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Target induced local leion IN genome (Tilling) and plant breeding

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Abstract: TILLING which stands for Targeting induced local lesions in genomes can be described as a general reverse-genetic strategy which is employed to locate allelic series of induced point mutations in the genes that are the target of our focus and interest. Identifying induced point mutations in a group of chemically mutagenize individuals is faster and more cost-saving by the highly effective TILING. It is not only the organisms which can be modeled by this technique but also the organisms which are economically significant among the plants can be used too. Admitting the fact that this technique has many various advantages such as simple procedure, high sensitivity, and high efficiency, TILLING can also present a strong approach for gene discovery, DNA polymorphism assessment, and plant improvement. A joined genomic resources, TILLING and Eco TILLING can be used instantly as a haplotyping tool in the domain of plant breeding to detect allelic variation in genes showing gene expression correlating with phenotypes and building an allelic series at genetic locations with the desirable specifications in germplasm or induced mutant.

Key words: *Tilling; Reverse genetics; Mutations; Plant breeding*

1. Introduction

Actually, the research domain of plant science has come to a new age of genomics by completing the project of genome sequence in Arabidopsis thaliana and rice (Oryza sativa L.) (Anonymous, 2000). In fact, the increased speed of saving the sequence information in public databases has led to a very urgent need to develop genome-scale reverse genetic strategies which are automated, widely applicable, with the capability of creating a wide range of mutant alleles required for functional analysis. Although most of the phenotypes are unknown, forward genetics can barely meet the demand of a comprehensive and large-scale survey of gene functions. TILLING which has been newly founded as a general reverse-genetic strategy contributes in locating an allelic series of induced point mutation in any desirable genes. Identifying induced points mutation in a population where the individuals are chemically mutagenized is quite fast and low cost by employing TILLING. Since TILLING contributes to locate an allelic series of induced point mutations, like missense and truncation lesions, we can't underestimates its usefulness both in organisms, for instance Arabidopsis, with complex gene knockout methods, and in organisms which do not have practical reverse-genetics tools, in which a knockout seems to be really of interest. All of the above merits shape TILLING an undeniably attractive strategy not only for a specific application but also for all range from basic to functional genomic study to practical crop breeding (Stemple, 2004).

2. Improvement in TILLING

Claire McCallum and colleagues were pioneers in presenting this strategy at the Fred Hutchinson Cancer Research Center in Seattle, WA, in the 1990s (McCallum, 2000). In a study, it was shown that TILLING is quite possible and they did it through identifying mutations of two chromomethylase (CMT) genes where the researchers used the traditional chemical mutagens to mutagenize Arabidopsis and found the mutations from the pooled DNA pulled out of each single plant [3]. Due to the easily scale up and high efficiency characteristic of TILLING as a type of reverse-genetics strategy, it has been quickly developed (Colbert et al., 2001); (Till, 2004). At first, the commercial 958 Acta Genetica Sinica Vol.33 No.11 2006 which changed the identity of high-performance liquid chromatography (dHPLC) was utilized to identify wrong matching in heteroduplex DNA produce by PCR amplification. Afterwards, a lower-cost faster modification of the TILLING protocol was released, which used a mismatch-specific celery nuclease, CEL1, joined to the LI-COR gel analyzer system which was quite suitable for this

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application (Oleykowski et al., 1998) Then the standard proposal was developed in 2001, and accordingly the practical software was investigated, the TILLING technique came to be a typical and customary method to identify mutations and finally satisfactory results were gained (Colbert et al., 2001). As the first stage of the highly efficient TILLING process, seeds are treated by a chemical mutagen (like ethyl methanesulfonate, EMS) to induce new point mutations. At first, the tissues of M2 plants extracted from each single M1 seed are gathered, and DNA samples taken from each plant are compiled to build a satisfactory representative population. For instance, in an 8-fold pooling, a 96-well plate was quite the same as 768 M2 individuals. In order to evaluate TILLING, two PCR primers get designed according to the particular area of a single target gene which is desirable by the researcher are labeled by 700 nm and 800 nm fluorescent dyes, respectively. Afterwards, the nature of PCR products which are produced from the dye-labeled primers and also pooled DNA of multiple individuals, are changed and re-annealed in order to let the mismatched base pairs shape. Following the formation of heteroduplex, the endonuclease, Cel I , which particularly identifies and separates mismatched DNA, is utilized to focus and zoom on the mismatched points and splits them. Then the broken pieces are separated by means of some denaturing electrophoresis gels and later identified in two separate channels by LI-COR gel analyzer system. The thorough length of the bands in the same band identified by various fluorescent dye channels ought to be the same as the length in the full-sized wild kind of bands at the end. The same method which suited the other individual mutations is employed for the rest of the bands on the electrophoresis gel. As PCR technique is employed, the bands are ordered to prove on the mutations. Then functional evaluation is utilized to analyze the identified mutations (Colbert et al., 2001).

3. Benefits of TILLING

3.1 Simple Technique

Reverse genetics is used to detect the function of a gene with known sequence by means of phenotypic analysis of cells or organisms where this gene functions defectively. Concerning the plants, insertion mutagenesis, anti-sense RNA, and double-stranded RNA interference (RNAi are the most frequently reverse-genetic approaches used. Despite this, we can't apply all these reverse-genetic methodologies to all organisms. For instance, T-DNA adjoin mutagenesis has changed the problem of obtaining a gene knockout to an in silico procedure for 70% of Arabidopsis genes (Alonso et al., 2003) but surprisingly, there's no comparable resource available for rice in spite of the easy access to its high-coverage genomic sequence. Like cereal model plants, rice has more than 200 000 T-DNA adjoin populations, but on the other hand only few reports are available about rice gene knockout by T-DNA insertion (An, 2005). In order to decrease expression of genes, Anti-sense RNA and RNAi techniques have been regularly employed (Graham, 1998). however, RNAi suppression merely produces some out puts which cannot be predicted. In addition, the entire procedure needs hard work due to its requirement to vector construction, transformation, and transgenic analysis (Que, 1998). From to the demands of high-throughput and of larger-scale of mutant detection the promise of using these reverse- genetic technologies is hampered. The TILLING technique is a mixture of the traditional chemical mutagenesis and the double-dye far-red fluorescent identifying technique. This technique does not need any complex manipulations and costly appliances. It provides the feasibility of displaying the mutant pools for studying the functions of special genes, and avoids the complicating gene separation process and boring tissue-culture process of anti-sense RNA and RNAi. Plenty of point mutants which were induced by chemical mutagenesis can produce series of mutant alleles relating to a particular gene. Incidentally, multi-allelic gene causes a decrease in inducing sub lethal mutant, and special genes function in a new way, like what WANG De-Kai et al. stated: "Application of TILLING in Plant Improvement 959 production of thermo-sensitive mutant and new phenotypes can be systematically investigated for gene functions (Thitamadee et al., 2002).

3.2. Great in sensitivity

McCallum and her colleagues showed TILLING was highly sensitive in their original study (Oleykowski et al., 1998). There were seven distinct PCR fragments in which their size ranged between 345 and 970 base pair in in the experiment studied for a total of 2 Mb of DNA sequence displayed by dHPLC to recognize mutations in CMT2 and CMT3 within 835 M2 plants in Arabidopsis. There were thirteen chromatographic alterations recognized to be mutations occurred by amplification and sequencing. There were not any PCR errors and accordingly showed an error rate of <10−6[1]. A combination of Cel I , double- end fluorescent dyes labeling and LI-COR system as an alteration to dHPLC was saved and it secured the modified efficient TILLING with high sensitivity. The appropriateness of celery (Cel I) to derive single-strand specific (sss) nucleases has been already proved for genotyping applications due to its ability to cleave almost all individual base-pair wrong matches being experimented in heteroduplexes extracted from various mutations whether in induced or naturally produced polymorphisms (Oleykowski et al., 1998). When multiple lanes exist in an individual channel, identifying the samples is completely easy since the identifying wavelengths are split by a wavelength of 110 nm in double

channel, which are 710 nm and 820 nm, respectively, without any mutual interference. Moreover, this method can identify the mutant by red dye quite clearly in big quantity. Distinguishing mutants from wild kinds is easy if the signal intensity which each of them produces differ from each other. McCallum compared the sensitivities of dHPLC and double-end labeling methods in his study, he could identify seven mutations in the same 8-fold pools in both approaches. Clearly, the LI-COR double end-labeling system for identifying Cel I cleavage products meets the requirements of large-scale mutant recognition.

3.3. High effectiveness

Actually the high efficiency of mutation-detecting characteristic of TILLLING can be linked to both high displaying capacity and also its high frequencies of the mutagenesis. In addition, estimating the densities of traditional chemical mutagenesis is quite possible. For instance, EMS, as a really stable alkylation which is usually used in order to induce point mutation in DNA generates primarily C to T changes which leads to mutation of C/G to T/A in Arabidopsis. Considering that all changes are C/G to T/A transitions, overall 5% of mutations will be a stop codon, 65% missense mutations, and 30% silent changes, in order (Colbert et al., 2001)); (Till, 2004). Ninety-nine percent of mutations from alkylation of guanine induced by EMS are reported as G/C-to-A/T transitions (Greene et al., 2003). Based on these results, the most suitable fragment is selected in a specific gene of interest. Due to the ability of chemical mutagenesis for inducing high density of mutations in several locus, genome wide saturated mutagenesis can be done by utilizing a rather small mutant population. Various species and receptors have different frequencies of mutagenesis and receptors [18]. In case the suitable mutagenic densities meet the requirements, then it will result in approving the size of mutant population. Based on some general estimations figured out by the Arabidopsis TILLING Project (ATP), almost 7 mutations can be detected per 1 Mb following screening the mutant Arabidopsis plant lines (Colbert et al., 2001). According to the afore-mentioned calculations, totally 10 000 mutant plants will get to the desirable mutant densities (Henik et al., 2004). Actually, integrating automatic sample-loading apartments, high-throughput gel, prominent LICOR analyzer system, and a standard commercial image processing program gives TILLING a really strong screening capacity, for example, the automated manipulation has been discovered in the ATP (Till et al., 2003). In case it is roughly 1 kb length for each given specific gene employed to target, 750 kb DNA sequence will be screened per gel altogether for 96 PCR reactions, as identifying 96-well plate equals to 96 M1 plants or 768 M2 plants (in 8-fold pools). Actually, each technician can simply carryout all of the operations as many as four gel runs in a day in Colbert's laboratory which is quite sufficient for screening the mutations in 3 000 plants. Imagining the rough rate of 10 000h mutation per genome as the highest amount observed in the ATP, this can be applied for over 960 Acta Genetica Sinica Vol.33 No.11 2006 20 mutations, sufficient enough to increase the probability better than at least one knockout lesion in a typical gene, further to allelic series of a dozen or even more missense mutations. TILLing with the rate of three to four genes in a day is feasible with standard 96-well pipettors, robotics substituting manual pipettors, PCR machines and four LI-COR scanners which lead to the increase in the capacity of screening to 16 runs per day.

4. Operation of TILLING

4.1. TILLING for gene detection

The first time in which the TILLING technique was used in Arabidopsis goes back to a workshop which aimed at developing TILLING as a service to the Arabidopsis community. It was better known as the Arabidopsis TILLING Project (ATP), which launched in 2001. Interestingly, ATP could identify, sequence, and deliver more than 1000 mutations in over 100 genes having ben ordered by the researchers in the first year of operating in public. Mutant materials, DNA samples, and mutant data were all completely shared by all researchers studying on Arabidopsis in the workshop. To analyze polymorphism, and to design primers for any kinds of organisms, and also to identify any types of mutagen according to DNA sequencing information which is counted as input for the users, CODDLE (standing for Codons Optimized to Detect Deleterious Lesions) was developed as a general tool to be employed for. Concerning TILLING and polymorphism analysis, it is quite necessary to evaluate the influence of missense mutations. For instance, in case special DNA sequence as long as 1 kb is aimed, CODDLE can assess whether a missense mutation may probably effect on the encoded protein or not. It can be totally efficient; for example, predicting the conservation- based SIFT program is quite feasible with the rough accuracy percentage of 75% accuracy, regardless of the change in amino-acid leading to a damage to a protein (Ng and Henikoff S, 2003). It has been proved that the ATP non-stop operation is a successful case for applying the TILLING in model system and it also encourages a boarder use of the technique for the other organisms. In fact, there has been the convenient access to well-developed and tested protocols for both genetic model organisms, like Arabidopsis (Till et al., 2003), (McCallum, 2000). Lotus japonicas (Perry et al., 2003) and some major crops, like maize (Zea mayz L (Till et al., 2003) wheat (Triticum aestivum L.) (Slade et al., 2005) and rice (Wuet al., 2005) Due to the prominent advantage of the model plant of legume, L.japonicus is highly useful in the studies focusing on root symbiosis with rhizobia and arbuscular mycorrhizal fungi, compound-leaf

development, and aspects of flower development. Perry and colleagues could get almost 45 600 M2 progeny out of 4 190 EMS-mutagenized M1 plants of L. japonicas and single isolated mutants influencing on metabolism, morphology, and also the root-nodule symbiosis. This makes the assembly of trait-specific or theme-based TILLING populations rich for mutants which are influenced in a special developmental process (Perry et al., 2003). Another application of TILLING is in maize, which is besides being an important crop; it also has a large genome in spite of its limited reverse-genetic resources presently accessible. Screening the pools of DNA samples was carried out targeting mutations in 1 kb segments out of 11 genes, and also 750 polle-mutagenized maize plans were selected as the population from which 17 independent induced mutations were drawn (Till et al., 2004) Interestingly, the result gained from maize complied with the one from Arabidopsis, showing that TILLING can be broadly applied as an efficient reverse- genetic strategy for large genome. Moreover, this strategy was also successful to create and also detect genetic variation in a major plant: wheat and it therefore proved to be potentially suitable tool for genomic research in polyploidy plants (Slade et al., 2005). Collaborating with ATP specialists Till, Comai, and Henikoff, the researchers working on rice at the International Rice Research Institute (IRRI) attempted to screen pooled DNA samples out of almost 2 000 lines having been induced by the 0.8% and 1.0% EMS. In their study, independent mutations could be identified in two genes out of 10 genes which were screened in the research: "pp2A4 encoding serine/ threonine protein phosphatase catalytic subunit and cal7 encoding callose synthase, yielding a mutation density of approximately 0.5 mutation per Mb. Sequencing mutated loci confirmed that they were G/C-to-A/T transition mutations" (Wuet al., 2005).

4.2 TILLING for identify DNA polymorphism

Actually, there is big number of DNA polymorphism in different species, and they clearly play a major role in biological WANG De-Kai et al.: "Application of TILLING in Plant Improvement 961 evolution". At the present, there are some methods applicable to identify DNA polymorphism including DNA sequencing, single-strand conformation polymorphism (SSCP), hybridization, and microarray. However, these methods have both advantages and limitations. In spite of the simple and clear characteristic of DNA sequencing, it is to some extent expensive and also time-consuming. SSCP prepares a highly efficient strategy for polymorphism identification, although it is less efficient in identifying new mutations limited to 200 to 300 bp length of aimed DNA sequence. Concerning Microarray, we can't deny the two disadvantages, one of them is the costly operation, and another one is the low identifying-frequency of about less than 50% (Tillib and Mirzabekov , 2001)., (Borevitz et al., 2003). Afterwards, another strategy was developed on the basis of TILLING which was called EcoTILLING. It referred to an Ecotypic TILLING strategy. Actually, it was developed to identify DNA polymorphism existing in naturally generating mutations (Comai et al., 2004). Eco TILLING is able to identify DNA variations out of individual nucleotide polymorphism (SNP), inserting little fragments, and omitting simple sequence repeat pattern (SSR). EcoTILLING can act like an efficient, cost-saving, with high-accuracy approach in comparison with the other methods explained before. Since it is only the sequencing of the unique haplotypes which needs to specify the exact nucleotide polymorphism at a locus, SNP identification can be consequently cost saved by Eco-TILLING, AND IT ONLY NEEDS a small amount o the total cost spent in the conventional approach of sequencing a genetic locus in each individual. Utilizing EcoTILLING helped Comai and colleagues to discover 55 haplotypes out of 150 individual plants in five genes which their sequences ranged from the ones differing by a single-nucleotide polymorphism to the ones showing complicated haplotypes (Comai et al., 2004). Detecting natural genetic variants among the species can lead to more information about gene function for many species like the tree Populus trichocarpa whose entire genome sequence data can be accessed, but it's not consistent with classical mutagenesis and genetic analysis. Moreover, it is also efficient for relating mapping and linkage disequilibrium analysis (Gilchrist, 2005).

4.3 TILLING for crop improvement

It has been proved that the production of many varieties like high-yielding rice, barley, and wheat is influenced by conventional mutation breeding, whether by radiation or chemical remedies [26]. In difference to conventional mutation breeding where the mutation frequency is not clear or it can be merely estimated from mutations transferring a visible phenotype, we see TILLING which prepares a direct measure of induced mutations. Moreover, TILLING not only lets a quick, parallel clear imaging of several genes but also it forecasts the number of alleles to be detected based on the mutation frequency and the size of the library. In fact, TILLING develops reverse genetics to mutation breeding, which is an over 50 year approach for improving crops. In an article published in Nature Biotechnology by Slade and colleagues in Anawah, a company where the activities were concentrated on the commercial applications of TILLING, employed a technique to recognize vast allelic series of the waxy genes existing in both hexaploid bread wheat and tetraploid pasta wheat to show the strength of TILLING to improve thecrops practically. The waxy genes, encoding granule bound starch synthase (GBSSI) waxy starches, are completely or almost completely made of amylopectin. They have low amount or even nothing of amylose and hold their unique physiochemical characteristic beside the

economically beneficial functional properties. A mutation frequency of one in 24 kb was discovered in hexaploid wheat and another frequency of 40 kb was recognized in tetraploid wheat in Slade's study. This resulting frequencies is roughly 5 folds more than what was found out in Arabidopsis. The researchers of the afore-mentioned study explained that the mutagenized population they were experimenting on had a small number of obviously visible phenotypes associated with the mutagenesis. Then they hypothesized that it was due to genetic redundancy which was inherent in this polyploid crop. As a result of this considerable mutation frequency, Slade and colleagues could detect 196 novel alleles in the A and D genome waxy genes just in 1 152 single plants could be screened in the hexaploid TILLING population they were studying, and further to this, 50 new alleles were identified just in 768 single ones of the TILLING population of tetraploid pasta wheat. Actually, these allelic series in hexaploid and tetraploid wheat comprised of multiple truncation and splice junction mutations like many missense mutations which have some foreseen harmful influences on the activity of the waxy enzyme. Fortunately, these new alleles existing in GBSSI of wheat provide a useful resource for growing, and improving different waxy wheat from partial to the whole. However, another significant point is this work prepares proof-of-concept for TILLING other genes which their modifications may be of interest in wheat or any other crops (Ahloowalia et al., 2004) 962 Acta Genetica Sinica Vol.33 No.11 2006

5. Conclusion

TILLING has been practiced as a new reverse-genetics technique since it was first created. TILLING has been proved to be a technique with a strong potentiality for crop improvement. It implies an extension of the using spontaneous and induced mutants in plant breeding. It provides the facility of direct recognition of beneficial nucleotide and amino-acid changes in genes which have known functions, they are used use as the genetic markers in order to select. It is not probably easy to find the range of alleles, developed by TILLING within a short time anywhere else in the pool of germplasm which is quite available for the plant breeders, from landraces to undomesticated relatives. The results obtained from the basic scientific research can be effectively adapted to crop improvement when the new data about the function of potential gene target gets accessible. We can enumerate at least two instant applications in plant improvement by the use of TILLING and EcoTILLING as a haplotyping tool for identifying genetic loci which are commonly linked with agronomically major specifications. Detecting allelic variations in genes showing expression which correlate or co-segregate with phenotypes is actually the first application. In this way gene expression will be connected to DNA variation. Resolving the major problems of discovering DNA variation on the basis of restriction-site polymorphism or connection to hypervariable markers like SSR will be more efficiently facilitated by identifying haplotypic variation generated by SNP or small indels. Concerning the second application, we can see establishing an allelic series at genetic loci with desirable specifications in germplasm or induced mutants. These Allelic series in such locations will put strong evidence on confirming the relationship between the phenotypes and candidate gene sequences. A broad group of alleles at a locus will show patterns of relation to comprehend the functional importance of special SNPs. It has been lately recommended that the recent developments in the field of plant molecular biology and plant genomics can result in a new Green Revolution. On the other hand, we should remember that these discoveries require being included in novel cultivars to comprehend the potentiality. TILLING which is a uniquely prominent approach utilized for genome-wide functional genomics, paired with the other lately developed genomic resources comprising detailed genetic maps, numerous of ESTs, deep-coverage large insert libraries with broad counting assemblies, both focused on entire genome sequencing and annotation, give the high efficiency in detecting gene(s) controlling phenotypes in both model systems and the prime plants which are economically beneficial. Applying TILLING seems to have much more direct or indirect advantages in near future.

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