VALIDATION OF TILLING POPULATIONS IN DIPLOID AND HEXAPLOID WHEAT

by

NOLAN ROTHE

B.S., Fort Hays State University, 2007

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Interdepartmental Genetics Program

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

Approved by:

Major Professor
Bikram S. Gill

Abstract

TILLING (Targeting Induced Local Lesions IN Genomes) is a high-throughput, reverse genetics strategy for scanning mutagenized populations for point mutations in loci of interest. Originally, TILLING was used to investigate gene function in *Arabidopsis* and has since been similarly applied for gene functional analysis in other organisms. TILLING also allows the generation of novel genetic variation in specific genotypes and, thus, has been implemented as a tool for crop improvement.

Ethyl methanesulfonate (EMS) is a widely used mutagen to induce point mutations in most TILLING protocols. M_1 plants are then self-pollinated and M_2 seed harvested. A single seed is grown from each M_2 progeny and tissue taken for DNA isolation. M_3 seed is catalogued. DNA is pooled to increase the efficiency and aid in mutation detection. Polymerase chain reaction (PCR) is used to amplify a locus of interest using the M_2 DNA pools as a template. The PCR products are digested with an endonuclease that cleaves mismatched, mutant DNA, and the digested products are visualized. The pools for which PCR products are positive for a mutation are deconvoluted to determine which individual plant of the pool was responsible for the mutation. DNA from the positive individual is sequenced to determine the type of mutation (missense, nonsense, synonymous). Individuals with mutations that are more likely to disrupt gene function (nonsense and certain missense) are studied further by growing the corresponding M_3 generation.

In bread wheat, *Triticum aestivum*, TILLING is complicated by polyploidy: genes that have homoeologs require that the functionality of each be studied. If functional homoeologs are present for all three genomes, mutants must be identified for each homoeolog, followed by successive intercrossing to produce a triple mutant plant. As a model for wheat genetics, we propose TILLING in diploid wheat.

EMS mutant populations were created in diploid wheat (*Triticum monococcum* ssp. *monococcum)* and the hexaploid bread wheat cultivar 'Jagger'. The diploid and hexaploid wheat populations were screened for mutations at the *waxy* locus, *GBSS1*, as a validation of our population and for comparative analysis of mutation rates in 2x and 6x wheat. For diploid wheat, *GBSSI* was screened in 716 M_2 plants, and one mutant was found for 1.9 Mb screened.

For hexaploid wheat, *GBSSI* was screened in 518 M_2 plants, and 30 mutants were identified within a total of 657 Kb screened, giving a mutation frequency of one mutation per 22 Kb. The reasons for this vast difference in mutation frequency between diploid and hexaploid wheat are discussed. The diploid wheat population was further examined by screening for mutations within four lignin biosynthesis candidate genes, for a total of 2 Mb screened. A single mutant was discovered for both of the lignin genes *PAL6* and *HCT*, giving a mutation frequency of one mutation per 1 Mb screened.

Table of Contents

List of Figures

List of Tables

Dedication

for Mama

the flowers bloom
the days pass by
a bird sings
a fish swims
a mother loves

Introduction

TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetics technique combining chemical mutagenesis with high-throughput mutation detection to identify point mutations within a specific locus. The method was created while studying gene function of chromomethylase homologues in *Arabidopsis* (McCallum 2000a). Two other reverse genetics strategies had fallen short: T-DNA insertions were not recovered, and antisense transgenics failed to produce a change in expression. Thus, another methodology, TILLING, was derived in response. This technique may be preferable to other reverse genetics methods for various reasons. EMS produces a broad range of alleles, including nonsense (truncation) and missense mutations (McCallum 2000b), allowing more flexibility than transgenics or insertional mutagenesis. Furthermore, mutagenesis is achieved without the addition of exogenous DNA and is applicable to a broad range of organisms.

TILLING has two major promises for plant genetics: gene functional analysis and crop improvement. The DNA sequence alone is often enough to infer something about a gene's function; however, the prediction must be validated phenotypically. TILLING can provide the empirical validation needed for this type of sequence-driven hypothesis (McCallum 2000b). The importance of this locus-to-phenotype approach is demonstrated by the success of the Arabidopsis TILLING Project, a high-throughput service for gene functional analysis (Henikoff et at. 2004). TILLING also finds application in crop improvement, and TILLING resources have been developed for numerous crop species. For the cereals alone, there are populations for maize (Till et al. 2004), barley (Caldwell et al. 2004, Talame 2008), rice (Wu et al. 2005, Till et al. 2007), sorghum (Xin et al. 2008), and hexaploid and tetraploid wheat (Slade et al. 2005, Dong et al. 2009, Uauy et al. 2009).

In wheat, TILLING has been applied to both hexaploid and tetraploid varieties and was used to create a near null *waxy* phenotype by targeting the loci of *GBSSI* (Slade et al. 2005). By intercrossing mutants of the *GBSSI* homoloci produced by TILLING, a complete *waxy* phenotype was produced (Dong et al. 2009). It was also used to identify a hard grain variant in a soft grain background by screening a *puroindoline* (*Pinb*) gene (Dong et al. 2009). These examples show the potential of TILLING for wheat improvement. This technology has come at a time when bioinformatics resources and sequence data are accumulating rapidly. Efforts are being coordinated by the International Wheat Genome Sequencing Consortium for physical

mapping and sequencing of the wheat genome (http://www.wheatgenome.org/). Techniques such as TILLING will be paramount in connecting gene sequences with plant phenotypes.

Although TILLING is of tremendous importance to the improvement of polyploid wheat, applying the technique for polyploid gene functional analysis presents challenges. Because polyploid wheat has three sets of seven chromosomes derived from related species, most loci are tripicated as a homoeologous set. This requires sequence information from all homoeoloci. For TILLING, genome-specific primers must be designed, and each functionally important homoeolocus must be targeted. Then, mutants for each target homoeolocus must be intercrossed to engineer a fully mutant plant, drawing out the number of generations for a genetic study. This process needs to be simplified to make gene functional analysis more efficient in wheat. Diploid wheat gives us the opportunity to efficiently dissect gene function, because mutants can immediately be associated with a phenotype. This knowledge can then be applied to 4x and 6x wheat.

The objective of this study was to develop a TILLING population in the diploid wheat ancestor, *Triticum monococcum* subsp. *monococcum* L., to overcome the barriers of working in polyploid wheat species. Mutation frequencies are reported for *T. monococcum* and hexaploid bread wheat, as validation of the populations and methodology.

Review of Literature

TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetics technique combining mutagenesis with a high-throughput mutation detection method to identify point mutations or indels within a specific locus. The method was created while studying gene function of chromomethylase homologues in *Arabidopsis* (McCallum et al. 2000a). Two other reverse genetics strategies had failed: T-DNA insertions were not recovered, and antisense transgenics failed to produce a change in expression. Thus, an alternate method was sought. This involved the creation of an EMS mutant population, amplifying PCR products from the mutant template DNA, and using DHPLC to detect mutants in the form of heteroduplexed products. The methodology successfully identified mutations within the chromomethylase homologues, and the new reverse genetics technique was coined as TILLING. EMS produces a wide range of alleles within a gene, including missense and truncation mutations, allowing flexibility and giving potential advantages over other reverse genetic methodologies (McCallum et al. 2000b). Since its inception, TILLING has been applied to various model organisms, including fruit fly, *Drosophila melanogaster* (Winkler et al. 2005); nematode, *Caenorhabditis elegans* (Cuppen et al. 2007, Gilchrist et al. 2006); rat, *Rattus norvegicus* (Smits et al 2006); and zebrafish, *Danio rerio* (Draper et al. 2004, Wienholds and Plasterk 2004). Aside from gene functional analysis in model organisms, TILLING has been adapted as a tool for crop improvement (Slade and Knauf 2005). Promise here can be measured by the sheer number of crops to which TILLING has been applied. For the cereals alone, there are populations for maize (Till et al 2004), barley (Caldwell et al. 2004, Talame 2008), rice (Wu et al. 2005, Till et al. 2007), sorghum (Xin et al. 2008), and hexaploid and tetraploid wheat (Slade et al. 2005, Dong et al. 2009, Uauy et al. 2009).

For TILLING in flowering plants, there are variations upon the mutagenesis strategy, but a common methodology is as follows. Mutagenesis is performed by imbibing seeds in a solution of ethyl methane sulfonate (EMS). Other chemical mutagens used include sodium azide, EMS alkylates the hydrogen-bonding oxygen of guanine bases, resulting in O-6 alkylguanines. Subsequently, O-6 alkylguanine will pair with thymine, rather than cytosine, ultimately causing G/C to A/T transitions (Hein et al. 2009). The EMS-treated M_0 seeds produce the M_1 plants, which are self-pollinated to produce M_2 seed. Only one plant is grown per M_1- derived M_2 seed

because M_1 plants are genetic chimeras (Parry et al. 2009). Tissue is collected from the M_2 plants (which are nonchimeric), and M_3 seed is harvested and cataloged. A sufficient population size depends on the size of the targeted locus, the mutation frequency, and the type of mutation sought. Parry et al. (2009) estimated the number of lines required for 95% confidence in recovering a nonsense mutation within a 1,000-bp amplicon. For the mutation frequency of hexaploid wheat, approximately 1,400 mutant lines are required. Although there have not been mutation frequencies reported for diploid wheat, diploid species may require much larger populations for saturated coverage of the genome; an EMS population of barley will require an estimated 60,000 individuals (Parry et al. 2009). DNA is isolated from the M_2 tissue and is pooled into groups of two to eight individuals. The depth of pooling depends on the sensitivity of the mutation detection method; deeper pools require higher sensitivity. Pooling serves not only to increase screening efficiency but is also necessary for the c*el*-I mutation detection method (Colbert et al. 2001). In this technique, PCR is used to amplify a locus of interest, which is then denatured and slowly reannealed to form heteroduplexes if amplicons contain point mutations or indels. The samples are then digested with *cel*-I, a mismatch-specific endonuclease that cleaves heteroduplexed DNA (Colbert et al. 2001). The digested PCR product is visually inspected for samples containing cleavage products, the lengths of which add up to the full-length product. Each individual from a pool containing a mutant is reexamined separately to identify the source of the variant. The amplicon of the identified individual may be sequenced to determine the type and location (exon or intron, synonymous or nonsynonymous site, splice junction) of the variant and, thus, its putative significance (McCallum 2000a). Tools are available to predict if mutations will affect protein function, such as the program SIFT (Sorting Intolerant from Tolerant) (Henikoff et al. 2004). From there, genotypes are investigated by growing the corresponding M_3 progeny to study phenotypes or making further crosses (McCallum et al. 2000b).

Since the advent of TILLING, there have been some alterations in mutation detection approach. McCallum originally used Denaturing High-Pressure Liquid Chromatography (DHPLC) for mutation detection, taking advantage of the reduced retention time of heteroduplexed PCR products, causing chromatographic alterations (McCallum 2000a). Colbert et al. (2001) modified the mutation detection method, using the mismatch-specific endonuclease, *cel*-I, to cleave heteroduplexed DNA, and used labeled primers along with Li-Cor (Omaha, NE)

polyacrylamide gel electrophoresis to visualize the cleaved PCR products. The *cel*-I digestion technique remains a common mutation detection method for TILLING, although others have used alternate visualization strategies such as agarose gel electrophoresis (Raghavan et al. 2006, Dong et al. 2009) and nondenaturing polyacrylamide gel electrophoresis (Uauy et al. 2009). Other projects have moved away from the enzymatic digestion method to a TILLING by sequencing approach (http://tilling.ucdavis.edu/index.php/TILLING-by-Sequencing).

In the original wheat TILLING publication, Slade et al. (2005) investigated the *waxy* genes encoding Granule-Bound Starch Synthase I (*GBSSI*) in both hexaploid bread wheat and tetraploid durum varieties. It was shown that TILLING can be used to generate and locate novel alleles for crop improvement. By identifying and crossing hexaploid wheat plants with a truncation mutation *Wx-D1* (D-genome waxy locus) and a missense mutation in *Wx-A1* (A-genome *waxy* locus), they were able to create a near-null *waxy* phenotype (the B-genome *waxy* homeolocus was naturally null). Dong et al. (2009) also TILLed within the *waxy* locus and succeeded in producing a full-null *waxy* phenotype by crossing a *Wx-A1* truncation mutant with a *Wx-D1* truncation mutant (*Wx-B1* was naturally null). Additionally, the puroindoline genes, *Pina* and *Pinb* were targeted. Mutations or deletions in either of these genes result in a hard seed phenotype (Giroux and Morris 1998). A truncation mutation was discovered within *Pinb*, resulting in a hard wheat in a soft wheat population. Uauy et al. (2009) developed both tetraploid and hexaploid populations. The tetraploid variety was characterized by TILLING in two *Starch Branching Enzyme II* genes, *SBEIIa* and *SBEIIb.* The hexaploid variety was screened with *Wheat Kinase Start* (*WKS1)*, *WKS2*, and *SBEIIa* genes. Over 275 mutations were found among the gene and genome combinations, although they were not characterized phenotypically.

Bread wheat, *Triticum aestivum* subsp. *aestivum* L., has long been important as a staple crop for humanity. Genetically, it is an allohexaploid, containing the genomes of three separate, yet related species with the genomes A, B, and D. *T. aestivum* is the result of the hybridization between the tetraploid *Triticum turgidum* subsp. *turgidum* L. (containing the A and the S-related B genome) and the D-genome diploid, *Aegilops tauschii* Coss. (Huang et al. 2002). *Triticum turgidum* subsp. *durum*, called durum wheat or macaroni wheat, is economically important for pasta production. *Triticum turgidum* is the result of the hybridization between diploid *Triticum urartu* Tumanian ex Gandilyan (A genome donor) and a B-genome donor closely related to *Ae. speltoides* Tausch. (S genome) (Dvorak et al. 1993). *Triticum monococcum* L. subsp.

aegilopoides is closely related to *T. urartu*. *Triticum monococcum* subsp. *monococcum* is a domesticated form of *T. monococcum* subsp. *aegilopoides* (Link) Thell. Although there are TILLING populations in hexaploid bread wheat and tetraploid macaroni wheat, to the author's knowledge, there is no literature applying TILLING to the diploid wheats.

Currently, there is increasing interest in cellulosic ethanol. Unlike corn ethanol production, the production of cellulosic ethanol is not a direct competitor with a food crop, because it can be produced from wheat stubble, corn stover, and other forms of biomass. Still, the production of cellulosic ethanol requires pretreatment steps that are absent in corn ethanol production. These additional steps are the largest obstacles towards economical cellulosic ethanol production (Sticklen 2008). Cellulose is bound by lignin, inhibiting the chemical pretreatment and enzymatic digestion steps. One promising method of circumventing this problem involves modifying lignin to make cellulose more available.

Lignin content has been modified in various species, albeit for purposes other than delignification for biofuels. In poplar, (*Populus* spp.), the lignin biosynthetic genes cinnamyl alcohol dehydrogenase (*CAD*) and caffeate/5-hydroxy-ferulate *O*-methyltransferase (*COMT*) were downregulated by introducing antisense transgenes, resulting in more efficient delignification for papermaking (Pilate et al. 2002). In alfalfa (*Medicago sativa* L.), 4-coumarate 3-hydroxylase (*C3H*) was downregulated, altering the lignin structure and improving digestibility in ruminants. Directly relating to ethanol production, Chen and Dixon (2007) showed that downregulating the lignin biosynthetic genes cinnamate 4-hydroxylase (*C4H*), hydroxycinnamoyl transferase (*HCT*), *C3H, S*-adenosyl-methionine caffeoyl-CoA/5-hydroxyferuloyl-CoA-*O*-methyltransferase (CCoA-OMT), ferulate 5-hydroxylase (*F5H*), or caffeate *O*-methyltransferase (*COMT*) could significantly increase the saccharification efficiency in alfalfa, reducing or potentially eliminating the need for chemical pretreatment. In bread wheat, lignin biosynthetic candidate genes were identified through comparative analysis with rice genes with putative function in the lignin biosynthesis pathway (Li et al. 2008). Approximately 275 unigenes from 11 gene families in wheat showed a very high homology with genes putatively annotated as lignin precursors in rice. Gene expression studies using hexaploid bread wheat identified 28 ESTs covering 11 gene families that were found to play a major role in the lignin biosynthetic pathway (Li et al. 2008).

Materials and Methods

Development of the TILLING Populations

The diploid relative of wheat *T. monococcum* subsp. *monococcum* (accession number TA4342) was used to develop a mutant population for TILLING. Ethyl methane sulfonate (EMS) was used for mutagenesis. The first experiment was to determine the dose of the EMS needed to achieve 40-60 % survival. Five sets of 50 seeds of TA4342 were soaked in water for 8h imbibition on a shaker at 75 rpm and then treated with five different doses (0.1, 0.2, 0.3, 0.4, and 0.5 %) of EMS for 16h on shaker at 75 rpm. The treated seeds were washed under running water for 8h and then planted individually into root trainers. Observations were made 15 days after planting to estimate the survival frequency.

It was observed that the survival rates of plants with EMS doses 0.2 and 0.25% were closest to the targeted percent survival (Table 1A). A second experiment was performed with six doses of EMS spanning the 0.2 to 0.25% interval using 100 seeds per treatment (Table 1B).

Table 1. A) Effect of 0.10-0.50% EMS dosage on survival for *T. monococcum*. **B)** Effect of 0.20-0.25 % EMS dosage on survival for *T. monococcum*.

A

Dosage EMS (%)	Survival (%)
0.10	90
0.20	70
0.30	21
0.40	5
0.50	3

B

Dosage EMS (%)	Survival (%)
0.20	73
0.21	68
0.22	60
0.23	58
0.24	51
0.25	32

The percent survival of the 0.24% EMS treatment was closest to the target. Thus, the 0.24% treatment was chosen for the development of the TILLING population. The M_1 population was self-pollinated. A single M_2 plant was grown per M_1 plant with M_1- derived M_2 seed to prevent genetic redundancy. Tissue was collected from the M_2 plants, and M_3 seed was harvested and cataloged.

The DNA of 518 individuals of a hexaploid TILLING population, cultivar Jagger, was provided by Michael Pumphrey (USDA-ARS, Manhattan, KS). The Jagger M_0 seeds were

mutagenized in the same manner as the the *T. monococcum* population, except that 0.7% EMS was used in the treatment. M_2 plants were grown by single-seed descent. M_2 tissue was collected, and M_3 seed was cataloged.

Development of DNA Pools

DNA was isolated from the M_2 tissue using a Biosprint 96 robot (Qiagen, Germantown, MD) as per the Biosprint 96 plant protocol. DNA was quantified on a NanoDrop (Wilmington, MD) and normalized to 20 ng/µl in 96-well blocks. DNA was pooled either two-fold or four-fold. For four-fold pooling, normalized DNA from four 96-well blocks was combined in equal proportions into one 96-well block (Figure 1).

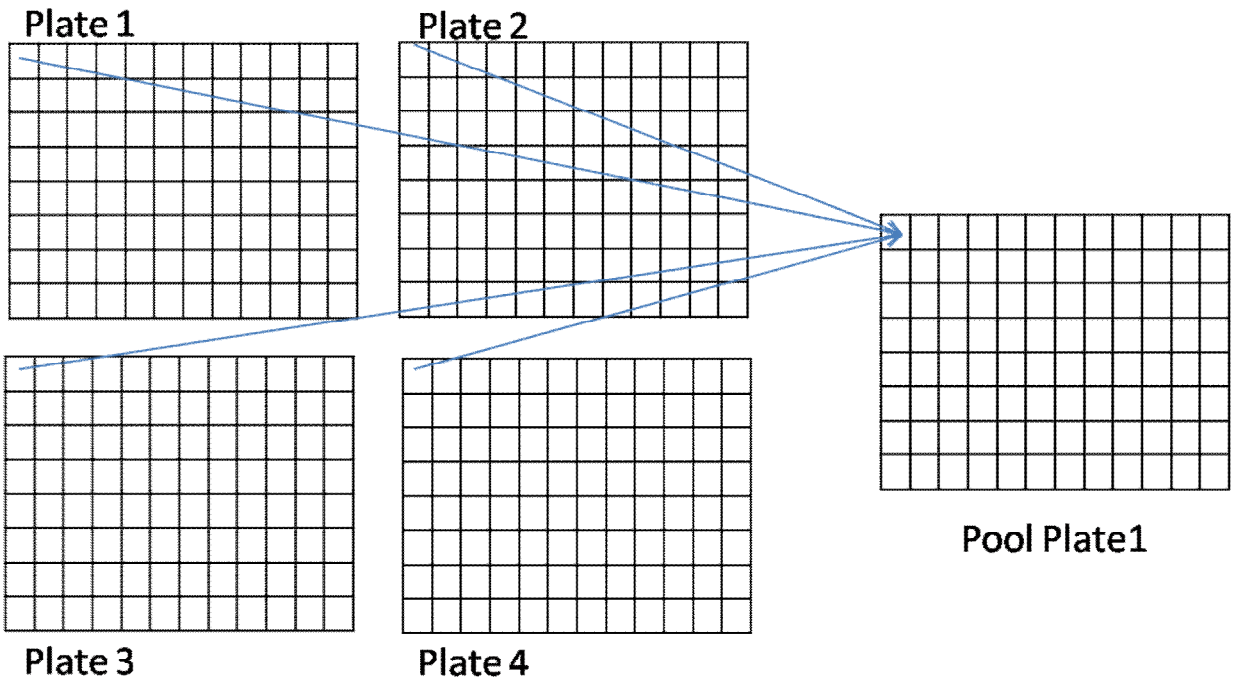


Figure 1. Four-fold pooling. DNA was normalized to 20 ng/µl in 96-well plates. Four 96-well DNA plates were added in equal proportions into one pool plate, so that each well of the pool plate contained the DNA of four M_2 individuals.

Identification of Lignin Biosynthesis Pathway Genes in *Triticum monococcum*

Rice genes with putative function in the lignin biosynthesis pathway were used to identify candidate genes for the lignin biosynthesis pathway in wheat through comparative analysis. Approximately 275 unigenes from 11 gene families in wheat showed a very high homology with genes putatively annotated as lignin precursors in rice. Gene expression studies using hexaploid bread wheat identified 28 ESTs covering 11 gene families, which were found to play a major role in the lignin biosynthesis pathway (Li et al. 2008). Based on real-time PCR results, these ESTs were divided into two groups. The first group includes 16 ESTs that showed a significant developmental regulatory pattern that were in close agreement with total lignin content. The second group is comprised of 12 ESTs that generally showed constitutive expression and lacked significant differences with stages of growth or in different tissues. The first group was the target of this study.

To confirm their presence and determine the sequence homology of the first group of ESTs in *T. monococcum*, primers were designed from 16 ESTs and amplified from *T. monococcum* accession TA4342. Fourteen ESTs amplified and were sequenced (Table 2). Sequence comparison of *T. monococcum* with known EST sequence showed high homology with six ESTs (TC248157, TC234479, TC233169, TC264767, TC269065, and TC252261).

To increase the likelihood of finding mutants with phenotypic differences, only the exonic regions for the genes of interest were targeted. Full length cDNA sequences or full length gene sequences were unavailable for the target genes. However, homology between rice and wheat allowed the use rice genomic DNA and cDNA sequences to identify exons from available wheat ESTs. SPIDEY was used for alignment: (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) (Figure 2). Using this approach, the exonic regions of genes were determined. Primers were designed for exons in which there was a high homology between the wheat EST and rice genomic DNA. Those that amplified are listed in Table 3.

Table 2. EST primers designed for lignin genes, spanning exon and intron regions.

Gene	*T. aestivum* accession	Rice locus	Forward (F) and reverse (R) primers
HCT 2	TC271108	LOC_Os02g39850	F: TCTGGAACTCCAACCTCGAC
			R: TGCAGCTAGGACTGCATACG
4CL1	TC252223	LOC_Os06g44620	F: AGATCCCCAACGAGCAGAC
			R: CATCCATCCTTGTCGATGGT
4CL2	TC269435	LOC_Os06g44620	F: ATGGCCAAGATCCCCAAC
			R: TCAGCTTTTGGACTGTGTGC
C3H	TC237096	LOC_Os05g41440	F: GCAGCCACAATGGACGTG
			R: ACACGTTCACCATCACGTTG
C4H1	TC234480	LOC_Os05g25640	F: GACCTGAACCACCGCAAC
			R: CGAAGGGCAAGTACCTGAAG
C4H2	TC234479	LOC_Os05g25641	F: GATGGACGTCCTCCTCCTG
			R: CGTTGACGAGGATCTTGGA
CAD4	TC247577	LOC_Os02g09490	F: CTCCCAAATCCACGCATC
			R: GCCTGGTTGACGTAGTCCAT
CCoAOMT1	TC233169	LOC_Os08g38910	F: ACAACCAGCCCAAACAGAAC
			R: GCGGCAGATGGTGACTCC
CCoAOMT3	TC246834	LOC_Os06g06980	F: AGCCCAGATCTCGTCTCCTT
			R: GACGGGTTCTGTGGTTCACT
CCR1	TC252568	LOC_Os03g60380	F: GGAGACGGTGCTGGTCAC
			R: TCACGGAACTTTGGGACTTT
CCR2	TC251530	LOC_Os09g25150	F: CTCGACTACGACGCCATCTG
			R: TGATCATCGAAGCCGATACA
CCR3	TC251533	LOC_Os09g04050	F: GGAGATGGGCATTACATCGT
			R: TGTGCGTCATACAGCACTGA
COMT1	TC264767	LOC_Os08g06100	F: GTACGCTCTCCAGCTCGTCT
			R: CGAACTCCCTCTCGTACCTC
F5H1	TC254699	LOC_Os09g26940	F: TCGAGTATCCGTCAATGGTG
			R: CTTCATCACGCCCAGCTT
F5H2	TC269065	LOC_Os08g43440	F: GGATGAGTCGGACCTCGATA
			R: CAGTGAGGCCAAAGATGTCA
HCT1	TC252261	LOC_Os02g39850	F: CTGCAGGTGACCCACTTCA
			R: CATCTTCCGGAACTTCTCCA
PAL6	TC233239	LOC_Os05g35290	F: CGAGGAGGTGAAGAAGATGG
			R: CGAACATCAGCTTGCCAATA
PAL8	TC247198	LOC_Os02g41630	F: AGCTCCTCAGGCATCTGAAC
			F: CATGAGCTTGAGGATGTCCA

Table 3. Primers designed for the exonic regions of lignin genes.

Gene	Primer	Sequence	Size (bp)
PAL8	TC247198e1F	ACGAGGTGAAGCGGATGGT	218
	TC247198e1R	GGTGGTGACGCCGTAGATGT	
PAL8	TC247198e2s1F	CTGAACGCTGGAATCTTTGG	714
	TC247198e2s1R	CATGGACTTGGTGGCAGACC	
PAL8	TC247198e2s2F	TCTACAACAACGGGCTGACCTC	676
	TC247198e2s2R	GGGAACGACCTGCTCTCCTT	
C3H	TC237096e1F	CCCATCATCTCCGTCTGGTTC	230
	TC237096e1R	AGGCGCTTCTGGGTGAAGAG	
C3H	TC237096e3F	TGAGGAATCACCTTGCTATGG	367
	TC237096e3R	AGAAGTCCGAAAACTGTGTCATC	
C3H	TC237096e4F	AGAAGAAGCTGCAAGAGGAGCTG	493
	TC237096e4R	GTGCCCATGAAGGTGACGAG	
CCoAOMT3	TC246834e3F	GACGAGGGCCAGTTCCTCAA	326
	TC246834e3R	GAGCAGGGAGTAGCCGGTGTAG	
CCoAOMT3	TC246834e4F	ACCGCGAGAACTACGAGCTG	723
	TC246834e4R	CTTGTTGAGGTCGAGGACGAAGT	
PAL6	TC233239e1s2F	CATCTTGGAGGGAAGCTCATACAT	689
	TC233239e1s2R	GTTGTTCATGCTCAGGGTCTTTCTT	
COMT1	TC264767e1/2F	ATGTACGCTCTCCAGCTCGTCT	357
	TC264767e1/2R	GCTCTCCATGAGGACCTTGTC	
COMT1	TC264767e1/2F	AGGGGATGAAGAACCATTCCAT	538
	TC264767e1/2R	CATGCGTTGGCGTAGATGTAAGTA	
HCT1	TC252261e1F	GGCCTGCACTTCATCAACTCAT	811
	TC252261e1R	TCCGGAACTTCTCCATATGCTC	
4CL1	TC252223e1F	GACATCGAGATCCCCAACGAG	235
	TC252223e1R	CGAGGAAGGAGAAGGCGAACT	
4CL1	TC252223e4F	CTGCCGCTGTTCCACATCTA	188
	TC252223e4R	GTCACCTGGGGGCTCTTG	
4CL1	TC252223e5F	GCCAGGGGTACGGGATGAC	134
	TC252223e5R	GGGGTCGACGATCTTGAGC	
4CL1	TC252223e6F	CAGACTCCACCAAGAACACCATC	135
	TC252223e6R	CCACCTGGAAGCCCTTGTACT	

A

EXON	Genomic coordinates	mRNA coordinates	length	identity	mismatches	gaps	Donor site	Acc. site
Exon 1	77-861	1-785	785	100.0%	0	0	d	
Exon 2	2498-2619	786-907	122	100.0%	0	0	d	a
Exon 3	3705-4300	908-1503	596	100.0%	0	0		a

B

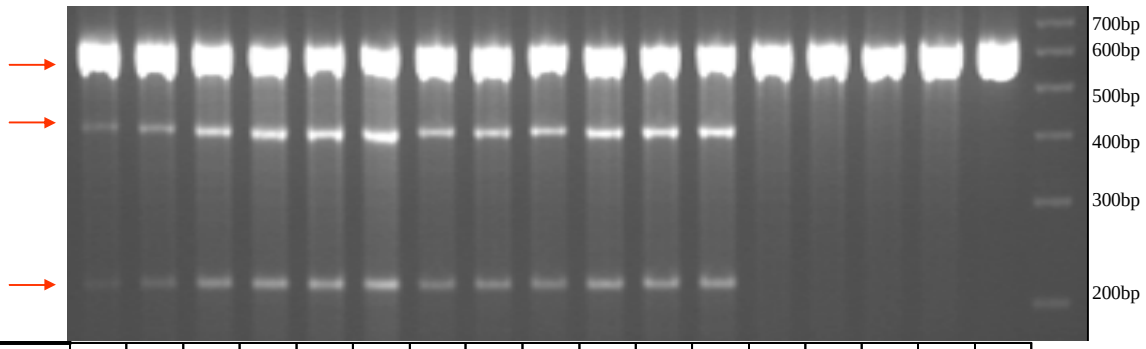
EXON	Genomic coordinates	mRNA coordinates	length	identity	mismatches	gaps	Donor site	Acc. site
Exon 1	77-862	1-788	788	88.8%	88	2		
Exon 2	2512-2616	802-906	105	94.3%	6	0	d	
Exon 3	3702-4287	907-1495	589	89.3%	63	3		a

Figure 2. (A) The alignment of rice genomic DNA and rice mRNA for gene *C4H2* by SPIDEY. **(B)** The alignment of rice genomic DNA for gene *C4H2* and with the wheat EST sequence for the corresponding gene (TC234479). The exon/intron boundaries are defined by this alignment, and using this information, primers were designed to span sufficiently large exons. All three exons present in the rice gene are represented by the wheat EST by comparing **B** to **A**. *Acc. indicates the presence of the splice acceptor site.

Target Amplificiation and *Cel*-I Endonuclease Digestion

The targeted loci were amplified from pooled DNA using Biolase PCR kits (Bioline, Taunton, MA). PCR was carried out in 25 µl volumes with 1x reaction buffer, 0.25 mM dNTP, 1.5 mM $MgCl_2$, 0.4 µM primers, 100 ng pooled or individual DNA, and 0.75 U Biolase Taq-polymerase. A touchdown PCR profile was conducted on an MJ Research DYAD (Emeryville, CA) thermal cycler for the amplicons w1, DOS, and TRES as follows: 5 min of 95C denaturation, 7 cycles of touchdown (95C for 30 s, 67C annealing for 45 s with 1C decrease per cycle, 2 min extension), followed by 30 cycles of PCR (95C for 30 s, 60C annealing for 45 s, 2 min extension), and finally 8 min of extension. A 60C annealing PCR profile was conduced on an ABI GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) thermal cycler for primers WX7A2, WX7A3, 233239e1s2, 247198e2s1, 247198e2s2, 264767, and 252261e1 as follows: 5 min of 95C denaturation, 35 cycles of PCR (95C for 30 s, 60C annealing for 45 s, 2 min extension), and finally 8 min of extension. After PCR, the products were denatured and slowly cooled to allow heteroduplex formation between mismatched DNA: 2 min of denaturation, ramping to 85C over 10 s, incubation at 85C 10 s, decrease by 0.1C per second until 25C reached, and finally incubation at 4C. Then, celery juice extract (CJE), produced according to the method of Till et al. (2006), was added to the heteroduplexed product and incubated at 45C for 45 min. Reactions were stopped with 5 µl 0.225M EDTA. The digested products were electrophoresed on 2% agarose gel with 0.5 µg/ml ethidium bromide. Gels were run at 110V for 2-4 hours, depending on product length, and products were visualized with a transilluminator.

CJE digestion was standardized using c*el*-I endonuclease (Transgenomic, Inc, Omaha, NE) and the control provided with the kit. The control consists of two DNA samples with a single nucleotide polymorphism (SNP) between the two. Samples digested which lacked the variant showed only one band when visualized on an agarose gel–the full-length, uncleaved product. Samples digested that contained the variant showed three bands when visualized–the full-length product, as well as two bands for the cleaved products, as expected (Figure 2). The pattern was the same for both CJE and *cel*-I digestion, showing the efficacy of the CJE.



CJE (ul)	1	2	3	4	5	6	0	0	0	0	0	0	2	2	0	0	0
cel-I (ul)	0	0	0	0	0	0	1	1	1.5	1.5	2	2	0	0	1.5	1.5	0
SNP?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	Y

Figure 2. Digestions using varying amounts of CJE and commercial *cel*-I were compared by adding the enzymes to a product containing equal amounts of PCR product with a guanine and cytosine SNP. Adding increasing volumes of CJE or *cel*-I resulted in stronger cleavage bands (lower two red arrows), whereas diminishing the intensity of full length product (upper red arrow). Samples lacking the SNP showed no cleavage bands when digested with CJE or *cel*-I. Samples with the SNP without the addition of CJE or *cel*-I produced no cleavage bands. The last lane (on the right) is the 100-bp ladder.

Mutation Detection

Lignin Biosynthesis Genes in Triticum monococcum

The *T. monococcum* population was scanned for mutations in the following genes: phenylalanine ammonia-lyase 6 (*PAL6*), phenylalanine ammonia-lyase 8 (*PAL8*), caffeic acid 3-O-methyltransferase 1 (*COMT1*), and hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (*HCT*) (Table 4).

Table 4. Primers and amplicon sizes of the lignin biosynthetic target genes screened for *T. monococcum*: Phenylalanine ammonia-lyase 6 (*PAL6*), phenylalanine ammonia-lyase 8 (PAL8), caffeic acid 3-O-methyltransferase 1 (*COMT1*), and hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (*HCT*).

Gene	Primer name	Primer sequence	Amplicon length (bp)
PAL6	TC233239e1s2F	CATCTTGGAGGGAAGCTCATACAT	689
	TC233239e1s2R	GTTGTTCATGCTCAGGGTCTTTCTT	
PAL8	TC247198e2s1F	CTGAACGCTGGAATCTTTGG	714
	TC247198e2s1R	CATGGACTTGGTGGCAGACC	
PAL8	TC247198e2s2F	TCTACAACAACGGGCTGACCTC	676
	TC247198e2s2R	GGGAACGACCTGCTCTCCTT	
COMT1	TC264767F	GTACGCTCTCCAGCTCGTCT	940
	TC264767R	CGAACTCCCTCTCGTACCTC	
HCT	TC252261e1F	GGCCTGCACTTCATCAACTCAT	811
	TC252261e1R	TCCGGAACTTCTCCATATGCTC	

Waxy Locus in Triticum monococcum

A region with prior characterization was examined to compare mutation frequencies and evaluate the new population. The *waxy* locus was selected, because TILLING had been successfully applied to this region in tetraploid and hexaploid wheat, and mutation frequencies had been calculated in multiple populations (Slade et al. 2005, Dong et al. 2009). The A-genome specific primers, WXA2 and WXA3 (Slade et al. 2005), failed to amplify in the population in *T. monococcum*. Therefore, *T. monococcum* primers from Yan et al. (2000) were amplified. Of the four primer pairs used to cover the *waxy* locus (w1, w2, w3, w4), only the w1 amplicon amplified to give a single band and strongly enough to be considered appropriate for TILLING. Two additional primer pairs (DOS, TRES) were designed to cover the majority of the gene along with w1. The primer pairs w1, DOS, and TRES cover 931, 1058, and 998 base pairs, respectively (Figure 3). Between the three regions are 47+182=229 base pairs of overlap. The *T. monococcum* population was scanned for mutations in each of the three amplicons to cover the majority of the *waxy* locus.

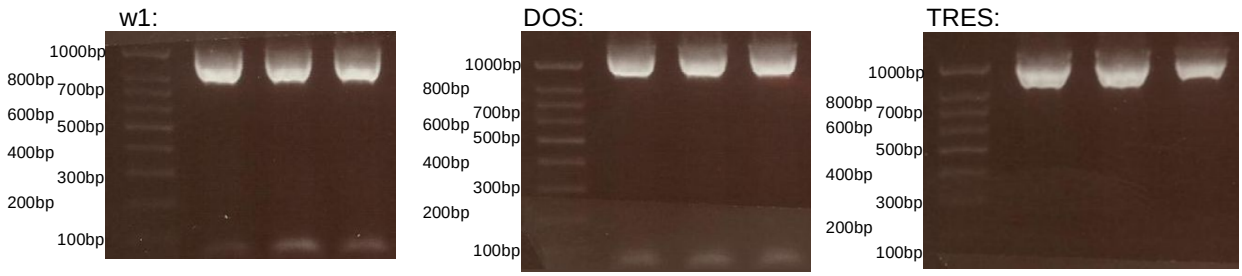


Figure 3. Amplicons w1 (931 bp), DOS (1,058 bp), and TRES (998 bp) were designed to span the *waxy* locus in *T. monococcum*. The primers amplified the wild type DNA of the parent of the TILLING population (TA4342 *T. monococcum* subsp. *monococcum*). Tests were performed in triplicate. The first lane of each gel image (on the left) is the 100-bp ladder.

Waxy Locus in Triticum aestivum var. Jagger

The surprisingly low identification of mutation events in *T. monococcum* led us to examine our technique by working with a region and species with prior characterization. The *GBSS1* gene, or *waxy* locus, was the subject of the original bread wheat TILLING project (Slade et al. 2005) and subsequently has been used as a standard for comparison (Dong et al. 2005). Thus, screening for *waxy* in a hexaploid population could provide more information on the effectiveness of the TILLING technique in our lab. To validate the CJE digestion mutation detection method on agarose, DNA from an M_2 population of hexaploid bread wheat, cultivar Jagger, was scanned. Two primer pairs specific to the A genome, WXA2 and WXA3 (Slade et al. 2005) with amplicon lengths of 577 bp and 793 bp, respectively, were used to screen 518 Jagger M_2s (Figure 4).

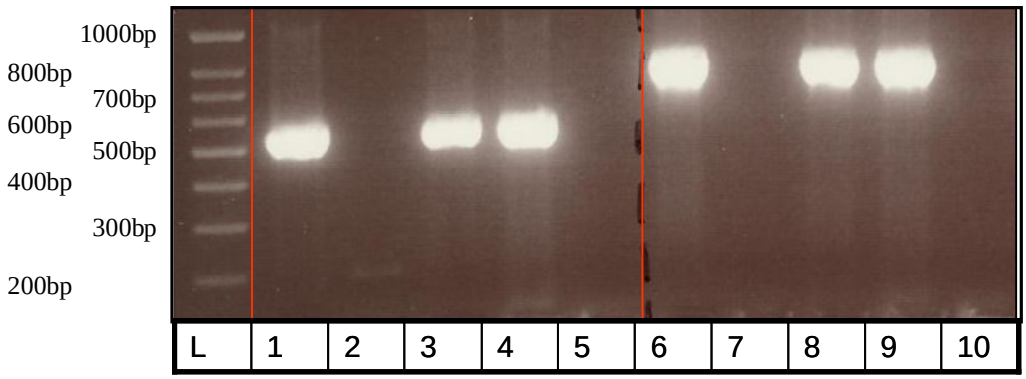


Figure 4. Genome specificity of WX7A2 and WX7A3. Lane L indicates the ladder. Lanes 1-5 were amplified with WX7A2 primers (577 bp), and lanes 6-10 were amplified with WX7A3 (793 bp). Chinese Spring DNA was used as the template DNA in lanes 1 and 6. Ditelosomic chromosome 7AL template (lacking 7AS) was used in lanes 2 and 7. Ditelosomic chromosome 4AS template (lacking 4AL) was used in lanes 3 and 8, and ditelosomic 7DL template (lacking 7DS) was used in lanes 4 and 9. No amplification occurred in lanes 2 and 7, showing that both WX7A2 and WX7A3 are specific to chromosome 7AS. Lanes 5 and 10 are nontemplate controls.

Results

Development of TILLING Population

A total of more than 1,400 *T. monococcum* M_2 lines were developed, and DNA has been isolated from 716 of the plants. The mutagenized population showed various mutant characters including albino, chlorina, and stunted plants, indicating that mutagenesis was effective. Phenotypic notes were taken on a sample of 756 M_2 plants and are summarized in Table 5.

Table 5. Phenotypes observed in EMS mutagenized *T. monococcum* population of 756 M_2s.

Phenotype	Plant count	Percentage
Small plant	38	5
Sterile	52	6.9
Late	29	3.8
No plant	74	9.8
Semi-sterile	22	2.7
Shattered	3	0.4
Chlorina (died)	10	1.3
Albino (died)	18	2.4
Total died	77	10
Chlorina (lived)	3	0.4
Two spikes	100	13.2
Three spikes	14	1.9
No spike	8	1.1
Grasslike	1	0.1

Mutation Detection for Lignin Biosynthesis Genes in *Triticum monococcum*

The *T. monococcum* population was screened for mutants within the *PAL6*, *PAL8*, *COMT1*, and *HCT* genes (Table 6). One *PAL6* mutant (Figure 7) and one *HCT* mutant were identified. The pools containing the mutants were deconvoluted to determine the individual responsible for the mutant genotype (Figure 8). PCR was performed on the template DNA of each individual of the pool, as well as on a 1:1 mixture of individual template to wild-type template (for heteroduplex formation). The products were heteroduplexed, CJE-digested, and visualized. Because the individuals' digested product produced the cleavage banding pattern, it

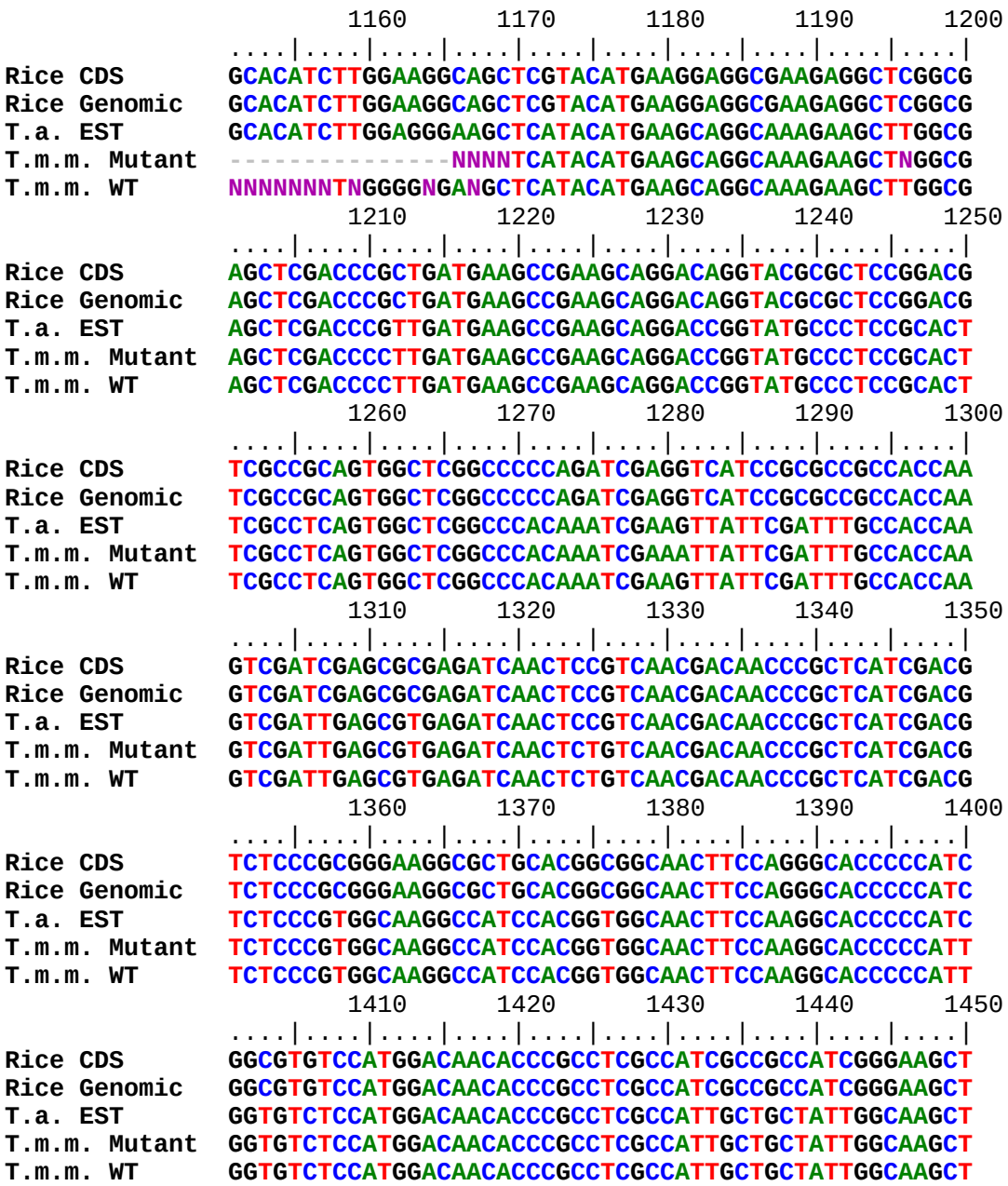


Figure 5. Multiple sequence alignment of gene *PAL6*. Sequences of rice genomic DNA, rice CDS, *T. aestivum* EST, *T. monococcum* mutant, and *T. monococcum* wild type are shown. A SNP at reference number 1,281 causes a missense mutation, changing the wildtype valine to isoleucine in *T. monococcum*.

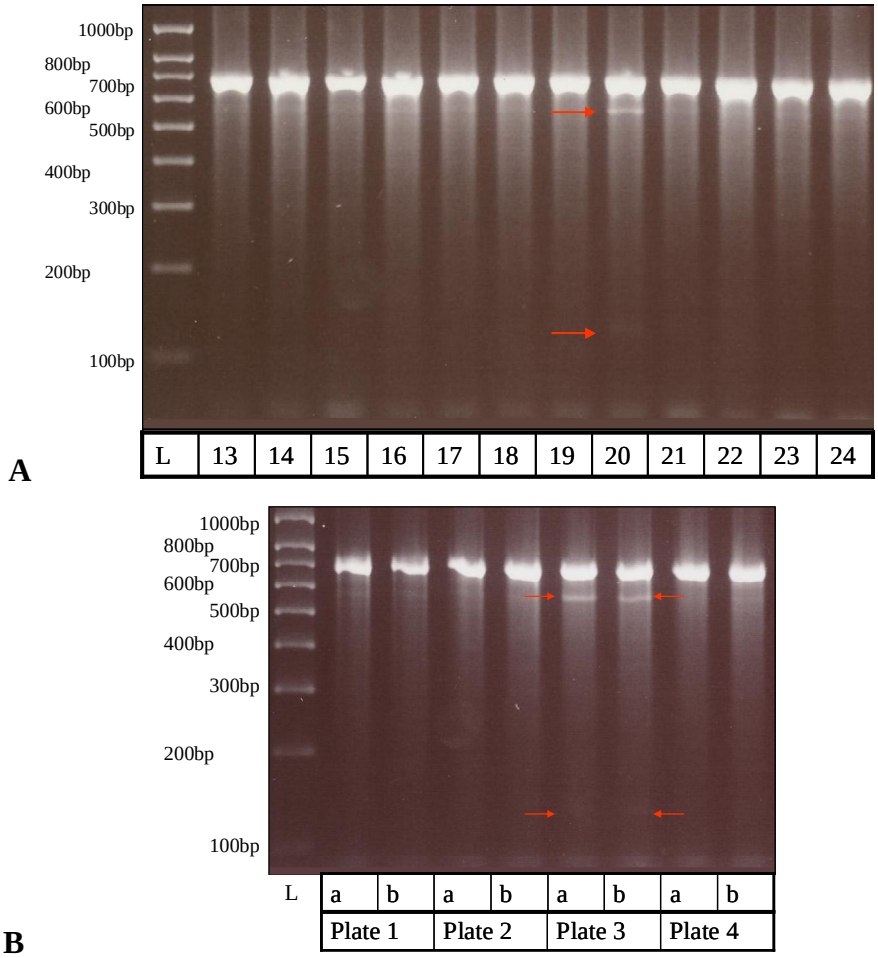


Figure 6. A) Phenylalanine ammonia-lyase (*PAL6*) mutant found in pool #20. Primer 233239e1s2 (689 bp) was used to amplify *PAL6* in pooled DNA #13-24. The products were digested with CJE, and pool #20 showed cleavage bands at ~120 bp and ~570 bp, which give a close approximation of the full-length product when summed. The cleavage bands are indicated by the red arrows. A 100-bp ladder is indicated by L. **B)** Deconvolution of *T. monococcum PAL6* mutant found in pool #20. Pool #20 includes the DNA of the 20th individual M_2 from DNA plates 1-4. The template DNA from individual 20 in plates 1-4 was amplified using primer 233239e1s2, and samples were digested to determine which M_2 individual was responsible for the positive pool. Lanes labeled "a" were amplified with the DNA of only one individual and those labeled "b" were amplified with both the individual and wildtype *T. monococcum* DNA. Here, it is shown that plate 3 contains a heterozygous mutant because the "a" lane shows the mutant banding pattern. The 100-bp ladder is indicated by an L.

was determined that they were heterozygotes (homozygous mutants would require the addition of wild-type DNA to produce the banding pattern) (Figure 8). Twenty M_3s of the mutants were grown for genotypic examination and for future analysis of lignin content. DNA was isolated from the *PAL6* mutant progeny, and *cel*-I digestion was used to determine their zygosity for the mutation. Of the 20 M_3 plants, six were homozygous, eight were heterozygous, and six were wild-type, close to the expected 1:2:1 ratio expected from a self-pollinated heterozygote. The *PAL6* mutant was sequenced and showed a missense mutation, changing a valine codon to isoleucine (Figure 7).

Table 6. Mutants identified for lignin candidate genes in *T. monococcum*.

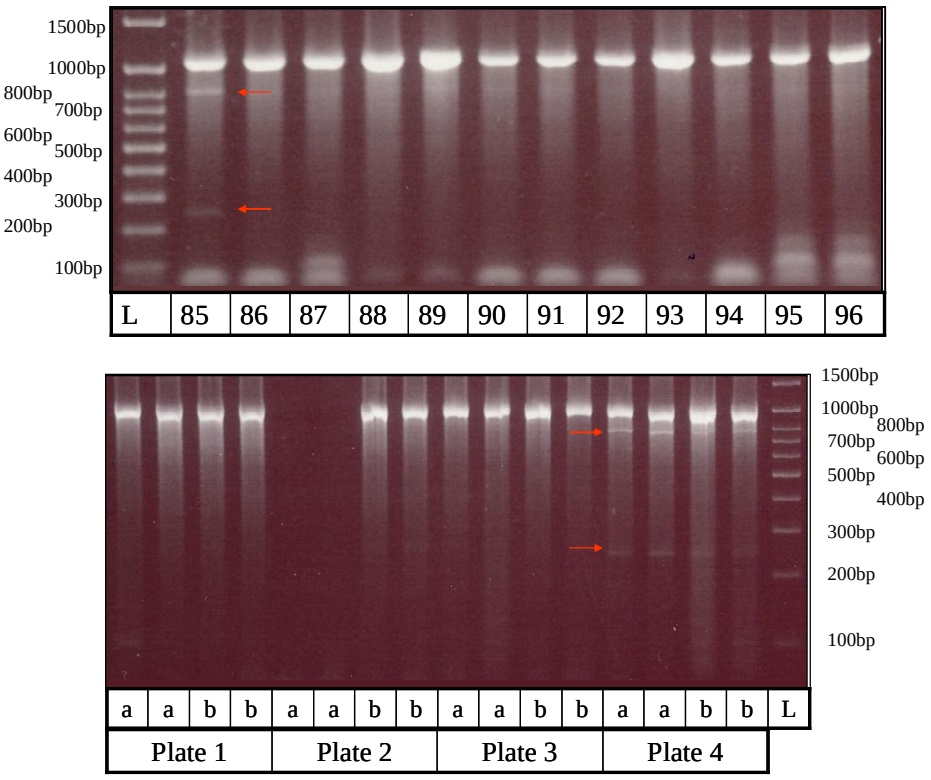
Gene	Primer	Length (bp)	M_2s screened	Mutants	Mutant ID
PAL6	233239e1s2	689	716	1	Idaho-352
PAL8	247198e2s1	714	716	0	-
PAL8	247198e2s2	676	716	0	-
COMT1	264767	940	384	0	-
HCT	252261ex1	811	384	1	Idaho-324

Mutation Detection for the *Waxy* Locus in *Triticum monococcum*

The *T. monococcum* population was screened for mutations with the primers w1, DOS, and TRES and 0, 1, and 0 mutants were found, respectively (Table 7). The mutation positive pool (#85) found by scanning within the DOS amplicon was deconvoluted to determine which individual from the pool gave the positive genotype (Figure 6). This was accomplished by PCR amplification of the DNA of each individuals in the pool in a 1:1 ratio of M_2: wild-type (wild-type DNA is needed for *cel*-I digestion in the case that the mutant is homozygous). The mutant individual responsible for the mutation within the positive pool was identified as accession 2010-277 through pool deconvolution and was shown to be heterozygous. Twenty M_3s from the positive individual are being grown for further analysis.

Table 7. Mutants found within *T. monococcum GBSSI*.

Primer	Length (bp)	M_2s screened	Mutants	Mutant ID
w1	931	716	0	
DOS	1058	716	1	2010-277
TRES	998	716	0	



B

Figure 7. A) *T. monococcum* Granule-Bound Starch Synthase I (*GBSSI*) mutant found in pool #85. *GBSSI* was amplified with primer pair DOS (1,058 bp) with DNA pools 85-96 as the template. When digested with CJE, pool 85 showed cleavage bands at ~800 bp and ~250 bp, as indicated by the red arrows. The 100-bp ladder is indicated by an L. **B)** Deconvolution of GBSSI mutant from pool #85. Individuals from DNA plates 1-4 were amplified with primer DOS and digested with CJE to determine the source of the mutant. Each sample was done in duplicate. Lanes labeled "a" were amplified with only the DNA of an M_2 individual, whereas those labeled "b" lanes were amplified with both the individual and wildtype *T. monococcum* DNA. Plate four contains a heterozygous mutant because the "a" lane shows the mutant banding pattern present in the positive pool, with cleavage bands at ~800 bp and ~250 bp indicated by red arrows. Plate 2 had no plant and no DNA isolated for individual #85. Lane L indicates the 100-bp ladder.

Mutation Detection for the *Waxy* Locus in *Triticum aestivum*

A total of 30 mutants were found. Within the WX7A2 region, 14 mutants were found, nine of which were heterozygous and five homozygous. Within the WX7A3 region, 16 mutants were found, of which all were heterozygous (Table 8). One mutant identified was sequenced as a confirmation and showed a missense mutation, from arginine to lysine (Figure 9).

Table 8. Jagger *WX-7A* mutants were catalogued according to the primer used to amplify the region, the accession number of the M_2, the estimated CJE cleavage fragment sizes in base pairs, and the zygosity of the mutation.

Primer	Accession number	Fragment size (bp)	Zygosity
WX7A2	39	450, 125	heterozygote
	44	400, 175	homozygote
	49	450, 125	homozygote
	145	375, 200	heterozygote
	156	375, 200	homozygote
	221	425, 150	heterozygote
	289	300, 275	heterozygote
	389	475, 100	heterozygote
	398	325, 250	heterozygote
	402	350, 225	homozygote
	448	375, 200	heterozygote
	468	375, 200	heterozygote
	521	350, 225	heterozygote
WX7A3	14	625, 175	heterozygote
	38	400, 375	heterozygote
	121	400, 375	heterozygote
	228	425, 350	heterozygote
	232	700, 75	heterozygote
	236	575, 225	heterozygote
	270	700, 100	heterozygote
	283	700, 75	heterozygote
	365	700, 100	heterozygote
	392	450, 325	heterozygote
	443	600, 200	heterozygote
	451	625, 150	heterozygote
	463	600, 200	heterozygote
	484	425, 150	heterozygote
	519	575, 200	heterozygote
	557	650, 150	heterozygote

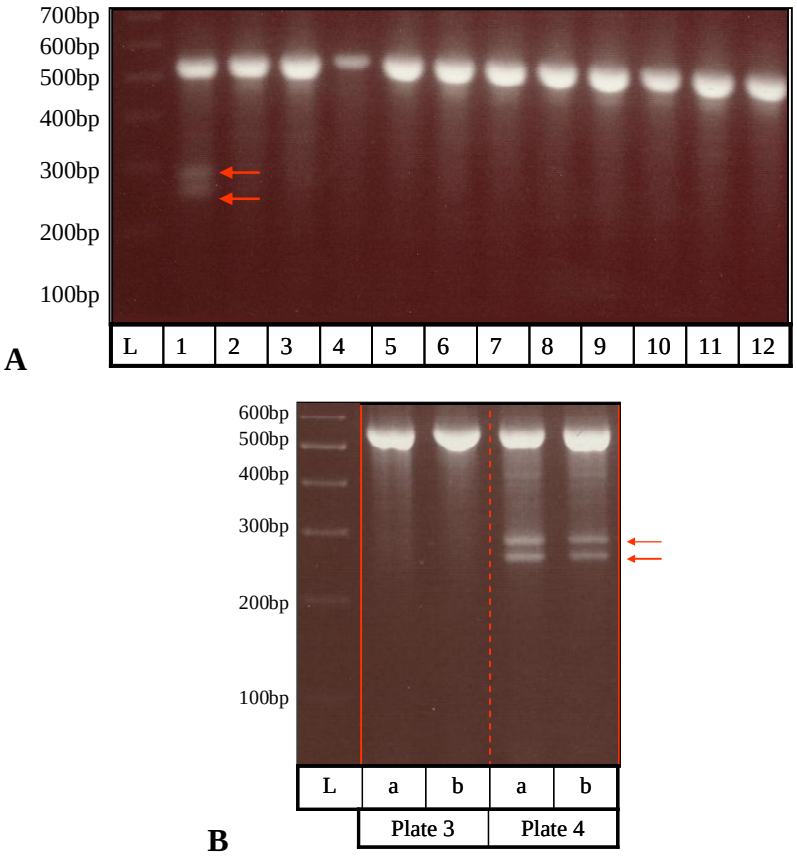


Figure 8. A) Digested WX7A2 product for Jagger M_2 pools 1-12 of a two-fold pool (a 1:1 mixture of DNA plates 3 + 4). Lane L indicates the 100-bp ladder. The template DNA of Jagger M_2s was amplified with WX7A2 primers for Granule Bound Starch Synthase I. Products were digested with CJE. Lane 1 shows cleavage bands (red arrows) at ~300 bp and ~270 bp, which, when summed, approximate the full-length WX7A2 product of 577 bp, indicating the presence of a mutation. **B)** Deconvolution of the positive Jagger pool of DNA plates 3+4, pool #1, containing a mutant in the WX7A2 region. Lane L indicates the 100-bp ladder. Individual #1 of plate 3 and plate 4 were amplified with WX7A2 primers. Lanes labeled "a" were amplified from the individual alone, and lanes labeled "b" were amplified with a 1:1 mixture of M_2 : wild-type DNA. Products were digested with CJE. Cleavage bands are present at ~300 bp and ~270 bp of individual #1 of plate 4, indicating the source of the mutation in pool #1 (Figure 8A). The presence of the cleavage bands in the "a" lane of plate 4 indicate that the mutant is heterozygous for the mutation.

CCTGCGCGCGCCATGGCGGCTCTGGTCACGTCCCAGCTCGCCACCTCCGGCACCGTCCTCAGCGTCACCG
ACAGATTCCGGCGTCCAGGTTTTCAGGGCCTGAGGCCCCGGAACCCGGCGGATGCGGCGCTCGGCATGAG
GACTGTCGGAGCGAGCGCCGCCCCAAAGCAAAGCAGGAAACCGCACCGATTCGACCGGCGGTGCCTCTCC
ATGGTGGTGCGCGCCACGGGCAGCGGCGGCATGAACCTCGTGTTCGTCGGCGCCGAGATGGCGCCCTGGA
GCAAGACTGGCGGCCTCGGCGACGTCCTCGGGGGCCTCCCCGCCGCCATGGCCGTAAGCTTGCGCCACTG
CCTTCTTATAAATGTTTCTTCCTGCAGCCATGCCTGCCGTTACAACGGGTGCCGTGTCCGTGCAGGCCAA
CGGTCACCGGGTCATGGTCATCTCCCCGCGCTACGACCAGTACAAGGACGCCTGGGACACCAGCGTCATC
TCCGAGGTATATATCCGCCACATGAATTATCACAATTCACATGCTCCTGCACATTTCTGCAAGACTTTAC
TGACTGGCTGGATCTCGCAGATCAAGGTCGTTGACAGGTACGAGAGGGTGAGGTACTTCCACTGCTACAA
GCGCGGGGTGGACCGCGTGTTCGTCGACCACCCGTGCTTCCTGGAGAAGGTGACCGATCGCTCGCCGTCG
ATCGATCAAGCTAGCTCCTCGTCGTCTCAACCCGCATGGTGTTTGATAATTTCAGTGAGTCTTTGCGTCT
GCTGGTTACAATTTCCAGGTCCGGGGCAAGACCAAGGAGAAGATCTATGGACCCGACGCCGGCACCGACT
ACGAGGACAACCAGCAGCGCTTCAGCCTTCTCTGCCAGGCAGCACTTGAGGTGCCCAGGATCCTCGACCT
CAACAACAACCCACACTTTTCTGGACCCTACGGTAAGATCAAGAACAACTAGAGTGTATCTGAAGAACTT
GATTTCTACTTGAGAGCACTGGATGATTATCATCTTCCTTGTATCTTGGTGCTGCCATGCTATGCCGTGC
CGTGCCGCGCCGCGCAGGGGAAGACGTGGTGTTTGTGTGCAACGACTGGCACACGGGCCTTCTGGCCTGC
TACCTCAAGAGCAACTACCAGTCCAATGGCATCTATAGGACGGCCAAGGTTTTGCATCTTCTGAAACTTT
ATATTCGCTCTGCATATCAATTTTGCGGTTCATTCTGGCAGCCTGAATTTTACATTGCAACTCCATTTCA
TGGCTAGGTGGCATTCTGCATCCACAACATCTCGTACCAGGGCCGCTTCTCCTTCGACGACTTCGCGCAG
CTCAACCTGCCTGACAGGTTCAAGTCGTCCTTCGACTTCATCGACGGCTACGACAAGCCGGTGGAGGGGC
GCAAGATCAACTGGATGAAGGCCGGGATCCTGCAGGCCGACAAGGTGCTGACTGTGAGCCCCTACTATGC
TGAGGAGCTAATCTCTGGCGAAGCCAGGGGCTGCGAGCTCGACAACATCATGCGCCTCACTGGGATCACC
GGCATCGTCAACGGCATGGACGTCAGCGAGTGGGACCCCATCAAGGACAAGTTCCTCACCGTCAACTACG
ACGTCACCACCGTGAGCACCCACCCACCCACACAAAGATTTCTTCCGGTGATCGCTGGTTCTGGGTGGAT
TCTGAGTTCTGACAAACGAGGCAAAGTGACAGGCGTTGGAGGGGAAGGCGCTGAACAAGGAGGCGCTGCA
GGCCGAGGTGGGGCTGCCGGTGGACCGGAAGGTGCCCCTGGTGGCGTTCATCGGCAAGCTGGAGGAGCAG
AAGGGCCCCGACGTGATGATCGCCGCCATCCCGGAGATCGTGAAGGAGGAGGACGTCCAGATCGTTCTCC
TGGTACGATCGACCGACATTGCTGACCCGTTCAGGAAAATCTCCTGATAGCTCGCCGTGGGGATGGGTGG
GTGACTGACTGATCGAATGCATTGCAGGGCACCGGGAAGAAGAAGTTTGAGCGGCTGCTCAAGAGCGTGG
AGGAGAAGTTCCCGACCAAGGTGTGGGCCGTGGTCAGGTTCAACGCGCCGCTGGCTCACCAGATGATGGC
CGGCGCCGACGTGCTGGCGGTCACCAGCCGCTTCGAGCCCTGCGGCCTCATCCAGCTCCAGGGAATGCGC
TACGGAACGGTAAACGCATCCTCCTTCAGTCCTTCTTGCCAGTTCCTCACCTCCTTTGCATATCCATGGC
CATGACCGAAGTTTCTTTCAAATTTTCAGCCGTGCGCCTGCGCGTCGACAGGCGGGCTCGTCGACACTAT
CGTGGAAGGCAAGACCGGGTTCCACATGGGCCGCCTCAGCGTTGACGTATGCTCATCGATCCTCTTGTAT
ACATTCATTCATCTTGTTCATCATGGCAGCTCAGACAGATCATGAAGTGGTGCACTTTTCTTGTTGGTGG
CCAGTGCAACGTGGTGGAGCCGGCCGACGTGAAGAAGGTGGTCACCACCCTGAAGCGCGCCGTCAAGGTC
GTCGGCACGCCGGCGTACCATGAGATGGTCAAGAACTGCATGATACAGGATCTCTCCTGGAAGGTAAGTC
GTCTCTGGTTCAGTATGCACTTCCTGGAACAACTAAGAGTGAAGGGCCGATGTATCCATTAATGGTGGCT
TGCGCATATGATGCAGGGGCCTGCCAAGAACTGGGAGGACGTGCTTCTGGAACTGGGGGTGGAGGGGAGC
GAGCCGGGCATCGTCGGCGAGGAGATCGCGCCGCTCGCCCTGGAGAACGTCGCCGCTCCCTGAAGAGAGA
AAGAA

Figure 9. A SNP transition from G to A was identified in the gene Granule Bound Starch Synthase I, of Jagger M_2 #14. This SNP results in an amino acid change from arginine to lysine. The primers used to amplify the region, WX7A3, are indicated in green.

Mutation Frequencies in *T. aestivum* and *T. monococcum*

Mutation frequencies were estimated by dividing the total base pairs screened by the number of mutants found. First, target amplicon lengths were trimmed by 50 bp (Dong et al. 2009) to compensate for the ends of the PCR products where mutants are undetectable. Overlap between amplicons was also subtracted from the amplicon lengths. The trimmed amplicon lengths were multiplied by the number of M_2s screened, and the products were summed to give the total number of base pairs screened. The total number of base pairs screened was divided by the number of mutants found. A total of 3,882,476 bp were screened for *T. monococcum.* Dividing this value by a total of three mutants gives a mutation frequency of ~1.3 Mb per mutation. A total of 657,860 base pairs were screened in *T. aestivum*, cultivar Jagger, and 30 mutants were found, giving a mutation frequency of 22 Kb per mutation.

Discussion

The results show a dramatic difference in the mutation frequencies between diploid and hexaploid wheat. *T. aestivum* had a mutation frequency of one mutant per 22 Kb, and *T. monococcum* show a mutation frequency of one per 1.3 Mb. The *T. aestivum* mutation frequency is similar to that of other *T. aestivum* TILLING populations (Table 9), giving validation to the TILLING methodology used. There are, however, no other *T. monococcum* TILLING populations with which to make a direct comparison to our observed *T. monococcum* mutation frequency. The most similar mutation frequency of one mutant per 1.0 Mb was observed in an EMS mutagenized barley population (Caldwell et al. 2004) (Table 9). To the authors' knowledge, barley is the only other diploid member of the Triticeae in which TILLING has been performed (Caldwell et al. 2004, Talame et al. 2008).

Table 9. EMS mutation frequencies of TILLING populations in the Triticeae. The genome, the mutation frequency (in base pairs per mutation), and the publication referenced are given for each species of Triticeae in which TILLING has been performed.

Species	Genome	BP/mutation	Reference
Einkorn wheat	AA	1,300,000	Author's data
Barley	HH	1,000,000	Caldwell et al., 2004
Durum wheat	AABB	40,000	Slade et al., 2005
Durum wheat	AABB	*51,000	Uauy et al., 2009
Bread wheat	AABBDD	24000	Slade et al., 2005
Bread wheat	AABBDD	**23,300-37,500	Dong et al., 2009
Bread wheat	AABBDD	*38,000	Uauy et al., 2009
Bread wheat	AABBDD	22,000	Author's data

*normalized to 50% G/C content
**depending upon cultivar and EMS treatment

The additional genomes of polyploid species are known to mask the effects of deleterious alleles (Soltis and Soltis 2000). Similarly, the tremendous tolerance of hexaploid wheat to a high mutation frequency has been attributed to the buffering capacity of polyploidy against deleterious mutations (Hein et al. 2009). Ploidy most likely plays a large role in the differences

observed in the mutation frequencies of *T. aestivum* and *T. monococcum*. The differences in lethality between diploid and hexaploid wheat during mutagenesis may be noted in support. For the diploid wheat, 0.24% EMS treatment was sufficient to cause the targeted 50% lethality in M_1 plants. A treatment of 0.5% EMS resulted in almost complete lethality, with only 3% survival. In hexaploid Jagger, 0.7% was used to achieve the targeted 50% lethality. The differences in tolerance to EMS support the differences observed in mutation frequencies for diploid and hexaploid wheat.

Although there is evidence to support why hexaploid wheat would have a higher mutation frequency than diploid species, there is still the question of why *T. monococcum* would have a mutation frequency so much lower than other diploid species. Caldwell et al. (2004) propose that the differences in mutation frequency between the diploids, barley and *Arabidopsis,* relate to the evolutionary histories: barley is considered a true diploid, whereas *Arabidopsis* contains numerous segmental duplications, providing functional redundancy.

Alternatively, the differences among diploids and polyploids may not be entirely genetic, but experimental phenomena. The mutation frequency observed in barley (1mutant/1 Mb screened) (Caldwell et al., 2004) and *T. monococcum* may not be representative of the true capacity for mutation. Talame et al. (2008) observed a much higher mutation frequency in their barley TILLING population, TILLMore, with one mutant per 374 kb screened. Talame et al. (2008) suggest the difference between the two barley projects may lie in experimental variation, different genetic backgrounds, or different mutagens used. Of interest to the author is the mutagen used for TILLMore, sodium azide. With sodium azide, a higher mutation frequency than that of an EMS population (Caldwell et al. 2004) was observed with only 20% mortality in the M_1, explained by the mutagen's low cell toxicity. This data is encouraging, but the mutagen's efficacy is variable across species, with low success in *Arabidopsis* (Talame et al., 2008). TILLING experiments using sodium azide in hexaploid wheat demonstrated a rate of 1 mutant per 127 kb screened–a low value by hexaploid wheat standards; furthermore, the same group achieved rates of 1 mutant per 23.3-37.5 Kb using EMS. Although the results of sodium azide mutagenesis are mixed, investigating its application in *T. monococcum* could be immensely rewarding, as seen in barley.

The Jagger TILLING population has great utility for mutation discovery, with a mutation frequency comparable and slightly higher than those of previously reported hexaploid wheat

varieties (Table 9). Further research is needed to make TILLING in *T. monococcum* a more efficient system. Despite scanning nearly 4 Mb, only three mutants were found. If the mutation frequency found is representative of the actual value, a much larger population will be needed. Parry et al. (2009) considered mutation frequencies in crops and calculated the number of lines that would be needed (95% confidence level) to find a nonsense mutation in a 1,000-bp coding target, considering that nonsense mutations account for 5% of EMS mutants. Based on the criteria of Parry et al. (2009) and the mutation frequency observed (one mutant per 1.3 Mb), a total of 76,700 M_2s would be needed for *T. monococcum*.

In principle, *T. monococcum* offers a simplified model of wheat genetics. Once a sufficient population is established, connecting phenotypes with mutants will be straightforward, offering great potential for the functional validation of genes. Although *T. aestivum* has the benefit of small TILLING populations, there will always be challenges in working in a polyploid system, potentially involving scanning the homoeologous genes of three genomes and the lag time of breeding full mutants. In future research, it will be important to explore new routes and further optimize the mutation frequency in *T. monococcum* to reduce the required population size if it is to be a viable system. At present, however, a wealth of resources would be required to develop a sufficient TILLING population in *T. monococcum*. Unless the mutation frequency can be significantly increased, the benefits of working in *T. monococcum* may not offset the costs.

References

Caldwell, D. G., McCallum, N., Shaw, P., Muehlbauer, G. J., Marshall, D. F., & Waugh, R. (2004). A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L.). *The Plant Journal : For Cell and Molecular Biology, 40*(1), 143-150.

Chen, F., & Dixon, R. A. (2007). Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology, 25*(7), 759-761.

Colbert, T., Till, B. J., Tompa, R., Reynolds, S., Steine, M. N., Yeung, A. T., et al. (2001). High-throughput screening for induced point mutations. *Plant Physiology, 126*(2), 480-484.

Cuppen, E., Gort, E., Hazendonk, E., Mudde, J., van de Belt, J., Nijman, I. J., et al. (2007). Efficient target-selected mutagenesis in *Caenorhabditis elegans*: Toward a knockout for every gene. *Genome Research, 17*(5), 649-658.

Dong, C., Dalton-Morgan, J., Vincent, K., & Sharp, P. (2009). A modified TILLING method for wheat breeding. *The Plant Genome, 2*(1), 39-47.

Draper, B. W., McCallum, C. M., Stout, J. L., Slade, A. J., & Moens, C. B. (2004). A high-throughput method for identifying N-ethyl-N-nitrosourea (ENU)-induced point mutations in zebrafish. *Methods in Cell Biology, 77*, 91-112.

Dvorak, J., Terlizzi, P., Zhang, H. B., & Resta, P. (1993). The evolution of polyploid wheats: Identification of the A genome donor species. *Genome, 36*(1), 21-31.

Gilchrist, E. J., O'Neil, N. J., Rose, A. M., Zetka, M. C., & Haughn, G. W. (2006). TILLING is an effective reverse genetics technique for *Caenorhabditis elegans*. *BMC Genomics, 7*, 262.

Giroux, M. J., & Morris, C. F. (1998). Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. *Proceedings of the National Academy of Sciences of the United States of America, 95*(11), 6262-6266.

Hein, I., Kumlehn, J., & Waugh, R. (2009). Functional validation in the triticeae. *Genetics and genomics of the Triticeae* (pp. 359)

Henikoff, S., Till, B. J., & Comai, L. (2004). TILLING. traditional mutagenesis meets functional genomics. *Plant Physiology, 135*(2), 630-636.

Huang, S., Sirikhachornkit, A., Su, X., Faris, J., Gill, B., Haselkorn, R., et al. (2002). Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum*/*Aegilops* complex and the evolutionary history of polyploid wheat. *Proceedings of the National Academy of Sciences of the United States of America, 99*(12), 8133-8138.

Li, X., Weng, J. K., & Chapple, C. (2008). Improvement of biomass through lignin modification. *The Plant Journal : For Cell and Molecular Biology, 54*(4), 569-581.

McCallum, C. M., Comai, L., Greene, E. A., & Henikoff, S. (2000a). Targeted screening for induced mutations. *Nature Biotechnology, 18*(4), 455-457.

McCallum, C. M., Comai, L., Greene, E. A., & Henikoff, S. (2000b). Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiology, 123*(2), 439-442.

Parry, M. A., Madgwick, P. J., Bayon, C., Tearall, K., Hernandez-Lopez, A., Baudo, M., et al. (2009). Mutation discovery for crop improvement. *Journal of Experimental Botany, 60*(10), 2817-2825.

Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leple, J. C., et al. (2002). Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnology, 20*(6), 607-612.

Raghavan, C., Elizabeth, M., Naredo, B., Wang, H., Atienza, G., Liu, B., et al. (2007). Rapid method for detecting SNPs on agarose gels and its application in candidate gene mapping. *Molecular Breeding, 19*(2), 87-101.

Slade, A. J., Fuerstenberg, S. I., Loeffler, D., Steine, M. N., & Facciotti, D. (2005). A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology, 23*(1), 75-81.

Slade, A. J., & Knauf, V. C. (2005). TILLING moves beyond functional genomics into crop improvement. *Transgenic Research, 14*(2), 109-115.

Smits, B. M., Mudde, J. B., van de Belt, J., Verheul, M., Olivier, J., Homberg, J., et al. (2006). Generation of gene knockouts and mutant models in the laboratory rat by ENU-driven target-selected mutagenesis. *Pharmacogenetics and Genomics, 16*(3), 159-169.

Soltis, P. S., & Soltis, D. E. (2000). The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America, 97*(13), 7051-7057.

Sticklen, M. B. (2008). Plant genetic engineering for biofuel production: Towards affordable cellulosic ethanol. *Nature Reviews.Genetics, 9*(6), 433-443.

Talame, V., Bovina, R., Sanguineti, M. C., Tuberosa, R., Lundqvist, U., & Salvi, S. (2008). TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotechnology Journal, 6*(5), 477-485.

Till, B. J., Reynolds, S. H., Weil, C., Springer, N., Burtner, C., Young, K., et al. (2004). Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biology, 4*, 12.

Till, B. J., Cooper, J., Tai, T. H., Colowit, P., Greene, E. A., Henikoff, S., et al. (2007). Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biology, 7*, 19.

Uauy, C., Paraiso, F., Colasuonno, P., Tran, R. K., Tsai, H., Berardi, S., et al. (2009). A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biology, 9*, 115.

Winkler, S., Schwabedissen, A., Backasch, D., Bokel, C., Seidel, C., Bonisch, S., et al. (2005). Target-selected mutant screen by TILLING in drosophila. *Genome Research, 15*(5), 718-723.

Wu, J. L., Wu, C., Lei, C., Baraoidan, M., Bordeos, A., Madamba, M. R., et al. (2005). Chemical- and irradiation-induced mutants of Indica rice IR64 for forward and reverse genetics. *Plant Molecular Biology, 59*(1), 85-97.

Xin, Z., Wang, M. L., Barkley, N. A., Burow, G., Franks, C., Pederson, G., et al. (2008). Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biology, 8*, 103.

Yan, L., Bhave, M., Fairclough, R., Konik, C., Rahman, S., & Appels, R. (2000). The genes encoding granule-bound starch synthases at the waxy loci of the A, B, and D progenitors of common wheat. *Genome 43*, 264-272.