Determination of the mutation frequency in a TILLING population of the highly Fusarium head blight resistant wheat line CM82036

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"The highest reward for a person's toil is not what they get for it, but what they become by it."

John Ruskin

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List of Terms and Abbreviations

APS:	Ammonium persulfate
bp:	base pair
CM-82036:	CM-82036-1TP-1OY-OST-1OY-OM-OFC
CTAB:	Hexadecyltrimethylammonium bromide
ddH2O:	double-distilled water
DHPLC:	Denaturing High Pressure Liquid Chromatography
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxynucleotide Triphosphates
DMSO:	Dimethyl sulfoxide
EDTA:	Ethylenediaminetetraacetic acid
EMS:	Ethyl methane sulfonate
ENU:	N-ethyl- N-nitrosourea
et al.:	and others
FHB:	Fusarium head blight
MMS:	Methylmethane sulphonate
MNU:	N-nitroso-N-methylurea
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
PCR:	Polymerase chain reaction
QTL:	Quantitative trait locus/loci
RNA:	Ribonucleic acid
SNP:	Single nucleotide polymorphism
TE buffer:	Mixed Tris and EDTA
TEMED:	Tetramethylethylenediamine
TILLING:	Targeting Induced Local Lesions in Genomes
Tris:	$(HOCH_2)_3CNH_2$
UV:	ultraviolet light

Abstract

Cereals are the most important sources of plant food for humans and livestock. As a result, the understanding of the genetics of resistance/susceptibility of wheat to pathogens is of great value for plant breeding purposes.

Fusarium head blight (FHB) is one of the most devastating fungal diseases of wheat worldwide. For functional validation of candidate genes for FHB resistance a TILLING (Targeting Induced Local Lesions in Genomes) population of the highly FHB resistant spring wheat line CM-82036 was generated. The current study intends to determine the mutation frequency in the TILLING population of CM-82036 by screening the *waxy* gene *Wx-A1* for mutations.

About 100,000 seeds of CM-82036 were treated with the chemical mutagen ethylmethane sulfonate (EMS) using two concentrations (0.053 and 0.058 mol/l) and from approximately 12,000 surviving M1 plants one head per plant was harvested. To create the M2 population 4,880 M2 seeds (one seed per head), preferably those exposed to the higher EMS concentration, were sown in the greenhouse. Subsequently, the DNA of 760 M2 plants, 380 M2 plants per EMS concentration, was extracted and analyzed in pools of four. Two gene-specific primer pairs, *Wx7A2* and *Wx7A3*, were used to identify single base mutations in a 1,205 bp fragment of the *waxy* gene *Wx-A1* by Endo-I-mediated heteroduplex cleavage. Mutations identified in the four-way pools were confirmed in individual samples.

In total 20 mutations were detected by screening a total sequence of 915,800 bp. The mutation frequencies for the two different EMS concentrations were calculated: for samples treated by 0.053M EMS concentration it was one mutation per 57.23 kb, while for 0.058M EMS concentration one mutation per 38.15 kb was identified. These results are in a similar range as detected by other studies in hexaploid wheat varying from one mutation per 24 kb to one per 84 kb. The results indicate that this mutant population represents a useful resource for the wheat research community, especially for validating candidate genes for FHB resistance.

Zusammenfassung

Getreide sind die wichtigsten Quellen der pflanzlichen Nahrung für Menschen und Vieh. Daher ist das Verständnis der Genetik der Resistenz /Anfälligkeit von Weizen gegenüber Krankheitserreger von großem Wert für die Pflanzenzüchtung. Die Ährenfusariose ist eine der verheerensten Weizenkrankheit weltweit. Zur funktionellen Validierung von Kandidatengenen für Ährenfusarioseresistenz wurde eine Mutantenpopulation von der fusariumresistenten Weizenlinie CM-82036 hergestellt. Ziel der aktuellen Arbeit ist die Bestimmung der Mutationsfrequenz in der Mutantenpopulation mit Primern spezifisch für das *waxy* Gen *Wx-A1*.

Etwa 100,000 Körner wurden mit Ethylmethansulfonat (EMS) in zwei Konzentrationen (0.053 und 0.058 mol/l) behandelt und von 12,000 überlebenden M1 Pflanzen konnte eine Ähre pro Pflanze geerntet werden. Für die M2 wurden 4,880 M2 Körner (ein Korn pro Ähre) im Glashaus angebaut, M2 der höheren EMS Konzentration wurden bevorzugt. Anschließend wurde die DNA von 760 M2 Pflanzen, 380 pro EMS-Konzentration, extrahiert und in vierfach Pools mit zwei Primerpaaren, *Wx7A2* und *Wx7A3* analysiert um Mutationen in einem 1,205 bp Fragment des *waxy* Gen *Wx-A1* zu identifizieren.

Mutationen, die in den vierfach Pools gefunden wurden, wurden in Einzelproben bestätigt. Insgesamt konnten 20 Mutationen detektiert werden bei der Analyse von 915,800 bp. Die Mutationsfrequenzen wurden für beide EMS-Konzentrationen berechnet: für Stichproben, die mit 0.053M EMS Konzentration behandelt wurden, war es eine Mutation pro 57.23 kb, während für die 0.058M EMS Konzentration eine Mutation pro 38.15 kb identifiziert wurde. Die Ergebnisse liegen in einem ähnlichen Bereich wie in anderen Studien in hexaploidem Weizen, die von einer Mutation pro 24 kb bis zu einer Mutation pro 84 kb variieren. Die Ergbnisse zeigen, dass diese Mutantenpopulation eine brauchbare Ressource für die Weizenforschung darstellt, besonders für die Validierung von Kandidatenengenen für Fusariumresistenz.

1 Introduction

1.1 Aims and Objectives

Wheat (*Triticum aestivum*) is the most important cereal crop for the majority of world's populations. The crop not only enjoys an extraordinary weight in the diet of humankind, since wheat products are almost the greatest widely used articles of the individual diet, but also it is the most broadly grown cereal cultivated over a wide range of climatic conditions. Its exceptional ecological adaptability to different climatic conditions and strong competitive ability against other species provides other evidences.

As a result, the understanding of the genetics of resistance/susceptibility of wheat to pathogens is of great value for plant breeding purposes. In response to this need, the current study intends to investigate the spring wheat line CM-82036. CM-82036 is highly resistant to fungal pathogens of the genus *Fusarium* and major resistance quantitative trait loci (QTL) have been mapped. To validate candidate genes for Fusarium resistance QTL a TILLING (Targeting Induced Local Lesions in Genomes) population of the highly resistant CM-82036 was generated. In specific, the objectives of this study are:

- Give a brief history about hexaploid wheat
- Provide a literature review about TILLING in wheat
- Determination of the mutation frequency in a TILLING population of CM-82036 mutagenized with ethylmethane sulfonate (EMS)

1.2 Literature

1.2.1 General Information about Wheat

Cereals are unquestionably the most important sources of plant food for humans and livestock. It should be mention that, the development of all the major cereals occurred long before recorded history, so, all the oldest civilizations were familiar with several kinds of barley, wheat and other grains. As it is recorded, in ancient Rome, some festivals were held at seeding time and harvest, in honor of the goddess of Ceres. People brought offerings of wheat and barley to these festivals, the "cerealia munera" or gifts of Ceres, from which the name "cereals" was derived (Sargent, 1899).

Although archeological evidence indicates that wheat had already been cultivated by earlier than 4,000 B.C, and has be grown under a wide range of climates and soils, its native home is not definitely known. There are some indications that highlands in Syria and Palestine

might be the place of its origin, however, the Central Asian plateau and the Tigris and Euphrates valleys might be included. It was also cultured in the Fertile Crescent, a geographical region extending from, Jordan, Lebanon, and western Syria into southeastern Turkey and along the Tigris and Euphrates rivers into Iraq and Iran (Zohary and Hopf, 2000).

It is worth mentioning that, wheat, as an edible grain has always been one of the most important nutrient corns containing a high percentage of carbohydrates as well as a considerable amount of protein and some fats and vitamins (Küster et al., 1999). Table 1 gives an overview of the quantitative distribution of the constituents in a wheat grain (Belderok et al., 2000).

Table 1: Chemical components of a whole-wheat grain with its various parts.

Out of 100%	Whole grain	Endosperm	Bran	Embryo
Proteins	16	13	16	22
Fats	2	1.5	5	7
Carbohydrates	68	82	16	40
Roughage	11	1.5	53	25
Minerals	1.8	0.5	7.2	4.5
Additional components	1.2	1.5	2.8	1.5
Total	100	100	100	100

On the other hand, wheat not only provides an essential food of many countries, but also has been known as a high-quality and main export product. Therefore, it has been grown both in the Western industrialized countries, and in many so-called developing countries in large scale; especially in China, India, United States of America, Russian Federation and France, which are among the largest wheat producers (FAO, the statistics division 2010).

Considering its botanical taxonomy, wheat (*Triticum L.*), together with other cereals, belongs to the class of monocotyledons (Monocotyledoneae) and within these, of the Gramineae (Poaceae) family. While numerous inbred lines formed the variations of wheat, all wheat varieties are almost entirely self-fertilized (Zohary and Hopf, 2000). Wheat species are also allopolyploid crops; despite the existence of two or more sets of chromosomes from different parents, they behave genetically as diploids and show normal fertility (Becker, 2011).

Although there are several types of wheat classifications, in 1918, a chromosome number set (genomes) was selected for each commonly recognized type. This was a turning point in *Triticum* classification. The taxonomy separated wheat into three groups. Diploids included 14 (n=7), Tetraploids contained 28 (n=14), and the Hexaploids which were consisted of 42

(n=21) chromosomes. In this fashion, (*Triticum aestivum L*) which is a hexaploid, consists of three diploid AA, BB and DD, originating from three different wild species (Becker, 2011).

According to Zohary, and Hopf (2000), and Aufhammer (1998), apart from wild varieties of wheat, diploid, tetraploid, and hexaploid, hexaploid varieties have been the most useable cultivars. In sum, different species of the genus (*Triticum L*.) were created by culturing. Among virtually all modern wheat cultivars, however, the hexaploid wheat *Triticum aestivum*, known as common bread wheat valued for bread making, plays the dominant role.

1.2.2 The Advent of TILLING

Crop improvement referring to the genetic alteration of plants to satisfy human needs has a long history. Through a long period of trial and error, some elements of different species have been transformed into other ones. However, the selection process mostly happened unconscious in many cases.

For example, in wild wheats, the grains scatter by disarticulation, separation of the seed from the seed head. When these grains were harvested by cutting the heads with a sickle, an unconscious selection occurred for "nonshattering" types that would be continually replanted. More recently, this progress has been accelerated as the green revolution has brought about great increases in crop yields (Khush, 2001). With the advent of genomics during the last 25 years, opportunities for crop improvement have continued to grow aiming to meet future challenges of food production and land sustainability (Slade et al., 2005a).

One of the most important advances in the history of genetics was the discovery of planed induced mutations (Muller, 1930; Stadler, 1932). The method through high ionizing radiation frequency and applying certain chemicals to cause induced mutations has opened a window to perform genetic studies that were not feasible when only spontaneous mutations were available.

As a result, much of our understanding of genetics of higher organisms, including gene functions, has been based upon studies utilizing induced mutations. Alkylating agents, that yield predominantly point mutations, have been especially valuable, since the resulting altered and truncated protein products help to precisely map gene and protein function (McCallum et al., 2000b).

One of the most important tools in biological research is mutational analysis. Our understanding of the basic mechanisms of disease, development, cell biology and metabolism has been transformed by systematic application of this method (Stemple, 2004). As a tool for breeders, mutagenesis has been used in breeding programs in forward genetic screening and the selection of individual mutant genotypes with improved traits.

Nevertheless, since 70 years ago, more than 2,500 varieties have been developed by means of mutagenesis, including large numbers of rice, wheat and maize lines (http://www-infocris.iaea.org/MVD/).

Because of the high mutational density and the great utility of point mutations, traditional chemical mutagenesis methods have continued to be popular in phenotypic screens despite the development of other mutagenic tools such as transposon mobilization (Bingham et al., 1981).

Traditionally, forward genetics, driven by the identification of mutant phenotypes, has been the most widely used approach. Most of our understanding about the development in genetic of animal and plant derives from systematic screens for mutations that produce visible phenotypes resulting in defective developmental processes. Consequently, the forward-genetic approach has been so powerful that mouse geneticists while having the tools to generate individual mutations at will, at times find large-scale chemical mutagenesis more informative (Stemple, 2004).

Although forward-genetic method has been used successfully, it also has several disadvantages. For example, it is difficult to identify novel genotypes when there are small numbers in a large population, whereas the presence of gene duplication and polyploidy results in genetic redundancy in many plant species. Consequently, many mutations have no phenotypic effects on the plant. In conclusion, the mentioned problems have led to the development of reverse genetic strategies, such as TILLING (McCallum et al., 2000b).

1.2.3 Reverse Genetics

In the post-genomic sequencing era, an expanding portfolio of genomic technologies has been applied to the study of gene function (Comai et al., 2006). Reverse genetics generally refer to approaches discovering the function of a gene by analyzing the phenotypic effects of specific gene sequences obtained by DNA sequencing. This investigative process proceeds in the opposite direction of so-called forward genetic screens of classical genetics. With the recent expansion of sequence databanks, (locus-to-phenotype) reverse genetic strategies have become an increasingly popular alternative to phenotypic screens for functional analysis.

Simply, while forward genetics seeks to find the genetic basis of a phenotype or trait, reverse genetics seeks to find what phenotypes arise as a result of particular genes (McCallum et al., 2000b). Therefore, the method by using several techniques developed to screen the point mutations and small insertions/deletions (indels) in speciefic genes, and can even detect point mutations to relate sequences information to the biological functions of the genes in

individual plants (Parry et al., 2009). In short, this method attempts to connect a given genetic sequence with specific effects on the organism.

In plants generally, the two most common methods for producing reduction-of-function mutations have been Antisense RNA suppression and Insertional mutagenesis. Since, Antisense RNA suppression requires considerable effort for any given target genes before knowing whether it works or not, insertional mutagenesis occurs at a low frequency per genome (Waterhouse et al., 1998).

However, their efficacy is not yet clear; because their usefulness as general reverse genetics methods is limited to very few plant species. On the other hand, these techniques rely either on T-DNA vectors such as in Agro bacterium for transmission or on an endogenous tagging system producing a very limited range of allele types. Therefore, as the amount of sequence data grows for organisms, it is important to develop genome-scale reverse genetic strategies that are automated, broadly applicable, and capable of creating the wide range of mutant alleles needed for functional analysis (McCallum et al., 2000b).

Henikoff and his colleagues assembled a process that has been a useful solution to the problem of identifying mutations in genes known only by their sequence. They introduced a new reverse genetic strategy combining the high density of point mutations provided by traditional chemical mutagenesis with rapid mutational screening to discover induced lesions. The method was named TILLING (Targeting Induced Local Lesions IN Genomes), partly to reflect their first application of the method to the identification of mutations in the plant *Arabidopsis thaliana* (Stemple, 2004).

Actually, the motivation for TILLING arose when a graduate student frustrated with the limitations of reverse genetic methods available for Arabidopsis in the late 1990s. Claire McCallum went on to demonstrate the feasibility of TILLING by discovering mutations in two chromomethylase genes that were the subject of her research (McCallum et al., 2000a). Currently, TILLING is known as a ‘reverse’ counterpart to what is the most common tool for traditional genetic analysis: chemical mutagenesis and screening (Comai et al., 2006).

1.2.4 Definition, Applications and Advantages of TILLING

Although most of the genes of an organism are known from sequence, a lot of phenotypes are obscure. In response, reverse genetics has become an important goal for many biologists. However, reverse-genetic methodologies are not similarly applicable to all organisms but TILLING promises to be generally applicable (Henikoff et al., 2004). Novel DNA sequence information allows the development of additional molecular markers for breeding as well as providing targets for transgenic alteration of gene expression and introduction of new traits. Therefore, TILLING method was developed to take advantage of

this new DNA sequence information and to investigate the functions of speciefic genes (Slade et al., 2005a).

Generally, TILLING is a process combining an efficient ,rapid and low-cost technique of mutagenesis with a chemical mutagen such as ethylmethanesulfonate (EMS) with a sensitive DNA screening-technique to identify single base mutations (also called point mutations) in a target gene for investigating the functions of speciefic genes. TILLING, as well, performs as a nontransgenic tool to improve domesticated crops by introducing and identifying novel genetic variation in genes that affect key traits. (Till et al., 2007; Haughn et al., 2005; McCallum et al., 2000b and Slade et al., 2005b).

Since the first applications of this strategy in *Arabidopsis thaliana* (McCallum et al., 2000a) and *Drosophila melanogaster* (Bentley et al., 2000), the method has emerged as a robust approach (Gilchrist and Haughn, 2005 and Stemple, 2004). In fact, the success with Arabidopsis has inspired researchers working with other model organisms to use the method, which has the potential for applying to many other genetic systems, especially organism species for which there are no or limited, reverse genetic methods (Stemple, 2004).

The general applicability of TILLING makes it appropriate for genetic modification of crops, and there may be agricultural interest in producing phenotypic variants without introducing foreign DNA of any type into a plant's genome (McCallum et al., 2000b). As a result, TILLING is attractive not only for functional genomics but also for agricultural applications and also be applied to species for which genomic resources are limited (Comai et al., 2006 and Henikoff et al., 2004).

1.2.5 TILLING in Wheat

Wheat (*Triticum spp*.) is an important source of food worldwide and the focus of considerable efforts to identify new combinations of genetic diversity for crop improvement. While the ability to understand and modify gene function for crop improvement has been hindered by the lack of available genomic resources, TILLING has shown a powerful reverse genetics approach that combines chemical mutagenesis with a high-throughput screen for mutations. As it has noted, TILLING was originally developed for using in the fully sequenced diploid model organism, *Arabidopsis thaliana*, and thousands of mutations in hundreds of Arabidopsis genes have since been identified by this method (Till et al., 2003).

Could TILLING be applied successfully to species with more complex genomes and much less sequence information? It was the question emerged next. However, wheat as an economically important crop with a hexaploid genome 140 times more than the size of Arabidopsis has been such a challenging candidate. In addition, desirable genetic changes in

wheat are difficult to identify based on phenotypic screens because these changes are often masked by redundant copies of genes within its hexaploid genome (Slade et al., 2005a).

In this fashion, Slade and her colleagues (2005a) created a TILLING library for both bread and durum wheat. They targeted a well-characterized gene in wheat of economic importance encoding granule bound starch synthase I, or the waxy locus. These experiments demonstrated that TILLING can generate and identify novel alleles that some of them have a phenotypic effect. In addition, these alleles represent a rich resource of genetic diversity at the wheat waxy loci for potential modulation of starch quality and characteristics.

In short, According to Uauy and his colleagues (2009) wheat is specially well-suited for TILLING due to the high mutation densities tolerated by polyploids allowing for very efficient screens. However, few TILLING populations are currently available, current TILLING screening protocols require high-throughput genotyping platforms, limiting their use.

However, base on Fitzgerald and his colleagues (2010) recently modified TILLING protocols have been developed specifically for mutation detection in wheat. While extremely powerful in detecting single nucleotide changes and small deletions, these methods are not suitable for detecting whole gene deletions. Therefore, high-throughput methods for screening of candidate homoeologous gene deletions are needed for application to wheat populations generated by the use of certain mutagenic agents (e.g. heavy ion irradiation) that frequently generate whole-gene deletions.

Currently, apart from wheat, the development of mutation detection techniques such as TILLING has renewed interest in the generation of diversity through mutagenesis. Therefore, mutant populations have recently been created for many crops, including rice (Suzuki et al., 2008), maize (Weil et al., 2005), sorghum (Xin et al., 2008), barley (Caldwell et al., 2004 and Talame et al., 2008), tomato (Menda et al., 2004) and soybean (Cooper et al., 2008), mostly by treating seeds or pollen with chemical mutagens (Weil, 2009).

1.3 The TILLING Technique

According to Figure 1, the steps of this procedure are:

1) EMS mutagenesis to produce M1 plants
2) DNA preparation and pooling of individuals in the M2 generation
3) PCR amplification of a region of interest
4) Denaturation and annealing to allow formation of heteroduplexes
5) Identification and visualization of pool mutant samples by different gel analyzer systems such as DHPLC (Denaturing High Pressure Liquid Chromatography) or LI-COR

6) Identification of the mutant individual and sequencing of the mutant PCR product (McCallum et al., 2000b)

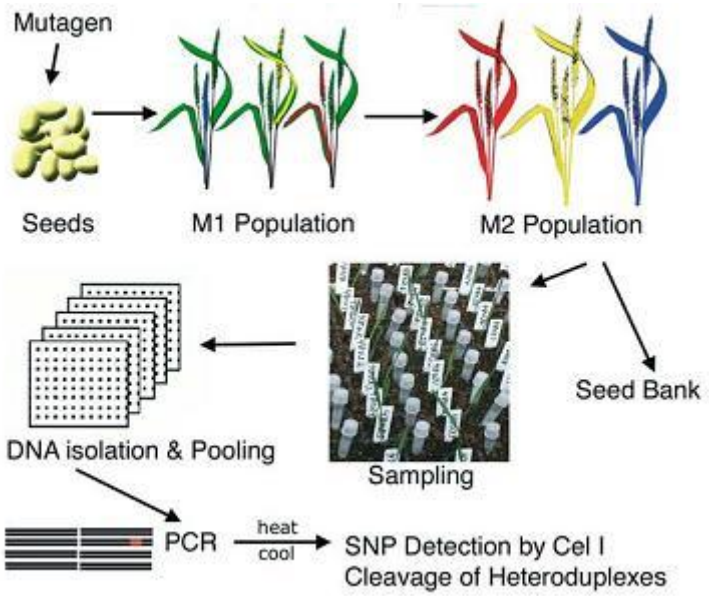


Figure 1: The TILLING method.
Seeds treated with a chemical mutagen to induce genetic variation are then planted. The resulting M1 population of plants is chimeric for mutations. Therefore, one seed from each M1 is planted to create the M2 population. M2 DNA is extracted from leaf tissue and M3 seeds from each plant are stored in a seed bank. DNA samples are pooled to increase throughput and PCR amplified with dye-labeled PCR primers specific to a target gene of interest. PCR products are denatured and allowed to reanneal to form heteroduplexes. Heteroduplex DNA is then cleaved by Cel I and analyzed (Slade et al., 2005a).

1.3.1 EMS Mutagenesis

TILLING applies advances in molecular biology and genomics to identify the genetic variation at the level of a single base pair. Beginning the process, novel single base pair changes are induced in a population of plants by treating seeds (or pollen) with a chemical mutagen, causing a new generation where mutations will be stably inherited (Figure 1) (Slade et al., 2005a). Mutations can be induced by using physical or chemical mutagens. Physical mutagenesis, by irradiation with non-ionizing (e.g. UV) or ionizing (e.g. X and gamma rays, alpha and beta rays, fast and slow neutrons) radiation, often results in the large-scale deletion of DNA and visual effects on chromosome structure.

In contrast, chemical mutagens used most widely in plants including ethylmethane sulphonate (EMS), methylmethane sulphonate (MMS), hydrogen fluoride, sodium azide, N-nitroso-N-methylurea (MNU) and hydroxylamine, predominantly change single nucleotide pairs. The extent of mutation is dependent on the tissue, the kind of mutagen used, and the degree of exposure (dosage and time). Mutations of single nucleotide pairs are generally of most interest to breeders (large-scale changes to chromosome structures usually have

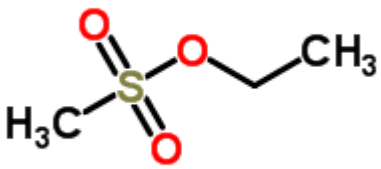
severely negative effects). Thus, the useful chemical mutagens are those that generate point mutations (Rakszegi et al., 2010).

Alkylating agents, such as EMS, and N-ethyl- N-nitrosourea (ENU), directly modify bases of the DNA and, on replication, these bases pair inappropriately change. The ethyl group of EMS that its chemical formula is shown in Figure 2A, reacts with guanine in DNA forming the abnormal base O-6-ethylguanine. During DNA replication, DNA polymerases catalyzing the process frequently place thymine, instead of cytosine (Figure 2B).

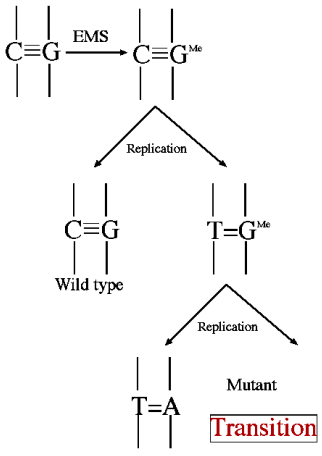
Following subsequent rounds of replication, the original G:C base pair can become the A:T pair (Greene, 2003). By contrast, whereas ENU can also generate O6-ethylguanine modifications, several studies indicate that numerous A:T →T:A transversions occur, resulting of O4-ethylthymidine modifications (Jansen et al.,1995 and Skopek et al., 1992). Therefore, different alkylating mutagens can produce different constellations of mutations that lead to different projected codon changes (Stemple, 2004).

Through most of TILLING experiments, especially those conducted on *Triticum aestivum*, EMS has been applied as a mutagen. In addition, EMS has been used as the mutagen in experiments performed on *Brassica napus*, *Brassica oleracea*, *Glycine max*, *Hurdium vulgare*, *Lotus japonicus*, *Medicago truncatula*, *Oryza sativa*, *Sorghum bicolor*, *Arabidopsis thaliana*, *Triticum durum* and *zea mays* (Gilchrist and Haughn, 2005; Martín et al., 2009).

EMS-induced mutations are randomly distributed in the genome and a high degree of mutational saturation can be achieved without excessive DNA damage (Gilchrist and Haughn, 2005 and Kurowska et al., 2011). Another alkylating agent, (MNU, MNH), induced only G/C to A/T transitions in several TILLING experiments in *G. max* and *O. sativa* (Cooper et al., 2008 and Suzuki et al., 2008) respectively.



A)



B)

Figure 2: A) The 2D chemical formula of EMS **B)** Screening for models of SNPs using EMS mutagenesis

1.3.2 DNA Preparation and Pooling of Individuals

Although the absolute number of gametes or individuals carrying mutations depends on several factors, such as: the size of the gene, and the frequency of induced changes, it will be in the order of thousands (Stemple, 2004).

Throughout the basic TILLING method seeds are mutagenized by EMS treatment; so, the resulting M1 plants are self-fertilized, and the M2 generation of individuals is used for mutational screening while their seeds are inventoried (Colbert et al., 2001 and Stemple, 2004). After DNA extraction, seeds are stored from all members of the population to create a resource that can be used repeatedly over time. Establishing a good population and preparing DNA samples from what typically constitutes thousands of plants for a TILLING 'library' could easily take the better part of two years for crop plants (Slade et al., 2005a).

Following DNA samples preparation, they are pooled and arrayed on microtiter plates subjected to gene-specific PCR (Colbert et al., 2001 and Stemple, 2004 and McCallum et al., 2000b). Such pooling not only increases screening efficiency but, since only one plant of the pool is likely to carry a mutation in the target sequence, but also ensures that any pool with a mutated target sequence will also have the wild type target sequence. Therefore, amplification of the target sequence from a pool where at least one plant has a nucleotide polymorphism produces a mixture of both mutant and wild type PCR products (Haughn et al., 2005).

1.3.3 PCR Amplification of a Region of Interest

In PCR process, forward and reverse primers are designed to amplify 1,500 bp or less of genomic DNA from a locus of interest (target sequence). The primers are synthesized such that the forward and reverse primers are tagged with different fluorescent markers. The PCR template consists of genomic DNA isolated from individual plant lines derived from a population carrying point mutations throughout the genome. Typically, the genomic DNAs from 2-8 plants are pooled prior to PCR amplification (Haughn et al., 2005).

1.3.4 Denaturation and Annealing to Allow Formation of Heteroduplexes

The PCR products are melted and re-annealed and then digested in the PCR mix using CEL1 endonuclease.

1.3.4.1 Explanation about CEL1/Endo1 Endonuclease

Oleykowski and his colleagues (1998) described a member of the S1 nuclease family, CEL1, a plant-specific extracellular glycoprotein which is identified from celery (Stemple, 2004).

Heterduplexes are detected by treatment of the amplified DNA with CEL1 endonuclease or any one of a number of single strand endonucleases - such as Endo1- (Till et al., 2004).

TILLING uses heteroduplex analysis to identify individuals having single DNA base pair (bp) differences in a specific target sequence. A second round of TILLING is then used to determine which of the plant lines in the pool carries the polymorphism (DNA from individuals in the pool is mixed with wild type DNA to ensure heteroduplex formation when the mutation is present). TILLING identifies the position of the nucleotide change in the amplified fragment within a few base pairs. Typically, DNA sequencing of the amplified fragment from the individual is used to verify the nature and exact position of any mutation detected. The overview of the procedure is shown through Figure **3** (Haughn et al., 2005).

CEL1 has been shown to be suitable for genotyping applications because of its preferentially mismatched cleaves of all types (Oleykowski et al., 1998) used to detect heterozygous polymorphisms in DNA pools (Kulinski et al., 2000). Following PCR amplification of genomic DNA in 96-well plates, a solution contained CEL1 was added and incubated (Colbert et al., 2001 and Henikoff et al., 2004). Then Henikoff group used CEL1 to detect mutations in TILLING instead of other methods like CHROMATOGRAPHY (DHPLC) used in TILLING in Arabidopsis to increase throughput and decrease costs (McCallum et al., 2000b and Colbert et al., 2001).

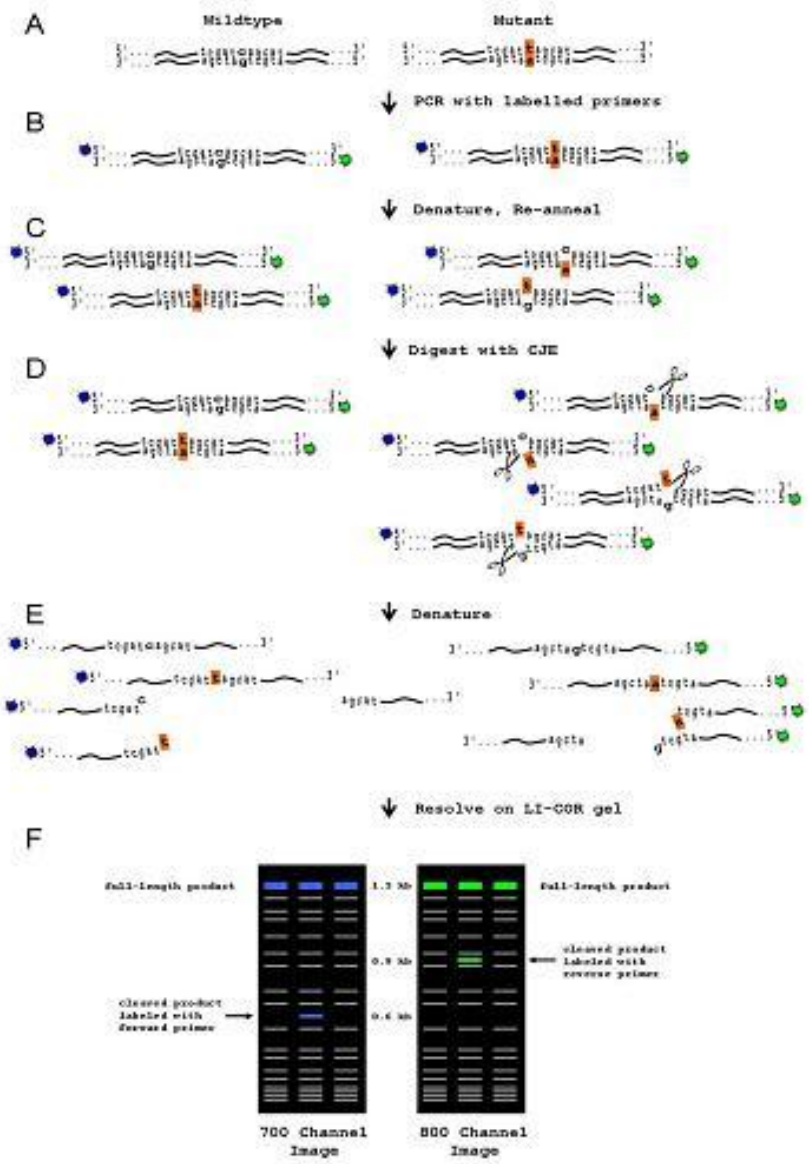


Figure 3: Overview of the TILLING procedure

Pooled DNA (**A**) is amplified using fluorescently tagged, gene-specific primers (**B**). The forward and reverse primers are labeled with different fluorophors (IRD700 and IRD800) The amplified products are denatured by heating and then allowed to cool slowly so that they randomly re-anneal (**C**). Heteroduplex molecules form when single DNA strands of mutant and wild-type PCR products anneal together. These heteroduplexes become targets for a single-strand-specific nuclease as found in celery juice extract. (**D**). The PCR products that retain one of the labeled primers can be detected on polyacrylamide denaturing LICOR gels (**E, F**). The position of the mutation within the PCR amplicon can be calculated from the size of the fragments carrying the IRD700-labeled forward primer and the IRD800- labeled reverse primer. (Haughn et al., 2005)

As it is shown in Figure 4, the other factor to consider is the choice of genotyping method. Although CEL1 is clearly, an effective way to detect heterozygous mutations, even in pools of individuals, other methods might be more applicable for an existing set-up or might provide more information. Perhaps the most complete information is given by resequencing the target exons of each individual in the array (Stemple, 2004).

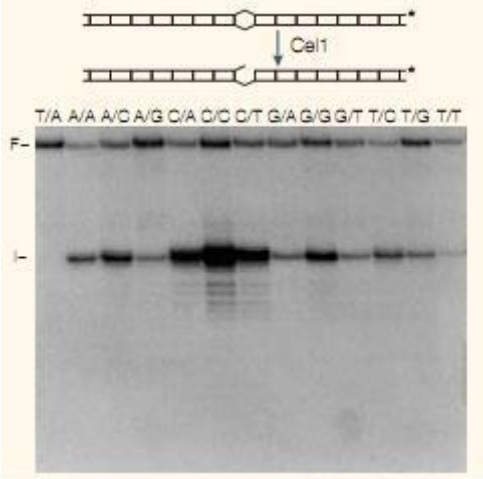


Figure 4: The Cel1 endonuclease

1) CEL1 endonuclease will cleave heteroduplex DNA at any single base-pair mismatch.
2) The top panel depicts a single base mismatched heteroduplex DNA end-labeled on one strand.
3) CEL1 cuts single strands leaving both full-length substrate (F) and cut fragment (I).
4) All possible mismatch combinations are effectively cleaved by CEL1 (Stemple, 2004).

1.3.5 Identification and visualization of pool mutant samples by different gel analyzer systems such as DHPLC or LI-COR

Although, the original TILLING method has used a commercial denaturing HPLC (DHPLC) apparatus for mutation discovery, Henikoff and his colleagues predicted that this method would not be scaled up easily, so they looked for alternatives. Briefly, the method described by Tony Yeung (Enzymatic Mismatch Cleavage) has attracted them so much that they proceeded to adapt it (Oleykowski et al., 1998). They found that the LI-COR gel analyzer system (Lincoln, NE; Middendorf et al., 1992) is ideally suited for this application. During the first months of 2001, they established the robust protocols and its software to begin a TILLING production operation on their mutagenized Arabidopsis populations (Colbert et al., 2001).

LI-COR gel electrophoresis is well suited for large scale mutation detection. In addition, the two-dimensional readout facilitates the detection of rare events; because a new band will stand out above the wild-type background and can be easily spotted. As well, the size of

each new band is also obtained, which it's another advantage over other methods based on detection of mismatches or conformational changes (Nataraj et al., 1999).

It should be mention that, cleavage electrophoresed products use the LI-COR gel analyzer system and a standard commercial image processing program (Adobe Photoshop; Adobe Systems, Mountain View, CA) is used to examine the gel readout. As it was emphasized, differential double-end labeling of amplification products allows a rapid visual confirmation because mutations are detected on complementary strands and so can be easily distinguished from amplification artifacts (Henikoff et al., 2004). One of the important advantages of double-end labeling for detecting both CEL I cleavage products is its avoidance to identify false positive bands. There are two types: those that appear in multiple lanes in a single channel and the ones that appear in a single lane but in the same position in both channels.

Since, it is highly unlikely that the same mutation appears in two different plants, it is assume that certain homoduplex sites are especially sensitive to variability in CEL I digestion causing bands to appear in multiple lanes above the background pattern. Bands that appear in both channels are likely to be examples in which priming leads to a large amount of double-end labeled product of a single size. However, smaller products have a selective advantage over larger products during cycling, leading to sporadic low bands. It has been found that PCR product yield is typically low and inconsistent using both IR Dye 700 and IR Dye 800 dyes on opposing primers; yet, consistent results have been obtained using a mixture of IR Dye-labeled and unlabeled primers (Colbert et al., 2001).

The sensitivity of DNAs separated by denaturing gel electrophoresis and detected in two separate channels by scanners (LI-COR, Lincoln, NE; Middendorf et al., 1992) is sufficient to detect the approximately 100 attomoles of cleavage product generated by CEL I in an 8-fold pool, or one in 16 genomes for a heterozygous mutation. Opposed PCR primers carry different dye labels. Because there is no detectable overlap between the infrared IR Dye 700 and IR Dye 800 dye labels, images can be examined directly for the presence of novel bands in either channel. Colbert and his colleagues (2001) used a UNIX perl program ("grab") to retrieve and archive the image files from the LI-COR scanners via a file transfer protocol and process them (using Image Magick for UNIX, www.imagemagick.org) to create compressed JPEG files on a central server for Adobe Photoshop (Adobe, Seattle) analysis on networked local computers (Macintoshes and personal computers).

1.3.1 Identification of the Mutant Individual and Sequencing of the Mutant PCR Product

Upon detection of a mutation in a pool, the individual DNA samples are similarly screened to identify the plant carrying the mutation. This rapid screening procedure determines the location of a mutation within a few base pairs for PCR products up to 1 kb in size.

A key advantage of high-throughput TILLING over competing methods is that the approximate position of each detected mutation based on the size of the fragment greatly facilitates subsequent sequencing. Furthermore, the double-end labeling strategy provides validation within the pool screen, and further confirmation comes from identifying the same fragments in tracking down individuals. Therefore, sequencing is done with near certainty that a mutation exists within a small interval. Examination of a sequencing gel trace in the predicted location is sufficient to identify the mutated base and the substitution, for example Sequencer trace analysis software (Gene Codes, Ann Arbor, MI) is used to facilitate this step (Henikoff et al., 2004).

Henikoff and his colleagues, (2004) identified 3,000 Arabidopsis mutations in this way, typically using the readout from only the strand in which the primer is closer to the detected mutation. By contrast, methods that do not provide an approximate location for a detected mutation, such as DHPLC, require the full amplified segment to be interrogated by sequencing. Detection of heterozygotes under such circumstances can be challenging, especially when peak heights vary, moreover, false positives will greatly exacerbate this problem.

A second round of TILLING is then used to determine the plant lines through the pool which carry the polymorphisms (DNA from individuals in the pool is mixed with wild type DNA to ensure heteroduplex formation when the mutation is present). TILLING identifies the position of the nucleotide change in the amplified fragment or within a few base pairs. Typically, DNA sequencing of the amplified fragment from the individual is used to verify the nature and exact position of any visible mutation (Haughn et al., 2005).

2 Materials and Methods

2.1 Plant material

Through this experiment the highly Fusarium head blight (FHB) resistant line CM-82036 with the complete name CM-82036–1TP-10Y-OST-10Y-OM-OFC' has been used (Buerstmayr et al., 2002). CM-82036 is a spring wheat line originating from the cross 'Sumai#3'/'Thornbird-S' developed in a shuttle breeding program between CIMMYT Mexico and South-America (Buerstmayr et al., 1996). 'CM-82036' has been evaluated during several seasons for resistance to FHB and proved to have a very high level of resistance, similar to that of 'Sumai#3' (Buerstmayr et al., 1996), but showed a better agronomic adaptation.

In the highly resistant 'CM-82036' two major FHB resistance QTL (Fhb1 and Qfhs.ifa-5A) have been genetically mapped (Buerstmayr et al. 2002). Two different components of FHB resistance are associated with the two QTL: Qfhs.ifa-5A governs resistance against fungal penetration, whereas, Fhb1 confers resistance to fungal spread. Although 'CM-82036' has a high level of resistance to FHB, it has several undesirable agronomic characteristics such as low yield (Salameh and Buerstmayr 2004).

2.2 Creation of mutated populations with chemical mutagenesis

In spring 2010 about 100,000 seeds were treated with EMS using two concentrations 0.053 and 0.058 mol/l. Therefore approximately 3.2 kg seeds of CM82036 (thousand kernel weight 32 g) were divided in 24 batches of 133 g and filled in nylon stockings. The batches of seeds were soaked in osmosis water for 3 hours at room temperature. The water was changed every hour. Then the stockings with the seeds were placed under the fume hood in two beakers with the two EMS solutions (0.053 and 0.058 mol/l EMS, 0.1 M Tris-HCl, pH 8), and soaked in EMS for 18 hours. The beakers were placed on magnetic stirrers and covered with cling films to protect against evaporation (Figure 5).

Figure 5: EMS treatment of CM-82036 seeds with two different EMS concentrations (the right side beaker with 0.053mol/l and the other one with 0.058mol/l).

The EMS solution was drained and inactivated before disposal (sodium hydroxid was added to a concentration of 1M). To neutralize the effect of EMS on the seeds the stockings with the seeds were soaked in sodium thiosulfate (100 mM) for 20 minutes. The process was repeated one more time. Finally, the seeds were rinsed in water for 8 hours and dried on filter paper prior to planting.

2.3 Development of M1 and M2 generations

The 100,000 M1 seeds were planted in the field. From approximately 12,000 surviving M1 plants (Table 2) one head per plant was harvested, 7,524 treated with the lower EMS concentration and 4,480 treated with the higher EMS concentration. From all heads obtained with the higher concentrated EMS treatment and from 400 randomly chosen heads from the lower concentrated EMS treatment one seed per head was sown in the greenhouse to create the M2 population. In spring 2011, in total 4,880 M2 seeds were germinated on a mixture of compost and sand in trays. Temperature in the greenhouse was on average 14/12°C (day/night) during tillering with 12 h of light. From heading to harvesting the conditions in the greenhouse were set to 20-22°C during daytime and 18-20°C during night with a 12-14 h photoperiod. Mineral fertilization was applied four weeks after planting with Nitrophoska perfect (15-5-20).

Table 2: The effect of EMS treatment on germination of M1 seeds in 2010

2010	[EMS mol/l]	Treated grains	Harvested heads	% survived
Set 1	0.053	50,000	7,524	15.05
Set 2	0.058	50,000	4,480	8.96
Total		100,000	12,004	

2.4 DNA Extraction from Individual M2 Plants

From both EMS concentrations 380 plants were randomly selected for DNA extractions and mutation screening to determine the mutation density. About 10 cm of the leaf material was cut off from young M2 plants and put in individual paper bags for subsequent drying. The bags were labeled according to the consecutive plant number from 1 to 380, and the EMS concentration and were dried at 38°C for 2 days in a drying oven. Subsequently, the 2 x 380 samples were arranged in eight 96-well plates. Therefore, 2 to 3 cm of each dry leaf sample were fragmented into tiny pieces (2 to 3 mm) and put into the corresponding well of the 96 deep-well plate; then, 3 to 5 glass beads were added to each of them. The samples were grinded for 10 minutes using a Retsch-mill machinery with a vibrational frequency of 300 min-1.

2.4.1 DNA Extraction – Protocol

The DNA extraction was performed according to the CTAB-extraction method (Hoisington et al. 1994). The compositions of the solutions used for DNA-extraction are summarized in Table 3:

1. 600 µl of CTAB buffer (hexadecyltrimethyl ammonium bromide) were pipetted in each tube under fume hood to increase the separation speed of polysaccharides from the DNA.
2. The plates were incubated in water bath along with gentle shaking at 65˚C for 60-90 min.
3. Proteins were precipitated by adding 300 µl of Chloroform and Isoamyl-Alcohol mixture (24:1) to each sample under the fume hood. Samples were mixed by gentle inversion for 5 min, and then they were spun down for 10 min at moderate speed.
4. Chloroform is known as a deproteinizing agent of DNA (Laulhere et al. 1975); so after centrifugation, DNA and RNA were located in the upper water phase, whereas proteins and other cell-debris were placed in the lower part of the tube. To avoid blending of these two phases, plates were handled carefully. Finally, 400 µl of the water phase was taken off and transferred to the fresh tubes.

5. 10 µl of RNAs A (1.2 mg/ml) was added to the extracts. After changing the caps, they were mixed by inversion. Subsequently, samples were incubated for 30 minutes at room temperature to facilitate the degradation of RNA. Lastly, they were spun down at high speed (3000 RPM).
6. 400 µl of Isopropyl alcohol were added to the samples and mixed well by gentle inversion. Next, the samples were spun down to precipitate DNA at 400 x g for 8 min.
7. The supernatant solution in each tube was poured out.
8. DNA-pellets were purified 100 µl of wash1 and wash2 respectively. DNA-pellets were incubated with wash1 and wash2 for 5 min, spun down at 400 x g for 8 min and then the washing solution was poured out.
9. DNA pellets were dried for 2 hours at room temperature (they can be also left over night, although DNA will not dissolve easily when it is too dry).
10. 50 µl of 0.1x TE buffer were added to the samples and mixed for a few hours (or overnight). It was done at room temperature with a shaker to solve DNA in this buffer. Then the dissolved DNA was stored at 4˚C.

Table 3: The compositions of the solutions used for DNA-extraction

CTAB-Buffer		
100 mM	Tris-7.5	Tris (hydroxymethyl)amino-methane
700 mM	NaCl	Sodium chloride
50 mM	EDTA-8.0	Ethylendiamintetraacetate
1%	CTAB*	Hexadecyltrimethyl ammonium bromide
140 mM	BME*	ß-Mercaptoethanol
Wash1		
76%	EtOH	Ethanol
25 mM	NaOAc	Sodium acetate
Wash2		
76%	EtOH	Ethanol
10 mM	NH4OAc	Ammonium acetate
TE-Buffer		
10 mM	Tris-8.0	Tris (hydroxymethyl) amino-methane
1 mM	EDTA-8.0	Ethylendiamintetraacetate

***)** Before the addition, buffer solution was heated to 60-65˚C.

2.5 Creation of DNA Pools of Four M2 Plants

The DNA concentration was determined by a BioSpec-nano UV-VIS Spectrophotometer. Using the machine, a sample is exposed to ultraviolet light; as a consequence, the photo-detector can measure the light passing through the sample. The absorbance of more light by the samples identifies the higher nucleic acid concentration.

The DNA concentration of all 760 samples was measured and the ratio of absorbance at 260nm/280nm was used to control the DNA purity. Subsequently, all samples were diluted with ddH_2O to the same concentration of 4 ng /µl. To avoid extra expenses and time for doing PCR, samples were four-way pooled. Therefore, 10 µl of 4 samples were pooled in one well resulting in two pool plates, one per pool plate per EMS concentration.

2.6 Detection of Mutations in the waxy gene *Wx-A1*

2.6.1 PCR Amplification of the waxy gene *Wx-A1* Using Pooled DNA and heteroduplex formation

Prior to PCR, 2 µl of the DNA samples were pipetted from the two pooled plates into a 384-well microtiter plate. Then a PCR reaction mixture was prepared according to Table 4, vortexed and centrifuged briefly. 8 µl of the reaction mixture were added to the 2 µl DNA samples, giving a total of 10 µl in each tube, and was vortexed and spun down again. Finally, the microtiter plates were placed in a thermocycler and the corresponding PCR-program was started (see Table 5).

Table 4: Reaction mixture for the PCR of the pooled samples

Component	Concentration (stock)	Concentration (final)	1RX[µl]
10x PCR Buffer	10x	1x	1
dNTPs	2 mM (each)	0.2 mM (each)	1
Primer (forward)	2 µM	0.1 µM	0.5
Primer (reverse)	2 µM	0.1 µM	0.5
Polymerase (proof-reading)	2.5 u	0.1u	0.04
DNA pooled mutants	4 ng/ µL	0.8 ng/ µL	2
ddH2O		Fill to 10 µL	4.96

Two primer pairs, *Wx7A2* and *Wx7A3*, specific for the waxy gene *Wx-A1* located on the 7A chromosome of hexaploid wheat were used for mutant screening (Slade et al., 2005b). *Wx-A1* has the GenBank entry number AB019622.

The sequence of the two primer pairs and the amplified fragments are given below, forward and reverse primer sequences are highlighted in yellow and green. The *Wx7A2* and *Wx7A3* primer pairs amplify a 548 bp and 793 bp fragment respectively, with a 82 bp overlap (sequence given in red letters) so, totally 1,205 bp of the waxy gene *Wx-A1* were analyzed.

Wx7A2:
acccgcatggtgtttgataatttcagtgagtctttgcgtctgctggttacaatttccaggtccggggcaagaccaaggagaagatctatggacccg
acgccggcaccgactacgaggacaaccagcagcgcttcagccttctctgccaggcagcacttgaggtgcccaggatcctcgacctcaacaa
caacccacacttttctggaccctacggtaagatcaagaacaactagagtgtatctgaagaacttgatttctacttgagagcactggatgattatcat
cttccttgtatcttggtgctgccatgctatgccgtgccgtgccgcgccgcgaggggaagacgtggtgtttgtgtgcaacgactggcacacgggc
cttctggcctgctacctcaagagcaactaccagtccaatggcatctataggacggccaaggttttgcatcttctgaaactttatattcgctctgcatat
caattttgcggttcattctggcagcctgaattttacattgcaactccatttcatggctaggtggcattct

Wx7A3:
cgctctgcatatcaattttgcggttcattctggcagcctgaattttacattgcaactccatttcatggctaggtggcattctgcatccacaacatctcgta
ccagggccgcttctccttcgacgacttcgcgcagctcaacctgcctgacaggttcaagtcgtccttcgacttcatcgacggctacgacaagccgg
tggaggggcgcaagatcaactggatgaaggccgggatcctgcaggccgacaaggtgctgactgtgagcccctactatgctgaggagctaat
ctctggcgaagccaggggctgcgagctcgacaacatcatgcgcctcactgggatcaccggcatcgtcaacggcatggacgtcagcgagtgg
gaccccatcaaggacaagttcctcaccgtcaactacgacgtcaccaccgtgagcacccacccacccacacaaagatttcttccggtgatcgct
ggttctgggtggattctgagttctgacaaacgaggcaaagtgacaggcgttggaggggaaggcgctgaacaaggaggcgctgcaggccga
ggtggggctgccggtggaccggaaggtgcccctggtggcgttcatcggcaggctggaggagcagaagggccccgacgtgatgatcgccgc
catcccggagatcgtgaaggaggaggacgtccagatcgttctcctggtacgatcgaccgacattgctgacccgttcaggaaaatctcctgatag
ctcgccgtggggatgggtgggtgactgactgatcgaatgcattgcagg

Fluorescence dye-labeled primers were used for PCR amplification. Both primers were directly labeled at the 5' with the fluorochrome IRD700 or IRD800.

The Primer Sets Used

Wx7A2:

WX7A2 forward, 5´-ACCCGCATGGTGTTTGATAATTTCAGTG-3´, IRD700 labeled

WX7A2 reverse, 5´-AGAATGCCACCTAGCCATGAAATGGAGT-3´, IRD800 labeled

Wx7A3:

WX7A3 forward, 5´-CGCTCTGCATATCAATTTTGCGGTTC-3´, IRD700 labeled

WX7A3 reverse, 5´-CCTGCAATGCATTCGATCAGTCAGTC-3´, IRD800 labeled

Unlabeled primers and identical primers labeled at the 5´end with the fluorescent dye (IRD700 or IRD800) were mixed and used in PCR amplification as follows:

-Forward Primer IRD700 labeled primer: 50% labeled, 50% unlabeled. Reverse Primer IRD800 labeled primer: 90% labeled, 10% unlabeled.

Table 5: The steps done during the PCR Program

	Temp	Time
initial denaturation step	95°C	2 min
Loop: 8 cycles		
Denaturation	94°C	20 sec
annealing with touch-down -1°C/cycle	68°C	30 secs
Extension	72°C	1 min
Loop: 36 cycles		
Denaturation	94°C	20 sec
Annealing *	61°C	30 sec
Extension	72°C	1 min
Last cycle	72°C	8 min
	4°C	Forever

***** The Annealing Temperature for both primers are the same

Heteroduplex program:

After the PCR amplification PCR products are denatured and re-annealed to form heteroduplexes between the mutated sequence and its wild-type according to following data:

95˚C	5 min	(ramp -0.1˚C/s)
5˚C	forever	

2.6.2 Restriction with Endo I

Heteroduplexes are substrates for cleavage by the endonuclease Endo I. To start this part, 1 µl of 10x reaction buffer was added to samples and centrifuged at moderate rpm. After that, 1 µl of fresh made Endo I (1:50) concentration was added to the samples. The new mixtures were vortexed, spun down, and incubated at 37˚C for 40 min. Immediately after the incubation 1.7 µl of stop buffer solution was added to the samples, mixed and spun down once more.

The buffers following mixtures:

10x-reaction buffer:
100 mM Hepes buffer pH 7.5
100mM MgSO4
100 mM KCl
0.02% Triton X-100

stop buffer:
25 mM EDTA

2.6.3 Sephadex Purification

Sephadex powder “Sephadex G-50 DNA Grade fine” or alternatively “Dextran crosses linked G-50 (20-80 µm)” was poured inside the Millipore MultiScreen Column Loader and the wells of loader were covered by Sephadex powder using one scraper. Afterwards, an empty Millipore MultiScreen-HV (MSHVN4510) 96-well plate was slid upside down into the loader in order that the two plates were held together and turned over.

After pouring the sephadex powder into Millipore multi screen-HV, 300 µl of distilled water were added to it and the plate was left at room temperature for 3 hours to gain its formation (the plate can also be stored up to 2 days at 4˚C). Accordingly, the MultiScreen plate was spun down for 5 min at 910 g (2250 rpm) using a waste plate being able to hold up to 300 µl. The water inside the waste plate was disposed and spun down for 5 min with the same speed again.

Following changing the waste plate with a fresh one, the samples were pipetted on top of the “mini-column” and centrifuged at 910 g for 5 min. To avoid evaporation process of solutions in the wells, plates were covered with PCR sealing film and put in the refrigerator at 4˚C.

2.6.4 Polyacrylamide LI-COR Gel Electrophoresis

The detection of DNA fragments was performed on a LI-COR 4200 DNA analyzer. The device was used for two distinct concurrently aims: the electrophoresis separation of the fragments and the detection of the fluorescence-labeled fragments with a wavelength of 700 or 800 nm.

The result was shown through a digital image of electrophoretograms by the connected PC. It should be mention that the gel could be loaded during a day several times.

The Requisite Instruments for Preparing the LICOR-Gel:

Front and rear glass plates
Clamps holding the glass plates (right and left side)
64 well shark tooth comb
Glass holding comb
Small Erlenmeyer with 125 ml size
Magnetic stirrer
Pipette
Plastic tape with 2 mm thickness, 2 cm width, 30 cm length as spacers
Paper towels
Buffer tanks

Preparation of Gels (25 cm long, 0.25 mm thick, 7% Acrylamid, Denaturing):

1) The insides of front and rear glasses were cleaned two times; first by distilled water, and next by 70% ethanol.
2) Assembling the gel apparatus and placing the glass plates in a flat angle.
3) 3.5 ml of 50% acrylamid stock (Long ranger gel solution, FMC Bio products), (10.5 g of urea, and 2.5ml of 10xTBE-buffer were weighed and pipetted respectively.)
4) (After dissolution of the urea, it was filled with dH2O to 25 ml.)
5) 250 µl DMSO (Dimethyl sulfoxide, Sigma# D8418), 25 µl of TEMED (N,N,N´,N´-Tetramethylethanediamine) , and 175 µl of APS (Ammonium persulfate) 10% were added to solution respectively and then mixed well.
6) The solution was poured into the space between the 2 glasses until the gap was filled completely. Afterward, the comb was inserted conversely in the space (on the upper part of glass) and held by using a place holder.
7) The glass was located horizontally under the fume hood for 45 min to polymerize the gel.
8) After removing the place holder for the comb the gel was placed in the LI-COR device, subsequently the 64 well shark toothcomb was inserted while the buffer chambers were filled with 1x TBE-buffer up to the mark.

Device-Settings:

Voltage:	1500 Volt
Amperage:	50 mA
Power:	40 W
Temperature:	48 ˚C
Motor speed:	3

At least 10 Frames for *Wx7A2* and 13 Frames for *Wx7A3* primer.

In order to increase the concentration of the PCR fragments the samples were incubated the samples for about 20 min at 72°C without the sealing film. Then, prior to loading 3-5 µl of loading buffer solution were added to samples; next, they were denatured (about 5 minutes at 95°C) and subsequently were put on the ice. By using the Hamilton-8-channel-gel-loading syringe, 0.8 µl of the samples were loaded on the gel.

Composition of Solutions Used in Loading Buffer:

950 µl	95% deionized formamide
50 µl	0.5 mM EDTA (Ethylendiamintetraacetate)
4 µl	0.1 mg/ml Fuchsine

10x TBE-Buffer

1340 mM	Tris (hydroxymethyl)-aminomethane
450 mM	Boric acid
25 mM	EDTA Ethylendiamintetraacetate

The gel was poured between two glass plates of different lengths. The glass plates were fixed by the fixing brackets. These brackets are used in the LI-COR analyzer as a holder for the upper buffer chamber, placing directly on the gel to secure the device. The main function of the shark tooth comb was to form wells with little space in-between. Then, the samples were loaded into the space between the teeth using a Hamilton-8-channel-gel-loading syringe to prevent samples from spreading. The lower end of the gel immersed into the lower buffer chamber. The electricity was applied to the upper and lower parts of buffer chamber; so, negatively charged DNA fragments are moved from top to bottom through the gel (on the basis of the PH of the buffer solution).

A few centimeters above the lower edge of gel was illuminated by a laser light through the gel at short intervals, alternating with 700 or 800 nm wavelength. If a labeled fragment passed by? This place, the receptor received a signal change, which was forwarded to the connected computer and displayed as a black band on a digital image.

2.6.5 Analysis of the Gel

The digital images were printed or evaluated visually on screen. Figure 6 shows an example of a fourfold pooled TILLING assay using *Wx7A3* primers for the *Wx-A1* locus. Mutations are visible as dark bands (red – IRD700 and green – IRD800 circles) resulting from Endo1 endonuclease cleavage, are shorter than the full-length PCR product (which for *Wx7A2* is 548 bp and for *Wx7A3* is 793 bp) at the top of each panel. The IRD700-*Wx7A3* image is shown on the left panel whereas the IRD800-*Wx7A3* image is on the right panel.

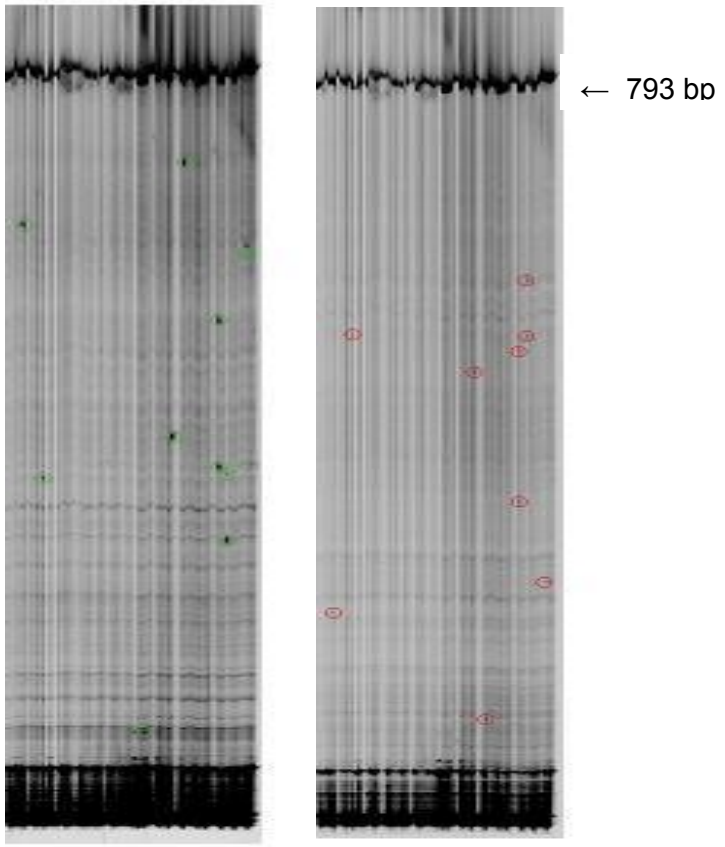


Figure 6: A view of Licor-Gel results of pool samples

EMS-induced mutations were distinguishable from PCR artifacts by the appearance of cleavage products in both IRD700 and IRD800 channels at reciprocal sizes added up to the full-length PCR product (artifacts appear at the same size in both panels).

According to Table 6, after recognition of mutations within pool samples, the PCR was done for all the four pooled samples separately, to identify the mutant sample after gel analysis. All the steps were done as previous ones, except that, DNA from wild type CM-82036 was added to each mutant sample to ensure heteroduplex formation. In this stage at least one plant of the fourfold-pooled samples must be recognized to contain a nucleotide polymorphism.

Table 6: Reaction mixture for the PCR to screen the individual samples

Component	Concentration(stock)	Concentration(final)	1RX[µL] (single line)
10x PCR Buffer	10x	1x	1
dNTPs	2 mM (each)	0.2 mM (each)	1
Primer (forward)	2 µM	0.1 µM	0.5
Primer (reverse)	2 µM	0.1 µM	0.5
Polymerase (proof-reading)	2.5 u	0.1u	0.04
DNA Wildtype	222 ng/ µL	0.4 ng/ µL	0.018
DNA Single mutant	4 ng/ µL	0.4 ng/ µL	1
ddH2O		Fill to 10 µL	5.942

The average mutation frequency was calculated as: (length of analyzed fragment × total number of individuals screened) / total number of identified mutants.

3 Results and Discussion

3.1 Detection of Mutations in fourfold-pooled Samples

The following results are presented according to the information including in previous parts, meant to aid in finding mutations in a CM-82036 mutant population by TILLING at the *Wx-A1* locus.

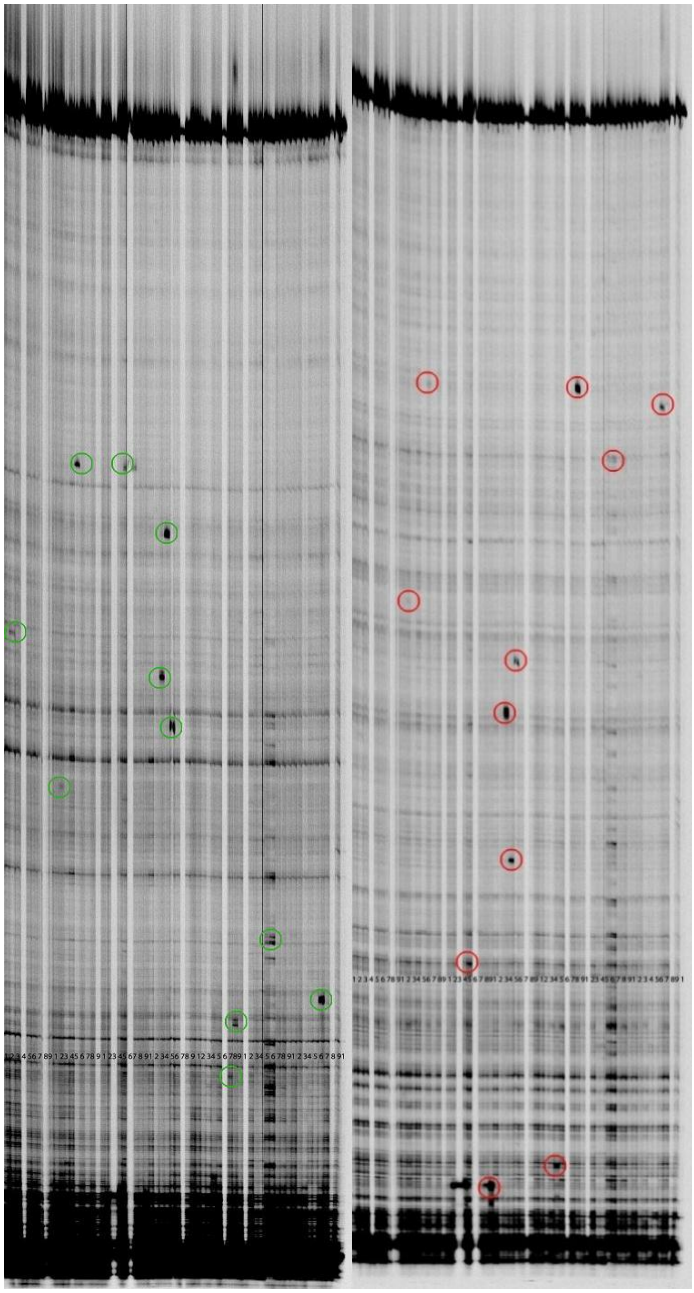


Figure 7: Distinguishing the mutations by color circles in order to determine the mutant samples for primer *Wx7A2* for the *Wx-A1* gene in pool plates.

Figure 7 reveals the mutations for 64 pool samples (total 256 samples), visible as dark bands (green and red circles) resulting from Endo1 endonuclease cleavage, are shorter than the full-length PCR product at the top of each panel. All DNA pool samples were screened for mutations at the *Wx-A1* locus with two primer pairs; the images were analyzed by Photoshop program and summarized through Microsoft Excel program. 15 probable mutations were detected in the pools using the *Wx7A2* primer and 28 pools for the *Wx7A3* primer.

3.2 Identifying Mutations in Individual DNA Samples

Results from the probable mutant pool samples were separately screened and analyzed for individual mutant samples. Accordingly, statistical analysis was applied for determining the quantities of mutations identified in the two different EMS concentrations for the two primer pairs.

It was assumed that the effect of EMS mutagenesis had been random (which means, any G-residue within the DNA sequences screened by TILLING was equally likely to be mutated) (Slade et al., 2005b). So, based on the sequence length and the number of observed mutations screened by LI-COR electrophoresis, the key parameters were: mutants quantities, plates names, and location of well, and approximate fragment sizes of the mutations at the IRD700 and IRD800 gel images for the two different primer pairs *Wx7A2* and *Wx7A3* (Table 7).

Table 7: Identifying of individual samples with mutations in two different EMS concentrations according to their numbers, position in plates and sequence length.

	Mutant ID	Plate	Well	Primers	Fragment size 700nm	Fragment size 800nm
EMS 0.053 mol/l	38	A	C10	Wx7A2	~330	~260
	131	B	A11	Wx7A2	~260	~310
	146	B	B10	Wx7A2	~410	~170
	255	C	A11	Wx7A2	~410	~170
	43	B	C3	Wx7A3	~300(375)	~590(520)
	53	A	D9	Wx7A3	~385(550)	~?(330)
	73	B	E4	Wx7A3	~800	?
	245	C	H1	Wx7A3	~670	~210
EMS 0.058 mol/l	402	E	A6	Wx7A2	~300	~275
	403	E	A7	Wx7A2	~380	~205
	404	E	A8	Wx7A2	~290	~290
	468	E	E8	Wx7A2	~160	~405
	523	F	H3	Wx7A2	~195	~375
	776	H	H8	Wx7A2	~180	~400
	532	F	A12	Wx7A3	~350	~490
	658	H	A2	Wx7A3	~340	~480
	705	H	D1	Wx7A3	~610	~210
	754	H	G2	Wx7A3	~480	~360
	756	H	G4	Wx7A3	~480	~360
	779	H	H11	Wx7A3	~530	~290

The following Table summarizes the distribution of all mutations identified in the waxy gene *Wx-A1* located on the 7A chromosome in the CM-82036 mutant population. A total of 760 individuals from the library of 4,880 M2 plants were screened over 1,205 bp of the *Wx-A1* locus, giving a total surveyed DNA length of 915.8 kb.

Table 8: Existence's probability of mutations in different concentrations for two different primer pairs

Line	generation	EMS concentration	individual plants screened	primers	bp of amplified fragment	mutations found	1 mutation/kb
CM-82036	M2	0.053M	380	Wx7A2	548	4	52.06
	M2	0.053M		Wx7A3	793	4	75.33
	M2	0.058M	380	Wx7A2	548	6	34.70
	M2	0.058M		Wx7A3	793	6	50.22

The obtained data presented in Table 8 identifies 8 mutations among 380 individual plants for the 0.053 EMS concentration and 12 mutations for the 0.058M EMS concentration.

Consequently, the mutation density was calculated for samples treated by 0.053M EMS concentration with 1 mutation per 57.23 kb while for 0.058M EMS concentration 1 mutation per 38.15 kb was identified (Table 9).

Table 9: The number of detected mutations in range of both nucleotide primers and existence's probability mutation per kb for different concentrations of EMS

CM-82036 **M2/2010**	EMS concentration	individual plants	combined Wx7A2 + 3	Bp of both primers screened sequence	bp of totally screened sequence	Total mutations found	1 mutation per x kb
	0.053M	380	Wx7A2 +Wx7A3	1,205	457,900	8	57.23
	0.058M	380	Wx7A2 +Wx7A3	1,205	457,900	12	38.15

According to the statistic data, percent of probability GC-content for combined primer is 56.43%. The results of mutation densities in both populations for 50% GC targets are as follows:

0.053M (EMS) ⟶ For 50% GC 57.23 x 56.43 / 50 = 64.58 kb

0.058M (EMS) ⟶ For 50% GC 38.15 x 56.43 / 50 = 43.05 kb

3.3 Discussion

3.3.1 TILLING in general

The main advantage of TILLING as a reverse genetics strategy is that, it can be applied to any plant species, regardless of its genome size, ploidy level or method of propagation (Kurowska et al., 2011). It also takes advantage of classical mutagenesis, sequence availability and high-throughput screening for nucleotide polymorphisms in a targeted sequence.

This approach provides a rich and convenient resource for both basic functional genomics researches and commercial crop developments. Polyploid species, such as wheat, are able to have a high level of tolerance regarding mutations due to the complementation of essential genes by homoeologous copies, and smaller populations are therefore required for saturation than with diploid species.

As a consequence of this strategy, the current study used a collection of approximately 800 mutagenized M2 hexaploid wheat plants for mutation frequency determination. Going over the steps and data including this research, the results of the study, demonstrate that TILLING offers a non transgenic alternative for the rapid generation of novel genetic variation. In addition, these results explain that how wheat can be mutagenized as efficiently as diploid plants without undue problems related to fertility or ability to recover mutations.

Hence, the application of TILLING for many target genes could help the improving of nutrition, quality and yield of wheat and other agriculturally important plants.

3.3.2 The Comparison of Obtained Results with Previous Ones

EMS-induced mutations are randomly distributed in the genome (Gilchrist and Haughn 2005). Therefore, it can be possible to determine whether there is a significant difference between the mutation densities in samples experimented a few years ago with the ones of this study. However, it should be noted that, the samples were treated with different EMS concentrations.

In previous studies at our institute, the mutation frequencies varied between one mutation per 20.1 kb (treated 2008 with 0.05M EMS) to one mutation per 39 kb (treated 2009 with 0.05M EMS) in M2 generations. The mutation rate of one mutation per 38.1 kb received in this study for the 0.058 M EMS concentration is in a similar range. As expected, for the lower EMS concentration, 0.053 M, a lower mutation density was detected, one mutation per 57.2 kb. Table 10 summarizes the different results:

Table 10: Comparison of the results with results from previous studies at our institute

Line	Generation	EMS concentration	1 mutation per x kb	Bp screened	Mutations found	Individual plants screened
CM-82036	M2	0.05M_2007	38.8	1.395,756	36	1,132
	M2	0.06M_2007	29.2	87,543	3	71
	M2	0.05M_2008	20.1	462,375	23	375
	M3	0.05M_2008	31.2	468,540	15	380
	M3	0.05M_2009	39.0	468,540	12	380
	M3	0.055M_2009	24.7	468,540	19	380
	M2	0.053M_2010	57.2	457,900	8	380
	M2	0.058M_2010	38.1	457,900	12	380

3.3.3 The Comparison of Obtained Results with the Results of other Researches

Slade and her colleagues (2005b) developed a TILLING population of hexaploid wheat, cultivar Express, and tetraploid wheat, cultivar Kronos, by treatment with EMS. According to them, the mutation frequency determined by screening ~800 M2 plants over several regions of the genome, had been approximately one mutation per 24 kb in hexaploid wheat and one mutation per 40 kb in tetraploid wheat .

However, according to Fitzgerald and his colleagues (2010), the mutation frequency determined by screening 4,500 M2 mutant wheat lines generated by heavy ion irradiation, had been approximately one mutation per 84 kb in hexaploid wheat. They developed a method for automated, high-throughput screening to identify deletions of individual homoeologues of a wheat gene. This method is also potentially applicable to other polyploidy plants.

On the other hand, Uauy and his colleagues (2009) generated reverse genetics TILLING resources for pasta and bread wheat which developed a non-denaturing polyacrylamide gel set-up. They achieved a high mutation density in both populations. Each mutant library was characterized by TILLING multiple genes, revealing high mutation densities in both the hexaploid one mutation per 38 kb and tetraploid one mutation per 51 kb populations for 50% GC targets. In total, they identified over 275 novel alleles in eleven targeted gene/genome combinations in hexaploid and tetraploid wheat and have validated the presence of a subset of them in their seed stock.

Taken together, the mutation frequency in our CM-82036 TILLING population is comparable to other results from the literature. The TILLING population is established and ready to use for mutant screening in candidate genes for FHB resistance.

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