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 M1 plant, storage of M2 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 M2 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.

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 $0.33 \times 10^{-9}$ 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 homoeologs for a single gene function. For a recessive trait, all three
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 NaNO₃), growth of M₁ plants and self-fertilization to produce M₂ seeds, collection of M₂ DNA for screening, and storage of M₁ 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 M₁ plant, storage of M₂ 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-DI, 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 amyllose. Knockouts of all three GBSSI genes will produce a waxy starch that is composed almost entirely of amylopectin with little or no amyllose (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. M₁ seeds were harvested as five separate spikes per M₁ plant (QAL2000), with each spike being threshed and bagged individually; or one spike per M₁ plant (Ventura).

DNA Extraction

One M₁ 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 PfuUlraII 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 MgSO4, 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 etidium bromide. Images were analyzed visually for the presence of cleavage products using Adobe Photo- shop 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 M3 to identify homozygous lines, which were then intercrossed. The F2 seeds of this cross were iodine stained (Nakamura et al., 1995) to identify waxy phenotypes.

The pina and pinb mutants were selfed and M4, or M4 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 (355 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 M2 fertility of the most heavily treated population was about 70%. Among M2 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 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;
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).

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

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 M1 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 M1 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 M1 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.

**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 QA158, 164, and 209, had different mutations as listed in Table 3, indicating different meristem origins.

Apart from these natural variations, we found that Wx-BI (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 1. TILLING (Targeting Induced Local Lesions IN Genomes) results and mutation frequencies.

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

†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.

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

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

DONG ET AL.: WHEAT TILLING 43
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.

<table>
<thead>
<tr>
<th>Gene/fragment</th>
<th>Line</th>
<th>Zygosity1</th>
<th>Nucleotide change</th>
<th>Codon change</th>
<th>Amino acid change</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wx7A3</td>
<td>QA49.5 Het C1372T</td>
<td>ATC-ATT</td>
<td>I287I</td>
<td>Repeat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA49.4 Het C1372T</td>
<td>ATC-ATT</td>
<td>I287I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA49.3 Hom G1860A</td>
<td>GTG-ATG</td>
<td>V420M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA49.2 Hom G1860A</td>
<td>GTG-ATG</td>
<td>V420M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA49.1 Hom G1860A</td>
<td>GTG-ATG</td>
<td>V420M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA53.2 Hom G1762A</td>
<td>GGG-GAG</td>
<td>G387E</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA53.3 Hom C1485T</td>
<td>TCT-TTT</td>
<td>S325F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA61.4 Het C1874T</td>
<td>GAC-GAT</td>
<td>D424D</td>
<td>Repeat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA61.2 Hom C1874T</td>
<td>GAC-GAT</td>
<td>D424D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA152.4 Het G1339A</td>
<td>CTG-CTA</td>
<td>L276L</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA152.5 Het C1372T</td>
<td>ATC-ATT</td>
<td>I287I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA161.3 Het C1342T</td>
<td>CCC-CCT</td>
<td>P277P</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA161.2 Het G1413A</td>
<td>TGG-TAG</td>
<td>W301Stop</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA191.3 Het G1872A</td>
<td>GAC-AAC</td>
<td>D424N</td>
<td>Repeat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7A3</td>
<td>QA191.4 Het G1872A</td>
<td>GAC-AAC</td>
<td>D424N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QA154.2 Hom C1561T</td>
<td>TCT-CTC</td>
<td>L323F</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QA154.4 Het G1748A</td>
<td>Intron</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QA201.1 Het G1860A</td>
<td>AGG-AGA</td>
<td>R402R</td>
<td>Multiple/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QA201.4 Hom C1703T</td>
<td>ACC-ATC</td>
<td>T370I</td>
<td>Repeat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QA201.5 Het C1703T</td>
<td>ACC-ATC</td>
<td>T370I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QB3.3 Het G1474A</td>
<td>GTG-ATG</td>
<td>V294M</td>
<td>Repeat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QB3.4 Het G1474A</td>
<td>GTG-ATG</td>
<td>V294M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QB1.1 Het G1912A</td>
<td>GTG-GTA</td>
<td>V411V</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QB1.3 Het G1912A</td>
<td>GTG-GTA</td>
<td>V411V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QB1.3 Het G1656A</td>
<td>TGG-TGA</td>
<td>W354Stop</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QB163.1 Het G1800A</td>
<td>GGG-GAG</td>
<td>G374E</td>
<td>Repeat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx7B3</td>
<td>QB163.5 Het G1800A</td>
<td>GGG-GAG</td>
<td>G374E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pina</td>
<td>QA209.3 Het G206A</td>
<td>TGG-TAG</td>
<td>W415Stop</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pina</td>
<td>QA209.4 Het G65A</td>
<td>AGG-AAG</td>
<td>S(-7)N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pina</td>
<td>QA158.1 Het C118T</td>
<td>CCT-CCT</td>
<td>P125S</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pina</td>
<td>QA158.4 Het G127A</td>
<td>GAG-AAG</td>
<td>E14-K14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pina</td>
<td>QA164.1 Het G375A</td>
<td>CAG-CAA</td>
<td>E14-K14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pina</td>
<td>QA164.2 Het A318C</td>
<td>CAA-CAC</td>
<td>Q77H</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Het, heterozygote; Hom, homozygote.
2†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 mutations 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 F2 seeds screened by iodine staining, 101 waxy individuals were identified (Fig. 2), indicating a two-gene segregation ($x^2 = 1:1.5 = 0.0431$, $P > 0.90$). 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 F3 of the cross of Mutant 17 (truncation mutant of Wx-A1) and Mutant 34 (truncation mutant of Wx-D1) were grown to F3. Other mutations giving dwarf, tall, late-maturing, partial sterility, and stripe-rust-resistant phenotypes were observed among the individual F3 plants, while the waxy phenotype was maintained. This clearly indicates other background mutations following the EMS mutagenesis. Through selection and further self-fertilization to F3, 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 F2 and nonmutant F2, were tested by SKCS as shown in Table 5. Our data show that the HI was similar in nonmutant QAL2000 plant. Seeds of the homozygous gene. The QA232.2 mutant was crossed to a wild-type QAL2000; however, mutant F2 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 M1 population can be further reduced. The M2 seeds, if stored appropriately, can be used for repeated screening of a large number of targets.

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

<table>
<thead>
<tr>
<th>Sample†</th>
<th>Hardness index (HI)‡</th>
<th>Diameter (mm)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. SD</td>
<td>Avg. SD</td>
<td></td>
</tr>
<tr>
<td>Australian Hard</td>
<td>67.99  19.25</td>
<td>2.46  0.51</td>
<td>Hard</td>
</tr>
<tr>
<td>Sunco</td>
<td>86.96  14.72</td>
<td>2.51  0.42</td>
<td>Hard (Pinb-D1a, Pinb-D1b)</td>
</tr>
<tr>
<td>Janz</td>
<td>84.52  16.64</td>
<td>2.48  0.41</td>
<td>Hard (Pinb-D1a, Pinb-D1b)</td>
</tr>
<tr>
<td>Rosella</td>
<td>40.05  13.31</td>
<td>2.60  0.35</td>
<td>Soft (Pinb-D1a)</td>
</tr>
<tr>
<td>Tincinur</td>
<td>47.37  17.22</td>
<td>2.32  0.35</td>
<td>Soft (Pinb-D1a)</td>
</tr>
<tr>
<td>QA51.2 WT</td>
<td>43.1   15.4</td>
<td>3.05  0.59</td>
<td>Pina-D1a, Pinb-D1a</td>
</tr>
<tr>
<td>QA51.2 M</td>
<td>44.73  13.92</td>
<td>3.05  0.56</td>
<td>Pina-D1a, Pinb(t-13I)</td>
</tr>
<tr>
<td>QA150.4 WT</td>
<td>43.06  18.33</td>
<td>2.63  0.53</td>
<td>Pina-D1a, Pinb-D1a</td>
</tr>
<tr>
<td>QA150.4 M</td>
<td>42.54  12.58</td>
<td>3.31  0.54</td>
<td>Pina-C11Y, Pinb-D1a</td>
</tr>
<tr>
<td>QA158.1 WT</td>
<td>34.29  13.87</td>
<td>2.44  0.52</td>
<td>Pina-D1a, Pinb-D1a</td>
</tr>
<tr>
<td>QA158.1 M***</td>
<td>45.1   15.71</td>
<td>2.24  0.55</td>
<td>Pina-P125, Pinb-D1a</td>
</tr>
<tr>
<td>QA232.2 WT</td>
<td>69.69  16.39</td>
<td>2.60  0.45</td>
<td>Pina-D1a, Pinb-D1a</td>
</tr>
<tr>
<td>QA232.2 M***</td>
<td>87.46  18.23</td>
<td>2.31  0.47</td>
<td>Pina-D1a, Pinb-R74Stop</td>
</tr>
</tbody>
</table>

***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 contain hard wheat. Sunco and Janz are both hard cultivars that carry the Pinb-D1b allele. Rosella and Tincinur 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hardness index</th>
<th>Diameter (mm)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. SD</td>
<td>Avg. SD</td>
<td></td>
</tr>
<tr>
<td>QAL2000$^*$</td>
<td>41 15</td>
<td>2.63 0.50</td>
<td>Pina-D1a, Pinb-R74Stop</td>
</tr>
<tr>
<td>F$_2$ WT</td>
<td>45 14</td>
<td>2.58 0.50</td>
<td>Pina-D1a, Pinb-D1a</td>
</tr>
<tr>
<td>F$_2$ M$^{***}$</td>
<td>75 16</td>
<td>2.60 0.34</td>
<td>Pina-D1a, Pinb-R74Stop</td>
</tr>
</tbody>
</table>

$^{***}$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.

### Acknowledgments

We thank Prof Bob McIntosh, University of Sydney, for valuable discussion on the project and critical reading of the manuscript. This work was supported by the Value Added Wheat Cooperative Research Centre, Australia and the Australian Centre for Plant Functional Genomics. Note: These mutant populations are available for collaborative research.

### References


