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The Potential Wheat Signaling Pathways in Response to Abiotic Stress

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Abstract: The *Q* gene of wheat is a member of the AP2 class of transcription factors and has been shown to influence numerous morphological and domestication-related characters including plant height, flowering time, leaf morphology and spike architecture. To identify candidate genes involved with the transcriptional regulation networks of *Q* gene signaling pathways, I used the yeast two-hybrid system to identify proteins that directly interact with the Q protein. Five potential Q interactors were identified and they include protein kinases, transcription factors and a stress responsive protein. This research provides the basis for unraveling the signaling pathways and genetic networks governed by the *Q* gene.

Key words: Q interactors, yeast two-hybrid analysis

INTRODUCTION

Wheat, including bread wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD genomes) and durum wheat (*T. turgidum* L., 2n = 4x = 28, AABB genomes), is one of three major cereal crops consumed throughout the world. The wheat *Q* gene pleiotropically affects characteristics, such as free threshing, heading time, plant height, spike length, spikelet size, seed fertility, rachis fragility and glume shape and tenacity (Muramatsu, 1986; Kato *et al*., 2003; Jantasuriyarat *et al*., 2004; Simons *et al*., 2006). Thus, Q is a major regulatory gene involved in both vegetative and floral development. The *Q* gene was recently cloned and found to belong to the AP2/ERF family of transcription factors (Simons *et al*., 2006).

The AP2/ERF family of transcription factors consists of three subfamilies (Riechmann *et al*., 2000). The RAV subfamily is characterized by one AP2 DNA-binding domain and a B3 DNA-binding domain. They are involved in ethylene response (Alonso *et al*., 2003) and brassinosteroid response (Hu *et al*., 2004). The ERF subfamily has one AP2 binding domain and its members have very diverse functions including hormonal signal transduction (Ohme-Takagi and Shinshi, 1995), biotic and abiotic stress response (Stockinger *et al*., 1997; Liu *et al*., 1998; Dubouzet *et al*., 2003), metabolism regulation (van der Fits and Memelink, 2000; Aharoni *et al*., 2004; Broun *et al*., 2004; Zhang *et al*., 2005) and developmental functions (van der Graaff *et al*., 2000; Banno *et al*., 2001; Chuck *et al*., 2002). The third subfamily is the AP2 subfamily having two AP2 binding domains and regulates various developmental processes. The Q gene is classified into this subfamily due to the presence of two AP2 DNA-binding domains (Simons *et al*., 2006).

Fourteen genes have been classified as belonging to the AP2 subfamily in *Arabidopsis* (Kim *et al*., 2006). Of these, *APETALA2* (*AP2*) has been the most extensively characterized. *AP2* is involved in establishing floral meristem identity and regulating floral homeotic gene expression (Komaki *et al*., 1988; Bowman *et al*., 1991; Jofuku *et al*., 1994). *AP2* also regulates the stem cell niche in shoot meristems in a dosage dependent manner (Wurschum *et al*., 2006) and it influences gibberellic regulation of metabolism in seed development, sucrose sensing, flowering time, leaf number, soluble sucrose metabolism and yield (Jofuku *et al*., 2005; Ohto *et al*., 2005). Analysis of mutants and/or over expression studies of other *Arabidopsis* genes within the *AP2* subfamily, such as *TOE1*, *TOE2*, *WRINKLED1*, *SCHLAFUMTZE*, *SCHNARCHZAPFEN*, *ANTEGUMENTA*, *PLETHORA1*,

PLETHORA2 and *BABYBOOM*, have implicated them in developmental roles (Boutilier *et al*., 2002; Aukerman and Sakai, 2003; Schmid *et al*., 2003; Aida *et al*., 2004; Cernac and Benning, 2004; Nole-Wilson *et al*., 2005).

Outside of *Arabidopsis*, the study of *AP2*-like genes has been very limited. In maize *INDETERMINATE SPIKELET1* suppresses indeterminate growth of the spikelet (Chuck *et al*., 1998) and *GLOSSY15* regulates the identity of leaf epidermal cells during the vegetative phase transition from juvenile to adult plants (Moose and Sisco, 1996). Other *AP2*-like genes have been identified in other flowering plants (Maes *et al*., 2001; Boutilier *et al*., 2002) and gymnosperms (Vahala *et al*., 2001), but thus far no genes within the AP2 subfamily have been reported to have functions other than developmental regulation. Yeast two-hybrid (Y2H) experiments were used to begin unraveling the signaling network associated with Q.

MATERIALS AND METHODS

Plant Materials

The *Q* gene cDNA was cloned from leaves of *T. aestivum* cultivar Chinese Spring (CS). Leaf tissue of the *T. durum* cultivar Langdon (LDN) was used to construct a cDNA library for yeast two-hybrid screening. Spike tissue of *T. aestivum* cultivar Bobwhite (*QQ* genotype) was used for the cloning of genes in the targeted Y2H approach.

Plasmid Vectors, Bacterial and Yeast Strains and Culture Conditions

E. coli strains DH5α and TOP10 (Invitrogen, Carlsbad, CA, USA) were used for transformation after DNA ligation and TOPO cloning, respectively. Constructs in the bait vector pGBKT7 were selected by 50 μg mL^{-1} kanamycin and constructs in the prey vector pGADT7 were under 200 μg mL^{-1} ampicillin selection. All *E. coli* cells were grown in standard Luria-Bertrani medium at 37°C.

Cloning of Full-length cDNAs of Q and Putative Q Interactors

Wheat total RNA was isolated using TRIzol® Reagent (Invitrogen). To clone the full-length coding region of the *Q* gene, reverse transcription polymerase chain reaction (RT-PCR) was performed according to the protocol of the SMART™ RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA) followed by PCR amplification using primers 5'-CATATG GTG CTG GAT CTC AAT GTG GAG TC-3' (created *Nde*I restriction enzyme site underlined) and 5'-GGATCC TCA GTT GTC CGG CGG GCG GGG GAA-3' (created *BamH*I site underlined) based on GenBank sequence AY702956. The cloned *Q* gene coding region was excised from the vector by *Nde*I and *Bam*HI restriction enzymes and ligated into pGBKT7 as the bait for yeast two-hybrid analysis. For the Q interactors, first-strand cDNA was synthesized from total RNA using a random hexamer primer followed by PCR amplification using the primers listed in Table 1. PCR amplifications were performed using a combination of Taq DNA polymerase (New England Biolabs, Beverly, MA, USA) and Pfu Turbo (Stratagene, La Jolla, CA, USA) with the GeneAmp PCR System 9700 (ABI, Applied Biosystems, Foster City, CA, USA). PCR products were cloned into pCR® 2.1-TOPO® vector (Invitrogen) and sequenced. Recombinant plasmid DNA was isolated using the QIAGEN (Valencia, CA, USA) mini-prep kit.

Yeast Two-hybrid Analyses for Identification of Q Interactors

The yeast two-hybrid Matchmaker™ Library Construction and Screening kit was used for analysis of protein-protein interactions according to the manufacturer's protocols (Clontech). The *Q* gene was ligated into pGBKT7 as bait and pGADT7 was used to construct prey of a cDNA library or was used in the targeted Y2H approach. Cells of yeast strain Y187 were transformed with the bait

Table 1: Primers used to clone putative wheat Q interactors. Restriction sites are underlined

Gene	Primer sequences
Stress responsive protein	
Forward (*Nde*I)	5'-CATATGGACTACTACCGCGAGACC-3'
Reverse (*Bam*HI)	5'-GGATCCTTATTCTGCGGGGTAGGTAGC-3'
TaERF7	
Forward (*Nde*I)	5'-CATATGGCGCCTAGAGCGGCGGAGA-3'
Reverse (*Bam*HI)	5'-GGATCCTAGTCCTCGGAAGGCGGCGG-3'
TaTGA2	
Forward (*Eco*RI)	5'-GAATTCATGGCAGATGCTAGCTCAAGGA-3'
Reverse (*Bam*HI)	5'-GGATCCTTATTCCCTGGGCCTAGCAAG-3'
TaPKS3	
Forward (*Nde*I)	5'-CATATGGAGAACAGTGGGAAGATCGT-3'
Reverse (*Bam*HI)	5'-GGATCCTCAGCTCCATGCCATGCCCA-3'
TaSOS2	
Forward (*Eco*RI)	5'-GAATTCATGGCGGCGGCGGCGGGCA-3'
Reverse (*Bam*HI)	5'-GGATCCTATTTCGCTATTGTGTTGGATTCA-3'

plasmids and AH109 cells were used for the prey plasmids. The Fast Yeast Transformation Kit™ (G-Biosciences/Genotech, St. Louis, MO, USA) was used, according to the manufacturer's protocols.

For Y2H library screening, a cDNA library was constructed in pGADT7, according to the instructions provided by the manufacturer (Clontech). Total RNA from leaves of wheat seedlings at the 2-leaf-stage was used as template for the cDNA library construction. For targeted Y2H analysis, cDNAs were generated from total RNA isolated from immature spikes of wheat. Yeast strains Y187 and AH109 were mated on YPD plates overnight. Then the yeast cells were cultured in SD medium without leucine (L) and tryptophan (W) for two days. To test the protein interactions, the SD/-L-W cultured yeast cells were inoculated on SD plates containing X-Gal but lacking leucine, tryptophan, histidine (H) and adenine (A). The empty vectors pGBKT7 and pGADT7 were used as negative controls and the interaction between p53 and LTA (large T-antigen) served as a positive control. Prey plasmids were isolated from yeast and transformed into *E. coli*. The isolated plasmid from *E. coli* was then retransformed back to yeast AH109 and the mating assays were repeated using SD medium with X-Gal and under -L-W-H-A stringent selection. The Q interactors of positive colonies from the cDNA library screening were sequenced. Sequence data were queried onto the NCBI database (http://www.ncbi.nlm.nih.gov/) and the DFCI (http://compbio.dfci.harvard.edu/tgi/) *Triticum aestivum* Gene Index (TaGI) using BlastN.

RESULTS AND DISCUSSION

The *Q* gene is present in all domesticated wheat and it most profoundly affects spike architecture and grain threshability (Muramatsu, 1986). However, the *Q* gene is also expressed in other tissues, such as roots and leaves and it is known to influence morphology of the vegetative tissues (Simons *et al*., 2006). Whereas members of the AP2 subfamily of AP2 genes have not been associated with stress, many members of the other AP2 subfamilies are stress-related genes. Therefore, I investigated the role of the *Q* gene in wheat in response to abiotic stresses.

Because the *Q* gene product is a transcription factor, I hypothesized that protein-protein interactions play a critical role for the regulation of transcriptional activity. Q is not auto-activated in the Y2H system, as confirmed by the absence of interaction with AD empty vector or LTA (Fig. 1). Similar to most transcription factors, Q has the ability to form a homodimer (Fig. 1). To further understand how the *Q* gene is involved in regulatory networks, I screened a cDNA library and also applied a targeted Y2H approach to identify gene products that directly interact with the Q protein.

After screening approximately 10 million yeast colonies from a cDNA library made from leaves, 52 initial putative positive clones were further validated by a second round of selection. Three

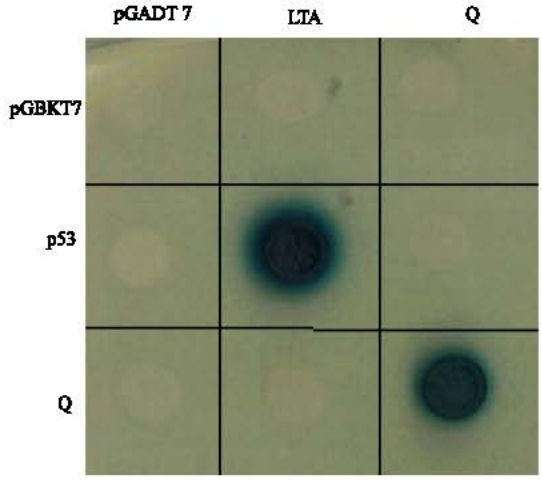

Fig. 1: Yeast two-hybrid analysis. Bait: pGBKT7 (empty vector), p53 and Q. Prey: pGADT7 (empty vector), LTA (large T-antigen) and Q. The interaction between p53 and LTA serves as a positive control. Yeast cells were incubated under highly stringent selection (the absence of leucine, tryptophan, histidine and adenine) and the medium contained X-Gal. The yeast cells can grow and show blue on the plate only when the bait and prey proteins interact with each other

independent clones of the same gene were confirmed to be positive for encoding a Stress Responsive Protein (SRP) (Table 2, Fig. 2). Interestingly, a rice homolog of the stress responsive protein was characterized to be induced by ABA and salt (Moons *et al*., 1995). The exact biochemical function of this stress responsive protein remains unknown.

Furthermore, I followed the targeted Y2H approach published by Menke *et al.* (2005), especially for the low expression levels of transcription factors. Because the Q protein is a member of the AP2 transcription factor family and is physiologically responsive to stresses, I hypothesize that the Q protein may interact with other proteins that are involved in stress stimuli. Therefore, I chose well-characterized transcription factors (ERF, TGA and WRKY) and protein kinases (SOS2 and PKS3) as candidates.

I did identify an ERF in wheat that interacted with the Q protein, while there were no positive interactions between any WRKY protein and the Q protein. The AP2/ERF family of transcription factors includes the ERF and AP2 subfamilies (Riechmann *et al*., 2000; McGrath *et al*., 2005; Nakano *et al*., 2006). AP2/ERF proteins are important for the transcriptional regulation of development and various responses to environmental stimuli (Riechmann and Meyerowitz, 1998). The ERF family proteins usually participate in the crosstalk of regulatory networks in response to different stimuli, such as salt, drought, cold and ABA (Shinozaki *et al*., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005; Fujita *et al*., 2006). I designated the wheat ERF-like protein that interacted with the Q protein as TaERF7 because of its similarity to AtERF7 (GenBank accession number NP-188666), which was found to have 38% identity at the amino acid level. In *Arabidopsis*, AtERF7 plays a critical role in drought and ABA responses and the transcriptional complex may be regulated by PKS3 (Song *et al*., 2005), which also interacts with the Q protein. AtERF7 interacts with and is thereby phosphorylated by PKS3. The regulation of the AtERF7 network may involve chromatin remodeling, because AtERF7 also interacts with AtSin3 that in turn interacts with the histone deacetylase HDA19 (Song *et al*., 2005).

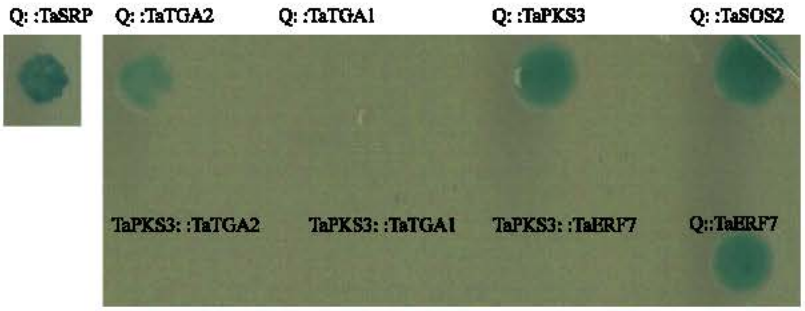

Fig. 2: Putative wheat Q interactors identified by library screening and the direct targeted approach. Each pair is shown as bait::prey combination. The selection condition was the same as in Fig. 1 and also described in the Materials and Methods

Table 2: Identified wheat Q interactors by yeast two-hybrid analysis

Gene	Database accession No.
Stress responsive protein	NCBI DQ022951
TaTGA2	TaGI TC235293
TaERF7	TaGI TC238185
TaPKS3	TaGI TC235974
TaSOS2	TaGI TC248207

Intriguingly, I also identified PKS3 and SOS2 homologs in wheat, designated TaPKS3 and TaSOS2, respectively, which interacted with the Q protein. Both SOS2 and PKS3 are Ser/Thr protein kinases. *Arabidopsis* SOS2 interacts with another protein kinase known as SOS3 and plays a role in salt tolerance (Guo *et al*., 2004). PKS3 physically interacts with the protein phosphatase ABI2 and is a negative regulator of ABA responses (Guo *et al*., 2002). SOS2 only weakly interacts with AtERF7, but PKS3 strongly interacts with AtERF7 (Song *et al*., 2005). Whereas both TaSOS2 and TaPKS3 can interact with Q protein, no detectable interaction was identified between TaPKS3 and TaERF7 (Fig. 2).

The TGA family of transcription factors belongs to a class of basic leucine zipper (bZIP) proteins. NPR1 is a critical regulator of the SA-mediated systemic acquired resistance (Cao *et al*., 1994; Delaney *et al*., 1995). It has been shown in *Arabidoposis* that members of the TGA family proteins differentially interact with NPR1 in the Y2H system, with TGA2, TGA3 and TGA6 showing strong binding (Zhang *et al*., 1999; Zhou *et al*., 2000), whereas TGA1 and TGA4 do not bind to NPR1 in Y2H assays. These results show that the Q protein also interacts differentially with TGA transcription factors because Q interacted with TaTGA2 but not with TaTGA1 (Fig. 2). In addition to the results of *Arabidopsis*, accumulating evidence suggests that NPR1 is pivotal for the regulation network in response to pathogens in rice (Chern *et al*., 2005) and wheat (Makandar *et al*., 2006). Usually there are cross-talking networks among responses to ABA, JA, SA and ethylene (Pieterse and Van Loon, 2004; Fujita *et al*., 2006). How the Q protein integrates the signaling pathways is largely unknown so far. However, the identification of Q interactors, including protein kinases and transcription factors, provides the basis for unraveling the pathways and mechanisms of Q-mediated regulation.

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