Cloning of a down-regulated gene encoding small GTP binding protein in hybrid wheat*

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Abstract Previous studies showed that differential gene expression between wheat hybrids and their parents was responsible for the heterosis. To provide an insight into the molecular basis of wheat heterosis, one cDNA, designated *TaRab*, was identified from the cDNA library of wheat seedling leaves. The sequence comparison in GenBank revealed that *TaRab* is homologous to a group of genes encoding Rab-GTP binding protein. Semi-quantitative RT-PCR analysis indicated that *TaRab* was expressed in all plant tissues examined, but at slightly higher level in leaves. Further analysis exhibited that *TaRab* displayed lower expression in hybrid than in its patents in both roots and leaves, which was in agreement with the original results of suppression subtractive hybridization. *TaRab* was located on chromosome 7B and C-7DS5-0.36 by in silico mapping. The relationship between differential expression of *TaRab* and the molecular basis of wheat heterosis was also discussed.

Keywords: wheat, Rab protein, gene expression, heterosis.

Hybrid cultivars have been used commercially in many crops and have made significant contribution to the world food supply. However, molecular mechanism of heterosis remains to be revealed[1]. It has been suggested that molecular foundation of phenotypic changes could reside in the variability of genome expression. Although all the genes in hybrid F_1 are derived from its parental inbreds, hybrid performance is quite different from its parental inbreds. Therefore, it is reasonable to speculate that differential gene expression between hybrids and their parents should be responsible for the observed heterosis[2]. Previous studies detected significant difference in mRNA quantity and expression patterns between hybrids and their parental inbreds. Further analysis indicated that differential gene expression patterns between hybrid and its parents in rice, maize and wheat were correlated with heterosis[3—6]. Recently, attempts have been made to clone and characterize differentially expressed cDNAs between hybrid and its parents. Hu et al.[1)] identified three genes which are differentially expressed between hybrid and its parents. Ni et al.[7] cloned a cDNA encoding for a novel RNA-binding protein that was specifically expressed in F_1 but not in the parents and discussed its possible role in wheat heterosis. Wu et al.[8] reported the cloning of 24 differentially expressed leaf cDNAs between wheat hybrids and their parents. Here we report the identification of a hybrid down-regulated gene encoding GTP-binding protein in seedling leaves.

GTP-binding proteins (G proteins) have the ability to specifically bind and subsequently hydrolyze guanine nucleotides, thereby use the GTP/GDP cycle as a molecular switch in which the active/inactive state depends on the binding of GTP or GDP, respectively[9]. Small GTP-binding proteins are monomeric G proteins with molecular masses of 20—40 kD. Rab proteins exist in all eukaryotic cells and form the largest branch of the small G protein superfamily, and they have been recognized as molecular switches and the central regulators involved in endocytotic and exocytotic pathways, cytoplasmic Ca^{2+} concentration, movement of the secretory vesicles and/or regulate the fusion of these vesicles with cellular membrane compartments[10]. A large body of evidence has accumulated in support of a role for Rab proteins in vesicle trafficking from yeast to human. In plants,

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1) Hu J. G. Isolation and cloning of the genes differentially expressed between hybrid and its parents in maize. Ph.D. Dissertation, Fudan university, 1997

such transport pathways are important for storage protein accumulation, cell growth and differentiation, secretion of protein and biosynthesis of polysaccharide components of the cell wall and cell plate. The complete members of Rab family in the *Caenorhabditis elegans*, *Drosophila melanogaster*, *Homo sapiens* and *Arabidopsis thaliana* have been identified, and this family is extremely well conserved among divergent eukaryotes, including vertebrates, invertebrates, fungi and plants, indicating their essential roles in all eukaryotes[11—13]. Recently, a novel member of the Arabidopsis Rab family, Ara6, was isolated representing a plant-specific subtype of Rab protein[14]. Up to date, no GTP-binding protein gene that is differentially expressed between hybrid and its parent has been reported. In addition, to our knowledge, no gene encoding Rab protein has been cloned in wheat.

In this study, using one EST obtained by suppression subtractive hybridization (SSH) as probe, we identified one cDNA with a complete open reading frame (ORF), and designated it *TaRab*. The spatiotemporal expression pattern of *TaRab* was analyzed by semi-quantitative RT-PCR. At the same time, differentially expressed patterns of *TaRab* between hybrid and its parents were also examined. Based on the above analysis, together with the results of in silico mapping, the relationship between differential expression of *TaRab* and the molecular basis of wheat heterosis was also discussed.

1 Materials and methods

1.1 Materials

One highly heterotic interspecific hybrid 3338/2463 and its female parent 3338 (*Triticum asetivum*), male parent 2463 (*Triticum spelta*) were cultivated in vermiculite that was watered by nutrient solution every 3 days. Seedling leaves of common wheat 3338 were collected for the cDNA library construction. The tissues of hybrid and its parents such as roots and leaves at the trefoil and tillering stage, roots, leaves and shoots at the jointing stage and flag leaves, spikes and internode below spike at the booting stage were collected for RT-PCR analysis.

1.2 Methods

1.2.1 RNA isolation Total RNA was isolated according to the method described by Ni et al.[7]. Poly (A) RNA was purified from total RNA using the PolyATract mRNA isolation system (Promega) according to the manufacturer's instruction.

1.2.2 Construction and screening of cDNA library Oligo (dT)-primed cDNA was synthesized from 5 μg of the poly (A) + RNA preparation of 3338 seedlings using Super Script cDNA synthesis kit (Pharmacia) according to the instruction of the supplier. cDNAs were size-fractioned by pre-packed oligo (dT)-cellulose spun column (Pharmacia) and then ligated into the *Sal* I-*Not* I sites of λ gt 22A vector. The primary library size was 2.0×10^5. The size of cDNA inserts was between 0.5 and 7.0 kb. The library was screened with reamplified cDNA fragment using coventional hybridization methods as described by Sambrook et al.[15]

1.2.3 Sequencing and bioinformatics analysis cDNA clone obtained from the library was sequenced. The sequences were used for a BLAST search against NCBI (http://www.ncbi.nlm.nih.gov) protein database using BLASTX. Sequence analysis was performed using the software DNAMAN (Version 3.0, Lynnon BioSoft). Protein alignments were performed with the CLUSTALX program. Putative amino acid sequences analysis and structure prediction were performed at ExPaSy (http://www.expasy.org). In silico mapping on the Chinese Spring deletion map by BLASTN alignment was carried out at Graingenes (http://www.graingenes.org/blast_databases.html).

1.2.4 Semi-quantitative RT-PCR analysis Gene-specific RT-PCR primers were designed according to the nucleotide sequences of *TaRab*: 5′-GGCGAAACCAAAGAATGGCGTACC-3′; 5′-ACGGATTAGGATGAGCAGCATCCAGCC-3′. Three RT-PCR replications were conducted using independently isolated total RNAs with the following thermal cycling parameters: 94°C for 30 sec, 68°C for 1 min, and 72°C for 1 min. Various numbers of PCR cycles (25, 30, 35) were tested to ensure that the reactions had not reached the plateau. A 350 bp β-actin gene fragment was amplified as a positive control using the primer pair 5′-CAGCAACTGGGATGATATGG-3′ and 5′-ATTTCGCTTTCAGCAGTGGT-3′. The RT-PCR products were sequenced to verify the PCR amplification fragment.

2 Results

2.1 Cloning of the wheat *TaRab*

SSH was performed between wheat hybrid leaves

and its parents leaves. Equal amount (1 μg) ploy (A) mRNA of two parents was mixed and used as "tester" and 2 μg ploy (A) mRNA from hybrid was used as "driver". One hybrid down regulated EST was identified from the subtractive library (Our unpublished results). Using this EST as the probe, one cDNA, designated *TaRab*, was identified from the wheat leaf cDNA library. Fig. 1 shows nucleotide sequence of *TaRab* and its deduced amino acid sequence. The 1280 bp cDNA contains a complete open reading frame of 651 bp, which begins at nucleotide 246 and ends at position 896 and encodes a protein of 216 amino acid residues, with a predicted molecular mass of 24125.24 Da and a pI of 5.51.

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1       GCGGCCGCTCTTCCTGCCCCTTGCCATTCCAAGAACGTTCTCCGGGGCGTCTCCTCCTCC
61      TCCCACCACCCCATCCCGTTGCTGTAACTCCGATAAGCGCGCGCCGCCGCCGACGGCCCC
121     AGAAGCCACCTCCGAGCTGCCATCCGCGGGAAGAGCGGGTGGCTTGACCGATTCTGCGAG
181     GACGAAGAGGAGGACGAACGTACGAGGGCGTGGCCTACACGTGACCACCGCGGCGAAACC
241     AAAGAATGGCGTACCGGGCGGACGACGACTACGACTACCTCTTCAAGGTGGTGCTCATCG
            M  A  Y  R  A  D  D  D  Y  D  Y  L  F  K  V  V  L  I
301     GCGACTCGGGCGTCGGGAAATCCAACCTCCTCACCCGGTTCACGCGCAACGAGTTCAGCC
        G  D  S  G  V  G  K  S  N  L  L  T  R  F  T  R  N  E  F  S
361     TGGAGTCAAAGTCCACAATCGGGGTCGAGTTCGCCACCAGGAGCATTAGGGTCGACGATA
        L  E  S  K  S  T  I  G  V  E  F  A  T  R  S  I  R  V  D  D
421     AGGTCGTCAAGGCGCAGATCTGGGATACGGCTGGCCAAGAGAGATATCGTGCAATTACAA
        K  V  V  K  A  Q  I  W  D  T  A  G  Q  E  R  Y  R  A  I  T
481     GCGCATATTACAGAGGAGCTGTCGGTGCGCTTGTTGTGTATGATGTGACACGCCATGTGA
        S  A  Y  Y  R  G  A  V  G  A  L  V  V  Y  D  V  T  R  H  V
541     CCTTCGAGAATGTGGAGAGGTGGCTGAAGGAGCTCAAGGACCATACCGATGCTAACATTG
        T  F  E  N  V  E  R  W  L  K  E  L  K  D  H  T  D  A  N  I
601     TGATCATGCTAGTCGGCAACAAGGCTGACCTGCGCCACTTGCGGGCTGTCTCTGTGGAGG
        V  I  M  L  V  G  N  K  A  D  L  R  H  L  R  A  V  S  V  E
661     ATGCCAAGGCATTTGCGGAGAGGGAATACACCTTTTTCATGGAAACCTCTGCCCTGGAGT
        D  A  K  A  F  A  E  R  E  Y  T  F  F  M  E  T  S  A  L  E
721     CCATGAATGTAGAGGACGCTTTCACTGAGGTGTTAACCCAGATCTACCGAGTGGTCAGCA
        S  M  N  V  E  D  A  F  T  E  V  L  T  Q  I  Y  R  V  V  S
781     AGAAAGCTCTTGACATCGGCGACGACCCTGCCGCTCCACCACGAGGGCAGACCATCAACG
        K  K  A  L  D  I  G  D  D  P  A  A  P  P  R  G  Q  T  I  N
841     TAGGGTCCAAAGACGATGTCTCTGCCGTAAAGAAGGCTGGATGCTGCTCATCCTAATCCG
        V  G  S  K  D  D  V  S  A  V  K  K  A  G  C  C  S  S  *
901     TAGACTTTGAAAAGTATACCGGGCCCTTGTTTATCATTGATGGATGGGTAGCTGCGTCTT
961     GGGCGTGCTTTTGGAGGCCCATACTTAACCCAGGGGGTTGGAAACGATATAAAACACTAA
1021    CAGTAATAGAACTAGCAGCCGTTTTTTGGTGGCAGTCATGGTATCCTAATTGTTGCAGTC
1081    TTGGTTCATATCGGACGCCAAGGATCCCTTCCACAATCGATTATGTATGTACCCTATTAA
1141    GTATGTGTAACTGAAGCTTTCATCCTTTTCTCCTTTCTTTTGAGAGAAACTGTTGTTATC
1201    TTGTTTGTATGATTCTTCACATTTTGCTAATCAGTGACATAATGTAAATCTTATGGAGGA
1261    ATACTATTAAAAAAAAAAAA
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Fig. 1. The nucleotide and putative amino acid sequences of *TaRab*.

2.2 Sequence analysis of TaRab protein

BLASTX analysis revealed that *TaRab* has a high similarity to a group of Rab-GTP binding proteins of different species, and its amino acid sequence similarity with *Arabidopsis thaliana* (BAB10106), *Pisum sativum* (BAA02112), *Nicotiana tabacum* (T03620) and *Oryza sativa* (BAB90506) reached 90%, 88%, 86% and 79%, respectively.

Structure analysis showed that TaRab protein contains four conserved functional domains, which has been found among many small GTP-binding proteins (Fig. 2). Among them, domain GXXGXGKS from 19 to 27 residue is considered to be the phosphoryl binding sites; domain DXXG from 67 to 70 residue is involved in the Mg^{2+}-binding; the domain from 125—128 NKXD participates in the binding of guanine; domain ESTA (153—156) is also related to GTP binding[16]. It has been shown that these domains are critical for binding of GTP, GDP, dGTP, ppGpp and analogs of GTP and GDP[17]. Besides the four typical GTP-binding protein domains, a specific domain in Rab subfamily (YYRGA)[18] was also found in TaRab protein. In addition, the residues from 41 to 49 are highly conserved, which can be recognized by several GTPase-activation proteins (GAPs), and is essential for regulation of GTPase[19].

Different from other small G proteins, Rab proteins exhibit a variety of prenylation motifs at their C-termini, containing either one or, more frequently, two cysteine residues, both of which are modified by geranylgeranyl groups and Rab escort protein[10]. Interestingly, *TaRab* also had this C-terminal motif with two cysteine residues, which is a prenylation site and important for small GTP-binding protein to membrane attachment.

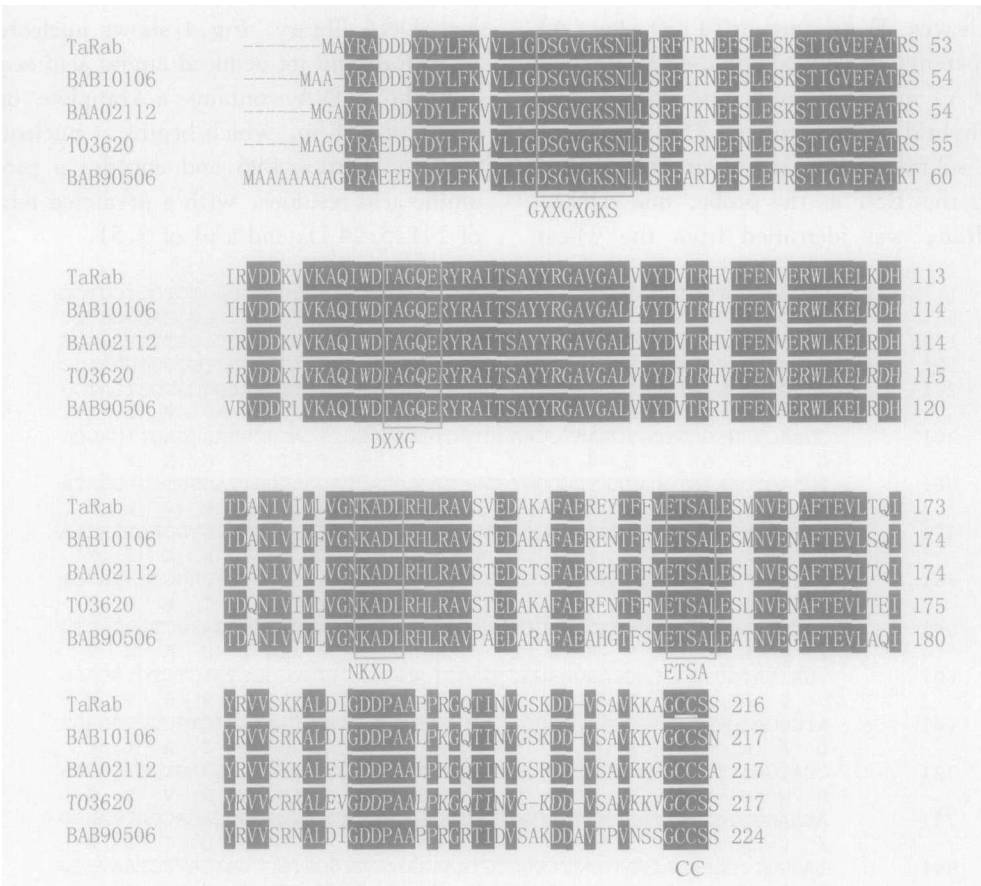

Fig. 2. The deduced amino acid sequence comparison of *TaRab* with GTP-binding proteins in other plants. Alignment with other species *Arabidopsis thaliana* (BAB10106), *Pisum sativum* (BAA02112), *Nicotiana tabacum* (T03620) and *Oryza sativa* (BAB90506) was performed using the program CLUSTALX. The shaded amino acids represent identical amino acids. Four highly conserved domains in all small GTP-binding proteins are boxed. Italics show domain YYRGA that is specific for Rab family.

2.3 Expression pattern of *TaRab*

2.3.1 Expression patterns in different tissues

Expression pattern of *TaRab* in roots and leaves of trefoil and tillering stages, roots, leaves and shoots of jointing stage and flag leaves, spikes and internode below spike of booting stage was examined by semi-quantitative RT-PCR. The results revealed that *TaRab* showed a constitutive expression pattern in root and leaf at the trefoil stage. While at the tillering and jointing stages, *TaRab* had a higher expression level in leaf. At the booting stage, the expression level of *TaRab* in flag leaf was higher than that in root, spike, and internode blow spike (Fig. 3). BLASTX analysis showed that *TaRab* exhibits a high amino acid sequence identity to Rab11 (*Nicotiana tabacum*, Q40521) and rb1b (*Arabidopsis thaliana*, Q39222), and the expression patterns have demonstrated that Rab11 had a higher expression level in shoots[20], and rb1b is detected mostly in root hairs[21], which differ obviously from that of *TaRab*. Recently, it was reported that each member of Rab proteins had a specific subcellular localization and many with specific patterns of tissue distribution. Therefore, the functions of *TaRab* may be different from that of Rab11 and rb1b.

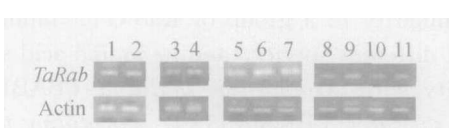

Fig. 3. Tissue expression pattern of *TaRab*. 1, root at the trefoil stage; 2, leaf at the trefoil stage; 3, root at the tillering stage; 4, leaf at the tillering stage; 5, root at the jointing stage; 6, leaf at the jointing stage; 7, shoot at the jointing stage; 8, root at the booting stage; 9, flag leaf the at booting stage; 10, spike at the booting stage; 11, internode below spike at the booting stage.

Temporal expression analysis in root and leaf of *TaRab* was also determined. Semi-quantitative RT-PCR indicated that *TaRab* was transcribed constitutively in root of four stages (trefoil, tillering, jointing and booting stage). In addition, the expression level of *TaRab* in leaf was higher at the booting stage than

that at the other three stages (Fig.4).

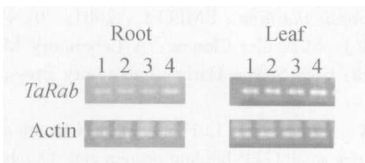

Fig. 4. Expression analysis of *TaRab* at root and leaf at different developmental stages. 1—4 represent trefoil, tillering, jointing and booting stage, respectively.

2.3.2 Differential expression between hybrid and its parents Since the EST probe for cDNA library screening was derived from subtracted library of parent by SSH, which can enrich genes that are down-regulated in hybrid or equally expressed to the lowly expressed parent, to further confirm the differential expression patterns of *TaRab* between hybrid and its parent, RT-PCR experiment was performed (Fig. 5). The results indicated that *TaRab* was expressed at a lower level in root of the trefoil stage and leaf of the tillering stage in hybrid, and equally expressed to the lowly expressed male parent 2463 in root of the tillering stage and leaf of the trefoil stage. This down-regulated expression pattern in hybrid was in good agreement with the results of SSH.

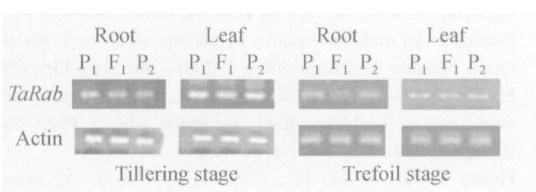

Fig. 5. Differential expression between hybrid and its parents of *TaRab*. P1, female parent 3338; F1, hybrid 3338/2463; P2, male parent 2463.

2.4 In silico mapping of *TaRab*

Up to date, over 16000 ESTs had been mapped to wheat chromosome deletion lines, and can be used for in silico mapping analysis[22]. BLASTN search showed that *TaRab* has 98% identity to EST BG263021, which was located at centromere of 7B and C-7DS5-0.36 on wheat chromosome. In addition, *TaRab* showed high amino acid sequence identity to three Rab proteins of rice (NP-916817, NP-916116, NP-915496), which were located at the locus of 114.1, 129, and 143.7 cM on chromosome 3, respectively.

3 Discussion

Wheat hybrids demonstrate heterosis in terms of biomass, productivity and better stability of performance over environments. It has been shown that wheat hybrids were different from their parental inbreds in many biochemical properties, such as mitochondrial oxidation and phosphorylation, nucleic acid synthesis, phytohormone levels and enzyme activities[2]. Recently, by using differential display of mRNA, we detected significant differences in mRNA quantity and patterns between wheat hybrids and their parental inbreds. Further analysis showed that genes occurred in either of the parents but not in F1 was positively correlated with heterosis in some traits[23]. He et al.[24] globally compared gene expression of synthetic hexaploid wheat with its diploid (*Aegilops tauschii*) and tetraploid (*T. turgidum*) parents by cDNA-AFLP display. The results demonstrated that the expression of a significant fraction of genes altered in the synthetic hexaploid, and most of them appeared to be diminished. Therefore, it is worthy to isolate the down-regulated genes in hybrid, and *TaRab* identified in this study belongs to this category. RT-PCR results exhibited that *TaRab* had a lower expression level in hybrid as compared to their corresponding parents.

By in silico mapping, *TaRab* was preliminarily located at wheat centromere of 7B and C-7DS5-0.36, in which two QTLs for wheat thousand-grains-weight were detected[25,26]. Although the relation between *TaRab* and thousand-grains-weight needs more analysis, a combination of systematic identification of differentially expressed genes with comparative mapping should provide a tool to understand molecular basis of heterosis.

Rab family protein was shown to participate in intracellular transport and secretion and regulate vesicle docking and fusion events during both endocytic and biosynthetic transport. In plants, these vesicle-mediated transports are important for storage protein accumulation, cell growth and differentiation, secretion of protein and polysaccharide components of the cell wall and cell plate. Tobacco plants transformed with *rgp1*, a gene encoding a Ras-related small GTP-binding protein, were previously shown to exhibit a distinct reduction in apical dominance with increased tillering[27,28]. *TaRab* was found to be abundantly expressed in leaf, shoot, spike and internode below spike, which are highly active tissues in vesicle traffic for biosynthesis and cell division. Therefore, *TaRab* may be involved in transport in these processes. However, our speculation about the function of

TaRab protein is only based on analysis of other previously reported Rab proteins, further studies are needed to investigate its role in vesicle-mediated transports and its relation to wheat heterosis.

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