RNA Interference-Based Gene Silencing as an Efficient Tool for Functional Genomics in Hexaploid Bread Wheat

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Insertional mutagenesis and gene silencing are efficient tools for the determination of gene function. In contrast to gain- or loss-of-function approaches, RNA interference (RNAi)-induced gene silencing can possibly silence multigene families and homoeologous genes in polyploids. This is of great importance for functional studies in hexaploid wheat (*Triticum aestivum*), where most of the genes are present in at least three homoeologous copies and conventional insertional mutagenesis is not effective. We have introduced into bread wheat double-stranded RNA-expressing constructs containing fragments of genes encoding *Phytoene Desaturase* (PDS) or the signal transducer of ethylene, *Ethylene Insensitive 2* (EIN2). Transformed plants showed phenotypic changes that were stably inherited over at least two generations. These changes were very similar to mutant phenotypes of the two genes in diploid model plants. Quantitative real-time polymerase chain reaction revealed a good correlation between decreasing mRNA levels and increasingly severe phenotypes. RNAi silencing had the same quantitative effect on all three homoeologous genes. The most severe phenotypes were observed in homozygous plants that showed the strongest mRNA reduction and, interestingly, produced around 2-fold the amount of small RNAs compared to heterozygous plants. This suggests that the effect of RNAi in hexaploid wheat is gene-dosage dependent. Wheat seedlings with low mRNA levels for *EIN2* were ethylene insensitive. Thus, *EIN2* is a positive regulator of the ethylene-signaling pathway in wheat, very similar to its homologs in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*). Our data show that RNAi results in stably inherited phenotypes and therefore represents an efficient tool for functional genomic studies in polyploid wheat.

A major challenge in the postgenome era of plant biology is to determine the functions of all the genes in a plant genome. A straightforward approach to this problem is to reduce or knock out expression of a gene to induce a mutant phenotype that is indicative of the gene function. Insertional mutagenesis is a useful tool for this type of study and is based on transposon/T-DNA insertions (Page and Grossniklaus, 2002). However, this approach is limited by the time required to saturate a genome, by lethal knockouts, and is restricted to a few plant species. In addition, it is complicated by the problem of genetic redundancy caused by multigene families and polyploidy. In contrast to insertional mutagenesis, RNA interference (RNAi) is based on sequence-specific RNA degradation that follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Marx, 2000; Carthew, 2001; Baulcombe, 2004). RNAi allows silencing one, several, or all members of a multigene family or homoeologous gene copies in polyploids by targeting sequences that are unique or shared by several genes (Lawrence and Pikaard, 2003; Miki et al., 2005). dsRNA is detected by the host plant genome as aberrant and is cleaved by the action of Dicer-like enzymes (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Djikeng et al., 2001; Tang et al., 2003) into two distinct classes of small interfering RNA (siRNA): long and short siRNAs (Hamilton et al., 2002; Tang et al., 2003). These two classes of small RNAs were proposed to have distinct RNA silencing functions: approximately 21-mers to direct posttranscriptional signaling via mRNA degradation and the approximately 24-mers to trigger systemic silencing and the methylation of homologous DNA (Hamilton et al., 2002). Biochemical experiments in wheat (*Triticum aestivum*) germ and cauliflower (*Brassica oleracea*) extracts provided clear evidence that each class of siRNAs is generated by distinct Dicer-like enzymes (Tang et al., 2003). Through sequence complementarity, siRNA in association with an RNA-induced silencing complex directs the cleavage of endogenous RNA transcripts. siRNAs are also responsible for amplifying the silencing signal by priming endogenous RNA, which can be converted to dsRNA by the action of RNA-directed RNA polymerase (RdRP) encoded in the plant genome (Lipardi et al., 2001; Sijen et al., 2001). Tang et al. (2003) showed that wheat germ extracts contained an RdRP activity. Thus, a few trigger dsRNA molecules suffice to inactivate a continuously transcribed target.
RNA Interference for Functional Genomics in Hexaploid Wheat

RESULTS

Identification of Wheat Expressed Sequence Tags for PDS and EIN2

The two genes selected for testing RNAi in hexaploid wheat, *PDS* and *EIN2*, have not yet been described in the wheat genome. A BLASTN search using the barley *PDS* cDNA sequence (AY062039; Holzberg et al., 2002) identified several wheat expressed sequence tags (ESTs) showing high nucleotide (nt) identity (*E* < 10^-100) to the barley sequence. Seven wheat ESTs that were homologous to the 5’ end of the barley sequence were used in a multiple sequence alignment to find out whether more than one *PDS* gene is expressed in wheat. Two groups of three and four ESTs, with more than 97% nt identity, could be clearly
distinguished based on characteristic simple nt polymorphisms (SNPs). A pair of primers (Table I) was designed within the conserved region and based on the BG908924 hexaploid wheat sequence that shared the highest nt identity ($95\%$) with the barley PDS cDNA (AY062039). This BG908924 wheat sequence (785 bp) belongs to the consensus sequence TC236658 of 2,464 bp (http://www.tigr.org/tigr-scripts/tgi/tc_report.pl?tc=TC236658&species=wheat) and aligns with the full-length cDNA of PDS of rice (AF049356; 2,027 bp) between nts 311 and 1,075 with an $89\%$ identity (Fig. 1A). A 480-bp fragment of wheat PDS ($wPDS$) was amplified by reverse transcription (RT)-PCR from wheat leaf cDNA and was used for the RNAi construct. The RT-PCR product was also cloned, and several independent transformants were sequenced. Following a multiple sequence alignment, three distinct groups of sequences were identified based on SNPs (Supplemental Fig. 1A; the same SNPs were detected in three independent RT-PCR reactions), indicating that there are at least three active copies of the PDS gene in the wheat genome. The same RT-PCR fragment was used as a probe to estimate the number of copies of $wPDS$ in the wheat genome by hybridization to hexaploid (cv Bobwhite and cv Chinese Spring) and diploid (Triticum monococcum cv DV92) wheat DNA digested with several restriction enzymes (Supplemental Fig. 2A). The hybridization pattern was simple for several enzyme combinations, showing between one and two bands in the diploid $T. monococcum$ species and between two and four bands in hexaploid wheat. Southern hybridizations to nulli-tetrasomic lines of Chinese Spring (Sears, 1966) digested with HindIII allowed mapping $wPDS$ on chromosomes 4A and 4D (Supplemental Fig. 2B). In a recent study, Cenci et al. (2004) localized durum wheat (Triticum durum) bacterial artificial chromosome clones containing the PDS genes on chromosome 4A as well as 4B. The nulli-tetrasomic 4B line used in our study was probably not genetically pure. Indeed, several SSR markers specific of chromosome 4B could be amplified (data not shown), suggesting the presence of chromosome 4B in this nulli-tetrasomic 4B line. Therefore, additional fragments of $PDS$ could not be mapped on chromosome 4B.

A BLASTN search using the barley EIN2 cDNA sequence (BM816947) identified eight wheat EST sequences, but only one EST (AL816731) showed high nt identity ($E < 10^{-100}; 93\%$) to the barley sequence. This AL816731 wheat sequence (564 bp) belongs to the

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with *HindIII* allowed mapping *wEin2* on chromosomes 5A, 5B, and 5D (Supplemental Fig. 2D). These results suggest that for both *wPDS* and *wEin2* genes, there are three expressed copies in hexaploid wheat, with one copy on each of the homoeologous genomes.

**Specific Silencing of the PDS Genes Induces Photobleaching in Hexaploid Wheat**

One hundred and fifty three putative transgenic plants were produced by particle bombardment with the intron-spliced self-complementary construct containing the sense and antisense repeats of the *wPDS* cDNA sequence. Genomic DNA from these plants was digested with *HindIII*, and the *wPDS* sequence was used as a probe for DNA Southern-blot hybridization. Eighty-two lines, which correspond to 53% of the analyzed plants, contained the expected three intact *HindIII* fragments of the construct (0.5, 1, and 2.2 kb; Fig. 1C; Supplemental Fig. 4A) and 64 of them (78%) exhibited photobleaching at the seedling stage, indicating specific silencing of endogenous *PDS* (Fig. 2). The *PDS*-RNAi transgenic lines were arranged into a phenotypic series based on the severity of the photobleached phenotype (strong, intermediate, and weak). Most of the lines (41 lines) developed a strong photobleached phenotype, resulting in a lethal albino phenotype (Fig. 2A). Fifteen lines showed an intermediate phenotype with continuous parallel streaks (Fig. 2, B and C) where photobleaching affected either one-half of the leaves (Fig. 2B) or only the middle part of the leaf, whereas the leaf margins maintained the wild-type phenotype (Fig. 2C). The remaining eight lines produced a weak phenotype where only a small part of the leaves was affected by photobleaching (Fig. 2D). Both single and multiple copy lines with extensive DNA rearrangements showed photobleaching (Supplemental Fig. 4A; Fig. 3A).

Thus, *PDS* dsRNA-mediated genetic interference causes photobleaching in hexaploid wheat in a similar manner.
way to plants treated with a chemical inhibitor of PDS (Böger and Sandmann, 1998) or to virus-induced PDS gene silencing (VIGS) in barley (Holzberg et al., 2002). The consistent appearance of bleached tissue in the large number of regenerated plants suggests that the endogenous PDS genes are no longer functional in these tissues due to RNAi.

dsRNA Interferes with wPDS mRNA Accumulation of All Three Homoeologous Genes

The expression of PDS in the RNAi transgenic wheat lines was determined by real-time quantitative RT-PCR. Primers were designed to specifically measure effective endogenous PDS mRNA levels and not the transgene transcripts. Primers were located on the
wheat EST sequence CK163183 (Table I; Supplemental Fig. 1A) upstream of the conserved nt sequence regions used to design the primers for the RNAi construct. Cloning and sequencing of the products produced by RT-PCR revealed that the activity of all three genes described above was measured. The primer binding sites were completely conserved in the three expressed genes, and three distinct sequences were identified based on SNPs (Supplemental Fig. 1A; the same SNPs were detected in three independent RT-PCR reactions). The SNPs allowed the design of additional primers for specifically amplifying each of the homoeologous copies of the wPDS gene (Supplemental Fig. 1B). The reduction of relative mRNA levels of wPDS was very similar in each of the three homoeologous genes (Fig. 3A). Therefore, the RNAi silencing mechanism affects all three copies of the gene in the same way.

The severity of the photobleached phenotype inversely correlated with PDS mRNA expression levels in the leaves of all the transgenic RNAi lines analyzed. As shown in Figure 3A, expression levels of lines that exhibited an albino phenotype (lines 1, 3, 9, and 10) were 11%, 8.4%, 15%, and 7.2% of the wild type, respectively. A transcript reduction relative to the wild type was also observed in lines showing streaks of photobleaching (5 and 11 in Fig. 3A), but they accumulated more mRNA than the albino lines (38% and 48% of the wild type, respectively). In all the other transgenic RNAi lines not showing any photobleaching, PDS mRNA expression levels were not significantly different from the wild type.

These results indicate that PDS mRNA levels decline with increasingly severe phenotypes and suggest that endogenous PDS mRNA is a target of dsRNA-mediated genetic interference that equally silences the three homoeologous copies.

Phytoene Accumulates in Bleached Tissue as a Result of RNAi-Based Silencing of Endogenous Wheat PDS

Blockage of the carotenoid pathway can be mimicked chemically with the herbicide norflurazon, a specific inhibitor of the PDS enzyme. This inhibition causes accumulation of phytoene in treated tissues (Böger and Sandmann, 1998). To confirm that the bleached phenotype in our experiments was due to PDS silencing, we used HPLC to determine phytoene levels in RNAi transgenic lines with or without bleached tissues and in norflurazon-treated and -untreated wild-type plants. In addition to a specific retention time on reverse-phase columns, carotenoids are characterized by their spectral properties, consisting of three peak maxima with a unique spectral shape (ratio of peaks II/III), which are influenced by the solvent used (Wurtzel et al., 2001). As shown in Supplemental Figure 5A, leaf tissue from wild-type norflurazon-treated plants contained two spectral peaks at 286 nm, with a retention time of 3.65 min (peak 1) and 5.61 min (peak 2; Supplemental Fig. 5G). These two peaks were absent in wild-type untreated plants (Supplemental Fig. 5B), which contained another type of spectral peak absorbing at a maximum of 446 nm, with a retention time of 4 min (Supplemental Fig. 5G). The spectra of numbered peaks 1 and 2 (Supplemental Fig. 5, D and E) show the characteristics of the compound phytoene (three peaks maxima and peaks shape), which is identical to previously published spectral profiles of phytoene (Li et al., 1996; Wurtzel et al., 2001). The spectrum detected in wild-type untreated plants (peak 3, Supplemental Fig. 5F) showed no phytoene peaks but the characteristics of the compound lutein (Suzuki and Shioi, 2003), which is derived from subsequent series of desaturations and cyclizations in the carotenoid biosynthesis pathway when PDS is active. PDS-RNAi transgenic lines, which showed an albino phenotype, also contained two peaks with virtually identical spectra to the spectrum of phytoene from norflurazon-treated wild-type tissue (Supplemental Fig. 5, A, D, and E). Leaves with photobleached streaks contained a mixture of peaks corresponding to the two compounds phytoene and lutein that were present in norflurazon-treated and -untreated wild-type tissue, respectively (Supplemental Fig. 5C). PDS-RNAi transgenic lines, which did not show any phenotype, produced a spectrum identical to the one detected in wild-type untreated plants (Supplemental Fig. 5B). Moreover, as shown in Supplemental Figure 5G, a comparison of peak retention times, peak maxima, and peak ratios II/III between the norflurazon-treated wild-type plants and the RNAi transgenic lines corroborated the presence or absence of phytoene in relation to the efficiency of silencing. These results demonstrate that phytoene accumulation and photobleaching are correlated and provide biochemical confirmation of PDS gene inactivation in hexaploid wheat.

Inheritance of the Genetic Interference of wPDS and Detection of siRNA in Silenced Plants

T₀ PDS-RNAi lines that showed an albino phenotype did not set seeds and therefore could not be analyzed further. The progeny from each PDS-RNAi line that had intermediate phenotypes (streaks on leaves; 23 lines) all showed a 3:1 segregation for the presence and absence of the hp transgene, as shown in Figure 3B, with an example of PCR analysis in the progeny of line 5. This result suggests that the primary transformants integrated the hp transgene at a single locus, although separate integration at very closely linked loci cannot be excluded. All the T₁ progeny showed Mendelian segregation for the photobleached phenotype on the leaves. Surprisingly, the intermediate phenotype with continuous parallel streaks (Fig. 2, B–D) of some of the T₀ transgenic lines was never observed again in the following generations. Twenty-five percent of the T₁ plants containing the hp transgene showed strong photobleaching of the leaves with large albino areas, which in a few cases eventually developed into completely albino plants (Fig. 3D). The
other T1 plants that had integrated the transgene (approximately 50%) showed a weak phenotype, where photobleaching was affecting only the base of the leaves (Fig. 3D). Quantitative real-time PCR revealed again a correlation between the level of mRNA and the severity of the photobleached phenotype. As shown in Figure 3C, the T1 plants (derived from T0 line no. 5) having a reduction of endogenous wPDS transcripts to less than 20% of wild type (T1 plants 4, 8, 10, and 20) showed strong photobleaching. Among the T1 plants that showed a weak phenotype, plants 3, 5, 6, 13, 14, 16, 17, 18, and 19 accumulated mRNA levels between 30% and 70% of wild type (medium reduction). Plants 1, 2, 9, and 12, which did not contain the hp transgene, had the same mRNA levels as the wild type.

We subsequently analyzed each of the PDS-RNAi lines in the T2 generation by selfing different T1 plants showing strong/weak photobleaching and wild-type phenotypes. The T1 plants that showed strong photobleaching were homozygous, since all the T2 progenies contained the transgene, showed large albino areas, and had their mRNA levels of endogenous wPDS genes reduced to less than 40% of the wild type (data not shown). The T1 plants that showed weak photobleaching were heterozygous, since all the T2 progenies were still segregating for the phenotype and the transgene.

Small RNA-gel blots were used to test whether the amounts of siRNA were different in homozygous and heterozygous lines. Small RNAs were recovered from a subset of the T1 plants used as the source of total RNA for quantitative real-time PCR. We detected sequence-specific siRNAs of around 24 bp in both homozygous and heterozygous plants showing strong/weak photobleaching (Fig. 3E). Wild-type plants did not accumulate small PDS RNAs. Thus, small PDS RNAs accumulated only upon silencing of the PDS genes. The relative intensity of the hybridization signals in the transgenic versus wild-type plants indicated that the homozygous plants contain around double the amount of small RNAs compared to heterozygous plants. It is therefore likely that the strong photobleached phenotype observed in the homozygous plants is in part related to a higher accumulation of siRNAs.

This genetic analysis indicates that RNAi of wPDS is stably inherited over at least two generations in a Mendelian fashion of a single locus. Small RNAs specific for the silenced gene were detected and their accumulation was quantitatively different in homozygous and heterozygous lines. In homozygous plants, accumulation of siRNAs was significantly higher to give effective gene silencing and to develop the most severe phenotype. These results suggest that the effect of RNAi in hexaploid wheat is probably gene-dosage dependent. The reason for the discrepancy of hemizygous and heterozygous phenotypes at T0 versus later generations is not known, but it is intriguing that the streak phenotype of the T0 generation was never recovered in later generations.

### EIN2 dsRNA-Mediated Genetic Interference in Hexaploid Wheat

To assess whether the EIN2 signal transducer of ethylene can also be effectively silenced by RNAi in wheat, 33 putative transgenic plants were produced by particle bombardment with the intron-spliced self-complementary construct containing the sense and antisense repeats of the wEIN2 cDNA sequence. Genomic DNA from these plants was digested with EcoRI, and blots were hybridized with a probe corresponding to the wEIN2 cDNA sequence of the hpRNA construct. Eighteen lines, which correspond to 54% of the analyzed plants, contained the expected intact EcoRI fragment of the construct (2 kb; Supplemental Fig. 4B; Fig. 1C).

We identified by quantitative real-time PCR six T0 primary transgenic lines (33% of the lines that contained the full-length RNAi fragment) with a significant reduction of mRNA expression of the endogenous wEIN2 genes (Fig. 4A). As for the wPDS genes, the RT-PCR primers were designed to specifically detect the endogenous EIN2 and not the transgene transcripts. Primers were located on the wheat EST sequence CD925940 (Table I; Supplemental Fig. 3A) upstream of the conserved nt sequence regions used to design the primers for the RNAi construct. Cloning and sequencing of the RT-PCR products revealed that the activity of all three homoeologous genes was measured (Supplemental Fig. 3A). Three distinct sequences were identified based on SNPs (the same SNPs were detected in three independent RT-PCR reactions). Quantitative real-time PCR with primers specific to each of the homoeologous copies of the wEIN2 gene (Supplemental Fig. 3B) revealed that the RNAi silencing mechanism is affecting all three homoeologous genes in the same way (Fig. 4A).

For five lines, wEIN2 transcript level was reduced to between 30% and 50% compared to the wild type (intermediate reduction in lines 2, 7, 10, 13, and 19 of Fig. 4A). Line 18 had a strong reduction of the wEIN2 mRNA level, which was only 1% of the level detected in the wild-type plant (Fig. 4A). As for the wPDS genes, both single and multiple copy lines with extensive DNA rearrangements showed a significant reduction of wEIN2 mRNA levels (Supplemental Fig. 4B; Fig. 4A). The six T0 primary transgenic lines with lower EIN2 expression showed a normal phenotype, and their ethylene response signaling was studied in the next generations.

### Ethylene Response Signaling in Wheat

**EIN2-RNAi Lines**

EIN2 was identified as a central component of the ethylene signaling pathway both in Arabidopsis (Alonso et al., 1999) and in rice (Jun et al., 2004). All six T0 EIN2-RNAi transgenic wheat lines with lower EIN2 expression set seeds, and we could therefore examine whether their ethylene response was altered.
Figure 4. RNAi-mediated specific silencing of the wEIN2 genes in hexaploid wheat. A, Quantitative real-time PCR of eight wEIN2-RNAi transgenic T₀ primary transformants with primers designed within the conserved nt sequence region identified on wheat EST CD925940 and with primers specific to each of the homoeologous wEIN2 genes (see Supplemental Fig. 3). Relative mRNA levels of wEIN2 were normalized to the mRNA level of wild-type (Wt) plants (=1). The GAPDH gene was used as an internal standard. Data are the average of triplicate samples (±SD). The numbering of the transgenic lines analyzed by quantitative real-time PCR refers to the Southern-blot data shown in Supplemental Figure 4, and the Southern analysis is summarized at the bottom of A. +, Transgenic; −, nontransgenic T₀ lines. B, C, and D, Analysis of the ethylene response in the T₁ generation of the T₀ EIN2-RNAi transgenic line 10. B, Wild-type seeds germinated in presence and in absence of ACC. C, T₁ seeds germinated in presence of ACC. Bars in B and C = 4 cm. D, Relative mRNA levels of wEIN2 that were normalized to the mRNA level of wild-type (Wt) plants (=1). Line T₀ from which this population is derived is also shown. Length measurements of each T₁ plant are shown on the same diagram. PCR analysis for the presence/absence of the transgene is shown in D.
Twenty T1 seeds of each of these six lines, wild-type plants, and five primary transgenic lines that did not show any reduction of the \( wEIN2 \) transcript were germinated at 25°C under light (16-h light, 8-h dark) on Murashige and Skoog medium in the presence of 20 \( \mu M \) 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene. Plant length was determined 15 d after sowing. All T1 families showed a 3:1 segregation for the presence and absence of the hp transgene, suggesting that the primary transformants integrated the hp transgene at a single locus, although separate integration at very closely linked loci cannot be excluded. The six T1 families derived from the T0 lines with lower \( EIN2 \) expression showed Mendelian segregation for normal and stunted growth when grown in the presence of 20 \( \mu M \) ACC (e.g. in Fig. 4C, six T1 plants derived from T0 line 10 are shown). Wild-type plants and the other five transgenic lines tested (negative controls) showed a stunted morphology on ACC-containing medium (Fig. 4B). As observed with the \( mPDS \) genes, 25% of the T1 plants containing the hp transgene showed complete insensitivity to ethylene (Fig. 4C; T1 plant 1) and a normal growth in presence of ACC. Seedling lengths of these ethylene-insensitive plants were significantly higher \( (p = 1.18 \times 10^{-3}) \) than the rest of the population in the presence of ACC. The other T1 plants that had the transgene (approximately 50%) showed a growth only partially affected by the presence of ACC (Fig. 4C; T1 plants 3 and 4), and the length of the seedlings was also significantly higher \( (p = 2.78 \times 10^{-5}) \) than the T1 plants that did not contain the hp transgene (Fig. 4C; T1 plants 2, 5, and 6).

There was a very high negative linear correlation \( (r^2 = -0.976) \) between the length of the seedlings in the presence of ACC and the amount of \( wEIN2 \) transcript measured in the T1 progenies. As observed with the \( wPDS \) genes, \( EIN2 \) mRNA levels declined with increasingly severe phenotypes (Fig. 4D). T1 plants completely insensitive to ethylene showed the strongest mRNA reduction (around 70%) compared to wild type. T1 plants with an intermediate phenotype accumulated mRNA levels around 50% of wild type, whereas all the other T1 plants that did not contain the hp transgene and were sensitive to ethylene with a stunted morphology had the same mRNA levels as the wild type.

By analyzing the T2 generation, we confirmed that those T1 plants that had the strongest mRNA reduction and were completely insensitive to ethylene were homozygous plants, because all their T2 progeny contained the transgene (Fig. 5A), had their mRNA levels reduced to less than 40% of the wild-type (Fig. 5B), and showed complete ethylene insensitivity in the presence of ACC (Fig. 5, B and C). T1 plants that were partially affected by ethylene were heterozygous, since their T2 progeny were still segregating for normal and stunted growth when grown in the presence of ACC.

These results demonstrate that the \( EIN2 \)-RNAi transgenic wheat lines produced in this study are ethylene insensitive. This is consistent with the hypothesis that \( EIN2 \) is a positive signal component in ethylene signaling and that inhibiting its expression reduces the ethylene response. We conclude that RNAi silencing in hexaploid wheat is also effective for the silencing of a gene involved in a signaling process.

**DISCUSSION**

We have demonstrated that RNAi-mediated gene silencing is effective in hexaploid wheat and can efficiently induce reduction of mRNA levels of three homoeologous genes. Expression of the three homoeologous genes was reduced to the same extent, suggesting that RNAi can resolve the issue of genetic redundancy in hexaploid wheat in an efficient way, as it was also suggested by studies in the two allotetraploid species \( Arabidopsis suecica \) (Lawrence and Pikaard, 2003) and cotton (\( Gossypium hirsutum \); Liu et al., 2000).

Knowledge of the complete and exact sequence of the target genes was not essential to induce specific gene silencing, as sequence information from ESTs was sufficient. This is important for the development of high-throughput methods for functional genomics. Trigger-dsRNAs as short as 23 to 26 bp have been shown to induce degradation of target mRNAs (Hamilton and Baulcombe, 1999; Thomas et al., 2001), and several studies reported that sequences that are 88% to 100% identical to the endogenous gene target caused silencing (Jones et al., 1998; Ingelbrecht et al., 1999; Schweizer et al., 2000; Xu et al., 2001; Holzberg et al., 2002). In our study, we used wheat EST sequences of around 500 bp to construct the RNAi vectors. A specific EST is derived from a specific gene, but it is known that homoeologous genes in wheat share up to 99% identity at the nt sequence level in the coding regions (Kimbara et al., 2004). Therefore, there is a high chance that homoeologous genes will retain regions of identity, resulting in silencing of all the genes. The suppression of the homoeologous genes was probably also facilitated by the use of a sequence within the conserved regions identified among homologous wheat ESTs. Concerns have arisen that siRNA could cause other effects than those related to the knockdown of the target gene due to cross hybridization or binding in a sequence-dependent manner to various cellular proteins (off-target effects; Jackson et al., 2003; Scacheri et al., 2004). Standard software can now be used for improving detection of sequence identity to accurately and systematically evaluate and minimize RNAi off-target effects between siRNA sequences and target genes (Qiu et al., 2005).

We have used two different genes to assess the effectiveness of RNAi in hexaploid wheat, the enzyme \( PDS \) and the ethylene signaling component \( EIN2 \). These genes were chosen because mutant alleles have been reported to give distinct phenotypes in diploid plant species. It was also important to assess whether a gene that encodes a regulatory factor can be effectively silenced in wheat. Indeed, a reduction of enzyme
amount might allow the detection of a mutant phenotype, whereas silencing of a signaling component might not be sufficient to cause a mutant phenotype, because even a strongly reduced amount of protein might still be sufficient for proper function. We have shown that RNAi-mediated silencing of both genes results in the reduction of transcripts by up to 93% for PDS and 99% for EIN2. Therefore, dsRNAs corresponding to these two genes caused strong and specific genetic interference, suggesting that dsRNA-mediated gene silencing under the control of the ubiquitin promoter can occur in the tissues where these genes normally function.

We found a strong correlation between decreased levels of mRNA and increased severity of phenotypes. A phenotypic series was obtained from the RNAi lines with a full spectrum of the effect of RNAi (weak, intermediate, and strong) on gene expression, which is in agreement with the results in Arabidopsis (Chuang and Meyerowitz, 2000; Wang et al., 2005) and tomato (Xiong et al., 2005). Part of the variation of the RNAi effect can be explained by transgene copy number and/or positional effects of particular DNA insertion events. However, our results suggest that the severity of phenotypes is not related to the transgene copy number, because both single and multiple copy lines with extensive DNA rearrangements showed a mutant phenotype. Chuang and Meyerowitz (2000) and Wang et al. (2005) also found no relationship between severity of the phenotype and transgene copy number in Arabidopsis. In contrast, Kerschen et al. (2004) described that multicopy RNAi Arabidopsis lines reduced target mRNA levels to a lesser extent and with more variability between lines than did single-copy lines. A number of studies in rice and wheat have reported that there is no evidence for any direct relationship between transgene copy number and transgene expression or stability (Kohli et al., 1999;
Stoger et al., 1999). Thus, we can assume that the phenotypic series observed in the T0 transgenic lines is due to positional effects of the DNA insertion event. The variation in the degree of silencing observed in the transformants showing both reduction and loss of function may be a useful feature for gene discovery and functional genomics. Complete silencing of genes encoding a key element in basic cell functions or at particular developmental stages may result in lethality, whereas the reduced gene expression may give viable plants with phenotypes indicative of the role of the target gene. Systems to deliver inducible RNAi offer the advantage of silencing gene expression at specific developmental stages or in specific tissues, because they provide flexibility for the timing and the degree of gene inactivation and have the potential for reversal of silencing by withdrawal of the inducer (Guo et al., 2003; Wielopolska et al., 2005).

It has been shown by Smith et al. (2000) and Wesley et al. (2001) that 66% to 100% of the plants (tobacco [Nicotiana tabacum], Arabidopsis, cotton, and rice) transformed with an intron-containing hp construct can be silenced. In our investigation, 78% of the wheat PDS-RNAi and 33% of the wheat EIN2-RNAi transgenic lines showed silencing. One explanation for the difference in efficiency of silencing between the two genes may be that some phenotypes (such as silencing of EIN2) are less sensitive to the level of gene expression and that a reduced amount of protein is still sufficient to confer the proper function. The effectiveness of silencing appears to be gene dependent and could reflect accessibility of target mRNA or the relative abundance of the target mRNA and the hpRNA in cells in which the gene is active (Helliwell and Waterhouse, 2003). Kerschen et al. (2004) and Wang et al. (2005) suggested that RNAi efficiency and the endogenous transcription level of the target gene are not necessarily related.

We confirmed that the expression of the RNAi phenotype is stably inherited over at least two generations for both wPDS and wEIN2 genes, possibly making this approach a reliable tool not only for functional genomics but also for the genetic modification of agronomically interesting traits. In our study, dsRNA-expressing constructs were inherited in a Mendelian fashion as a single locus, and the most severe phenotype was observed in homozygous plants that showed the strongest mRNA reduction. Among the phenotypic series that we obtained from the transgenic PDS-RNAi lines, we have also observed T0 lines showing a strong phenotype in a hemizygous state. This strong phenotype was lethal for the wPDS genes but had no effect in the case of the wEIN2 genes (Fig. 5A, plant 18). The intermediate phenotype of wPDS with continuous parallel streaks (Fig. 2, B–D) of some of the T0 transgenic lines was never observed again in the following generations. This suggests that in the early development of newly transformed T0 seedlings, RNAi is not fully established, and therefore, the mutant phenotypes differ from later generations. Thus, it might be advisable to not only study the T0 generation in wheat RNAi projects but also in later generations. Our results indicate that the most efficient silenced phenotypes are stably recovered in homozygous lines, suggesting that the effect of RNAi in hexaploid wheat is gene-dosage dependent. This is possibly due to the progressive repression of the target gene with increasing allelic concentration of the transgene. This hypothesis is supported by our observation of gene-dosage dependence of specific siRNAs that were approximately double the amount in homozygous plants compared to heterozygous plants. These results are in agreement with Garcia-Perez et al. (2004), who reported that tobacco leaves of homozygous rootstocks have accumulated approximately double the amount of secondary siRNAs than the heterozygous rootstocks, suggesting that the induction of systemic silencing was strikingly dosage dependent. Similarly, several studies have suggested that transgene dosage can affect resistance of a transgenic line carrying transgenes homologous to viral sequences, because homozygous, but not hemizygous, R2 progenies were able to confer high level of resistance to different strains (Dinant et al., 1997; McDonald et al., 1997; Tenllado and Diaz-Ruiz, 1999; Tennant et al., 2001).

New studies provide convincing biochemical and genetic evidence that RdRP plays a critical role in amplifying the RNAi effect, explaining the extreme efficiency and the self-sustaining nature of RNAi (Lipardi et al., 2001; Sijen et al., 2001). An RdRP can use the siRNAs as primers and the target mRNAs as templates to produce newly formed dsRNAs that are subsequently cleaved to produce secondary siRNAs. Recently, it has been shown (Himber et al., 2003; Garcia-Perez et al., 2004) that secondary siRNAs belong exclusively to the 21-nt siRNA class. This observation argues against the previously proposed role for the 25-nt siRNAs as systemic silencing signal (Hamilton et al., 2002) and contrasts with the observation that de novo dsRNA synthesis in wheat germ extracts is linked to production of siRNAs that are almost exclusively 25-nt long (Tang et al., 2003). In addition, experiments with dsRNA suggested that the 21-mers are produced in wheat at about one-quarter the rate of the 24 to 25-nt small RNAs (Tang et al., 2003). Our results are in agreement with the observations of Hamilton et al. (2002) and Tang et al. (2003) because the RNAi wheat transgenic lines produced in our study have accumulated only one class of siRNAs, which is the 25-nt siRNAs. Identification of the RdRP responsible for systemic silencing signal in vitro will help to address the issue with the contrasting findings of Himber et al. (2003) and Garcia-Perez et al. (2004).

Down-regulation of EIN2 by RNAi resulted in ethylene-insensitive wheat plants that showed normal wild-type growth in presence of ACC, the immediate precursor of ethylene. This is consistent with the hypothesis that EIN2 is a positive regulator of the ethylene-signaling in wheat, very similar to its homologs in Arabidopsis (Alonso et al., 1999) and rice.
transgenic plants are necessary to successfully use RNAi constructs for plant functional genomics. Gene transfer technology is still limited in wheat by the low frequency of generation of transgenic plants. With further development and increase of the efficiency of the wheat transformation methods, an exciting perspective will be opened up to improve tools for functional analysis of wheat genes, with a strong impact on wheat breeding.

MATERIALS AND METHODS

Plant Material

Wheat (Triticum aestivum) cv Bobwhite, accession SH98 26 (provided by Dr. A. Pellegrineschi, International Center for Development of Maize and Wheat, Mexico) was used for all experiments. Donor plants for embryonic transformation and transgenic plants were grown in a greenhouse with 16-h light at 21°C and 8-h night at 16°C. Every 10 to 15 d, routine fertilizer treatments were applied to the donor plants.

Generation of RNAi Lines

PDS and EIN2 nt sequences were obtained from The Institute for Genome Research database (www.tigr.org). Using barley (Hordeum vulgare) sequences, a BLASTN search was carried out to find hexaploid wheat ESTs. Multiple sequence alignments were done with ClustalW (Thompson et al., 1994). PCR primers were designed on the basis of wheat ESTs similar to the target genes using the Primer3 WWW primer tool (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3/www.cgi; Whitehead Institute for Biomedical Research). Vector pAHHC17 containing the maize (Zea mays) ubi-1 promoter and the nopaline synthase terminator (Christensen et al., 1992) were used to make the self-complementary intron-containing hp RNA constructs. Fragments of 480 and 518 bp corresponding to the wheat cDNAs of wPDS and wEIN2, respectively, were isolated by RT-PCR using specific primers with incorporated BamHI and BgIII restriction sites (Table I), which produced ends compatible with each other. This allowed the gene fragments to be directionally cloned within the unique BamHI site of vector pAHHC17. PCR amplification was carried out for 35 cycles of 45 s, denaturation at 94°C, 45 s annealing at 62°C, and 90 min extension at 72°C. The amplified fragments were then subcloned into pGEM-T vector (Promega) and sequenced. The cDNA fragments recovered by BamHI and BgIII digestion were cloned in the BamHI restriction site of plasmid pAHHC17. Each RNAi construct contained a cDNA fragment derived from the respective target gene and oriented in the antisense and sense directions at the 5′ and 3′ ends of the construct, respectively (Fig. 1C), separated by an intron derived from the wheat TAK14 gene (AF325198). The intron TAK14 fragment was produced with the same cloning strategy as described for the two RNAi target genes. The resulting plasmids were named pPDS-RNAi and pEIN2-RNAi for the wPDS and wEIN2 genes, respectively.

RNAi lines were produced using particle bombardment-mediated transformation of immature embryos as described (Pellegrineschi et al., 2002). Plasmid pPDS-RNAi or pEIN2-RNAi was cotransformed with a plasmid containing phospho-Man isomerase as selectable marker (Wright et al., 2001). Plasmid pPDS-RNAi, pEIN2-RNAi, or pAVE2-RNAi was cotransformed with a plasmid containing phospho-Man isomerase as selectable marker (Wright et al., 2001). Regeneration and selection of the transformed plants were performed essentially as described (Wright et al., 2001; Pellegrineschi et al., 2002). Transformants were identified by DNA Southern hybridization (Stein et al., 2000) using plant genomic DNA digested with HindIII for pPDS and EcoRI for pEIN2 and probes corresponding to the selected cDNA sequences of the hpRNA construct (Fig. 1C). Hybridizations were performed overnight at 65°C in 50 mM sodium chloride/sodium phosphate/EDTA, 0.5% SDS, and 5× Denhardt’s solution. Membranes were washed at 65°C three times for 20 min in 0.5× SSC and 0.1% SDS, and then they were autoradiographed.

To determine the inheritance of the RNAi construct and the phenotype, transgenic lines were allowed to self pollinate, and segregation analyses were performed in the T1 and T2 generations by means of PCR, phenotype observation, and quantitative real-time PCR. Rapid DNA extractions at the two-leaf stage (modified for small volumes from Stein et al., 2000) and PCR techniques accelerated the screening of the T1 and T2 plants. The presence of the RNAi construct was determined using a forward primer at the 3′ end of the
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