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ALLELE MINING AND ITS IMPORTANCE IN SEED QUALITY

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ABSTRACT

Enormous sequence information is available in public databases as a result of sequencing of diverse crop genomes. It is important to use this genomic information for the identification and isolation of novel and superior alleles of seed quality. These important genes from different gene pools to suitably deploy for the development of improved seeds with better quality attribute. Allele mining is a promising approach to dissect naturally occurring allelic variation at candidate genes controlling key traits which has potential applications in seed improvement programs. It helps in tracing the evolution of alleles, identification of new haplotypes and development of allele-specific markers for use in marker-assisted selection. Realizing the immense potential of allele mining, concerted allele mining efforts are underway in many international crop research institutes. This review examines the concepts, approaches and applications of allele mining in relation to the seed quality attributes. How best we can use allele mining in improving the quality attributes of seed.

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INTRODUCTION

Progress in plant breeding in terms of development of superior and high yielding varieties of agricultural crops was made possible by accumulation of beneficial alleles from vast plant genetic resources existing worldwide. Still, a significant portion of these beneficial/ superior alleles were not utilized as these were left behind during evolution and domestication. This untapped genetic variation existing in wild relatives and land races of crop plants could be exploited gainfully for development of agronomic ally superior cultivars. Introgressions of novel alleles from wild relatives of crop plants into cultivated varieties (deVicente and Tanksley, 1993; Xiao et al., 1996; 1998; McCouch et al., 2007) have clearly demonstrated that certain alleles and their combinations potentially make dramatic changes in trait expression when moved to a suitable genetic background by overcoming the genetic bottlenecks which restricted their introgression to cultivars. Hence, the vast germplasm resources need to be relooked for novel alleles to further enhance the genetic potential of crop varieties for various seed quality traits. Enormous progress has been made in the last 15 years in depositing an exponential amount of sequence information into Gene Bank (Chan, 2005; Mardis, 2008).

With rapid accumulation of sequence and expression data in various genomic databases, accelerated discovery and annotation of new genes can be expected which would enable the development of allele-specific markers (Spooner et al., 2005). Based on gene and genome sequences, polymerase chain reaction (PCR) strategies are devised to isolate useful alleles of genes from a wide range of species (Latha et al., 2004). This capability enables direct access to key alleles conferring resistance to biotic and abiotic stresses, greater nutrient use efficiency, enhanced yield and improved quality. Using novel genomic tools, similar alleles responsible for a given trait and their variants in other genotypes can be identified. This is often referred to as 'dissection of naturally occurring variation at candidate genes/loci' or simply 'allele mining'. Identification of allelic variants from germplasm collections not only provides new germplasm for delivering novel alleles to targeted trait improvement but also categorizes the germplasm entries for their conservation. Realizing the importance of allele mining in traits like resistance to biotic and a biotic stresses, greater nutrient efficiency, enhanced yield and improved quality. Now we are giving more emphasis on the traits like enhanced yield and improved seed quality.

Seed quality

In general terms we will define the seed quality as degree of excellence in regard to the seed characteristics that determine quality, the standards fixed for certified seeds are considered

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as quality standards. It implies that if seed lot meets the certification standards it is good quality seed. The seed quality characteristics are Improved variety which is superior than existing ones, Genetic purity it should be -true to type, Physical purity (seeds of same kind), Seed germination and vigour, Planting value, Freedom from weeds and other crop seeds, Seed health, Seed moisture, Other characteristics like seed size, weight, specific gravity and seed colour. There will be different parameters quality in terms of different people, as seed analyst quality attributes are those which we discussed earlier, for normal purchaser the seed quality means good uniform colour without any contamination, in relation to a breeder the quality of seed means

1. marketable seed (the characters like Freedom from defective, shriveled (or) diseased seed, It involves development of cultivars with bright seed colour, Plump seeds, Commercial cultivars have yellow seed coats, High germination) and resistance to field weather conditions (pre harvest injury by seed pathogens, mechanical damage to harvest seed, improper storage causes, seed deterioration rapidly, selection for hard seed coat and resistance to fungal sps infection, reducing the field weathering, pre harvest pathogen injury).
2. Protein and starch content and quality.
3. Oil content and quality
4. Food and industrial uses

Knowing the importance of allele mining in genomics-driven plant breeding era, we discuss the concept of allele mining and its strategies along with the prospects and challenges.

Allele mining

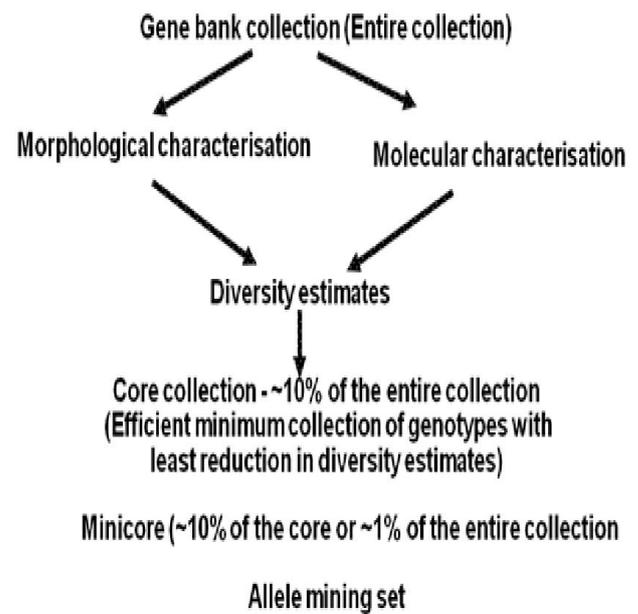
Before undergoing in detail about allele mining, we should know the allele first, An allele is one of two or more forms of a gene or a genetic locus (generally a group of genes). Mining is nothing but searching the new alleles in the wild germplasm. Allele mining is a research field aimed at identifying allelic variation of relevant traits within genetic resources collections.

Evolution of new alleles

Mutation is considered as an evolutionary driving force which underlies existing allelic diversity in any crop species. For creation of new alleles or causing variations in the existing allele and allelic combinations, mutations in the genic regions of the genome either as single nucleotide polymorphism (SNP) or as insertion and deletion (InDel) are important. The mutations in coding regions and/or regulatory regions may have tremendous effect on the phenotype by altering the encoded protein structure and/or function while those that occur in noncoding regions of a gene could often be silent without any effect on the phenotype. Even though most of the mutations are deleterious, in general 0.1% of the mutations are supervital leading to alterations in gene function which may be highly necessary for the survival of the plant (Singh, 2005)

Steps in the development of allele mining set

From the gene bank collection we have different allele resources that we called as entire collection, from the entire collection, characterisation should be done by morphologically



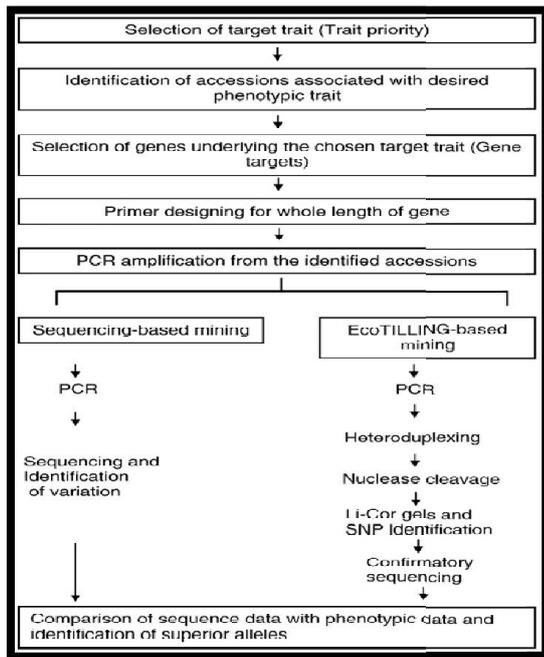
and molecularly then we should estimate the diversity, then we make entire collection in to core collection (10% of entire collection) then we should go for diversity analysis for core collection, and develop mini core collection (10% of core collection or 1% of entire collection) in this way we should develop allele mining set.

'True' allele mining

Initial studies of allele mining have focused only on the identification of SNPs/InDels at coding sequences or exons of the gene, since these variations were expected to affect the encoded protein structure and/or function. Ample examples are available to demonstrate the effect of such sequence variations in genic regions in altering the phenotypes. However, recent reports indicate that the nucleotide changes in non-coding regions (5' UTR) including promoter, introns and 3' UTR) also have significant effect on transcript synthesis and accumulation which in turn alter the trait expression. Role of intronic mutations in gene regulation was evident in the expression of some genes like tubulin (components of microtubules) (Fiume *et al.*, 2004) and rubi3 (polyubiquitin gene) (Samadder *et al.*, 2008) in rice as well VRN-1 (which affect vernalization response) in barley and wheat (Fu *et al.*, 2005). A mutation in 5' splice site of the first intron of the waxy (Wx) gene had resulted in tenfold increase in the gene activity in rice (Isshiki *et al.*, 1998).

Intronic mutations including insertions of transposons or differential expansion of microsatellite repeat sequences were correlated with alteration of gene expression (Sureshkumar *et al.*, 2009). In recent years, mining for sequence variation especially in regulatory region of genes is gaining more importance in context of gene expression. Since, it is well known that promoter elements play a key role in gene regulation and any changes in their sequences will dramatically influence gene expression resulting in variable trait expression. Such differential gene expression is usually due to sequence polymorphism in cis-acting/trans-acting regulatory elements binding sites present in upstream region.

Steps involved in allele mining:



A comparison between ecoTilling and sequence based allele mining given in the Table 1

Parameters	EcoTilling	Sequencing-based allele mining
Technical expertise	Requires high technical expertise starting from DNA pooling, to detection of cleavage of hetero duplexes	Require less expertise with direct sequencing of PCR products
Complexity	More	Less
Efficiency	Less efficient due to high chances of false positives, nonspecific cleavage and chances of non-detection in DNA pools	Highly efficient since it involves one step direct identification of sequence variations.
Utility	Proposed as effective in detection of SNPs rather than InDels	Effective in detection of any type of nucleotide polymorphism
Cost per data point	Comparatively high as it needs the intervention of confirmatory sequencing step	Comparatively less cost is involved
Time	Requires more time especially for sample preparation	Comparatively less time is required
Throughput	Associated complexity reduces the throughput and only less samples can be processed	Throughput and sample size increases with massively parallel sequencing platforms

Approaches

Two major approaches are available for the identification of sequence polymorphisms for a given gene in the naturally occurring populations. They are (i) modified TILLING (Targeting Induced Local Lesions in Genomes) procedure called EcoTilling and (ii) sequencing based allele mining.

EcoTilling

TILLING is a technique that can identify polymorphisms (more specifically point mutations) resulting from induced mutations in a target gene by heteroduplex analysis (Till *et al.*, 2003). A variation of this technique, EcoTilling, represents a means to determine the extent of natural variation in selected genes in crops. The method is essentially same as TILLING except that the mutations are not induced artificially and are detected from naturally occurring alleles in the primary and secondary crop gene pools (Comai *et al.*, 2004; Comai and Henikoff, 2006). Like TILLING, EcoTilling also relies on the enzymatic cleavage of heteroduplexed DNA (formed due to single nucleotide mismatch in sequence between reference and test genotype) with a single strand specific nuclease (i.e., Cel-1, mung bean nuclease, S1 nuclease, etc.) under specific conditions followed by detection through Li-Cor genotypers (Li-Cor, USA). At point mutations, there will be a cleavage by the nuclease to produce two cleaved products whose sizes will be equal to the size of full length product. The presence, type

and location of point mutation or SNP will be confirmed by sequencing the amplicon from the test genotype that carry the mutation. Although TILLING and EcoTilling were proposed as cost effective approaches for haplotyping and SNP discovery, these techniques require more sophistication and involve several steps starting from making DNA pools of reference and test genotypes, specific conditions for efficient cleavage by nuclease, detection in polyacrylamide gels using LiCor genotyper and confirming through sequencing. Recently, a rapid and cost-effective method for detecting novel allelic variants of known candidate on agarose gels and its utility in candidate gene mapping has been described (Raghavan *et al.* 2007). We can anticipate that the cost of EcoTilling can be significantly reduced by the adoption of such innovative strategies for allele mining.

Sequencing-based allele mining

This technique involves amplification of alleles in diverse genotypes through PCR followed by identification of nucleotide variation by DNA sequencing. Sequencing-based allele mining would help to analyze individuals for haplotype structure and diversity to infer genetic association studies in plants. Unlike EcoTilling, sequencing-based allele mining

does not require much sophisticated equipment or involve tedious steps, but involves huge costs of sequencing.

Considerations for allele mining

s.no	Activities/steps	Criteria for selection/design
1	Germplasm identification	Reference collection representing maximum genetic diversity in a minimum possible number.
2	crop species	Availability of genetic map, segregating populations, ESTs or gene/genome sequences Relatedness to model species for which target gene sequence is available Availability of comparative map/sequence co-linearity Availability of mini core collections or 200–300 reference accessions covering all varietal groups selected from mini core
3.	Target trait	Traits of interest
4	Phenotypic characterization	Precise phenotyping using efficient protocols in suitable environmental condition
5.	Candidate gene targets	genes with known homologues in related crops within species genes for important agronomic traits
6.	Primer design	may be suitable for amplification of the homologs in related species Covering the coding and upstream regulatory regions
7.	Database of candidate gene diversity	Well classified and easily accessible database for each nucleotide polymorphism associated with trait expression

Tools required for allele mining

An important step in allele mining, irrespective of the methods used, essentially involves the identification of polymorphism by comparative analysis of sequences of various genotypes. Several software tools are available for handling the complex nucleotide data, prediction of putative functional or structural components of complex macromolecules, prediction of transcription factor binding sites, identification of sequence polymorphisms and to predict the amino acid changes which are responsible for changes in encoded protein structure and/or function. These tools simplify the access and analysis of DNA sequence; help in identification of sequence polymorphism, and probe whether the variation correspond to the variation in TFBMs. These tools are also useful to identify the over-represented motifs in the upstream regions while delimiting the core promoter region and such identification narrows down the focus to identify new variation in regulatory elements. A typical output of many such computational methods will be a list of motifs, which are most likely to correspond to the actual binding sites, along with a large number of random variations. It should be noted that, the computational based predictions depend on the pre-designed algorithms which are largely developed based on the pre-characterized sequences available in the databases. Hence, such predictions should always be validated through systematic wet-lab experimental approaches.

Applications of allele mining

1. Gene prediction
2. Expression study
3. Evolution study
4. Discovery of superior alleles
5. Identification of new haplotypes
6. Similarity analysis –inter and intra species
7. Functional molecular marker development for MAS

Challenges in allele mining

Considering the huge number of accessions that are held collectively in various gene banks, genetic resources collections are deemed to harbour a wealth of undisclosed allelic variants. Now the challenge is to efficiently identify and exploit the useful variation for crop improvement. Here, we describe the challenges in allele mining and suggest the ways to overcome them in order to increase the efficiency.

Selection of genotypes

The foremost challenge in unlocking the existing variation is the selection of germplasm to be 'mined'. Given a reliable protocol for characterizing a gene, screening the entire collection would certainly be helpful to find rare alleles, but this is an enormous and inefficient way of screening. Hence, we need to find a way forward from genotyping the whole composite collections to efficiently screening the accessions to discover new alleles and allele combinations while minimizing the number of accessions to be screened. Several approaches through which the prioritization of genotypes for allele mining can be done are described they are:

- 1) Development of core/mini core collections
- 2) Accurate phenotyping methods
- 3) Flexible computational tools

Handling genomic resources

To keep pace with rapid accumulation of nucleotide and gene expression data, computational tools need to be developed for analyzing the functional nucleotide diversity and to predict specific nucleotide changes responsible for altered function. Exploiting the developments in allele mining, association genetics and comparative genomics by combining expertise from several disciplines, including molecular genetics, statistics and bioinformatics is the suggested way

Demarcation of promoter region

Promoters and regulatory elements are spread across the upstream region and their locations are variable from one gene to another. There is a need to develop specific software tools which can accurately predict the core promoter region based on the representation/over-representation of regulatory motifs.

Characterization of regulatory region

In contrast to identifying variation in coding regions of the genome, characterizing the extent of cis-acting regulatory variation presents a much greater challenge, since it is not possible to discern this even in the fully sequenced genomes. Screening for regulatory variants based on differences in transcript levels between individuals is confounded by potential trans-acting factors or environmental differences (Dai *et al.*, 2007). As the promoter regions can function in bi-directional pathway, prediction of the orientation will be difficult even by the software's.

Higher sequencing costs

One of the important challenges is to minimize the time and efforts required while reducing the cost per data point. These challenges may partly be overcome by resorting to cheaper and faster sequencing platforms for high throughput detection of allelic variations. Allele mining in relation to the seed quality can be best explained by taking rice, the number of superior alleles found in rice by different authors summarizing below:

Since rice is the first crop species sequenced, superior alleles like

1. Sh4 for grain shattering (Li and Sang *et al.*, 2006)
2. Rc7 for grain pericarp color (Sweeney *et al.*, 2007)
3. Wx for granule-bound starch synthase (GBSS) (Wang *et al.*, 1995)
4. GS3 grain size/shape (Fan *et al.*, 2006)
5. Disease resistant alleles like bacterial leaf blight resistance gene Xa21 from *Oryza longistaminata* (Khush *et al.*, 1991).
6. blast resistance genes like Pi9 from *Oryzaminuta* (Sitch *et al.*, 1989; Amante-Bordeos *et al.*, 1992)
7. Pi40 from *Oryza australiensis* (Jeung *et al.*, 2007)
8. Trait-enhancing alleles like grain filling GIF1 (Wang *et al.*, 2008)

These are important superior alleles from different germ plasms of rice. So, incorporating these alleles in to the desired cultivar, we can enhance the better quality parameters, and efficiency of crop can be increased.

Seed quality: It is the degree of excellence in regard to the seed characteristics that determine the seed quality. Generally, the standards fixed for certified seeds are considered as quality standards. It implies that if seed lot meets the certification standards it is good quality seed. The good seed have these quality attributes they are

- Improved variety- superior than existing ones
- Genetic purity -trueness to type
- Physical purity - seeds of same kind
- Seed germination and vigour
- Planting value
- Freedom from weeds and other crop seeds
- Seed health
- Seed moisture
- Other characteristics like seed size, weight, specific gravity and seed colour.

Status of allele mining in crop plants

S.No.	Crop	Allele/locus	Trait/name of the protein	Author
1	Aegilops sp.	GluDy y	subunit of glutenin	Giles and Brown (2006)
2	Agropyron sp.	HMW-GS	Endosperm storage proteins	Liu <i>et al.</i> (2008)
3	Barley	Bmy1 β -amylase I —	starch break down enzyme	Chiapparino <i>et al.</i> (2006)
4	Barley	Gpc-B1	Grain protein content	Distelfeld <i>et al.</i> (2008)
5	Phaseolus sp.	Lectin locus	Storage and defense proteins	Lioi <i>et al.</i> (2007)
6	Potato	Rpi-blb1	Late blight resistance	Wang <i>et al.</i> (2008)
7	Rice	Badh2	Fragrance	Amarawathi <i>et al.</i> (2008)
8	Rice	Pi ta	Blast resistance	Huang <i>et al.</i> (2008)
9	Rice	wx locus	Granule-bound starch synthase	Mikami <i>et al.</i> (2008)
10	Rice	Rc locus	Pericarp color	Brooks <i>et al.</i> (2008)
11	Soybean	SKTI	Soybean Kunitz trypsin inhibitor	Wang <i>et al.</i> (2008d)
12	Tomato	Pto	Disease resistance	Rose <i>et al.</i> (2005)
13	Wheat	Pm3	Powdery mildew resistance	Yahiaoui <i>et al.</i> (2006)
14	Wheat	Wx-A1	Waxy gene and amylose biosynthesis	Saito and Nakamura (2005)
15	Wheat	SSII	Endosperm starch synthesis	Shimbata <i>et al.</i> (2005)
16	Wheat	Wx-B1	Waxy protein	Monari <i>et al.</i> (2005)
17	Wheat	PSY-1 and PSY-2	Grain yellow pigment content (GYPC)	Zhang and Dubcovsky (2008)
18	Wheat	Viviparous-1	Pre-harvest sprouting Tolerance	Xia <i>et al.</i> (2008)

Conclusion

allele mining can be visualized as a vital link between effective utilization of genetic and genomic resources in genomics. It is certainly expected that sequencing-based allele mining would emerge as a method of choice in revealing natural variations and in providing novel and effective alleles and would take center stage for all seed improvement activities.

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