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Gene expression polymorphisms and ESTs associated with gravitropic response of subterranean branch meristems and growth habit in *Leymus* wildryes

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ABSTRACT

Negatively orthogeotropic (NOGT) tiller and diageotropic (DGT) rhizome meristems develop from the same type of lateral axillary meristems and phytomer structure. Although subterranean NOGT and DGT buds appear similar, they display different responses to gravity and perhaps other cues governing branch angle and overall growth habit (GH). *Leymus* wildryes show remarkable variation in GH and include some of the largest native grasses in western North America. Previous studies detected GH QTLs on homoeologous regions of LG3a and LG3b controlling differences between caespitose *Leymus cinereus* and rhizomatous *Leymus triticoides* allotetraploids. Heterologous barley and wheat microarrays in conjunction with bulk segregate analysis were used to find gene expression polymorphisms associated with GH QTLs. Approximately 34% and 25% of the probe sets showed detectable signals on the barley and wheat arrays, respectively. Overall gene expression patterns of NOGT and DGT meristems were remarkably similar, consistent with the assertion that *Leymus* NOGT and DGT buds develop from homologous meristems. Only 28 and 27 genes on barley and wheat gene chips, respectively, showed more than twofold differential expressions between NOGT and DGT tissues. One expression polymorphism genetically mapped in the *Leymus* LG3 rhizome QTL region.

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1. Introduction

Growth habit (GH) is a functionally important, adaptive trait in perennial grasses. In general, grasses with caespitose and rhizomatous GH are adapted to xeric and mesic environments, respectively [1]. Caespitose grasses form a compact tussock of upright aerial tiller stem branches, whereas sod-forming grasses typically spread via prostrate stolons or underground rhizome stem branches. The rhizomatous GH is also related to competitive ability and invasiveness of perennial grasses [2]. Rhizomes provide protection from herbivory and trampling, storage tissues for vegetative propagation, and dispersal, which is problematic in perennial grass weeds such as quackgrass (*Elymus repens*) [3].

Despite differences in appearance, the aerial tiller and subterranean rhizome branches are homologous in that they develop from the same type of axillary meristem and eventually form the same basic phytomer organization and structure [4]. Initially, the only obvious difference between the subterranean axillary meristems of caespitose and rhizomatous grass buds involves the direction of branch growth. The axillary meristems of caespitose grasses grow upwards (negative orthogeotropism), emerging within the leaf sheath, whereas the axillary meristems of rhizomatous grasses cut through the leaf sheath and grow outwards perpendicular to gravity (diageotropism). However, intermediate branch angles (plagiotropism) are also common. Rhizomes and tillers differentiate, at least temporarily, in that rhizomes may develop reduced scale-like leaves, roots at the nodes, and a sharp apical cap with lignified epidermal cell walls [5], whereas aerial tillers obviously display fully developed leaves and photosynthetic capacity with exposure to light. Nevertheless, most rhizomes eventually emerge as fully functional aerial stems and retain the ability to flower.

Although plants are essentially sessile in nature, they show different types of movements in response to their environment. For

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Abbreviations: DGT, diageotropic; EST, expressed sequence tags; GH, growth habit; NOGT, negatively orthogeotropic; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; QTL, quantitative trait locus.

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example, the direction of growth in subterranean axillary meristems is primarily governed by gravity, but can be affected by light and perhaps other environmental cues [6]. It has been established that plants perceive gravity by specialized statocyte cells, particularly in the apical meristems of roots and shoots, which contain a special class of amyloplasts known as statoliths [7]. Stems generally show negative gravitropism mediated in part by increased cell expansion of the lower side of a horizontally placed stem in response to auxin, whereas roots generally show positive gravitropism and decreased cell expansion of the lower side of a horizontally placed root in response to auxin. Genetic studies suggest that different genes are involved in positive and negative gravitropism. For example, at least eight different loci have been shown to be involved in root gravitropism and six independent loci named SGR1 to SGR6 are related to shoot gravitropism in *Arabidopsis thaliana* [8–10].

The genus *Leymus* includes about 30 long-lived perennial grass species with remarkable variation in GH, stature, and adaptation to harsh cold, dry, and saline environments of Europe, Asia, and the Americas. Basin wildrye (*Leymus cinereus*) and several other large-statured *Leymus* species, including Altai wildrye (*Leymus angustus*) and mammoth wildrye (*Leymus racemosus*), have high biomass accumulation potential across a wide range of high-elevation or high-latitude growing environments of western North America [11–13], ideal for stockpiling forage or bioenergy feedstocks. *L. cinereus* is one of the largest native grasses (some ecotypes exceed 2 m tall) and the most abundant *Leymus* species in the Great Basin, Rocky Mountain, and Intermountain regions of western North America. However, caespitose *L. cinereus* is susceptible to damage by intense grazing of early season and fall regrowth. Once abundant on the floodplains of major rivers, alluvial gullies, and other watered areas with deep, well-drained soils in the Great Basin and Intermountain regions, *L. cinereus* has been eliminated from much of its former range due to grazing, harvesting, and cultivation of field crops. Cultivars of *L. cinereus* are commonly used in rangeland seed mixtures in western North America, but have limited use in pastures or hay crops. The second most common *Leymus* species in western North America is creeping wildrye (*Leymus triticoides*). *L. triticoides* is a shorter (0.3–0.7 m), but highly rhizomatous grass specifically adapted to poorly drained alkaline sites in the Great Basin, California, and other regions of western North America. Creeping wildrye is cultivated using vegetative propagules as a saline biomass crop in California, but poor seed production limits widespread use of this species.

Two experimental TTC (*triticoides* × (*triticoides* × *cinereus*) mapping families, TTC1 and TTC2, derived from interspecific hybrids of caespitose *L. cinereus* and rhizomatous *L. triticoides* have been developed for plant improvement and genetic investigations of functionally important traits in perennial forage grasses [14–16]. These interspecific hybrids are very robust plants exhibiting heterosis through increased plant height, large stems and leaves, prolific seed production, and improved seed germination from *L. cinereus* with vigorous proliferation of tillers, rhizome production, regrowth potential, and plant resiliency from *L. triticoides*. In terms of applied breeding, breeding populations derived from interspecific hybrids of *L. cinereus* and *L. triticoides* show excellent potential for high biomass production, reduced susceptibility to grazing or harvest, and improved regrowth potential. The linkage maps include 67 cross-species anchor markers (i.e., markers mapped in other grass species) used to identify and compare the 14 linkage groups of allotetraploid *Leymus* ($2n = 4x = 28$) based on synteny of corresponding markers in closely related wheat (*Triticum* spp.), barley (*Hordeum vulgare*), and cereal rye (*Secale cereale*) Triticeae cereals [14,16]. Moreover, genome-specific markers have been used to distinguish several homoeologous linkage groups corresponding to the *Ns* and *Xm* genomes of *Leymus* [14]. The *Ns* genome originates from *Psathyrostachys* [17,18], whereas the *Xm* genome is considered unknown [19]. It was speculated that the other (*Xm*) genome originates from *Thinopyrum bessarabicum* [20], but chromosome pairing in *Thinopyrum* × *Leymus* hybrids has been ambiguous [21] or very low [18,22].

Significant GH QTLs were detected on LG3a, LG3b, and LG6a in the *Leymus* TTC1 family [16]. Likewise, significant GH QTLs were detected on LG3a, LG3b, and LG5Xm in the *Leymus* TTC2 family. The LG3a and LG3b QTLs were evidently conserved between TTC1 and TTC2 families. Moreover, the LG3a and LG3b QTLs were located on homoeologous regions of the allotetraploid *Leymus* subgenomes [16]. Although the TTC1 LG6a and TTC2 LG5Xm showed relatively large log likelihood ratios (LOD test statistics) effects in the third or last evaluation year, the most consistent QTL effects over all three evaluation years were located on LG3a in both TTC1 and TTC2 families. Chromosome 3 is highly conserved within the Triticeae [23], which includes LG3a and LG3b of *Leymus* [14,16]. Collinear from end to end with rice chromosome 1, Triticeae group 3 is also the most conserved of all chromosome groups when compared to rice [24]. Rhizome and tiller angle QTLs also map to rice chromosome 1 [25,26].

Gene expression is the fundamental mechanism that controls the developmental processes in a plant and can be measured by microarray technologies [27]. Moreover, differences in mRNA levels of individual genes between genotypes are potentially used as an expression QTL (eQTL) to identify genotypic variation [28,29]. Although microarrays are a powerful method of transcriptome analysis, this technique is restricted to few model species, mainly due to lack of extensive sequence information of every species of interest. Manufacturing high-density microarrays is time-consuming and expensive, involving sequencing transcripts on a large scale from various tissues [30]. It was hypothesized that the sequence conservation between closely related species could be used for cross-species hybridizations, without having to design and construct microarrays for the many possible species of interest [27]. Based on the unique feature of the Affymetrix GeneChips®, where multiple probes represent each gene, it was hypothesized that sequence conservation within genera or families may be high enough to generate sufficient signals from some of the probes for expression analysis [27]. This approach is still at the early stage of research development for the identification of genes with relatively small differences in expression levels between closely related genotypes from a segregating population. Recently, a number of studies of this kind have been reported in plants [31–34].

Based on the assumption that NOGT and DGT subterranean meristems arise from homologous axillary buds, we speculate that overall gene expression profiles are predominantly similar between DGT and NOGT meristems during similar stages of early subterranean development. However, a relatively small number of genes may show mRNA expression polymorphisms specifically associated with branch angle differences in these same NOGT and DGT bud meristems. Thus, the objectives of this study were to compare overall gene expression profiles of *Leymus* DGT and NOGT meristems in a similar genetic background, identify gene expression polymorphisms specifically associated with the *Leymus* LG3a rhizome QTL by bulk segregate analysis, and identify other possible genes specifically involved in branch angle differences of otherwise similar DGT and NOGT meristems. At least some of these genes may be fundamentally important determinants of GH variation in grasses.

2. Materials and methods

2.1. Plant materials and RNA samples used for gene expression analysis

Full-sib mapping populations TTC1 and TTC2 were derived from one *L. triticoides* accession 641 plant (T-tester) pollinated by two different *L. triticoides* accession 641 × *L. cinereus* accession 636 F1 hybrids (TC1 and TC2). Accession 636 was received from the Agriculture Research Centre, Lethbridge, Alberta, Canada, and presumably originates from a natural population in Alberta or Saskatchewan. Accession 641 was collected from a natural population near Jamieson, Oregon, USA. The TTC1 and TTC2 families have very similar genetic backgrounds in that both families were derived from the same T-tester maternal parent genotype. Because more than two plants were used to derive the hybrid population from which the TC1 and TC2 genotypes were selected, it is not known for certain if they share any other relationship other than the fact that they were derived from crosses of the same two accessions. The 164-sib TTC1 and 170-sib TTC2 mapping populations, TC1 and TC2 hybrids, and T-tester clones are maintained by the United States Department of Agriculture, Agriculture Research Service, Forage and Range Research Laboratory (Logan, UT).

Diageotropic and negatively orthogeotropic apical meristems of subterranean branches were harvested from greenhouse source clones of the TTC1 and TTC2 families during the spring of 2005 and 2006. The length of lateral branches varied remarkably at the time of apical meristems tissue collection (5–18 cm) with only the distal region (less than 2 cm) being harvested, weighed, and quickly placed in liquid nitrogen. NOGT branch meristem samples were selected from a total of six TTC1, and six TTC2 segregant progenies that carry the caespitose *L. cinereus* LG3a GH QTL alleles (Table 1) using genotypic data [14,16]. Likewise, DGT meristems were selected from six TTC1 and six TTC2 clones, which carry the rhizomatous *L. triticoides* LG3a GH QTL allele (Table 1). NOGT and DGT meristem visually appear similar, and are only distinguishable by branch angle. Therefore, we arbitrarily set morphological selection criteria that the NOGT meristems were growing more than 135° opposite the center of gravity, whereas DGT meristems were growing less than 90° opposite the center of gravity. Genotypic selection criteria required that progeny did not display any recombination events in the entire LG3a GH QTL region, based on graphical genotypes displayed using Graphical GenoTyping software [35]. Although many other TTC1 and TTC2 progeny met the genotypic selection criteria, the clones used in this experiment (Table 1) were the only clones that actually displayed DGT and NOGT subterranean branch meristems suitable for the intended experiments. Moreover, some different TTC1 and TTC2 progeny genotypes were used in 2005 and 2006 because of variance in the initiation and phenotype of subterranean branch meristems.

Subterranean branch meristems from different TTC1 and TTC2 clones were bulked (Table 1) so that approximately 100 mg of tissue was available for each RNA extraction. Total RNA was isolated using Qiagen RNeasy® plant mini kit (Qiagen, Valencia, CA). RNA quality was assessed by BIO-RAD automated electrophoresis station (Biocompare, Inc., San Francisco, CA). Total RNA was used to generate labeled, fragmented cRNA using one-cycle target labeling and control reagents (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's protocol. Total RNA (5 μg), spiked with appropriately diluted Poly-A controls (1:10,000), was used in the synthesis of first and second-strand cDNA. Following second-strand synthesis, half of the purified cDNA (12 μL) was used in synthesis of biotin-labeled cRNA. Purification of labeling reaction typically yielded 45–55 μg labeled cRNA, as determined by spectrophotometric analysis of A_{260}. cRNA (20 μg) was fragmented in 5× fragmentation buffer (40 μL) by incubating at 94 °C for 35 min. Quality of labeled cRNA and fragmented cRNA was determined by gel electrophoresis and analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA).

2.2. Microarray analysis

Microarray experiments were performed using one Affymetrix GeneChip Wheat Genome Array and one Affymetrix GeneChip® Barley Genome Array for each of the 12 samples (Table 1). The Affymetrix GeneChip® Wheat Genome Array contains 61,127 probe sets representing 55,052 transcripts, whereas the Affymetrix GeneChip Barley Genome Array has 22,840 probe sets. The Biotin-labeled, fragmented cRNA was hybridized to GeneChip® Wheat Genome Arrays (Affymetrix, Inc., Santa Clara, CA) for 16 h according to the manufacturer's protocol. GeneChips were washed and stained with the fluidics script EukGE-WS2v5-450, using the Fluidics Station 450, and scanned using the GeneChip Scanner 3000, housed at the Center for Integrated BioSystems (Utah State University, Logan, UT). The total number of informative probe sets was determined by the number of signals present as determined by the Affymetrix GCOS software. Otherwise, data analysis of all gene expression arrays was conducted using ArrayAssist 3.3 software (Stratagene, La Jolla, CA). Probe-level analysis, normalization and summarization were performed by ArrayAssist's GC-RMA algorithm [36]. NGOT samples consisted of six pooled RNA extractions (SL01, SL02, SL03, SL07, SL08, SL09) and the DGT samples consisted of six pooled RNA extractions (SL04, SL05, SL06, SL10, SL11, SL12),

Table 1
Description of *Leymus* clones used for comparison of gene expression in diageotropic (DGT) and negatively orthogeotropic (NOGT) subterranean branch meristems

GeneChip ID	Year	LG3a GH-QTL genotype	Branch meristem phenotype[a]	Leymus clones[b]
SL01	2005	T/C	NOGT	A092, A171*, B2062
SL02	2005	T/C	NOGT	A141*, B1056*
SL03	2005	T/C	NOGT	A133*, B1083*, B1062
SL04	2005	T/T	DGT	A053, A086*, B1078
SL05	2005	T/T	DGT	A106*, A183, B1085*
SL06	2005	T/T	DGT	A072*, B2014*, B2037
SL07-PK7	2006	T/C	NOGT	A133*, B1083*
SL08-PK8	2006	T/C	NOGT	A187, B1108, B2009
SL09-PK9	2006	T/C	NOGT	A141*, A122, A171*, B1056*
SL10-PK10	2006	T/T	DGT	A086*, B2014*, B2083
SL11-PK11	2006	T/T	DGT	A106*, A072*, B1085*
SL12-PK12	2006	T/T	DGT	A119, B2044

*Clones used in both 2005 and 2006 experiments.
[a] Selected based on genotype of rhizomatous *L. triticoides* (T) or upright *L. cinereus* (C) LG3a growth habit QTL (GH-QTL) alleles.
[b] A and B clones are from TTC1 and TTC2 mapping families, respectively.

as shown in Table 1. Significance analysis was conducted using *P*-value of 0.001 and twofold differential expression between the six NOGT and six DGT samples.

2.3. Leymus EST library

The *Leymus* EST library was constructed, normalized, sequenced, filtered, and assembled using the same methods described by Anderson et al. [37] with the following modifications. Tissues were immediately flash-frozen in liquid nitrogen and stored at −80 °C and ground to a fine powder in liquid nitrogen.

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) reagent following the manufacturers' protocol. Samples were purified using the RNA cleanup procedure with Qiagen Midi columns (Qiagen, Valencia, CA). The poly $(A)^+$mRNA was converted to double-stranded cDNA using different NotI/oligo(dT)-tagged primers for two independent RNA samples as follows: [5′-AACTGGAAGAATTCGCGGCCGCTCCGA$(T)_{18}$V-3′] for purple and green aerial regrowth (less than 10 cm above ground), from field evaluations of the *Leymus* TTC1 and TTC2 rhizomes [16], harvested under freezing cold temperatures about 2 h after sunrise, and [5′-AACTGGAAGAATTCGCGGCCGCTCGCA$(T)_{18}$V-3′] for NOGT, DGT, and plagiotropic subterranean branch meristems of TTC1 and TTC2 plants sampled during March of 2005 (Table 1). Double-stranded cDNA samples ≥600 bp were selected by agarose gel electrophoresis and an equal mass of subterranean branch meristem cDNA and aerial spring regrowth cDNA was used for cloning. The total number of white colony forming units (cfu) before amplification was 3×10^6, whereas the total number of clones with insert was 2×10^6 following normalization. A total of 15,000 clones were sequenced from the 5′ end using the T7 primer and from the 3′ end using the T3 or M13R primer.

The gene sequences of differentially expressed genes on wheat and barley chips were blasted to the *Leymus* EST database to find the *Leymus* homologs that were aligned to rice sequences using the Gramene database to identify the location of *Leymus* ESTs on the rice genome.

2.4. Validation of array results with qRT-PCR and Leymus ESTs

A subset of the differentially expressing genes between the two groups in the gene-chip experiment was verified using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The nucleotide sequences for the differentially expressing genes on wheat and barley chips were extracted from wheat and barley consensus sequence files (www.Affymetrix.com). These sequences were blasted against the *Leymus* EST database (http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=Leymus). The *Leymus* EST database sequences were used to design PCR primers specific to each EST using Primer3 Input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

For qRT-PCR, the new tissue samples were recollected using the same method as for the microarrays, with the same plants used for microarray experiments in 2006. This new preparation of RNA provided for validation of differential expression independent of potential extraction artifacts. qRT-PCR was carried out in a two-step reaction in real time. Total RNA (2.0 μg) was reverse-transcribed from an oligo-dT primer using a First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD) in a 20 μL reaction at 37 °C for 60 min followed by 70 °C for 10 min. Reactions were diluted to 100 μL with 0.1× TE, and stored at −20 °C. First-strand cDNA (2 μL) was used as a template for PCR amplification of specific transcripts using GoTaq DNA Polymerase (Promega, Madison, WI) in a 25-μL reaction (1× reaction buffer, 25 mM $MgCl_2$, 0.2 μM gene specific primers, 0.25 mM dNTP, 1.25 U Taq polymerase, 0.2X SYBR Green 1 (Molecular Probes, Inc., Eugene, OR)). Thermal cycling was carried out in DNA Engine Opticon2 System (MJ Research, Waltham, MA) for 36 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 75 s. Opticon Monitor 2 version 2.02.24 software (MJ Research, Waltham, MA) was used to analyze the data. All measured samples were amplified in three replicates, and standard curves for each primer pair were established to quantify the samples. Standard curves had slopes of $y = -0.28$ to -0.44 and $r^2 = 0.829$–0.995. The normalized values for each transcript were determined by dividing their average copy value by the average tubulin value. The specificity of PCR amplification was confirmed by the following criteria: (1) a single peak in the melting-curve analysis of real-time PCR-amplified products and (2) a single band as determined by agarose gel electrophoresis. The fold changes of expression values between NOGT and DGT subterranean meristems were compared for array and qRT-PCR experiments. For this, the average copy number value over six corresponding chips for NOGT and DGT tissues and the average copy number value over three bulks each of NOGT and DGT tissues for qRT-PCR were calculated and normalized by dividing the average values by the corresponding average tubulin expression (Table 4). Tubulin expression values from wheat (Ta.812.1.S1_x_at) and barley (Contig902_x_at) arrays were used to normalize array expression values. The tubulin PCR primers (5′ ATCTCGATCCACATCGGCCA 3′ and 5′ ATCACAGTGGGCTCAAGATC 3′) were designed from the *Leymus* EG385442 EST.

3. Results

3.1. Analysis of GeneChip expression data

An average of 7,813 (34%) and 15,132 (25%) positive signals were detected by hybridization of the 12 *Leymus* subterranean branch meristem cRNA samples (Table 1) to the barley and wheat arrays, respectively. Thus, a total of 22,945 wheat and barley probe sets were interrogated. However, at least some of the wheat and barley probe sets were designed from homologous genes. Thus, the actual number of expressed sequences that were interrogated was greater than 15,132 but less than 22,945.

Twenty-seven wheat array probe sets detected significant ($P < 0.001$) differences in mRNA levels between the NOGT and DGT subterranean branch meristem samples, including 18 probe sets detecting greater expression in DGT samples and nine probe sets detecting greater expression in the NOGT samples (Table 2). Using $P < 0.05$, $P < 0.01$, and $P < 0.005$ as cutoff values, 552, 234, and 141 probe sets were identified, respectively, on the wheat array (data not shown). Using the barley array, 28 probe sets detected significant ($P < 0.001$) differences in mRNA levels between the NOGT and DGT subterranean branch meristem samples, including 8 probe sets detecting greater expression in DGT samples and 21 probe sets detecting greater expression in the NOGT samples (Table 3). Using $P < 0.05$, $P < 0.01$, and $P < 0.005$ as cutoff values, 268, 148, and 96 probe sets were identified on the barley array, respectively (data not shown). Several genes were identified from both wheat and barley arrays including ATP synthase-like proteins (barley contig835_s_at and wheat Ta.2412.1.S1_at), putative glycine dehydrogenase (barley contig1483_at and wheat Ta.30795.1.S1_at), as well as 40S and 60S ribosomal subunits (Tables 2 and 3). Major genes identified as differentially expressing were those related to putative auxin-induced proteins, calcium-binding, calmodulin and calmodulin-like proteins, H^+ exchange proteins, chloroplast precursors and light harvesting proteins, MAP kinases and centromere/microtubule-binding proteins, and genes regulating glycine biosynthesis. Other genes were related to nucleolar proteins such as fibrillarin, Nop56, and NopA64, and

Table 2
Description of Wheat Genome Array probe sets that detected different gene expression levels between negatively orthogeotropic (NOGT) and diageotropic (DGT) subterranean branch meristems of *Leymus* (*triticoides* × (*triticoides* × *cinereus*)) progeny selected for upright *L. cinereus* or rhizomatous *L. triticoides* LG3a growth habit QTL alleles (see Table 1)

Probe set ID	Probe set description	Expression level NOGT branch meristem[a]	Expression level DGT branch meristem[a]	Expression ratio NOGT/DGT	Differential expression *P*-value
Ta.636.1.S1_s_at	Weakly similar to NP_566086.1 expressed protein [*A. thaliana*]	31	359	11.1^{-1}	0.00061
Ta.22762.2.S1_at	Strongly similar to XP_477140.1 putative mRNA binding protein precursor [*O. sativa* (japonica cv-gp.)]	11	106	9.09^{-1}	0.00032
Ta.2431.1.S1_x_at*	Strongly similar to XP_462957.1 putative hydrolase [*O. sativa*]	22	158	7.14^{-1}	0.00031
Ta.20878.1.S1_at	Moderately similar to XP_479480.1 putative ultraviolet-B-repressible protein [*O. sativa* (japonica cv-gp.)]	11	72	6.67^{-1}	0.00026
Ta.27751.3.S1_at	Cdpk1 protein	68	321	4.76^{-1}	0.00074
Ta.641.1.S1_at	Weakly similar to XP_507201.1 predicted P0426E02.15-2 gene product [*O. sativa* (japonica cv-gp.)]	16	71	4.55^{-1}	0.00014
Ta.2412.1.S1_at	Moderately similar to NP_194953.1 ATP synthase family [*A. thaliana*]	354	1519	4.35^{-1}	0.00034
Ta.18087.1.S1_at	Transcribed locus	18	70	3.85^{-1}	0.00032
Ta.28292.1.S1_at	Moderately similar to NP_918616.1 OSJNBa0094H06.16 [*O. sativa* (japonica cv-gp.)]	115	397	3.45^{-1}	0.00099
Ta.485.1.A1_at	Strongly similar to XP_464447.1 putative Lipoxygenase 2.3, chloroplast precursor [*O. sativa* (japonica cv-gp.)]	107	353	3.33^{-1}	0.00099
TaAffx.33924.1.S1_at	Strongly similar to XP_483046.1 putative CfxY protein [*O. sativa* (japonica cv-gp.)]	21	70	3.33^{-1}	0.00087
Ta.2431.1.S1_at*	Strongly similar to XP_462957.1 putative hydrolase [*O. sativa*]	37	118	3.23^{-1}	0.00069
Ta.7338.1.A1_at	Moderately similar to NP_919061.1 putative N5′-nucleotidase [*O. sativa* (japonica cv-gp.)]	79	249	3.13^{-1}	0.00006
Ta.3366.1.S1_at	Clone wlm4.pk0022.h2:fis, full insert mRNA sequence	109	325	3.03^{-1}	0.00062
Ta.12341.1.S1_at	Moderately similar to XP_483757.1 putative auxin-regulated protein [*O. sativa* (japonica cv-gp.)]	240	632	2.63^{-1}	0.00078
Ta.28357.1.A1_s_at	Transcribed locus	633	1602	2.50^{-1}	0.00014
Ta.30795.1.S1_at	Strongly similar to NP_916596.1 putative glycine dehydrogenase [*O. sativa* (japonica cv-gp.)]	267	601	2.27^{-1}	0.00096
Ta.3581.1.S1_x_at	Strongly similar to NP_909865.1 photosystem-1 F subunit precursor [*O. sativa* (japonica cv-gp.)]	90	190	2.13^{-1}	0.00087
Ta.27377.1.S1_x_at	Ubiquitin/ribosomal fusion protein	8287	3880	2.14	0.00078
Ta.9201.3.S1_x_at	Moderately similar to NP_176007.1 nucleolar protein Nop56, putative [*A. thaliana*]	67	29	2.32	0.00882
Ta.8431.1.S1_at	Strongly similar to XP_469398.1 silencing gp. B protein [*O. sativa* (japonica cv-gp.)]	361	144	2.51	0.00006
Ta.28703.2.S1_x_at*	Strongly similar to XP_472792.1 OSJNBb0048E02.13 [*O. sativa* (japonica cv-gp.)]	1239	479	2.58	0.00083
Ta.28703.2.S1_at*	Strongly similar to XP_472792.1 OSJNBb0048E02.13 [*O. sativa* (japonica cv-gp.)]	1163	448	2.60	0.00067
Ta.6315.1.S1_a_at	Strongly similar to XP_469984.1 putative RNA helicase [*O. sativa* (japonica cv-gp.)]	306	98	3.12	0.00086
Ta.912.1.S1_at	Moderately similar to NP_192960.1 esterase/lipase/thioesterase family protein [*A. thaliana*]	9653	3091	3.12	0.00068
Ta.9549.3.S1_x_at*	Strongly similar to XP_479248.1 putative centromere/microtubule binding protein [*O. sativa* (japonica cv-gp.)]	373	95	3.92	0.00085
Ta.9549.1.S1_x_at*	Strongly similar to XP_479248.1 putative centromere/microtubule binding protein [*O. sativa* (japonica cv-gp.)]	908	160	5.69	0.00011

* Significant differences detected by different probe sets designed from same sequence.
[a] Expression levels shown are the average normalized data from six GeneChips.

heat-shock proteins, in addition to some unknown genes or transcribed loci.

3.2. Identifying Leymus ESTs homologous to gene-expression polymorphisms

The GH QTLs in *Leymus* have been assigned to chromosome 3 [16], which is syntenic with rice chromosome 1. *Leymus* sequences, EG392086, EG390562, and EG383468 correspond to rice chromosome 1L, while EG375403 corresponds to rice chromosome 7L (Table 4). Three of these *Leymus* sequences were mapped on pre-existing linkage maps of *Leymus* [14,16]. Primers designed from EG392086 (5′ GTGAATCGCAAGATCAGCAA 3′ and 5′ AGCGTTAACGTTCTCGTCGT 3′) produced polymorphic PCR amplicons that mapped to LG5Ns. Primers designed from EG390562 (Table 4) produced PCR amplicons that contained an AluI restriction site polymorphism that mapped to LG3b. Primers designed from EG375403 (Table 4) produced polymorphic PCR amplicons that mapped to LG2a. EG383468 was not polymorphic and could not be mapped.

3.3. qRT-PCR analysis of differentially expressed genes

Eight differentially expressed genes showing high-fold differences between NOGT and DGT branch meristems were analyzed by qRT-PCR. Of the genes selected for validation by qRT-PCR, four genes showed higher expression in NOGT subterranean branch meristems and four genes showed higher expression in DGT meristems (Table 4). Probeset Ta.22762.2.S1_at (wheat array) had the smallest normalized NOGT/DGT ratio of 6.67^{-1} and qRT-PCR resulted in a ratio of 11.11^{-1}. The largest normalized NOGT/DGT ratio was Contig3457_at (barley array) at 7.14 with a qRT-PCR ratio of 3.66. Quantification of transcript levels of all eight genes assayed by qRT-PCR resulted in ratios that supported the differential expression shown by the array data, although four of the probe sets (Ta.22762.2.S1_at, Ta.28292.1.S1_at, Contig2369_s_at, Conti-

Table 3
Description of Barley Genome Array probe sets that detected different gene expression levels between negatively orthogeotropic (NOGT) and diageotropic (DGT) subterranean branch meristems of *Leymus* (*triticoides* × (*triticoides* × *cinereus*)) progeny selected for upright *L. cinereus* or rhizomatous *L. triticoides* LG3a growth habit QTL alleles (see Table 1)

Probe Set ID	Probe set description	Expression level NOGT branch meristem[a]	Expression level DGT branch meristem[a]	Expression ratio NOGT/DGT	P-value
Contig1523_at	Chlorophyll *a*/*b*-binding protein CP24 precursor [*Vigna radiata*]	8	57	7.14^{-1}	0.00074
Contig835_s_at	H+-transporting ATP synthase-like protein [i]	18	90	5.00^{-1}	0.00053
Contig2083_s_at	Photosystem I reaction center subunit III, chloroplast precursor	21	103	4.76^{-1}	0.00044
Contig2985_s_at	(AY039003) Mg-chelatase subunit XANTHA-F [*H. vulgare*] [*H. vulgare* subsp. vulgare]	22	69	3.13^{-1}	0.00054
Contig25506_at	None	46	138	3.03^{-1}	0.00065
HO04H12S_s_at	None	57	171	3.03^{-1}	0.00075
Contig6148_at	50S ribosomal protein L24, chloroplast precursor; protein id: At5g54600.1, supported by cDNA: 27973[*A. thaliana*]	55	160	2.86^{-1}	0.00045
Contig1483_at	Glycine dehydrogenase (decarboxylating) (EC 1.4.4.2) [imported]-H. sp. × *Triticum* sp.	1896	3959	2.08^{-1}	0.00034
Contig3268_at	Beta 5 subunit of 20S proteasome [*O. sativa* (japonica cv.-gp.)]	581	285	2.04	0.00031
Contig1810_at	40S ribosomal protein S9-like; protein id: At5g39850.1, supported by cDNA: 41408. [*A. thaliana*]	1801	858	2.10	0.00017
HVSMEg0010B23r2_at	7e-32 putative ribosomal protein; protein id: At3g23390.1, supported by cDNA: 13557, gi_17065557	277	132	2.11	0.00009
Contig1343_at	Putative ribosomal protein L7; protein id: At2g01250.1, supported by cDNA: 13298, gi_13877566	2619	1171	2.24	0.00083
Contig1139_s_at	Putative 60S ribosomal protein [Sorghum bicolor]	1102	490	2.25	0.00076
Contig1819_s_at	Unnamed protein product [*O. sativa* (japonica cv.-gp.)]	781	344	2.27	0.00069
Contig3176_at	Probable DNA-binding protein GBP 16-rice gb|AAB80919.1| [*O. sativa*]	550	237	2.32	0.00057
Contig2290_s_at	60S ribosomal protein L31 gb|AAF42953.1| AF237624_1 80S ribosomal protein L31 [*Perilla frutescens*]	1524	651	2.34	0.00039
Contig1962_at	Putative 40S ribosomal protein S26 [*O. sativa* (japonica cultivar-group)]	2251	959	2.35	0.00015
Contig2369_s_at	ESTs AU077873 (S1878), D40121 (S1878), Similar to *A. thaliana* 60S ribosomal protein	645	267	2.42	0.00047
Contig1248_s_at	60S Ribosomal protein L6 (YL16-LIKE) pir||S28586 ribosomal protein ML16, cytosolic-common ice plant	464	190	2.44	0.00021
Contig1809_at	Acidic ribosomal protein P2a-2 [*Zea mays*]	2852	1141	2.50	0.00075
Contig1950_at	40S ribosomal protein S12 gb|AAD39838.1 |AF067732_1 ribosomal protein S12 [*H. vulgare*]	1333	514	2.59	0.00076
Contig2468_at	Putative 40S ribosomal protein S24 [*O. sativa* (japonica cultivar-group)]	1899	717	2.65	0.00021
Contig6096_at	DnaK-type molecular chaperone HSC70-11	343	126	2.72	0.00090
Contig3821_s_at	SAR DNA binding protein [*O. sativa*]	101	34	3.01	0.00064
HA08n04r_s_at	5e-36 60S Ribosomal protein L34 pir||S48027 ribosomal protein L34, cytosolic-common tobacco	121	38	3.21	0.00056
Contig11344_s_at	Putative protein; protein id: At4g25340.1, supported by cDNA: gi_15982871 [*A. thaliana*]	134	41	3.24	0.00074
Contig3457_x_at	Probable fibrillarin [*P. mariana*]	534	59	8.99	0.00026
Contig3457_at	Probable fibrillarin [*P. mariana*]	471	51	9.32	0.00020

[a] Expression levels shown are the average normalized data from six GeneChips.

g3457_at) had about twofold differences in array and qRT-PCR derived expression ratios.

4. Discussion

In this project, we demonstrated the usefulness of heterologous wheat and barley GeneChips to compare gene expression between NOGT and DGT subterranean meristems in the perennial Triticeae. The wheat GeneChip was designed from expressed sequences representing the A, B, and D genomes of wheat, whereas the barley array was designed from sequences representing the H genome of barley. Thus, it is possible that up to three homeologous genes are represented on the wheat array and one homeologous gene on the barley array. Neither array contains probe sets representing all possible genes because their complete genomes have yet to be sequenced. Additionally, single nucleotide polymorphisms in the probe set design can effect whether or not an expressed transcript will be detected. For these reasons it is expected that some transcripts from a heterologous species would be detected on one array but not the other. Approximately 25 and 34% of probe sets on wheat and barley GeneChips, respectively, were classified as "present" by Affymetrix GCOS software when hybridized with labeled *Leymus* cRNA. Given that hybridizing barley cRNA to barley chips resulted in 49% present calls [38], and hybridizing wheat cRNA to wheat chips resulted in 48% present calls [39], the results obtained using *Leymus* cRNA on these heterologous arrays seem

Table 4
Validation of gene expression polymorphisms detected using wheat or barley genome arrays (Tables 2 and 3) based on expression levels of homologous *Leymus* ESTs detected by qRT-PCR

Microarray probe set	Array level (SD) NOGT[a]	Array level (SD) DGT[b]	Array ratio NOGT/DGT	*Leymus* EST (rice genome alignment)	Primers Designed from *Leymus* EST	qRT-PCR level (SD) NOGT[c]	qRT-PCR level (SD) DGT	qRT-PCR ratio NOGT/DGT
Wheat Ta.22762.2.S1_at	0.08 (0.002)	0.55 (0.36)	6.87^{-1}	EG386159 (7S)	GGGATTTACCTTCCCACTGA, TTCTGTCGAAGAACCACTCCT	1.29 (1.04)	21.12 (0.51)	11.11^{-1}
Wheat Ta.28292.1.S1_at	1.28 (0.29)	5.42 (3.28)	4.17^{-1}	EG392086 (1L)	GCAGCTTGACAGAGACAAGG, CTTGGTTTGGCGTTTGAGAT	0.14 (0.23)	1.21 (0.72)	9.09^{-1}
Barley HO04H12S_s_at	0.006 (0.002)	0.01 (0.006)	1.66^{-1}	EG383468 (1L)	TTCTTCCGTTCATCATTTCG, GTGGTAGTCACCTGGCAAGA	0.73 (0.47)	1.07 (0.67)	1.47^{-1}
Wheat Ta.30795.1.S1_at	2.66 (0.73)	6.13 (2.83)	2.33^{-1}	EG390562 (1L)	ATCCTGCAAGTGCTGCTATG, TCTCATCAATGCCTTCTTCG	0.34 (0.22)	0.69 (0.45)	2.04^{-1}
Wheat Ta.27377.1.S1_x_at	91.61 (0.92)	44.21 (0.39)	2.07	EG382389 (4L)	GGGAAGACCATCACCCTAGA, GAGTCCACCCTCCATCTTGT	0.52 (0.14)	0.30 (0.03)	1.74
Wheat Ta.28703.2.S1_at	1.92 (0.07)	0.86 (0.18)	2.23	EG375403 (7L)	GTCAACACATACCGCAGGAC, TGCAAGCAGCTTCTGGTACT	2.56 (0.48)	1.24 (0.19)	2.06
Barley Contig2369_s_at	0.08 (0.01)	0.03 (0.01)	2.66	EG376293 (6S)	GCCCTACCTCTGGACCTCTT, CATACCCTCAAGCGACCTGT	2.49 (0.71)	0.40 (0.37)	6.26
Barley Contig3457_at	0.05 (0.02)	0.007 (0.004)	7.14	EG387031 (5S)	GAGAAGTTCGTCCACAAGCA, CCCACCTTCTCGATGTTCTT	4.53 (0.35)	1.24 (0.82)	3.66

[a] Negatively orthogeotropic (NOGT) subterranean branch meristems of the *Leymus* TTC1 and TTC2 progeny selected for upright *L. cinereus* LG3a growth habit QTL alleles (see Table 1).
[b] Diageotropic (DGT) subterranean branch meristems of the *Leymus* TTC1 and TTC2 progeny selected for rhizomatous *L. triticoides* LG3a growth habit QTL alleles (see Table 1).
[c] Array and qRT-PCR values shown are average gene specific expression values normalized to the average tubulin expression.

reasonable. The results also suggest that the *Leymus* genome has higher similarity to the barley genome than to the wheat genome. Somewhere between 15,132 and 22,945 probe sets were interrogated by the *Leymus* cRNA between the two arrays. Of those, only 55 (~0.29%) of the probe sets had greater than twofold difference in expression between NOGT and DGT meristems, demonstrating the remarkable similarity of the two structures.

Among the identified differentially expressing genes were those whose gene products have been previously implicated in plant gravitropism, such as putative auxin-induced proteins, Ta.12341.1.S1_at [40,41], calcium-binding, calmodulin, and calmodulin-like proteins represented by Ta.27751.3.S1_at [42–44], MAP kinases, Ta.1130.1.S1_a_at, Ta.1130.2.S1_x_at, Ta.1130.3.S1_x_at [45–48], and H^+ exchange proteins, Contig835_s_at, Contig442_at [49,50].

One differentially expressed gene, wheat Ta12341.1.S1_at, was related to a putative auxin-regulated protein and showed higher expression levels in DGT subterranean meristems with an NOGT/DGT expression ratio of 2.63^{-1} (Table 2). Since auxin has been implicated in the inhibition of lateral bud growth [51], it is possible that auxin and some auxin-regulated proteins, such as Ta12341.1.S1_at, are involved in suppressing formation of NOGT or maintaining DGT. Since the asymmetric distribution of auxin at the elongation zone is considered to be the causative factor for asymmetric growth of curvature induced by gravity [40,52,53], our results suggest that auxin may play an important role in determining what type of subterranean meristems develop, either NOGT or DGT. SAR (suppressor of auxin resistance) DNA-binding protein and GBP16 (gibberellin-binding proteins) have not been shown to be directly involved in plant gravitropism. However, it has been demonstrated that auxin controls the growth of *Arabidopsis* roots through the modulation of the cellular response to phytohormone gibberellin (GA), which has long been known to regulate shoot growth [54]. Also, it was proposed that SAR1 is a regulator of cell growth and AXR1 (auxin resistant) acts to modify the activity of SAR1. AXR1 is likely to have a role in protein degradation and may promote the degradation of SAR1 in an auxin-dependent manner [55]. Both SAR-binding proteins and GBP16 are related to auxin action and their expression was found to be higher in NOGT meristems as opposed to auxin-induced genes, which were more expressed in DGT meristems. These may be the novel genes involved in apical bud differentiation to DGT or NOGT meristems. It may also suggest that a balanced action of auxin and GA is involved in determining NOGT growth.

Cdpk1, a gene coding for a calcium-dependent protein kinase (Ta.27751.3.S1_at), was expressed more in DGT meristems with an NOGT/DGT expression ratio of 4.76^{-1}. Ca^{2+}/calmodulin-dependent protein kinases have been thought to be involved in light dependent orthogravitropic response of roots in some cultivars of corn [44]. It was suggested that the gravitropic response is the main cause of increase in concentration of Ca^{2+} in young *Arabidopsis* seedlings [56]. High amounts of calmodulin associated with amyloplasts in statocytes [57,58] and expression of calmodulin-like genes usually known as touch genes are induced by mechanical stimulation [59,60]. Lateral growth of DGT meristems and the mechanical hindrance of soil while growing through the ground may be responsible for the higher expression levels of *Cdpk1* in the DGT meristems. Ca^{2+} is also important for lateral distribution of auxin in maize roots [61]. It was also hypothesized that the kinase is associated with the establishment of auxin gradients in corn roots via activation of proton pumps causing auxin redistribution [61]. The putative Ca^{2+}-channel blocker, $LaCl_3$ (lanthanum chloride) inhibits stem curvature of snapdragon (*Antirrhinum majus* L.) spikes by preventing several gravity-dependent processes indicating that $LaCl_3$, which modulates cytosolic Ca^{2+}, does not influence general stem-growth processes but may specifically affect other gravity-associated processes occurring at the stem-bending zone [62].

Higher expression of MAP kinases (Ta.1130.1.S1_a_at, Ta.1130.2.S1_x_at, Ta.1130.3.S1_x_at) in the DGT tissues in the array experiments can be related to previous findings [45], where it was stated that there is possibility that changes in MAPK activity in the gravistimulated maize pulvinus are a part of a signal cascade that may distinguish between minor perturbations in plant orientation and more significant and long-term changes, and may also help to determine the direction of bending. MAP kinases have also been shown to phosphorylate microtubule-associated proteins, thereby altering their ability to stabilize microtubules [46]. Ta.9549.1.S1_x_at and Ta.9549.3.S1_x_at are related to putative centromere/microtubule-binding proteins and showed higher expression in NOGT meristems. The organization of microtubules is gravity-sensitive [63]. It was established that

the cortical microtubule might act as a strain gauge for amplification and stabilization of environmentally induced changes in direction of growth elongation [47,48].

Genes on both wheat and barley chips were found to be the associated with chloroplast precursors and light-harvesting proteins. This family of genes including lipoxygenases and esterase/lipase/thioesterase may be involved in plant gravitropism. The starch statolith hypothesis indicates that the starch-filled chloroplasts are gravisensors in shoots of plants [64–67]. Some of these genes from this category were expressed more in NOGT meristems whereas others were expressed more in DGT meristems. Both gravity and light interact with each other to enhance or reduce the other's effectiveness and they share common elements in the transduction pathways [68]. The presence of the differential expression related to chlorophyll binding and light-harvesting proteins is also supported by the literature [69,70]. They established that physical stimuli, such as phytochromes (photoreceptors), also interfere with some gravitropic responses and may play a crucial role in the expression of gravisensitivity.

Decrease in quantity of nucleolar proteins fibrillarin and NopA64 in roots of *Lepidium sativum* under altered/reduced-gravity conditions was discovered [71]. We found transcripts for these two pre-rRNA processing proteins, Nop56 (Ta.9201.3.S1_x_at) and fibrillarin (Contig3457_at, Contig3457_x_at), were highly expressed in NOGT meristems, suggesting again the involvement of the nucleolous in the gravitropic signaling.

A putative glycine dehydrogenase gene (contig1483_at, Ta.30795.1.S1_at) had higher expression values in DGT branch meristems. A search for the conserved domains using the NCBI blast (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed that Ta.28357.1.A1_s_at, which was also expressed higher in DGT branch meristems, was related to glycine cleavage system aminomethyltransferases and the glycine cleavage T-protein C-terminal barrel domain. Expression levels of genes related to the cytoskeleton, such as glycine hydroxymethyltransferase changed in response to gravitational signals [72]. Finding three different genes involved in glycine biosynthesis with higher expression in DGT branch meristems suggests that glycine may play a role in gravitropism.

The role of heat-shock proteins HSC70-11, Hsp60p (Contig6096_at, Ta.6964.1.S1_at, respectively) in gravitropism has not been elucidated yet, but HSC70-11 was found to be differentially expressing during the transcription profiling of the early gravitropic response in *Arabidopsis* [73].

Genes functioning as transcription factors may indirectly affect the GH QTLs. This might explain why only one out of three expression polymorphisms (EG390562) that aligned to rice chromosome 1 (Table 4) mapped on LG3b of *Leymus*. However, we cannot exclude the possibility that EG390562 is also on LG3a, as both LG3a and LG3b show the presence of the GH QTL and are suggested to be homoeologous due to the presence of VP1 loci occurring near the QTL region on both groups [16]. Also, the progeny-selection criterion for the gene-expression experiments was no recombination events in the LG3a GH QTL region, which may explain why EG390562 did not map on LG3a. Additionally, wheat linkage group 5 is the least conserved among all the chromosomes, with genes scattered across all 12 rice chromosomes; however, it shows some regions of homology to rice chromosomes 3, 9, and 12 [74]. This may explain the expression polymorphism aligning to rice 1L on *Leymus* 5Ns. Rice chromosome 9 also contains a major QTL (*Ta*) for tiller angle [26]. Alignment of a gene expression polymorphism on rice chromosome 7L and its mapping on *Leymus* LG2a can be supported by the previous findings [74] that rice chromosome 7 shows 62% homology with short arm of wheat chromosome 2.

The directional growth of axillary meristems determines the caespitose or rhizomatous nature of grasses. Despite the disparate phenotypes expressed by these two growth habits, NOGT and DGT meristems appear visually similar and are only visually distinguishable by the direction of their growth. Our results have confirmed the semblance of negative orthogeotropic and diageotropic shoot meristems, with less than 0.18% of transcripts showing differential expression between the two meristems types. This is remarkable given that gene expression difference between wheat shoot and root tissue exceeds 53% under similar statistical parameters [39]. Many of the differentially expressing genes we found in this study were involved directly or indirectly in gravitropism pathways. This supports the interpretation that differences in GH of NOGT and DGT meristems can be due to their responses towards earth's gravitational force, achieved probably through the auxin-signaling pathway. Continued efforts to assess differential expression in these meristems and fine genetic mapping and positional cloning should advance the discovery of genes related to GH.

5. Supplementary materials

Array data files, including text files of normalized expression data for each probe set on both wheat and barley genome arrays, can be accessed via ArrayExpress at the European Bioinformatics Institute (www.ebi.ac.uk) with the assigned accession number E-MEXP-1265.

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