Breeding desired quality wheat by reverse genetics

Dong C, Dalton-Morgan J, Vincent K, Sharp P

Plant Breeding Institute, University of Sydney, PMB11, Camden, NSW2570, Australia
Value Added Wheat Cooperative Research Centre, Australia

ABSTRACT

TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetics tool which uses traditional chemical mutagenesis methods and high-throughput mutation detection techniques. It identifies a series of point mutations in a gene of interest. Here we show that TILLING is suitable in wheat for identifying useful mutants for wheat breeding. Screening of Waxy genes *Wx-A1* and *Wx-D1* in 1,025 EMS-treated M2 plants or 2,200 heads has found 119 mutants including truncation mutations in *Wx-A1* and *Wx-D1*. Due to the null-4A (*Wx-B1*) nature of this population, a waxy wheat has been bred by crossing the two truncation mutants (*Wx-A1-trunction* and *Wx-D1-truncation*). Screening of two puroindoline (*pin*) genes (a and b) has identified 20 mutants. Some of them showed either harder or softer phenotype when compared to untreated wild-type. As wheat genomics is producing a large amount of information on potential target genes, many genes of interests can be screened for mutations in this TILLING population and mutants can be used for breeding wheat with desired quality.

INTRODUCTION

TILLING was first developed in *Arabidopsis* to study gene function after the completion of *Arabidopsis* genome sequencing (McCallum et al. 2000; Colbert et al. 2001). This reverse genetics tool combines traditional chemical mutagenesis methods with high-throughput mismatch detection techniques, and yields an allelic series of point mutations including knock-out and partial inactivation of the gene of interest. Wheat has a large and complex genome. Its polyploid nature makes it difficult to identify desirable genetic changes based on phenotypic screens due to the redundant copies of genes. Therefore forward genetics is more difficult in wheat than in other diploid plants. It has been shown that TILLING is suitable for wheat (Slade et al. 2005). We used TILLING to produce new mutant alleles of quality-related genes in adapted backgrounds of wheat cultivars. If new alleles can be found with extra benefits to end-users, these mutants can be used as potential commercial lines, or new parental materials for wheat breeding.

MATERIALS AND METHODS

The Value Added Wheat CRC cultivar QAL2000, a soft wheat, and Ventura, a hard wheat, were chosen for mutagenesis and mutation screening. The *waxy* (granule-bound starch synthase I, or GBSS I) and *puroindoline* (*pina* and *pinb*) genes were chosen for screening mutations. Wheat contains three homoeologous *waxy* genes, *Wx-A1*, *Wx-B1* and *Wx-D1*, located on 7A, 4A and 7D respectively. *Pina* and *pinb* genes are located on 5D.

Seeds were treated with the mutagen EMS (ethyl methanesulfonate) (SIGMA, Australia) at 0.5%, 0.6% and 0.7% (w/v) overnight and then washed extensively for at least 2 hr. The treated seeds were germinated in pots and then transplanted to a field as M1 plants. M2 seeds were harvested as five heads per M1 plant (for QAL2000), with each head bagged individually; or one head per M1 plant (for Ventura). One seed of each head was sown in a 100-cell seedling tray. Leaves of 2.5-week-old seedlings were collected for DNA extraction in a 96-plate format using an ABI6100 machine according to the manufacturer's instructions.

The DNA concentrations were normalized to 5-15 ng/µl after gel electrophoresis with a known mass standard. DNA samples from individual heads were pooled threefold or fourfold. PCR amplification was carried out in a 10 µl volume containing 2 µl of pooled DNA, 1 µl 10x Pfu buffer (Stratagene), 0.2 mM dNTPs, 0.4 µM primers and 0.25 U PfuUltra®II Fusion HS DNA Polymerase (Stratagene). PCR was conducted using a thermal cycler (Eppendorf) as follows: 95˚C for 2 min, followed by 6 cycles of touchdown PCR (94˚C for 30 s, an annealing step starting at 72˚C for 30 s and decreasing 1˚C per cycle, a temperature ramp increasing 0.5˚C per second to 72˚C, and 72˚C for 1 min), then 35 more 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.

PCR primers used for *waxy* mutation screening were Wx7A2, Wx7A3, Wx7A4, Wx7D3 and Wx4A6 of Slade et al. (2005). After PCR amplification, the product was denatured and annealed in the thermal cycler (95˚C 8 min, 85˚C 1 min, followed by 99 cycles of 84˚C for 30 s decreasing 0.5˚C per cycle). Primers for *pina* and *pinb* are according to Gautier et al. (1994) with the modification of pinb-F to 5'-ATGAAGACCTTATTCCTCCTAGCTCTC-3'. The PCR profile is the same as for the *waxy* PCR except annealing temperature for *pina* is 58˚C and for *pinb* is 60˚C.

After PCR amplification, denaturation and annealing, samples were digested with CelI enzyme. This celery juice extract was prepared as described by Till et al. (2004) except the last step; the dialysed extract was mixed with glycerol to a 50% glycerol enzyme solution. The digestion reaction was performed at 45ºC for 30 min in a 20 µl reaction volume containing 8 µl PCR product, 2 µl CelI enzyme and 10 µl buffer (20 mM HEPES pH7.5, 20 mM $MgSO_4$, 20 mM KCl, 0.004% TritonX-

100 and 0.4 µg/ml BSA). The samples were then electrophoresed in a thin agarose gel. Images were analysed visually for the presence of cleavage products using Adobe Photoshop software (Adobe Systems Inc.).

If a pooled sample was identified as having CelI cleavage bands, individual samples from this pool were tested by PCR together with the wild-type DNA sample, and then CelI digestion. Thus an individual sample was identified as a mutant. The PCR product of this sample was then sequenced. Sequences were analysed using Web-based programs CODDLE (http://www.proweb.org/coddle) and PARSESNP (http://www.proweb.org/parsesnp) to identify deleterious mutations.

A truncation *Wx-A1* (7A) mutant and a truncation *Wx-D1* (7D) mutant (both lines were identified as null-4A in nature) were grown to M3 to identify homozygous lines. Both homozygous lines were grown and crossed with each other. The F1 of this cross were grown to maturity and seeds were harvested and iodine stained (Nakamura et al, 1995) to identify waxy seeds.

RESULTS AND DISCUSSION

Mutation frequencies in TILLING populations

In the QAL2000 TILLING population, more than 2000 samples, ~2200 kb DNA were screened in the *Waxy* gene fragments, the mutation frequency of different mutagenesis treatment could be estimated. In the most heavily treated population, 0.7% EMS treatment, mutation frequency is about one in 22 kb. This population had about 30% sterility in the M2. The 0.6% EMS treated population has mutation frequency at about one in 29 kb. Table 1 shows examples of mutation occurrence in the screened population.

In the Ventura TILLING population, single head was harvested, which is different from QAL2000. A total of 16 mutants were identified in 7A3 and 7D3 fragments from 369 0.7% EMS treated plants and 14 mutants identified in 308 0.6% EMS treated plants, mutation frequency being about one in 33 kb.

These mutation frequencies are quite high compared to Arabidopsis TILLING (1 in 170 kb, Colbert et al. 2001; Greene et al. 2003) and barley TILLING (1 in 1 million bp, Caldwell et al. 2004), and similar to what was found in wheat (1 in 24 kb, Slade et al. 2005). The sterility and lethality of these populations are about 30%, at an acceptable level. The polyploid nature of wheat may make it partially resistant to EMS mutations.

Harvesting of five heads per plant in QAL2000 has the advantage of getting more mutants from the same number of plants, if screened by single heads. In addition, as five heads are sister lines with same parental origin, mutant identified in one head can be characterised by comparing with other sister lines.

Screening of Waxy genes *Wx-A1* and *Wx-D1* in 1,025 EMS-treated M2 plants or 2,200 heads has found 119 mutants including truncation mutations in *Wx-A1* and *Wx-D1*. Table 2 gave an example of identified mutants.

Production of waxy QAL2000

Truncation mutants QA199.2 and QA209.1 (both lines were null-4A in nature) were grown and crossed. F1 of this cross were grown and the resultant F2 seeds were screened for waxy phenotype by iodine staining. In 617 screened seeds, 37 waxy seeds were identified; this fits well to the expect 1:15 two gene segregation. Figure 1 shows one waxy line.

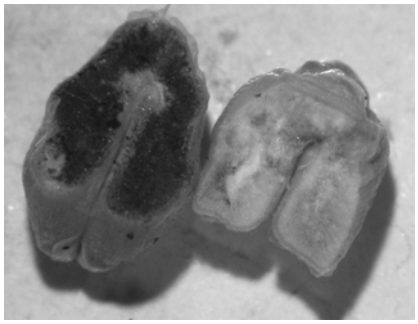

Figure 1. Waxy QAL2000 (right) was produced by crossing two mutants identified in TILLING. Normal QAL2000 in left has dark iodine staining.

Screening of *waxy* mutants and producing waxy QAL2000 wheat is a proof of concept of using TILLING in wheat breeding. With the development of more efficient screening methods, TILLING is practical and useful for wheat. As wheat genomics is producing a large amount of information of target genes, many genes of interest can be screened for mutations in this and other TILLING populations, enabling breeding for desired quality traits in wheat.

Identification of puroindoline mutants

Screening of 864 QAL2000 samples identified 20 mutants in *pina* and *pinb* genes. Estimated mutation frequency is one in 30 kb. Both *pina* and *pinb* truncation mutants were identified. Other interesting mutants included missense mutations in the signal peptide and TRR (Tryptophan-Rich Region). Preliminary hardness test (SKCS) showed phenotypes with harder or softer grain character were identified in these mutants.

A recent survey by Pickering and Bhave (2007) of the genotypes of Australian hard wheats noted the presence of only the *Pina-D1b* and *Pinb-D1b* types. The TILLING work allows for the expansion of the gene pool by methods faster than introgression of desirable alleles into Australian material, while also expanding the set of puroindoline alleles that are available to global breeders.

REFERENCES

Caldwell, D.G., McCallum, N., Shaw, P., Muehlbauer, G.J., Marshall, D.F., and Waugh, R. (2004). A structured mutant population for forward and reverse

genetics in Barley (Hordeum vulgare L.). *Plant Journal* 40: 143-150.

Colbert, T., Till, B.J., Tompa, R., Reynolds, S., Steine, M.N., Yeung, A.T., McCallum, C.M., Comai, L., and Henikoff, S. (2001). Hugh-throughput screening for induced point mutations. *Plant Physiology* 126: 480-484.

Gautier, M. F., Aleman, M. E., Guirao, A., Marion, D. and Joudrier, P. (1994). *Triticum aestivum* puroindolines, two basic cysteine-rich seed proteins: cDNA sequence analysis and developmental gene expression. *Plant Molecular Biology* **25**(1):43-57.

Greene, E.A., Codomo, C.A., Taylor, N.E., Henikoff, J.G., Till, B.J., Reynolds, S.H., Enns, L.C., Burtner, C., Johnson, J.E., Odden, A.R., Comai, L., and Henikoff, S. (2003). Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. *Genetics* 164: 731-740.

McCallum, C.M., Comai, L., Greene, E.A., and Henikoff, S. (2000). Targeted screening for induced mutations. *Nature Biotechnology* 18: 455-457.

Nakamura, T., Yamamori, M., Hirano, H., Hidaka, S., and Nagamine, T. (1995). Production of waxy (amylose-free) wheats. *Molecular and General Genetics* 248: 253-259.

Pickering, P. A. and Bhave, M. (2007). Comprehensive analysis of Australian hard wheat cultivars shows limited *puroindoline* allele diversity. *Plant Science* **172**(2):371-379.

Slade, A.J., Fuerstenberg, S.I., Loeffler, D., Steine, M.N., and Facciotti, D. (2005). A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology* 23: 75-81.

Till, B.J., Burtner, C., Comai, L., and Henikoff, S. (2004). Mismatch cleavage by single-strand specific nucleases. *Nucleic Acids Research* 32: 2632-2641.

Table 1. Mutation occurrence in the screened QAL2000 TILLING population.

				Mutants[2]					
Sample[1] plates	No. heads	gene/PCR	Mutants	Repeat-ed	Mis-sense	Trun-cation	silent	Un-known	Frequency
QA3,8,10	288	A3 (743bp)	14	2	4	2	8		
3,8,10		D3 (710bp)	9	1	3	1	4	1	
7,12,13	288	D3	8	0	1	1	2	4	
7,12,13		A4 (708bp)	6	1	2		2	2	
Total	576	2871bpx288	37						1/22.3kb
QB1,2,3,4	384	D3	9	1	2	2	4	1	
5,6,7,8	384	D3	9	1	4	0	4	1	
Total	768	1420bpx384	18						1/30kb

[1] QA is 0.7% EMS treated population, and QB is 0.6% EMS treated population.

[2] Mutants were identified by sequencing. Repeated mutant is the same mutation identified in one plant but different heads; missense mutation is a mutation causing an amino acid change; truncation mutant is a mutation causing a premature STOP codon; silent mutation is a mutation not causing an amino acid change or a mutation in an intron region; unknown mutations are mutations identified by CelI digestion but not sequenced.

Table 2. Examples of mutants identified in screening *Wx-A1* and *Wx-D1* in a QAL2000 TILLING population.

PCR fragment	Plant	Cel I bands	Zygosity	Base Change	Code Change	Effect (a.a. change)	Mutant
Wx7A3	QA199.2	560+230	Hom	G1414A	TGG-TGA	W301Stop	truncation
	QA165.4	450+340	Het	G1534A	GGG-GGA	G323G	silent
	QA191.3	700+100	Het	G1872A	GAC-AAC	D430N	missense
	QA207.5	400+390	Het	G1626A			intron
Wx7D3	QA201.4	410+350	Hom	C1703T	ACC-ATC	T370I	missense
	QA209.1	540+220	Het	G1497A	TGG-TGA	W318Stop	truncation
	QA132.3	560+200	Het	G1792A	GCG-GCA	A371A	silent
	QA258.4	520+240	Het	C1516T	CAG-TAG	Q308Stop	truncation