Identification and Expression Analysis of Wheat TaGF14 Genes

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The 14-3-3 gene family members play key roles in various cellular processes. However, little is known about the numbers and roles of 14-3-3 genes in wheat. The aims of this study were to identify TaGF14 numbers in wheat by searching its whole genome through blast, to study the phylogenetic relationships with other plant species and to discuss the functions of TaGF14s. The results showed that common wheat harbored 20 TaGF14 genes, located on wheat chromosome groups 2, 3, 4, and 7. Out of them, eighteen TaGF14s are non-ε proteins, and two wheat TaGF14 genes, TaGF14i and TaGF14f, are ε proteins. Phylogenetic analysis indicated that these genes were divided into six clusters: cluster 1 (TaGF14d, TaGF14g, TaGF14j, TaGF14h, TaGF14c, and TaGF14n); cluster 2 (TaGF14k); cluster 3 (TaGF14b, TaGF14l, TaGF14m, and TaGF14s); cluster 4 (TaGF14a, TaGF14e, and TaGF14r); cluster 5 (TaGF14i and TaGF14f); and cluster 6 (TaGF14o, TaGF14p, TaGF14q, and TaGF14t). Tissue-specific gene expressions suggested that all TaGF14s were likely constitutively expressed, except two genes, i.e., TaGF14p and TaGF14f. And the highest amount of TaGF14 transcripts were observed in developing grains at 20 days post anthesis (DPA), especially for TaGF14j and TaGF14l. After drought stress, five genes, i.e., TaGF14c, TaGF14d, TaGF14g, TaGF14h, and TaGF14j, were up-regulated expression under drought stress for both 1 and 6 h, suggesting these genes played vital role in combating against drought stress. However, all the TaGF14s were down-regulated expression under heat stress for both 1 and 6 h, indicating TaGF14s may be negatively associated with heat stress by reducing the expression to combat heat stress or through other pathways. These results suggested that cluster 1, e.g., TaGF14j, may participate in the whole wheat developing stages, e.g., grain-filling (starch biosynthesis) and may also participate in combating against drought stress. Subsequently, a homolog of TaGF14j, TaGF14-JM22, were cloned by RACE and used to validate its function. Immunoblotting results showed that TaGF14-JM22 protein, closely related to TaGF14d, TaGF14g, and TaGF14j, can interact with AGP-L, SSI, SSII, SBEIIa, and SBEIIb in developing grains, suggesting that TaGF14s located on group 4 may be involved in starch biosynthesis. Therefore, it is possible to develop starch-rich wheat cultivars by modifying TaGF14s.

Keywords: Triticum aestivum, 14-3-3, phylogenetic analysis, tissue-specific expression, starch biosynthesis
INTRODUCTION

The ubiquitous 14-3-3 proteins, as one of the families of regulatory proteins, have been found in all eukaryotic organisms and tissues. The family consists of dimeric α-helical pSer/Thr binding proteins that play key roles in various cellular processes, such as signal transduction, biotic and abiotic stress responses, and carbon and nitrogen metabolism, by mediating protein–protein interactions (Aitken et al., 1992; Fulgosi et al., 2002). However, little is known about the numbers and roles of 14-3-3 genes (TaGF14s) in wheat. Therefore, it is necessary to develop elite wheat cultivars to explore the numbers and to study the functions of TaGF14s.

Different species may have different numbers of GF14s. For example, humans have seven 14-3-3 genes (Iwata et al., 2000), while Arabidopsis, rice and maize have thirteen GF14s and two pseudogenes, eight GF14s and twelve GF14s, respectively (Wu et al., 1997; Rosenquist et al., 2001; Lai et al., 2004; Sehnke et al., 2006; Yao et al., 2007; Alexandrov et al., 2009), which suggested that plants maybe have more GF14s than animal. 14-3-3 proteins, binding a range of transcription factors and signaling proteins, have roles in regulating carbon and nitrogen metabolism, plant development, and biotic and abiotic stress responses (Roberts, 2000, 2003; Fulgosi et al., 2002; Maraschin et al., 2003). For example, BdGF14f were associated Cr and cold stresses in Brachypodium distachyon (Cao et al., 2016). Different 14-3-3 protein isoforms have different roles. For example, 14-3-3A processing and 14-3-3C initiation of specific cell type differentiation (Maraschin et al., 2003). And 14-3-3 proteins were also reported to be involved in starch biosynthesis in plants. For example, Alexander and Morris (2006) identified 54 14-3-3 binding proteins by MALDI-TOF MS, and the largest category was for carbohydrate metabolism, including plastidic enzymes for starch synthesis and modification. Out of them, four enzymes, i.e., GSBSI, SSI, SSII and SBEIIa were involved in starch biosynthesis. Presently, only four GF14s have been reported in common wheat (Ikeda et al., 2000; Yao et al., 2005; Wang et al., 2008). It is unknown whether there are more GF14s in common wheat than rice and maize. Due to the roles of GF14 reported previously, it is necessary to study the 14-3-3 genes and their functions in wheat.

The allohexaploid bread wheat (Triticum aestivum, 2n = 6x = 42; genome AABBDD) is one of the largest crop worldwide. Due to two times of heterologous hybridization and two times of chromosome self-doubling, modern common wheat have a larger genome size (17 gigabase) than rice (466 megabases) and maize (2.3 gigabase) (Yu et al., 2002; Schnable et al., 2009; International Wheat Genome Sequencing Consortium [IWGSC], 2014). Because of its genome complexity and its big genome size, wheat chromosome sequencing is not possible in the last decade. However, with the advances of technologies, e.g., chromosome follow sorting and sequencing technology (next generation sequence/de novo assemble and pacbio), a reference genome of common wheat version TGACv1 is obtained by next generation sequence/de novo assembly (International Wheat Genome Sequencing Consortium [IWGSC], 2014), which is very attractive to wheat geneticists and breeders and highlights wheat genetic improvement.

In this study, we are aimed to identify TaGF14 numbers in wheat by searching the wheat whole genome through blast, to study the phylogenetic relationships with other plant species and to discuss the functions of TaGF14s.

MATERIALS AND METHODS

Plant Materials

The hard white winter wheat cultivar Jimai 22, released by our lab, was used in this study and was sown in a field at the Experimental Station of Shandong Academy of Agricultural Sciences (SAAS), Jinan, Shandong Province, China. The plot size was 12 m². Soil fertility was high. Weeds and diseases were controlled. Developing wheat ears were tagged at the onset of anthesis. Endosperm tissue was obtained from developing wheat grains (at Z71 and Z75) taken from the mid-ear region of the head (Zadoks et al., 1974).

RNA Extraction and Cloning of TaGF14-JM22

The total RNA was isolated from the developing grains or kernels at Zadok scale 71 according to the instructions of an RNaseasy Plant Mini Kit (Qiagen, Germany). RNase-free DNase I (Promega, United States) was used to remove any contaminating genomic DNA. Quality and integrity of the total RNA were determined by running the appropriate amount of RNA in a formamide denaturing gel. TaGF14-JM22 was cloned from wheat according to the methods described in the Supplementary Material. The cDNA sequence of TaGF14-JM22 obtained was submitted to GenBank, and the accession number is GenBank JF957590. The 3D structure of TaGF14-JM22 was predicted using the ExPASy proteomics online server and Swiss-Model⁴.

Construction of the Phylogenetic Tree and Expression of the TaGF14-JM22 Genes in Developing Grains

To determine the 14-3-3 gene numbers in wheat and to construct the phylogenetic tree of 14-3-3 genes from cereal crops and Arabidopsis, the coding sequence of TaGF14-JM22, cloned from wheat in this study, was used as the query to search the NCBi database² and the genome sequence databases of Sorghum¹, wheat⁴, and Brachypodium⁵ with a cut-off parameter of E-value ≤ 1E−10 for homologous GF14s. The phylogenetic tree was constructed using the maximum likelihood method with a Poisson distribution model and 1000 bootstrap replicates.

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¹https://swissmodel.expasy.org/
²https://www.ncbi.nlm.nih.gov/
³http://www.plantgd.b.org/S6GDB/
⁴https://urgi.versailles.inra.fr/blast/
⁵http://www.brachypodium.org/
by MEGA 6.0 (Tamura et al., 2013) based on the amino acid sequences of 14-3-3 proteins with a cut-off value of 50% for the condensed tree. In addition, the silicon expression profiles of TaGF14 in Root_Z13, Stem_Z30, Leaf_Z23, Spike_Z65, and the developing grains at 10, 20, and 30 days post anthesis (DPA) were obtained through WheatExp (Pearce et al., 2015) and analyzed. Data was analyzed with SAS software version 9.0. The mean expression values of every gene in different tissues were compared with each other, respectively. Duncan’s multiple range test was used to test for significant differences.

Expression and Purification

For cloning in pET29c, the TaGF14-JM22 sequence was amplified using the primers BamHI F and HindIII R. Amplicons were digested together with the pET29c vector and BamHI and HindIII enzymes at 37°C for 3 h. The digested products were purified and ligated together with T4 DNA ligase (Promega, United States) at 4°C overnight. The ligation mix was then transformed into Escherichia coli BL21 (DE3) for protein expression. The positive clones were screened for correct insertion by colony PCR and sequencing. The successful constructs were expected to express a TaGF14-JM22 fusion protein with an S-tag at the N-terminus. The recombinant proteins were purified using the S-tag rEK Purification Kit (Novagen, United States) according to the manual’s protocol. For more details, please see the Supplementary Material.

Amyloplast Isolation

The amyloplasts were isolated from the developing endosperm obtained from wheat grains (at Zadok scale 75) taken from the mid-ear region of the head as described by Tetlow et al. (2008). Starch granules were washed, and the granule-associated proteins, e.g., AGPase and GBSS, were extracted as described by Denyer et al. (1995). The protein content was measured using the Bio-Rad protein assay according to the manufacturer’s instructions and using thryoglobulin as a standard (Bio-Rad Lab., Canada).

Preparation of Peptides and Antisera

Polyclonal antibodies of starch biosynthetic enzymes were raised in rabbits against synthetic peptides, which were obtained from wheat grains (at Zadok scale 75) taken from the mid-ear region of the head as described by Tetlow et al. (2008). Starch granules were washed, and the granule-associated proteins, e.g., AGPase and GBSS, were extracted as described by Denyer et al. (1995). The protein content was measured using the Bio-Rad protein assay according to the manufacturer’s instructions and using thryoglobulin as a standard (Bio-Rad Lab., Canada).

Preparation of Peptides and Antisera

Polyclonal antibodies of starch biosynthetic enzymes were derived from N-terminal sequences of wheat AGP-L (CIIDMNARIGRVDVIVSN, Ainsworth et al., 1995), AGP-S (AIIDKNARIGENVKIIN, Rösti and Denyer, 2007), SSI (APAQPAPTQPLPDAG, Li et al., 1999), SSI (ARVDDDAASARQPRARRG, Li et al., 1999), GBSSI (QDLSWKPGAKNWEDV, Vrinten and Nakamura, 2000), SBEI (VSAQPDYTMATAEDGV, Rahman et al., 2001), SBEIIa (AASPGKVLHDPGDSEDDLAS, Rahman et al., 2001), SBEIIb (AGGPSGEMIDPGSS, Regina et al., 2005), DE (SVGVGEDLPEGYEQM, Bresolin et al., 2006), and SP (NYDELMGSLEGNYGRADYFLV, Tickle et al., 2009). The antigen was prepared by coupling the synthesized peptide to keyhole limpet haemocyanin using the heterobifunctional reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (Tetlow et al., 2008).

SDS-PAGE and Immunoblotting

The methods of SDS-PAGE and immunoblotting were according to Tetlow et al. (2008), for more detail, please see the Supplementary Materials. Gels were stained with a colloidal Coomassie Brilliant Blue G250 kit (Neuhoff et al., 1988).

RESULTS

Numbers of TaGF14 Genes and Phylogenetic Tree Construction

To explore the chromosomal locations and numbers of TaGF14 in wheat, the complete coding sequence of TaGF14-JM22 was used as the query to search the wheat whole-genome sequences published by IWGSC4. In total, 20 genes were obtained through Blast, and the coding sequences and chromosomal location of these genes are listed in Supplementary Table S1. In addition, these genes were located on wheat chromosome groups 2, 3, 4, and 7 (Figure 1). However, the 14-3-3 genes were not equally distributed on the wheat chromosome groups. In this study, eight genes were located on the wheat chromosome group 4 and the remaining 3 chromosome groups harbored equal numbers (four genes per group) of TaGF14 genes.

To investigate the evolutionary relationship among TaGF14-JM22 and other 14-3-3 genes and proteins derived from Oryza sativa, B. distachyon, Zea mays, Hordeum vulgare, and Arabidopsis thaliana, phylogenetic trees were constructed using the maximum likelihood method with a Poisson model and with 1000 bootstrap replicates by MEGA 6.0 (Tamura et al., 2013) based on the amino acid sequences of 14-3-3 proteins with a cut-off value of 50% for the condensed tree (Figure 2). The results showed that the 20 wheat TaGF14s could be divided into six clusters: cluster 1, including six genes (TaGF14d, TaGF14g, TaGF14j, TaGF14h, TaGF14c, and TaGF14n); cluster 2, including one gene (TaGF14k); cluster 3, including four genes (TaGF14b, TaGF14l, TaGF14m, and TaGF14s); cluster 4, including three genes (TaGF14a, TaGF14e, and TaGF14r); cluster 5, including two genes (TaGF14i and TaGF14f); and cluster 6, including four genes (TaGF14o, TaGF14p, TaGF14q, and TaGF14i). The results also showed that eighteen TaGF14s are non-ε proteins, except two wheat GF14 genes, TaGF14i and TaGF14j, which are ε proteins (Figure 2).

Expression of TaGF14s in Wheat

In order to investigate the gene expression levels of TaGF14 in the wheat root, stem, leaf, spike and developing grains at 10, 20, and 30 DPA, the silicon expression dataset was downloaded from WheatExp. As shown in Figure 3, it appeared that all TaGF14s were constitutively expressed, except two genes, i.e., TaGF14p, which was not observed, and TaGF14f, which showed tissue-specific expression in the root (Figure 3H and Supplementary Figure S6). In addition, TaGF14i was also likely expressed in a tissue-specific manner in stem_230, the
developing grains at 20 and 30 DPA. TaGF14o, TaGF14q, and TaGF14t, which belong to cluster 6, were expressed less in developing grains than in other investigated tissues (Supplementary Figure S6). These results indicated that TaGF14j, TaGF14l, and TaGF14i may play important role in the wheat grain-filling stage.

In addition, the gene expressions of TaGF14s were also determined in the wheat seedling stage treated with drought stress and heat stress. The results showed that five genes, i.e., TaGF14c, TaGF14d, TaGF14g, TaGF14h, and TaGF14j, were up-regulated expression under drought stress for both 1 and 6 h (Figure 4), suggesting these genes played vital role in combating against drought stress. However, all the TaGF14s were down-regulated expression under heat stress for both 1 and 6 h, which indicated that TaGF14s may be negatively associated with heat stress by reducing the expression to combat heat stress or through other pathways. Furthermore, the heatmap of TaGF14s were also drawn based on the gene expression data of TaGF14s. The results showed that TaGF14s in Root_Z13, Stem_Z30, Spike_Z65 and the developing grains at 20 DPA had the similar gene expression, while the rest had the similar gene expression pattern (Figure 5). And the TaGF14s clustered into three clusters based on gene expression in different samples or tissues, i.e., CL1, CL2, and CL3. And TaGF14h, TaGF14l, TaGF14m, and TaGF14s belonged to CL1. TaGF14d and TaGF14j belonged to CL3, while the rest belonged to CL2. These results suggested that TaGF14d and TaGF14j, both constitutively expressed, may participate in the whole wheat developing stages, e.g., grain-filling (starch biosynthesis) and may also participate in combating against drought stress.

**Cloning and Sequence Analysis of TaGF14-JM22**

To validate the gene function of TaGF14j, a homologous gene, TaGF14-JM22 was cloned and used for further analysis. The full-length cDNA of TaGF14-JM22, containing 786 nucleotides, was obtained using the RACE-PCR technique (Supplementary Table S3 and Supplementary Figures S1, S2) and submitted to GenBank (Accession number: JF957590). Multiple alignments showed that this sequence shared high identity with 14-3-3 proteins from other species ranging from 31 to 98% (Supplementary Figure S3), e.g., 98% identity with Brachypodium
Phylogenetic analysis of TaGF14s in common wheat with 14-3-3 proteins in other plant species. A rooted phylogenetic tree based on the sequence alignment using the MEGA 6.0 software from the CLUSTALW multiple sequence alignment. The scale represents estimated branch length. The TaGF14 genes from common wheat were marked in blue color.

(BdGF14f) and Oryza (OsGF14f) and 31% identity with Oryza (OsGF14h). TaGF14-JM22 was predicted to encode 261 amino acids (AA), with a predicted molecular mass of 29.27 kDa and an isoelectric point (pI) of 4.82. Structure analysis revealed that the predicted TaGF14-JM22 protein contained two 14-3-3 protein signatures and six functional motifs (Supplementary Table S2), such as a cAMP- (or cGMP-) dependent protein kinase phosphorylation site and a tyrosine kinase phosphorylation site, which were highly conserved in 14-3-3 homologs. Based on a WoLF PSORT analysis7, TaGF14-JM22 was located in the plasma membrane or nuclear plasma membrane. In addition, the three-dimensional (3D) structure prediction was analyzed by comparative protein modeling. The coding sequence of TaGF14-JM22 was submitted to the Swiss-Model online server8, and six 14-3-3-like proteins with sequence similarities of 90.60, 90.34, 90.17, 85.83, 84.86, and 84.52% were selected as templates to build models. Subsequently, nine models were generated using the abovementioned 14-3-3 proteins as models for the Swiss-Model homology modeling (Supplementary Figure S5). In addition, the QMEAN Z-score evaluations for the models were −1.12, −0.95, −1.50, −1.03, −0.50, −0.97, −1.33, −1.39, and −1.75, respectively, showing that the predicted models were of good quality. Furthermore, the phylogenetic results indicated that TaGF14-JM22, cloned in this study and belonging to non-ε protein, was closely related to three wheat genes (TaGF14d, TaGF14g, and TaGF14j) as well as OsGF14f and HvGF14f (Figure 2 and Supplementary Figure S3).

Validation the Function of TaGF14-JM22 in Developing Grains
To validate TaGF14-JM22 similar to TaGF14j participating in starch biosynthesis in developing grains, the coding sequence of TaGF14-JM22 was sub-cloned into pET29c. After induction by 1 mM IPTG at 37°C for 1, 3, 5, and 7 h, the highest

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7 https://www.genscript.com/wolf-psort.html
8 https://swissmodel.expasy.org/
expression occurred with 1 mM IPTG in both 5 and 7 h inductions at 37°C. SDS-PAGE was used for induction and purification of the TaGF14-JM22 protein. The protein with the highest abundance was found in the *E. coli* extracts. The molecular mass of the induced protein was about 29 kDa, which was in accordance with the predicted amino acid sequence (Supplementary Figure S4).

The purified recombinant TaGF14-JM22 protein was bound to S-protein agarose as a biochemical bait and then incubated with wheat amyloplast extract. Protein–protein interactions between the TaGF14-JM22 protein and ten key starch biosynthetic enzymes from amyloplasts, i.e., AGP-L, AGP-S, SSI, SSII, GBSSI, SBEI, SBEIIa, DE, SBEIIb, and SP, were investigated and analyzed by SDS-PAGE and western blotting. As shown in Figure 6, BSA, as a control, could not bind any starch biosynthetic enzymes, but protein–protein interactions between the TaGF14-JM22 protein and starch biosynthetic enzymes were observed. AGP-L, SSI, SSII, SBEIIa, and SBEIIb interacted with the TaGF14-JM22 protein (Figure 6). However, AGP-S, GBSSI, SBEI, DE, and SP could not interact with the TaGF14-JM22 protein. These results suggested that TaGF14-JM22 indeed participated in starch biosynthesis by binding to biosynthetic enzymes.

**DISCUSSION**

The family of 14-3-3 proteins is one of the families of regulatory proteins in plants (Aitken et al., 1992). Previous studies showed that plants have more 14-3-3 genes than animals. For example, human has seven 14-3-3 genes, while *Arabidopsis* and maize have thirteen 14-3-3 genes and twelve 14-3-3 genes, respectively (Wu et al., 1997; Iwata et al., 2000; Rosenquist et al., 2001). However, the number of 14-3-3 genes in common wheat and their relationships with other species are still unknown. In the present study, it was determined by Blastn against the whole genome sequences of Chinese Spring wheat released by International Wheat Genome Sequencing Consortium [IWGSC],
2014. The results indicated that common wheat harbored 20 GFI4s (Supplementary Table S1 and Figure 2), which was much more than rice and Brachypodium (Wu et al., 1997; Iwata et al., 2000; Rosenquist et al., 2001). Of all the genes, eight (40%) were located on wheat chromosome group 4 (Supplementary Table S1). In addition, the phylogenetic tree was constructed based on 14-3-3 protein sequences, which revealed that most of the TaGF14s, including five clusters (clusters 1–5), are non-$\varepsilon$ proteins, except cluster 6 (TaGF14f and TaGF14i) which are $\varepsilon$ proteins (Figure 2 and Supplementary Table S1).

**FIGURE 4** Wheat TaGF14s expression induced by drought and heat. Red, green, blue, and purple column represent drought stress for 1 h, drought stress for 6 h, heat stress for 1 h, and heat stress for 6 h. Bar represents the standard error. *At the top of each column indicates significant difference at $P = 0.05$.

**FIGURE 5** Heatmap of TaGF14s drawn with software R program (gplot) based on its expression data in different tissues or samples treated with drought and heat stresses. St, Stem_Z30. Sp, Spike_Z65. R, Root_Z13. L, Leaf_Z23. DG10, Developing grains_10 DPA. DG20, Developing grains_20 DPA. DG30, Developing grains_30 DPA.
The 14-3-3 proteins play important roles in diverse cellular processes by mediating protein-protein interactions in plants (Fulgosi et al., 2002). Previous studies indicated that HvGF14a was a protein induced by powdery mildew fungus, suggesting that it was involved in plant resistance to fungus infection in *H. vulgare* (Brandt et al., 1992). In *Brachypodium*, BdGF14f was significantly induced by Cr and cold stress (Cao et al., 2016). In addition, OsGF14f was constitutively expressed in rice (Yao et al., 2007). Previous studies indicated that starch was synthesized through the coordinated interactions of a suite of biosynthetic enzymes in plants (Zeeman et al., 2010). However, whether 14-3-3 as a regulatory protein involved in starch biosynthesis was known in wheat. And very little was also known about the functions of 14-3-3 proteins in wheat. In this study, TaGF14-JM22, which is similar to TaGF14d, TaGF14g and TaGF14j, the most highly expressed genes among the twenty TaGF14s in developing wheat grains, was used to investigate the protein-protein interactions between 14-3-3 proteins in wheat. In this study, TaGF14-JM22, which is similar to TaGF14d, TaGF14g and TaGF14j, the most highly expressed genes among the twenty TaGF14s in developing wheat grains, was used to investigate the protein-protein interactions between 14-3-3 proteins in wheat. In this study, TaGF14-JM22, which is similar to TaGF14d, TaGF14g and TaGF14j, the most highly expressed genes among the twenty TaGF14s in developing wheat grains, was used to investigate the protein-protein interactions between 14-3-3 proteins in wheat. 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and TaGF14j may also have the similar functions, e.g., resistance to pathogen and Cr stress, with other plant species. Furthermore, *Arabidopsis* GRF6 was linked to the “stay green” phenotype and drought tolerance by cotton transformation experiments (Yan et al., 2004). Our results indicated that the TaGF14s, closely related to GRF6, on group 7 that belong to cluster 6 may also be linked to the “stay green” phenotype (Figure 2). So, in our next project, the functions of TaGF14s, especially TaGF14s located on group 4 and group 7 will be further analyzed by gene overexpressing or gene silencing in wheat.

**AUTHOR CONTRIBUTIONS**

JS and JG conceived and designed the experiments. JS, JG, SD, and HL performed the experiments. JG, CL, XsC, and JS analyzed the data. DC, AL, XYc, SZ, ZZ, and JL contributed reagents/materials/analysis tools. JG and JS wrote the paper.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2018.00012/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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