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REVIEW

# RNA interference for wheat functional gene analysis

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**Abstract** RNA interference (RNAi) refers to a common mechanism of RNA-based post-transcriptional gene silencing in eukaryotic cells. In model plant species such as *Arabidopsis* and rice, RNAi has been routinely used to characterize gene function and to engineer novel phenotypes. In polyploid species, this approach is in its early stages, but has great potential since multiple homoeologous copies can be simultaneously silenced with a single RNAi construct. In this article, we discuss the utilization of RNAi in wheat functional gene analysis and its effect on transcript regulation of homoeologous genes. We also review recent examples of RNAi modification of important agronomic and quality traits in wheat and discuss future directions for this technology.

**Keywords** Wheat · RNA interference · Gene silencing

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## Introduction

Wheat (*Triticum* spp.) is one of the world's most important crop species, providing over 20% of the calories consumed by humans (FAO 2006). In spite of its economic importance, functional gene analysis in wheat is still in its early stages due to several limitations. Common wheat is a polyploid species with three homoeologous genomes (A, B, and D genomes) that share ~95% sequence similarity. This translates into many wheat genes having three homoeologous copies that are functionally redundant. Therefore, efforts to produce loss of function mutants through phenotypic screenings (chemical or insertional mutagenesis) are less effective in polyploid wheat. In addition, the large genome size of hexaploid wheat (16,000 Mb, Bennett and Leitch 2004) and its high content of repetitive DNA (83%, Flavell et al. 1974) have delayed efforts to sequence its entire genome.

Despite these limitations, useful genomic resources have been developed for wheat. Efforts to sequence expressed sequence tags (EST) have generated over one million wheat EST reads (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) that cover ~60% of the expressed genome (Gill et al. 2004). The construction of multiple diploid, tetraploid, and hexaploid bacterial artificial chromosome (BAC) libraries (Moullet et al. 1999; Lijavetzky et al. 1999; Cenci et al. 2003; Allouis et al. 2003; Akhunov et al. 2005) has facilitated the recent positional cloning of several

agronomically important genes in wheat and the assembly of physical maps for chromosome arm 3AS (Dr. B. Gill, Kansas State University, USA), 3BS (Dr. C. Feuillet, INRA, France) and the wheat D genome progenitor, *Aegilops tauschii* (http://www.wheatgenome.org). At present, the International Wheat Genome Sequencing Consortium is sequencing multiple BAC clones from these physical maps, generating unprecedented amounts of wheat genomic sequence, and annotating numerous wheat genes.

To accelerate the functional characterization of wheat genes, Sallaud et al. (2004) proposed using T-DNA/transposon insertion mutants of the orthologous rice genes. However, rice and wheat orthologues do not always have identical functions. After a common ancestral polyploidization event that occurred ~70 million years ago (Guyot and Keller 2004; Paterson et al. 2004; Vandepoele et al. 2003; Wang et al. 2005) wheat and rice lineages have followed independent evolutionary paths for the last 50 million years (Gaut 2002). Therefore, genes duplicated in the ancestral polyploidization event have been subjected to independent mutations and functional differentiation in the two lineages. To complicate this further, high frequencies of duplication and deletion events in the large wheat genomes have further eroded the colinearity between these two genomes (Dubcovsky et al. 1996; Wolfe 2001; Akhunov et al. 2003; Dubcovsky and Dvorak 2007). These changes can alter the functional relationships between orthologous genes in wheat and rice, making it difficult to predict the function in one species based on the function of the orthologue in the other species. *Brachypodium* is emerging as a better model system for wheat because of the more recent divergence of these two lineages (35–40 million years) compared to the wheat–rice divergence (Draper et al. 2001; Hasterok et al. 2006; Vogel et al. 2006). Although the successful sequencing of *Brachypodium* will most likely be a valuable resource for wheat, the first comparative genomic studies are showing multiple alterations of colinearity between these two genomes (Bossolini et al. 2007; Valarik et al. 2007) suggesting that a final validation of gene function directly in wheat will still be necessary.

Most of the techniques currently available to study gene function in plants involve knockouts of target genes followed by the phenotypic characterization of the mutant plants. In *Arabidopsis* and rice, large gene knockout collections have been assembled from chemical mutagenesis and T-DNA or transposon insertional mutagenesis (Jeon et al. 2000; Alonso et al. 2003; Sallaud et al. 2004). In wheat, a powerful reverse genetics approach was recently implemented through the combination of EMS-mediated mutagenesis and TILLING technology (Slade et al. 2005). Because of the tolerance of polyploid wheat to high-mutation rates, this method is very efficient in identifying mutations in the target genes. However, the effect of single-gene knockouts can be masked by the functional redundancy of homoeologous genes present in the other wheat genomes (Lawrence and Pikaard 2003). This limitation can be overcome by generating double and triple mutants, although this process is cumbersome and time consuming. Therefore, faster alternatives are required for functional gene analysis in polyploid wheat.

The discovery of RNA mediated gene silencing creates a viable alternative strategy for gene functional analysis through the simultaneous knockdown of expression of multiple related gene copies. This phenomenon was discovered in the late nineties in *Caenorhabditis elegans* (Fire et al. 1998) and plants (Jorgensen et al. 1996; Meyer and Saedler 1996) and involves formation of double stranded RNA (dsRNA) that subsequently leads to the cleavage of homologous mRNAs at the post-transcriptional stage [RNA interference (RNAi)] or to the blockage of these mRNAs at the transcriptional stage.

Even though there are a limited number of RNAi studies in wheat, some general trends are emerging from its first applications that have successfully modified important agronomic and quality traits. In this article, we will first review the early transient and stable RNAi studies, followed by more recent examples that characterized the effect of RNAi on each of the homoeologous copies of the targeted wheat genes. Finally, we will compare RNAi and virus induced gene silencing (VIGS), summarize strategies to optimize wheat RNAi experiments, and discuss future directions for this technology.

## Early studies on wheat RNAi

The molecular aspects of RNAi have been described in detail elsewhere (Tijsterman et al. 2002; Denli and Hannon 2003; Baulcombe 2004; Meins et al. 2005).

Briefly, dsRNA is cleaved by a Dicer-like enzyme into 21–26 bp small RNAs (smRNA) which are then recruited by the RNA-induced silencing complex (RISC). This complex mediates the cleavage of the specific target mRNA, resulting in post-transcriptional gene silencing.

The earliest report of RNAi in wheat was a single cell based transient RNA-silencing assay using wheat epidermal cells (Schweizer et al. 2000). In this study, in vitro synthesized dsRNA was cotransformed through particle bombardment with either the *gusA* (*GUS*) reporter gene or with the fusion of a germin-like protein (*GLP*) and reporter gene green fluorescence protein (*GFP*) (*TaGLP2a::GFP*). Through phenotypic analysis, Schweizer et al. (2000) showed that dsRNA of *GUS*, *TaGLP2a*, and *GFP* were all able to interfere with their respective target genes in a sequence specific manner. In another transient RNAi study in wheat, Christensen et al. (2004) used a hairpin construct to target *TaGLP2a* (renamed *TaGLP4* in this later study) to determine the effect of this gene on plant defense responses. The transient silencing of *TaGLP2a* reduced wheat basal resistance to powdery mildew suggesting that this gene participates in the resistance response. These promising preliminary studies were followed by stable RNAi experiments in wheat.

## Application of stable RNAi in wheat functional studies

RNA interference was recently applied to study the effect of specific genes at the whole organism level in wheat. To induce stable RNAi, it is necessary to produce stably transformed wheat plants that carry a construct expressing a double-stranded RNA. These constructs consist of a trigger sequence homologous to the endogenous target gene cloned in the sense and antisense orientations separated by a linker sequence. This allows the formation of dsRNA hairpin structures that elicit the RNAi response.

The first stable RNAi transformation in wheat targeted a candidate for the vernalization gene *VRN2* (Yan et al. 2004, Table 1). *VRN2* is a zinc-finger CCT domain gene that represses flowering and is down-regulated by long exposures to cold temperatures (vernalization) and by short day photoperiods (Yan et al. 2004; Dubcovsky et al. 2006). Yan et al. (2004) silenced *VRN2* by using a 347-bp trigger sequence from the *Triticum monococcum* L. *VRN2* gene, excluding the conserved zinc finger and CCT domain. This strategy was chosen to avoid degradation of non-target mRNA transcripts that shared sequence identity to these conserved motifs. The relative expression levels of *VRN2* in transgenic and non-transgenic lines were studied using quantitative reverse-transcriptase PCR (qRT-PCR). These experiments demonstrated a reduction of endogenous *VRN2* mRNA levels in the transgenic plants to 40% of the levels found in non-transgenic control lines. The reduction of *VRN2* transcripts accelerated flowering of the transgenic winter lines more than 40 days, confirming that *VRN2* is a strong flowering repressor.

To determine the sequence specificity of the *VRN2* RNAi construct, the expression level of *ZCCT2*, a close homologue to *VRN2*, was examined. *ZCCT2* shares both the zinc finger and CCT domain with *VRN2* and has no contiguous stretches of identical sequence of over 18-nt with *VRN2* within the 347-bp trigger sequence (83% identity, Fig. 1a). No differences were detected in *ZCCT2* expression between transgenic and non-transgenic lines, confirming that sequence-specific RNAi could be used successfully in polyploid wheat.

Using a similar strategy, Loukoianov et al. (2005) targeted *VRN1*, a MADS-box transcription factor that promotes flowering and is up-regulated by vernalization (Yan et al. 2003). They designed a 294-bp trigger sequence from *T. monococcum VRN1* that excluded the conserved MADS- and K-box domains. Using a conserved primer pair for all three genomes (A, B, and D genomes), qRT-PCR analysis of transgenic lines revealed a reduction of total *VRN1* transcripts to 19% of that observed in the non-transgenic controls. As a result, the flowering time in transgenic spring lines was delayed by 2 weeks compared to the non-transgenic controls, confirming that *VRN1* promotes flowering in wheat.

RNAi has also been used to modulate starch content in wheat. According to its degree of polymerization (DP), starch can be classified into amylose (low DP) or amylopectin (high DP). Amylose synthesis requires an active granule bound starch synthase (GBSS), whereas amylopectin synthesis requires starch branching enzyme (SBE) activity. In an effort to reduce amylose content in wheat, Li et al. (2005) silenced the *GBSSI*

**Table 1** Main characteristics of stable RNAi studies in wheat

| Target loci | Gene and function | Promoter | Trigger | Linker | Eff. (%)[a] | Homoeologues | Detection | Expression (relative to control) | Phenotype | Transf. Method[b] | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *VRN2* | *ZCCT1*, Zinc finger, CCT domain protein | 35S + *Adh1* intron | 347 bp (excluding Zinc finger and CCT domain) | 1,163 bp (rice *Waxy*) | 33 | A,B, D genomes (not specified)[c] | qRT-PCR | 40% average | Accelerated flowering time | B | Yan et al. (2004) |
| *VRN1* | *AP1*, MADS, K-box domain protein | 35S + *Adh1* intron | 294 bp (excluding MADS and K-box domains) | 1,163 bp (rice *Waxy*) | n.r.[d] | A, B, D genomes (not specified)[c] | qRT-PCR | 20% average | Delayed flowering time | B | Loukoianov et al. (2005) |
| *GBSSI* | Granule bound starch synthase enzyme | Maize *Ubi* | 683 bp (including conserved domains) | 150 bp (*GBSSI* intron) | 50 | A, B, D genomes (not specified)[c] | RT-PCR | Non-quantitative | Reduced amylose content | A | Li et al. (2005) |
| *SBE-IIa* | Starch branching enzyme | HMWG[e] | 540 bp (exons 1–3: non-conserved) | 512 bp (intron 3 *SBE-IIa*) | n.r.[f] | A, B, D genomes (not specified)[c] | Immuno-blotting | <1–10% via immunodetection | Reduced amylopectin content | A | Regina et al. (2006) |
| *SBE-IIb* | Starch branching enzyme | HMWG | 528 bp (exons 1–3 non-conserved) | 595 bp (intron 3 *SBE-IIb*) | n.r.[f] | A, B, D genomes (not specified)[c] | Immuno-blotting | <1% Via immunodetection | No detectable phenotype | A | Regina et al. (2006) |
| *EIN2* | Transmembrane protein | Maize *Ubi* | 518 bp | 548 bp (*TAK14*) | 33 | A, B, D genomes | qRT-PCR | 30–50% for each homoeologue | Ethylene insensitivity | B | Travella et al. (2006) |
| *PDS* | Phytoene desaturase enzyme | Maize *Ubi* | 480 bp | 548 bp (*TAK14*) | 78 | A, B, D genomes | qRT-PCR | <10–70% for each homoeologue | Photobleaching | B | Travella et al. (2006) |
| *GPC* | *NAM*, NAC transcription factor | 35S + *Adh1* intron | 475 bp (excluding NAC domains) | 1,163 bp (rice *Waxy*) | 29 | A, D genomes & B, D paralogues | qRT-PCR | 40–70% For each homoeologue | Delayed senescence, reduced grain protein, *Zn* and *Fe* | B | Uauy et al. (2006b) |
| *1Dx5* | Seed storage protein | Maize *Ubi* | 210 bp (N-terminal coding region) | 160 bp (wheat *Waxy*) | 100 | A, B, D genomes & B, D paralogues | SDS-PAGE, RT-PCR | <1% for *1Dx5*, 60–80% for *1Bx7*, no effect for *By9*, *Dy10* | Reduced gluten and mixing quality | B | Yue et al. (2007) |

[a] Efficiency (%): percent of independent transgenic events that had the expected phenotype

[b] Transformation Method (A: *Agrobacterium*, B: bombardment)

[c] Studies did not analyze homoeologous genomes separately

[d] *n.r.* not reported. The *VRN1* RNAi transgene showed poor transmission making it difficult to measure efficiency accurately

[e] *HMWG* High molecular weight glutenin *Dx5* subunit

[f] Phenotypes for each transgenic event were not reported, although 92 and 33% of the transgenic plants had reduced *SBEII-a* and *SBEII-b* protein levels, respectively

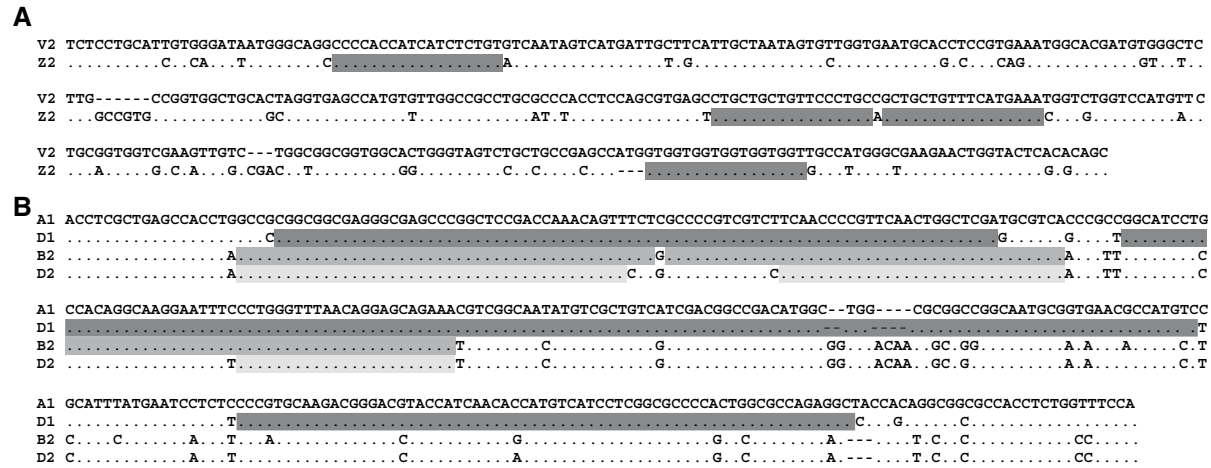


**Fig. 1** (**A**) Sequence alignment of *VRN2* RNAi trigger sequence (V2) and of the closely related *ZCCT2* gene (Z2). The longest contiguous stretches of identical nucleotides (three 17-nt and one 18-nt stretch) are highlighted in *dark gray*. (**B**) Sequence alignment of *NAM* RNAi trigger sequence (A1) and corresponding homoeologous (D1) and paralogous copies (B2 and D2). Stretches of over 21 identical contiguous nucleotides between trigger (A1) and corresponding homologous sequence are highlighted in different shades of *gray*. *Dots* and *letters* represent similar or polymorphic nucleotides between the RNAi trigger sequence and targets, respectively. *Dashes* represent insertion/deletion events

gene. They produced four independent transgenic lines with reduced levels of *GBSSI* transcript with respect to control lines (measured by RT-PCR) and two of these lines showed the expected reduction in seed amylose content (Table 1).

An alternative approach was taken by Regina et al. (2006) who silenced each of two isoforms of *SBEII* (a and b) in independent transgenic lines using isoform-specific trigger sequences. In the case of *SBEII-b*, protein levels were reduced to <10% of the control level (as detected by immunoblotting), while no reduction of SBEII-a protein levels was detected. Despite the reduction in SBEII-b protein levels in the transgenic lines relative to the controls, there was no significant effect on amylopectin content. In the *SBEII-a* RNAi experiment, SBEII-a protein levels were also reduced to <10% of the control, and interestingly, SBEII-b protein levels were also reduced. The authors suggest that the co-suppression of *SBEII-b* by the *SBEII-a* construct was due to a mechanism different from RNAi (Regina et al. 2006), but the presence of an identical 21-nt stretch between the *SBEII-a* trigger sequence and the *SBEII-b* mRNA suggests that more research would be necessary to clarify this mechanism. The transgenic lines with reduced levels of the two SBEII proteins showed a sharp decrease in amylopectin content and an increase in amylose content relative to the non-transgenic controls. Rats fed with the high amylose flour from the transgenic wheat showed improved indices of gastrointestinal health compared to rats fed with regular flour from the control wheat lines. These results suggest a potential health benefit of high-amylose wheat (Regina et al. 2006).

## RNAi-mediated silencing of individual wheat homoeologous genes

Three recent wheat studies shed new light on the potential of RNAi silencing to simultaneously knock-down transcripts of homoeologous genes in polyploid wheat (Travella et al. 2006; Uauy et al. 2006b; Yue et al. 2007). In the first study, Travella et al. (2006) targeted the phytoene desaturase (*PDS*) and ethylene insensitive 2 (*EIN2*) genes, which both have three homoeologous copies that are expressed in wheat. Silencing of these genes generates phenotypes that are easy to visualize making them ideal targets for evaluating silencing of individual homoeologous genes. *PDS* is an important enzyme in carotenoid biosynthesis (Cunningham and Gantt 1998) and reduction or inhibition of this enzyme in plants results in variegated green/white leaves (Bartley and Scolnik 1995). *EIN2* is a central component of the ethylene signaling pathway (Alonso et al. 1999) and

*Arabidopsis ein2* mutants show a clear ethylene insensitivity phenotype.

For the *PDS* genes, Travella et al. (2006) used a 480-bp trigger fragment from the wheat D genome that shares over 96% nucleotide identity with the other two homoeologous copies. Using particle bombardment, they generated 64 independent transgenic lines showing the photobleaching phenotype at the seedling stage. These $T_0$ lines were grouped into a phenotypic series based on the affected leaf surface, which was correlated with the reduction of endogenous *PDS* transcript levels. Albino plants (strong phenotype) had *PDS* transcript levels reduced to ~10–15% of the non-transgenic control levels, whereas streaked plants (intermediate phenotype) had ~40–50% relative *PDS* transcript levels with respect to non-transgenic controls. This suggests that RNAi in wheat will produce phenotypic series when multiple transgenic events are generated, as described before in *Arabidopsis* (Chuang and Meyerowitz 2000).

For the *EIN2* gene, a 518-bp fragment from the *EIN2* B genome allele (96% nucleotide identity to homoeologous copies) was used as the RNAi trigger (Travella et al. 2006). Six independent transgenic $T_0$ lines displayed a significant reduction in endogenous *EIN2* expression levels. Once again, a phenotypic series was generated in which the reduction of *EIN2* transcript was correlated with the severity of the phenotype. In this case, the average reduction in *EIN2* transcript levels in the different events was ~30–50% compared to non-transgenic controls, with one extreme event reducing *EIN2* transcript levels to only 1% of the control levels.

This study detailed for the first time the relative silencing of each of the three homoeologous target genes. Using genome-specific primers and qRT-PCR, Travella et al. (2006) measured the endogenous *PDS* or *EIN2* mRNA levels of the individual homoeologous copies, as well as the total transcript levels for all three homoeologues. They found that all three copies of each gene were equally affected by the RNAi and that the transcript levels detected by the consensus primers (which amplify all three copies) were indicative of the three individual genomes. This suggests that RNAi affected each of the three homoeologous copies in a similar manner.

The study further confirmed the stable co-inheritance of the transgene and the RNAi induced phenotype over at least two generations, a phenomena also observed for wheat *VRN2* (J. Dubcovsky et al., unpublished data) and recently for *NAM* genes (Uauy et al. 2006b) and *1Dx5* (Yue et al. 2007). However, this has not always been the case as the *VRN1* RNAi transgene (Loukoianov et al. 2005) showed poor transmission through the pollen (M. Bonafede and J. Dubcovsky, unpublished data). Progeny tests additionally demonstrated that homozygous transgenic plants appear to have a stronger reduction of the target transcripts, accumulate more small interfering RNA (siRNA), and have more severe phenotypic changes than the heterozygous plants, suggesting a dosage effect of RNAi in hexaploid wheat (Travella et al. 2006). It is also interesting to mention that in both cases (*PDS* and *EIN2*), RNAi induced phenotypic changes were similar to mutant phenotypes described in diploid model plants (Bartley and Scolnik 1995; Alonso et al. 1999).

The second study characterized the effect of RNAi on individual homologous gene copies of wheat *NAM* genes, which encode NAC transcription factors affecting senescence and grain protein, zinc and iron content (Uauy et al. 2006a, b). The ancestral wild wheat allele on chromosome 6B encodes a functional transcription factor that accelerates senescence and increases nutrient remobilization from leaves to developing grains, whereas modern wheat varieties carry a non-functional *NAM-B1* allele. In addition to the 6B locus, modern bread wheat has two functional homoeologous copies on chromosomes 6A and 6D and two additional paralogous copies on chromosomes 2B and 2D. Expression and sequence analysis suggested that the reduction in overall *NAM* transcript levels, due to the inactivity of the 6B allele in modern wheat, was responsible for the observed phenotypes (Uauy et al. 2006b).

To test this dosage effect, an RNAi construct was designed to target the last exon and the 3′ untranslated region (UTR) of the four functional *NAM* genes present in hexaploid wheat (*NAM-6A, 6D, 2B*, and *2D*). The 475-bp trigger sequence from *NAM-6A* was designed outside the NAC domain, to avoid interference with other NAC transcription factors, and shares at least three contiguous stretches of over 21-nt with the other three copies (Fig. 1b) (96, 89, and 88% sequence similarity with the 6D, 2B, and 2D copies, respectively). Using qRT-PCR and genome-specific primers, all four *NAM* genes showed significant

transcript reduction in flag leaves (40–60% relative transcript level compared to non-transgenic control at 4 or 9 days after anthesis). As in the *PDS* and *EIN2* cases, the four *NAM* transcripts exhibited similar reduction levels and the consensus primers, which amplified all four copies, were good indicators of the average reduction of the individual targets. The knockdown of the multiple *NAM* transcripts resulted in delayed senescence (over 3 weeks) and a significant reduction in grain protein, zinc, and iron content (over 30%) compared with the non-transgenic controls (Uauy et al. 2006b).

A second transgenic line showed smaller reductions in *NAM* transcript levels than the previous one. Relative transcript levels were 50–70% of the non-transgenic control for each individual copy and for the *NAM* genes overall. This translated into less severe phenotypic differences relative to the non-transgenic control in this second transgenic line (2 weeks delay in senescence and <30% reduction in grain protein, zinc, and iron content). Both transgenic events showed similar results over two generations, confirming the heritable nature of the RNAi response.

The third study targeted a gene encoding the high-molecular-weight (HMW-) glutenin subunit 1Dx5, a seed storage protein associated with dough strength and elasticity in wheat flours (Yue et al. 2007). Six independent transformants were obtained and two transgenic lines, one with complete and one with partial *1Dx5* silencing, were analyzed during seed development by RT-PCR. Transcripts from the *1Dx5* gene were present at reduced levels in seeds from both lines 10 days after pollination (DAP) but were not detectable by 15 DAP in the completely silenced line. These results show that the *1Dx5* gene is functional in these lines, but that total or partial silencing set in shortly after the start of transcription during normal development. Flours from these two lines showed reduced gluten and dough quality, confirming the importance of the 1Dx5 glutenin subunit in wheat bread-making properties (Yue et al. 2007).

In addition to the reduction or elimination of the 1Dx5 protein subunit, these two transgenic lines showed a significant reduction of the homoeologous 1Bx7 protein subunit (60–80% of the protein level found in the non-transgenic control). The gene coding for the *1Bx7* subunit shares a 28-nt stretch of perfect homology and is 86% identical to the 210-bp *1Dx5* trigger sequence, suggesting RNAi mediated gene silencing of *1Bx7* by the *1Dx5* construct. However, no differences in protein or RNA transcript levels were observed for the homoeologous *1Ax2** gene, in spite of its 91% identity with the *1Dx5* trigger fragment and the presence of an identical continuous stretch of 32-nt. Furthermore, no effect was observed for the paralogous *1By9* and *1Dy10* genes which both share a 22-nt stretch of perfect homology with the *1Dx5* trigger sequence. These results suggest that the presence of continuous nucleotide stretches of over 21-nt and high sequence identity with the trigger sequence may not always be sufficient to trigger the RNAi process.

## Strategies for RNAi in wheat

Some general trends are starting to emerge from the first successful wheat RNAi studies. We describe some of these trends below with the hope of helping future users of RNAi in wheat to better define their strategies.

### Sequence identity

When choosing the RNAi trigger, it is important to consider the presence of continuous stretches of perfect identity between the trigger sequence and its target genes. During the RNAi response in plants, cleavage of dsRNA produces siRNAs of 21–26 nt (Hamilton et al. 2002; Llave et al. 2002; Tang et al. 2003; Qi et al. 2005). Therefore, the presence of a continuous stretch of at least 21 identical nucleotides between the trigger and the target gene is required, although it is not always sufficient to produce efficient silencing (Miki et al. 2005; Yue et al. 2007; McGinnis et al. 2007).

Several studies report overall sequence identity as a criterion for RNAi trigger selection (as opposed to stretches of contiguous identical sequence), but this parameter should be interpreted with caution. Both measurements are related, because the higher the overall sequence identity, the higher the chance of encountering at least one identical contiguous stretch of 21-nt. For example, homoeologous genes in wheat share at least 95% sequence identity, which translates into an average of one polymorphism every twenty nucleotides. The likelihood that all polymorphisms

within the target sequence would be perfectly spaced every 20-nt is almost zero (e.g., Fig. 1b). Therefore, in a normal sized trigger of 200–500 bp (see below), wheat homoeologues will naturally have uninterrupted identical stretches of at least 21-nt among them. For this reason, it is safe to predict that using one homoeoalelle as the trigger sequence should usually suffice to silence all three wheat homoeologues simultaneously. This has been confirmed in recent wheat studies where a unique construct was sufficient to down-regulate all three homoeologues (*PDS*, *EIN2* and *NAM*) and even closely related paralogues (*NAM*). However, exceptions have been reported for the HMW-glutenin x-type subunits (Yue et al. 2007). This may be related to the unusually large divergence between the homoeologous glutenin genes (86–91% sequence identity) or their highly repetitive structure.

The silencing of the *NAM* paralogous copies provides an example where identical stretches of 40, 31, and 23-nt between the RNAi trigger and the target region (Fig. 1b) were sufficient to effectively reduce transcript levels. On the other hand, RNAi down-regulation of the *VRN2* vernalization gene showed no effect on transcript levels for the paralogous *ZCCT2*, which was more divergent and showed identical contiguous stretches of only 18-nt or less with the *VRN2* trigger (Fig. 1a, Yan et al. 2004). Results from the previous two studies indicate that in order to trigger RNAi the two sequences need to share a continuous stretch of identical nucleotides longer than 18-nt, which is consistent with the "21-nt rule" that indicates that at least 21-nt of continuous perfect identity are required to trigger RNAi (Miki et al. 2005; McGinnis et al. 2007).

Even though an uninterrupted stretch of 21 identical nucleotides may be necessary to trigger RNAi, the presence of these stretches alone is not always sufficient. Regina et al. (2006) used RNAi triggers for *SBEII-a* and *SBEII-b*, which each have an identical 21-nt stretch to the paralogous gene. The *SBEII-a* transgenic plants showed reduced levels of both proteins, whereas *SBEII-b* transgenic plants showed reduced protein levels only for SBEII-b. In addition, two recent studies in wheat (Yue et al. 2007) and maize (McGinnis et al. 2007) presented examples of genes whose transcript levels were not significantly affected by RNAi in spite of including 21-nt of perfect identity with the RNAi trigger sequence. Other factors, such as GC content and predicted melting temperature of the RNA hairpin, have been suggested to play a role in determining the efficacies of RNAi triggers (Reynolds et al. 2004). However, these characteristics were not significantly correlated to non-target silencing efficiency in a comprehensive study in maize (McGinnis et al. 2007), suggesting that additional experiments will be required to identify the factors that affect RNAi silencing efficiency beyond the "21-nt rule." For example, it would be interesting to create artificial RNAi triggers with high sequence identity to the different homoeologues, but with mutations spaced out evenly at intervals of 19–23-nt. An alternative approach would be to create several different RNAi triggers with a unique 19–23-nt identical continuous stretch to the target gene, but lacking sequence identity throughout the rest of the trigger. These unique identical stretches could be shifted to complement different regions along the target sequence to better understand how the trigger sequence itself affects the RNAi efficiency. These experiments would help define the limits of the RNAi silencing response and would aid future users to design more effective RNAi triggers, especially for genes with conserved domains or with duplications elsewhere in the genome. This is especially important in polyploid wheat, which has been shown to have a high rate of gene duplication (Dubcovsky et al. 1996; Akhunov et al. 2003), and therefore a high chance of targeting undesired (or unknown) paralogous genes.

## Selected gene region

The region of the gene selected as the RNAi trigger depends on the objective of the RNAi experiment. If the aim is to down-regulate only the targeted gene and its homoeologous copies, but not other members of the gene family, it is important to exclude the conserved domains from the RNAi trigger to avoid silencing a large number of paralogous genes. Both non-conserved coding regions and 3′ UTRs have been used successfully in wheat as RNAi triggers for individual members of multigene families (e.g., MADS-box, NAC domain, etc.) However, if the objective is to silence all members of a particular multigene family, the conserved region would be preferred for the RNAi trigger in order to target multiple members of the family simultaneously. This

was recently accomplished for seven members of the rice *OsRac* gene family (Miki et al. 2005) whose transcript levels were all simultaneously down-regulated using a single RNAi construct targeting a conserved region. These types of constructs are still difficult to design in wheat because its complete genome sequence is still not available.

### RNAi trigger size

Trigger size may place a major limitation on the design of the RNAi construct when the target gene contains only short pieces of non-conserved regions. In these cases it is important to know the minimum trigger size that can be used to induce effective gene silencing. Successful RNAi studies in wheat have used trigger regions ranging in size from ~200 to 550-bp (Table 1), although trigger dsRNA as short as 23-bp have been shown to induce the degradation of target mRNAs in *Nicotiana benthamiana* (Thomas et al. 2001). Transient RNAi studies using VIGS showed that a trigger of 120-bp was sufficient to produce significant gene silencing (Scofield et al. 2005). In the same experiment, trigger sequences of 80-bp were less effective for gene silencing and no detectable silencing was seen when using trigger sequences of 40-bp. Assuming that the VIGS data can be extrapolated to stable RNAi transformed plants, RNAi trigger sizes of at least 120-bp are desirable.

The upper limit for RNAi trigger size is less defined, with successful examples in wheat of up to 683 bp (Li et al. 2005) and in barley VIGS of up to 1,215 bp (Holzberg et al. 2002). The determination of the upper limit for RNAi trigger size may be also related to the number of targets that can be effectively included in a single RNAi construct to knockdown multiple target transcripts. For example, Miki et al. (2005) reported a progressive decrease in silencing efficiency in rice when increasing the size of a chimeric construct from 553 (two targets) to 1,089-bp (four targets).

### Silencing efficiency

RNAi is often associated with varying silencing efficiencies. Approximately half of the studied wheat RNAi transgenic lines showed a phenotype that differed significantly from the non-transgenic controls. The actual ratio may be gene-dependent since the reported efficiency ranged from 29 to 100%, making it difficult to predict a priori the number of independent transgenic events required to obtain the modified phenotype. Therefore, it is fair to conclude that multiple independent transgenic events must be analyzed in order to obtain reliable and robust results with RNAi in wheat.

## Transient RNAi in wheat through virus induced gene silencing (VIGS)

A faster alternative to stable transformation is transient RNAi silencing by VIGS. This system is based on the plant RNA-mediated antiviral defense mechanism, which targets viral dsRNA, and any target gene incorporated within the viral construct, for degradation (Vance and Vaucheret 2001). This response is transient since it depends on the virus replicating within the plant.

To date, VIGS has had broad application in dicots (Burch-Smith et al. 2004) and has also proven to be feasible in barley when using engineered Barley Stripe Mosaic Virus (BSMV) vectors (Holzberg et al. 2002). Using *PDS* and the visible photobleaching phenotype, Holzberg et al. (2002) showed that the BSMV-VIGS system elicits target-directed gene silencing in barley in a sequence dependent manner. Similar results were extended recently to common wheat (Scofield et al. 2005). Using barley *PDS* as the RNAi trigger (96% sequence homology to the corresponding wheat *PDS* region), they found photobleaching in wheat similar to that seen before in barley (Scofield et al. 2005). *PDS* transcript levels were reduced to at least 40% of the non-infected control during the duration of the study (3–21 days after infection).

To further determine the usefulness of the BSMV-VIGS system for wheat functional genomics, Scofield et al. (2005) targeted the leaf rust resistance gene *Lr21*. They reduced *Lr21* transcript levels to 17% relative to the non-infected control, leading to increased susceptibility of infected wheat plants to *Puccinia triticina*, the causal agent of leaf rust. Empty control vector inoculations were used as a control to eliminate putative confounding effects due to the virus infection. Other recent examples include

the BSMV-VIGS silencing of the leaf rust resistance gene *Lr1* candidate gene, *RGA567-5* (Cloutier et al. 2007), and of three receptor-like kinase genes (*TaRLK*) conferring resistance to *Puccinia striiformis* f. sp. *tritici*, the causal agent of stripe rust (Zhou et al. 2007). These examples suggest that the BSMV-VIGS system has the potential to aid in the functional analysis of wheat disease resistance genes, among others.

Despite its advantages, there are a number of limitations associated with the VIGS system. First, genes of interest may be expressed only under environmental conditions that are unsuitable for virus replication or in tissues or developmental stages that are not infected by the BSMV virus. In addition some wheat varieties possess host resistance to the BSMV virus, limiting the genotypes that can be used with this system. Finally, implementation of VIGS can be subjected to regulations which may limit its utilization in regions where the BSMV virus is absent and cannot be introduced.

Based on the previous considerations, VIGS would be favored over stable RNAi transformation when the response of the target genes can be tested in leaves of a susceptible wheat genotype and the phenotype can be observed under the environmental conditions in which the virus can grow. On the other hand, when the effect of the target gene has to be modified over a long developmental processes, as in the cases of vernalization genes (Yan et al. 2004) or senescence genes (Uauy et al. 2006b), the use of stable RNAi transformation would be a better strategy. An additional advantage of the stable transformation is that the transgenic plants can then be used in crosses to test the epistatic interactions between different genes or to test transgene effects in different genetic backgrounds.

## Conclusions and future directions

To date, wheat RNAi has been used successfully to target a wide range of genes including those encoding transcription factors, starch biosynthetic enzymes, storage proteins, and proteins involved in signaling and developmental processes. Likewise, the RNAi response has been documented in different tissues (seeds and leaves) and developmental stages (vegetative, grain filling, and senescence) and has been shown to be stably inherited. Similar to RNAi in other species, wheat RNAi is sequence specific. Multiple genes sharing stretches of identical sequence can be effectively silenced with a single RNAi construct. This feature is especially important in polyploid wheat, which has multiple homoeologous copies for each gene. Therefore, RNAi is coming of age as a useful and flexible tool to study gene function in polyploid wheat species, although more research is necessary to establish the additional factors that determine a construct's silencing efficiency in addition to the "21-nt rule."

Transcript levels have been reduced to at least 50–60% of those found in the non-transgenic controls, but the degree of silencing varies in different transgenic lines (Table 1). One caveat of this statistic is that the expression levels are usually measured only in transgenic lines showing a visible phenotype. Therefore, this value is an overestimation as it does not consider the expression change of transgenic lines showing subtle differences for the expected phenotype. To better define the RNAi effect in wheat, it would be beneficial for future studies to characterize the target expression in all transgenic lines, independent of the presence of the expected phenotype.

The phenotypic series obtained in most RNAi experiments provide valuable information on the effect of quantitative differences in transcript levels of the target gene, allowing a more precise understanding of the gene's function. In addition, the variable quantitative response generated by RNAi is an advantage when the complete knockdown of the target genes might result in lethality or extreme phenotypes with multiple pleiotropic effects. In these cases, intermediate levels of transcript down-regulation could have less severe effects facilitating a better definition of gene function.

In the coming years, wheat researchers will have access to unprecedented amounts of sequence information from the different whole-genome sequencing efforts. The ability to determine the function of these sequences will ultimately depend on the establishment of robust and flexible reverse genetic tools, such as RNAi. In reviewing the recent application of RNAi technology in wheat, we are confident that this technology will have significant impacts on wheat functional gene analyses in the upcoming years.

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