Genome Wide Association Study of Frost Tolerance in Wheat

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Abstract

Winter wheat growing areas in the Northern hemisphere are regularly exposed to heavy frost. Due to the negative impact on yield, the identification of genetic factors controlling frost tolerance (FroT) and development of tools for breeding is of prime importance.

Here, we detected QTL associated with FroT by genome wide association studies (GWAS) using a diverse panel of 276 winter wheat genotypes that was phenotyped at five locations in Germany and Russia in three years. The panel was genotyped using the 90K iSelect array and SNPs in FroT candidate genes. In total, 17,566 SNPs were used for GWAS resulting in the identification of 53 markers significantly associated (LOD ≥4) to FroT, corresponding to 23 QTL regions located on 11 chromosomes (1A, 1B, 2A, 2B, 2D, 3A, 3D, 4A, 5A, 5B and 7D). The strongest QTL effect confirmed the importance of chromosome 5A for FroT. In addition, to our best knowledge, seven FroT QTLs were discovered for the first time in this study comprising QTLs on chromosomes 3A, 4A, 1B, and two on chromosomes 2D, 3D, and 7D.

Identification of novel FroT candidate genes will help to better understand the FroT mechanism in wheat and to develop more effective combating strategies.

Introduction

Bread wheat (*Triticum aestivum* L.) is an allohexaploid (2n = 6x = 42, AABBDD) species derived from two hybridization events in the region of the Near Eastern Fertile Crescent (Dvorak and Akhunov 2005). The spread of domesticated hexaploid wheat from the Near Eastern Fertile Crescent to today's growing regions required phenological adaption to different environments, e.g., selection of spring/winter types or phenotypes with reduced photoperiod sensitivity (Peng et al. 2011; Faris 2014). In general, three different types of bread wheat adapted to specific environments according to the vernalization requirement, which is necessary for the transition from the vegetative to the generative stage, are known (spring, facultative and winter type (Li et al. 2013)). The exposure to cold temperature needed for the vernalization response varies between 2-4 weeks and 4-6 weeks in semi and strong winter types, respectively (Yan et al. 2015). Winter wheat types are higher yielding compared to spring wheat types, because they can take advantages of autumn rainfall (Armoniene et al. 2013) and have a longer vegetation period (Ozturk et al. 2006). Today, wheat is cultivated on around 220M ha worldwide, which resulted in an annual production of 757M tones in 2018 (FAO 2019). It is estimated, that approximately one-third of the wheat growing area is cultivated with winter or facultative wheat types (Braun and Sãulescu 2002). Winter hardy wheat varieties are mostly needed in the Great Plains of North America, the Russian Federation, as well as Eastern Turkey, Northwestern Iran and China (Braun and Sãulescu 2002).

Low temperatures at early growth stages can significantly reduce yield performance e.g., yield losses of more than 85% were reported in some seasons in the USA (Zheng et al. 2015). Frost damage is observed when sensitive tissue of plants is faced with low temperature in different growth stages (Wu et al. 2014). Economic damage of frost events on crop performance depends on the time point of occurrence (Nuttall

et al. 2019). Due to the cold acclimation phenomenon, winter cereals survive frost by regulating their metabolism at low temperatures and protecting critical structures of cells against freezing temperatures (Vagujfalvi et al. 2003, Visioni et al. 2013).

In order to reduce the negative effects of frost on crop production, it is necessary to identify genes or genomic regions involved in FroT (Liu et al. 2014). The mechanism of plants that describes the response to low temperature by increasing the freezing tolerance is called cold acclimation. Some physiological and biochemical changes occur during cold acclimation, e.g., soluble sugars, proline and cold-resistance proteins are synthesized to protect proteins at the physiological level (Kaplan et al. 2007). These substances play a role in cold stress response of plants through the regulation of the osmotic potential, ice crystal formation, stability of cell membranes and reactive oxygen species (Ding et al. 2019). Several components encompassing messenger molecules, protein kinases, phosphatases and transcription factors assumed to be involved in cold-stress signaling pathways have been reported during the last decades (Ding et al. 2019).

Two important FroT loci namely FROST RESISTANCE 1 (FR-A1) and 2 (FR-A2) are located on the long arm of wheat chromosome 5A. These loci influence freezing tolerance and winter hardiness. The first locus is closely located to the *VRN-A1* gene, but no information on the effect of this gene on the FR1 locus is known. *Vrn-B1* and *Vrn-D1* genes are mapped on the long arm of chromosome 5B and 5D, respectively. *VRN-A1* has the major effect in the determination of spring/winter habit (Todorovska et al. 2014) and plays a major role in FroT (Vagujfalvi et al. 2003). Regarding other genes involved in FroT, the *ICE (inducer of CBF expression*) - *CBF* (*C-repeat binding factor*) - COR (cold-responsive or cold-regulated) pathway has been known as the main cold signaling pathway in many plant species (Guo et al. 2019; Jin et al. 2018; Shi et al. 2015). Under low temperatures, DELLA releases ICE1 from its JASMONATE ZIM-DOMAIN (JAZs) enabling the induction of CBF genes, which are members of the AP2/ERF (APETALA2/ETHLENE RESPONSIVE FACTOR) family (Ritonga and Chen 2020). CBF genes bind to C-repeat/ dehydration-responsive elements (CRT/DRE) and regulate the expression of cold-responsive/late embryogenesis-abundant (COR/LEA) genes (Babben et al. 2018).

In addition to the ICE – CBF – COR pathway, vernalization genes are involved in the flowering pathway, e.g., VRN1, VRN2 and VRN3 responding to low temperature in plants through changes in the regulatory regions (VRN1 and VRN3) or in coding regions (VRN2), which cause delays in flowering time (Galiba et al. 2009). For instance, VRN1 reduces FroT by decreasing the transcript level of CBF and COR genes (Dhillon et al. 2010). Therefore, a delay in flowering time increases FroT, which indicates a connection between the flowering and cold response pathway by the interaction of VRN1 with CBF and COR genes.

Genome wide association studies (GWAS) are widely applied in many crop plants to identify quantitative trait loci (QTLs) associated with traits of interest (Varshney et al. 2021; Li et al. 2021; Chen et al. 2017; Korte and Farlow 2013). Development of high-throughput single nucleotide polymorphism (SNP) genotyping platforms, e.g., Illumina (Wang et al. 2014) and Affymetrix (Allen et al. 2017) enabled the conduction of GWAS in plants and became a useful approach to detect QTL and allelic variation for

complex traits (Liu et al. 2018; Turuspekov et al. 2017). GWAS was successfully applied in wheat to identify QTL regions associated with abiotic stress tolerance (e.g. Valluru et al. 2017), yield components (e.g. Sukumaran et al. 2015; Zanke et al. 2014), grain quality (e.g. Arora et al. 2017) or diseases resistance (e.g. Naruoka et al. 2015). However, up to now, only a few studies were published dealing with the identification of QTL regions associated with FroT by GWAS (Zhao et al. 2020).

During the last decade, several QTLs associated with FroT were identified on different wheat chromosomes (with the exception of chromosome 4D) (Båga et al. 2007; Zhao et al. 2013; Case et al. 2014; Fowler et al. 2016; Kruse et al. 2017). However, the majority of these QTL regions was identified by bi-parental QTL studies. Vagujfalvi et al. (2003) pointed out that ten wheat chromosomes were assumed to be involved in the regulatory gene networks associated with FroT. However, until now, the majority of genes, which are assumed to be involved in FroT have been identified on chromosomes 5A, 5B and 5D (Babben et al. 2018; Todorovska et al. 2014; Würschum et al. 2017).

Therefore, the aims of the present study were (i) to conduct GWAS to identify genome regions associated with FroT, (ii) to investigate potential candidate genes from QTL regions using the wheat reference genome and (iii) to compare our results with previously published FroT regions and genes in winter wheat.

Material And Methods

Phenotypic data

A panel of 276 bread wheat genotypes from 31 countries was evaluated for FroT. This panel comprised 83, 4, 143 and 46 genotypes from Asia, Australia, Europe and USA, respectively (Supplementary Table S1). Out of these 216, 48, and 12 were cultivars, breeding lines and doubled haploid lines, respectively. In a previous study, Babben et al. (2018) used 235 out of the 276 genotypes under investigation to identify polymorphism in known FroT genes and to conduct a candidate gene based association study (CGAS).

All genotypes were tested in four environments during 2012 and 2013 (Gatersleben, Germany; Ranzin, Germany; Puskin, Russia; Roshchinskiy, Russia) and one environment in 2012 and 2014 (Novosibirsk, Russia), according to Babben et al. (2018). The genotypes were tested in a random design in double rows and two replications per genotype. However, in Roshchinskiy, genotypes were tested as a miniplot ($2.5m^2$) trial with only one replication. FroT was assessed as percentage winter survival of plants per plot for each genotype after winter (0% = all plants died, 100% = no plant died; for further information see Babben et al. (2018)). The quality check of phenotypic data was done as described by Babben et al. (2018). Least Square means (LSmeans) per genotype were estimated by fitting a mixed linear model in SAS 9.4 (SASInstitute 2019) (for further information please see Babben et al. 2018).

Genome wide association studies (GWAS)

Genotyping of the 276 wheat genotypes was conducted at Trait Genetics, Gatersleben (Germany), by using the 90K iSelect array (Illumina Inc., San Diego, USA). Flanking sequences of the 90K array were mapped against the reference genome of Chinese Spring RefSeqv1.0 (IWGSC 2018). All mapped markers with more than 30% missing values were excluded. The resulted marker set was imputed by using the Beagle 4.1 software (Browning and Browning 2007). Next, the imputed marker data set was filtered for minor allele frequency (MAF) ≥ 3%, maximum percentage of missing values <10% and heterozygosity ≤12.5%. The filtered marker data set was combined with 182 SNP markers indicating polymorphisms in 15 candidate genes for FroT known from a previously published study (Babben et al. 2018). This final marker data set consisting of 17,566 SNP markers was used for LD decay and GWAS.

To estimate linkage disequilibrium (LD) and to determine LD decay the software package R was used (R Core Team. 2014, packages “genetics” and “LDheatmap” (Shin et al. 2006; Warnes et al. 2013)). The LD was estimated as squared allelic correlation (r^2) between all pairs of markers within a chromosome. For graphical display, the genetic distances between markers in base pairs were plotted against the estimated r^2. The critical value of r^2 was set to r^2=0.2 as described by Voss-Fels et al. (2015). Furthermore, a smooth locally weighted polynomial regression (LOESS) curve was fitted to calculate the LD decay (Sannemann et al. 2015). Finally, the LD decay was determined as intersection point of the LOESS curve and the critical r^2 value (Sannemann et al. 2015). LD decay was estimated for each chromosome and across all 21 wheat chromosomes.

In order to get comparable results with previously published analysis on the same material, a reduced marker set (249 markers, for further information see (Babben et al. 2018)) was used to determine population structure and to calculate kinship matrix.

Kinship matrix was calculated based on Roger’s distance for each pairwise genotype – genotype combination (Reif et al. 2005). Population structure was investigated by using Bayesian cluster analysis implemented in Structure (Pritchard et al. 2000) and principal coordinates analysis (PCoA) implemented in the DARwin 6 software (Perrier and Jacquemoud-Collet 2006). To determine the population structure by using the software package STRUCTURE (source), ten independent runs were performed setting the number of populations (k) from 1 to 10. Furthermore, the number of burn-in and Markov Chain Monte Carlo (MCMC) iterations was set to 100,000. To determine the optimal number of subpopulations, the Evanno method (ΔK method) implemented in the software package STRUCTUTRE HARVESTER version 2.3.4 (Earl 2012) was used.

GWAS was conducted by using the software package TASSEL 5 (Bradbury et al. 2007). A compressed Mixed Linear Model (CMLM) was used to examine associations between SNP markers and FroT data. Two association models were tested: 1) Q + K model (CMLM with Q-matrix and K-matrix as correction for population structure and kinship relationship); 2) K model (CMLM with K-matrix as correction for kinship relationship). All marker trait associations with LOD ≥4 ($-\log_{10}$ of *P* value) were assumed to be significantly associated with FroT according to Babben et al. (2018) and Zhao et al. (2020). All markers, which were significantly associated with FroT, were assigned to QTL regions according to their

chromosomal position and the estimated LD decay (3.5 million base pairs). The peak marker of each QTL region is defined by the highest LOD value. Markers within a distance of ±3.5 million base pairs to the QTL peak marker were assigned to one QTL region. Genes located within the QTL regions were identified based on their position on the reference genome of Chinese Spring (IWGSC 2018). All high and low confidential (HC and LC) genes located within a QTL region were identified. Additionally, published functional gene annotations (IWGSC 2018) were used to identify gene onthology (GO) terms associated with FroT. All GO terms associated with frost or cold tolerance were downloaded from the QuickGO website (https://www.ebi.ac.uk/QuickGO/). Genes within QTL regions were filtered based on GO terms associated with frost or cold tolerance (GO, Supplementary Table S2).

Identification of candidate genes via BLASTn

The sequences of associated candidate genes to FroT were used to identify gene IDs. For this purpose, a BLASTn analysis (nucleotide Basic Local Alignment Search Tool) from National Center of Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) based on the whole coding sequence (CDS) of candidate genes was used. These sequences were aligned to *Triticum* species with default settings. Perfect match or high similarity was identified based on 100% query coverage, an Expect (E) value of 0 and an identity higher 99%.

Results

Genotyping of the 276 wheat accessions resulted in a raw data set of 81,587 SNP markers. In total, 54,340 markers were excluded from further analyses, due to the absence of a hit or no unique map position according to the reference genome sequence of Chinese Spring (IWGSC 2018). The 27,247 uniquely mapped markers were filtered for minor allele frequency (≥3%) and heterozygosity (≤12.5%), resulting in a set of 17,566 informative (17,384 90K SNPs and 182 CG polymorphic sites) markers.

The LD decay ranged between 609,686bp (chromosome 6B) and 8,434,298bp (chromosome 1D). However, it was not possible to estimate LD decay for chromosome 4D. The LD decay across all chromosomes was 3,377,883 bp (Supplementary Table S3). The calculated LD decay was used to define QTL regions.

Population structure was determined by a Bayesian cluster analysis implemented in in the software package Structure and a PCoA implemented in DARwin 6. The Bayesian cluster analysis revealed an optimal number of K=2 or K=3 subpopulations. Due to the origin of the genotypes (North America, Asia and Europe), K=3 was used as optimal number of subpopulations (Figure S1). Genotypes were assigned to one of the three subpopulations based on their membership coefficients. However, genotypes with membership coefficients <0.7 to a subpopulation were considered as admixture (Spataro et al. 2011). In total, 23, 78, 88 and 87 genotypes were assigned to subpopulation 1, 2, 3 or the admixed group. Additionally, a PCoA plot was used to visualize the results of the Bayesian cluster analysis. The first and the second PCoA explained 8.4% and 4.7% of the whole variance. Results of both analyses pointed to a weak to moderate population structure (Figure S2).

The compressed mixed linear model with correction for kinship relatedness (CMLM with K) turned out to be the most appropriate GWAS model (Figure S3). Therefore, GWAS was conducted based on the CMLM K model.

In total, 53 markers were found to be significantly associated with FroT at a significance threshold of LOD≥ 4 (Supplementary Table S4). Out of these, 16 SNPs were associated with polymorphisms in a candidate gene for FroT (*CBF-14* on chromosome 5A) previously published by Babben et al. (2018) (Supplementary Table S4). The significantly associated markers were assigned to 23 QTL regions, which explain 6.1–16.2% of the total phenotypic variance. These QTLs were located on 11 chromosomes (1A, 1B, 2A, 2B, 2D, 3A, 3D, 4A, 5A, 5B and 7D) (Table 1, Figure 1 and S4), whereby the majority of significantly associated markers was detected on chromosome 2B (13 markers, Supplementary Table S4 and Figure 1).

All identified QTL regions were screened for known functional genes located within the QTL regions. In total, 3,112 HC and 2,004 LC genes were identified within the QTL regions (Supplementary Table S2). The number of LC and HC genes per interval ranged between 111 and 589. As mentioned above, 16 of the markers significantly associated with FroT indicate polymorphisms in the *CBF-A14* gene located within the QTL region QTL_5A_2 on chromosome 5A. The *CBF 14* gene is known as a putative candidate gene for FroT (Babben et al. 2018). Additionally, all LC and HC genes located within the QTL regions were screened for GO terms associated with cold stress or FroT. In total, 11 QTL regions on six chromosomes (1B, 2A, 2B, 3A, 5A and 5B) include candidate genes (52) associated with GO terms for cold stress or FroT (supplementary Table S5).

Three of these identified genes were located on chromosome 2A. Interestingly, two of these genes code for a *cold-responsive protein* (*WCOR15*, QTL_2A_3) and the other one was a *cold shock domain protein 1* (QTL_2A_2). In addition, a gene coding for a low temperature and salt responsive protein was identified within QTL_2A_1 on chromosome 2A. Furthermore, genes coding for 16 *basic helix-loop-helix* (*bHLH*) transcription factors were detected on chromosome 1B, 2A, 2B, 3A, 5A and 5B. Three and four genes coding for *flowering locus T-like proteins* (*FT-like*) and *flowering time control proteins* (*FPA*) were identified on chromosome 2A and 5A, respectively. An important gene involved in the FroT pathway, i.e., *ICE1* was observed on chromosome 3A. In addition, 25 *CBF* genes have been identified. Fifteen of them were located on chromosome 5A (specifically on the locus of frost resistance A2 (FR-A2)) and 10 on chromosome 5B. Seventeen specific gene names out of these 25 *CBF* candidate sequences were identified via BLASTn (supplementary Table S6). Twelve and five out of these 17 identified genes were located at the FR-A2 locus on chromosome 5A and 5B, respectively.

Based on obtained BLASTn results, 13 out of 16 transcription factor candidate sequences were identified (supplementary Table S6) on five different wheat chromosomes. One, three and two out of six identified *MYC2*-like transcription factors were located on chromosome 1B, 5A and 5B, respectively. The five *bHLH* transcription factors comprised three *bHLH14-like* transcription factors (5A and 5B), one *bHLH35-like* (3A)

and one *bHLH122-like* (2A), respectively. The genes *ROOT HAIR DEFECTIVE6-LIKE 4* (*RSL4*) and *anthocyanin regulatory R-S protein-like* were identified on chromosome 2B.

Additionally, we identified further possible candidate genes like a dehydration responsive element binding transcription factor (1B), a *late embryogenesis abundant* (*LEA*) *19-like protein* (2A), a *MYB8* transcription factor (2B), a *AP2/B3-like transcriptional factor protein* (*REM18*, 5A) and *Phytochrome A* (*PHYA*, 5A) (supplementary Table S6).

Discussion

Bread wheat is grown worldwide in temperate latitudes and subtropical regions (Royo et al. 2014) and constitutes the main source of proteins and calories in human diets (Venske et al. 2019). However, many wheat-growing areas are regularly exposed to heavy low temperature events during the early stage of wheat development (Braun and Sãulescu 2002) causing severe yield losses (Kajla et. al. 2015). Therefore, FroT is an important trait in breeding, in order to improve winter hardiness of wheat (Visioni et al. 2013).

In the recent years, high-throughput sequencing technologies fostered the availability of large SNP data sets and therefore the conduction of population genetic studies and GWAS (Wang et al. 2014). Furthermore, these technologies made the first fully annotated reference genome sequence of wheat available (IWGSC 2018). Altogether, this progress in plant genetics and genomics helps to increase the understanding of wheat biology and the molecular basis of important agronomic traits (Muthamilarasan and Prasad 2014; Li et al. 2020). Based on this progress, this study aimed to identify QTL regions and candidate genes associated with FroT in wheat.

Recently, QTL mapping studies or GWAS identified several QTL regions associated with FroT in wheat on all wheat chromosomes except chromosome 4D (Zhao et al. 2020; Fowler et al. 2016; Zhao et al. 2013; Case et al. 2014; Kurse et al. 2017; Chen et al. 2019; Sieber et al. 2016; Babben et al. 2018). In general, it is difficult to compare QTL regions identified by different studies using different marker systems and different genetic maps. Therefore, to compare the records from literature with findings of this study, known flanking sequences of markers associated with QTL for FroT in literature were mapped to the reference genome sequence of Chinese Spring (IWGSC 2018). However, flanking markers were not available for all published QTL regions. Hence, these QTLs could not be anchored on the reference genome sequence of Chinese Spring. Furthermore, for some flanking marker sequences no unique position on the reference genome sequence of Chinese Spring (IWGSC 2018) could be identified. Therefore, for markers and QTL regions that could be not uniquely mapped to the reference genome, comparison with the results of this study was conducted based on the chromosome (supplementary Table S7).

To get comparable results, identified candidate genes in the current study with previous study (Babben et al. 2018), LOD≥ 4 was subjected as threshold for significantly associated markers with FroT. In addition, Zhao et al. (2020) reported associated significant markers with FroT with LOD≥ 4. The *P* value was also adjusted by Bonferroni-Holm (LOD≥ 5.5) correction in the present study. Finally, since only three markers

were identified at Bonferroni-Holm threshold at LOD≥ 5.5 (result are not shown), we considered markers with LOD≥ 4 for further analysis. In total, 53 markers were found to be significantly associated (LOD≥ 4) with FroT in this study. These markers were assigned to 23 QTL regions on 11 chromosomes (1A, 1B, 2A, 2B, 2D, 3A, 3D, 4A, 5A, 5B and 7D).

In the present study, three out of 23 identified QTL regions namely QTL_5A_1, QTL_5A_2 and QTL_5B are overlapping with previously reported QTL regions (Case et al. 2014; Fowler et al. 2016; Kruse et al. 2017 and Zhao et al. 2020). It is known that important genes associated with FroT are located on chromosome 5A, e.g., *CBF* genes and *VRN* genes. In this study, 17 *CBF* genes, three *bHLH* family transcription factors and one *FPA* gene were identified within QTL regions associated with FroT on chromosome 5A and 5B. Polymorphisms within two out of 17 identified *CBF* genes (*CBF-A14* and *CBF-A15*) were previously detected by CGAS (Babben et al. 2018). In total, 16 markers associated with polymorphic sites for *CBF-A14* were significantly associated with FroT in the present study.

In plants, Hormones and photoreceptors, such as phytochromes, are regulated by CBFs (Kurepin et al. 2013). The positive role of *PHYA* on the transcription levels of *CBF* pathway genes is reported in tomato (Wang et al. 2020). In the present study, *PHYA* was associated with a QTL for FroT on chromosome 5A.

Furthermore, twelve QTL regions, i.e., QTL_1A, QTL_2A-1, QTL_2A-2, QTL_2A-3, QTL_2A-4, QTL_2B_1, QTL_2B_2, QTL_2B_3, QTL_2B_4, QTL_2B_5, QTL_2B_6 and QTL_2B_7 are potentially overlapping with previously reported QTLs on the same chromosomes. (Båga et al. 2007; Motomura et al. 2013 and Sofalian et al. 2009). However, due to the unavailability of flanking markers or due to the fact that the flanking sequences of available markers could not be anchored on the reference genome (IWGSC 2018); it was not possible to confirm this assumption. Three candidate genes associated with cold or low temperature tolerance were identified on chromosome 2A. The QTL_2A_1, QTL_2A_2 and QTL_2A_3 were co-localized with genes coding for *Low temperature and salt responsive protein*, *cold shock domain protein 1*, *WCOR15* and *LEA 19-like protein*, respectively.

CORs are referred to as proteins encoded by cold-responsive or cold-regulated genes, which are involved in the cold tolerance acquisition and subsequent freezing tolerance. These genes, i.e., LEA, stress responsive protein (SRP), cold induced (KIN) and low temperature induced (LTI) (Guo et al. 2019), are increasing cold tolerance in plants. For instance, accumulation of COR/LEA proteins during cold acclimation protects cell structures and functions from freezing damage (Motomura el al. 2013). The *Wcor15* is expressed under low temperature (Takumi et al. 2003) and encodes a chloroplast-targeted protein in wheat and barley.

QTL_2A_4 includes a gene coding for Flowering Locus T-like protein. It has been shown, that flowering time genes are not only responsible for the transition from the vegetative to the reproductive phase, but are also involved in various environmental stress responses. The relation between flowering and cold response is well known (Limin and Fowler. 2006).

For eight of the QTLs associated with FroT in this study, we did not find any evidence that these QTL were previously reported in literature. These QTLs are located on chromosome 1B, 2D, 3A, 3D, 4D and 7D and will be discussed in the following.

One out of two identified QTL regions on chromosome 1B contains a gene encoding a *bHLH* transcription factor. *bHLH* transcription factors play diverse roles in different physiological processes (Huang et al. 2013). Several studies have been shown that *bHLH* is involved in different responses, which are provoked by cold and other abiotic stresses in Arabidopsis and rice (Jiang et al. 2009; Kiribuchi et al. 2005; Ogo et al. 2006; Wang et al. 2003; Xu et al. 2014). Wang et al. (2019) identified 159 *bHLH*-encoding genes in wheat, which are involved in abiotic and biotic stress response. Furthermore, they pointed out that 98.7% of these genes are associated with more than one stress. In addition, the expression of these genes under different stresses was evaluated. In total, 38.44% of these genes were upregulated under cold stress in wheat (Wang et al. 2019).

The identified *MYC2-like transcription factor* is also called *JAM* (*JASMONATE ASSOCIATED MYC2-LIKE*) and *bHLH14* is called *JAM1*. Both transcription factors are members of the *IIId bHLH* subfamily, which is phylogenetically closely related to *MYC* proteins interacting with *JAZ* proteins. These *bHLH* subfamily acts as transcription repressor of *MYC2* and so as a negative regulator of jasmonate mediated response (Goossens et al. 2017; Sasaki-Sekimoto et al. 2013). Furthermore, Xiang et al. (2019) described the role of cold induced transcription factor *bHLH112*, which promotes a positive regulation of *AP2/ERF* transcription factor in *Artemisia annua* and Jiang et al. (2019) identified that *bHLH35* is involved in cold tolerance in *Anthurium andraeanum*.

In addition, we identified one QTL region on chromosome 3A. This region comprises transcription factor ICE1 and *bHLH* transcription factor genes. The transcription factor *ICE1* is known as an important gene involved in freezing tolerance (*ICE-CBF-COR*) pathway. *ICE1* genes are known in wheat, but until now, no *ICE1* gene was found to be located within a QTL region associated with FroT. Two *ICE* homologs, i.e., *TaICE41* (accession no. EU562183) and *TaICE87* (accession no. EU562184) have been identified in wheat (Guo et al. 2019). The identified AP2/B3-like transcriptional factor protein (REM18) on chromosome 5A is also a member of the DREB/ERF subfamily and it is accordingly maybe involved in FroT (Yamasaki et al. 2004; Chen et al. 2016).

The *bHLH35-like* gene was identified on chromosome 3A. Less knowledge is available for this gene. Jiang et al. (2019) have reported the positive role of *bHLH35* in response to abiotic stresses in Arabidopsis. They reported that *bHLH35* from *Anthurium andraeanum* (*AabHLH35*) increases stress tolerance to cold and drought in Arabidopsis. The expression of *CBF1* and *COR15A* in wild type (WT) and *AabHLH35* transgenic lines of Arabidopsis was significantly increased under cold stress compared to control plants. Expression of *COR15A* was 3-fold higher in *AabHLH35* transgenic lines relative to WT lines. Therefore, they assumed that *AabHLH35* might promote *COR15A* expression in response to cold stress. Furthermore, *OsbHLH35* increased salinity tolerance in rice (Chen et al. 2018) and *PebHLH35* from *Populus euphratica* increases drought tolerance in Arabidopsis (Dong et al. 2014).

As mentioned above, several genes are involved in the enhancement of FroT in plants. In addition to the *ICE-CBF-COR* pathway and flowering time genes, we identified nine genes of Jasmonates (JA) in the present study, which play a major role in the *ICE-CBF-COR* pathway (Figure 2) by activating transcription factors. Activated transcription factors bind to the cis-acting element in the promoter of target genes to increase FroT in plants (Ritonga and Chen, 2020).

Conclusion

This study dealt with the identification of QTL regions and putative candidate genes associated with FroT in wheat. GWAS resulted in the identification of 23 QTL regions associated with FroT. The identified QTL regions on chromosome 5A and 5B are in accordance with known genomic regions and candidate genes previously described for FroT in wheat. Moreover, the findings reported here, confirm the results of the previous study of Babben et al. (2018) in regard to polymorphisms in candidate genes for FroT (*CBF-14)* on chromosome 5A. To the best of our knowledge, eight of the detected QTL regions can be assumed to be novel, as these regions were not described in literature before. Furthermore, within the QTL regions on chromosome 1B, 2A, 2B, 3A, 5A and 5B genes with GO terms associated with cold stress response or FroT were identified. The findings reported here give hints to known and previously unknown genome regions and candidate genes, which are putatively associated with FroT in wheat and therefore mark the starting point for further research. Prospectively, these findings will help to develop diagnostic markers for FroT in wheat and to fine map putative candidate genes. Both will foster the better understanding of FroT in wheat and the improvement of winter hardiness and FroT in bread wheat elite breeding pools.

Declarations

Authors' contributions:

DP, FO, AB and MK conceived and designed the experiments. AB, MK, TP and YC provided the experimental material. SB, FA, YC, TP, AB and MK performed the experiments. BS, SB, HL, JK and DP analyzed the data. BS, SB, HL, JK, DP and FO wrote the paper. DP, MK and FO did study design, subject recruitment and sample preparation. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

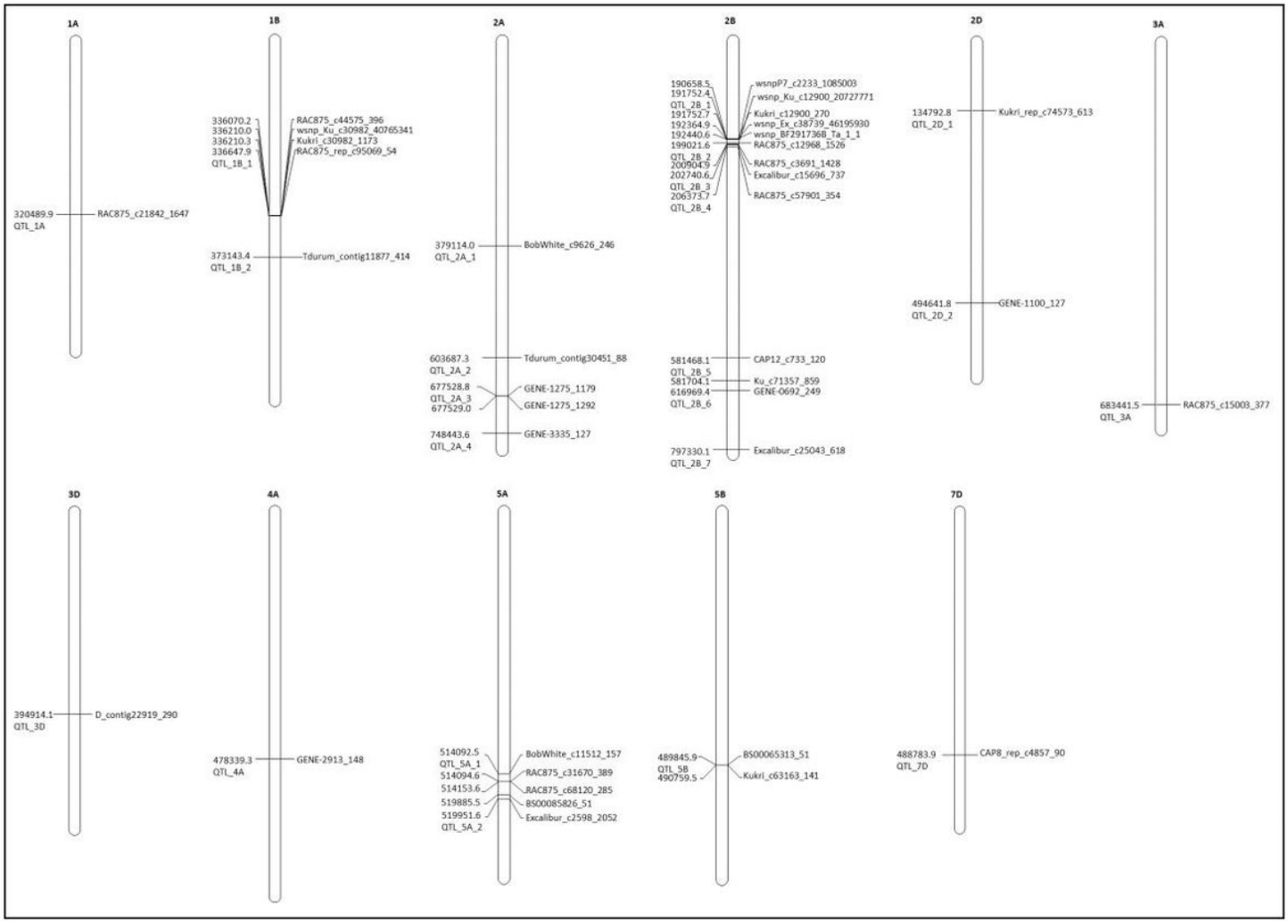

Figure 1

Genetic map of identified QTLs for FroT on wheat chromosomes: The identified markers are shown on the different chromosomes.

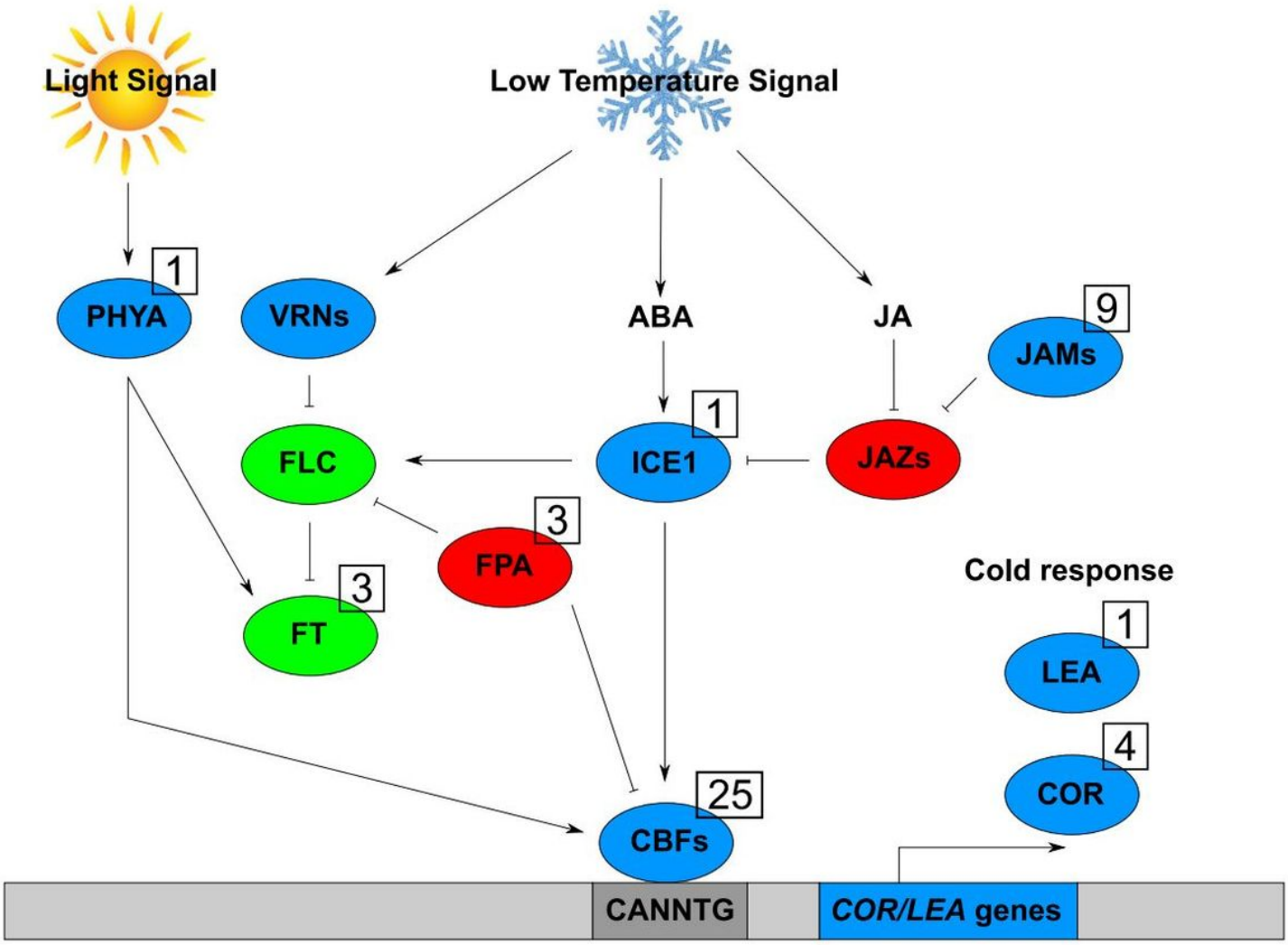

Figure 2

Cold response regulatory pathway based on identified genes for FroT in the current study.

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