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Mapping of STS Markers Developed from Drought Tolerance Candidate Genes and Preliminary Analysis of their Association with Yield-related Traits in Common Wheat *(Triticum aestivum)*

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Drought is a severe abiotic stress that affects wheat production worldwide. In order to identify candidate genes for tolerance to water stress in wheat, sequences of 11 genes that have function of drought tolerance in other plant species were used to identify the wheat ortholog genes via homology searching in the wheat EST database. A total of 11 primer pairs were identified and amplified PCR products in wheat. Of them, 10 STS markers were mapped on 11 chromosomes in a set of nulli-tetrasomic lines of 'Chinese Spring' wheat; six were mapped on chromosomes 1A, 1B, 4B, 7A, 2B and 5D, respectively, in a spring wheat mapping population (POP1). The marker *XTaABH1* mapped on 7A in POP1 was the only one mapped but characterized in a winter wheat mapping population (POP2) for grain yield, kernel weight and diameter, and height in four-field trials applied different water stress or irrigation. The marker *XTaABH1* was significantly associated with grain yield under rainfed condition, with kernel weight under terminal stress and non-irrigation conditions, with kernel diameter and height under non-irrigated condition. The STS primers, map information and marker-trait association produced in the currently study would be of interest to researchers working on drought tolerance.

Keywords: wheat, SSR, drought, candidate gene

Introduction

Wheat is an important food crop that can be cultivated in diverse environments in the world. Water stress severely affects grain yield and productivity of wheat, especially in the scenario of recently predicted climate change (Porter and Gawith 1999). Drought toler-

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ance is a complex trait that can be estimated as the ratio of yield under water stress vs. non-stressed conditions (Reynolds et al. 1999). However, measuring yield is expensive and time consuming. Using forward genetics approach, some QTLs associated with yield and related traits were identified, but the use of these QTL in breeding programs were still limited due to the small effect and/or inconsistent presence of the QTL across different populations and environments (Cuthbert et al. 2008; McIntyre et al. 2010). Using reverse genetics such as candidate gene (CG) approach offers opportunities to identify functional QTL or ortholog genes related to the drought tolerance, and the CG approach can develop closely linked markers for marker-assisted selection (MAS) and/or in cloning of the candidate genes (Pflieger et al. 2001). Pflieger et al. (2001) proposed three chronological steps in the CG approach: first, identify previously sequenced genes of known function that could correspond to major loci of agronomic traits; second, localize the CGs on a genetic linkage map to look for genetic linkage between the CG markers and the loci being characterized; and the last, confirm the actual involvement of the CG in the trait variation.

Transcription factors are known to play important roles in the drought responsive pathways. In *Arabidopsis, CBF3/DREB1A* is a transcription factor well characterized in the ABA-independent pathway (Gilmour et al. 1998; Shinwari et al. 1998); while *ABF3* has been characterized for its function in the ABA-dependent pathway (Kang et al. 2002). Molecular characterizations of the transcription factors illustrated that they interact with the promoter regions of many stress responsive genes (Baker et al. 1994; Giraudat et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994; Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kang et al. 2002; Maruyama et al. 2004). Sakuma et al. (2006) expressed another transcription factor of *DREB2A* (dehydration responsive element binding protein 2A) under the constitutive promoter in *Arabidopsis,* and the transgenic plants clearly showed resistance to the drought stress treatment compared to the corresponding wild type plants. Over-expression of *MYB* gene caused ABA sensitive phenotype in *Arabidopsis* and improved drought tolerance (Abe et al. 2003). Enhanced proline biosynthesis might reduce the damage of oxidation reactions under the osmotic stress condition and could represent an alternative direction in stress studies (Hong et al. 2000). Though the original genetic studies were conducted mostly in *Arabidopsis,* testing functions of genes that related to drought tolerance has been expanded into economic crops. For instance, Nelson et al. (2007) over-expressed the nuclear transcription factor of NF-YB2 in maize under the constitutive promoter of CaMV-35S, and found the transgenic plants showed significantly improved drought tolerance based on parameters including chlorophyll content, stomatal conductance, leaf temperature, reduced wilting and maintenance of photosynthesis. In another experiment, two transcription factors, CBF3 and ABF3, were expressed in rice plants under an ubiquitin constitutive promoter, and the transgenic plants showed resistance to drought and salt stress (Oh et al. 2005).

The objectives of current study were: i) to identify wheat ortholog gene sequences of the drought tolerance genes described above; ii) to develop primers that can amplify these genes in wheat; and iii) to map the STS markers and identify marker-traits associations using a set of nulli-tetrasomic lines of 'Chinese Spring' wheat and two wheat mapping populations.

Materials and Methods

Plant materials

Two mapping populations and one set of nulli-tetrasomic lines (NTLs) of Chinese Spring Wheat (Sears 1954 and 1966) were used in this study. One spring wheat mapping population (POP1) comprised of 120 doubled haploid lines derived from a cross between a synthetic hexaploid wheat line TA4152-60 and a hard red spring wheat line ND495 (Chu et al. 2008). Another winter wheat mapping population (POP2) comprised of 159 recombination lines (RILs) derived from a cross between a hard white winter wheat cultivar 'RioBlanco' (PI 531244) and a hard red winter breeding line 'IDO444' (PI 578278) (Chen et al. 2012). Rio Blanco is a semi-dwarf *(Rht-B1b, Rht-D1a)* hard white winter wheat cultivar released by Agripro Biosciences, Inc. Mission, KS. IDO444 is a tall (*Rht-B1a* and *Rht-D1a*) hard red winter wheat germplasm developed by University of Idaho, Aberdeen, ID. IDO444 was released as germplasm based on its disease resistance and improved grain yield under rain-fed production conditions in the Pacific Northwest (Windes et al. 1995).

Identification of wheat orthologs and corresponding STS markers

The drought tolerance candidate genes were selected based on the literature in which gene function in drought stress was reported in other plant species. DNA and protein sequences of the candidate genes were used to search the wheat expressed sequence tag (EST) database. The top hits were identified and sequences were retrieved from the database for the wheat ortholog genes. Detailed information for each candidate gene and corresponding wheat tentative consensus (TCs) were summarized in Table S1*. The TCs from the wheat EST database (http://compbio.dfci.harvard.edu/tgi/plant.html) were also used to do BLAST search in the database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to make sure the selected TCs were aligned with sequences from other species with the same annotation.

To develop sequence tagged site (STS) markers for the ortholog genes, the retrieved EST sequences were used to design PCR primers through the online computer program Primer 3 (http://frodo.wi.mit.edu/primer3/). The major parameters for designing primers were about 50% GC content and 60ºC Tm value. Primer pairs were selected from 2–4 regions of each EST sequence, and a total of 40 primer pairs were obtained from the selected 11 wheat ortholog gene sequences. The sizes of the PCR products were estimated based on the 100 bp DNA ladder (M) on the gel (Bio-Rad Lab, Hercules, CA, USA).

DNA extraction and PCR amplifications

Total genomic DNA of each line was extracted from seeds using the method described by Pallotta et al. (2003) except that the SDS was replaced by CTAB buffer. Seeds were grounded using 2000-115 Geno Grinder (SPEX SamplePrep, Metuchen, NJ). The DNA concentration was measured by ND-1000 Spectrophotometer (NanoDrop Technologies,

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Inc. Wilmington, DE) and adjusted to 40 ng/µl for PCR reaction. PCR reactions were performed as described by Röder et al. (1998) except that 36 cycles were used. Electrophoresis was carried out on 6% polyacrylamide gels (0.4 mm thick) in 0.5 × TBE (90 mM Tris–borate, 2 mM EDTA) at 300 volts for 3 h using C.B.S.gel electrophoresis system (C.B.S. SCIENTIFIC CO., Del Mar, CA) and the PCR products were visualized with GEL Doc^{TM} XRsystem (Bio-Rad Lab, Hercules, CA).

Determination of chromosomal locations of STS markers using NTLs

To assign STS markers onto specific wheat chromosomes, a set of NTLs were analyzed. When a given STS marker was absent in a particular nulli-tetrasomic line but present in all other 20 lines, this STS marker was then assigned to the chromosome that is absent in the NTL (Fig. S1). Because of the homoeologous nature of common wheat, a given PCR product can be located on more than one homoeologous chromosomes (Fig. S2).

Mapping of STS markers derived from wheat ortholog genes and validating the PCR amplicons

Genome-wide linkage maps of the two mapping populations were previously constructed (Chu et al. 2008; Chen et al. 2012). The previous mapped marker data were used to assign the chromosomal location of the newly developed STS markers. The computer program MAPMAKER (V2.0) for Macintosh (Lander et al. 1987) was used to perform linkage analysis with a minimum LOD threshold of 3.0 and the Kosambi mapping function (Kosambi 1944). The ''two-point/group'' command with a minimum LOD = 3.0 and a maximum $\theta = 0.40$ was first used to identify linkage groups that the STS markers were belonged to, the TRY command was then used to determine the most plausible position of the STS markers, and the RIPPLE (LOD > 3.0) command was finally used to verify the order of markers within linkage groups. To assure the PCR products are from the target genes, all the PCR amplicons were excised, purified and sequenced. The sequence data was used in the BLAST search to confirm if the PCR products were right gene products.

Characterization of mapped candidate genes for yield and other agronomic traits

Because POP1 has un-adapted agronomic performance such as non-free threshability, this population was not used in phenotype analysis of the mapped candidate genes. However, the entire POP2 was evaluated in two locations one in Aberdeen in 2010 (hereafter as AB10) and another in Rockland in 2011 (hereafter as RK11), respectively. The water stress in the AB10 was applied after flowering was completed. The AB10 trial received rainfall water of 82 and 101 mm in early (from planting to February) and late (from March to harvesting) growing seasons, respectively, irrigation water of 208 mm (estimated) before flowering. The RK11 trial received only rainfall water of 205 and 225 mm in early and late growing seasons, respectively.

A subset of 64 RILs of the POP2 was planted in two blocks side by side in Aberdeen, ID in 2011, one block with irrigation water of 512 mm from April to July (hereafter as AB11W) and another with limited irrigation water of 73 mm from April to anthesis (here-

after as AB11D). Both AB11Wand AB11D trials received 230 mm of rainfall from planting to harvesting based on the record of weather station. Most of the rainfall water was from snow in winter and early spring. Therefore, the order of water applied and presumably the water stress experienced by the trials were AB11D (303 mm), AB10 (391 mm), RK11 (416 mm), and AB11W (742 mm). Historically, Rockland and Aberdeen received annual precipitation about 180 and 150 mm, respectively. The 2011 growing season was wetter and cooler than usual.

In all four trials (AB10, RK11, AB11D and AB11W), parents and the RILs were planted in a randomized complete block design with two replications in RK11 and three replications in AB11W and AB11D. Seeding rate was 1.98 M kernels per ha in RK11 and 2.47M kernels per ha in both AB11W and AB11D, respectively. Plot size was 3 m long and 1.5 m wide in all three trials with four-row plots planted in RK11 and seven-row plots in AB11W and AB11D.

Grain yield (GY, kg/ha) of each plot was estimated at harvesting by using HM-400 HarvestData system (HarvestMaster, Inc., Logan, UT). Grain volume weight (GVW, kg/hL) was measured for each plot after harvesting. Kernel weight (KW, mg) was determined using the single kernel characterization system (SKCS 4100, Perten Instruments North America, Inc., Springfield, IL) by taking a sample of 100 sound kernels from each plot. Heading date (HD, d) was recorded as days from Jan. 1 to the date when 50% of spikes had completely emerged above the flag leaf in a plot. Plant height (HT, cm) was determined after maturity as the height of the stem from the ground to the tip of the spike excluding awns.

Allelic effect of candidate genes on the traits evaluated was analyzed by T-test using PROC TTEST in SAS (SAS Institute, Cary, NC, version 9.3). The p value < 0.05 was used if the differences of the trait values between two alleles were significant.

Results

Amplification of candidate gene in wheat genome for developing new STS markers

A total of 40 primer pairs were synthesized and tested in the four parental lines of the two mapping populations. Primers that produced clear PCR products either monomorphic or polymorphic among the parental lines were selected, and the PCR products were then sequenced and confirmed as the correct ortholog gene sequence. Under this criterion, a total of 11 primer pairs were selected and used in mapping analysis (Table S2).

The observed PCR products from the selected primer pairs were shown in Table 1. Fragment size of the observed fragments were varied from the expected may be due to the possible variations of the sequences in three sub-genomes (Table 1). Of the 11 selected primer pairs, six (TaDREB3b, TaNFYb, TaERA1, TaABH, TaABF and TaMYB) detected the polymorphisms between the two parental lines in POP1. Of those, TaABH also amplified polymorphic products between parental lines of POP2 (data not presented). The remaining five primer pairs were monomorphic among the four parental lines of the two populations.

Table 1. STS markers for wheat ortholog genes mapped either by linkage mapping (LM) or nulli-tetrasomic analysis (NT). PCR product sizes were estimated based on the comparison to the 100 bp DNA ladder (Bio-Rad)

Ortholog gene	STS markers	Chromosome location	Mapped fragments (bp) by	
			LM	NT
CDPK2	*XTaCDPK2*	2A, 2B		900
CBF3/DREB1a	*XTaCBF3*	5A, 5D	180, 230	290
DREB3b	*XTaDREB3b*	1A	210	210
NF-YB1	*XTaNFYB1a*	1A, 1D		900
	XTaNFYB1b	1B	250	
TsVP1	*XTaVP1a*	7A		300, 400
	XTaVP1b	7B		260, 350, 400
	XTaVP1d	7D		300, 350
LEA	*XTaLEA*	1A		280
NCED3	*XTaNCED3*	5A		280
ERA1	*XTaERA1*	4B	400	400
ABH1	*XTaABH1*	7A	340	340
ABF3	*XTaABF3*	2B	350	350
MYB2	*XTaMYB2*	5D	280	

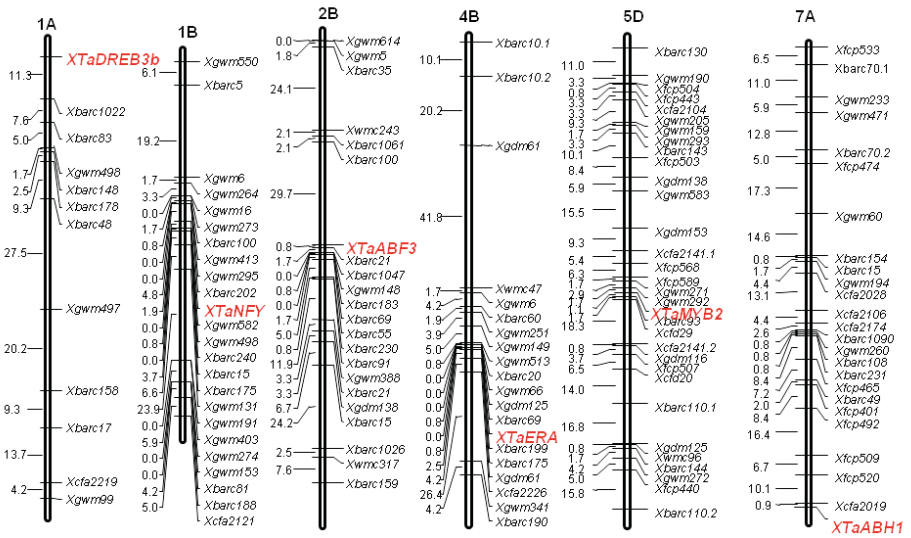

Figure 1. STS markers of the six candidate genes were integrated to the previous genetic linkage maps constructed in POP1 of TA4152-60 × ND495 DH mapping population. The positions of marker loci are shown to the right of the linkage groups and centi Morgan distances between loci are shown along the left

Mapping of CG sequences in NTLs

The 11 primer pairs were first used and all amplified PCR products in the 21 NTLs. Six of the 11 primer pairs (TaDREB3b, TaLEA, TaNCED1, TaERA1, TaABH and TaABF) amplified fragments that were located on chromosomes 1A, 1A, 5A, 4B, 7A and 2B, respectively (Table 1, Fig. S1), while other four of them (TaCDPK2, TaDREB1a, TaNF-YB1 and TaTsVP1) produced fragments located on more than one homoeologous chromosomes. For example, TaCDPK produced fragments that were mapped on chromosomes 2A and 2B; TaDREB3b amplified products on chromosomes 5A and 5D; TaNFYb generated amplicons on 1A and 1D; and TaVP1b amplified fragments on chromosomes 7A, 7B and 7D (Fig. S2). However, the products from the primer pair of TaMYB could not be located on any chromosomes using NTLs because the primer pair amplified products in all NTLs.

Mapping of CG sequences in two mapping populations

Of the six STS markers showing polymorphism between two parental lines of POP1, the *XTaDREB3b* was co-dominant (Fig. S3A), while the other five (Fig. S3B) of *XTaABF3, XTaNFYb, XTaERA, XTaABH1* and *XTaMYB* were dominant. Those six STS markers were mapped to six chromosomes on 1A, 1B, 2B, 4B, 5D and 7A in POP1 (Table S3, Fig. 1), respectively. The STS marker of *XTaABH1* was also mapped on same chromosomal regions on 7A in POP2 (Fig. S4).

Allelic effects of the candidate gene ABH1 on grain yield and other agronomic traits

Based on the genotypes of marker *XTaABH1,* all RILs in POP2 were classified into two groups based on the allele from either RioBlanco or IDO444. Mean differences of the two groups for traits GY, HT, KW and KD were compared and presented in Table S3. In Aberdeen location in 2011, HT, KW and KD of I allele (allele from IDO444) group were significantly ($P < 0.05$) higher than that of R allele (allele from RioBlanco) group under drought condition (AB11D) but not under irrigation condition (AB11W). GY and HD of the two groups were not significant under either drought or irrigated conditions. In Rockland location in 2011, GY of the I group was significantly ($P < 0.05$) higher than that of R group, while the traits of HT, KW, KD and HD were not significant different in two groups. In AB10 trial, KW of the I group was significantly ($P < 0.05$) higher than that of R group, confirmed that in AB11D trial.

Discussion

This study developed 11 primer pairs that can amplify drought candidate genes in wheat. The amplified STS markers were mapped and saturated the current genetic maps in two mapping populations POP1 and POP2. This provides a starting point to develop functional maps of these candidate genes when marker-trait associations are identified.

Some of the selected candidate genes have been confirmed their function in transgenic experiments (Gilmour et al. 1998; Shinwari et al. 1998; Kang et al. 2002; Abe et al. 2003; Oh et al. 2005). However, their chromosomal positions in wheat were unclear. Results from this report linked the functional candidate genes and physical locations. The results also confirmed the practical usefulness of steps proposed by Pflieger et al. (2001) for the CG approach in development of stress related functional markers in wheat.

Grain yield and KW evaluated under water limited environments have been commonly used to evaluate drought tolerance in crops (McIntyre et al. 2010). This study evaluated yield, KW, KD, HT and HD in RILs of POP2 in four diverse trials. The order of water applied and presumably the water stress experienced by the four trials are AB11D, AB10, RK11 and AB11W. One STS marker *XTaABH1* was associated with KW in two (AB10 and AB11D) of the three stressed trials, with yield in one stressed trial (RK11), with KD, HT and HD in one stressed trial (AB11D). Interestingly the marker of *Xgwm332* in the same chromosomal region of wheat detected a QTL of grain yield and tiller number per plant (Kumar et al. 2007) although the growth environment was not very clear. Because of the limited DNA polymorphism detected in POP2 we were not able to distinguish the remaining marker-trait association in the current study. Therefore, we could not determine if the STS marker *XTaABH1* is associated with drought tolerance in terms of yield and KW based on the limited and inconsistent marker-trait associations obtained. However, more STS markers mapped in POP1 suggest that the remaining 10 STS markers can be mapped when additional mapping populations are evaluated using primer sequences and map information produced in the current study.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1.* Wheat ortholog genes identified by homology search of the original drought tolerance candidate genes in other plants

Electronic Supplementary *Table S2.* Sequences of the selected primer pairs that are designed based on wheat ortholog genes and were used for genetic linkage analysis

Electronic Supplementary *Table S3.* Allelic differences of the candidate gene marker *XTaABH1* contributing to grain yield (GY, kg/ha), plant height (HT, cm), kernel weight (KW, mg), and kernel diameter (KD, mm) evaluated in irrigated and water stressed environments in RioBlanco (R) × IDO444 (I) RIL population

Electronic Supplementary *Figure S1*. STS marker *XTaLEA* was located on chromosome 1A using a set of nulli-tetrasomic lines of Chinese Spring (CS) labelled as indicated. The arrow on the right side indicated the dominant fragments sized by comparing them to the 100 bp DNA ladder (M) from Bio-Rad

Electronic Supplementary *Figure S2*. STS marker *XTaVP1* was located on chromosomes 7A, 7B and 7D, respectively. The nulli-tetrasomic lines and Chinese Spring wheat were labelled as indicated. The arrows on the right side indicated the fragment size by comparing them to the 100 bp DNA ladder (M) from Bio-Rad

Electronic Supplementary *Figure S3*. Co-dominant STS marker *XTaCBF3b* (A) and dominant marker *XTaABF3* (B) detected polymorphic fragments between parental lines of ND495 (P1) and TA4152-60 (P2) and among the 23 DH lines in POP1. The arrows on the right side indicated the fragments sized by comparing them to the 100 bp DNA ladder from Bio-Rad

Electronic Supplementary *Figure S4.* STS marker *XTaABH1* was mapped on wheat 7A chromosome and associated with GY, HT, KW and KD in POP2 of RioBlanco × IDO444 population evaluated under drought and water limited condition