An Efficient Approach for the Development of Locus Specific Primers in Bread Wheat (*Triticum aestivum* L.) and Its Application to Re-Sequencing of Genes Involved in Frost Tolerance

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Abstract

Recent declines in costs accelerated sequencing of many species with large genomes, including hexaploid wheat (*Triticum aestivum* L.). Although the draft sequence of bread wheat is known, it is still one of the major challenges to develop locus specific primers suitable to be used in marker assisted selection procedures, due to the high homology of the three genomes. In this study we describe an efficient approach for the development of locus specific primers comprising four steps, i.e. (i) identification of genomic and coding sequences (CDS) of candidate genes, (ii) intron- and exon-structure reconstruction, (iii) identification of wheat A, B and D sub-genome sequences and primer development based on sequence differences between the three sub-genomes, and (iv); testing of primers for functionality, correct size and localisation. This approach was applied to single, low and high copy genes involved in frost tolerance in wheat. In summary for 27 of these genes for which sequences were derived from *Triticum aestivum*, *Triticum monococcum* and *Hordeum vulgare*, a set of 119 primer pairs was developed and after testing on Nullitetrasomic (NT) lines, a set of 65 primer pairs (54.6%), corresponding to 19 candidate genes, turned out to be specific. Out of these a set of 35 fragments was selected for validation via Sanger's amplicon re-sequencing. All fragments, with the exception of one, could be assigned to the original reference sequence. The approach presented here showed a much higher specificity in primer development in comparison to techniques used so far in bread wheat and can be applied to other polyploid species with a known draft sequence.
Introduction
Genomic resources in wheat

Wheat (Triticum aestivum L.) is the cereal with the largest acreage worldwide [1]. It belongs to the family Poaceae and has a complex allohexaploid genome of about 17 Giga-base pairs (Gbp). The repeat content is approximately 80% which consists primarily of retroelements. The gene density is between 1 per 87 Kilo-base pairs (Kbp) and 1 per 184 Kbp [2, 3]. During evolution wheat became an allohexaploid organism (2n = 6x = 42) with the A, B and D genome. In brief, 300,000–500,000 years ago the first hybridisation between the wild diploid wheat (Triticum urartu, 2n = 2x = 14, genome AuAu) and an ancestor closest related to goat grass (Aegilops speltoides, 2n = 2x = 14, genome SS) took place [4, 5] leading to the generation of wild emmer wheat (Triticum dicoccoides, 2n = 4x = 28, genome A^A^BB) [6]. Tribal communities formerly making a living of gathering and hunting began to cultivate the wild emmer about 10,000 years ago. Human selection led to cultivated emmer (Triticum dicoccum). By a spontaneous hybridisation of cultivated emmer with another goat grass (Aegilops tauschii 2n = 2x = 14, genome DD) in combination with a natural mutation, bread wheat (Triticum aestivum, 2n = 6x = 42, genome AABBDD) was created [7]. Due to the hexaploid genome and a very high homology of the three sub-genomes in wheat, the genome sequence information has an inestimable value for molecular breeding, comparative genomics and association studies.

Nowadays, the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) database is a key virtual library of genomic, transcriptional and protein sequence data for more than 33,000 organisms [8]. NCBI serves as a web-platform for the identification of target gene sequences in organisms of interest, e.g. Triticum aestivum, Triticum monococcum, Hordeum vulgare etc. An additional wheat database is the CerealsDB web page created by members of the Functional Genomics Group at the University of Bristol (http://www.cerealsdb.uk.net), which includes online resources of genomic information, i.e. varietal SNPs, DArT markers, and EST sequences all linked to a draft genome sequence of the cultivar Chinese Spring [9]. Another web based portal is URGI, which includes datasets such as chromosome survey sequences, reference sequences, physical maps, genetic maps, polymorphisms, genetic resources, many phenotypic data and various genomic arrays (http://wheat-urgi.versailles.inra.fr). The chromosomal sequence information is granted by the International Wheat Genome Sequencing Consortium (IWGSC). All mentioned databases are suitable for the identification of homologous chromosome sequences in bread wheat. In addition to these resources, an important tool for wheat is the upcoming Genome Zipper of wheat (http://wheat-urgi.versailles.inra.fr). In the past few years, a lot of sequence information of wheat-sorted chromosome arms [10–12], T. urartu [13] and Ae. tauschii [14] became available and was integrated in the above mentioned databases.

Function and structure of frost tolerance genes

Low temperature is one of the most important limiting factors of wheat cultivation in North America and Eastern Europe. To ensure high yields in these areas, introduction of efficient frost tolerance alleles into elite cultivars is a prerequisite. Cold stress inhibits metabolic reactions and prevents wheat from fulfilling its genetic potential. To avoid yield losses, wheat needs acclimatisation to low temperatures, which prevents premature transition to the reproductive phase. This must happen before the threat of freezing stress during winter has passed [15]. Frost tolerance is a complex system involving many genes out of which six gene families/groups have been analysed in this study. According to their function, these genes belong to two separated metabolic pathways. The Ppd and Vrn genes are responsible for flowering, whereas
the Cbf, Ice, Tacr7, Dem, Cab and Dhn genes are involved directly in frost tolerance. Regarding copy number, the analysed genes could be assigned as follows: Dem and Tacr7 are single copy; Ppd, Vrn and Ice are low copy, while Cbf, Cab and Dhn are high copy genes.

A high number of low temperature-induced genes was identified and characterized in plants [16, 17]. These are referred to as LATE EMBRYOGENESIS-ABUNDANT (LEA), Dehydrin (Dhn), Responsive to Abscisic Acid (RAB), Low Temperature–Responsive (LT) and Cold-Responsive (COR) genes. Several of the COR genes are dehydrins, which are a distinct biochemical group of LEA proteins [18–20] for which 54 different unigenes are described, of which 23 are involved in frost tolerance [21]. Dehydrins have either one but mostly two exons [22]. Cab genes or CAM-like (CML) genes, encoding proteins composed mostly of EF-hand Ca2+-binding motifs, may contain one to six exons [23]. Cbf genes are very important in the induction of COR genes through binding of C-repeat/dehydration-responsive elements (CRT/DRE) [15]. The complex Cbf gene family consists of 27 paralogs with 1–3 homologous copies per sub-genome. In total, the family contains at least 65 Cbf gene family members [24]. Knox et al. [25] detected that approximately half of the eleven Cbf orthologues at the FR-H2 locus in barley are duplicated. In addition, they reported that the variation in Cbf genes, which do not carry any introns, is widespread in the Triticeae [26]. This gene family is regulated by two wheat specific Ice genes under cold conditions [27, 28]. Both Ice genes have four exons [29, 30]. Tacr7 belongs to the group of LT genes [31]. The Dem genes have an important role in the development of apical meristems and are thereby involved in the vegetative/reproductive transition of the shoot apex [27].

Flowering genes may be involved in frost the tolerance pathway because the flowering pathway contains vernalization and photoperiod response genes at crucial positions [32]. This pathway is regulated by five major Vrn genes (Vrn1, Vrn2, Vrn3, Vrn4 and Vrn5) and two Ppd genes (Ppd1 and Ppd2) [33]. The gene structure of the five vernalization genes varies from Vrn1 having eight exons [34], via Vrn3 with three exons [35] and Vrn2 with two exons [36] to Vrn4 and Vrn5 of which the structure is unknown. The Ppd1 gene shows eight exons [37], while the structure of Ppd2 is unknown. The interaction between the flowering and the frost tolerance pathway is based on Vrn1 and Cbf genes. The Vrn1 gene may reduce transcript levels of Cbfs and COR genes under long day conditions.

The draft wheat sequence and development of genomic markers

Nowadays, molecular markers, i.e. marker-assisted selection (MAS), are basic tools in plant breeding during germplasm characterization and cost efficient selection of important traits/genes. Furthermore, after gene isolation re-sequencing of specific fragments allows efficient allele mining [38]. However, the development of gene specific primers in wheat is hampered by the large genome size of 17 Gbp, the high repeat content of about 80% [2, 3], by the close homology of the three genomes (A, B and D) and by the high rate of similarity within genes and gene family members [10]. Comparative analysis of wheat sub-genomes shows high sequence homology and structural conservation and no significant differences in the rate of duplications between the sub-genomes are observed [11]. Recent efforts of the scientific community and the IWGS in sequencing of the 3 donor genomes as well as of the hexaploid wheat offer a solution in deciphering the intron-exon-structure of genes. By using differences of intron sequences among the homologous and paralogous copies of the various genes, it is possible to reconstruct the gene structure and identify differences between homologues. Continuous improvements of BLAST algorithms enhance the use of the above mentioned wheat genomic resources facilitating efficient primer development. Furthermore, specific primers are the basis for the development of molecular marker assays based on SNPsi.e. cleaved amplified
polymorphic sequence (CAPS) [39], pyrosequencing [40] or competitive allele-specific polymerase chain reaction (KASP) [41], which are the base for marker assisted selection (MAS) procedures, anchoring physical and sequence contigs [12], germplasm characterization [42].

Material and Methods

Plant material and DNA extraction

In this study three cultivars (`Chinese Spring`, `Moskovskaya 39` and `VAKKA`) were used in initial testing of designed primer pairs, while a set of 24 genotypes, comprising two spring and 22 winter wheat cultivars, was used for re-sequencing of amplicons of frost tolerance genes (Table 1). For the physical assignment to chromosomes and chromosome segments 21 NT-lines [43] and 46 deletion-lines [44] were used (S1 Table) having the genetic background of `Chinese Spring`. The DNA was extracted at the three leaf stage according to Stein et al. [45].

Sequence retrieval of genes involved in frost tolerance

As a starting point a set of 27 genes involved in frost tolerance was selected. 9 *Triticum aestivum* sequences together with 9 sequences from *Triticum monococcum* and 9 from *Hordeum*

| No. | Genotype          | Country       | Variety | Variety |
|-----|------------------|---------------|---------|---------|
| 1   | Chinese Spring*  |               | spring  |         |
| 2   | Zentos           | Germany       | winter  |         |
| 3   | Simila           | Czech Republic| winter  |         |
| 4   | Roughrider       | USA           | winter  |         |
| 5   | Norstar          | USA           | winter  |         |
| 6   | Moskovskaya 39*  | Russia        | winter  |         |
| 7   | Bezenchuckskaja 380 | Russia    | winter  |         |
| 8   | Cheyenne         | USA           | winter  |         |
| 9   | ÄRING II         | Sweden        | winter  |         |
| 10  | VAKKA*           | Finland       | winter  |         |
| 11  | Bezostaja 1      | Russia        | winter  |         |
| 12  | Capelle Desprez  | France        | winter  |         |
| 13  | Centurk          | USA           | winter  |         |
| 14  | Mironovska 808   | Ukraine       | winter  |         |
| 15  | Pobeda           | Serbia        | winter  |         |
| 16  | Renesansa        | Serbia        | winter  |         |
| 17  | Sava             | Serbia        | winter  |         |
| 18  | Triple Dirk B (GK 775) | Australia | winter  |         |
| 19  | Triple Dirk S    | Australia     | spring  |         |
| 20  | ISENGRAIN        | France        | winter  |         |
| 21  | APACHE           | France        | winter  |         |
| 22  | SKAGEN           | Germany       | winter  |         |
| 23  | JULIUS           | Germany       | winter  |         |
| 24  | Biryuza          | Russia        | winter  |         |
| 25  | Moskovskaya 40   | Russia        | winter  |         |

Table 1. Plant material for PCR amplification and re-sequencing.

Complete set of 24 genotypes (without `Chinese Spring`) were used for sequencing.

* Genotypes for primer testing

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vulgare, known to be involved in frost tolerance from previous studies, served as a backbone for the identification of bread wheat frost tolerance candidate gene sequences (Table 2). If only the coding regions (mRNA-, EST- or protein-sequences) were available, the data bases of the International Wheat Genome Sequencing Consortium (IWGSC, http://www.wheatgenome.org/) and/or the Bristol Wheat Genomics (http://www.cerealsdb.uk.net/) were used for the identification of the full genomic sequence and subsequent reconstruction of the gene structure. The BLAST algorithm parameters were set as default.

Reconstruction of intron-exon-structure and gene specific primer development

The reconstruction of the gene intron-exon-structure was performed using the internet platform 'Spidey' (http://www.ncbi.nlm.nih.gov/spidey/spideyweb.cgi) from NCBI, which allows alignment of mRNA to genomic sequence. The intron/UTR regions sequences were used for primer development. The next step was the identification of the best hits to the three different wheat genomes on the IWGSC and/or the Bristol Wheat Genomics website via BLASTn. After collecting three homologue sequences of each targeted gene the gene structure was reconstructed for each one separately and then used for multiple alignments. Multiple alignments were constructed by using Sequencer 5.1 (Gene Codes Corporation, Ann Arbor, USA) and

| Candidate gene | Gene   | Species         | Cultivar      | Accession | Type     | Citation |
|----------------|--------|-----------------|---------------|-----------|----------|----------|
| Cbf1           | TaCBF1 | Triticum aestivum | Winoka       | AF376136  | Gene/CDS | [72]     |
| Cbf4           | TrCBF4 | Triticum monococcum | n.a         | AY951945  | Gene/CDS | [73]     |
| Cbf5           | TrCBF5 | Triticum monococcum | n.a         | AY951947  | Gene/CDS | [73]     |
| Cbf7           | TrCBF7 | Triticum monococcum | DV92        | AY785904  | Gene/CDS | [26]     |
| Cbf8           | HvCBF8 | Hordeum vulgare  | Tremois      | DQ445252  | Gene/CDS | [25]     |
| Cbf10          | TrCBF10| Triticum monococcum | n.a        | AY951950  | Gene/CDS | [73]     |
| Cbf13          | TrCBF13| Triticum monococcum | n.a        | AY951951  | Gene/CDS | [73]     |
| Cbf14          | TrCBF14| Triticum monococcum | n.a        | AY951948  | Gene/CDS | [73]     |
| Cbf15          | TaCBF15| Triticum aestivum | Norstar      | EF028765  | Gene/CDS | [74]     |
| Cbf16          | TrCBF16| Triticum monococcum | G3116      | EU076384  | Gene/CDS | [75]     |
| Cbf17          | TrCBF17| Triticum monococcum | n.a        | AY951945  | Gene/CDS | [73]     |
| Cbf18          | TrCBF18| Triticum monococcum | n.a        | AY951946  | Gene/CDS | [73]     |
| Dhn1           | HvDhn1 | Hordeum vulgare  | Dicktoo      | AF043087  | Gene/CDS | [76]     |
| Dhn3           | HvDhn3 | Hordeum vulgare  | Dicktoo      | AF043089  | Gene/CDS | [76]     |
| Dhn4           | HvDhn4 | Hordeum vulgare  | Barke        | BQ466915  | EST      | [77]     |
| Ice2           | HvIce2 | Hordeum vulgare  | Morex        | DQ113909  | Gene/CDS | [29]     |
| Vrn-A1         | TaVRN-A1| Triticum aestivum | Triple Dirk C Line | AY747600 | Gene/CDS | [34]     |
| Vrn-B1         | TaVRN-B1| Triticum aestivum | Triple Dirk B Line | AY747603 | Gene/CDS | [34]     |
| Vrn-D1         | TaVRN-D1| Triticum aestivum | Triple Dirk C Line | AY747606 | Gene/CDS | [34]     |
| Vrn2           | TaVRN2 | Hordeum vulgare  | Dairokkaku   | AY485977  | partial CDS | [78]     |
| Vrn3           | TaVRN3 | Triticum aestivum | Chinese Spring | DQ890162 | Gene/CDS | [35]     |
| Cab            | HvCab  | Hordeum vulgare  | Barke        | BQ465487  | EST      | [77]     |
| Dem            | HvDem  | Hordeum vulgare  | Barke        | AL504294  | EST      | [79]     |
| Tacr7          | HvTacr7| Hordeum vulgare  | Golden Promise | BQ659345 | EST      | [77]     |
| Ppd-A1         | TaPpd-A1| Triticum aestivum | Chinese Spring | DQ885753 | Gene/CDS | [37]     |
| Ppd-B1         | TaPpd-B1| Triticum aestivum | Chinese Spring | DQ885757 | Gene/CDS | [37]     |
| Ppd-D1         | TaPpd-D1| Triticum aestivum | Chinese Spring | DQ885766 | Gene/CDS | [37]     |

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CLC Main Workbench 7.6 (CLC Bio, Aarhus, Denmark) software and visually inspected for unique stretches among three homologues. The polymorphisms between the three homologous genomes of each gene were detected and used for specific primer development. The primers were developed by using Primer3 (v. 0.4.0) [46, 47]. Parameters utilized for primer development were set to a maximal 3’ stability of 50, primer size between 19 and 28 bp and primer melting temperature between 57° and 63°Celsius. The maximal fragment length was set up to 1200 bp, while optimal fragment length was 900 bp. Other parameters remained as default. Specificity of primers was based on two nucleotide differences within the primer binding site or one difference within the last seven nucleotides at the 3’ end of the primer based on the analyses of the three homologue target sequences [48]. All primers were designed to bind locus specific sequences within the introns/UTR regions of selected genes. At least one primer of a primer pair had to be locus specific for single band amplification.

**PCR amplification and fragment analysis**

Newly designed PCR primers were amplified in two different reaction volumes i.e. firstly, in a volume of 10 μl for functionality testing and chromosomal assignment, and secondly in a 20 μl reaction volume for re-sequencing. The PCR reactions comprised two different polymerases, FIREPol™ DNA polymerase (Solis BioDyne, Tartu, Estonia), in a first round of testing, and MyTaq™ DNA polymerase (BIOLINE, Luckenwalde, Germany), in a second round of testing in case the FIREPol product was very weak, with 50 ng of genomic DNA. The master mix for one PCR reaction comprised 0.4 U FIREPol™ DNA Polymerase, 1 x Buffer B, 2.5 mM MgCl2 (Solis BioDyne, Tartu, Estonia), 0.2 mM dNTPs (Fermentas, St. Leon-Rot, Germany) and 0.25 pmol primers (Microsynth, Balgach, Switzerland) or 0.4 U MyTaq™ DNA Polymerase, 1 x My Taq Reaction Buffer B (that comprised 1 mM dNTPs and 3 mM MgCl2) (BIOLINE, Luckenwalde, Germany) and 0.25 pmol primers. The fragment amplification was conducted in a thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems, Darmstadt, Germany) under various PCR profiles (S2 Table). PCR fragments were separated by using agarose gel electrophoreses and analysed using the imaging system Gel Doc™ XR and the Quantity One® 1-D analysis software (4.6.2) (Bio-Rad, Hercules, USA).

**PCR fragment mapping by using NT- and deletion lines**

All specific and single banded PCR fragments were assigned to chromosomes by using 21 nullisomic-tetrasomic (NT) lines [43] and by a set of 46 deletion-lines [44]. The information about chromosomal localisation of these gene specific amplicons was compared to published results. The map of specific PCR fragments was printed via LaTeX 4.4.1 software (freeware).

**In silico analysis of primer sub-genome specificity**

A set of 98 primers used for amplification of 65 PCR fragments with correct chromosomal localisation were in silico validated for sub-genome specificity by aligning to the draft sequence of wheat. The primers were aligned via Multiple Alignment using Fast Fourier Transform (MAFFT, http://www.ebi.ac.uk/Tools/msa/mafft/), CLC and Sequencher. Parameters for the Sequencher based alignment were as follows: clean data with minimum overlap of 19 nucleotides and minimum match percentage of 90%, while CLC and MAFFT parameters were as default. The differences between the sub-genome sequences and designed primers were manually inspected. Primers with sub-genome specificity were those having two or more differences in binding site or at least one difference at the last seven nucleotide bases at 3’ end of primer.
Re-sequencing of frost tolerance candidate genes and BLAST verification

Sequencing of PCR fragments was performed by Microsynth AG (Balgach, Switzerland) using the Sanger sequencing method [49]. First sequencing reactions were performed with primers used for amplification and if quality was lower than 70% an optimisation with redesigned oligos was conducted. Subsequently all fragment sequences were compared to reference sequences and/or candidate genes of related species by using NCBI MegaBlast function [50]. The results were limited to five hits, minimum expect threshold of $e^{-100}$ and minimum identity of 85%. All other parameters remained as default. The haplotype diversity (Hd), the nucleotide diversity and the average number of nucleotide diversity in a set of 24 analysed wheat cultivars were calculated using the DnaSP 5.1 freeware software [51, 52].

Results

Alignment of candidate gene sequences with corresponding genomic sequences retrieved from the International Wheat Genome Sequencing Consortium, the Bristol Wheat Genomics and NCBI allowed the identification of exon-intron splicing positions, and the identification of coding and non coding regions. Therefore, reconstruction of the intron-exon structure by using newly available genomic sequences is the basic step towards the development of gene specific primers in polyploid plants such as hexaploid wheat.

Reconstruction of intron-exon-structure and development of gene specific primers

The workflow for the development of gene specific primers and validation regarding PCR specificity, chromosomal localisation and sequence homology contains four steps (Fig 1). In short, the procedure starts with collecting sequences of candidate genes, followed by the reconstruction of intron and exon structure and sub-genome sequence identification, until primer development and PCR fragment testing. Functionality and correctness of PCR fragments were assessed by NT mapping, sequencing and BLASTing by using three databases, six tools ('Spidey', 'Primer3', BLASTn, BLASTx, CLC Main Workbench and Sequencer) and two cytological stocks of wheat.

For all of the 27 candidate genes we were able to re-construct the gene structure or at least a part of it. A set of 119 PCR products was obtained from 157 primers pairs designed in this study. 13 of them have recently been published in Keilwagen et al. [53]. Additional 12 primers from literature were used for the amplification of targeted genes. By combining the primers from this study and the 12 primers from literature a total of 169 primers were analysed. As an example the reconstruction of the three copies of the Vrn1 gene structure, primer positions, intron length differences and exon SNPs are shown in Fig 2.

Testing primers for specificity and chromosomal assignment of PCR products

In total, a set of 169 primers representing 119 PCR products from 27 candidate genes was tested for functionality and specificity. A set of 86 primer combinations from 23 candidate genes showed single band amplification (72.27%).

Chromosomal localisation via Nulli-tetrasomic (NT)-lines of Chinese Spring [43] of a set of 86 single band PCR amplicons revealed that 65 fragments were located on expected chromosomes according to the literature. Out of these 65 fragments, six were products of combination of already published and newly designed primers. The remaining 19 fragments showed an
incorrect localisation (literature vs. NT-lines) or no localisation was possible as all NT-lines showed a fragment. Correctly assigned amplicons originated from 19 genes and were located on 11 wheat chromosomes (Table 3, Fig 3). A set of 10 out of 19 analysed genes were located on wheat chromosome group 5, out of 119 PCR fragments 65 single bands were correctly localised. That is equivalent to a success rate of 54.6%. These 65 amplicons represent 19 frost tolerance genes, are gene specific and were therefore selected for further studies (Table 4, S2 Table).

Furthermore, a set of 40 amplicons was physically assigned using a set of 46 available deletion-lines [44] (Fig 3, Table 3). All six genes, which are localised on chromosome 5A via NT-lines, are map to a large cluster between sector AL-12 and AL-17 on the long arm of chromosome 5.

Fig 1. Workflow of development gene specific primers and PCR fragments in wheat. The method comprises four steps, i.e. (i) identification of genomic and coding sequences (CDS) of candidate genes, (ii) intron- and exon-structure reconstruction, (iii) identification of wheat A, B and D sub-genome sequences and primer development on sequence differences between the three sub-genomes, and (iv); primer and PCR fragment testing for functionality, correct size and localisation. The dashed lines show optional applications.

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Table 3. Overview of chromosome localisation of candidate genes (PCR fragment) via NT-lines, deletion-lines and literature.

| Gene   | NT-lines | PCR signal present via deletion-lines | Deletion-line localisation section | Literature location | Reference |
|--------|----------|---------------------------------------|------------------------------------|---------------------|-----------|
| Cbf1   | 5D       | 5DS-5; 5DL-5,-7                       | proximal from 5DL-5 and distal from 5DL-1 | n.a                 | [73]      |
| Cbf5   | 7A       | 7AL-1                                 | distal on short arm from 7AS-1     | 7Am                 | [81]      |
| Cbf7   | 2B       | 2BS-1,-3,-4                           | distal on long arm from 2BL-6      | n.a                 | [73]      |
| Cbf10  | 5A       | 5AS-3,-10; 5AL-17,-23                 | proximal from 5AL-12 and 5AL-17    | 5Am                 | [73]      |
| Cbf13  | 5A       | 5AS-3,-10; 5AL-17,-23                 | proximal from 5AL-12 and 5AL-17    | 5Am                 | [73]      |
| Cbf14  | 5A       | 5AS-3,-10; 5AL-17,-23                 | proximal from 5AL-12 and 5AL-17    | 5Am                 | [73]      |
| Cbf15  | 5A       | 5AS-3,-10; 5AL-17,-23                 | proximal from 5AL-12 and 5AL-17    | 5Am                 | [73]      |
| Cbf18  | 6A       | 6AS-1                                 | distal on long arm from 6AL-8      | 6Am                 | [73]      |
| Dhn1   | 5D       | 5DS-5; 5DL-5                          | proximal from 5DL-5 and distal from 5DL-1 | 5H                  | [80]      |
| Vrn-A1 | 5A       | 5AS-3,-10; 5AL-17,-23                 | proximal from 5AL-12 and 5AL-17    | 5A                  | [81]      |
| Vrn-B1 | 5B       | 5BS-4,-5,-6,-8; 5BL-16                | proximal from 5BL-16 and distal from 5BL-9 | 5B                  | [81]      |
| Vrn-D1 | 5D       | 5DS-5; 5DL-7                          | distal on long arm from 5DL-5      | 5D                  | [81]      |
| Vm2    | 4D       | everywhere (4DS-1,-2,-3; 4DL-9,-13)   | proximal from 4DS-1 and 4DL-9      | 5AmL                | [82]      |
| Vm3    | 7B       | 7BL-7,-6,-10                          | distal on short arm from 7BS-1     | 7BS                 | [35]      |
| Cab    | 5A       | 5AS-3,-10; 5AL-17,-23                 | proximal from 5AL-12 and 5AL-17    | 5HL                 | [55]      |
| Dem    | 6B/6D    | 6BS-2; 6BL-3,-5,-6; 6DS-4,-6; 6-DL 10 | distal on short arm from 6BS-3 and proximal from 6DL-11 and distal from 6DL-12 | 6HL                 | [55]      |
| Tac7   | 2B       | everywhere (2BS-1,-3,-4; 2BL-6)       | proximal from 2BS-1 and 2BL-6      | 2HL                 | [55]      |
| Ppd-B1 | 2B       | 2BS-3; 2BL-6                          | proximal from 2BS-1 and distal from 2BS-4 | 2B                  | [83-85]   |
| Ppd-D1 | 2D       | 2DL-9                                 | distal on short arm from 2DS-5     | 2D                  | [84, 85]  |

The table shows the analysed frost tolerance candidate gene, their chromosomal localisation and fine mapping via NT and deletion-lines. The column deletion-line localisation section shows the approximate chromosomal position of respective genes based on deletion break points.

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In silico analysis of primer sub-genome specificity

The draft sequence of wheat and related species allows detailed in silico analysis of oligos used in this study by doing simple BLAST comparison. Out of 98 oligos that were used for the amplification of 65 PCR fragments, 54 turned out to be specific to one sub-genome, 21 specific to two sub-genomes, and 14 were unspecific. For 9 oligos the comparison could not be performed due to non availability of sub-genome sequences (S2 Table). 57 out of 65 amplicons comprise at least one sub-genome specific primer. For five PCR fragments (Cbf5, Dhn1, Cab b, Cab d and Dem) no wheat sub-genome sequences could be identified. Both primers of PCR fragments Cbf7, Ppd-B1f and Ppd-D1b showed no-specificity to one sub-genome in reference to Wu et al. [48]. Nevertheless, all three fragments showed single bands and correct chromosome localisation via NT-lines (S1 Fig). The primer sequences of Ppd-B1f and Ppd-D1b were derived from a specific sub-genome. At least one of the primers showed one or more differences to corresponding regions on the chromosomes in alignments with the other two sub-genomes. Special cases are the primers of fragment Cbf7. The forward primer has no sub-genome specificity and the reverse primer is specific to sub-genomes A and B (S3 Table).

Re-sequencing of genes involved in frost tolerance and homology validation via BLAST

Five out of 40 amplicons revealed a presence/absence polymorphisms (dominant) and were therefore not sequenced. These five dominant markers were directly used for genotyping of a Ppd-D1 deletion in the promoter and a transposable element (TE) in intron1 [54]. One PCR fragment (Cbf7) could not be sequenced due to very low quality. Finally, 34 amplicons,
Table 4. Primer sequences used for amplification of candidate genes.

| Fragment | Forward primer name | Forward primer sequence (5'→3') | Reverse primer name | Reverse primer sequence (5'→3') |
|----------|---------------------|----------------------------------|---------------------|----------------------------------|
| Cbf1     | AF376136_s1         | TTTTGTACGCTGCAACTGAT            | AF376136_as709      | TTTACCGAGGGTAGTTTCCA            |
| Cbf5     | TmCBF5_F            | CAGTGGCAAAATGCCAGAATCC          | AF376136_as709      | ACTAGCTCATCGGAAATATGGT          |
| Cbf7     | AY785904_s4         | TTCTAGTCACACTTACTAGGC          | AY785904_as926      | CACTAGCAAAGACATTCTAGG           |
| Cbf10    | AY951950_s1522      | ATACCTCCACACTCCACAGATG         | Cbf4B_R            | GCAGATCTCGCTACAAGCTCC           |
| Cbf13    | Cbf5_F              | CAGAGGACAAGAGCTAGGGGAATC       | AY951951_as1691     | GCTAGCTCATCCTACCTGGAAT         |
| Cbf14    | AY951948_s656       | TAAACTCTGCTTATATATCACC         | AY951948_as1312     | ATATTTGGTGGAACAGACAGGA         |
| Cbf15    | EF028765_s90        | ACCCAGCAGACTCTCGACTG           | EF028765_as875      | TTGGTCCATCTAGCTAGCTAAG         |
| Cbf18    | AY951946_s400       | CGTATAAATACGCACACGCACTA        | AY951946_as1445     | ACATGGTGAGGGATTTTATT            |
| Dhn1     | ScDhn1_F            | CATCAACACACGACACTTGAGAAGAAGAAG | AY951946_as1445     | ACATGGTGAGGGATTTTATT            |
| Vrn-A1b  | VRN1-A_F            | GCAAGGAGAATACGCTGCAAAGGAGG    | AY747606_as1083     | TTTACCGAGGGTAGTTTCCA            |
| Vrn-A1c  | AY747600_s9072      | TTCTCTGAGTCTCTCTCTGGTTGAAGG    | AY747600_as10169    | CAGTAGACTGCTGTGCTGCAAAGGAGG    |
| Vrn-A1d  | AY747600_s10718     | TTGCTCAGGCTGCTGCTGCTGCTGCTGCTG | AY747600_as11318    | ACGCTTAGCTGCTGCTGCTGCTGCTGCTG |
| Vrn-A1e  | AY747600_s11297     | CTCTCTGAGTCTCTCTGCTGCTGCTGCTG | AY747600_as12066    | GCTGACGCTCTGCTGCTGCTGCTGCTGCTG |
| Vrn-B1b  | AY747603_s18        | AGCGCTAGGGACCTGAGAAGGAGGAGG   | AY747606_as820      | CAAACGGAATACGCTGCAAAGGAGG    |
| Vrn-B1c  | AY747603_s3097      | TCTGTGCAGATATCTCTCTCTCGCTGCTG | AY747606_as9488     | GATGCTAGCTGCTGCTGCAAAGGAGG   |
| Vrn-B1d  | AY747603_s4783      | CTTTCTTCTTACGCTGCTGCTGCTGCTG | AY747606_as5249     | TTTTACTGCTGAGAGCATATGACTAAA   |
| Vrn-B1e  | AY747603_s5134      | AAAAAGGAAATACGCTGCAAAGGAGG   | AY747603_as6211     | ATTACGTTGAAATACGCTGCAAAGGAGG   |
| Vrn-D1b  | AY747606_s6         | TTTTCTTCTTCTCTCTCTCTCTCTCTCTCT | AY747606_as6280     | CAAACGGAATACGCTGCAAAGGAGG   |
| Vrn-D1c  | AY747606_s8129      | GTGATTCAGGCTGCTGCTGCTGCTGCTG | AY747606_as9488     | GATGCTAGCTGCTGCTGCAAAGGAGG   |
| Vrn-D1d  | AY747606_s10719     | GACTCTAGCGCAATTTGCTGCTGCTGCT | AY747606_as11608    | TTAGAAACAAATACGCTGCAAAGGAGG |
| Vrn-D1e  | AY747606_s11586     | CAAGGGGCTCAGATATCTCTCTCGCTGCTG | AY747606_as12291    | TTAGAAACAAATACGCTGCAAAGGAGG |
| Vrn-B2a  | AY485977_s306       | AAAAAGGAAATACGCTGCAAAGGAGG   | AY485977_as1282     | AAAAACGGAATACGCTGCAAAGGAGG   |
| Vrn-B2b  | AY485977_s1542      | AAAAAGGAAATACGCTGCAAAGGAGG   | AY485977_as1985     | AAAAACGGAATACGCTGCAAAGGAGG   |
| Vrn-B2c  | AY485977_s1542      | AAAAAGGAAATACGCTGCAAAGGAGG   | AY485977_as1985     | AAAAACGGAATACGCTGCAAAGGAGG   |
| Vrn-B2d  | AY485977_s1439      | AAAAAGGAAATACGCTGCAAAGGAGG   | AY485977_as1985     | AAAAACGGAATACGCTGCAAAGGAGG   |
| Vrn-3a/b | DQ890162_s1430      | AAAAAGGAAATACGCTGCAAAGGAGG   | DQ890162_as1915     | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Vrn-3a/b | DQ890162_s1552      | AAAAAGGAAATACGCTGCAAAGGAGG   | DQ890162_as1915     | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Vrn-3b   | DQ890162_s2159      | AAAAAGGAAATACGCTGCAAAGGAGG   | DQ890162_as3153     | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Vrn-3c/b | DQ890162_s2396      | AAAAAGGAAATACGCTGCAAAGGAGG   | DQ890162_as3153     | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Vrn-3c/b | DQ890162_s1552      | AAAAAGGAAATACGCTGCAAAGGAGG   | DQ890162_as3153     | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Cab b    | contig22616_s209    | TTTTCTGAGGCTGCTGCTGCTGCTGCTG | contig22616_as938    | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Cab c    | contig22616_s209    | TTTTCTGAGGCTGCTGCTGCTGCTGCTG | contig22616_as828    | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Dem      | CD837801_s29        | AAAAAGGAAATACGCTGCAAAGGAGG   | CD837801_as520      | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Tac7 b   | contig4120743_s26   | AAAAAGGAAATACGCTGCAAAGGAGG   | contig4120743_as455  | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Tac7 c   | contig4120743_s271  | AAAAAGGAAATACGCTGCAAAGGAGG   | contig4120743_as455  | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Tac7 d   | BJ246882_s196       | AAAAAGGAAATACGCTGCAAAGGAGG   | contig4120743_as455  | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Ppd-B1c  | DQ885757_s11028     | AAAAAGGAAATACGCTGCAAAGGAGG   | DQ885757_as12453    | AAAAAGGAAATACGCTGCAAAGGAGG   |
| Ppd-B1d  | DQ885757_s11883     | AAAAAGGAAATACGCTGCAAAGGAGG   | DQ885757_as12453    | AAAAAGGAAATACGCTGCAAAGGAGG   |

(Continued)
representing 18 frost tolerance genes, were selected for sequencing and all 34 obtained sequences were compared to retrieved gene models via MegaBlast. In case of Tacr7, Kocsy et al. [55] identified BQ659345 of Hordeum vulgare as reference, but the fragments of 24 sequenced genotypes of Tacr7 do not exceed an identity of 84% to the published wheat reference sequence L28093 for Tacr7 [31]. The best BLAST hit of the 24 sequences is still the initial barley sequence BQ659345 with an identity of 92.3%, but the second best barley BLAST hit of 91.8% identity is to X97916, the barley low temperature gene 14.1 abbreviated as blt14 [1]. The rest of 33 BLAST results show very high identities from 88.8% to 100% to initial gene sequences (Table 5).

In 12 genes out of a set of 18 sequenced candidate genes represented by 16 unique PCR amplicons, differences between the 24 genotypes were determined, revealing a high level of polymorphism of 66.67%. The number of polymorphic sites ranged from 1 to 37, the haplotypes (h) from two to three, the haplotype diversity (Hd) from 0.08 to 0.61 and the nucleotide diversity (π) from 0.00008 to 0.00757 (Table 6).

The results of the workflow for locus specific primer development presented in this paper are very promising. The main workflow step is the identification of sub-genome sequences and the design of primers on sub-genome sequence differences. This is the essential step of this development of PCR markers for frost tolerance genes in wheat.

### Table 4. (Continued)

| Fragment | Forward primer name | Forward primer sequence (5’- 3’) | Reverse primer name | Reverse primer sequence (5’- 3’) |
|----------|---------------------|---------------------------------|---------------------|---------------------------------|
| Ppd-B1e  | DQ885757_s_12390    | CTTTGTGAATCCTAATACTCC          | DQ885757_as_13162   | AACAGAGAACAAGAATCCGG           |
| Ppd-B1f  | DQ885757_s_13184    | GGGCTATATCCAGATACTGTA          | DQ885757_as_13562   | ATCGACTCCGACTTCTACTG           |
|          | DQ885757_s_13148    | CTGTTGTCTCCTGTTCTCGTTG         | DQ885757_as_13625   | ACCGTACACAGTTACAGAC            |
|          | DQ885757_s_13184    | GGGCTATATCCAGATACTGTA          | DQ885757_as_13625   | ACCGTACACAGTTACAGAC            |
|          | DQ885757_s_13148    | CTGTTGTCTCCTGTTCTCGTTG         | DQ885757_as_13625   | ATCGACTCCGACTTCTACTG           |
| Ppd-D1 Prom | DQ885766_s3601     | CTTGCTCAACTCCAATCTAGTG         | DQ885766_as6489     | TCCTCCCTGTTCITTTTTTACTC       |
|          | DQ885766_s4578     | TCAGCTCACTCAGAGATCTGAT         | DQ885766_as5712     | AGTACGCTCGGAGTGAATAT           |
|          | DQ885766_s4450     | CATACTCCCTCCTGTTCTTGGT        | DQ885766_as5712     | AGTACGCTGCCGAGTGAATAT          |
| Ppd-D1a  | DQ885766_s5689     | ATTATCTACACGCCAGCTACT         | DQ885766_as6299     | TACTGAACATTGTTAGGCCAAAG       |
| Ppd-D1a2 | DQ885766_s5766     | CAACATGTTTCTCTTGGAGC          | DQ885766_as6535     | GAACAGACTCAACACACTCAGA         |
| Ppd-D1b  | DQ885766_s6298     | TATCAGGTTCATTTTCTCTAGTG       | DQ885766_as7002     | ATGGCAAAATATGCTACTAGGC        |
|          | DQ885766_s6277     | CTTGCCCTAAATGTTCTGCA          | DQ885766_as7002     | ATGGCAAAATATGCTACTAGGC        |
|          | DQ885766_s6277     | CTTGCCCTAAATGTTCTGCA          | DQ885766_as7002     | ATGGCAAAATATGCTACTAGGC        |
| Ppd-D1c  | DQ885766_s7244     | TGACGACTACGTCATCTGAC          | DQ885766_as8033     | GATTCGGAAGAGACATGATT          |
| Ppd-D1d  | DQ885766_s6939     | GGAACGCTAGATAAACGCTAGG         | DQ885766_as8033     | GATTCGGAAGAGACATGATT          |
| Ppd-D1e  | DQ885766_s8011     | AATACGATGTCCTCGGGACT          | Ppd-D1exon8_R1*     | gtltaaatagtagtagtactagg       |
| Ppd-D1f  | DQ885766_s8771     | CTGCTCTGCTTTCTGTTCAT          | DQ885766_as9720     | ACCTCCCTACGGAAGACTC           |

Primer names with † are developed in course of this work but published from Keilwagen et al. [53]. Primer names with * as already published were used in combination with primers with † and without labels.

1[86] 2[73] 3[87] 4[81] 5[37] 6[53]

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Table 5. BLASTn results of sequenced PCR fragments versus NCBI nucleotide collection (nr/nt) and NCBI candidate gene reference EST.

| Gene   | PCR fragment | Database | Subject Seq-id (ID of the database hit) | Percentage of identical matches | Expectation value (E-value) | Bit score | Subject discription |
|--------|--------------|----------|----------------------------------------|-------------------------------|---------------------------|-----------|---------------------|
| Cab    | Cab b        | EST      | gi|21273269|gb| BQ465487.1 | 93,71                 | 0          | 643                 | HU03M14 HU Hordeum vulgare subsp. vulgare cDNA clone HU03M14 5-PRIME, mRNA sequence. |
| Cbf1   | Cbf1         | nucleotide | gi|17226800|gb| AF376136.1 | 100                      | 0             | 1279               | Triticum aestivum putative CRT/DRE-binding factor (CBF1) mRNA, complete cds |
| Cbf5   | Cbf5         | nucleotide | gi|404415276|gb| JN987194.1 | 100                      | 0             | 1519               | Triticum aestivum AP2 domain CBF protein (CBFI) mRNA, CBFI-5.4 allele, complete cds |
| Cbf10  | Cbf10        | nucleotide | gi|404415286|gb| JN987199.1 | 99,53                    | 0             | 1548               | Triticum aestivum AP2 domain CBF protein (CBFIic) mRNA, CBFIic-10.1 allele, complete cds |
| Cbf13  | Cbf13        | nucleotide | gi|404415320|gb| JN987217.1 | 100                      | 0             | 1493               | Triticum aestivum AP2 domain CBF protein (CBFIic) pseudogene, CBFIic-13.1c allele, complete sequence |
| Cbf14  | Cbf14        | nucleotide | gi|158999375|gb| EU076382.1 | 99,01                    | 0             | 902                | Triticum monococcum CBF14 gene, complete cds |
| Cbf15  | Cbf15        | nucleotide | gi|404415321|gb| JN987218.1 | 100                      | 0             | 1325               | Triticum aestivum AP2 domain CBF protein (CBFIId) gene, CBFIId-15.2b allele, complete cds |
| Cbf18  | Cbf18        | nucleotide | gi|63098599|gb| AY951946.1 | 94,92                    | 0             | 1559               | Triticum monococcum CRT/DRE binding factor 18 (CBF18) gene, complete cds |
| Dem    | Dem b        | nucleotide | gi|241986478|dbj| AK333739.1 | 97,37                    | 0             | 713                | Triticum aestivum cDNA, clone: WT008_O03, cultivar: Chinese Spring |
| EST    | EST           |          | gi|12030509|emb| AL504294.1 | 90,37                    | 7,00E-128    | 466                | AL504294 Hordeum vulgare Barke roots Hordeum vulgare subsp. vulgare cDNA clone HW04N07 5’, mRNA sequence. |
| Dhn1   | Dhn1         | nucleotide | gi|58994280|gb| AY895879.1 | 88,79                    | 3,00E-176    | 625                | Hordeum vulgare subsp. spontaneum voucher NPGS PI 559556 dehydrin 1 (Dhn1) gene, partial cds |
| Ppd-B1 | Ppd-B1c      | nucleotide | gi|456359289|dbj| AB745620.1 | 100                      | 0             | 1679               | Triticum turgidum subsp. pyramidalae Ppd-B1 gene for pseudo-response regulator, complete cds, strain: KU-9982 |
|        | Ppd-B1d      | nucleotide | gi|456359289|dbj| AB745620.1 | 99,82                    | 0             | 1009               | Triticum turgidum subsp. pyramidalae Ppd-B1 gene for pseudo-response regulator, complete cds, strain: KU-9982 |
|        | Ppd-B1e      | nucleotide | gi|456359289|dbj| AB745620.1 | 99,3                     | 0             | 1297               | Triticum turgidum subsp. pyramidalae Ppd-B1 gene for pseudo-response regulator, complete cds, strain: KU-9982 |
|        | Ppd-B1f      | nucleotide | gi|383215299|gb| JF946486.1 | 99,74                    | 0             | 693                | Triticum aestivum transposon TREP 3040_Harbinger, complete sequence; pseudo-response regulator (Ppd-B1) gene, Ppd-B1a allele, complete cds; and retrotransposon Gypsy TREP 3457_Danae, complete sequence |
| Ppd-D1 | Ppd-D1a      | nucleotide | gi|395759126|dbj| AB646977.1 | 99,91                    | 0             | 1965               | Triticum aestivum PRR gene for pseudo-response regulator, complete cds, allele: Ppd-D1b.2 |
|        | Ppd-D1e      | nucleotide | gi|395759124|dbj| AB646976.1 | 100                      | 0             | 1731               | Triticum aestivum PRR gene for pseudo-response regulator, complete cds, allele: Ppd-D1a.1 |
|        | Ppd1 3 UTR   | nucleotide | gi|118638641|gb| DQ885766.1 | 100                      | 0             | 1629               | Triticum aestivum cultivar Chinese Spring chromosome 2D pseudo-response regulator (PRR) gene, complete cds |
| Tacr7  | Tacr7 b      | nucleotide | gi|1418967|emb| X97916.1 | 91,75                    | 0             | 778                | H.vulgare bl14.1 gene |

(Continued)
workflow and is crucial for the success of this approach. The primer amplification test for single bands and the fragment mapping via NT-lines are a simple way to verify locus specificity. The sequencing of selected locus specific amplicons and the BLAST analysis of these fragment sequences versus initial data bases is the last step of safe-guarding the correct amplification. The results of this BLAST search showed no critical differences to the initially selected sequences.

### Discussion

New bioinformatic platforms and data bases containing recent genomics data are a powerful resource for the development of tools for molecular plant breeding.
Gene specific primer development and chromosomal assignment of specific PCR fragments by using NT- and deletion lines

The rapid progress in sequencing of plant genomes leads to the accumulation of whole genome sequence data, allowing the fast development of locus/genome specific markers in complex plant genomes (e.g. wheat) with a high success rate. Up to now, high homology of the hexaploid wheat genome hampered the success in gene specific primer development. Gene structure is important for marker development, because wheat introns have more sequence differences between the homologous chromosomes than exons [56, 57]. Therefore, gene structure reconstruction and comparison of homologue sequences by using three genomes facilitate an improved development of molecular markers as well as re-sequencing of targeted genes/loci.

Specificity of developed primers

Specificity of primers is the non-recurring binding in the target genome. This is reflected in a single PCR and a correct or syntenically localised amplicon. Fig 4 shows an example of the Cbf1 amplicon localisation via NT- and deletion-lines.

The inspection of primer functionality and single PCR product generation is a standard for the development of primers and therefore is the first necessary step of the presented approach.

| Gene   | No. accessons | No. of bp | No. of polymorphic sites | Percentage polymorphism | h  | Hd   | k   | π   | k (i) | π (i) |
|--------|---------------|-----------|--------------------------|-------------------------|----|------|-----|-----|-------|-------|
| CBF5   | 23            | 824       | 2                        | 0.24                    | 3  | 0.49 | 0.95 | 0.00115 | 0.44 | 0.00054 |
| CBF10  | 23            | 776       | 2                        | 0.26                    | 2  | 0.47 | 0.95 | 0.0012 | n/a  | n/a    |
| CBF13  | 23            | 773       | 4                        | 0.52                    | 2  | 0.47 | 1.42 | 0.00193 | 0.95 | 0.00123 |
| CBF14  | 22            | 1184      | 6                        | 0.51                    | 3  | 0.48 | 1.91 | 0.00163 | 0.46 | 0.00038 |
| CBF15A | 24            | 755       | 7                        | 0.93                    | 2  | 0.49 | 2.94 | 0.00395 | 0.49 | 0.00065 |
| CBF18  | 22            | 951       | 37                       | 3.89                    | 2  | 0.09 | 3.09 | 0.00328 | 0.27 | 0.00029 |
| Vrn-A1 | 24            | 2954      | 9                        | 0.30                    | 2  | 0.08 | 0.42 | 0.0014  | 0.33 | 0.00011 |
| Vrn-D1 | 23            | 3093      | 1                        | 0.03                    | 2  | 0.24 | 0.24 | 0.00008 | n/a  | n/a    |
| Vrn3   | 24            | 1566      | 1                        | 0.06                    | 2  | 0.52 | 0.52 | 0.00033 | n/a  | n/a    |
| Ppd-B1 | 24            | 3971      | 2                        | 0.05                    | 3  | 0.36 | 0.37 | 0.00009 | n/a  | n/a    |
| Ppd-D1 | 24            | 2642      | 1                        | 0.04                    | 2  | 0.23 | n/a  | n/a    | 0.23 | 0.00009 |

h haplotypes
Hd haplotype diversity
k average number of nucleotide differences
π nucleotide diversity
(i) InDel

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Gene specific primer development and chromosomal assignment of specific PCR fragments by using NT- and deletion lines

Fig 4. Example of fragment localisation from Cbf1 via NT- and deletion-lines. (A) The missing PCR fragment on NT-line N5D-T5B indicated the location on wheat chromosome 5D. (B) The missing PCR fragment on Csdel SDL-1 indicated the location on wheat long arm of chromosome 5D between the deletion segments 1 and 5.
Via the first inspection step we have eliminated 27.73% of studied primer pair combinations. Most of these showed no PCR amplification probably due to non-binding of target sequences. The second important step of checking the amplicon specificity is the mapping of the PCR products via NT-lines to get information about the correct amplification on the correct target chromosome template and sub-genome. By using NT-mapping of PCR amplicons we have eliminated 18.49% of primer pair combinations. One part of the eliminated PCR products shows a chromosome localisation that differs from what has been reported in the literature. In this case, we assume a non-specific binding in the wheat genome. That can occur if primers are derived from related organisms and not from wheat itself. For seven of eight discarded candidate genes, sequences of related organisms (Triticum monococcum and Hordeum vulgare) were used for primer development. The other part of eliminated primer pair combinations showed a PCR product on all NT-lines which may be due to the fact that both primers (forward and reverse) bind at least to two sub-genomes.

By using the draft wheat chromosome arm sorted sequences [10–12] and simple comparative methods we were able to develop gene specific primers in hexaploid wheat with a high success rate of 58.60%. Also a very high rate of 54.62% for specific fragment amplification confirmed the usefulness of wheat genomic sequence. To our knowledge such high rate is not yet described in literature for specific primer/marker development in polyploid plants. An overview of published success rates revealed a variation in microsatellite amplification in wheat between 22.88 and 45.0% [58–61]. In cotton this rate was 23.3% [62]. Contrary, Wang et al. [63] describe the development of effectively derived primers for sequence tagged sites (STS) with 24.56% and for STS primer combinations of only 3.7% in wheat. Chen et al. [64] achieved a rate of 27.5% for STS marker development in wheat. In Brassica oleracea (which is a paleohexaploid plant) a success rate of 29.1% is described in allele specific PCR primer development [65]. The highest success rate reported in literature is for potato [56]. In this study a rate of 51.79% developed intron targeting (IT) markers was achieved. With the ongoing genome sequencing projects and subsequent development of genome-wide physical maps in wheat and related plants an increase in the success of specific primer development may be expected.

Sequencing of frost tolerance candidate genes and BLAST based verification

In this study 18 out of 19 (94.74%) frost tolerance genes were sequenced using the same primers used for PCR amplification. For gene Cbf7, for which initial sequencing failed, a set of newly designed sequencing primers improved the sequencing, therefore optimisation for single band products could be recommended as a part of the verification procedure. Concerning the gene Tac7, Kocsy et al. [55] claimed BQ659345 of Hordeum vulgare is identical to the Tac7 gene in wheat. However, the analysis of the generated sequences presented in this paper showed an identity of 84% to the reference sequence L28093 for Tac7 of wheat and 92% to BQ659345. In contrast, our sequences reveal an identity of 92% to X97916 of Hordeum vulgare which is annotated as the barley low temperature gene 14.1 (Blt14.1). BLT14.1 shows a considerable homology to WLT10, as described by Ohno et al. [66]. Matching BQ659345 against X97916 results in an identity of 99%. Furthermore Tac7, Blt14.1 and Wlt10 are located on chromosome 2 of barley and wheat, respectively [55, 66, 67]. We also mapped PCR fragments derived from Tac7 on chromosome 2B. Further BLAST results indicate that the sequence of our Tac7 is with 92% the initial sequence BQ659345. Furthermore, it was shown recently that the newest sequence of Tac7 [55] is very similar to the sequences of the genes Blt14.1 and Wlt10, in contrast to the L28093 sequence (described also as Tac7 [31]). The nucleotide identity of 99% between Blt14.1 (X97916) and the initial reference sequence (BQ659345), which is
published as *Tacr7* [55], backed this hypothesis. All other PCR fragment sequences have shown a very good sequence identity to the original gene of interest (97.5%).

The sequencing of single bands and correct chromosome assigned PCR amplicons followed by BLAST based verification is the last check-up step in the workflow presented in this study. The results of the BLAST based verification demonstrate that the selection of PCR single products and the assignment to the correct chromosomes of the PCR amplicons is an efficient instrument of locus specific primer selection. The combination of sequencing and BLAST based verification using the presented approach leads to very robust results with an error rate tending to zero.

The identified SNPs at 11 polymorphic candidate genes can be used for developing SNP based marker. Also the InDels in eight candidate genes are suited for marker development based on size polymorphisms. Based on these PCR amplicons can be employed for genetic mapping of corresponding candidate genes in biparental mapping populations, thereby allowing for the first time their genetic localization.

This paper describes an efficient approach for the development of locus specific primers in wheat. With the aid of this locus specific primers are necessary for locus specific sequencing and detection of genes specific polymorphisms (SNPs and InDels) between genotypes of interest. The detected polymorphisms can follow up the use for genetic mapping, but also for gene editing via sequence information for transcription activator-like effector nucleases (TALENs) [68–70] or clustered regularly interspaced short palindromic repeats (CRISPR/Cas) systems [71]. Therefore our approach of development of locus specific primers is a base for many downstream applications i.e. detection of new polymorphisms, development of new markers, genetic mapping and gene editing in wheat.

**Conclusion**

It is still difficult to develop molecular markers in *Triticum aestivum* due to the very complex genome. In this study we presented an efficient approach for gene and genome specific primer development by using sequence data of wheat. Altogether, we have developed specific primers for 19 out of 27 selected frost candidate genes. For 27 candidate genes 119 primer pairs were generated of which 65 were specific. Out of candidate gene specific primer fragments 36 fragments were selected, corresponding to 19 genes, for validation via sequencing. Finally, 35 amplicons could be successfully sequenced and only one specific sequence showed a low identity of approximately 83% to the original reference sequence.

By using the presented approach for gene specific primer/PCR development, it is possible to sequence and analyse interesting candidate genes in wheat by using gene information of related sequenced plant species. The wheat genome sequences currently available, in combination with the wheat physical map, are well suited for the development of specific primers. The approach for primer design, developed within this study turned out to be very efficient by using available wheat genomic resources and it is expected to perform even better once new versions of wheat genomic sequences will be available.

**Supporting Information**

S1 Fig. Chromosome localisation and cycler programs of the PCR fragments Cbf7, Ppd-B1f and Ppd-D1b via NT-lines.

(TIF)

S1 Table. Plant material of NT- and Deletion-lines.

(XLSX)
S2 Table. Candidate gene specific primers, primer specificity, PCR fragments, used polymerases, cycler programs and primers for fragment re-sequencing. Primer names with † are developed in course of this work but published from Keilwagen et al. [53]. Primer names with * as already published were used in combination with primers with † and without labels. 1[86]; 2[73]; 3[87]; 4[81]; 5[37]; 6 [53]. †Primers published in Keilwagen et al. [53]. *Already published primers (XLSX)

S3 Table. Primer specificity and mismatches to compared the three sub-genomes of functional and correct localised PCR fragments via in silico alignments. Primers assigned † are developed in course of this study and published in Keilwagen et al. [54]. Already published primers with * assigned were used in combination with primers in green and black. The column differences describe the numbers of SNPs/InDels between primers at sequence level of A, B and D genomes. The columns position of InDels and SNPs in 5’ to 3’ direction describes the position of the differences between primers at sub-genomes (InDels and SNP) from primer 5’ to 3’ end direction. 1[86]; 2[73]; 3[87]; 4[81]; 5[37]; 6 [53]. †Primers published in Keilwagen et al. [53]. *Already published primers. (XLSX)

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Author Contributions

Conceived and designed the experiments: DP FO. Performed the experiments: SB. Analyzed the data: SB DP. Contributed reagents/materials/analysis tools: DP MK FO. Wrote the paper: SB DP FO. Read and approved the final manuscript: SB DP MK FO.

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