400, TraesCS2D02G538100	*FT8*	R	GTATGTCTGTGACCAACC
TraesCS2A02G347000, TraesCS2B02G365300, TraesCS2D02G345700	*FT9*	F	TGGTTGGTGACAGACATC
TraesCS2A02G347000, TraesCS2B02G365300, TraesCS2D02G345700	*FT9*	R	CTCCATCCTGGTGCATAT
TraesCS5A02G297300, TraesCS5B02G296600, TraesCS5D02G304400	*FT10*	F	GTTTATGCACCAGGATGGC

TraesCS5A02G297300, TraesCS5B02G296600, TraesCS5D02G304400	*FT10*	R	CACCACTCTCCTTTTGGCA
TraesCS4B02G073800, TraesCS4D02G072300	*FT11*	F	TTGGGGATATTGTGGACC
TraesCS4B02G073800, TraesCS4D02G072300	*FT11*	R	CAGATTCCTCATGTCTCG
TraesCS6A02G214400, TraesCS6B02G244400, TraesCS6D02G197100	*FT12*	F	ATGGATCTGAGCTCAAGCC
TraesCS6A02G214400, TraesCS6B02G244400, TraesCS6D02G197100	*FT12*	R	CTTGGACTTGGTGAGTCAG
TraesCS2A02G132300	*FT4A*	F	TCGACCAGCAATGATCCTCA
TraesCS2A02G132300	*FT4A*	R	CATGGCTGATCAACCAGCTA
TraesCS2B02G154800	*FT4B*	F	GATCTGTCTAACTGATCGAT
TraesCS2B02G154800	*FT4B*	R	ATCATGGGTGTACGTATTAG
TraesCS2D02G134200	*FT4D*	F	ACCCAACGTTGACATAGG
TraesCS2D02G134200	*FT4D*	R	GATGGGATATCAGTCACC
TraesCS2A02G347000	*FT9A*	F	CAACGAATGCCAGTTACGG
TraesCS2A02G347000	*FT9A*	R	TGTTTGCCGGACAGATTGG
TraesCS2B02G365300	*FT9B*	F	ATACAACGGCAAGGAACTAACC
TraesCS2B02G365300	*FT9B*	R	TAACTGGCGTTCGTTGATTCTG
TraesCS2D02G345700	*FT9D*	F	CACTTTATGGTCTTGGACCCCCT
TraesCS2D02G345700	*FT9D*	R	AACCTCCTGCTTGTAGAGGATGG
TraesCS5B02G020300	*FT9B5*	F	AAACCCAACTGCAGGAATGC
TraesCS5B02G020300	*FT9B5*	R	ATCTGATGTACCGCTTGCCT
TraesCS5A02G297300	*FT10A*	F	TGCCTAGTCGACCAAAGTGT
TraesCS5A02G297300	*FT10A*	R	CGACCTTCAATCTGCACCTG
TraesCS5B02G296600	*FT10B*	F	CAAGTTATGTCACGGGGCAG
TraesCS5B02G296600	*FT10B*	R	AGCAGATGATCGTACCCCAG
TraesCS5D02G304400	*FT10D*	F	GGGGCTCTAGAACAAGGCAGC
TraesCS5D02G304400	*FT10D*	R	CGCCCTTCTTTGGTGGACTATAT
TraesCS6A02G214400	*FT12A*	F	AATTTATGCACCAGGATGGAGACCA
TraesCS6A02G214400	*FT12A*	R	TTGCAGTGTTGGAATGCAGTTATCC
TraesCS6B02G244400	*FT12B*	F	TTGCCATGTCAAGGGATCCAC
TraesCS6B02G244400	*FT12B*	R	TGAGTCAGGATCCACCATCAC
TraesCS6D02G197100	*FT12D*	F	TGGTGACAGACATTCCGGAAT
TraesCS6D02G197100	*FT12D*	R	GTTGAAATTCGGTCTCCATCC

4.2 Results

4.2.1. Testing for expression in *FT3-12*

In wheat, *FT* expression beyond *FT1* and *FT2* is a relatively new area of research, and little is known about when and where they are expressed and their function. It is assumed that some may be regulated through interaction with *Ppd-1* as occurs with *FT1* and *FT2*, but this is also unknown. The research conducted in this study attempts to investigate when the *FT* genes are expressed in wheat in apex and mature spike samples in germplasm with differing *Ppd-1* alleles in the spring wheat background variety Paragon. To do this, generic primers were designed, covering each of the genomes, for each of the *FT* genes (*FT1-12*) as described in materials and methods (4.1.3.) and the primers for *FT3-12* were used to map stage specific expression of the *FT* genes. Floral developmental stages were targeted as I wanted to identify which other *FT*'s may be regulated by Ppd-1. To understand if genes had been turned on during floral meristem developmental stages the expression first needed to be measured in the vegetative meristems. In vegetative meristems there was no expression of *FT3, FT4, FT6, FT7, FT8* and *FT12*D seen in any of the genotypes (Figure 4.1). This may be due to these genes being localised in other tissue and not in the apex and/or that they may have a function beyond flowering regulation. Expression was observed for *FT9*, *FT10* and *FT12* and so genome specific primers were designed and used to identify the different contributions for each of the genomes. For *FT9*B, *FT9*D, *FT9B5* and *FT10A* there was expression in all of the *Ppd-1* NILs tested in vegetative meristems (Figure 4.1). For *FT9*A, expression was only seen in the TK and Paragon wild-type (WT) samples and for *FT10A* expression was seen in TK, WT and TI and not in SI. *FT12*A was expressed in WT and SI vegetative meristem samples but not TI and TK. (Figure 4.1). No expression was seen in WT vegetative meristems for *FT12*B but was present in all the other genotypes.

In meristems at the double-ridged stage, there was also no expression seen for *FT3*, *FT4*, *FT6*, *FT7* and *FT8* for WT, TK and SI (Figure 4.2). However, for TI there was expression seen in *FT3*, *FT6* and *FT8*. No expression was seen for *FT4* and *FT7* in TI. For *FT9*B, *FT9D* and *FT9B5* and *FT10B*, there was expression seen in all double-ridged meristem samples across all four genotypes. *FT9*A was only expressed in TK and WT samples and *FT10A* was only seen to be expressed in TK, WT and TI but not SI. In *FT12*A expression was only seen in WT and TI, for *FT12*B, there was expression in SI and TI and for *FT12*D, expression in SI and TI was seen. For samples taken at the terminal spikelet stage, there was no expression seen in *FT3, FT4, FT6, FT7, FT8*, *FT9*A and *FT12*B. For all other genes, *FT9*B, *FT9*D, *FT9B5*, *FT10A*, *FT10B*, *FT12*A and *FT12*D expression was seen in all four *Ppd-1* genotypes (Figure 4.3).

In glume, no expression for *FT3, FT4, FT6, FT7* and *FT8* was seen. For *FT9*D, *FT9B5*, *FT10A*, and *FT10B*, expression was seen in all samples across the four genotypes (Figure 4.4). In *FT9*A, expression was only seen in SI glume samples and for *FT9*B, expression was only seen in WT and SI. In *FT12*A, expression was seen in TK, WT and SI, for *FT12*B, expression

was only seen in TK and for *FT12*D, there was only expression detected in WT and SI (Figure 4.4). Similarly, to the glumes, floret samples, there was no expression seen in *FT3, FT4, FT6, FT7* and *FT8*. Expression was seen across all four genotypes for *FT9*D, *FT10A* and *FT10B* (Figure 4.5). Expression for *FT9*A was only seen in SI, whereas for *FT9*B, expression was only seen in TK and WT. *FT9B5* was expressed in WT, SI and TI but not in TK floret samples. For *FT12*A, expression was detected in TK and SI but not in WT and TI, for *FT12*B, expression was only seen in SI and TI floret samples and *FT12*D was only detected in WT and SI samples.

In the rachis taken there was no expression of *FT3, FT4, FT6, FT7* and *FT8* detected for any of the genotypes (Figure 4.6). When testing for *FT9*D, *FT9B5*, *FT10A*, *FT10B* and *FT12*A however, expression was seen in all the four genotypes tested (Figure 4.6). *FT9*A was only detected in SI rachis samples and *FT9*B was seen to be expressed in WT, SI and TI samples. *FT12*B was detected in TK and SI samples and *FT12*D was detected in TK, WT and SI but not in TI. *FT3, FT4, FT6, FT7* and *FT8* being only expressed in TI double-ridged samples across all 6 sample locations taken seems to further suggest that they are not localised to the apex and polymorphisms around the *Ppd-1* alleles may be the reason for it being expressed in triple insensitive.

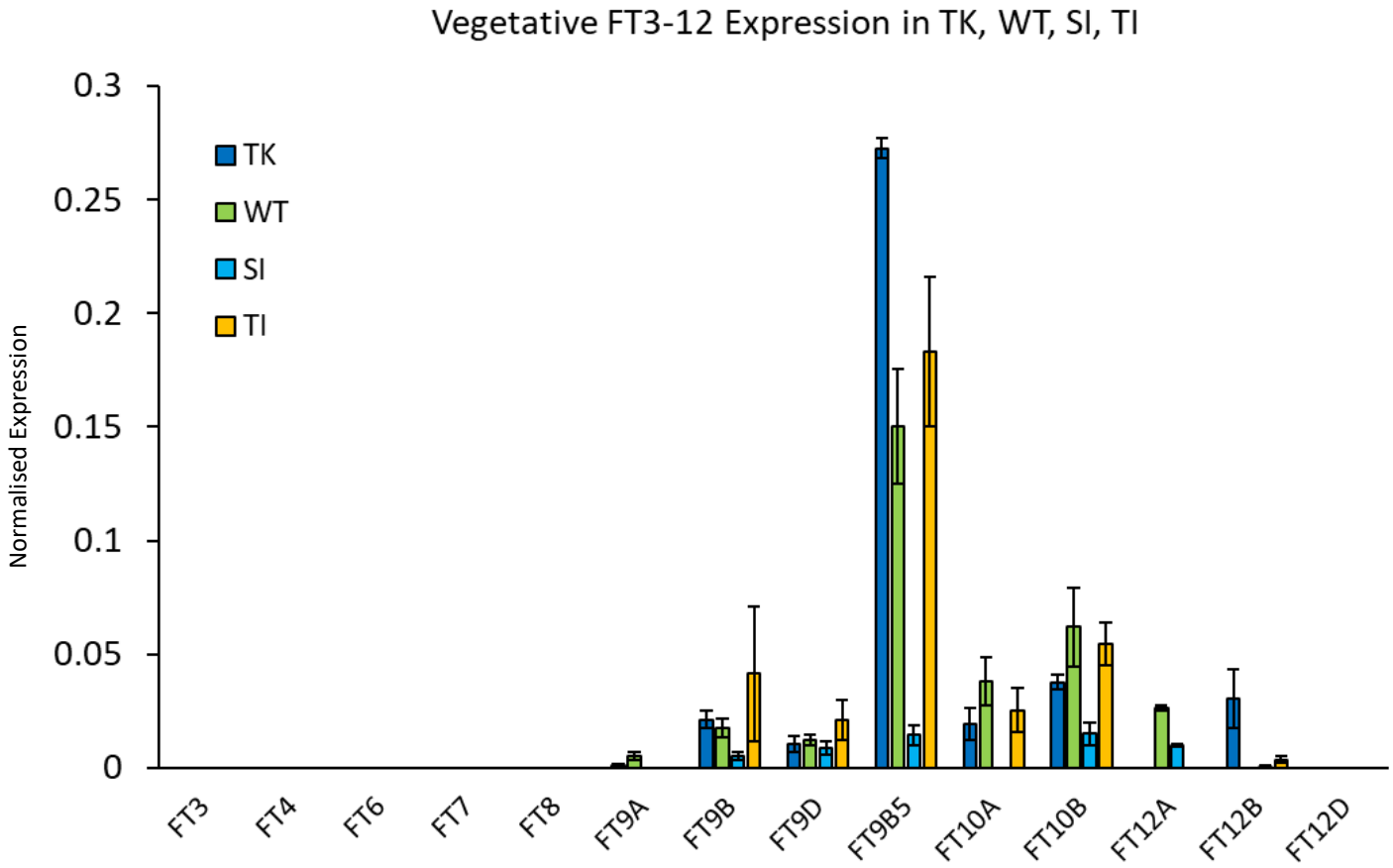

Figure 4.1: *FT3-12* expression in vegetative apical meristems across the Ppd-1 NILs WT (Paragon wild-type), TK (Triple Knockout), SI (Single Insensitive) and TI (Triple Insensitive) in glasshouse LD conditions. Dark blue represents TK, light green represents WT, turquoise represents SI and orange represents TI. Error bars represent standard error mean and sample number = 3. Expression was normalised against *Traes_5AS_019ECA143.1*.

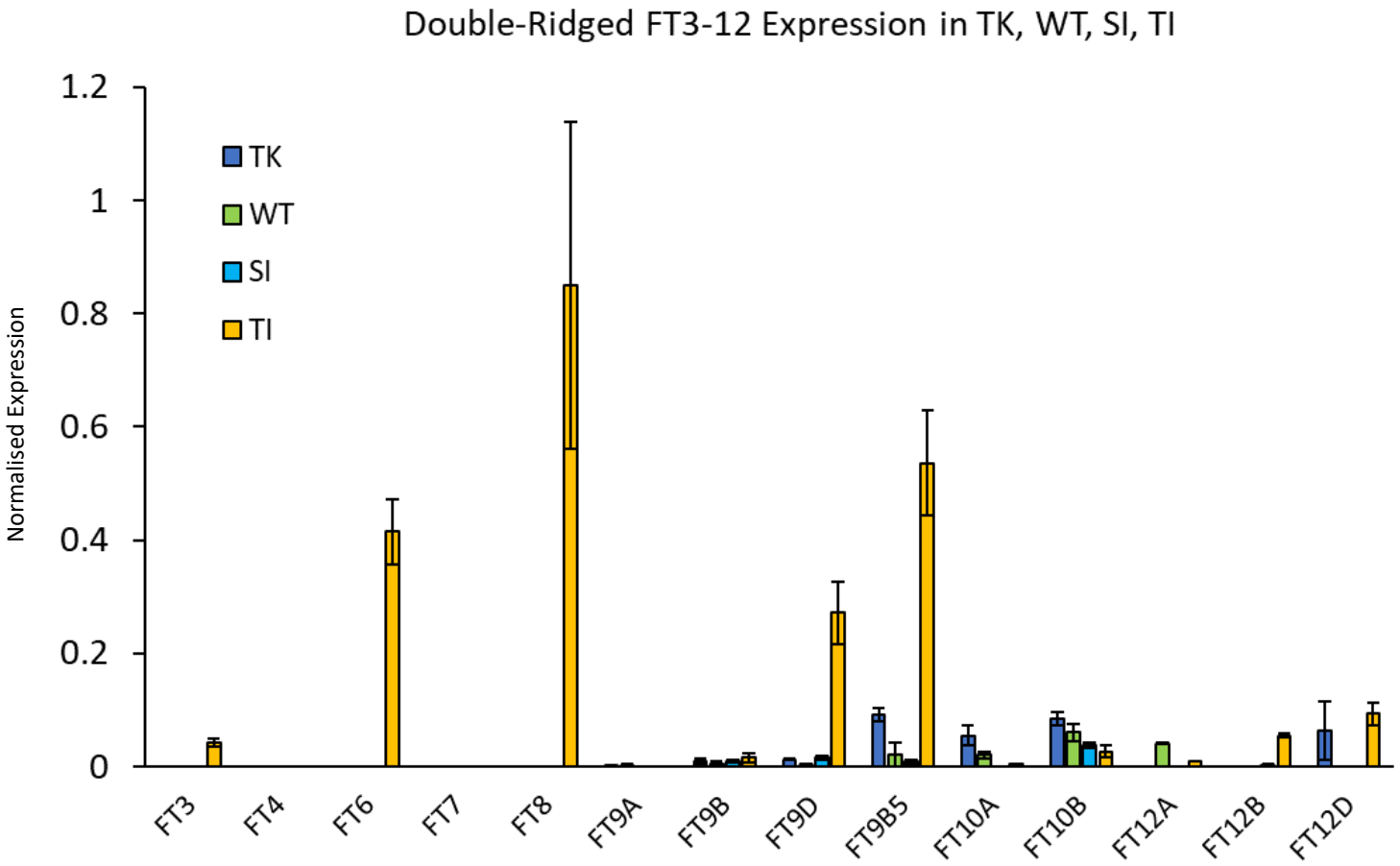

Figure 4.2: *FT3-12* expression in double-ridged apical meristems across the Ppd-1 NILs WT (Paragon wild-type), TK (Triple Knockout), SI (Single Insensitive) and TI (Triple Insensitive) in glasshouse LD conditions. Dark blue represents TK, light green represents WT, turquoise represents SI and orange represents TI. Error bars represent standard error mean and sample number = 3. Expression was normalised against *Traes_5AS_019ECA143.1*.

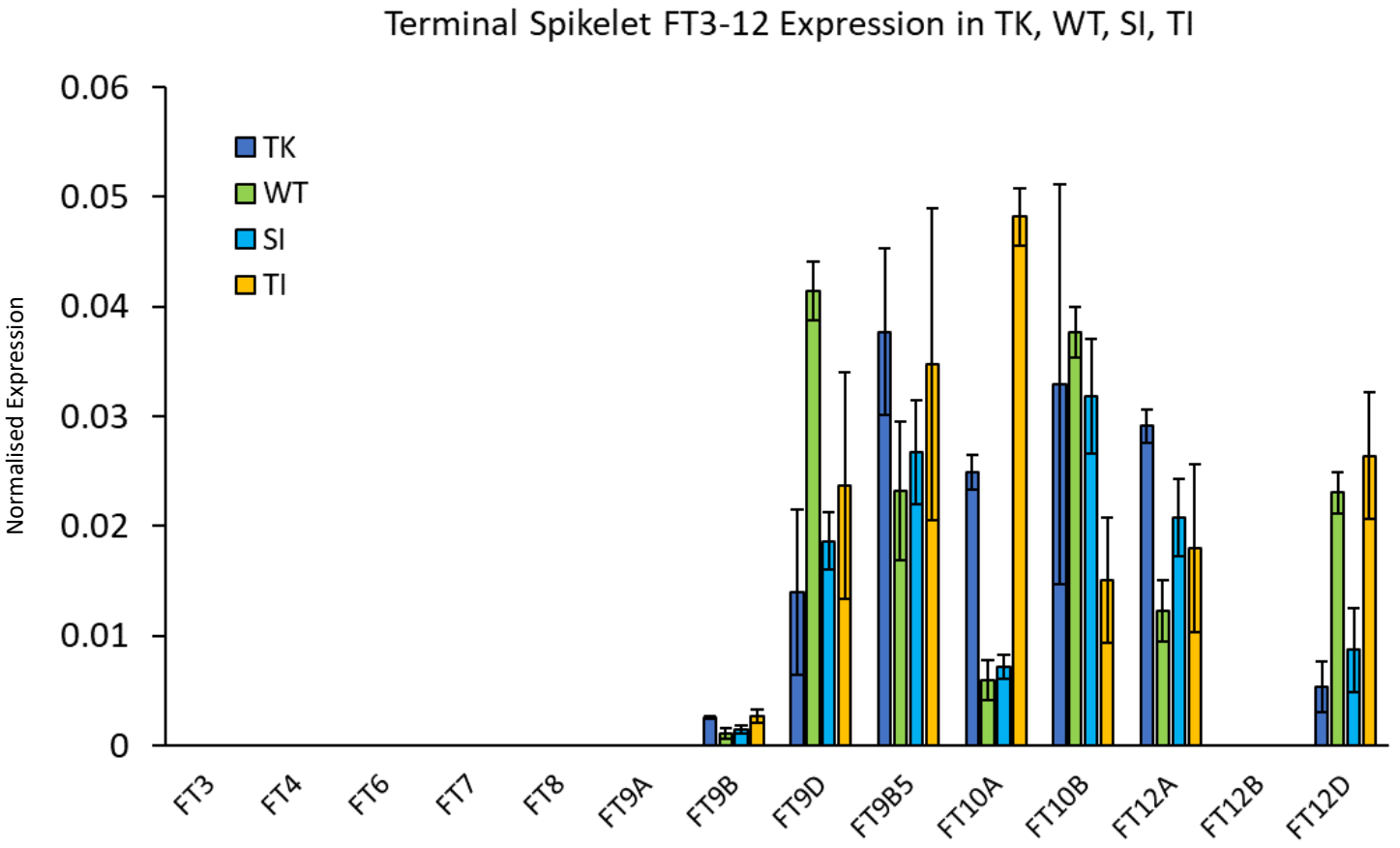

Figure 4.3: *FT3-12* expression in terminal spikelet apical meristems across the Ppd-1 NILs WT (Paragon wild-type), TK (Triple Knockout), SI (Single Insensitive) and TI (Triple Insensitive) in glasshouse LD conditions. Dark blue represents TK, light green represents WT, turquoise represents SI and orange represents TI. Error bars represent standard error mean and sample number = 3 . Expression was normalised against *Traes_5AS_019ECA143.1*.

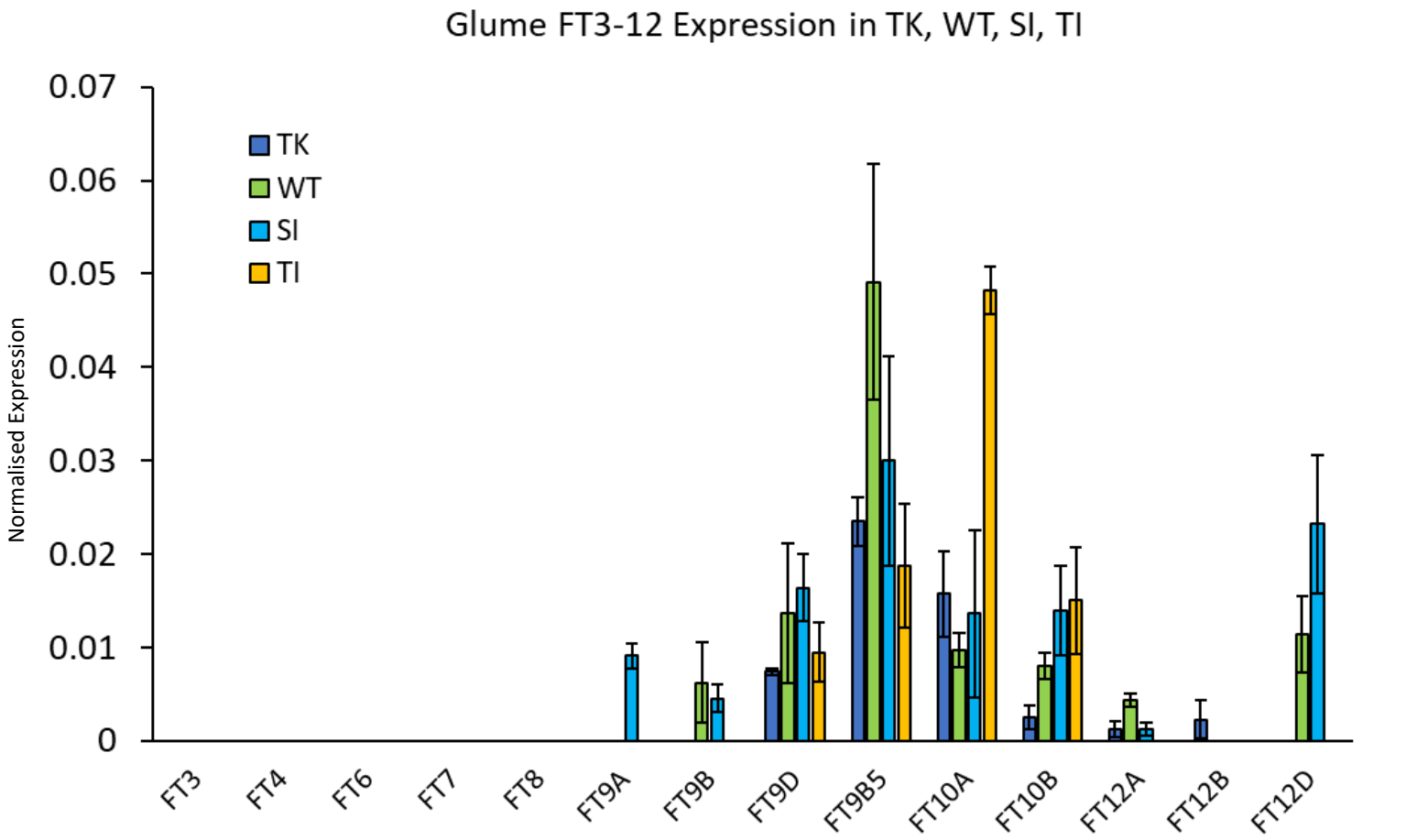

Figure 4.4: *FT3-12* expression in glumes across the Ppd-1 NILs WT (Paragon wild-type), TK (Triple Knockout), SI (Single Insensitive) and TI (Triple Insensitive) in glasshouse LD conditions. Dark blue represents TK, light green represents WT, turquoise represents SI and orange represents TI. Error bars represent standard error mean and sample number = 3. Expression was normalised against *Traes_5AS_019ECA143.1*.

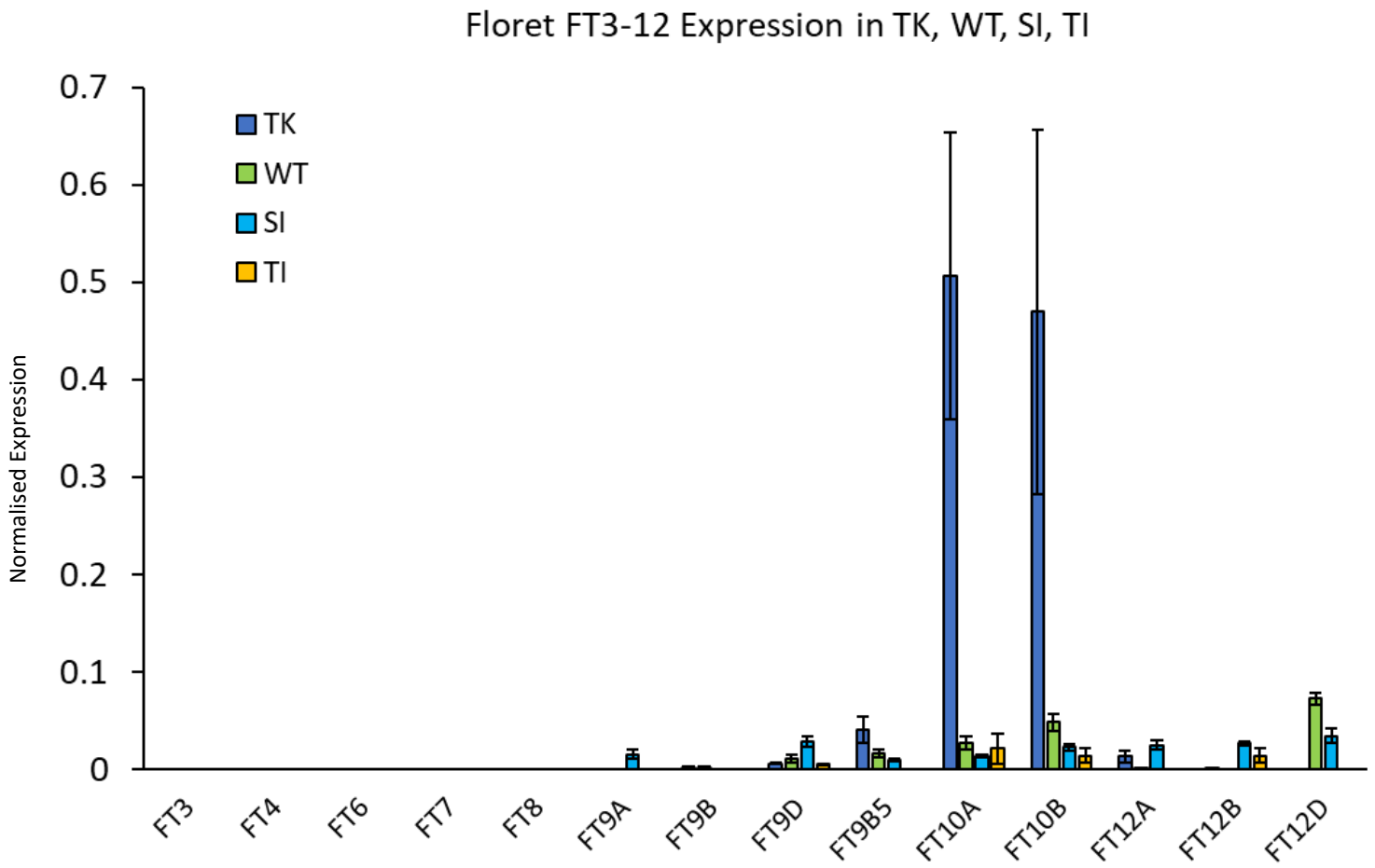

Figure 4.5: *FT3-12* expression in florets across the Ppd-1 NILs WT (Paragon wild-type), TK (Triple Knockout), SI (Single Insensitive) and TI (Triple Insensitive) in glasshouse LD conditions. Dark blue represents TK, light green represents WT, turquoise represents SI and orange represents TI. Error bars represent standard error mean and sample number = 3. Expression was normalised against *Traes_5AS_019ECA143.1*.

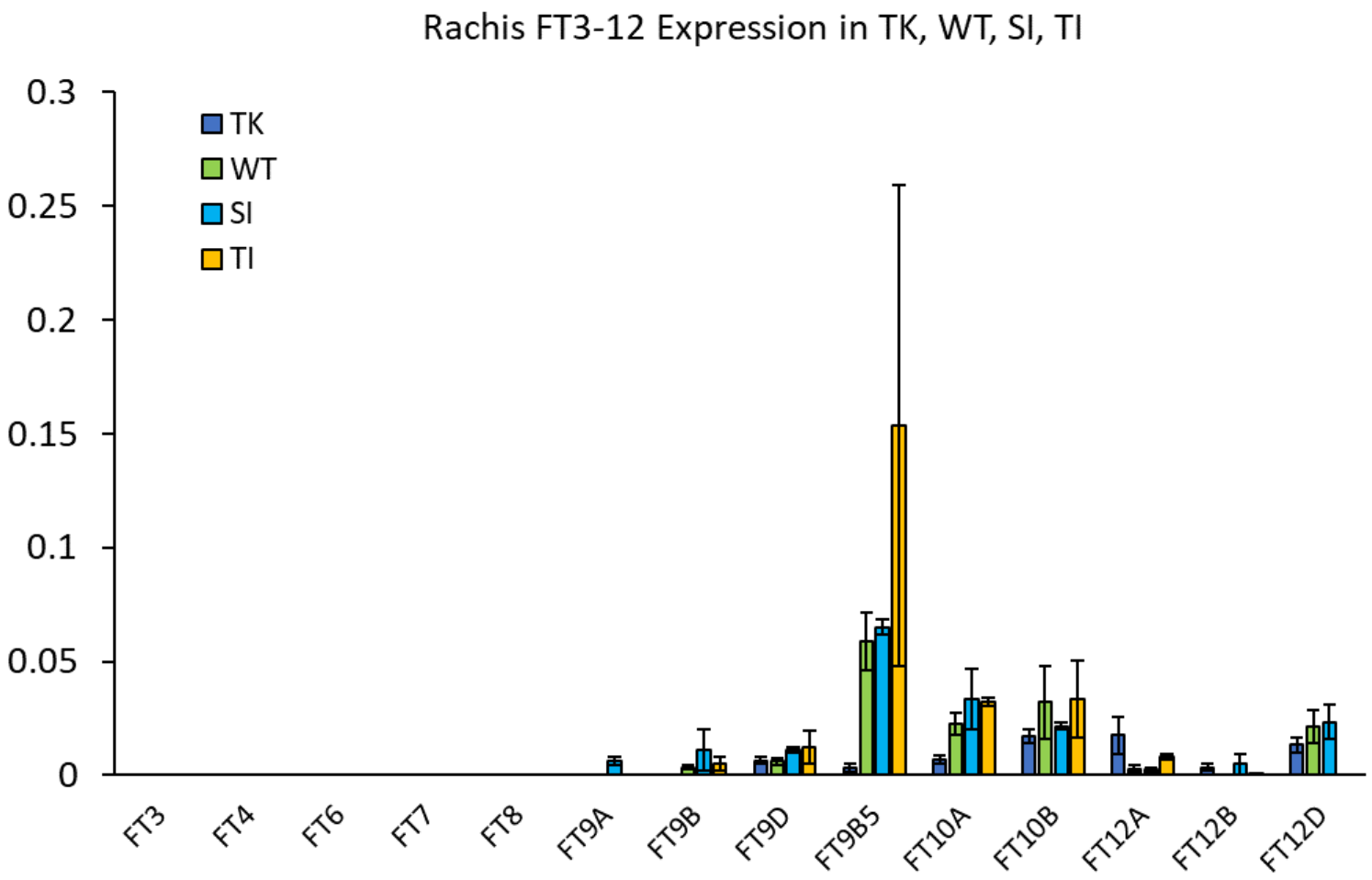

Figure 4.6: *FT3-12* expression in rachis across the Ppd-1 NILs WT (Paragon wild-type), TK (Triple Knockout), SI (Single Insensitive) and TI (Triple Insensitive) in glasshouse LD conditions. Dark blue represents TK, light green represents WT, turquoise represents SI and orange represents TI. Error bars represent standard error mean and sample number = 3. Expression was normalised against *Traes_5AS_019ECA143.1*.

4.2.2. Analysing genome specific *FT* expression in the context of apical stages and Ppd-1 NILs

From studying expression of the *FT*'s, it was clear that the genes that were present for the majority, if not all of the floral development process were *FT9*, *FT10* and *FT12*. FT3, *FT6* and *FT8* were also expressed at double ridged in TI samples but this could be due to polymorphisms around the A and B genomes where *Ppd-1* is being mis-regulated and therefore could be activating genes where they usually would not be turned on. Within *FT9* and *FT10*, significance between the apical stages (DR, TS, Glume, Florets, Rachis) and vegetative was calculated to see if these genes were more highly expressed, thereby potentially aiding in the suggestion of a function. Apical stages were compared between the *Ppd-1* NILs (TK, SI and TI) and Paragon wild-type to try to ascertain if *Ppd-1* is regulating their expression. For both major comparisons, two-tailed T tests were used to measure significance and analysis was represented in a table format (Table 4.1-4.3) to display these values.

4.2.3. Identifying significant changes in expression between the tissue stages for each of the *Ppd-1* NILs.

FT9B5

For *FT9*, *FT9B5* was the gene from which the majority of the transcript was expressed and so the significance calculated was between expression levels of *FT9B5*. In WT, *FT9B5* was seen to be significantly differently expressed comparing the reproductive stages (DR, TS, glume, florets, rachis) of apex development to vegetative with only the rachis showing no significant difference (Figure 4.7) (Table 4.1A). For all the reproductive stages of apex development where there was a significant difference seen, there was higher expression in vegetative meristems in comparison. This could indicate a role for *FT9* in the repression of apical reproductive transformation. For TK, all reproductive stages of development had significantly lower expression of *FT9B5* when compared to vegetative meristems. This further indicates a role in repression of the apex transitioning to reproductive. In SI, there was no significant difference seen between vegetative and reproductive stages apart from in the rachis where *FT9B5* was seen to have significantly higher expression compared to vegetative meristems. Finally, in TI there was no significant difference between any of the reproductive meristem samples and vegetative samples. When testing TI florets there was no *FT9B5* expression detected and therefore there was significantly higher expression in vegetative meristems in comparison.

FT10A

FT10A expression was analysed between the stages in each genotype and between the genotypes to measure significant differences in levels of expression. In WT, there was only a significant difference between vegetative and TS where *FT10A* expression was higher in the vegetative samples (Figure 4.8) (Table4.2A). In TK, there was a significant difference between vegetative and floret samples where there was significantly higher expression in florets. No expression of *FT10A* was detected in vegetative samples in SI and therefore comparisons between the rest of the apical stages could not be made. In TI samples there was no significant difference in *FT10A* expression between any of the reproductive stages (DR, TS, glume, floret, rachis) and vegetative.

FT10B

FT10's B genome transcript was measured in all samples collected for levels of expression. In WT, only one significant difference was detected in the apical stages comparison and that was between Veg and glume (Figure 4.8) (Table 4.2C). Here, more expression was seen in vegetative samples. In TK, there were significant differences in levels of expression between Veg and DR, Veg and glume and Veg and rachis. In TK more *FT10B* expression was seen in DR compared with Veg, however, less expression was seen in the glume and rachis in comparison. In SI samples, no significant differences were seen between any of the reproductive apical stages and Veg. For TI, two significant differences in *FT10B* expression levels were observed and these were between Veg and DR and Veg and glume. Here, there was significantly more expression seen in DR and significantly less expression seen in the glume.

FT10D

FT10D was not included in the overall expression due to extremely high expression in all samples collected. It was clear that the majority of the transcript for *FT10* was emanating from the D genome in the samples I collected (Figure 4.9) (Table 4.3). The first comparison made was between the reproductive stages of apical development (DR, TS, glume, floret, rachis) and vegetative for each genotype. In WT, there was no significant difference in *FT10D* expression found between any of the stages and vegetative. In TK, there were significant differences in *FT10D* expression observed between Veg and DR, Veg and glume and Veg and florets with no significant difference in other comparisons made. In all TK samples where significant differences in *FT10D* expression were found, more expression was seen in DR, glume and floret samples compared with Veg. In SI, all reproductive apical stages besides DR had significantly different levels of *FT10D* expression compared with Veg. All reproductive apical samples found to be significantly different in SI had significantly lower levels of *FT10D* expression when compared with Veg. Finally, in TI, significant differences in expression levels of *FT10D* were found between Veg and DR, Veg and TS and Veg and glume with no significant differences between Veg and florets and Veg and rachis. Higher levels of *FT10D* were found in TI Veg samples when compared with the reproductive apical stage samples that were found to be significant (DR, TS, glume).

FT12

As discussed earlier in this chapter, *FT12* is being expressed from at least one of the three genomes in T. aestivum in most of the samples taken (Figure 4.10). However, in some cases there is no expression of some of the genes such as *FT12*B in WT apical samples but in the Ppd-1 NILs it is expressed. *FT12*A and *FT12D* is also seen to be relatively highly expressed in some genotypes but not in others. Furthermore, it is unclear as to where the dominant transcript for *FT12* is coming from due to sporadic expression amongst the genomes which could be evidence of background expression as opposed to active expression. One observation of note is especially high *FT12*D expression in TK vegetative samples and little/no expression present in WT, SI and TI. This could point to Ppd-1 repressing *FT12* at this apical stage.

4.2.4. Identifying significant expression changes between the *Ppd-1* NILs

FT9B5

The first stage to be analysed for differences in *FT9B5* expression between the Ppd-1 NILs and WT was vegetative (Figure 4.7) (Table4.1B). Here, there was significantly more expression in TK and significantly less expression in SI when compared to WT but no significant difference between WT and TI *FT9B5* expression. Higher expression in TK and lower in SI could indicate Ppd-1 repressing *FT9B5* expression. In the double-ridged stage, there was only seen to be a significant difference between WT and TI where more expression was seen in TI. In TS, glume and floret samples there was no significant difference in *FT9B5* expression between the Ppd-1 NILs and WT. In rachis samples there was only a significant difference between WT and TK measured with there being higher expression in WT in comparison.

FT10A

When comparing the *Ppd-1* NILs to WT for *FT10A*, there was no significant difference between the expression levels seen in the Veg and DR stages (Figure 4.8) (Table 4.2B). In TS, there was a significant difference between WT and TK, with there being more expression in the TK TS samples. For glume and florets samples, there was no significant differences measured. Finally, when comparing rachis samples, there was only one significant difference recorded and that was between WT and TK. Here, there was more *FT10A* expression seen in WT.

FT10B

Ppd-1 NILs were then also compared with WT at each apical stage. No significant differences between the Ppd-1 NILs and WT were seen in Veg, TS, florets, and rachis

(Figure 4.8) (Table 4.2D). In DR, there was a significant difference between WT and TI *FT10B* expression levels but not between WT and TK and WT and SI. Here, significantly more expression was measured in TI DR samples when compared to WT. In glume samples, only WT and TK were found to have significantly different levels of expression, with there being significantly more *FT10B* expression in WT glume samples.

FT10D

Comparisons were then made between the Ppd-1 NILs and WT for each of the apical stages for *FT10D*. In Veg samples, there was a significant difference between WT and TI levels of *FT10D* expression with considerably more seen in TI samples (Figure 4.9) (Table 4.3B). In DR samples there was a significant difference seen between WT and TK and WT and TI. Here, more expression of *FT10D* was seen in TK and less seen in TI when compared to WT. For samples taken at TS, there was no significant differences seen between the *Ppd-1* NILs and WT. Glume samples showed only a significant difference between WT and SI with there being significantly more *FT10D* expression in WT. The same was seen for florets and rachis samples with the only significant difference in *FT10D* expression seen between WT and SI. In both cases, there was also more expression in the WT when compared to SI.

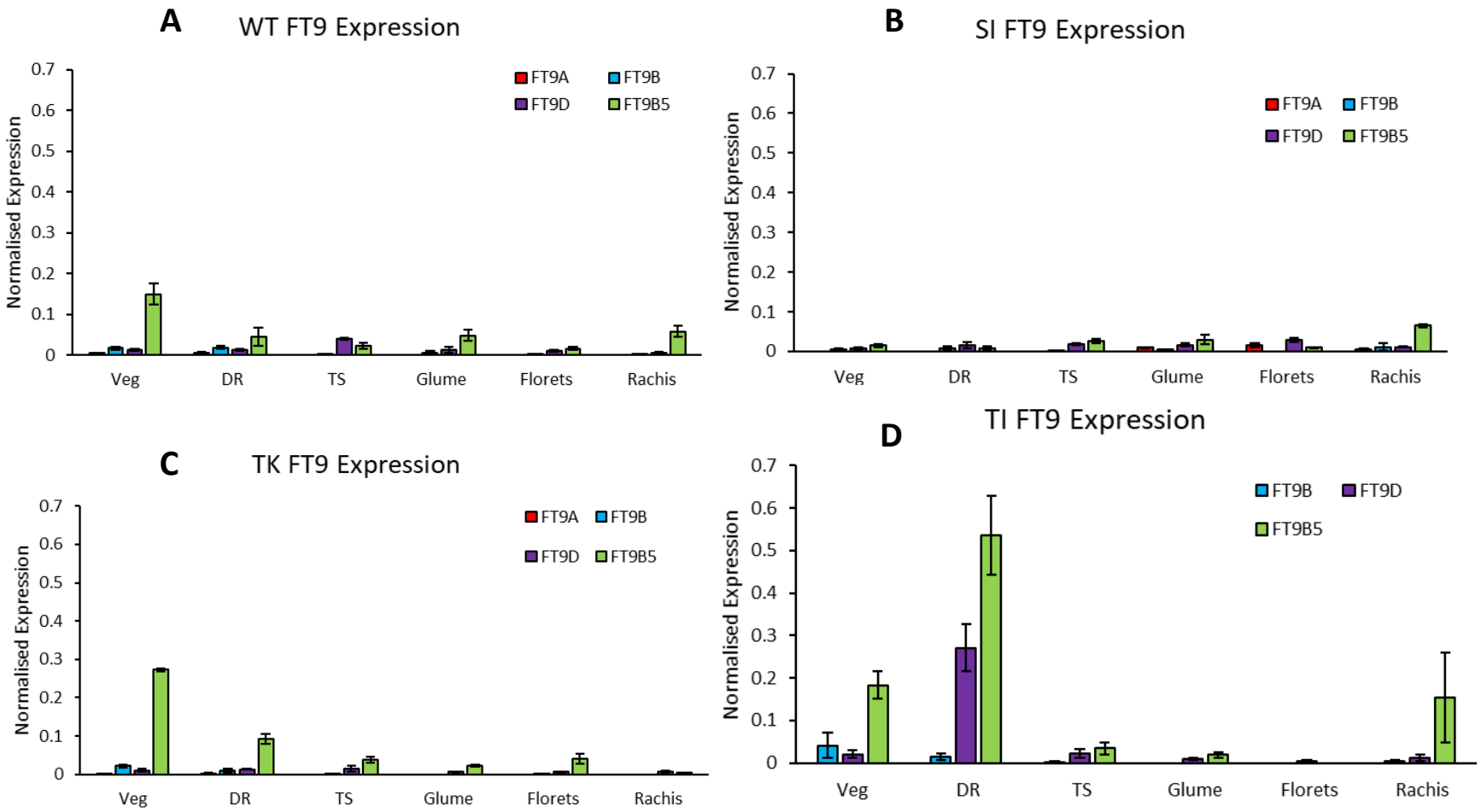

Figure 4.7: *FT9A,9B,9D,9B5* expression over the apical stages Veg (vegetative), DR (double-ridged), TS (terminal spikelet), Glume, Florets, Rachis in glasshouse LD conditions across the Ppd-1 NILs (A) WT (Paragon wild-type), (C) TK (Triple Knockout), (B) SI (Single Insensitive) and (D) TI (Triple Insensitive). Red represents *FT9A*, turquoise represents *FT9B*, purple represents *FT9D* and light green represents *FT9B5*. Error bars represent standard error mean and genes were normalised against *Traes_5AS_019ECA143.1*. Sample number = 3.

A

WT	FT9B5				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.034517	005303	0.023108	0.006272	073954
	*	**	*	**	
TK	FT9B5				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	001589	0.00005	000418	001067	00001
	**	***	***	**	***
SI	FT9B5				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	42473	0.126444	0.264554	0.354797	0.000793
					***
TI	FT9B5				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.062088	0.220448	0.18055	N/a	0.378095

B

FT9B5						
Veg				DR		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.033655	0.00606	0.76945		0.138211	0.767277	006822
*	**					**
TS				Glume		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.341475	0.052852	0.161428		0.108783	0.160537	0.05044
Florets				Rachis		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.167121	0.20457	N/a		0.011244	0.587323	0.537028
				*		

Table 4.1: Measuring the significance of different expression levels of *FT9B5* between different apical stages in each genotype (WT, TK, SI, TI) and between each apical stage tested across the genotypes Paragon wild-type (WT), triple knockout (TK), single insensitive (SI) and triple insensitive (TI). Stages Veg= vegetative, DR= double-ridged, TS= terminal spikelet. (A) shows interactions between the apical stages compared to vegetative in each genotype. (B) shows interactions between the genotypes at each apical stage where samples were taken. Tables (A) and (B) show significance values for the interactions tested. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

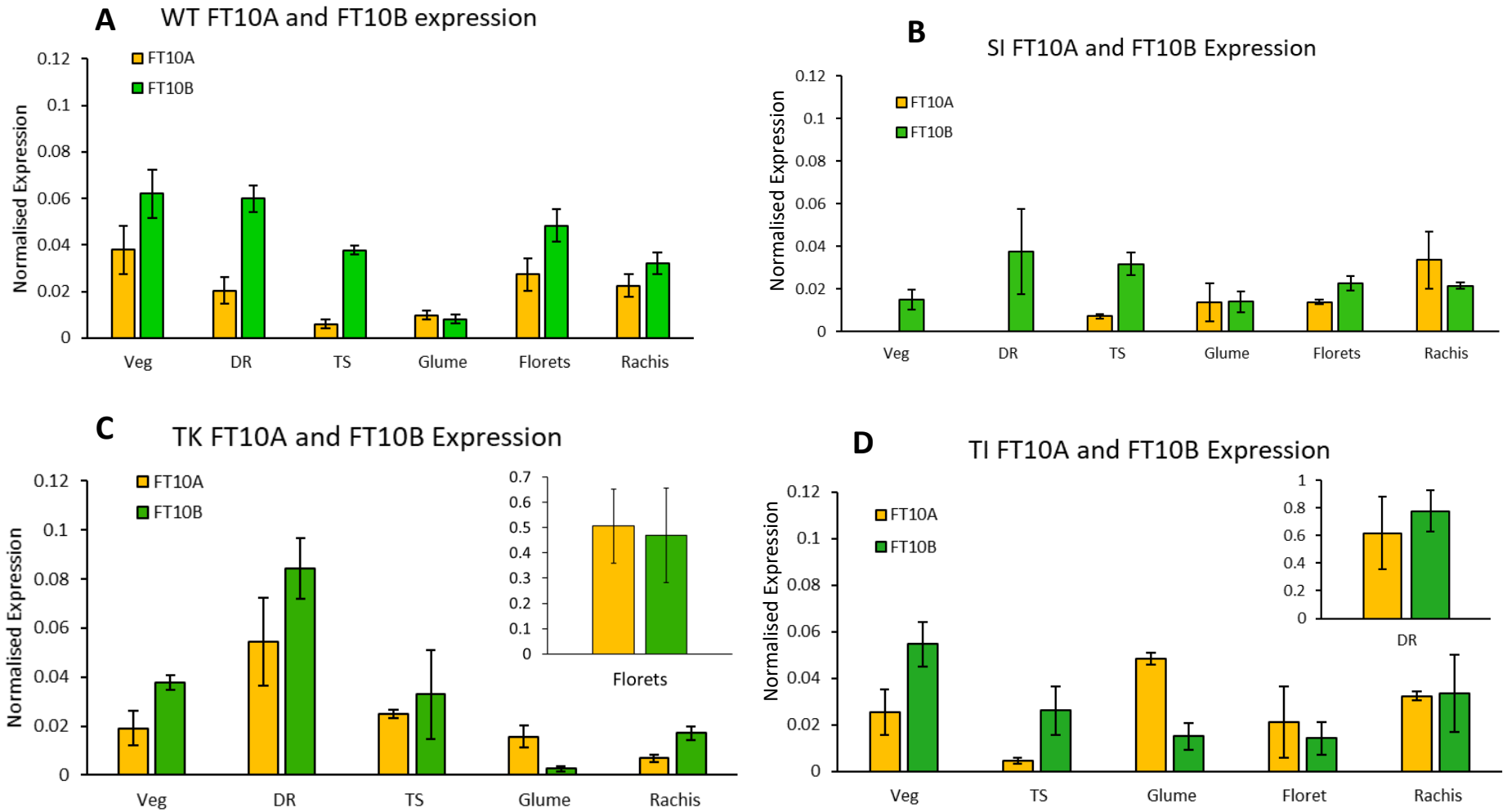

Figure 4.8: *FT10A* and 10B expression over the apical stages Veg (vegetative), DR (double-ridged), TS (terminal spikelet), Glume, Florets, Rachis in glasshouse LD conditions across the Ppd-1 NILs (A) WT (Paragon wild-type), (C) TK (Triple Knockout), (B) SI (Single Insensitive) and (D) TI (Triple Insensitive). Orange represents *FT10A* and lime green represents *FT10B*. Error bars represent standard error mean and genes were normalised against *Traes_5AS_019ECA143.1*.

A

WT	FT10A				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	□217937	□03456	0.093122	□193284	□251715
		*			
TK	FT10A				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.226453	0.089211	0.149749	0.02423	0.059795
				*	
SI	FT10A				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	N/a	N/a	N/a	N/a	N/a
TI	FT10A				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	□086108	0.097611	0.587969	0.819679	0.620314

B

FT10A						
Veg				DR		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.92849	N/a	0.430096		0.892383	N/a	0.084238
TS				Glume		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.03326	0.270738	0.995555		0.302414	0.253783	0.071237
*						
Florets				Rachis		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.078489	0.970249	0.69892		0.035566	□0.47779	0.684896
				*		

C

WT	FT10B				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	□936899	0.061552	□036333	□203524	0.275895
			*		
TK	FT10B				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.038296	0.399333	0.009376	0.095778	0.005207
	*		**		**
SI	FT10B				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.332403	0.076965	0.886217	0.263708	0.260498
TI	FT10B				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.008181	0.111919	□023554	0.940069	0.331342
	**		*		

D

FT10B						
Veg				DR		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.945067	0.059359	0.729617		0.231948	0.422816	0.008499
						**
TS				Glume		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.052358	0.060427	0.433556		0.036127	0.151012	0.150776
				*		
Florets				Rachis		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.16233	0.861855	0.449444		0.406338	0.543181	0.961822

Table 4.2: Measuring the significance of different expression levels of *FT10A* and *FT10B* between different apical stages in each genotype (WT, TK, SI, TI) and between each apical stage tested across the genotypes Paragon wild-type (WT), triple knockout (TK), single insensitive (SI) and triple insensitive (TI). Stages Veg= vegetative, DR= double-ridged, TS= terminal spikelet. Tables (A), (B), (C), (D) show significance values for the interactions tested. (A) and (C) show interactions between the apical stages compared to vegetative in each genotype. (B) and (D) show interactions between the genotypes at each apical stage where samples were taken. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

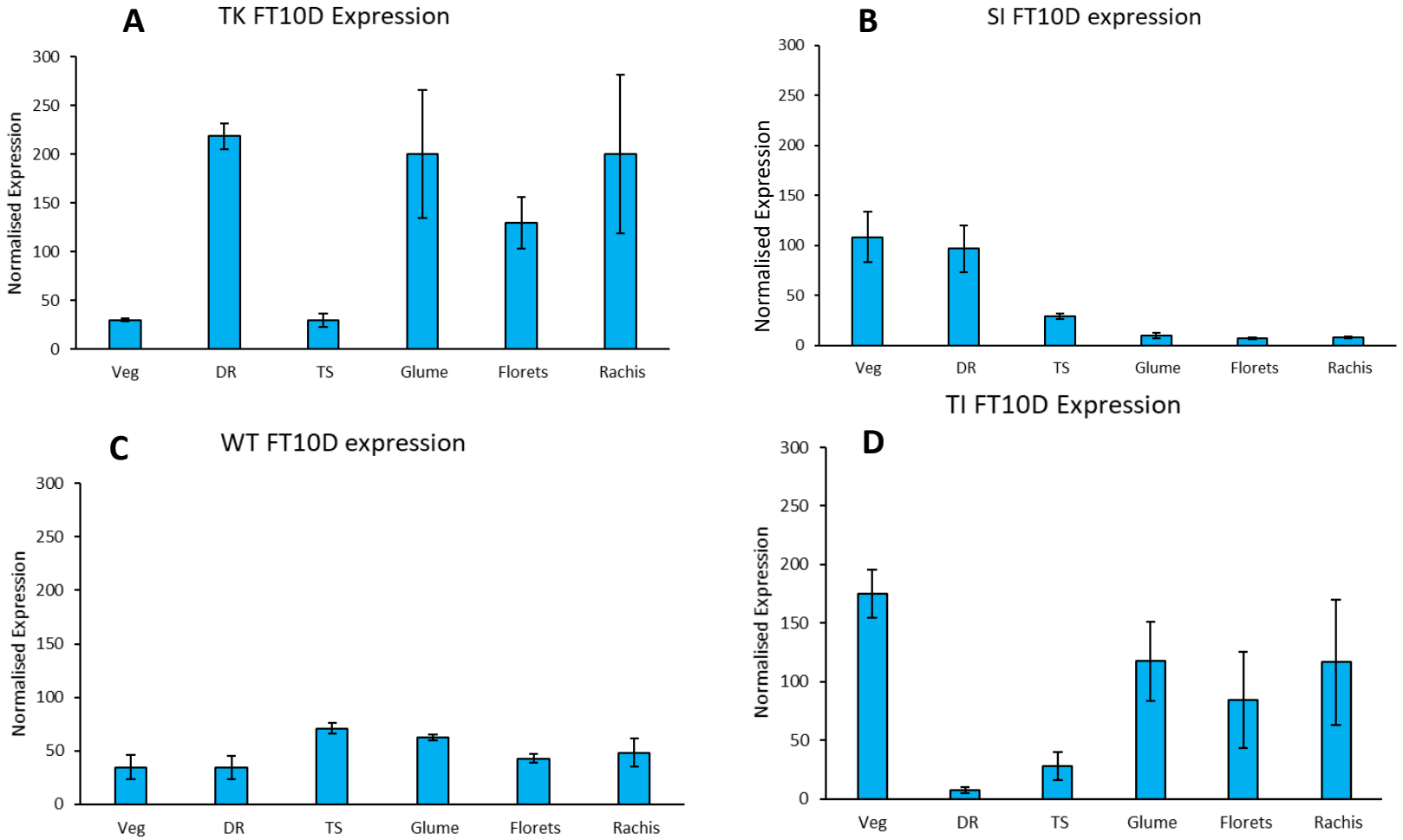

Figure 4.9: *FT10D* expression over the apical stages Veg (vegetative), DR (double-ridged), TS (terminal spikelet), Glume, Florets, Rachis in glasshouse LD conditions across the Ppd-1 NILs (A) WT (Paragon wild-type), (C) TK (Triple Knockout), (B) SI (Single Insensitive) and (D) TI (Triple Insensitive). Error bars represent standard error mean and genes were normalised against *Traes_5AS_019ECA143.1*. Sample number = 3

A

WT	FT10D				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.989213	0.978513	0.826259	0.426226	□059447
TK	FT10D				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.000325	0.092563	0.0397	0.020657	0.105946
	***		*	**	
SI	FT10D				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.479777	0.013013	0.007177	0.01277	0.013202
		*	**	*	*
TI	FT10D				
	Veg-DR	Veg-TS	Veg-Glume	Veg-Florets	Veg-Rachis
	0.001251	0.003396	0.048994	0.109877	0.36229
	**	**	*		

B

FT10D						
Veg				DR		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.712978	0.050829	0.003676		0.002842	0.824036	0.003167
		**		**		**
TS				Glume		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.059599	0.314789	0.6361		0.122405	0.012704	0.497558
					*	
Florets				Rachis		
WT-TK	WT-SI	WT-TI		WT-TK	WT-SI	WT-TI
0.188851	0.022077	0.268093		0.139898	0.038578	0.281656
	*				*	

Table 4.3: Measuring the significance of different expression levels of *FT10D* between different apical stages in each genotype (WT, TK, SI, TI) and between each apical stage tested across the genotypes Paragon wild-type (WT), triple knockout (TK), single insensitive (SI) and triple insensitive (TI). Stages Veg= vegetative, DR= double-ridged, TS= terminal spikelet. (A) shows interactions between the apical stages compared to vegetative in each genotype. (B) shows interactions between the genotypes at each apical stage where samples were taken. Tables (A) and (B) show significance values for the interactions tested. A Two-Tailed T test was performed to analyse significance where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

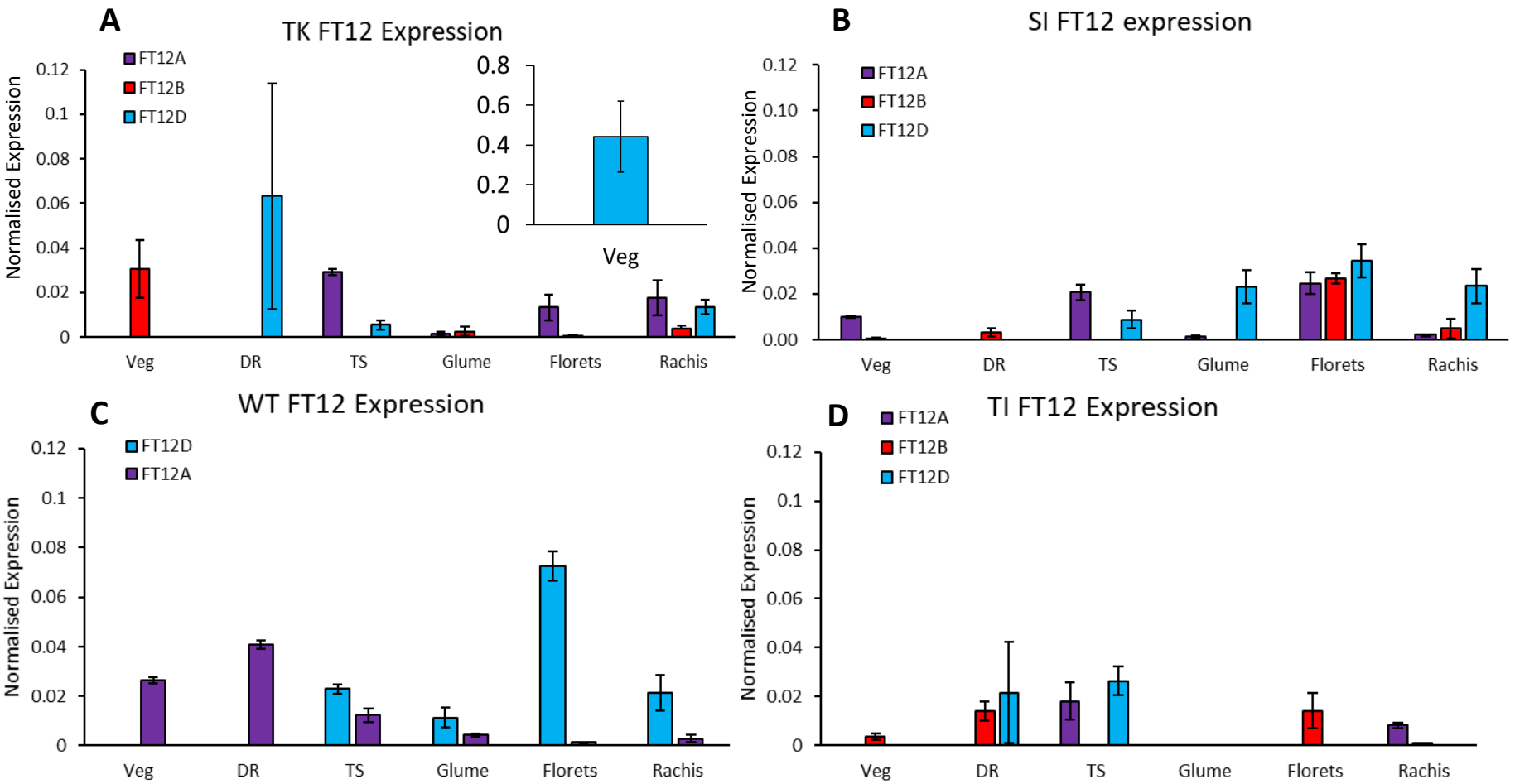

Figure 4.10: *FT12*A,*12B,12D* expression over the apical stages Veg (vegetative), DR (double-ridged), TS (terminal spikelet), Glume, Florets, Rachis in glasshouse LD conditions across the Ppd-1 NILs (A) WT (Paragon wild-type), (C) TK (Triple Knockout), (B) SI (Single Insensitive) and (D) TI (Triple Insensitive). Purple represents *FT12*A, red represents *FT12*B and turquoise represents *FT12*D. Error bars represent standard error mean and genes were normalised against *Traes_5AS_019ECA143.1*.

4.3 Discussion

4.3.1. Identifying expression in *FT3-12*

Expression analysis of the *FT* family outside of *FT1* and *FT2* is a new area of study and functions of these genes are largely unknown. Studies into *FT3* and FT4 have suggested roles such as being a short-day inducer of flowering in *FT3* (Zikhali, et al., 2017), but beyond this, the vast majority of *FT* genes have completely unknown functions within wheat. This study has focused on studying these genes within the context of the apical meristem and all samples collected have been associated with it. At all stages and throughout the *Ppd-1* NILs and WT Paragon, there was *FT9*, *FT10* and *FT12* expression in some form i.e., at least one of the genomes (A, B or D) being actively transcribed (Figures 4.1-4.6). In *FT9*, the dominant genome transcript seemed to be *FT9*B5, with much higher expression seen when compared to the other genomes in most case. This dominance was why statistics were carried out on just *FT9*B5 as differences in expression across apical stages and genotypes (WT, TK, SI, TI) were deemed more significant.

In TI double-ridged samples, there was expression seen in *FT3, FT6* and *FT8*, with high expression seen in all three. Interestingly, these genes have only appeared to be expressed in this stage and genotype which implies that increased *Ppd-1* may have a role in their regulation (Figure 4.2). This anomaly of expression was not seen in SI at the same stage which may imply one of two scenarios. Either the *Ppd-A1* and *Ppd-B1* genomes have a role in regulating *FT3*, *FT6* and *FT8* expression, or increased *Ppd-1* expression from the additional mis-regulation of *Ppd-A1* and *Ppd-B1* could outcompete repressive regulatory proteins that also interact with their promoter; thereby activating their expression. Previous studies in *FT3* have suggested it's function as a short-day inducer of flowering (Zikhali, et al., 2017) (Muhammed, et al., 2018) and the samples in these studies were taken from leaf tissue as opposed to the apical meristem tissue that was used in this experiment. The lack of *FT3* expression in the apex in all samples of WT, TK and SI compared to high expression seen in the leaf (Zikhali, et al., 2017), further implies its role as a florigen that, as seen in *FT1* (Corbesier, et al., 2007), may be able to travel from the leaf to the meristem and carry out its function. However, the samples in this study were taken in a long-day (LD) 23^{o}C setting so if *FT3* is a short-day inducer of flowering, expression would be expected to be low in long-days. Further study into apex *FT3* expression could be done to confirm whether it is not localised to the meristem as predicted.

4.3.3 Differences in expression between the *Ppd-1* NILs at each apical stage

It is heavily speculated that Ppd-1 has a role in the direct regulation of *FT1* through interaction with its promoter. Evidence for this comes from studies into *Ppd-1* NILs that have either decreased *FT1* expression when *Ppd-1* is knocked out (Shaw, et al., 2013) or increased *FT1* when there is a deletion of the promoter of *Ppd-1* causing its upregulation (Shaw, et al., 2012). Furthermore, the yeast-1-hybrid study in chapter 3 (Figure 3.5) has

shown evidence of a direct interaction between the Ppd-1 protein and FT1 promoter. This interaction is also speculated to occur between Ppd-1 and *FT2* and a study using *Ppd-1* NILs has shown that increased *Ppd-1* expression leads to higher expression of *FT2* (Boden & Gauley, 2020). With these clear trends between *Ppd-1* and *FT1* and *FT2* expression being evident, it brings us to the hypothesis that other genes in the *FT* family may also be transcriptionally regulated via Ppd-1.

This study attempted to decipher if Ppd-1 regulation of *FT3-12* did occur through the use of the *Ppd-1* NILs in the spring background Paragon and found two results of note. The first, is that in *FT9*B5, which from these studies is considered to be the dominant origin of transcript, there is low expression in SI vegetative samples and high expression in TK samples taken at the same apical stage (Figure 4.7). WT vegetative samples were observed to have expression levels in the middle of TK and SI. This implies that Ppd-1 may have a role at repressing *FT9*B5 as increased *Ppd-1* causes lower amounts of *FT9*B5 expression and lack of *Ppd-1* causes it to be higher. There are no spikes in expression seen at other apical stages and expression there could be just background. However, in TI samples there are large spikes in expression at the vegetative and double ridged stages which would be expected to be low if Ppd-1 did repress *FT9*B5. On the other hand, this could be due to a slip of regulation when *Ppd-1* is extremely high, and it could also act as an activator in this situation.

One of the more interesting results found in this study was from *FT10*. All three of the genomic transcripts of *FT10* were seen to be frequently expressed (Figures 4.8, 4.9) in most of the samples analysed, however, one that stood out was *FT10D*. This gene had huge levels of expression, being far more expressed than the housekeeping gene used to analyse it against. This implies that *FT10D* has an important role in wheat plant function and especially in the floral tissue it was observed to be expressed in. Results from this study also seem to imply that there may be some evidence for Ppd-1 regulation. In glume, floret and rachis samples there is extremely high expression in TK and extremely low in SI, with WT displaying expression in the middle of TK and SI (Figure 4.9). This suggests that *Ppd-D1* could be acting as a repressor of *FT10D* expression in these tissues. Similarly to *FT9B5*, TI shows an expression pattern as seen in TK glume, floret and rachis samples and this could be also due to mis-regulation when *Ppd-1* is expressed highly. For further confirmation of potential Ppd-1 regulation of *FT9*B5 and *FT10D*, overexpressed *Ppd-1* lines will be grown and sampled at the same apical stages collected in this study to see what effect extremely high levels of *Ppd-1* have on these genes. *FT10A* and *FT10B* were seen to be expressed (Figure 4.8) in most samples analysed, however, there seemed to be no pattern between the *Ppd-1* genotypes that implied *Ppd-1* having a role in their expression. It is clear that the main route of expression for *FT10* is through the D genome in wheat.

4.3.4 Limitations of this study and future experiments

This study provides a small insight into the expression of genes in the *FT* family outside of *FT1* and *FT2* and has identified expression in *FT9, FT10* and *FT12* in floral tissue. However, there are many limitations to this study and more needs to be done in order

to fully understand the roles and functions of *FT*s. Firstly, as shown in this study, *FT9, FT10* and *FT12* may be localised to the apical meristem and to investigate this further, samples would need to be taken from other tissues in the plant such as in the leaf to see if expression exists there also. Leaf samples have been taken and relate to each apical stage; for example, when taking the apex at double-ridged, leaf samples were simultaneously taken. These samples will be processed to have RNA extracted and subsequently analysed to measure *FT3-12* expression as was conducted in the apex. This would give us a better idea of how *FT* genes work in the context of the whole plant and may indicate if there are others that act as floral initiation signals, as seen in *FT1* (Corbesier, et al., 2007).

To investigate *FT10D* and *FT9B5* further, TILLING lines lacking these genes could be grown against wild-type counterparts to study the effect that these genes have on wheat phenotypes. Tilling lines for *FT10D* have been searched for but none were found, which implies that the removal of this gene may produce a lethal phenotype. However, wheat plants in the Watkins Collection that are missing *FT10D* have been selected and will be grown to study the effect that this has on growth. One limitation of this study was that only Veg, DR, TS, glume, florets and rachis were looked at for expression for genes in the *FT* family. To further these expression studies, samples of other stages may bring to light spikes in FT expression that could provide more indication of their functions. Examples of stages that should be taken are nodes, glume/lemma/floret primordia and even roots as FT has been shown to move to other plant roots such as in potato (Navarro, et al., 2011). Furthermore, 24-hour sampling of *FT*s of interest such as *FT10D* may show cyclic expression over this period which may further indicate regulation via external environmental cues such as photoperiod. Overexpression of Ppd-1 may also supplement data provided by analysis of FT expression in the Ppd-1 NILs studied in this experiment and should give more insight into which are regulated through Ppd-1 and which are not.

This study has been valuable for investigating when certain *FT*s are expressed in floral apical tissue - showing *FT* expression in key stages of apical development. However, functions for these genes have not been suggested and experiments to discover these roles within the plant need to be carried out. One experiment could explore the differences in development rates between the main stem and tillers. Here, samples of tiller apices could be collected alongside samples taken from the main stem and qPCR could be conducted using primers for the *FT*s that I designed. This could enable the observation of potential repressive and activating *FT*s that may be working to slow tiller apex development.

Chapter 5: Discussion

This study has set out to look into three key areas of research surrounding the action of *Ppd-1*. These were: the effect that it has on certain phenotypic traits in wheat, whether Ppd-1 directly regulates *FT1* through interaction with its promoter and if other members of the *FT* family are expressed in the apex and whether they may be also regulated through Ppd-1.

Phenotypic Traits influenced by *Ppd-1*

This part of the study was conducted to further evidence the effect that *Ppd-1* has on traits in wheat phenotype such as spikelet number and to confirm the phenotypes under our plant growth conditions (Perez-Gianmarco, et al., 2019). It also investigates traits that have been previously not a major focus such as internode length to understand the effect that *Ppd-1* has on these also. It was found that Ppd-1 did indeed influence all traits analysed and this was ascertained through the comparison of these traits of *Ppd-1* NILs in the background Paragon in wheat (Figures 2.2-2.7). Short and long-day (SD, LD) experiments investigating differences in apex length suggest that in LD glasshouse conditions, SI (*Ppd-D1* single insensitive) develop quicker than in TI (*Ppd-1* triple insensitive) which would contradict what is currently believed regarding the NILs. However, one hypothesis for this difference could be explained by what was seen in TI double-ridged samples recorded in chapter 4 (Figure 4.2) Here, *FT*s that were not seen to be expressed in other apex samples from the other genotypes at this stage were expressed. These *FT*s were *FT3*, *FT6* and *FT8* could be repressive and not activating. This could be caused by the *Ppd-A1* and *Ppd-B1* alleles (that do not carry an insensitive phenotypic mutation in SI) interacting with *FT3*, *FT6* and *FT8* promoters and upregulating them. These may have repressive functions thereby slowing apex growth in TI when compared to SI.

Ppd-1 interaction with the *FT1* promoter

Ppd-1 is speculated to interact with the *FT1* promoter or other parts of the gene such as intragenic introns and regulate its expression via this route. However, no direct interaction has ever been observed and evidence for it comes from expression studies in *Ppd-1* and *FT1* in *Ppd-1* NILs in Paragon background (Shaw, et al., 2013) (Shaw, et al., 2012). Ppd-1 is also suspected to regulate *FT2* (Boden & Gauley, 2020) and its own expression (unpublished data from Dixon group). This part of the study focused on identifying potential candidate motifs for Ppd-1 interaction in the *FT1* promoter and testing to see if this interaction occurs at all. Potential *FT1* promoter motifs were identified through alignment of the promoter regions where Zinc Finger Homeodomain (ZF-HD) and DNA binding with One zinc Finger (Dof) motifs were found to be highly conserved between *Ppd-1*, *FT1* and *FT2* promoters (Figure 3.2). These domains could be used by *Ppd-1* to regulate *FT1*, *FT2* and its own expression and further studies with yeast-1-hybrid should be conducted to investigate this. Also, a yeast-1-hybrid study looking into Ppd-1 protein interaction with the *FT1* promoter was conducted. This investigation

found an interaction between the two and narrowed down the 500bp region in the *FT1* promoter where Ppd-1 binds (Figure 3.5). This region lies within the 2kb deletion upstream of the *Ppd-D1* allele where binding regions for the regulation of *Ppd-1* expression reside. This 500bp region also contains the Dof domain that was found to be conserved between *FT1, FT2* and *Ppd-1* which provides evidence of its use as a potential site of binding for regulation.

Novel *FT* expression studies in the apical meristem

Expression studies in *FT* have primarily focused on *FT1*, with some studies in *FT2* (Boden & Gauley, 2020), *FT3* (Zikhali, et al., 2017)and *FT4* (Pieper, et al., 2021). The majority of these studies have been conducted in barley due to its diploid nature which make gene expression studies more accessible compared to hexaploid wheat. There are 12 identified members of the *FT* family in wheat (Bennett. & Dixon, 2021) and functions for the majority of these have not been identified. This chapter of the study first investigated the expression of the *FT*s (*FT3-12*) in tissue associated with the wheat apex. It was found that *FT9*, *FT10* and *FT12* were all expressed in the majority of samples analysed (Figures 4.1-4.6). From this data, it was hypothesised that these genes could be localised to floral tissue, with other genes such as *FT1* and *FT3* acting as florigen signals that react in response to external environmental cues. These florigen signals may interact with localised *FT*s in order to regulate their expression. It was also found that *FT3*, *FT6* and *FT8* were only expressed in TI double-ridged samples (Figure 4.2). This suggests that increased *Ppd-1* expression found in TI NILs may initiate abnormal expression of these genes in tissue where it is not usually expressed and provides evidence of *Ppd-1* regulation. Evidence for *Ppd-1* regulation of *FT9B5* and *FT10D* was also observed with higher expression seen in TK and low expression seen in SI (Figures 4.7, 4.9). Future studies into this area should focus on leaf *FT* expression to investigate if any are expressed there. Furthermore, *FT* expression studies into other florally active tissue such as nodes and other stages of the apex that were not included in this study (glume-floret primordia) should be conducted. This should aid in the identification of function as some *FT*s may only be expressed at very specific points in floral reproductive development. To further investigate the functions of genes within the FT family, knock-outs should be produced and grown in order to study their effect on certain phenotypes.

In summary

In terms of the genetic control of flowering in wheat, we have barely scratched the surface. There is still lots that we do not yet know but as each year goes by, more and more studies are being published that hold the key to understanding this complex process. With the wheat genome being 40 times larger than that of rice due to its hexaploid nature, it was considered an insurmountable task to sequence the full genome and was therefore only fully sequenced very recently in 2018 (IWGSC, 2014) (IWGSC, 2018). This means that research using bioinformatics into key processes like flowering is only just taking off.

*FT*s are involved in determining how many spikelets are produced on each apex (Boden & Gauley, 2020). The termination of production of spikelets in bread wheat is key to understanding the yields we can potentially gain from each crop as the more grain per spike we obtain in dry weight, the larger the yield we obtain and the higher the output of wheat we get. It is generally accepted that photoperiod insensitive wheat produces lower yields compared to their sensitive cousins due to them producing less tillers and rushing through apex development as described earlier in this review (Jones, et al., 2016). However, as they are more suited to growing in a shorter season that is experienced in countries like Spain, the yield becomes greater. As we delve deeper into how *FT*s organise flowering, we might find which genes promote and which repress in terms of spikelet and floret formation leading to the development of crops that flower quickly whilst simultaneously being fruitful. With global population expected to reach 9.7 billion by 2050 it is more important than ever that we source better solutions to feeding people, especially with climate change making it more difficult to do so (United Nations: Department for Economic and Social Affairs, 2019). Understanding the genetics of processes like flowering in our staple crops such as wheat will be a key component to solving this issue.

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Glossary:

Photoperiod – The length of daylight in a 24-hour period. This becomes longer or shorter depending on the season which therefore affects flowering time in winter wheat.

Vernalization – the period of cold that winter wheat must sense to begin the flowering process in the spring.

Tiller – a growth of another stem from the initial parental stem produced in grass species.

Glume – membranous, protective layer that surrounds spikelets found in grass species.

Spikelet – The arrangement of florets and glumes by grass species after flowering has been initiated.

Circadian clock – interpretation of signals by an organism to keep processes oscillating around 24 hours.

Knock-out – the removal of part of or a whole gene in order to make it non-functional

Locus – physical location of a gene in an organism's genome.

Phenotype – the physical manifestation of a gene in an organism.

Plasmid – circular loop of DNA that naturally exist in bacterial cells and can be transferred to some eukaryotic cells such as yeast to be used in gene studies.