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Divergent Functions of orthologous NAC Transcription Factors in Wheat and Rice

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Abstract

The wheat *GPC-B1* gene located on chromosome 6B is an early regulator of senescence and affects remobilization of protein and minerals to the grain. *GPC-B1* is a NAC transcription factor and has a paralogous copy on chromosome 2B in tetraploid wheat, *GPC-B2*. The closest rice homolog to both wheat *GPC* genes is *Os07g37920* which is located on rice chromosome 2 and is colinear with *GPC-B2*. Since rice is a diploid species with a sequenced genome, we initiated the study of *Os07g37920* to develop a simpler model to study senescence and mineral remobilization in cereals. We developed eleven independent RNA interference transgenic rice lines (*Os07g37920-RNAi*) and 10 over-expressing transgenic lines (*Os07g37920-OE*), but none of them showed differences in senescence. Transgenic *Os07g37920-RNAi* rice plants had reduced proportions of viable pollen grains and were male-sterile, but were able to produce seeds by cross pollination. Analysis of the flower morphology of the transgenic rice plants showed that anthers failed to dehisce. Transgenic *Os07g37920-OE* lines showed no sterility or anther dehiscence problems. *Os07g37920* transcript levels were higher in stamens compared to leaves and significantly reduced in the transgenic *Os07g37920-RNAi* plants. Wheat *GPC* genes showed the opposite transcription profile (higher transcript levels in leaves than in flowers) and plants carrying knock-out mutations of all *GPC-1* and *GPC-2* genes exhibited delayed senescence but normal anther dehiscence and fertility. These results indicate a functional divergence of the homologous wheat and rice *NAC* genes and suggest the need for separate studies of the function and targets of these transcription factors in wheat and rice.

Keywords

male sterility; senescence; NAC transcription factor; wheat; rice

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INTRODUCTION

Senescence is a complex and tightly regulated degeneration process controlled by multiple environmental and developmental signals (Breeze et al. 2011). In monocarpic plants, such as wheat, senescence acts at the whole-plant level. During senescence, genetically-programmed and developmentally-controlled catabolic activities convert cellular material into exportable nutrients that are remobilized from the leaves to the grain (Hörtensteiner and Feller 2002; Matile et al. 1996). Thus, senescence has a large impact on grain nutrient and mineral concentration, which, in turn, significantly affects plant survival, fitness and the nutritional value of these grain crops (Yoshida 2003). In spite of its importance, the underlying mechanisms controlling the initiation and progression of terminal senescence remain unclear, largely due to their complex nature and the limited number of studies focused on this developmental stage.

We have recently shown the existence of a close connection between senescence and nutrient remobilization through the map-based cloning of the *GPC-B1* (*Grain Protein Content 1* or *TtNAM-B1*) gene from wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*). The 6B chromosome segment including the *GPC-B1* gene was initially introgressed into durum and common wheat as a source of genetic variation for grain protein content (Joppa and Cantrell 1990) and was later shown to accelerate senescence (Uauy et al. 2006a) and to increase the grain protein (N), zinc and iron concentrations (Distelfeld et al. 2007). Positional cloning of this gene showed that *GPC-B1* is a NAC transcription factor related to the Arabidopsis *NO APICAL MERISTEM* (*NAM*) gene. A paralogous gene, designated *GPC-2*is present on chromosomes 2B (*GPC-B2*) and 2D (*GPC-D2*) of hexaploid wheat and shows a similar transcription profile to *GPC-B1* (Uauy et al. 2006b). Low levels of transcripts from all *GPC* genes are first detected in flag leaves seven days prior to anthesis (Zadok 45-49) and increase progressively towards maturity, a transcription profile which is consistent with a role in senescence. Reduction of the transcript levels of the multiple *GPC* homologues by RNA interference (RNAi) delayed senescence by several weeks and reduced nitrogen, zinc, and iron content in the grain by over 30 % (Uauy et al. 2006b).

The NAC domain genes are plant-specific transcriptional regulators known to be involved in several processes including developmental programs, defense and abiotic stress responses and senescence (Olsen et al. 2005). Computational analyses identified 151 and 117 non-redundant NAC genes in rice and Arabidopsis, respectively (Nuruzzaman et al. 2010). In these two species, the NAC genes were classified according to putative function into two groups and several subgroups (Nuruzzaman et al. 2010; Ooka et al. 2003). NAC proteins have a characteristic NAC domain (**N**AM; **A**TAF1,2; **C**UC2) that is divided into five sub-domains in the N-terminal region. This domain has been shown to mediate DNA binding as well as protein-protein interactions (Ernst et al. 2004).

The senescence-associated expression pattern of dozens of NAC family members (Buchanan-Wollaston et al. 2005; Breeze et al. 2011; Guo et al. 2004; Cantu et al. 2011) suggests that they play a prominent role in senescence. Several members of the NAC family

from Arabidopsis and rice such as *ORE1*/*ANAC092*/*AtNAC2*, *ORS1*, *AtNAP* and *OsNAC5* have been functionally characterized and shown to be involved in the regulation of leaf senescence (Balazadeh et al. 2008; Sperotto et al. 2009; Guo and Gan 2006; Kim et al. 2009; Balazadeh et al. 2011). Interestingly, the rice *OsNAP* gene was able to restore the Arabidopsis *atnap* null mutant when engineered under the regulation of the *AtNAP* promoter suggesting these genes share a similar regulatory role in plant leaf senescence (Guo and Gan 2006).

The long-term goal of this project is to provide a better understanding of the genes and transcriptional networks controlling senescence and nutrient remobilization in grasses. In the current research we identified the orthologous gene to the wheat *GPC* genes in rice, functionally characterized it and evaluated its relevance for studying senescence and mineral remobilization processes in cereals.

MATERIALS AND METHODS

Phylogenetic analysis

Based on previous comprehensive phylogenetic analyses of the NAC family, we selected sequences from rice proteins belonging to the SNAC subgroup (Nuruzzaman et al. 2010) and from Arabidopsis proteins belonging to the TAPNAC subgroup (Alvarado 2007). We excluded sequences with incomplete NAC domains (Os05g34830), very divergent NAC domains (Os02g12310 and Os05g34310), or almost identical sequences (Os12g03040 was eliminated and the closely related Os11g03300 retained). Only amino acids within the five NAC domains were used in the phylogenetic analysis (as reported in Uauy et al. 2006b), because other regions of these proteins cannot be precisely aligned among all proteins. The NAC domain sequences were aligned using ClustalW (Thompson et al. 1994). Phylogenetic trees were constructed using four different methods to test the robustness of the group including the GPC proteins: Neighbor-Joining, Minimum Evolution, Maximum Parsimony, and Unweighted Pair Groups Using Arithmetic Averages (UPGMA) as described in MEGA software version 4 (Tamura et al. 2007). The stability of the different nodes was tested using bootstrap analysis with 1000 replicates as implemented in the same version of MEGA.

Generation of RNA interference and over-expression lines for *Os07g37920*

RNA interference (RNAi) lines—We used a 293-bp segment from *Os07g37920* (GenBank *Os07g0566500*) including the region between 1239-bp to 1531-bp from the start codon. We excluded the five conserved domains characteristic of the NAC family of transcription factors from our RNAi trigger to avoid down-regulation of other NAC genes. BLASTN of this 293-bp stretch confirms that there are no stretches of 21-bp identical to other genes in rice and that therefore, the trigger sequence is specific to the *Os07g37920* gene. This segment was cloned in forward and reverse orientations into the vector pANDA provided by Daisuke Miki and Ko Shimamoto as described previously (Miki et al. 2005).

Over-expression (OE) lines—The complete coding sequence from cDNA of *Os07g37920* was amplified by PCR using adapter primers to incorporate Gateway compatible recombination attB1 and attB2 sites on the 5’ and 3’ ends of the amplified

product, respectively. This fragment was subsequently cloned into pDONR207 (Invitrogen) using the BP recombination reaction (GATEWAY™, Invitrogen) and sequenced. An LR reaction (GATEWAY™, Invitrogen) between *Os07g37920*-pDONR207 and the NTAPi vector (Rohila et al. 2004) resulted in a plasmid consisting of the *Os07g37920* gene fused to a tandem affinity purification (TAP) tag under the control of a constitutive promoter (2-kb maize ubiquitin promoter and intron 1). Transgenic plants (Oryza sativa spp. Japonica cv. Kitaake) were produced at the Ralph M. Parsons Foundation Plant Transformation Facility, University of California at Davis using *Agrobacterium*.

Wheat mutant screen

GPC-A1—Specific primers (TAGCTTGCTAGGGGGAACGAAGAAGATCC and CGTCCAACTGATGAGACGACGTACAGAA, the underlined G is an intentional mismatch introduced to achieve homoeologue-specific amplification) were designed to amplify the first 953-bp of the *GPC-A1* gene. This primer pair was used to amplify DNA pools from Ethyl methanesulfonate (EMS) treated population of tetraploid wheat (cv. Kronos) and the detection of mutants was performed as described before (Slade et al. 2005).

GPC-B2—Specific primers (ACAGTCCACAGGGGTGCT and TGGTGATGGAGCAGTGAATC) were designed to amplify the first 1038-bp of the *GPC-B2* gene. This primer pair was used to amplify DNA pools from EMS treated population of tetraploid wheat (cv. Kronos) and the detection of mutants was performed as described before (Uauy et al. 2009).

Genetic markers, genetic map and physical maps

Genetic markers—We developed a derived cleaved amplified polymorphic (dCAP) marker for the *GPC-B2* gene using primers CATGCACGCAACAAATGATA and GCCACATATATCCATGCAATGT, where T is the degenerate nucleotide used to generate a *Taq*I restriction site. These primers amplified a 149-bp product from the 3'UTR of *GPC-B2* in tetraploid wheat. Digestion of the amplified product with the restriction enzyme *Taq*I yielded two fragments of 20 and 129-bp in durum wheat (cv. Langdon) and an undigested fragment in wild emmer wheat, *Triticum turgidum* ssp. *dicoccoides*(acc# G18-16).

We also developed a CAP marker for a Single Nucleotide Polymorphism (SNP) detected in the expressed sequence tag (EST) BF291736 to establish an additional reference point in the comparison between wheat and rice. Primers GCAATGGGGAATGTAAGAATCT and CAGATGACCATATAAATGCACG were used to amplify 413-bp from tetraploid wheat. Digestion of the amplified product with the restriction enzyme *Rsa*I yielded three fragments of 38, 144 and 231-bp in Langdon and two fragments of 144 and 269-bp in G18-16.

Wheat genetic map—A population of 152 recombinant inbred lines (RILs) was produced from a cross between wild emmer wheat, *Triticum turgidum* ssp. *dicoccoides*, accession 'G18-16' and durum wheat 'cv. Langdon' (Peleg et al. 2008). Multi-locus ordering and validation were carried out using the procedures described before (Mester et al. 2003) and implemented in 'MultiPoint' package (http://www.MultiQTL.com).

The wheat physical map was adapted from previously published maps (Sourdille et al. 2004; Conley et al. 2004). The rice physical map was adapted from the Rice Functional Genomics Database website (http://signal.salk.edu/cgi-bin/RiceGE).

Quantitative PCR experiments

Quantitative PCR experiments were performed on Kitaake rice and Kronos wheat grown under long-day greenhouse conditions. RNA was extracted using Spectrum plant total RNA kit (Sigma-Aldrich, Saint Louis, MO). First strand cDNA was synthesized from 1 μg of total RNA with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). For Q-PCR amplification in wheat, we used the previously published primer sets for *GPC-B2* (Uauy et al. 2006b) and *TEF1* as endogenous control (Distelfeld et al. 2009). For Q-PCR amplification in rice, we developed new primers sets for *Os07g37920* (CGTCTCTCTCAGGCTGGATG and CCGTTTACGAGGAAGGTGTC, efficiency= 99%) and for *OsTEF1* (GCAGGAACTACTGCACCAAGG and GAGCCTGCTGAATCACATCA, efficiency=99%). Q-PCR was performed on an ABI PRISM 7000 SDS (Applied Biosystems, Foster City, CA, USA) using SYBR® GREEN. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to normalize and calibrate transcript values relative to the endogenous control.

Pollen viability test

In vitro germination was estimated following the method of Kariya (1989) at 20 °C in an artificial germination medium containing 20% sucrose, 1% agar and 20 ppm boric acid. In addition pollen grain starch was stained with Lugol iodine solution (Sygma-Aldrich) to estimate the proportion of viable pollen grains.

Chlorophyll measurements

Relative chlorophyll levels in senescing leaves were measured using a hand-held chlorophyll meter (SPAD) from wild-type, Δ*GPC-A1*, Δ*GPC-B2* and Δ*GPC-A1*/Δ*GPC-B2* double mutant wheat plants at 40, 50 and 60 days after anthesis (DAA). The averages of ten SPAD measurements per Flag-1 leaf were obtained for at least ten biological replicates of each genotype.

RESULTS

Identification of the orthologous rice gene to the *GPC* genes

To identify the orthologous rice NAC gene to the *GPC* genes we used 10 and 12 non-redundant NAC proteins from rice and Arabidopsis, respectively. The NAC domains of GPC-B1 and GPC-B2 proteins were aligned with these NAC sequences and a combined phylogenetic tree was constructed. Examination of the tree revealed that the *GPC* genes cluster with the rice gene *Os07g37920* (Fig. 1). *Os07g37920* is the closest rice homologue to both *GPC-1* and *GPC-2*. It is also possible that an orthologous copy of *GPC-1* was deleted in the rice lineage. The *Os07g37920* and *GPC* branch includes 3 Arabidopsis genes; *At1g52880*, *At3g15510* and *At1g61110* (Fig. 1). This cluster was supported by a bootstrap value of 76 in the Neighbor Joining tree (Fig.1). The same group of six proteins was clustered together in all three additional phylogenetic analyses used in this study (Minimum

Evolution, bootstrap 78; Maximum Parsimony, bootstrap 60; and UPGMA, bootstrap 87, data not shown) providing additional support to the close relationship among these proteins.

GPC-B2 and *Os07g37920* are located in syntenic wheat-rice regions

The *GPC-B2* gene was previously mapped to the centromeric region of the short arm of chromosome 2B (BIN C-2BS1-0.53) using nulli-tetrasomic, ditelocentric and deletion lines (Uauy et al. 2006b). To map this gene more precisely in wheat, we developed a derived cleaved amplified polymorphic (dCAP) marker for the *GPC-B2* gene (see Materials and Methods). In addition, we developed a CAP marker for the expressed sequence tag (EST) BF291736 previously mapped in the physical bin C-2BS1-0.53 (http://probes.pw.usda.gov:8080/snpworld/Map) (see Materials and Methods). These two markers were mapped on a population of 152 F6 recombinant inbred lines (RILs) from Langdon×G18-16 cross, kindly provided by Dr. Tzion Fahima from the University of Haifa, Israel.

GPC-B2 and *BF291736* loci were mapped within a 20 cM interval in the centromeric region of chromosome arm 2BS, flanked by SSR markers *gwm1171* and *gwm374* (Fig. 2). The *BF291736* locus was mapped 9.5 cM distal to *GPC-B2* and 3.9 cM proximal to *gwm374*. Sourdille et al. (2004) mapped *gwm374* to the proximal region of chromosome 2BS using wheat deletion lines (C-2BS1-0.53 bin) confirming our previous assignment of *GPC-B2* to this centromeric bin. Conley et al. (2004) further dissected the C-2BS1-0.53 centromeric bin into 3 consensus bins (designated CS, 1S and 2S from proximal to distal, Fig. 2) and mapped BF291736 and other wheat ESTs to CS, the most proximal bin (Fig. 2). Genes in this wheat 2BS region are colinear to genes in the long arm of rice chromosome 7, which includes *Os07g40320* (the rice orthologue of wheat EST BF291736) and *Os07g37920* (the rice gene showing greatest similarity to wheat *GPC*Fig. 1). Figure 2 shows the location of *Os07g37920*the flanking rice genes and their relationship with wheat ESTs mapped within the CS bin. Taken together, the genetic map and the phylogenetic analysis suggest that *GPC-B2* and *Os07g37920* are orthologous genes.

Transgenic RNAi and OE lines for *Os07g37920*

To functionally characterize the rice NAC transcription factor *Os07g37920*an RNAi construct was transformed into the rice variety Kitaake, selected for its high transformation efficiency. The RNAi construct was designed to target the 3’ end of the *Os07g37920* gene, outside the NAC domain to avoid interference with other NAC transcription factors.

Surprisingly, eleven independent transformation events (out of fourteen) produced no viable seeds. Plants from two of these events were pollinated with pollen from non-transgenic Kitaake plants and developed fertile seeds (backcross1 generation), indicating that the pistils of the transgenic plants were functional. The sterility of the male flower organ was correlated with an abnormal anther phenotype during anthesis (Fig. 3). The anthers of the transgenic plants were able to emerge from the glumes, and although they contained pollen, the grains were not released from the pollen sacs. At a later stage, as the anthers dried out, they retained their yellow color, showing that the pollen remained inside. Some pollen grains of the *Os07g37920* RNAi transgenic plants were able to germinate on artificial media (Fig. 4A-B). However, when treated with Lugol iodine, the proportion of stained pollen

grains in the transgenic plants was lower than in wild-type plants (64.2% ± 8.02 in RNAi versus 98.4% ±2.5 in wild-type, average stained pollen grains ± standard deviation) (Fig. 4C-D). The sterile *Os07g37920* RNAi transgenic plants senesced normally in comparison with wild type Kitaake plants and no other phenotypic differences were detected. The male sterile phenotype was repeated in all the seven backcrossed plants. Southern blot analysis showed at least two copies of the RNAi construct in each of the plants (data not shown).

Ten independent transformation events were obtained for the *Os07g37920* over expression construct. The plants were grown in a glasshouse and, in contrast to the transgenic RNAi lines there was no evidence of any effect on fertility or anther dehiscence. In addition, all plants exhibited normal growth and senescence and no phenotypic differences were detected between the transgenic and control lines.

The rice *Os07g37920* and wheat *GPC-B2* transcription profiles

Fang et al. (2008) showed that *Os07g37920* transcript levels were highest in the stamen (1 day before flowering) relative to other tissues. Our quantitative PCR experiments confirmed this finding and show that the expression of *Os07g37920* is up-regulated during flower development and leaf senescence (Fig. 5A). The expression of *Os07g37920* in senesced rice leaves was about half of that in the stamens. The expression of *Os07g37920* in the RNAi lines was reduced by 50% confirming an efficient silencing in the rice transgenic lines (dashed line, Fig. 5A). In wheat, *GPC-B2* expression was low in the stamen compared to senesced leaves and did not increase with flower development (Fig. 5B). As previously reported by Uauy et al (2006b), *GPC-B2* expression in the leaves increased dramatically during senescence (Fig. 5B).

Wheat *GPC* knock-out mutants show delayed senescence but normal anther dehiscence

All tetraploid durum cultivars analyzed so far (including Kronos) have a natural deletion of the *GPC-A2* gene and a non-functional copy of *GPC-B1* (Uauy et al. 2006b). Therefore, when screening for deleterious mutations for these paralogous genes, we needed only to target the *GPC-A1* and *GPC-B2* copies to eliminate all functional copies of both *GPC-1* and *GPC-2*.

GPC-A1—The screening of the tetraploid mutant population resulted in the identification of seventeen independent mutations in the amplified fragment of the *GPC-A1* gene. Among these mutations we selected the substitution of guanine to adenine in position 561 from the start codon (G561A) because this mutation results in a premature stop codon at position 114 of the GPC-A1 protein. This protein truncation will be referred hereafter as Δ*GPC-A1*. A CAP marker based on the *Bsr*I restriction site lost in the G561A mutation was developed to facilitate the selection of this mutation in subsequent crosses.

GPC-B2—a similar screen to identify mutations for the *GPC-B2* gene revealed 6 independent mutations that affect the GPC-B2 protein sequence. The most promising mutation was a substitution of guanine to adenine in position 516 from the start codon (G516A) that is translated into a premature stop codon at position 109 of the GPC-B2 protein. This protein truncation will be referred hereafter as Δ*GPC-B2*. The G516A

substitution also resulted in the loss of a *Bsr*I restriction site, and we used this difference to develop a CAP marker to facilitate the selection process.

Seeds of the Δ*GPC-A1* knock-out mutants were sown and later crossed with non-mutagenized Kronos to reduce the mutation load. The F_1 plants were self-pollinated and homozygous plants for Δ*GPC-A1* mutation were selected from the F_2 progeny. Selected homozygous *GPC-A1* mutant plants were crossed with the Δ*GPC-B2* mutants to combine both mutations. The hybrids of this cross were backcrossed twice to Kronos in order to further reduce the mutation load. BC_2F_2 plants were used to select single (Δ*GPC-A1* and Δ*GPC-B2*) and double (Δ*GPC-A1*/Δ*GPC-B2*) mutants. The selected plants were self-pollinated (BC_2F_3) and homozygous mutants were selected and evaluated under field conditions in the winter of 2010-2011 in Israel. Plants grown in the glasshouse were used to determine relative rates of senescence.

Flower and anther morphology of the knockout mutants compared to the wild type sister line were normal (data not shown). The number of seeds per spikelet was measured to detect and quantify any potential effect of the null mutants on the plant's fertility (Fig. 6A). No significant differences were detected between the genotypes although the double mutant showed a small decrease in number of seeds per spikelet. SPAD measurements to quantify relative leaf chlorophyll content were taken from each genotype at 40, 50 and 60 DAA (Figure 6B). An ANOVA including time, *GPC-A1*, *GPC-B2* and all possible two-way interactions showed highly significant differences for *GPC-A1* ($P<0.0001$) between wild type and mutant alleles. The adjusted means of the chlorophyll content in the lines with the *GPC-A1* mutant allele were almost twice as high as the means in the lines with the wild type alleles. The differences between wild type and mutant alleles for the *GPC-B2* locus were non-significant ($P= 0.06$), and the interaction between the two loci were also non-significant ($P= 0.08$). Differences in chlorophyll content in time were highly significant ($P= 0.0008$) (Figure 6B).

DISCUSSION

Os07g37920 is the orthologous rice gene to the *GPC* genes

All phylogenetic analyses using rice and Arabidopsis NAC genes show that *Os07g37920* is the most similar rice gene to the *GPC* genes in wheat, suggesting that it is the orthologous gene. This hypothesis is also supported by the genetic and physical mapping of *GPC-B2* and *Os07g37920* to colinear regions in wheat and rice (Fig. 2). These results suggest that the copy on chromosome 6B likely originated from a duplication that occurred in the wheat lineage after its divergence from the rice lineage. However, we cannot rule out the possibility of a deletion of a gene colinear to *GPC-1* in the rice lineage.

All the phylogenetic analyses also showed a group of three related Arabidopsis genes (*At1g52880*, *At3g15510*, and *At1g61110*) that are grouped with the cluster including the wheat *GPC* and rice *Os07g37920* genes, suggesting that they are the closest homeologs of the wheat and rice genes. This hypothesis is also supported by the increased transcript levels of the rice and Arabidopsis genes from this group during anther development (see next section).

Rice *Os07g37920* and wheat *GPC* genes have divergent functions

The results of our study demonstrate the importance of *Os07g37920* for anther development in rice. Our results show that *Os07g37920* is expressed at the highest levels in rice stamens and that a reduction of *Os07g37920* transcript levels resulted in male sterility in eleven independent transgenic lines. This phenotype was not observed in any of the ten *Os07g37920* over-expressing transgenic lines, suggesting that the sterility observed in the RNAi lines is caused specifically by this construct and was not the result of the transformation events themselves. The closest Arabidopsis gene to *Os07g37920* and the wheat *GPC* genes, *At1g61110* is expressed specifically in the tapetum and maximal expression occurs when the pollen grain undergoes maturation. Interestingly, Arabidopsis *At1g61110* null mutants failed to show any phenotype, most likely due to gene redundancy (Alvarado 2007).

In wild type plants, the tapetum cell layer in the anther undergoes cellular degradation during late stages of anther development. This degradation process is important for pollen grain maturation and is considered to be a programmed cell death (PCD) event. Therefore, it is possible that the lack of anther dehiscence in our rice *Os07g37920* RNAi transgenic plants is related to a modification of the tapetum senescence. More detailed anatomical studies will be required to test this hypothesis.

In contrast to the *Os07g37920-RNAi* rice results, reduction of *GPC-1* and *GPC*-2 expression in the transgenic RNAi wheat plants had no effect on fertility (Uauy et al. 2006b). Since the *GPC*-RNAi only reduced the *GPC* transcript levels by 50%, it is possible that the residual activity was sufficient to trigger anther senescence. This does not seem to be the case, since the wheat mutant lines with no functional *GPC* copies showed normal anther dehiscence and set seed normally by self-pollination. In addition, senescence was significantly delayed in the Δ*GPC-A1* and Δ*GPC-A1*/Δ*GPC-B2* double mutant plants, confirming the earlier finding that in wheat, the *GPC-1* gene plays an important role in the initiation and progression of senescence. The Δ*GPC-B2* truncation showed a non-significant effect but the *P* values were close to the significance level ($P = 0.06$). In addition the double Δ*GPC-A1*/Δ*GPC-B2* mutant showed a slight increase in the delay of senescence compared with the Δ*GPC-A1* alone. Although these results show that the *GPC-B2* gene has a weaker role in terminal senescence than the *GPC-A1* gene, it is possible that the presence of the Δ*GPC-B2* mutation enhances the effect of the *GPC-A1* mutations in the double mutants. Testing this hypothesis will require further experimentation with a larger number of replications and higher statistical power.

These results indicate that, despite their orthologous relationship, the wheat *GPC* genes and the rice *Os07g37920* genes have divergent functions. It is possible that the duplication of the wheat *GPC* genes after the wheat-rice divergence facilitated the functional divergence of the wheat genes.

Interestingly, another NAC transcription factor, Arabidopsis *AtNAP* (Fig. 1, ATNAP), seems to play a role in both senescence and fertility. *AtNAP* T-DNA mutant lines showed delayed senescence (Guo and Gan 2006) while *AtNAP* RNAi transgenic lines showed inflorescences with short stamens and their anthers often did not dehisce (Sablowski and Meyerowitz

1998). The different phenotypes reported in the previous two studies might be due to the different Arabidopsis ecotypes that were used: Landsberg erecta (Sablowski and Meyerowitz 1998) versus Columbia (Guo and Gan 2006) or to the different approaches employed: T-DNA insertion mutation versus antisense RNAi.

Different expression patterns for *Os07g37920* and the wheat *GPC* genes

Os07g37920 and the wheat *GPC* genes show divergent expression patterns with different relative transcript levels in leaves and anthers. In wheat, the highest transcript levels of *GPC-B2* was observed in the leaves while in rice the highest transcript level of *Os07g37920* was found in the stamens. It is possible that the different transcription profiles of these orthologous genes are related to their divergent functions.

In summary, our results confirmed that the wheat *GPC* genes have a significant effect on terminal senescence and that the *GPC-A1* gene has a larger phenotypic effect than the *GPC-B2* gene. Our results also showed that the rice *GPC* orthologue has no effect on terminal senescence but that it could be useful to engineer male sterility in rice. The functional differentiation of these genes precludes the use of the rice *GPC* orthologue as a model to study the effect of the wheat *GPC* genes on terminal senescence. Therefore the characterization of the role of the *GPC* in terminal senescence and nutrient remobilization will require dedicated studies in wheat. This study also provides an example of the dynamic nature of the large NAC transcription factor family and their potential to evolve different and novel functions.

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References

Alvarado, V. Ph.D thesis. College Station: Texas A&M; 2007. Analysis of the Arabidopsis NAC gene superfamily in plant development; p. 154

Balazadeh S, Kwasniewski M, Caldana C, Mehrnia M, Zanor MI, XueZanor G-P, Mueller-Roeber B. ORS1, an H_2O_2-Responsive NAC Transcription Factor, Controls Senescence in Arabidopsis thaliana. Mol Plant. 2011; 4:346–360. [PubMed: 21303842]

Balazadeh S, Riaño-Pachón DM, Mueller-Roeber B. Transcription factors regulating leaf senescence in *Arabidopsis thaliana*. Plant Biol. 2008; 10:63–75. [PubMed: 18721312]

Breeze E, Harrison E, McHattie S, Hughes L, Hickman R, Hill C, Kiddle S, Kim YS, Penfold CA, Jenkins D, Zhang CJ, Morris K, Jenner C, Jackson S, Thomas B, Tabrett A, Legaie R, Moore JD, Wild DL, Ott S, Rand D, Beynon J, Denby K, Mead A, Buchanan-Wollaston V. High-Resolution Temporal Profiling of Transcripts during Arabidopsis Leaf Senescence Reveals a Distinct Chronology of Processes and Regulation. Plant Cell. 2011; 23:873–894. [PubMed: 21447789]

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ. Comparative transcriptome analysis reveals significant differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J. 2005; 42:567–585. [PubMed: 15860015]

Cantu D, Pearce SP, Distelfeld A, Christiansen MW, Uauy C, Akhunov E, Fahima T, Dubcovsky J. Effect of the down-regulation of the high Grain Protein Content (*GPC*) genes on the wheat transcriptome during monocarpic senescence. BMC Genomics. 2011; 12:492. [PubMed: 21981858]

Conley EJ, Nduati V, Gonzalez-Hernandez JL, Mesfin A, Trudeau-Spanjers M, Chao S, Lazo GR, Hummel DD, Anderson OD, Qi LL, Gill BS, Echalier B, Linkiewicz AM, Dubcovsky J, Akhunov ED, Dyorak J, Peng JH, Lapitan NLV, Pathan MS, Nguyen HT, Ma XF, Miftahudin, Gustafson JP, Greene RA, Sorrells ME, Hossain G, Kalavacharla V, Kianian SF, Sidhu K, Dijbirligi M, Gill KS, Choi DW, Fenton RD, Close TJ, McGuire PE, Qualset CO, Anderson JA. A 2600-locus chromosome bin map of wheat homoeologous group 2 reveals interstitial gene-rich islands and colinearity with rice. Genetics. 2004; 168:625–637. [PubMed: 15514040]

Distelfeld A, Cakmak I, Peleg Z, Ozturk L, Yazici AM, Budak H, Saranga Y, Fahima T. Multiple QTL-effects of wheat *Gpc-B1* locus on grain protein and micronutrient concentrations. Physiol Plantarum. 2007; 129:635–643.

Distelfeld A, Tranquilli G, Li C, Yan L, Dubcovsky J. Genetic and molecular characterization of the *VRN2* loci in tetraploid wheat. Plant Physiol. 2009; 149:245–257. [PubMed: 19005084]

Ernst HA, Olsen AN, Skriver K, Larsen S, Lo Leggio L. Structure of the conserved domain of *ANAC*, a member of the NAC family of transcription factors. EMBO Reports. 2004; 5:297–303. [PubMed: 15083810]

Fang YJ, You J, Xie KB, Xie WB, Xiong LZ. Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. Mol Genet Genomics. 2008; 280:547–563. [PubMed: 18813954]

Guo Y, Cai Z, Gan S. Transcriptome of Arabidopsis leaf senescence. Plant Cell Environ. 2004; 27:521–549.

Guo YF, Gan SS. *AtNAP*, a NAC family transcription factor, has an important role in leaf senescence. Plant J. 2006; 46:601–612. [PubMed: 16640597]

Hörtensteiner S, Feller U. Nitrogen metabolism and remobilization during senescence. J Exp Bot. 2002; 53:927–937. [PubMed: 11912235]

Joppa L, Cantrell R. Chromosomal location of genes for grain protein content in wild tetraploid wheat. Crop Sci. 1990; 30:1059–1064.

Kariya K. Sterility caused by cooling treatment at the flowering stage in rice plants. Jpn J Crop Sci. 1989; 58:96–102.

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG. Trifurcate Feed-Forward Regulation of Age-Dependent Cell Death Involving miR164 in Arabidopsis. Science. 2009; 323:1053–1057. [PubMed: 19229035]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Matile P, Hortensteiner S, Thomas H, Krautler B. Chlorophyll Breakdown in Senescent Leaves. Plant Physiol. 1996; 112:1403–1409. [PubMed: 12226455]

Mester D, Ronin Y, Minkov D, Nevo E, Korol A. Constructing large-scale genetic maps using an evolutionary strategy algorithm. Genetics. 2003; 165:2269–2282. [PubMed: 14704202]

Miki D, Itoh R, Shimamoto K. RNA silencing of single and multiple members in a gene family of rice. Plant Physiol. 2005; 138:1903–1913. [PubMed: 16172097]

Nuruzzaman M, Manimekalai R, Sharoni AM, Satoh K, Kondoh H, Ooka H, Kikuchi S. Genome-wide analysis of NAC transcription factor family in rice. Gene. 2010; 465:30–44. [PubMed: 20600702]

Olsen AN, Ernst HA, Lo Leggio L, Skriver K. NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci. 2005; 10:79–87. [PubMed: 15708345]

Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Kojima K, Takahara Y, Yamamoto K, Kikuchi S. Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Research. 2003; 10:239–247. [PubMed: 15029955]

Peleg Z, Saranga Y, Suprunova T, Ronin Y, Roder MS, Kilian A, Korol AB, Fahima T. High-density genetic map of durum wheat × wild emmer wheat based on SSR and DArT markers. Theor Appl Genet. 2008; 117:103–115. [PubMed: 18437346]

Rohila JS, Chen M, Cerny R, Fromm ME. Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. Plant J. 2004; 38:172–181. [PubMed: 15053770]

Sablowski RWM, Meyerowitz EM. A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. Cell. 1998; 92:93–103. [PubMed: 9489703]

Slade A, Fuerstenberg S, Loeffler D, Steine M, Facciotti D. A reverse genetic, non transgenic approach to wheat crop improvement by TILLING. Nat Biotechnol. 2005; 23:75–81. [PubMed: 15580263]

Sourdille P, Singh S, Cadalen T, Brown-Guedira GL, Gay G, Qi L, Gill BS, Dufour P, Murigneux A, Bernard M. Microsatellite-based deletion bin system for the establishment of genetic-physical map relationships in wheat (*Triticum aestivum*L.). Funct Integr Genomics. 2004; 4:12–25. [PubMed: 15004738]

Sperotto R, Ricachenevsky F, Duarte G, Boff T, Lopes K, Sperb E, Grusak M, Fett J. Identification of up-regulated genes in flag leaves during rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor. Planta. 2009; 230:985–1002. [PubMed: 19697058]

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis software (MEGA). Mol Biol Evol. 2007; 24:1596–1599. [PubMed: 17488738]

Thompson JD, Higgins DG, Gibson TJ. Clustal W - Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. Nucleic Acids Res. 1994; 22:4673–4680. [PubMed: 7984417]

Uauy C, Brevis JC, Dubcovsky J. The high grain protein content gene *Gpc-B1* accelerates senescence and has pleiotropic effects on protein content in wheat. J Exp Bot. 2006a; 57:2785–2794. [PubMed: 16831844]

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J. A NAC gene regulating senescence improves grain protein, zinc and irons content in wheat. Science. 2006b; 314:1298–1300. [PubMed: 17124321]

Uauy C, Paraiso F, Colasuonno P, Tran RK, Tsai H, Berardi S, Comai L, Dubcovsky J. A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. BMC Plant Biology. 2009:9. [PubMed: 19161601]

Yoshida S. Molecular regulation of leaf senescence. Current Opinion in Plant Biol. 2003; 6:79–84.

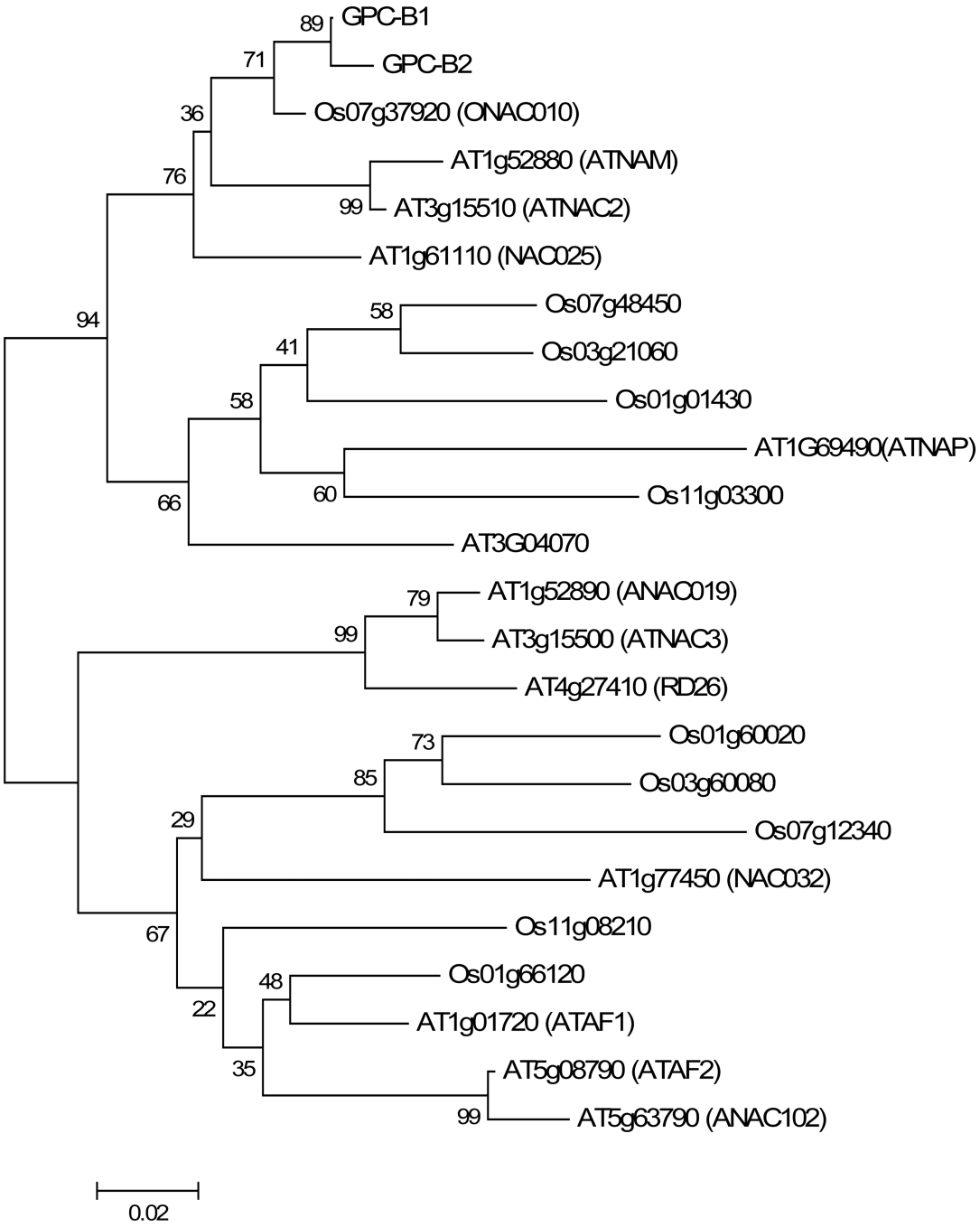

Figure 1.

Phylogenetic analysis of the NAC proteins closest to wheat GPC-1 and GPC-2 from Arabidopsis and rice. Amino acids within the five NAC domains were aligned using ClustalW (Thompson et al. 1994). The phylogenetic tree was constructed using the Neighbor-Joining method as described in MEGA software version 4 (Tamura et al. 2007). The stability of the different nodes was tested using bootstrap analysis with 1000 replicates as implemented in MEGA. The tree shows that the closest rice homologue to the *GPC* genes is *Os07g37920*.

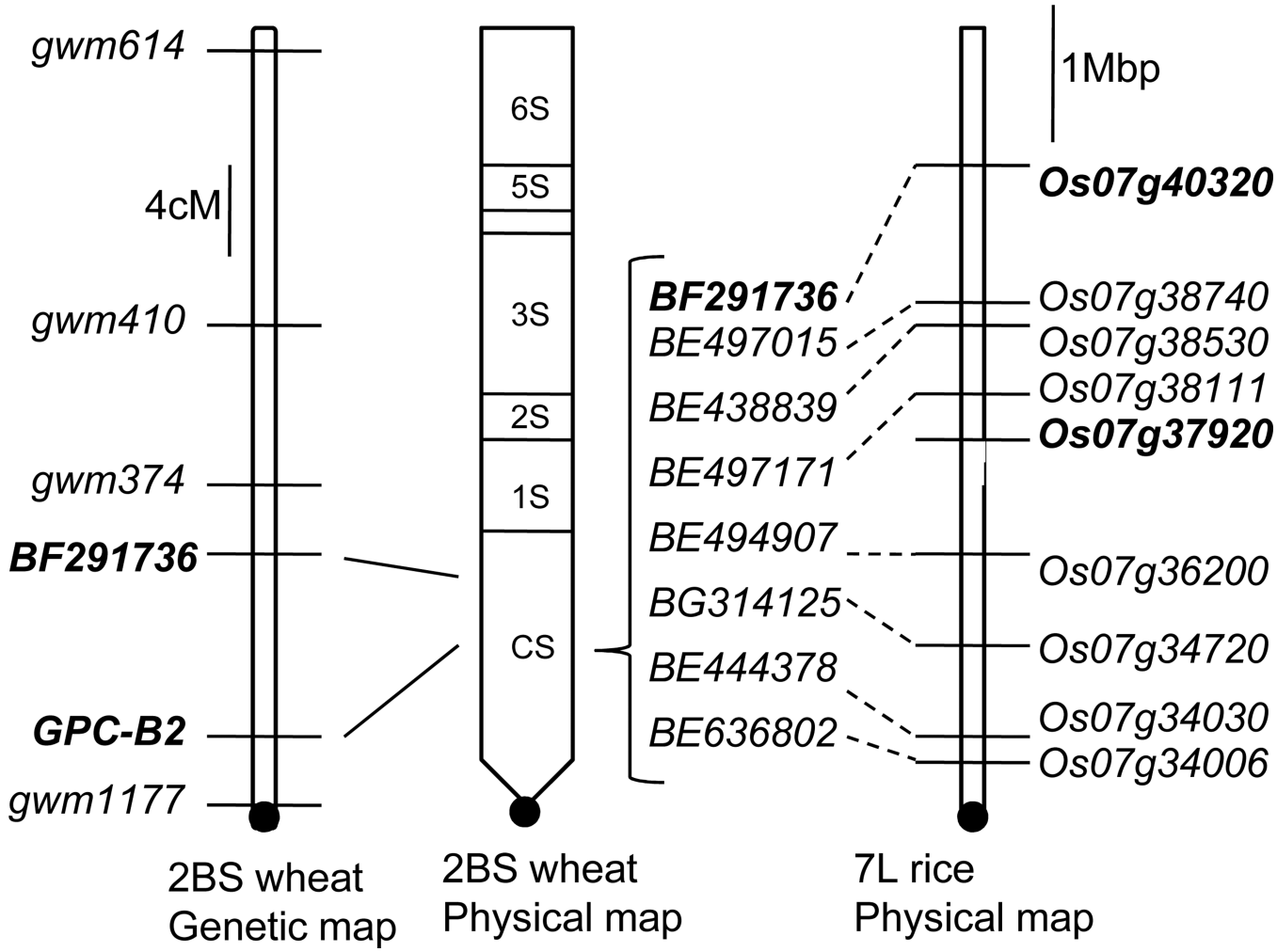

Figure 2.

Os07g37920 is orthologous to the wheat *GPC-B2* gene. Left: genetic mapping of *GPC-B2* to the proximal region of chromosome 2BS in a 20 cM interval flanked by *gwm374* and *gwm1177* SSR markers. *GPC-B2* was mapped 9.9 cM proximal to marker *BF291736*. Center: wheat chromosome 2BS physical map showing wheat ESTs including *BF291736* mapped to the CS centromeric bin (adapted from Sourdille et al. 2004; Conley et al. 2004). Right: rice chromosome 7L physical map showing *Os07g37920* flanked by genes with orthologous wheat ESTs mapped to consensus bins CS including *Os07g40320* (the orthologous gene to *BF291736*)

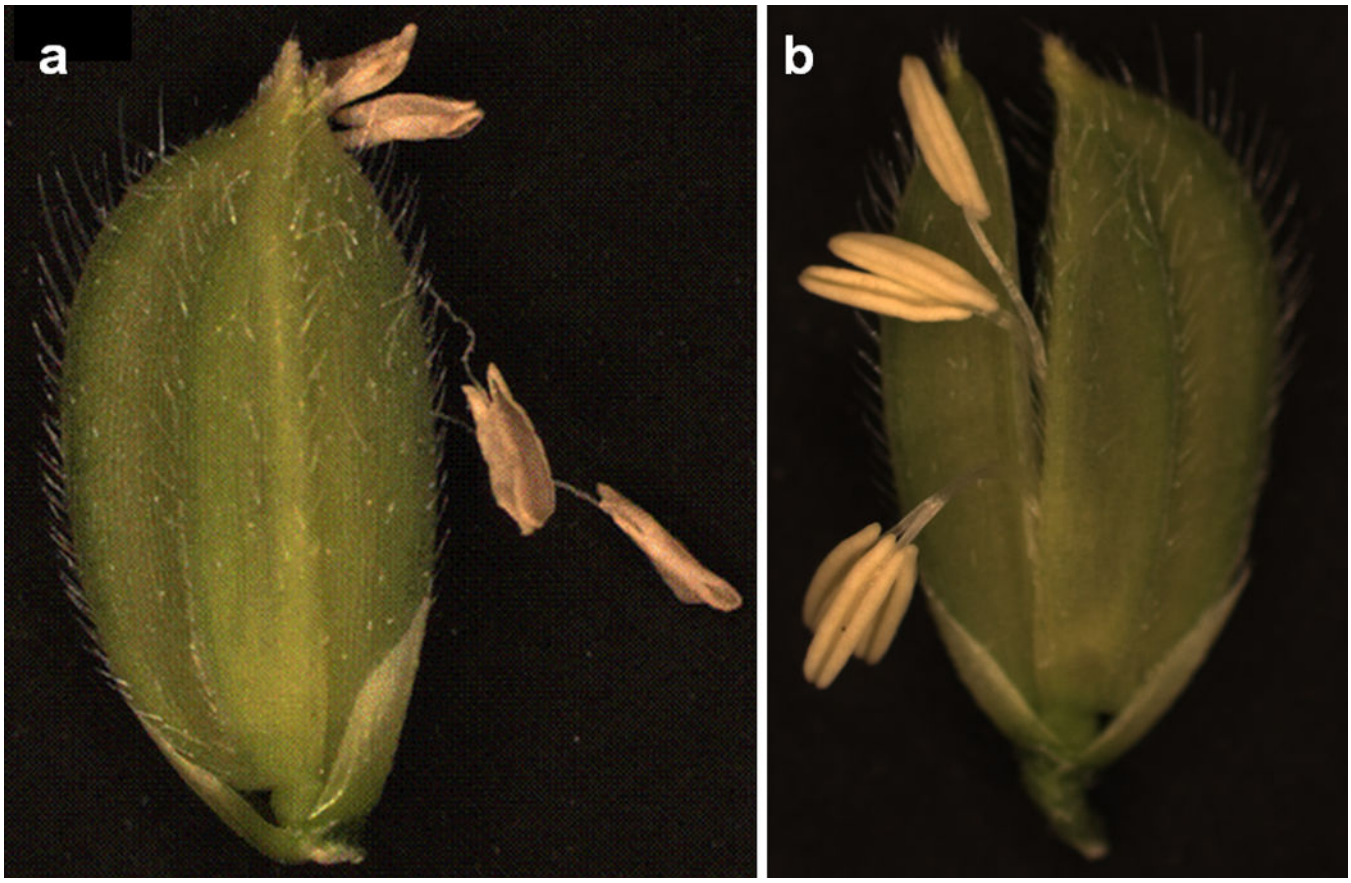

Figure 3.

(A) Wild type Kitaake rice flower at anthesis showing dehiscent anthers that released the pollen grains. (B) *Os07g37920* RNAi transgenic flower showing anthers that emerged from the lemma and palea at the same time as the wild type controls but remained intact and did not release pollen grains. Therefore, anthesis did not occur in the transgenic plants while the wild type flower continued its development.

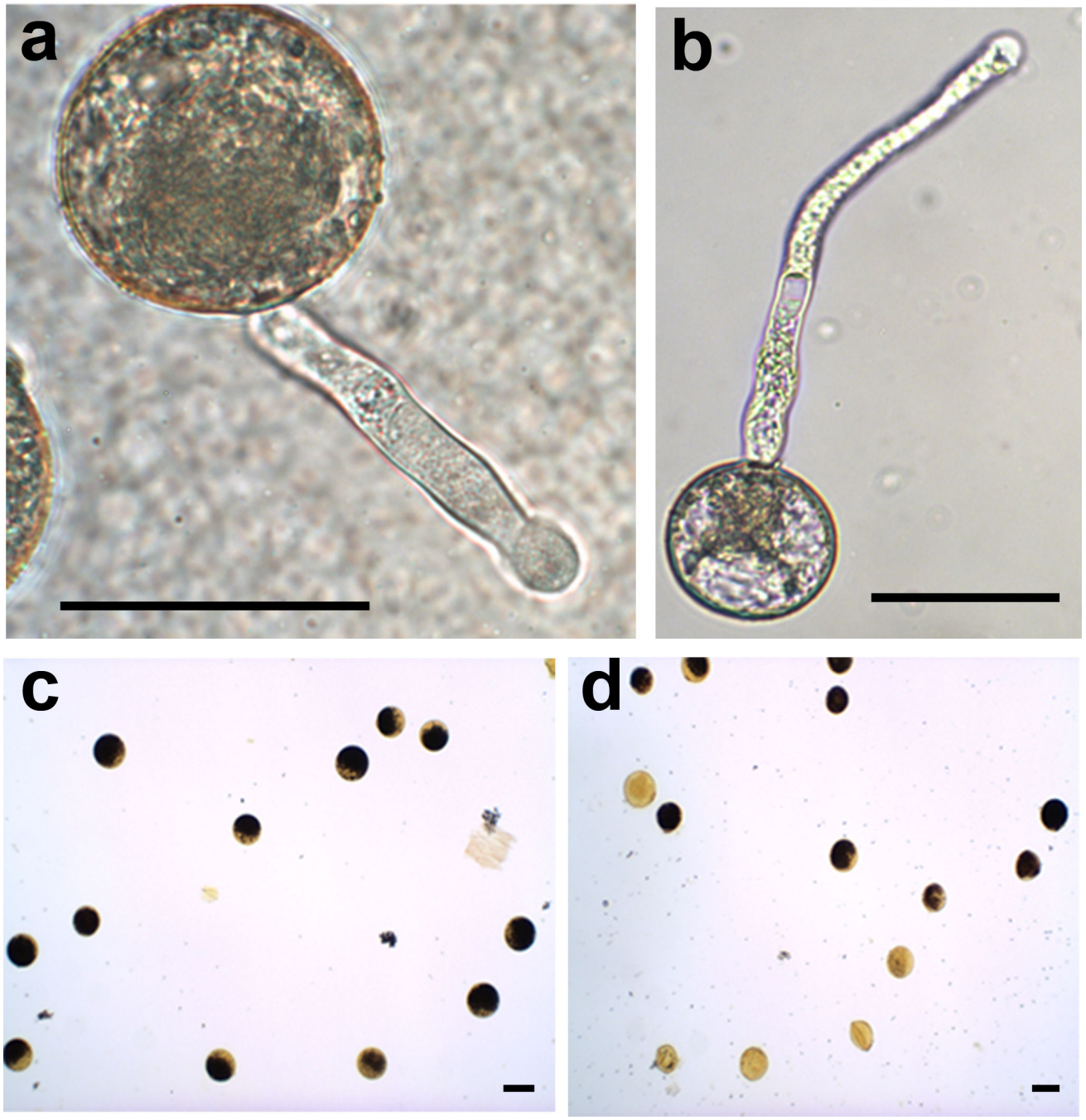

Figure 4.

Pollen viability tests. Pollen grain germinated on artificial medium (wild type – A, *Os07g37920* RNAi - B) and pollen grains stained with Lugol's iodine solution (wild type – C, *Os07g37920* RNAi - D). Scale bar = 15μM.

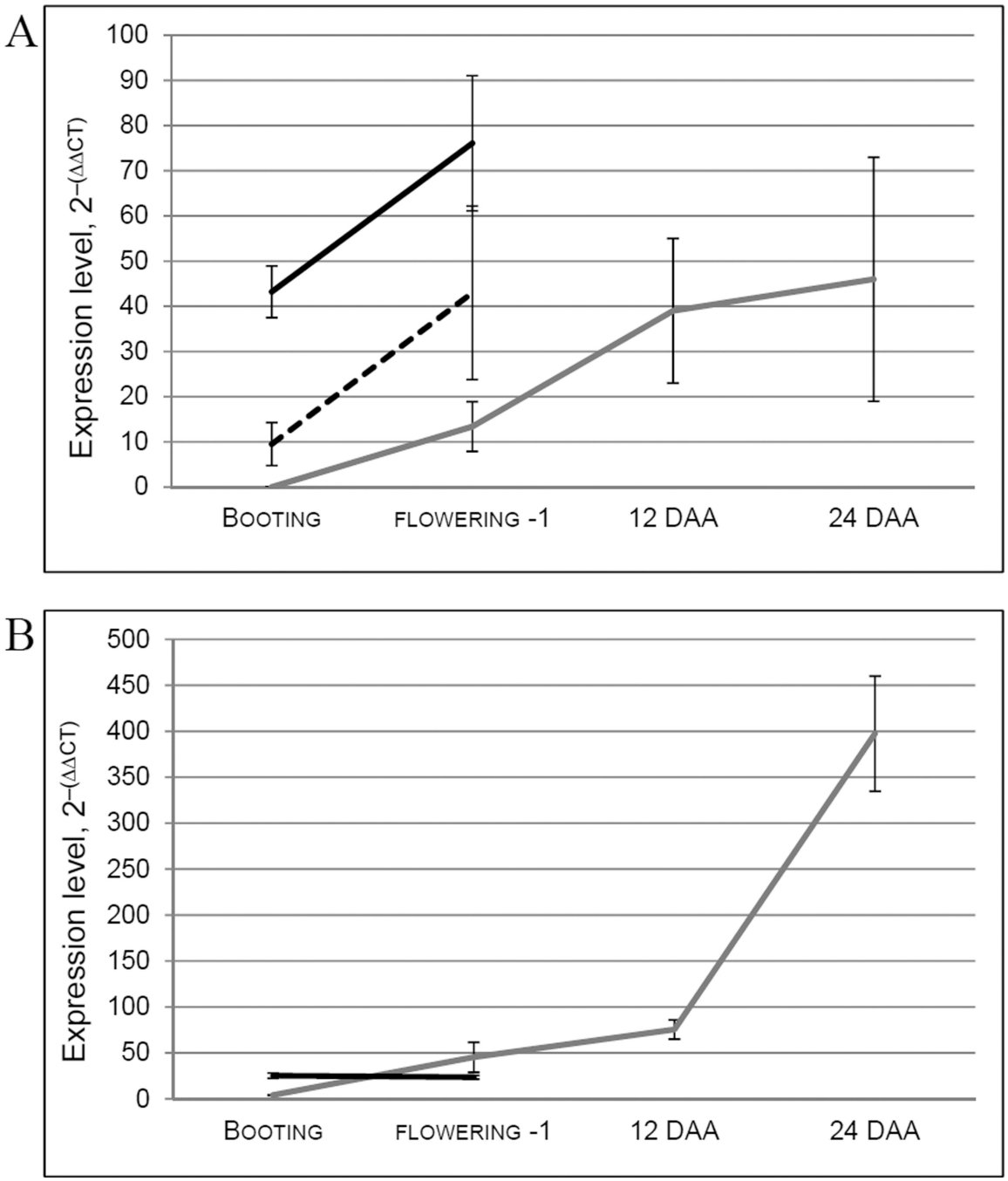

Figure 5.

Transcript levels of rice and wheat NAC transcription factors in the stamens (black lines) and leaves (grey lines) in different developmental stages: booting (10 days before anthesis), flowering-1 (1 day before anthesis), 12 and 24 DAA (days after anthesis). (A) Rice: transcript levels of *Os07g37920* in wild type Kitaake plants (solid lines) and transgenic RNAi- *Os07g37920* plants (dashed line). (B) Wheat: transcript levels of *GPC-B2* in tetraploid cultivar 'Kronos'. Values are averages of 4 to 8 individual plants and bars

represent standard errors of the mean. Since the same corrector value was used in the linearized transcript levels (10.9), the scales of the two graphs are comparable.

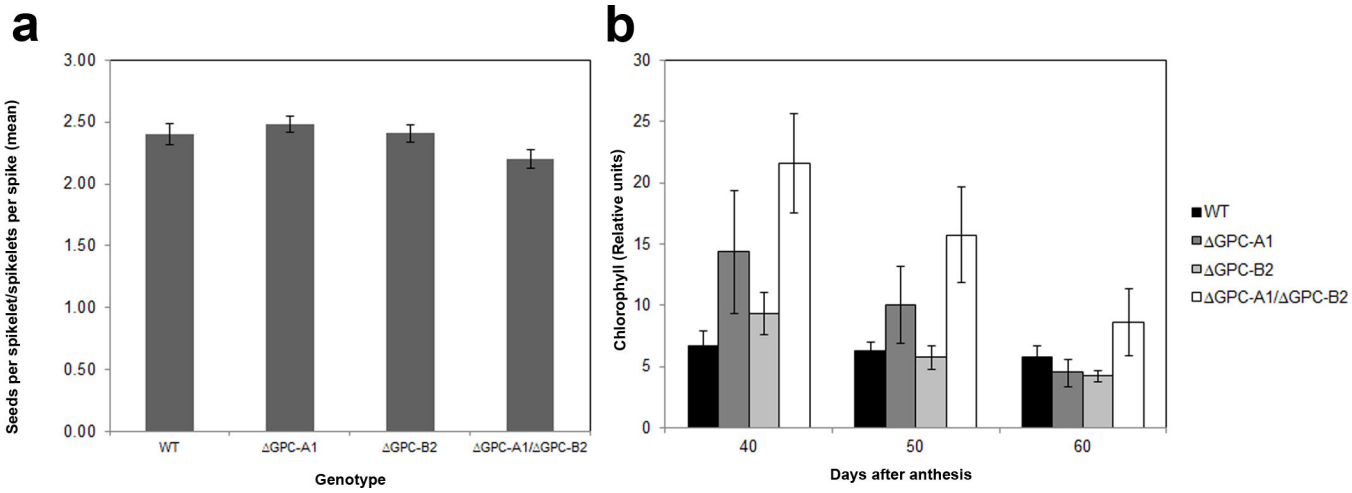

Figure 6.

Fertility and senescence phenotype of wheat *GPC* EMS mutants. (A) Number of seeds per spikelet calculated as number of seeds per spike divided by the number of spikelets in the spike. BC_2F_3 plants carrying single (Δ*GPC-A1* or Δ*GPC-B2*) and double (Δ*GPC-A1*/Δ*GPC-B2*) knock-out mutations for the *GPC* genes in tetraploid wheat were compared to a sister line with wild-type *GPC* alleles (WT). Pair-wise comparisons among the four genotypes using the Tukey-Kramer test revealed non-significant differences for all comparisons ($P<0.08$). (B) Relative chlorophyll levels measured by SPAD at 40, 50 and 60 DAA. Values are averages of ten independent measurements taken from between 10 and 20 biological replicates of each genotype ± SE of the mean.