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A Modified TILLING Method for Wheat Breeding

Chongmei Dong, Jessica Dalton-Morgan, Kate Vincent, and Peter Sharp*

Abstract

The large genome and polyploidy of wheat (*Triticum aestivum* L.) makes it difficult to identify desirable genetic changes based on phenotypic screening due to gene redundancy. Forward genetics is, therefore, more difficult in wheat than in diploid plants. A modified TILLING (Targeting Induced Local Lesions IN Genomes) method including the harvest of five heads per M_1 plant, storage of M_2 seeds, using unlabeled primers and agarose gels for mutation detection, and crossing of useful mutants for desired grain quality was explored in this report. A soft wheat cultivar, QAL2000, and a hard wheat cultivar, Ventura, were mutagenized with ethyl methanesulfonate (EMS). Screening of the *waxy* genes *Wx-A1* and *Wx-D1* in 2348 EMS-treated M_2 plants allowed identification of 121 mutants, including silent, missense, and knockout (truncation) mutations. A complete waxy wheat was successfully bred in 18 mo by crossing two truncation mutants (*Wx-A1-truncation* and *Wx-D1-truncation*; *Wx-B1* is naturally null in both mutants). Screening of two *puroindoline* genes (*Pina* and *Pinb*) in QAL2000 identified 19 mutants. A hard grain variant of a soft cultivar was identified due to a mutation in *Pinb* caused by a premature stop codon. Background mutations were observed and further self-fertilization or crossing with a wild type was performed to eliminate deleterious mutations. With the rapid accumulation of wheat genomics information, many potential target genes of interest can be screened for mutations in these TILLING populations.

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TRADITIONAL PLANT BREEDING involves incorporation of valuable traits from natural variation into agricultural genotypes by hybridization, recombination, and selection. This usually takes many years to achieve. The mutations that drive evolution are rare and random events. A recent study of spontaneous mutations in yeast revealed a base-substitutional mutation rate of 0.33×10^{-9} per site per cell division and estimated a rate of 10^{-10} in humans (Lynch et al., 2008). In plants, the frequency of point mutations was estimated at 10^{-7} to 10^{-8} events per base pair (Kovalchuk et al., 2000). In the era of genomics, it is increasingly possible to accelerate the evolutionary process. Technologies such as gene transformation can be used to improve crop traits by introducing useful foreign DNA (Chua and Tingey, 2006). However, transformation often leads to problems such as transgene silencing, species dependence, and a lack of acceptance by consumers. Traditional chemical mutagenesis (forward genetics) was used in plant breeding for many years, based on phenotypic screening. An excellent example is the breeding of imidazolinone-resistant wheat (Newhouse et al., 1992). Phenotypic screening for desired traits, however, is not always easy, especially for grain quality. Moreover, wheat is hexaploid, often containing three homoeoloci for a single gene function. For a recessive trait, all three

C. Dong, J. Dalton-Morgan, K. Vincent, and P. Sharp, and Plant Breeding Institute, Univ. of Sydney, PMB11, Camden, NSW, 2570, Australia; and Value Added Wheat Cooperative Research Centre, North Ryde, NSW, 1670, Australia; K. Vincent's alternate address is Australian Centre for Plant Functional Genomics, Plant Genomics Centre, The University of Adelaide, Waite Campus Hartley Grove, Urrbrae SA 5064, Australia. Received 9 Oct. 2008. *Corresponding author (p.sharp@usyd.edu.au).

Abbreviations: EMS, ethylmethane sulfonate; ENU, N-ethyl-N-nitrosourea; GBSSI, granule-bound starch synthase I; HI, hardness index; PCR, polymerase chain reaction; QA, QAL2000 plants harvested from the 0.7% EMS treatment; SKCS, Single-Kernel Characterization System; SNP, single nucleotide polymorphism; TILLING, Targeting Induced Local Lesions IN Genomes.

homoeoloci must be mutated and homozygous, an extremely rare event.

Modern genomics makes reverse genetics possible as large amounts of genomic and expressed sequence information become available. One reverse genetics method involves the use of chemical mutagenesis coupled with high-throughput screening to detect sequence variations in genes of interest, so called TILLING (Targeting Induced Local Lesion IN Genome). In contrast to typical reverse genetics techniques such as RNA interference and insertional mutagenesis, TILLING is nontransgenic and generates allelic series of mutations, including knockouts, in the desired gene (Henikoff and Comai, 2003). This technique is applicable to all organisms but is highly suited to plants. The TILLING method was first applied in plants (McCallum et al., 2000a) and has the advantage that seeds of mutant populations can be banked for later use.

The TILLING method is useful for both functional genomics as demonstrated in *Arabidopsis* (McCallum et al., 2000b) and crop improvement as demonstrated in wheat (*Triticum aestivum* L.) (Slade et al., 2005) and applied successfully to maize (*Zea mays* L.) (Till et al., 2004b); rice (*Oryza sativa* L.) (Wu et al., 2005; Till et al., 2007); barley (*Hordeum vulgare* L.) (Caldwell et al., 2004); sugar beet (*Beta vulgaris* L.) (Hohmann et al., 2005); *Lotus japonicus* (Regel) K. Larsen (Perry et al., 2003); and soybean [*Glycine max* (L.) Merr.] (Cooper et al., 2008). Wheat tolerates a much higher mutation frequency than other plants, possibly due to its polyploid nature (Slade et al., 2005). Compared with other plants, wheat therefore requires a relatively small mutant population for screening of desired traits.

In self-pollinating plants, the TILLING method involves chemical mutagenesis (e.g., ethylmethane sulfonate [EMS], N-ethyl-N-nitrosourea [ENU], or NaN_3), growth of M_1 plants and self-fertilization to produce M_2 seeds, collection of M_2 DNA for screening, and storage of M_3 seeds as a bank of mutants. This procedure requires a large resource. Here, we demonstrate a modified TILLING procedure including the harvest of five heads per M_1 plant, storage of M_2 seeds, using unlabeled primers and agarose gels for screening, and crossing of useful mutants for desired traits, which suits a small laboratory and budget.

We chose the *waxy* (*Wx-A1* and *Wx-D1*, encoding granule-bound starch synthase I [GBSSI]) and *puroindoline* (*Pina* and *Pinb*) genes for TILLING and breeding waxy wheat and hard wheat, as the sequences and functions of these genes were relatively clear. Functional GBSSI produces starch with amylose. Knockouts of all three GBSSI genes will produce a waxy starch that is composed almost entirely of amylopectin with little or no amylose (Nakamura et al., 1993, 1995; Yamamori et al., 1994). Varying amylose/amylopectin ratios lead to differences in granular structure, physicochemical properties, and quality of end-use products (Hung et al., 2006). Waxy wheats have not yet become commercially available, although many publications describe various research findings on waxy genes and the properties of waxy wheat starch. The closely linked *Puroindoline* genes, *Pina-D1* and *Pinb-D1*, located on chromosome 5D, are the main factors determining grain texture. Soft wheat contains wild-type *puroindoline* genes, *Pina-D1a* and *Pinb-D1a*; if either of these genes is mutated or deleted, such as the alleles *Pina-D1b* and *Pinb-D1b*, a hard wheat phenotype will result (Giroux and Morris, 1998). Different alleles of *Pina* and *Pinb* are known in different cultivars and landraces; however, only a few of the variations are in Australian germplasm (Cane et al., 2004). Through the TILLING study, we aim to produce a series of variations in *Pina* and *Pinb* genes and study their functional effects on the grain hardness, and to breed a waxy wheat by screening and crossing of *waxy* mutants.

Materials and Methods

Plant Materials

Breeders seed of the Australia bread wheat cultivars QAL2000, with soft-textured grain, and Ventura, with hard grain, was used.

Mutagenesis

Dry seeds were divided into treatment groups of ~300 grains wrapped in cheesecloth and soaked in the EMS solution with the ratio ~300 grains 100 mL^{-1} with gentle agitation overnight (~18 h). The mutagen EMS (SIGMA, Castle Hill, NSW, Australia) was prepared at 0.5, 0.6, and 0.7% (w/v). Treated seeds were washed extensively under running tap water for at least 2 h and then sown in pots at the rate of 20 ± 3 kernels pot^{-1} in a greenhouse (16–18°C). After seedling development, pots were then transferred outdoors for 2 wk before the seedlings were transplanted to the field. M_2 seeds were harvested as five separate spikes per M_1 plant (QAL2000), with each spike being threshed and bagged individually; or one spike per M_1 plant (Ventura).

DNA Extraction

One M_2 seed from each spike was sown in a 100-cell (10 × 10) seedling tray. Leaves of ~20-d-old seedlings were collected in a 96-well format and dried in a closed box with silica gel. DNA extraction was performed using the manufacturer's method on an ABI6100 PrepStation (Applied Biosystems, Melbourne, VIC, Australia), with the modifications of adding a centrifugation step after the cell lysis and adding RNase A to the elution buffer. All buffers were purchased from Applied Biosystems, Australia. Briefly, dried leaves were crushed into a powder using two ball bearings per sample on a Mix-Miller (MM300; Retsch, Germany). Extraction buffer (600 μl) was added to each sample and incubated at room temperature for 10 min before centrifuging at 5000 rpm (3857 × *g*) for 10 min. The supernatant (~550 μl) was loaded onto the DNA binding plate, and DNA was bound to the membrane by vacuum suction and washed in 600 μL washing buffer (three times), and eluted first in 100 μL of elution buffer 1 and then in 100 μL of elution buffer 2 (added RNase A to 50 μg mL^{-1}). The DNA concentrations were normalized to 5 to 15 ng μL^{-1} after gel electrophoresis with a known mass

standard. DNA samples from 3 or 4 individual spikes were pooled for initial screening.

PCR and Mutation Screening

Both QAL2000 and Ventura contain expressed alleles of *Wx-A1* and *Wx-D1*, but the allele *Wx-B1* on chromosome 4A is null in about 50% of the population; therefore, screening was only performed for *Wx-A1* and *Wx-D1*. The polymerase chain reaction (PCR) primers used for *waxy* mutation screening were Wx7A2, Wx7A3, Wx7A4, Wx7D2, and Wx7D3 as described by Slade et al. (2005). Amplification was performed in a 10-μL volume containing 2 μL of pooled DNA, 1 μL 10× Pfu buffer (Stratagene, La Jolla, CA), 0.2 mM dNTPs, 0.4 μM primers and 0.25 U PfuUltraII Fusion HS DNA Polymerase (Stratagene). Polymerase chain reaction was conducted using a thermal cycler (MasterCycler 5333; Eppendorf, North Ryde, NSW, Australia) as follows: 95°C for 2 min, followed by 6 cycles of touchdown at 94°C for 30 s, an annealing step starting at 72°C for 30 s and decreasing 1°C per cycle, and 72°C for 1 min, then 35 cycles of PCR (94°C for 20 s, 66°C for 20 s, and 72°C for 15 s), and finally extension at 72°C for 1 min.

The primers for *pina* and *pinb* were according to Gautier et al. (1994) with the modification of primer pinb-F to 5′-ATGAAGACCTTATTCCTCCTAGCTCTC-3′. The PCR profile was the same as for the *waxy* PCR, except the annealing temperature for *pina* was 58°C and for *pinb* was 60°C.

After PCR amplification, the products were denatured and annealed in the thermal cycler as follows: 95°C 8 min, 85°C 1 min, followed by 99 cycles of 84°C for 30 s, decreasing 0.5°C per cycle. Samples were then digested with Cel I enzyme, a celery juice extract prepared according to Till et al. (2004a) except the last step; the dialyzed extract was mixed with glycerol to a 50% (v/v) glycerol enzyme solution. The digestion reaction was performed at 45°C for 30 min in a 20-μL reaction volume containing 8 μL of PCR product, 2 μL of Cel I enzyme, and 10 μL of 2× buffer [20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid pH 7.5, 20 mM $MgSO_4$, 20 mM KCl, 0.004% (v/v) TritonX-100 (SIGMA, Castle Hill, NSW, Australia), and 0.4 μg/ml bovine serum albumin]. The samples were then electrophoresed in a thin 2% agarose gel (gel thickness ≤4 mm, distance between teeth of a comb and a gel tank ≤1 mm) for 30 min and stained with ethidium bromide. Images were analyzed visually for the presence of cleavage products using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

If a pooled sample was identified as having Cel I cleavage bands, individual samples from the pool were tested together with a wild-type DNA sample. The PCR products of identified mutants were then sequenced to determine the mutation.

Production of a Waxy Wheat and a Hard Grain Texture Wheat

Truncation mutants of the *Wx-A1* (7A) and *Wx-D1* (7D) loci (both lines were null-4A) were grown to M_3 to identify homozygous lines, which were then intercrossed. The F_2 seeds of this cross were iodine stained (Nakamura et al., 1995) to identify waxy phenotypes.

The *pina* and *pinb* mutants were selfed and M_3 or M_4 seeds from a homozygous derivative were subject to the Single-Kernel Characterization System (SKCS) test, a service provided by the BRI Australia (North Ryde, NSW, Australia) using an SKCS 4100 (Perten Instruments, Springfield, IL). Samples of 100 to 300 grains, including control soft and hard genotypes, were used in the test.

Results

Establishment of TILLING Population

To determine a suitable EMS concentration for mutagenesis, we conducted germination tests following treatment with EMS at concentrations of 0.7, 0.9, and 1.2% (w/v) according to Slade et al. (2005). We found that the germination rates of 30 and 10% following 0.9 and 1.2% treatments were too low. With 0.7% treatment, the germination rate was about 60%. McIntosh (1977) reported that 0.5% treatment was suitable. Therefore, we chose 0.5, 0.6, and 0.7% EMS concentrations for mutagenesis. About 600 seeds of each variety were treated at each concentration, leading to germination rates of 79, 69, and 63% for QAL2000, and 82, 75, and 65% for Ventura, respectively.

For QAL2000, 275 plants (1375 spikes) were harvested from the 0.7% EMS treatment, labeled as QA; 259 plants (1295 spikes) from the 0.6% EMS treatment, labeled as QB; and 335 plants (335 spikes) from the 0.5% EMS treatment, labeled as QC. From Ventura, 377, 308, and 483 spikes from individual plants were harvested from these treatments, labeled as VA, VB, and VC. The M_2 fertility of the most heavily treated population was about 70%. Among M_2 plants, we identified 4 to 7% phenotypic mutants (dwarf and albino) at the 2-wk-old seedling stage.

Detection of Mutations in *Waxy* and *Puroindoline* Genes

To develop a fast and cost-effective method for detecting mutations, plain agarose gels were tested. It was found that thin agarose gels (≤4 mm) can efficiently detect the Cel I–digested bands in up to eightfold pools (Fig. 1B). Commercial kits of Cel I (SURVEYOR nuclease) and special low-fluorescent agarose are available (Transgenomic, Omaha, NE), but testing showed that the methods used here was more efficient than that of the Transgenomic kits, due to higher Cel I activity in homemade celery extracts. Furthermore, the cost of homemade celery extracts was >100 times less than commercial kits. An example of the screening results of 48 3× pools screened with unlabeled Wx7D3 primer is shown in Fig. 1A where 7 of the pools contain mutations in the fragment. The individual members of the pools were screened again to determine the mutants. Seven mutants were sequenced and six of them had true genetic changes (1, Mutant 31, C1703T; 2, Mutant 32, C1703T; 3, Mutant 30, G1885A;

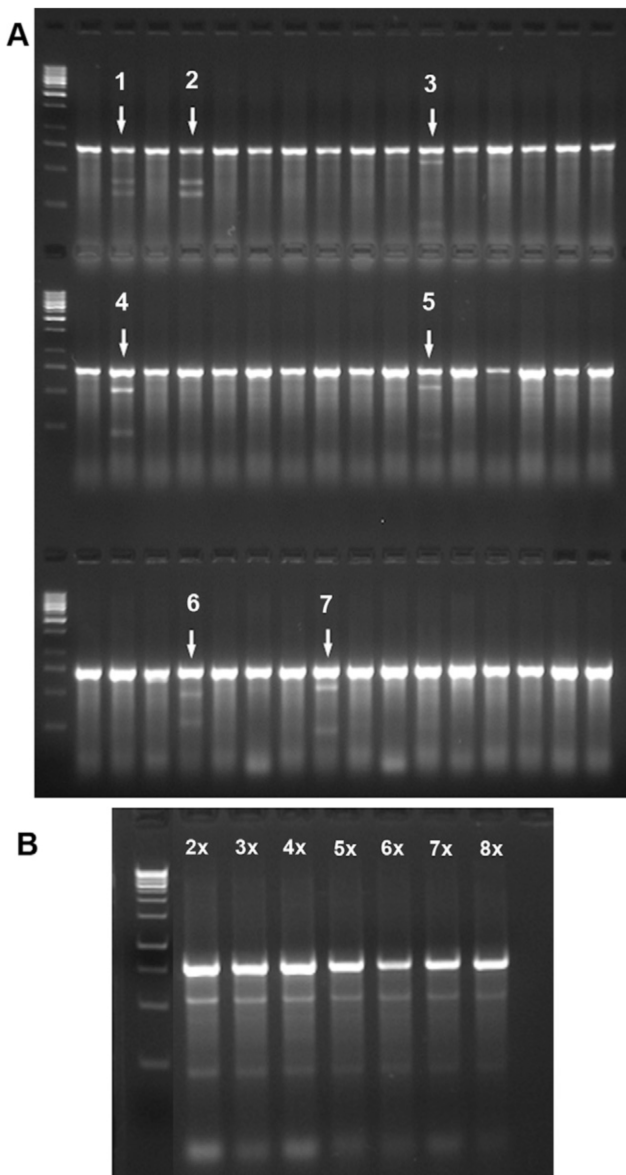

Figure 1. (A) A thin agarose gel showing results of mutation screening of 48 samples in the Wx7D3 fragment with three-fold pooling of DNA. Arrows indicate DNA pools containing mutations. (B) Mutant 4 identified in (A) was mixed with wild-type DNA to form 2×, 3×, 4×, 5×, 6×, 7×, and 8× pools, and screened in the Wx7D3 fragment.

4, Mutant 28, G1816A; 6, Mutant 33, G1557A; 7, Mutant 34, G1497A, shown in Supplementary Table S1) and one sample (5) had poor sequencing data. Number 4 sample (G1816A) was heterozygous so it was mixed with different ratio of wild-type DNA samples to test the sensitivity of the thin agarose gel as shown in Fig. 1B. Thus, a fast and efficient screening method was established. It takes about 1 h from preparation to finish a run of a thin agarose gel, while about 4 h are needed for a LI-COR (Lincoln, NE) gel from preparation to finish. The cost of fluorescent labeling of a primer is about five times more than that of an unlabeled primer.

From the total of 2348 samples screened for the *Wx-A1* and *Wx-D1* gene fragments, 121 mutants were detected; and by screening *Pina* in 864 samples and *Pinb* in 576 samples, 19 mutants were identified as listed in Supplementary Table S1. Table 1 lists mutations identified in *waxy* (7A and 7D), *pina*, and *pinb* genes in populations treated with different concentrations of EMS. Mutation frequencies ranged from 1 in 23.3 kb to 1 in 37.5 kb. A total of 60 mutants were identified in screened gene fragments in QA including knockout (truncation) mutants in each gene. A total of 1,398,528 bp were screened in this population; this was calculated by multiplication of the number of samples and the number of base pairs of the PCR fragment adjusted by subtraction of the primer base pairs (for uniformity, 50 bp were subtracted in all cases). The mutation frequency was determined by dividing the total base pairs screened by the total mutants detected. The 0.7% EMS–treated QAL2000 population had about 1 mutation per 23 kb, a value similar to that of Slade et al. (2005; 1 in 24 kb). In other populations, the 0.6% EMS–treated QAL2000 had about 1 mutant in 30 kb whereas Ventura had mutation frequencies of 1 in 36 kb, 1 in 32 kb, and 1 in 37.5 kb following 0.7, 0.6, and 0.5% EMS treatments, respectively. We also did mutagenesis by sodium azide treatment (5 mM), which yielded 1 mutation in 127 kb after screening 1,021,056 bp in the *waxy* fragments, and no mutant was detected in the *pina* or *pinb* genes (data not shown).

Among 119 sequenced mutations, we identified 30.3% silent, 54.6% nonsilent including missense and truncation mutations, and 15.1% mutations within introns (Table 2). No splice junction mutation was found. These mutations were 35.3% homozygous and 64.7% heterozygous. The majority of these mutations were transitions, 57.2% G to A and 42.0% C to T changes (Table 2), as expected from EMS alkylation (Greene et al., 2003). One mutant was identified as an A to C change (0.8%).

Some duplicate mutations (the same mutation in two independent individuals) were identified. In Wx7D3, mutants 44 and 104 (QB77.4 and VB92), mutants 33 and 113 (QA208.4 and VC344) had duplicated silent mutations (Supplementary Table S1). In Wx7A3, mutants 62 and 82 (VB241 and VA263), mutants 85 and 90 (VC6 and VC138) had duplicated missense mutations (Supplementary Tables S1 and S2). These duplicates may simply reflect the random nature of the EMS-induced mutation events. Other possible reasons for these duplicates could be a low level of heterogeneity in the source material (see below), contamination, or outcrossing. A similar phenomenon was also found by Slade et al. (2005).

Five Spikes vs. One Spike

Both repeated and nonrepeated mutations were identified among multiple spikes from QA and QB progenies (Table 3). In Wx7A3, three repeated mutations, G1860A, C1874T, and G1872A (one triple repeat and two double repeat; Table 3) originated from plants QA49, 61, and 191, respectively. It is likely that different tillers with the same mutation originated from the same meristem cell. However, different mutations were also found in sister spikes.

For example, plant QA53 had one spike (53.2) with mutation G1762A, a missense mutation causing a glycine 387 to glutamic acid change; whereas another spike (53.3) with mutation C1485T was a missense mutation causing a serine 325 to phenylalanine change. These two spikes obviously originated from different meristem cells. In Wx7D3, of mutations found in plant QA154, C1561T (154.2) and G1748A (154.4), one was a leucine 323 to phenylalanine change and the other was in the intronic region. Plant QA201 had one silent mutation (G1885A, 201.1), and two repeated missense mutations (C1703T, 201.4, homozygote and 201.5, heterozygote). This indicated that QA201.1 and 201.4 arose from different meristem cells, whereas QA201.4 and 201.5 may have originated from the same cell. Both *Pina* and *Pinb* included cases of different mutations arising from separate spikes of the same M_1 plant. Sister spikes of QA158, 164, and 209, had different mutations as listed in Table 3, indicating different meristem origins.

These results illustrate the chimeric nature of mutagenized M_1 plants. Spikes on the same plant can originate from different sections of the meristem, and screening of multiple spikes per plant does not only identify the same mutation. The advantage of screening multiple spikes per plant is that smaller M_1 populations are required. Moreover, nonmutant spikes on the same plant can be used as controls for mutant spikes, especially when breeder's bulk seed, rather than derivatives of a single plant, are used for mutagenesis.

Table 1. TILLING (Targeting Induced Local Lesions IN Genomes) results and mutation frequencies.

Sample†	No. plants or spikes	Fragment (bp screened)‡	Total mutants	Mutation type					Frequency¶
				Missense	Silent	Truncation	Intron	Unknown§	
QA	288	Wx7A3 (743)	18	7	7	2	2		
	576	Wx7D3 (710)	18	5	5	2	1	5	
	288	Wx7A4 (708)	5	2	1		1	1	
	864	Pina (397)	11	7	2	2			
	576	Pinb (397)	8	4	3	1			
		1,398,528	60						1/23.3 kb
QB	768	Wx7D3 (710)	18	6	5	2	3	2	
		545,280	18						1/30.3 kb
VA	332	Wx7A3 (743)	7	3	2			2	
	332	Wx7A2 (498)	5	2			2	1	
	332	Wx7D3 (710)	6	3				3	
		647,732	18						1/36.0 kb
VB	288	Wx7A3 (743)	8	3	3		1	1	
	288	Wx7A4 (708)	7	3	2		2		
	288	Wx7A2 (498)	3				2	1	
	288	Wx7D3 (710)	6	2	3	1			
		756,792	24						1/31.9 kb
VC	384	Wx7A3 (743)	7	4	1		1	1	
	384	Wx7A2 (498)	4	1			2	1	
	384	Wx7D3 (710)	9	2	2	1	1	3	
		749,184	20						1/37.5 kb

†Q, QAL2000; V, Ventura; A, B, and C, 0.7, 0.6, and 0.5% ethylmethane sulfonate treatments, respectively.

‡Amplicon size multiplied by the total number of samples screened.

§Sequence data were not good enough to determine the nucleotide change.

¶Total number of mutations divided by the total base pairs screened.

Natural Variations in the Population

In screening of the Wx7A3 fragment in QA, we identified variant C1372T 14 times and C1342T five times, both silent mutations, among 288 spikes (Supplementary Table S1). All five spikes on plant QA193 were homozygous C1372T, indicating it was present in the original seed, rather than an induced mutation. In Ventura, most screened plants had T instead of C at 1372. Interestingly, the T1372C mutation was identified six times with the C1342T variant also found in the same plant (Supplementary Table S1). With mutation frequencies in these populations at about 1 in 23 to 37 kb, the chance of double mutations in a 750-bp fragment should be very low, and the chance of repeated double mutations much lower. These mutations were therefore considered as natural variations (single nucleotide polymorphisms [SNPs]) in the original populations. Screening the Wx7A4 fragment in QA, we identified double mutations G2413A and T2508C repeated twice in 288 samples. These were likely natural variations for the same reasons.

Apart from these natural variations, we found that *Wx-B1* (4A allele) was deleted in about 50% of QAL2000 plants and >50% of Ventura. In *Pinb*, most plants of QAL2000 have the *Pinb-D1a* allele but a small proportion contain *Pinb-D1b*. These natural variations in the breeder's seed may cause problems in the later analysis of mutants, if the original population is used as control.

Table 2. Molecular changes in the mutations identified in the TILLING (Targeting Induced Local Lesions IN Genomes) population.

Sequenced mutants	Silent mutations	Nonsilent mutations	Intron mutations	Homozygous mutants	Heterozygous mutants	G to A change	C to T change	A to C change
Pin: 19	5 (26.3%)	14 (73.7%)	0	5 (26.3%)	14 (73.7%)	13 (68.4%)	5 (26.3%)	1 (5.3%)
Waxy: 100	31 (31%)	51 (51%)	18 (18%)	37 (37%)	63 (63%)	55 (55%)	45 (45%)	0
Total 119	36 (30.3%)	65 (54.6%)	18 (15.1%)	42 (35.3%)	77 (64.7%)	68 (57.2%)	50 (42.0%)	1 (0.8%)

Table 3. Mutants identified in QAL2000 treated with 0.7% ethylmethane sulfonate (EMS) and QAL2000 treated with 0.6% EMS sister spikes with repeated or multiple mutations.

Gene/ fragment	Line	Zygosity†	Nucleotide change	Codon change	Amino acid change	Type
Wx7A3	QA49.5	Het	C1372T‡	ATC-ATT	I287I	Repeat
	QA49.4	Het	C1372T‡	ATC-ATT	I287I	
	QA49.3	Het	G1860A	GTG-ATG	V420M	
	QA49.2	Hom	G1860A	GTG-ATG	V420M	
	QA49.1	Het	G1860A	GTG-ATG	V420M	
	QA53.2	Hom	G1762A	GGG-GAG	G387E	Multiple
	QA53.3	Hom	C1485T	TCT-TTT	S325F	
	QA61.4	Het	C1874T	GAC-GAT	D424D	Repeat
	QA61.2	Hom	C1874T	GAC-GAT	D424D	
	QA152.4	Het	G1339A	CTG-CTA	L276L	Multiple
	QA152.5	Het	C1372T‡	ATC-ATT	I287I	
	QA161.3	Het	C1342T‡	CCC-CCT	P277P	Multiple
	QA161.2	Het	G1413A	TGG-TAG	W301Stop	
	QA191.3	Het	G1872A	GAC-AAC	D424N	Repeat
	QA191.4	Het	G1872A	GAC-AAC	D424N	
Wx7D3	QA154.2	Hom	C1561T	CTC-TTC	L323F	Multiple
	QA154.4	Het	G1748A	Intron		
	QA201.1	Hom	G1885A	AGG-AGA	R402R	Multiple/
	QA201.4	Hom	C1703T	ACC-ATC	T370I	Repeat
	QA201.5	Het	C1703T	ACC-ATC	T370I	
	QB3.3	Het	G1474A	GTG-ATG	V294M	Repeat
	QB3.4	Het	G1474A	GTG-ATG	V294M	
	QB1.1	Het	G1912A	GTG-GTA	V411V	Multiple
	QB1.3	Hom	G1656A	TGG-TGA	W354Stop	
	QB163.1	Het	G1800A	GGG-GAG	G374E	Repeat
	QB163.5	Het	G1800A	GGG-GAG	G374E	
Pina	QA209.3	Het	G206A	TGG-TAG	W41Stop	Multiple
	QA209.4	Het	G65A	AGC-AAC	S(-7)N	
	QA158.1	Het	C118T	CCT-TCT	P12-S12	Multiple
Pinb	QA158.4	Hom	G127A	GAG-AAG	E14-K14	
	QA164.1	Hom	G375A	CAG-CAA	Q96Q	Multiple
	QA164.2	Het	A318C	CAA-CAC	Q77H	

†Het, heterozygote; Hom, homozygote.

‡C1372T and C1342T were detected >10 times in 288 samples, considered as natural variations.

Alternatively, nonmutant sister spikes of the mutant individual and the segregated progeny with mutant and nonmutant allele at the target locus can be used as controls. However, mutants identified in TILLING may have additional background mutations. Functional analyses of a gene of interest should include a number of similar mutants for phenotypic assessment or, alternatively, backcrossing or outcrossing to an unmutagenized line may be needed.

Production of a Waxy Wheat

Mutant 17 (Supplementary Table S1), QA199.2 (G1414A, W301stop in *Wx-A1*) and mutant 34, QA209.1 (G1497A, W301stop in *Wx-D1*) are truncation mutants chosen for producing waxy QAL2000, as both lines were null-4A and appeared phenotypically normal. Homozygous mutant individuals of each line were selected and intercrossed. Among 1580 F_2 seeds screened by iodine staining, 101 waxy individuals were identified (Fig. 2), indicating a two-gene segregation (χ^2 1:15 = 0.0431, $P > 0.90$). Progeny tests showed that the waxy individuals were homozygous as expected. The production of waxy wheat using EMS treatment, TILLING, and recombination took 18 mo and provided proof of concept for using TILLING as a rapid means of acquiring a predicted phenotypic outcome by plant breeding. Considering background mutations, however, further crossing with a wild-type plant may be needed for eliminating undesirable background mutations that may cause deleterious characteristics.

Production of a Hard Grain Texture Wheat

Screening of *Pina* and *Pinb* identified two truncation mutants in *Pina* (mutant 126, QA209.3 and mutant 131, QA241.2; Supplementary Table S1) and a truncation mutant in *Pinb* (mutant 139, QA232.2; Supplementary Table S1). The line QA232.2 was further studied for the effect of mutation on grain texture. This line has the nucleotide change C307T, causing arginine 74 (CGA) to become a premature stop codon TGA. Electrophoresis of both the Triton-X114-extractable and starch-associated friabilin proteins showed that this mutation led to a total lack of the PINB protein (data not shown). This change was expected to affect grain texture. The SKCS measurement revealed a high hardness index (HI) value for mutant QA232.2, similar to other hard genotypes used as controls (Table 4). Seeds with the QA232.2 wild-type allele (*Pinb-D1a*) also had an elevated HI value compared with other QA lines in the same test (Table 4). The plants of QA232.2 (both mutant and wild type) were weaker with smaller spikes and seeds than other QA plants. This mutant most likely carried a separate detrimental mutation resulting from the EMS treatment, and this condition may have had some effect on the measured texture of the kernels. However, grain from the mutant was significantly much harder than the wild-type sample, with the difference in HI between mutant and wild type being 17.77 ($P = 1.4 \times 10^{-9}$). To remove the influence of background mutations on the hardness test of this line, mutant QA232.2 was crossed to a wild-type QAL2000 plant.

Background Mutations

Waxy seeds identified in the F_2 of the cross of Mutant 17 (truncation mutant of *Wx-A1*) and Mutant 34 (truncation mutant of *Wx-D1*) were grown to F_3. Other mutations giving dwarf, tall, late-maturing, partial sterility, and stripe-rust-resistant phenotypes were observed among the individual F_3 plants, while the waxy phenotype was maintained. This clearly indicates other background mutations following the EMS mutagenesis. Through selection and further self-fertilization to F_5, we have obtained waxy lines with stable and good agronomic performance (data not shown).

Pinb truncation mutant QA232.2 had a high HI value; however, the same line with the wild-type *Pinb* allele also had an elevated HI value, although much lower than that of the mutant. Both mutant and wild type had weak growth compared with the original QAL2000. This suggests that background mutations are affecting plant growth and also hardness. Puroindolines are the major contributor to the grain hardness; however, other factors such as kernel moisture content, pentosans, and kernel lipid content have some influence on grain hardness (Turnbull and Rahman, 2002). Other minor quantitative trait loci on chromosome 1B and 5A related to grain texture were found (Turner et al., 2004). Elevated HI in line QA232.2 with *Pinb* mutation and without *Pinb* mutation indicated that there may be other mutations affecting hardness, as these two derivatives have the most similar genetic background. This is the reason we chose the mutant and nonmutant derivatives from a same line to study the phenotypic change caused by the mutated gene. The QA232.2 mutant was crossed to a wild-type QAL2000 plant. Seeds of the homozygous *Pinb* mutant F_2 and nonmutant F_2 were tested by SKCS as shown in Table 5. Our data show that the HI was similar in nonmutant F_2 seeds and wild-type QAL2000; however, mutant F_2 seeds exhibited a significant increase in HI.

Discussion

The TILLING method is a nontransgenic approach for functional genomics and crop improvement, based on mutagenesis followed by focused screening. The advantage of using this method is that it produces an array of lesions that contain a mixture of knockout and missense mutations, whereas techniques like posttranscriptional gene silencing and insertional mutagenesis will most likely produce only knockout mutations. Chemical mutagenesis was favored because of its propensity to cause point mutations. The TILLING method can identify a high density of mutations in a gene of interest and provide a more in-depth insight into protein function in the desired gene–domain. This study and other wheat TILLING (Slade et al., 2005) results indicate that wheat tolerates very high mutation frequencies (1 in 20–30 kb), presumably due to its polyploid nature. By comparison, EMS mutagenesis in *Arabidopsis* produced a 1 in 170 kb mutation frequency (Greene et al., 2003); barley had 1 in 1000 kb (Caldwell et al., 2004); and rice had 1 in 300 to 600 kb (Till et al., 2007). This means that TILLING of hexaploid wheat requires relatively small numbers of treated plants.

The TILLING method presented in this study is a modification of the strategy employed in the published literature but is compatible with a low-budget small laboratory. The common technique uses a different set of electrophoresis equipment (LI-COR DNA analyzer or ABI genetic analyzer [Applied Biosystems, Melbourne, VIC, Australia]), which requires the use of end-labeled primers. If there is no LI-COR equipment or other DNA analyzer available, agarose gels are still a cost-effective alternative for screening mutations. A detailed comparison between agarose gel and LI-COR gel was reported recently (Raghavan et al., 2007). Our method presents the first application of agarose gel screening for a large and complex genome in a high-throughput format. The screening technique is easily applied to either large- or smaller-scale analysis. The modified method simplifies the procedure by utilizing only equipment available in any basic molecular laboratory. It can analyze large sections of the desired genome with relative ease. If multiple spikes per plant are used for screening, the size of the M_1 population can be further reduced. The M_2 seeds, if stored appropriately, can be used for repeated screening of a large number of targets.

Figure 2. Waxy QAL2000 (right) was produced by crossing two truncation mutants identified in TILLING (Targeting Induced Local Lesions IN Genomes). Normal QAL2000 (left) shows dark staining with iodine; the selected recombinant waxy genotype has light color (right).

Table 4. Single-Kernel Characterization System hardness and grain size measurements of control wheat cultivars and mutant lines.

Sample†	Hardness index (HI)‡		Diameter (mm)		Genotype
	Avg.	SD	Avg.	SD	
Australian Hard	67.99	19.25	2.46	0.51	Hard
Sunco	86.96	14.72	2.51	0.42	Hard, Pina-D1a, Pinb-D1b
Janz	84.52	16.64	2.48	0.41	Hard, Pina-D1a, Pinb-D1b
Rosella	40.05	13.31	2.60	0.35	Soft, Pina-D1a, Pinb-D1a
Tincurrin	47.37	17.22	2.32	0.35	Soft, Pina-D1a, Pinb-D1a
QA51.2 WT	43.1	15.4	3.05	0.59	Pina-D1a, Pinb-D1a
QA51.2 M	44.73	13.92	3.05	0.56	Pina-D1a, Pinb-T(-13)I
QA150.4 WT	43.06	18.33	2.63	0.53	Pina-D1a, Pinb-D1a
QA150.4 M	42.54	12.58	3.31	0.54	Pina-C11Y, Pinb-D1a
QA158.1 WT	34.29	13.87	2.44	0.52	Pina-D1a, Pinb-D1a
QA158.1 M***	45.1	15.71	2.24	0.55	Pina-P12S, Pinb-D1a
QA232.2 WT	69.69	16.39	2.60	0.45	Pina-D1a, Pinb-D1a
QA232.2 M***	87.46	18.23	2.31	0.47	Pina-D1a, Pinb-R74Stop

***Significant difference of HI between M and WT at the 0.001 probability level.

† Australian Hard is an Australian marketing standard sample. It could be a mixture or a single cultivar but is certified to only contain hard wheat. Sunco and Janz are both hard cultivars that carry the *Pinb-D1b* allele. Rosella and Tincurrin are soft cultivars. Mutant seeds with segregated genotypes, homozygous mutant (M) and wild type (WT) at the mutation site, were tested.

‡ Hardness index is recorded as both an average and standard deviation.

Table 5. Single-Kernel Characterization System hardness and grain size measurement of wheat seeds from genotyped homozygous mutant (M) and nonmutant (WT) F_2 plants of the cross QA232.2 × QAL2000.

Sample	Hardness index		Diameter (mm)		Genotype
	Avg.	SD	Avg.	SD	
QAL2000†	41	15	2.63	0.51	Pina-D1a, Pinb-D1a
F_2 WT	45	14	2.58	0.50	Pina-D1a, Pinb-D1a
F_2 M***	75	16	2.60	0.34	Pina-D1a, Pinb-R74Stop

***Significant difference between mutant and wild-type lines at the 0.001 probability level.

†QAL2000 wild type grown in a greenhouse at the same condition and the same time as for the mutant lines.

The method presented here is effective in identifying a series of allelic mutations. Apart from truncation mutants identified in all screened gene fragments, which produced significant phenotype changes (waxy and hard grain), other missense mutants could possibly affect protein function, and thereby possibly produce partially waxy phenotypes or intermediate levels of hardness. There are a number of online tools available for evaluating the effects of mutation, for example, SIFT (Sorting Intolerant From Tolerant) and PARSESNP (Project Aligned Related Sequences and Evaluate SNPs) (http://www.proweb.org/parsesnp/ [verified 13 Jan. 2009]), which predict deleterious effects on an encoded protein, changed restriction sites by a mutation, and other information to facilitate phenotypic and genotypic analysis (Taylor and Greene, 2003). Supplementary Table S2 lists missense mutations in *Wx-A1* analyzed by PARSESNP. Mutations P317L, D350N, and G503E were predicted to confer possible deleterious effects. Further phenotypic analysis of these mutants may produce grain characteristics of potential interest to the wheat industry. Profiling of missense mutations with phenotypic analysis could be useful in determining the active sites of a protein.

The TILLING method results in a high-resolution map of mutations in a gene of interest, including silent, missense, and truncation mutations. As with other forward and reverse genetics techniques, mutations that would otherwise be silent can be identified. Some genes will not produce overt phenotypes; others could have overlapping functions with other genes that could mask the effects of mutation (Stemple, 2004). Genes like the *puroindolines* and *waxy* have a dominant wild type, requiring homozygosity of any variant to detect a phenotype. In contrast to TILLING, a recent study by Feiz et al. (2009) on generated allelic *pin* mutations by direct phenotyping of an EMS-mutagenized population followed by sequencing. Their results indicated that direct phenotyping was applicable for a single-copy gene conferring a detectable and targeted phenotype. This method may be effective for targeting desired phenotypes, whereas TILLING is more effective for targeting all mutations and genes without detectable phenotypes. The TILLING method allows for the streamlining of the screening–mutation detection part of the investigation, leaving more time for analysis of the candidate mutants by methods appropriate to the gene–trait affected. The production of waxy wheat and hard wheat from TILLING demonstrates the method is effective and useful for wheat breeding. Other targets, such as starch branching enzymes, starch synthase II and starch synthase III, can be TILLed in this TILLING population to produce novel grains with altered starch properties or for functional studies of genes of interest. Background mutations were observed during the production of waxy lines and hard grain lines; further self-fertilization and hybridization with a wild-type plant can eliminate the undesirable mutations. Information provided in this report is useful for both functional genomics and practical breeding.

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