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# 大麦 TILLING 群体的构建及 *EDR*1 基因突变的检测

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**摘　要**:该研究选用栽培大麦品种'西引 2 号',通过化学诱变剂甲基磺酸乙酯(ethyl methane sulfonate, EMS)处理,创建了 3 750 个 $M_1$ 及 2 012 个 $M_2$ 突变株系。结果表明:在 $M_2$ 中,有 115 株表现黄化,98 株表现矮化,101 株表现极早或极晚抽穗,其他 35 株表现分蘖少、叶片小或者不育等性状,所有突变株系占 $M_2$ 群体的 17.35%;$M_2$ 的基因组 DNA 用于突变频率的研究,用优化的 CEL Ⅰ酶切体系对 $M_2$ 株系进行了 *EDR*1 基因突变体筛选,获得了 *EDR*1 基因的 3 个突变体,其中一个发生在外显子区域,导致了氨基酸 Pro 到 Ser 的转换,突变频率平均为每 661 kb 一个点突变,这与前人研究的突变频率基本在一个范围内。该研究创建了大麦品种'西引 2 号'的 TILLING 筛选群体,并用于 *EDR*1 突变体的筛选。为大麦性状研究提供了不同的思路,搭建了新的资源和技术平台。

**关键词**:大麦;*EDR*1 基因;TILLING 技术

**中图分类号**:Q319$^+$.2　　**文献标志码**:A

# TILLING-population Analysis of *EDR*1 in Barley Cultivar 'Xiyin2'

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**Abstract**: To advance barley research, we produced a TILLING (Targeting Induced Local Lesions IN Genomes) population of the six-rowed barley cultivar 'Xiyin2'. Through ethyl methane sulfonate(EMS) mutagenesis treatment, we established a 3 750 mutagenised $M_1$ population and a 2 012 mutagenised $M_2$ population. Totally phenotypic variations in 17.35% of the $M_2$ population were observed. In which 115 lines showed chlorosis, 98 lines showed dwarfing, 101 lines showed very early/late flowering, and others 35 lines showed less tilling, small leaves, or produced no seeds. Likewise, genomic DNA was extracted from the $M_2$ lines for studying the mutation frequency. The estimated mutation frequency was one mutation per 661 kb by CEL Ⅰ-based screening, it is the same range as the previously studies. During our investigation, 3 different mutations in the *EDR*1 (AF305912) gene were identified, among which the Pro to Ser transition happened in extron region. The 'Xiyin2' TILLING population will be an important resource in the development of barley with specific characters.

**Key words**: barley (*Hordeum vulgare* L.); *EDR*1 gene; TILLING technology

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The barley genus *Hordeum* belongs to the grass family Poaceae[1]. Cultivated barley *Hordeum vulgare* L. is the economically important crop and ranks the forth in world cereal production after wheat, maize and rice[2]. Barley is mostly used as a food and in malting. In 2010, barley production was 123.7 Mt worldwide, with most of this production from Europe, Australia, USA and China[3]. The barley genome ($n = 7$) comprises more than 5 000 Mb, equivalent to approximately 12 times the size of the rice genome, and consists of about 80% repetitive DNA[4]. Due to its importance as a staple crop and as a model for other Triticeae genomes, comprehensive genetic and genomic resources have been established for barley over the past decades, including a large number of well-characterized genetic stocks and mutant collections (http://www.untamo.net/cgi-bin/ace/searches/basic)[5-6]. The first induced mutagenesis of barley was reported in 1928[7], only a year after Muller reported that ionizing irradiation could increase the mutation frequency in *Drosophila*[8]. This finding promoted barley as a model plant and one of the first crops in which mutations were induced for applied purposes[9-10].

*EDR*1 (Enhanced Disease Resistance 1) was reported as a key negative regulator of plant-powdery mildew interaction. The *EDR*1 mutant generates powdery mildew-induced cell death and increases resistance to powdery mildew infection. In addition, *EDR*1 results in increased leaf senescence when treated with ethylene. *EDR*1 encodes a protein kinase, which has a conserved C-terminal kinase domain and an N-terminal regulatory domain. The *EDR*1 in *Arabidopsis*, wheat and rice have been fully studied and cloned[11-14].

*EDR*1 may function as a negative regulator at the top of a MAPK cascade in wild-type plants[15]. The increased resistance conferred by the *EDR*1 mutation may provide a novel means of conferring resistance to powdery mildew. It has been suggested that the *EDR*1 pathway is conserved in crop species[16]. Putative orthologs of *EDR*1 are present in monocots such as *Oryza sativa* L. and *Hordeum vulgare* L., indicating that *EDR*1 may regulate defense responses in a wide range of crop species[16].

Usually, radiation or chemical mutagens such as EMS (ethyl methanesulfonate) were used to increase mutations in plant breeding. EMS is the most widely used mutagen due to its high mutation frequency, and most mutants are recessive[17]. The mutation process consists of two steps: 1) the O-6 location of guanine first becomes a quaternary ammonium group with positive charge by alkylation, then 2) the group base pairs thymine instead of cytosine in the subsequent DNA-replication step, which leads to base substitution (G-C→A-T)[18]. Mutations in coding regions can be silent, missense or nonsense, and mutations outside coding regions, such as promoter mutations, result in up-or down-regulation of transcription, aberrant splicing of mRNA, altered mRNA stability or changes in protein translation.

TILLING was first demonstrated in *Arabidopsis thaliana*[19] and *Drosophila melanogaster*[20], and has later been successfully applied to a number of plant systems including *Arabidopsis*[21], barley[6, 22], *Lotus japonicus*[23-24], wheat[25], maize[26], rice[26-28], pea[29], soybean[30] and naked oats[31]. Two large-scale EMS mutant populations from barley have been developed by Caldwell[6]. 16 — 32 alleles and 10 induced mutations in the coding sequences of two target genes were obtained. Gottwald[32] developed a barley TILLING population of 10 279 $M_2$ plants. The screening of six target genes demonstrated a moderate mutation frequency of approximately one mutation per 0.5 Mb. A detailed TILLING analysis was performed for the gene *HvHox*1, which is the major factor controlling the row-type morphology of the barley spike, multiple alleles causing phenotypic changes were obtained. Therefore, developing TILLING-populations in diploid barley with a reasonable mutation frequency could be productive in barley breeding.

In the present study, a TILLING population in a six-rowed barley cultivar 'Xiyin2', consisting

of 2012 different mutagenized seed lines, was developed and the mutation frequency was determined. To screen the genetic mutation of *EDR*1 gene in TILLING population, methods of optimized CEL Ⅰ screening system and RAPD-PCR were adapted to identify the different mutations on barley loci *EDR*1 (AF305912) for demonstrating the potential of the barley TILLING population. The study would be of great interest to barley breeders for increasing the resistance to powdery mildew.

## 1 Materials and Methods

### 1.1 Plant material

Cultivar 'Xiyin2' was a six-rowed barley (*Hordeum vulgare* L.) variety with the characteristics of mid-early maturity, high yield, and weak winter habit. This cultivar, introduced from Japan into China in 1980s by former Northwest Agriculture University, was mainly suitable cultivated in the Huanghuai areas of China. It was deposited in the College of Food Science and Engineering, Northwest A&F University at Shaanxi Province, China.

### 1.2 Methods

**1.2.1 EMS mutagenesis** For the screening of EMS (ethyl methanesulfonate) concentration stage, six groups of 600 barley seeds were treated at different EMS (Alfa Aesar Co.) concentrations (CK, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%). All seeds were put into a 1 000 mL beaker pre-soaked in $ddH_2O$ for 4 h at room temperature (25 ℃). Then each of 100 seeds were transferred to a 50 mL falcon tube with 20 mL EMS solutions followed by 15 h shaking at room temperature (150 r/min). Subsequently, seeds were washed 2 times in tap water by 1 h of vigorous shaking, then washed again with $ddH_2O$ two times. Next, seeds were gently wiped with filter paper and transferred to the fume hood where they were dried for use.

**1.2.2 Population cultivation** After treating seeds with mutagen, 600 barley seeds were immediately planted in a green house with a 17.5 h photo-period (19:30 pm—8:00 am) at aconstant temperature of 18 ℃. Germination data were collected 10 days after sowing. $M_1$ and $M_2$ populations were grown in experimental plots of the College of Plant Protection, Northwest A&F University. Genome DNA was collected.

**1.2.3 Genomic DNA isolation** Genomic DNA was extracted from young leaves at the three-leaf stage which is about 45 d after sowing using the standard CTAB protocol[33-34]. DNA samples were quantified with $OD_{260/280}$ test and adjusted to a final concentration of 20 ng/μL for RAPD PCR.

**1.2.4 RAPD analysis** Five primers which formed polymorphic bands were selected from the ten random 10-mer primers (Sangon Biotech Co., Ltd, Shanghai). The specific sequences as follows: S5(TGCGCCCTTC), S31(CAATCGCCGT), S86(GTGCCTAACC), S284 (GGCTGCAATG) and S1429 (AGAGCGTACC).

The genome DNA from a non-mutagenised sample was defined as CK (control sample). The 25 μL reaction volume of PCR amplification, including 2.0 μL 20 ng genome DNA, 1.5 μL 2.0 mmol/L $MgCl_2$, 2.0 μL 150 μmol/L dNTP, 1.0 μL 0.5 μmol/L primer, 0.25 μL 1.25 U *Taq* polymerase. The amplication program consisted of initial denaturation (94 ℃ for 5 min) followed by 40 cycles at 94 ℃ for 30 s, annealing (37 ℃ for 1 min), extension (72 ℃ for 1 min), and a final extension (72 ℃ for 10 min). PCR products were analysed on 1.5% agarose gels (Amresco, USA) in Tris-borate buffer at 5 V/cm. The gel was then stained with ethidium bromide and visualized under UV light and photographed.

**1.2.5 CEL Ⅰ-based mutation screening** CEL Ⅰ extraction followed Till's protocol[35]. The *EDR*1 of *Arabidopsis thaliana* (GenBank: AF305913) conserved in barley genome (*EDR*1: AF305912), gene-specific primers, based on sequence information from NCBI, designed by Primer Premier 5.0 software (Premier Biosoft Interntional). The primer of *EDR*1 (AF305912) is: F (5′GTGAACCTGGGACATTAG3′) and R (5′ATGGCTTTCTCAGTGTTG3′).

In all 2012 $M_2$ lines, every 4 line's DNA was merged into one pool and 503 DNA pools were ana-

lyzed.

RAPD PCR amplification was carried out in a 25 μL volume containing 2.0 μL 20 ng of individual pooled DNA, 2.0 μL 0.2 mmol/L dNTPs, 1.0 μL 0.4 μmol/L primers (Invetrogen), and 0.2 μL 1.0 U *Taq* polymerase (MBI). The PCR reactions were conducted using a thermal cycler (Applied Biosystems 9800 Fast Thermal Cycler, Foster City, USA) as follows: heat denaturation at 94 ℃ for 5 min was followed by 30 cycles at 94 ℃ for 30 s, 52 ℃ for 1 min, 72 ℃ for 1 min and extension at 72 ℃ for 1 to 1.30 min; and final extension at 72 ℃ for 8 min. The amplification step was followed by heteroduplex formation: inactivation and denaturation at 99 ℃ for 10 min; and a re-annealing process of 70 cycles for 20 s at 72 ℃ to 51 ℃, decreasing 0.3 ℃ per cycle.

CEL Ⅰ extraction followed Till's protocol[35]. CEL Ⅰ was diluted to 1/10, and using a 2 μL CEL Ⅰ dilution mixed with 2 μL buffer (20 mmol/L HEPES pH 7.5, 10 mmol/L KCl, 3 mmol/L $MgCl_2$) and 14 μL PCR products digested at 45 ℃ for 30 minu, then adding 5 μL EDTA (75 mmol/L) to end the reaction.

## 2 Results and Analysis

### 2.1 Optimal EMS mutagen concentration in the development of a barley TILLING-population

Based on previous study[6, 32], for choosing the optimal EMS treatment concentration, an EMS treatment curve for germination rates and full-sterile rates of barley seeds was established (Fig. 1). In total 5 different EMS concentrations, from 0.2% to 0.6% with the interval of 0.1%, were used and 100 seeds were treated at each concentration. According to Fig. 1, 0.4% should be the optimal EMS mutagen concentration for treatment, but in consideration of the balance of the maximum mutation density with an acceptable seed survival rate, a concentration of 0.3% was finally chosen for constructing the TILLING population. A total of 5 000 seeds were treated resulting in a 3 750 $M_1$ population (survival rate of 75.0%). Of these, only 2 534 fertile plants set seeds to produce $M_2$ lines. The rest died, didn't flower or produced empty seeds. $M_2$ seeds were harvested from each of the $M_1$ lines. We then planted every 4 seeds for 1 line and got 2012 $M_2$ lines, only keeping 1 strong plant after germination). An overview of the TILLING population procedure is given in Fig. 2.

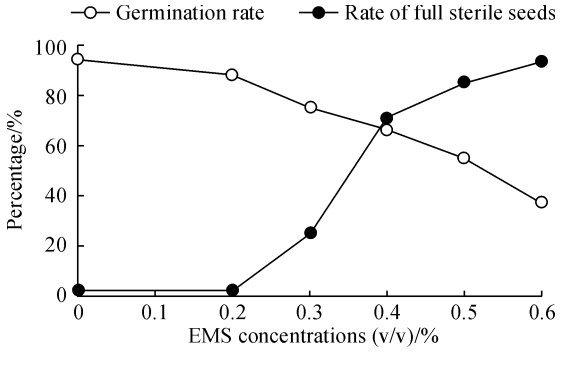



Fig. 1　Average germination rates by different EMS concentration

### 2.2 Phenotype evidence for occurrence of mutation in TILLING-population

The phenotype variations of the $M_2$ population were recorded in detail, in which 115 lines showed chlorosis(Fig. 3), 98 lines showed dwarfing, 101 lines showed very early/late flowering, and others 35 lines showed less tilling, small leaves, or produced no seeds. Totally phenotypic variations in 17.35% of the $M_2$ population were observed (Table 1).

### 2.3 Molecular level evidence for occurrence of mutation in TILLING-population achieved using RAPD

From the phenotypes of $M_2$, we observed apparent mutations in the population, and then used the effective and inexpensive system RAPD (Random Amplification of Polymorphic DNA) to seek molecular level evidence. Based on previous studies[36-37], we chose ten random primers using non-mutated 'Xiyin2' DNA as a control template, and found five of them showed stable fragments. We then used these five random primers amplified 256 samples of the $M_2$ lines, and detected the polymorphism. The results show the mutations in the $M_2$ lines that occurred (Fig. 4).

### 2.4 Estimation of the mutation frequency by CEL Ⅰ-based mutation screening

We developed a CEL Ⅰ (celery juice extract)-based screening system to explore the 2012 plants

of $M_2$. A plasmid pDRIVER (single-base variant) was used to optimize the temperature and digestion time of CEL Ⅰ activity, and 40 ℃, 30 min was chosen for the CEL Ⅰ-based screening system. In all 2012 plants of the $M_2$ population, each of the four lines was merged into one DNA pool, producing 503 DNA pools. Finally, three mutants were obtained after the $M_2$ lines of *EDR*1 gene were screened using the optimized CEL Ⅰ enzyme system (Table 2), among which the Pro to Ser transition happened in extron. By the 1 485 bp of the *EDR*1 fragment length and 3 mutations we detected, according to the previous studies[35, 38], we obtain an average mutation frequency of 1 per 661 kb.

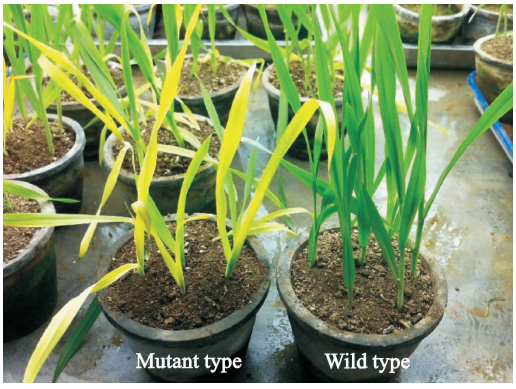



Fig. 3 Etiolated plants compared with normal plants

**Table 1 The phenotype variations of $M_2$**

| Type | Numbers |
|---|---|
| Etiolated | 115 |
| Dwarfing | 98 |
| Very early/late flowering | 101 |
| Less tilling, Small leaves, Sterility etc. | 35 |

**Table 2 Identified mutations in the *EDR*1 of $M_2$**

| Mutation | Transition of Amino acid | Position |
|---|---|---|
| C to T | Pro to Ser | 202 |
| G to A | Arg * | 251 |
| G to A | — | 295 |

Note: * This mutant didn't change the encoding of the Arg

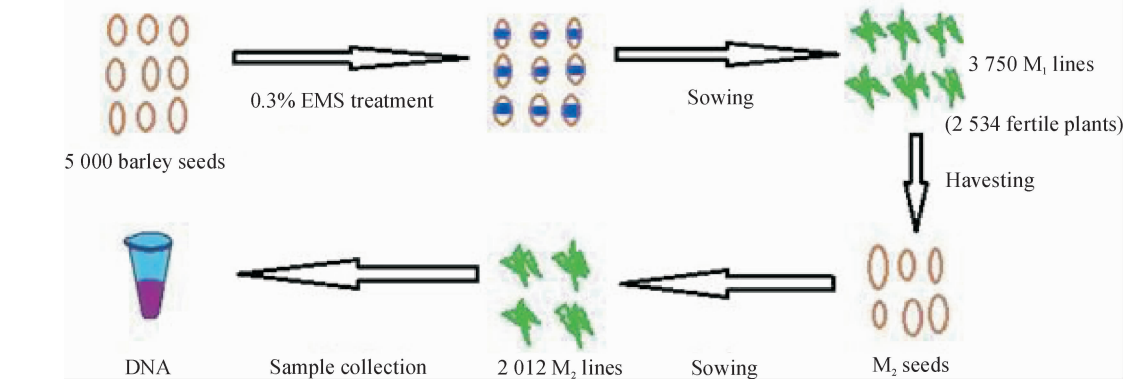
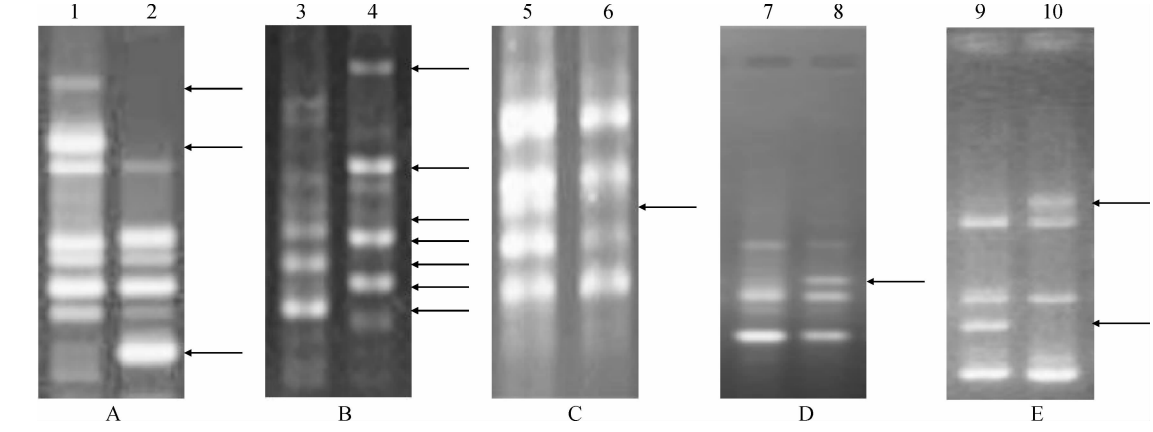



Fig. 2 Steps in the TILLING population procedure




A. S5; B. S31; C. S86; D. S284; E. S1429; 1, 3, 5, 7, 9. Wild type; 2, 4, 6, 8, 10. $M_2$ individuals. The arrows indicated variations

Fig. 4 RAPD profile produced in $M_2$ lines by the primers

## 3 Discussion

Triticeae crops are the most important human food resources in the world. However, the recalcitration of genetic transformation limits the development of relative functional genomics research. TILLING (Targeting induced local lesions in genomes) is a reverse-genetics method for performing functional genomics research and can be applied to both plants and animals[33]. EMS-induced genetic mutation is a powerful tool to generate and study mutants which have interesting characters or functions. The EMS-induced TILLING population is easy to maintain, and the mutagenesis is easy to identify. The TILLING technology induces diverse allelic variation, including missense mutations that can lead to altered protein function, as well as premature stop codons and splice site changes that can result in knockout or knockdown of a gene[39]. Our aim was to produce a population with relatively high mutation frequency and moderate size to avoid labor intensive costs. Therefore, we first investigated the optimal balance between mutation frequency and lethality in the development of a TILLING-population of barley. 0.3% was settled on after testing a series of different EMS concentrations; this gave a germination rate of 75.0% and a full sterile rate of 25.0%. In other related studies, the EMS concentrations used for wheat were (0.75%, 1.0% or 1.2%)[25], oats (0.9%)[40], maize (1.0%) and barley (0.25%－0.4%)[18, 28], while the EMS concentration used for rice was much higher (1.5%)[28]. The six-rowed Barley 'Xiyin2' is a mid-early maturity, high yield, weak winter barley variety, originally from Japan and introduced into China in 1980s by Northwest A&F University. It is mainly suitable for cultivation in the Huanghuai areas of China. By recording the phenotypes of the $M_2$ 'Xiyin2' TILLING population, about 17.35% phenotypic variation was observed, which is close to Sven Gottwald's "Barke" barley TILLING population (20%)[32]. According to other related studies on Triticeae crops, oat's visible phenotype variation is about 5%[40] while wheat's is only 0.5%[25]. Regarding the significant difference of the phenotype variation between barley, oat, and wheat, we presume that is because barley is a diploid plant in comparison to wheat and oats, which have relatively simple genomes. In wheat and oats, when one allele is mutated, other alleles in the genomes could compensate for the lost function[41].

In the present paper, two different approaches, RAPD and CEL Ⅰ-based screening, were used to detect the mutagenesis that occurred in the 'Xiyin2', and to estimate the mutation frequency at the DNA level, respectively. We detected stable polymorphism using five random primers. The specific genes of *EDR*1 were chosen as a model to estimate the mutation frequency of $M_2$, insofar as *EDR*1 mutation of *A. thaliana* results in resistance to powdery mildew caused by the fungus *Erysiphe cichoracearum*[42], and the *EDR*1 pathway is conserved in crop species[11] such as rice and barley. Subsequently, the *EDR*1 gene was isolated by positional cloning and was found to encode a putative MAP kinase kinase kinase (MAPKKK) which is similar to *CTR*1 (constitutive triple response), a negative regulator of ethylene responses in *A. thaliana*[11]. By adapting a CEL Ⅰ-based screening method to a barley TILLING population, mutations for the *EDR*1 target genes were identified. In total 1.485 kb were covered and 3 mutations were detected (Table 1) in 2 012 $M_2$ lines, by using 6% SDS-PAGE isolated digested products giving an average mutation frequency of 1 per 661 kb (with about a 0.25 kb mismatch happening at both ends). This is the same range as Gottwald and Hu's (1 mutation/500－862 kb)[32, 38].

Compared to other related studies, the mutation frequency of barley appears to be at about the same level as rice (1 mutation/530 kb)[28] and maize (1 mutation/500 kb)[26], but is much lower than that of wheat (1 mutation/24 kb)[25] and oats (1 mutation/33.3 kb)[40]. The reason for such differences remain unproven, but most likely, as earlier described, is because barley, rice and maize have fewer chromosomes and could tolerate less

mutagenesis before lethality occurs when a chemical mutation happens. In addition, the mutants in this study showed etiolation and senesce early after three-leave stage due to lacking of chloroplastids, however, if it is related to powdery mildew needs to be investigated further.

We choose the 0.3% EMS concentration to develop a TILLING population of the barley variety 'Xiyin2'. Among 2012 $M_2$ lines, we used two efficient methods to make sure mutations happened at the DNA level and estimated the mutant frequency is about 1 per 661 kb of *EDR*1 gene which is a key negative regulator of plant-powdery mildew interaction. By recording the phenotype variation of $M_2$, the mutations or genes of interest can be used in future forward or reverse genetics research.

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