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ORIGINAL RESEARCH

Identification of Candidate Genes Responsible for Stem Pith Production Using Expression Analysis in Solid-Stemmed Wheat

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Abstract

The wheat stem sawfly (WSS) is an economically important pest of wheat in the Northern Great Plains. The primary means of WSS control is resistance associated with the single quantitative trait locus (QTL) *Qss.msub.3BL*, which controls most stem solidness variation. The goal of this study was to identify stem solidness candidate genes via RNA-seq. This study made use of 28 single nucleotide polymorphism (SNP) makers derived from expressed sequence tags (ESTs) linked to *Qss.msub.3BL* contained within a 5.13 cM region. Allele specific expression of EST markers was examined in stem tissue for solid and hollow-stemmed pairs of two spring wheat near isogenic lines (NILs) differing for the *Qss.msub-3BL* QTL. Of the 28 ESTs, 13 were located within annotated genes and 10 had detectable stem expression. Annotated genes corresponding to four of the ESTs were differentially expressed between solid and hollow-stemmed NILs and represent possible stem solidness gene candidates. Further examination of the 5.13 cM region containing the 28 EST markers identified 260 annotated genes. Twenty of the 260 *Qss.msub.3BL* linked genes were up-regulated in hollow NIL stems, while only seven genes were up-regulated in solid NIL stems. An *O*-methyltransferase within the region of interest was identified as a candidate based on differential expression between solid and hollow-stemmed NILs and putative function. Further study of these candidate genes may lead to the identification of the gene(s) controlling stem solidness and an increased ability to select for wheat stem solidness and manage WSS.

Core Ideas

- Wheat stem solidness is associated with gene expression changes
- A wheat stem solidness candidate gene is involved in lignin biosynthesis
- Candidate genes can be identified via linked markers relatively easily.

Wheat stem sawfly, *Cephus cinctus* Norton (Hymenoptera: Cephidae), is an economically important pest in spring and winter wheat (*Triticum aestivum* L.) in the U.S. Northern Great Plains and Southern Canadian provinces. Adult female WSS lay eggs in wheat stems, and resulting larvae tunnel down the stem where they girdle and then notch the base of the stem. Economic yield losses occur from lodged stems and from damage caused by larval tunneling (Austin et al., 1977; Beres et al., 2007). The development of solid-stem wheat cultivars has been the best method for minimizing WSS economic losses. Solid stems are comprised of pith, largely consisting of parenchyma cells (Hansen et al., 2013). The solid-stem phenotype minimizes yield losses by reducing WSS egg deposition, increasing larval mortality (Holmes and Peterson, 1962), and inhibiting larval mining in the stem (Platt and Farstad, 1946). Solid stems can also reduce fecundity of the insect (Morrill et al., 1992).

Efforts to develop WSS resistance began by screening wheat germplasm from around the world for

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Abbreviations: cDNA, complementary DNA; EST, expressed sequence tag; NIL, near isogenic line; QTL, quantitative trait locus; RIL, recombinant inbred line; SNP, single nucleotide polymorphism; WSS, wheat stem sawfly.

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WSS-resistant genotypes (Kemp, 1934). These investigations identified S-615, a landrace from Portugal, as having consistently solid stems with reduced stem cutting as a source to be used in crosses to develop resistant varieties (Platt and Farstad, 1946). Current solid-stem varieties derive from this single germplasm source.

Early studies on the inheritance of the solid-stem trait pointed to a single major gene along with several minor genes differentiating hollow from solid-stem wheat (McNeal, 1956; McKenzie, 1965). Environmental factors influence the expression of the solid-stemmed trait. Cool temperatures, adequate moisture, and low light intensity tend to make stems less solid (Platt and Farstad, 1949; Holmes 1984). Plant breeders have historically treated stem solidness as a quantitative trait when considering the interaction between genetic control and varying environmental conditions (Lebsock and Koch, 1968). More recently, Cook et al. (2004) identified a microsatellite marker, *Xgwm340*, that explained 76% of the variation in stem solidness in a recombinant inbred line (RIL) population from the cross of a hollow and solid-stemmed parent. The QTL linked to the microsatellite marker was designated *Qss.msub-3BL*. In durum wheat, stem solidness is qualitative and controlled by a QTL, within which the gene is termed *Sst1*, which is linked to microsatellite marker *Xgwm340* on 3BL (Houshmand et al., 2007). *Xgwm340* was also previously reported by Cook et al. (2004) to be linked to stem solidness. However, the *Sst1* gene has not been sequenced or cloned, and whether solidness is dominant or recessive is not known.

Association mapping studies with 96 (Kalous et al., 2011) and 244 (Varella et al., 2015) spring wheat cultivars and breeding lines have shown that the *Qss.msub-3BL* QTL controls most variation for stem solidness in wheat. Sherman et al. (2010) made use of a RIL population from a cross between 'Reeder' (PI 613586), a hollow-stemmed WSS-susceptible parent, and 'Conan' (PI 607549), a semisolid-stemmed parent that showed greater resistance to WSS infestation and cutting than expected based on its stem solidness score. They found the stem solidness trait mapped to the *Qss.msub-3BL* QTL and identified additional QTL on chromosomes 2D and 4A associated with WSS cutting and infestation. Talbert et al. (2014) evaluated a RIL population derived from a cross between Conan and 'Scholar' (PI 607557), a parent deriving its stem solidness from the S-615 source. When genotyped with the *Xgwm340* marker, lines with the Conan allele had lower stem solidness but less WSS stem cutting and infestation than lines with the Scholar allele. This provided evidence for allelic variation at the *Qss.msub-3BL* locus (Varella et al., 2016).

Varella et al. (2015) associated the solid-stem trait with 28 SNP markers derived from mapped EST markers in a region spanning 5.13 cM on 3BL. Each marker contained a single, centrally located SNP within the marker sequence. They found these markers to be associated with stem solidness as early in development as Feekes stage 3. In fact, the *Qss.msub-3BL* conditions both early stem solidness expressed during jointing and booting, and late stem solidness expressed after anthesis (Varella et al., 2016). The presence of early stem solidness is likely key to WSS resistance (Varella et al., 2016) because it coincides with oviposition by WSS females. This results in reduced oviposition and increased neonate larval mortality (Varella et al., 2016, 2017).

A recent study examined haplotype diversity surrounding the *Qss.msub-3BL* QTL. Cook et al. (2017) used a subset of the 28 ESTs derived by Varella et al. (2015) to conduct haplotype analysis on a diverse array of tetraploid and hexaploid wheat lines from around the world expressing varying degrees of stem solidness. They found that most solid-stemmed hexaploid wheat lines had the same haplotype as the S-615 solid-stem source, although haplotype variation among solid-stem types did exist.

Although the *Qss.msub-3BL* QTL has been well documented, the genes responsible for the accumulation of pith in wheat stems are not known. The goal of this study was to identify candidate genes responsible for stem pith production using expression analysis of NILs differing for the *Qss-msub-3BL* QTL. Sherman et al. (2015) developed two NIL pairs for the *Qss.msub-3BL* QTL using the solid-stem spring wheat 'Choteau' (PI 633974) as the donor parent. The Choteau parent has the same haplotype as the S-615 source. These genetic materials provide a means to investigate the impact the *Qss.msub-3BL* QTL has on the underlying genetic mechanism that differentiates solid from hollow stems. This study made use of the 28 *Qss-msub-3BL* linked EST based SNP markers reported by Varella et al. (2015). Although relative position of the EST markers has been reported for a consensus map (Cavanagh et al., 2013; Cyrille et al., 2013; Wang et al., 2014), physical location on the 3B chromosome had not been reported. We determined physical locations for many of the EST markers as well as discovering if they were present within annotated gene regions. RNA-seq provided a useful approach to further characterize these markers. This technique allowed us to determine sequence specific expression of the ESTs in stem tissue. The 28 EST markers utilized in this study had previously been shown to be expressed in wheat tissue, however no stem libraries were used to develop these markers (Wang et al., 2014). Therefore, we expected to find only a subset of the markers to be expressed in stem tissue. The work presented here moves toward identification of the gene controlling most genetic variation for the stem solidness phenotype. Identification of the major gene controlling stem solidness would be useful to wheat breeders and geneticists by allowing for direct selection of individual allelic variants for enhanced WSS control.

Materials and Methods

Plant Material and Growth Conditions

In this study, two spring wheat NIL pairs were used for all experiments. The NILs were developed and tested in Sherman et al. (2015) by introgression of the solid-stem allele at *Qss.msub-3BL* from cultivar Choteau (Lanning et al., 2004)

Table 1. Expressed sequence tag (EST) markers significantly associated with stem solidness in a spring wheat association mapping panel from North America.†

EST no.	Marker‡	3B map location§	SNP A/B allele¶	Physical location#	Gene#
1	Kukri_c37097_1849	–	T/C	3B:761188585–761188685	TRAES3BF082800060CFD_g
2	Tdurum_contig59566_1534	143.29	T/C	3B:761747666–761747766	TRAES3BF026000070CFD_g
3	Kukri_c55981_194	143.29	T/G	3B:761748085–761748185	TRAES3BF026000070CFD_g
4	Tdurum_contig59566_2309	143.29	A/C	3B:761748441–761748541	TRAES3BF026000070CFD_g
5	IWA5892	143.29	T/C	3B:761749881–761750081	TRAES3BF026000070CFD_g
6	Tdurum_contig59566_4435	143.29	A/C	3B:761750567–761750667	TRAES3BF026000070CFD_g
7	Jagger_c3292_145	139.61	A/G	3B:762562052–762562069	
8	Ku_c16949_1230	–	T/C	3B:762897952–762897970	
9	BS00063160_51	143.29	A/G	3B:763226415–763226515	
10	BobWhite_c45118_495	140.5	A/G	3B:767461265–767461365	TRAES3BF074300080CFD_g
11	IWA2148	140.5	A/G	3B:767692971–767693171	TRAES3BF075300020CFD_g††
12	BS00079029_51	140.5	T/C	3B:768588119–768588219	TRAES3BF045500130CFD_g
13	GENE-1925_118	142.14	A/G	3B:769191940–769192010	TRAES3BF045500380CFD_g
14	RAC875_c37741_218	142.14	T/C	3B:770066313–770066413	TRAES3BF136700070CFD_g
15	BS00091257_51	140.5	A/C	3B:770213634–770213734	
16	IWA1756	143.44	A/G	3B:770216778–770216880	TRAES3BF266400030CFD_g
17	BS00073411_51	144.74	T/C	3B:773021313–773021413	
18	BS00074345_51	144.74	T/G	3B:773027051–773027151	TRAES3BF065400130CFD_g††
19	BS00065603_51	144.74	T/C	3B:773049018–773049079	
20	BS00071183_51	144.74	T/C	–	
21	GENE-1910_358	144.74	A/G		
22	RAC875_c8662_762	–	A/G	–	
23	Excalibur_c27675_1815	–	A/G	–	
24	RAC875_c3925_1043	–	T/C	–	
25	GENE-3471_298	–	T/C	–	
26	BobWhite_c8092_726	–	T/C	–	
27	IACX1477	–	T/C	–	
28	wsnp_BF473138A_Ta_2_3	–	C/G	–	

† EST associated with stem solidness phenotype in Varella et al. (2015), Supplemental Table S1.

‡ ESTs numbered 2–6 reside within the same gene.

§ Map positions are based on the consensus map SynOp_HD2013_3B with marker positions provided by Cavanagh et al. (2013), Cyrille et al. (2013), and Wang et al. (2014). A dash in this column indicates that EST was associated with the stem solidness trait on chromosome 3B, but the position is not known.

¶ Single nucleotide polymorphism (SNP) A allele/B allele designated according to The Triticeae Toolbox (T3, http://triticeaetoolbox.org/wheat, last accessed November 2015).

Physical location and gene annotations were determined using EnsemblPlant Blast (http://archive.plants.ensembl.org/Triticum_aestivum, last accessed July 2016). Location reflects top Blast hit within the region 3B:762,562,052–774,399,861.

†† EST located inside of an intron within the gene based on gene annotation.

into hollow-stem spring wheat lines ID0644 and WA008008 (released as Whit, PI 653841; Kidwell et al., 2009). Introgression occurred via five rounds of backcrossing, and resulted in NIL pairs within two genetic backgrounds. Theoretically, this resulted in hollow and solid-stemmed lines that were 98.4% genetically identical within each background, but segregated for the solid-stem locus. Near isogenic lines were grown in 2015 at the Arthur H. Post Research Farm near Bozeman, MT, in 3 m single-row plots at a seeding rate of 10 g per plot in a randomized block design with three blocks. Each block had solid/hollow NIL pairs randomized within it. Soil fertility consisted of 84 kg ha^{-1} residual N supplemented by applying 84 kg ha^{-1} during cultivation immediately prior to planting. Growing season precipitation was 16.75 cm supplemented with 3.75 cm of irrigation applied 1 wk prior to and after flowering.

Markers

Expressed sequence tag markers previously associated with stem solidness were characterized for gene expression in the two NIL backgrounds. The EST markers associated with early stem solidness (Table 1) are those identified in Varella et al. (2015; Supplemental Table S1). Single nucleotide polymorphism A and B allelic variation for EST markers was obtained from the Triticeae Toolbox (T3, triticeaetoolbox.org, accessed November 2015). Allelic differences across NIL backgrounds were not observed since the solid-stem locus was introgressed from the same source (Choteau). Therefore, allelic variation data was combined across NIL backgrounds. Allele presence of ESTs was determined for a subset of spring wheat lines included in the association mapping panel utilized in Varella et al. (2015) by querying the Triticeae

Toolbox. Wheat cultivars with a solid-stemmed phenotype included Rescue (Platt and Farstad, 1949), Fortuna (CI 13596, Lebsock et al., 1967), Choteau, and Duclair (PI 660981, Lanning et al., 2011). Hollow-stemmed cultivars included Hi-Line (PI 549275, Lanning et al., 1992), Newana (CI 17430, McNeal and Berg, 1977), McNeal (PI 574642, Lanning et al., 1994), Alturas (PI 620631, Souza et al., 2004), and Centennial (PI 537303, Souza et al., 1991). Physical location and gene annotations corresponding to EST marker positions were determined using the BLAST function of the EnsemblPlants database (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast, last accessed July, 2016). For annotated genes in which markers were located, gene names were queried within the UniProt Knowledgebase (http://uniprot.org/uniprot, last accessed July 2016) to find putative protein information for further characterization of markers that were found to be expressed in stem tissue.

Transcript Analysis

To examine expression of the SNP markers in stem tissue, stem tissue was collected at Feekes stage 7, just prior to appearance of flag leaves, from each of the 12 field plots. At this stage, there were three nodes with the bottom internode designated as one. The internode tissue between Nodes 2 and 3, along with the corresponding nodes, was harvested and combined from three plants within a single row for a single biological sample. Samples were collected from three replicated plots for a total of three biological replicates. Tissue was immediately frozen in liquid N_2 after removing the leaf sheath. Tissue was ground into a powder using liquid N_2 and a mortar and pestle, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. To ensure high quality RNA-seq data, RNA integrity was measured using a Bioanalyzer (Agilent Technologies, Santa Clara, CA) and complementary DNA (cDNA) libraries were created from 1 µg total RNA using TruSeq RNA Prep v2 kits (Illumina Inc., San Diego, CA). Resulting cDNA libraries were sequenced as single-end 100 bp reads using an Illumina High Scan-SQ platform.

RNA-seq expression analysis was performed as in Schlosser et al. (2014) and was performed using ArrayStar v12.3.1.4 (DNASTAR, Madison, WI). To examine allelic differences in expression, ESTs were modified to replace the variable base with a single base, resulting in two sequences representing the A and B alleles. This made it possible to examine allele specific expression in NIL pairs with presence or absence of the 5.13 cM region on 3B containing the stem solidness locus. Marker and coding sequences of interest were imported to ArrayStar, and expression values were obtained using default settings with match settings set to 50 bp. Additionally, to try to ensure that expression values were specific to 3B, match settings were set to 100%. Data was normalized using reads per kb of exon model per million mapped reads (RPKM) normalization (Mortazavi et al., 2008). Resultant expression data were additionally normalized between samples using the *ACT-1* gene (AY663392). Two-tailed, equal variance *t*-tests were used to determine significance between hollow- and solid-stem lines.

Results

Determining Physical Location of ESTs Associated with Stem Solidness on Chromosome 3B

Varella et al. (2015) demonstrated that 28 EST markers are associated with the stem solidness trait located within a 5.13 cM region on chromosome 3B. In this study, 19 of these ESTs were located within this region of 3B (Table 1). Five of the 28 ESTs resided within a single annotated gene (ESTs 2–6). Based on annotation status of the 3B chromosome (July 2016), 13 ESTs including ESTs 2 through 6 were located within annotated genes.

Allelic Expression of Stem Solidness Linked EST Markers

The 28 EST markers were associated with early stem solidness in Varella et al. (2015). Therefore, cDNA libraries were created from field harvested stem tissue at Feekes stage 7. Each cDNA library yielded approximately 23 Mb of sequence with 94.5% of reads achieving a quality score above Q30, exceeding Illumina's specification of 85%. Transcript expression analysis revealed that 10 of the 28 EST markers were expressed in stem tissue early in plant growth (Table 2). Several ESTs exhibited different patterns of expression between solid and hollow NIL genotypes. For example, for ESTs 5 and 15, the A allele is solely expressed in hollow stems, whereas the B allele is only expressed in solid stems, indicating both A and B alleles are specific to 3B (Table 2). Allele specific expression was also observed for ESTs 6, 11, 16, and 26. For instance, the B allele was only expressed in the solid-stem phenotype for ESTs 6 and 26, whereas the hollow-stem phenotype had expression of either A or B, indicating that the B allele was specific to 3B in solid stems. This observation was reversed for ESTs 11 and 16, in which the hollow stems only had expression of the B allele, whereas the solid stems varied for expression of either A or B. ESTs 24 and 28 did not show differences in expression in solid vs. hollow-stem phenotypes, whereas ESTs 12 and 23 were only detected in the hollow-stem phenotype. Additional allelic variation for the 28 EST markers is presented in Supplemental Table S1 for a subset of hollow- and solid-stemmed wheat lines from the association mapping panel used in Varella et al. (2015).

Whole Gene Expression

To further characterize genes corresponding to ESTs, expression of entire coding sequences was analyzed. Coding sequences corresponding to ESTs were obtained via the BLAST function of the EnsemblPlants database and represented the top hit within the 5.13 cM region of interest on chromosome 3B. Three genes were found to be significantly up-regulated in the solid NILs with solid to hollow expression ratios of 1.83, 3.34, and 8.61 for the

Table 2. Expressed sequence tag (EST) specific expression of A and B alleles in hollow and solid stem isolines with characterization of coding sequences corresponding to ESTs.

EST no.†	Allele‡	Solid expression avg. ± SE§	Hollow expression avg. ± SE§	Solid/hollow expression ratio¶	Solid allele#	Hollow allele#	Position in cDNA††	Amino acid change††
2	A	ND	ND	–			4504	Lys/Glu
	B	ND	ND	–				
3	A	ND	ND	–			4085	Ala/Glu
	B	ND	ND	–				
4	A	ND	ND	–			3729	Pro/Pro
	B	ND	ND	–				
5	A	ND	650 ± 231	>1**	B	A	2239	Ala/Thr
	B	385 ± 122	ND	<1*				
6	A	ND	314 ± 130	<1*	B	A/B	1603	Ser/Ala
	B	149 ± 104	242 ± 177	0.62				
11	A	1279 ± 480	ND	<1*	A/B	B	N/A	N/A
	B	787 ± 170	1300 ± 344	0.61				
12	A	ND	ND	–	–	B	954	Ala/Ala
	B	ND	155 ± 99	–				
15	A	ND	151 ± 96	–	B	A	N/A	N/A
	B	378 ± 267	ND	–				
16	A	2001 ± 266	ND	>1***	A/B	B	1657	Ala/Thr
	B	2761 ± 447	4438 ± 323	0.62*				
23‡‡	A	ND	151 ± 96	–	–	A	N/A	N/A
	B	ND	ND	–				
24‡‡	A	456 ± 175	211 ± 131	2.16	A/B	A/B	N/A	N/A
	B	192 ± 22	350 ± 95	0.55				
26‡‡	A	ND	407 ± 302	>1	B	A/B	N/A	N/A
	B	2068 ± 574	652 ± 186	0.06				
28‡‡	A	ND	ND	–	B	B	N/A	N/A
	B	194 ± 95	137 ± 89	1.42				
Housekeeping§§	*ACT-1*	282,897 ± 0	282,897 ± 0	1.0				
	Tubb4	173,709 ± 14,327	189,080 ± 17,349	0.92				
	GA3PD	502,400 ± 87,473	407,478 ± 42,946	1.23				

* Significant at $P < 0.05$ from two-tailed, equal variance *t*-tests in comparison of solid vs. hollow gene expression.

** Significant at $P < 0.01$ from two-tailed, equal variance *t*-tests in comparison of solid vs. hollow gene expression.

*** Significant at $P < 0.001$ from two-tailed, equal variance *t*-tests in comparison of solid vs. hollow gene expression.

† ESTs numbered 2 to 6 reside within the same gene.

‡ A and B alleles correspond to single nucleotide polymorphism presented in Table 1.

§ Values are RPKM (reads per kb of exon model per million mapped reads) normalized hits with additional normalization to *ACT-1*. Parameters were set at 50 bp and 100% match. Sample size is $n = 6$ for all genotypes. ND represents not detected at the level of sequencing employed.

¶ Solid/hollow expression ratio is the ratio of solid to hollow RPKM normalized hits.

Solid and hollow allele presences are derived from expression values in Columns 3 and 4.

†† Complementary DNA (cDNA) and amino acid information corresponds to "Gene" column in Table 1. N/A indicates no Ensembl annotations were available.

‡‡ EST does not blast to 3B region of interest, gene sequence unknown. The A allele contains the first listed amino acid.

§§ Housekeeping gene NCBI accessions are FN429985, TAU76745, and AY663392 for *ACT-1*, *Tubb4*, and *GA3PD*, respectively.

coding sequences associated with EST markers 11, 14, and 18, respectively (Table 3). The putative function for the coding sequence corresponding to EST 11 is as a RNA binding protein, while that of EST 14 is for protein transport (Table 4). The coding sequence corresponding to EST 18 is annotated as a pseudogene. A single gene, corresponding to EST 12, was significantly down-regulated in solid stems (Table 3); the putative function of this gene is a protein serine/threonine kinase (Table 4).

Additional *Qss.msub.3BL* Linked Genes

Further analysis of the 5.13 cM region of 3BL from which the EST markers were obtained revealed that there was a total of 260 annotated genes within this region (Table 5). These annotated genes together with location and putative protein function are summarized in Supplemental Table S2. Each coding sequence was downloaded and expression analysis revealed three categories of expressed genes (Supplemental Table S3). The first category consists

Table 3. Expressed sequence tag (EST) associated gene expression from complementary DNA (cDNA) annotated sequences.

EST no.†	Source sequence	Solid expression avg. ± SE‡	Hollow expression avg. ± SE‡	Solid/hollow expression ratio§
1	TRAES3BF082800060CFD	1309 ± 171	1232 ± 214	1.06
2–6	TRAES3BF026000070CFD	1368 ± 250	2040 ± 446	0.67
10	TRAES3BF074300080CFD	1390 ± 250	1484 ± 250	0.94
11	TRAES3BF075300020CFD	586 ± 72.9	320 ± 0.3 ± 60.6	1.83*
12	TRAES3BF045500130CFD	6.52 ± 1.67	83.6 ± 30.3	0.08*
13	TRAES3BF045500380CFD	ND	ND	–
14	TRAES3BF136700070CFD	922 ± 105	275 ± 0.7 ± 57.2	3.34***
16	TRAES3BF266400030CFD	2263 ± 252	2029 ± 245	1.12
18	TRAES3BF065400130CFD¶	124 ± 30.7	31.8 ± 7.0	3.90*
		342 ± 65.2	39.8 ± 25.1	8.61***

* Significant at $P < 0.05$ from two-tailed, equal variance *t*-tests in comparison of solid vs. hollow gene expression.

*** Significant at $P < 0.001$ from two-tailed, equal variance *t*-tests in comparison of solid vs. hollow gene expression.

† ESTs numbered 2 to 6 reside within the same gene.

‡ Values are RPKM (reads per kb of exon model per million mapped reads) normalized hits with additional normalization to *ACT-1*. Parameters were set at 50 bp and 100% match. Values represent the combination of phenotypes to give $n = 6$ for both genotypes. ND represents "not detected" at the level of sequencing employed.

§ Solid/hollow expression ratio is the ratio of solid to hollow RPKM normalized hits.

¶ Two splice variants were analyzed.

Table 4. Putative protein functional information for Expressed sequence tags (ESTs) corresponding to annotated genes.†

EST no.‡	Marker	UniProt ID	Molecular Function
1	Kukri_c37097_1849	W5CZ22	RNA binding
2	Tdurum_contig59566_1534	A0A080YUA1	ATP binding, protein kinase activity
3	Kukri_c55981_194		
4	Tdurum_contig59566_2309		
5	IWA5892		
6	Tdurum_contig59566_4435		
10	BobWhite_c45118_495	A0A080YUI8	zinc ion binding
11	IWA2148	W5D6E2	RNA binding
12	BS00079029_51	W5CU49	ATP binding, protein serine/threonine kinase activity
13	GENE-1925_118	A0A080YUK0	ATP binding, calcium ion binding, polysaccharide binding, protein kinase activity
14	RAC875_c37741_218	A0A090YUN9	intracellular protein transport, vesicle-mediated transport
16	IWA1756	A0A080YUP6	N/A
18	BS00074345_51§		pseudogene

† The UniProt Knowledgebase (http://uniprot.org/uniprot, verified 5 May 2017) was queried using the gene name corresponding to the "Source Sequence" column in Table 3 to determine putative protein functions of genes associated with stem solidness.

‡ ESTs numbered 2 to 6 reside within the same gene.

§ Pseudogene annotation provided by EnsemblPlants (http://plants.ensembl.org, verified 5 May 2017).

Table 5. Number of features in 5.13 cM marker region.

3B:761,183,585–774,399,861 Feature	13,316,276 bp No.
Unique gene regions	260
Pseudogenes	57
tRNA (transfer)	53
miRNA (microRNA)	10
ncRNA (noncoding)	6
snRNA (small nucleolar)	1
Nontranslated coding sequences	2

of genes only expressed in hollow stems. Eleven genes were found to be in this category. In the other two categories, genes are expressed in both hollow and solid stems, but up-regulated either in hollow or solid stems. Nine genes were found to be up-regulated in hollow stems, whereas seven genes were found to be up-regulated in solid stems.

Discussion

Development of solid-stem wheat cultivars has been the most widely used strategy to minimize economic loss from the WSS (Platt and Farstad, 1946; Platt et al., 1941). Unfortunately, the genes responsible for stem solidness are not known. To that end, we determined physical location of 19 of 28 EST markers shown to be associated with early stem solidness (Varella et al., 2015) within a 5.13 cM region of chromosome 3B (Table 1). Although ESTs 20 and 21 were also placed within this region based on consensus map SynOp_HD2013_3B (Table 1), we were not able to determine a physical location for these ESTs. In addition, we were also unable to determine physical location of seven other EST markers. The inability to place these markers within this region likely reflects the current annotation status of the 3B chromosome. It is unlikely that these markers fall outside of this region, as they were associated with *Qss.msub-3BL* (Varella et al., 2015). However, *Qss.msub-3BL* does not control all variation for the stem solidness locus. A secondary QTL on chromosome 3DL also explains a smaller amount variation for stem solidness (Lanning et al., 2006). Furthermore, plant height traits have been shown to impact stem solidness in wheat lines possessing *Qss.msub-3BL* (Lanning et al., 2012), and environmental factors have been shown to influence levels of stem solidness (Hayat et a., 1995).

Allele presence was compiled for a subset of solid and hollow-stemmed spring wheat varieties utilized by Varella et al. (2015). Allele presence was assigned by querying the T3 database and represent results from association mapping studies. Most of the EST markers segregated such that hollow- and solid-stemmed lines possessed alternate alleles (Supplemental Table 1).

An interesting occurrence is observed by examining ESTs 2 through 6. These EST-derived SNP markers

are all located within the same gene (Table 1), though expression values are only observed for ESTs 5 and 6. This is likely a sequencing artifact since ESTs 5 and 6 are located closer to the 3′ end of the sequence. Sequencing hits are skewed toward the 3′ end of a transcript, which indicates that deeper coverage in this RNA-seq experiment would have resulted in expression values for ESTs 2, 3, and 4. This may also apply to ESTs 1, 10, 13, 14, and 18 as well, since these markers did not have expression values, but the corresponding gene sequences did (Tables 2, 3). Therefore, ESTs without expression in stem tissue should not necessarily be ruled out as candidate genes for the solid-stem trait. Another observation regarding EST 5 and 6 is that the A and B alleles clearly segregate based on stem phenotype for EST 5. For EST 6, both the A and B alleles were detected in the hollow-stemmed lines, representing a limitation of this approach. Since wheat is a hexaploid, it is possible that expression values may represent hits from the A, B, and D genomes, as is the case for EST 6 in hollow stems. In this example, the solid-stem allele is 3B specific, whereas the hollow-stem allele is not.

Of the ESTs corresponding to annotated gene sequences, there were two markers in which the SNPs coded for silent mutations (Table 2), indicating that these SNPs would not be responsible for the stem solidness trait. An exception to this would be if there was an additional unknown SNP present within the coding sequence. Another interesting observation was that the B allele alone was present in both the hollow and solid phenotypes for EST 28. This contrasts with the allele reported for Choteau, the source of the solid-stem allele (Supplemental Table S1). Interestingly though, there were other instances in Choteau in which alternate alleles were detected by independent association mapping studies (e.g., EST 5 and 11, Supplemental Table S1). This would agree with Cook et al. (2017) that variation may exist within haplotype groups.

Coding sequences corresponding to ESTs 11, 12, 14, and 18 were found to be differentially expressed between hollow- and solid-stemmed NILs and may be considered candidate genes controlling stem solidness. Coding sequences for EST 11 and 14 were found to be up-regulated in solid stems (Table 3) and were characterized with putative functions involved in RNA binding and protein transport (Table 4). The sequence corresponding to EST 18 was also up-regulated; however, this sequence was annotated as a pseudogene, and although it is expressed it is not expected to have a molecular function. The coding sequence corresponding to EST 12 was characterized as a putative protein kinase and was down-regulated in solid stems (Tables 4, 3). Although these genes represent candidates responsible for stem solidness, they are not strong candidates based on putative protein function.

Since we did not discover a strong candidate gene responsible for the stem solidness trait, we performed expression analysis for all 260 annotated genes within the 5.13 cM region of interest on 3BL. We found a total of 20 genes to be up-regulated in hollow stems with an additional seven genes up-regulated in solid stems (Supplemental Table S3). Putative protein functions reveal that a gene that was up-regulated in hollow stems may be of particular interest. This gene is classified as a putative *O*-methyltransferase and contains the O-MeTrfase_COMT domain (Supplemental Tables S2 and S3). Caffeic acid 3-*O*-methyltransferase (COMT) functions in lignin biosynthesis and has been identified in wheat (Ma and Xu, 2007). This is of particular interest for the formation of pith inside the stem since this enzyme is intrinsic to the flavonoid and lignin biosynthesis pathway. Caffeic acid 3-*O*-methyltransferase has also been shown to affect stem strength and lodging in wheat (Ma, 2009), while the lignin biosynthesis pathway has been associated with the host plant defense mechanism in wheat (Bhuiyan et al., 2009). Further experiments are needed to validate this candidate gene in terms of its role in stem solidness.

In this study, we completed in-depth expression analysis of 28 EST markers previously associated with stem solidness and the *Qss.msub-3B* QTL. Four candidate genes were identified that vary in gene expression between hollow and solid-stemmed NILs. These include a putative RNA binding protein, protein transport protein, and protein kinase corresponding to ESTs 11, 14, and 12, respectively. Additionally, a putative *O*-methyltransferase located within the region of interest was identified as a promising candidate based on differential expression between solid- and hollow-stemmed NILs and putative protein function. RNA-seq provided useful gene expression data for the ESTs as well as for all coding sequences identified within the region of interest. Unfortunately, allele differences due to SNPs may not result in differences in gene expression, and effects may not be seen. This may include posttranscriptional changes that alter protein function. Although a single clear candidate gene responsible for the stem solidness trait was not conclusively identified, the dataset presented here provides a comprehensive list of genes for further study to lead to the discovery of the genes responsible for wheat stem solidness variation.

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