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Review Article

Allele mining for resistance gene analogs (RGAs) in crop plants: A special emphasis on blast resistance in finger millet (*Eleusine coracana* L.)

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Abstract

Finger millet a nutritionally rich underutilized crop requires more attention of research community. One of the major limitations of finger millet for wider agronomic acceptability is because of its susceptibility to blast fungus *Magnaporthe grisea*, which is also the causative agent of blast in rice. A large amount of sequence data available in the public domain has facilitated identification and isolation of novel genes for blast resistance and other agronomically important traits. Availability of such large genomic data has made allele mining a viable approach for detecting novel alleles for blast resistance in finger millet. However, very scarce genomic information is available in finger millet, being the major hurdle for such approaches. In the present review, we have summarized different strategic approaches suitable for allele mining of resistance gene analogs (RGAs) in finger millet by utilizing the large sequence data available for rice through comparative genomics. This paves the way for transfer of blast alleles into high yielding, blast susceptible and locally well adapted germplasm through molecular breeding and genetic engineering approaches.

Key words: Allele mining, finger millet, resistance gene analogs, NBS-LRR

Introduction

Finger millet (*Eleusine coracana* (L). G) (2n = 4X = 36), belongs to the family poaceae and tribe Eragrostidae. It is cultivated widely in the arid and semi-arid regions of the world, especially in east Africa, India and in other Asian countries (Fakrudin et al. 2004). It is believed that Uganda or a neighbouring region is the centre of origin of *Eleusine coracana* and it was introduced to India, probably over 3000 years ago (FAO 1995). It is a rich source of essential amino acids, fats, minerals, calcium and fibre (Barbeau and Hilu 1993). Finger millet genome consists of two groups A and B. The A genome donor is believed to be *E. indica* (2n = 18), while there are conflicting reports on the B genome donor. The species *Eleusine tristachya* and *E. floccifolia* have been considered as probable B genome donors to *E. coracana* based on rDNA restriction patterns (Hilu and Johnson 1992). Recently, increasing attention is being given to improve finger millet due to its inherent capacity to tolerate several abiotic stresses including moisture stress, high nutritional quality parameters and its adaptability to marginal soils with low fertility.

The grain yield in finger millet is highly affected by blast disease in the most parts of the world including East Africa and Southern India. The causal organism *Magnoporthae griseae* also infects the rice crop. The fungus affects finger millet in all the stages of plant development, from seedling to grain formation however, finger and neck blast cause more severe economic losses. Blast is both, economically significant and very destructive, causing over 50%

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yield losses, especially in wet seasons. Hence, serious efforts are required to understand molecular mechanisms of disease resistance and identify genes responsible for the blast disease. Though, finger millet genome sequence is not yet available, either complete genome sequence (in case of rice) or large amount of EST data are available in other major cereal crops in the public domain. Hence, comparative genomics can be made use of in finger millet to dissect the highly useful and agronomically important traits like blast resistance.

The genome composition of finger millet is AABB and has a basic chromosome number x = 9 with the genome size of about 1.8 pg as estimated by flow cytometry (Mysore and Baird 1997). Recently, Dida et al. (2007) generated a genetic map of the finger millet genome using different types of markers like RFLP, AFLP, EST and SSR. The map spans 721cM on the A genome and 787 cM on the B genome and covers all 18 finger millet linkage groups, at least partially. A set of 82 SSR markers were developed from small-insert genomic libraries for facilitating marker assisted selection in finger millet.

A considerable amount of molecular work has been carried out since the establishment of molecular biology techniques (Dida et al. 2007; Babu et al. 2007; Panwar et al. 2010). With rapid accumulation of sequence data in various genomic databases, accelerated discovery and annotation of new genes can be expected which would enable the development of allele-specific molecular markers for resistance gene analogs (Spooner et al. 2005). This is often simply referred as 'allele mining'. Identification of allelic variants from germplasm collections not only provides new germplasm for delivering novel alleles into targeted trait improvement but also categorizes the germplasm entries for their conservation. Since, blast disease is a major economic threat to major crop like rice and also for finger millet, and realizing the importance of allele mining in genomics-driven plant breeding era, here we discuss the allele mining strategies for improving the blast resistance in finger millet by taking advantage of fully sequenced rice genome.

Exploiting available genetic variation for blast resistance through molecular analysis

The foremost aspect of allele mining is exploitation of the existing genetic variation for blast resistance in finger millet. A comprehensive exploration of potential genetic resources and exploitation of natural genetic variations are proven source of useful genomic information. A rich diversity of landraces can be explored for their desirable traits like blast resistance and can be further utilised to develop new varieties through molecular plant breeding approaches. Molecular marker techniques have revolutionised the cereal genomics and understanding of structure and behaviour of cereal genomes. This will pave the way towards the detection of novel and superior genotypes and meet the ever increasing demands of food grains.

There are a few reports available on diversity studies of finger millet using molecular markers such as RAPDs (Babu et al. 2007; Salimath et al. 1995), SSRs (Dida et al. 2007; Panwar et al. 2010; Dida et al. 2008), isozymes (Hilu 1995) and RFLP (Parani et al. 2001). Panwar et al. (2011) studied the functional marker based molecular characterization of resistance gene analogs encoding NBS-LRR proteins from a large collection of finger millet genotypes for association of NBS sequences with the blast disease reaction. The study established genetic relationships among the closely related genotypes and thus helped to build a more complete picture of the molecular characterization of finger millet genotypes. Characterization of genetic variation within natural populations and among breeding lines is crucial for the effective blast management in finger millet or any other target crops. Molecular markers linked to the blast resistance genes offer a powerful tool not only for identification of resistant genotypes from vast germplasm resources but also for marker assisted breeding of resistance loci and gene-pyramiding strategies.

Resistance gene analogs (RGAs) for blast resistance

Plant disease resistance often results from the presence of a specific resistance gene (*R-gene*) in the host and a corresponding avirulence (*Avr*) gene in the pathogen (Flor 1956). The isolation and sequencing of disease resistance genes from various plant-pathogen interaction models has increased our understanding of the biochemical basis for induced plant innate defence responses. Several R genes corresponding to race-specific interactions have been isolated by map-based cloning and transposon tagging in rice. The similarity in the resistance gene sequences among the closely related plant species, both at the nucleotide and amino acid levels, has made it possible to isolate such resistance gene homologues (RGHs) using PCR primers being designed based on the conserved domains (Michelmore 1996). Most of the R genes has a series of leucine-rich repeats (LRRs), a

nucleotide-binding site (NBS) and a putative amino-terminal signaling domain and are termed as NBS-LRR proteins. This NBS-LRR class share conserved domains and structural similarities across diverse taxonomic groups, and confer resistance to viral, fungal or bacterial pathogens. The availability of rice genome sequence enabled the global characterization of NBS-leucine-rich repeat (NBS-LRR) genes (Goff et al. 2002). Reddy et al. (2012) isolated resistance gene homologues from finger millet (*Eleusine coracana* L.) using degenerate oligonucleotide primers designed to the conserved regions of the nucleotide binding site of the previously cloned plant disease resistance genes. Of the 107 clones sequenced, 41 showed homology to the known R genes, and were denoted as *EcRGHs* (*Eleusine coracana* resistance gene homologues), while 11 showed homology to pollen signalling proteins (*PSiPs*), and were denoted as *EcPSiPs* (*Eleusine coracana* pollen signalling proteins).

Allele mining

The term 'Allele mining' refers to exploitation of natural genetic variation in candidate genes for important agro-morphological traits. There were several reviews on allele mining and hence we skipped the general discussion on allele mining (Ramkumar et al. 2010). Briefly, there are two common and widely used strategies (Eco tilling and sequencing based allele mining) for the identification of allelic variation for a given gene in the naturally occurring population. Eco Tilling is a procedure that involves enzymatic cleavage of heteroduplex DNA with a single strand specific nuclease like *Cel1* under specific conditions followed by detection through Li-Cor genotypers (Li-Cor, USA) or any other appropriate detection methods. Sequencing based allele mining involves amplification of target locus among highly diverse genotypes by PCR followed by identification of nucleotide polymorphism using sequencing technologies. It would help to analyze individuals for haplotype structure and diversity to infer genetic association studies in crop plants.

Status of allele mining in rice with respect to resistance gene analogs (RGAs) for blast resistance

Rice blast is a well-studied disease and several reviews have focused on the biology of the fungal infection (Caracuel-Rios and Talbot 2007) and rice blast resistance (Dida et al. 2007). Moreover, with the completion of the rice and *M. oryzae* genome sequences, rice blast disease has become a model for plant-pathogen interactions for monocotyledons. The disease causes about 90% yield losses in rice and up to 50% yield loss in finger millet. Although some blast resistant varieties have already been developed through conventional plant breeding approaches; the breakdown of blast resistance is a serious concern and the cause of yield instability in several rice and finger millet accessions grown across the world. Different molecular breeding strategies to breed durable resistance have been proposed to counter the blast evolution. Strategies like pyramiding (Banman et al. 1992), lineage exclusion and multilines, are based on the use of complete and specific resistance genes. Of the various strategies tried, enhancement of host-plant resistance is considered one of the important approaches to tackle the blast disease. Identifying superior alleles of effective resistance genes can be immensely helpful in increasing the degree of resistance and can be valuable in breeding durable resistance for blast disease (Ramkumar et al. 2010). Forty-eight R genes have been cloned from numerous plant species (*Arabidopsis*, rice, wheat etc.) using map based cloning and transposon tagging. More than 85 blast resistance genes and 350 QTLs have been reported in rice (Ballini et al. 2008; Ramkumar et al. 2008) used sequencing based allele mining to isolate novel alleles of PiK^h and Pita from landraces and wild *Oryza* species and studied their effectiveness against blast. They used 27 landraces collected from north eastern India and 127 accessions of different *Oryza* species collected from IRRI and screened them for resistance against blast disease using the most virulent pathogen NLR-1. Das et al. (2012) isolated an orthologue of *Pi54* i.e. *Pi54rh* from the blast resistant wild species *Oryza rhizomatis* using allele mining approach and validated by complementation. This novel gene belongs to the CC-NBS-LRR family of disease resistance genes with a unique Zinc finger domain and a role in signal transduction process. The different reports on the status of allele mining for blast resistance in rice are given in Table 1.

Allele mining RGAs influencing blast resistance in finger millet: concept and prospects

A large amount of genetic information in the public data bases will be useful for isolating novel alleles of important genes, particularly in crops like finger millet. This helps in development of gene specific molecular markers (Spooner et al. 2005). The finger millet genome sequence is not available, rather only very few EST sequences (1956) were available at NCBI database

Table 1. The details of the status of allele mining for blast resistance in rice

Allele/ locus	Trait	References
Pita	Blast resistance	Huang et al. (2008)
Pita	Blast resistance	Wang et al. (1999)
Pikh	Blast resistance	Ramkumar et al. (2008)
NBS-LRR class R-genes	Disease resistance of the plant	Yang et al. (2008)
Pi54 (*Pi-kh*)	Blast resistance	Sharma et al. (2005)
Pi9	Blast resistance	Qu et al. (2006)
Pi-km	Blast resistance	Fukuoka et al. (2009)

till June, 2015. Hence, comparative genomics plays very important role in such under-utilized crops like finger millet. Dida et al. (2007) developed the genetic map of finger millet genome, where they identified 82 SSR markers and Hittalmani et al. (2013) developed SSR markers for drought tolerance in finger millet. Comparative genomics is a powerful tool for genome analysis, annotation with an objective to understand the detailed process of evolution at the gross level and to translate DNA sequence data into proteins of known functions. The rationale here is that DNA sequences encoding important cellular functions are likely to be conserved between species than sequences encoding non-coding sequences. With the availability of full sequence of the major cereal crop rice, identification of genes influencing the blast disease resistance in finger millet and thus further crop improvement has become easier. In rye, for aluminium tolerance superior homologous alleles were isolated using syntenic allele sequence information from the well studied crop wheat. The same technique has been employed to isolate agronomically superior alleles in grasses like *Phaseolous vulgaris* (Fontecha et al. 2007). Genes and/QTLs controlling quantitative resistance to the blast pathogen showed high similarity in their sequence in both rice and barley, suggesting a common evolutionary origin for these resistance gene (Chen et al. 2002). Wang et al. (2008) compared allelic frequency with two late blight resistance genes in *Solanum*. Similar approaches can also be used for identification of novel alleles for blast resistance through syntenic relationship with the data available from rice. By using syntenic relationships between rice and finger millet, Babu et al. (2014) recently mapped genes for blast resistance through association mapping approach by using the EST sequences of NBS-LRR, *M. grisea* and *Pi* genes of rice. They found QTLs of finger blast and neck blast were linked to genes like *Pi5*, *Pi21*, *Pi-d(t)*, NBS-LRR and *M. grisea*. Hence, blast resistance genes like *Pi5*, *Pi21*, *Pi-d(t)*, NBS-LRR and *M. grisea* may be targeted for allele mining in finger millet. Functional markers based molecular characterization of resistance gene analogs encoding NBS-LRR disease resistance proteins in finger millet (*Eleusine coracana*) have also been done in our laboratory by Panwar et al. (2011). The following is the detailed procedure involving in the sequence based allele mining for blast resistance in finger millet.

Selection of genotypes

Screening of suitable genotypes is a very challenging task for allele mining and hence requires a reliable protocol for characterizing a gene for finding rare alleles. Hence, we need to have efficient protocols to screen genotype(s) from a whole composite collection to discover new alleles and allele combinations while minimizing the number of accessions to be screened. Development of core collections plays an important role in allele mining. Based on the phenotypic data, highly resistant and susceptible landraces and, highly diverse finger millet accessions should be selected for allele mining.

Development of core collections

A core collection is a subset of accessions from the entire collection that capture most of the available genetic diversity of the species. Upadhyaya et al. (2006a) proposed several parameters pertaining to geographic, agronomic and botanical descriptions for development of core and mini core collections in finger millet for screening the genotypes for effective allele mining. They considered different parameters like means, variances, frequency distribution, Shannon-Weaver diversity index (H') and phenotypic correlations, in such a way that the core subset represents the entire collection. These tests indicated that sampling was optimal and the diversity has been captured very well in the core subset. The correlation analysis indicated that panicle exertion and finger length could be given lower priority in the future germplasm evaluation studies for finger millet. Generally, 10% of the crop accessions constitute the core collection representing the variability of the entire collection. Molecular marker based mini core development of cultivars were reported for many crops (Upadhyaya et al. 2006b). The data obtained from morphological and

molecular characterization studies could be used to define the genetic structure of the global composite collection and to finally select a reference sample of approximately 300 accessions representing maximum diversity. Upadhyaya et al. (2008) suggested a reference set of 300 accessions based on molecular and quantitative traits for finger millet. From a collection of 300 accessions, a mini-core was developed for finger millet comprising of 84 accessions. These core and mini-core accessions can be used for allele mining studies in finger millet.

Selection of genes for target traits

Of the various blast resistance genes (*R-genes*) characterized, *PiK*^{h} and *Pita* are the two major genes with NBS-LRR regions that confer broad-spectrum resistance to blast in India. Identification and molecular characterization of rice blast genes and NBS-LRR regions containing blast genes, *PiK*^{h} (Madhav et al. 2005) and *Pita* genes (Bryan et al. 2000), will pave the way to detect naturally occurring allelic variation from a wide range of germplasm through allele mining. NBS-LRR disease resistance genes belong to a large and diverse super family of genes which act as receptors in signal transduction pathways that are triggered in response to pathogen attack. The NBS region is thought to be important for ATP binding and overall functionality of the R-gene product. Finding the sequences similar to these genes in finger millet will also be useful for allele mining for blast resistance in finger millet.

In silico and comparative genomic analysis for blast resistance genes in finger millet with rice

Search for suitable genetic information regarding allele mining for blast resistance in finger millet can be obtained from several publicly available data bases like NCBI and TIGR. The coding and non-coding sequences will help in finding the syntenic relationships with different related genera for blast genes and such an approach was effectively used in barley for blast resistance by Chen et al. (2002). Recently Srinivasachary et al. (2007) made a comparative analysis of finger millet chromosomes with rice chromosomes and showed most of the chromosomes were highly collinear with 85% synteny (Table 2).

Table 2. The syntenic relationship observed between rice and finger millet chromosomes*

Finger millet chromosome	Rice chromosome	% synteny
1A and 1B	1	85
2A and 2B	2 long arm, 10 long arm	
3A	3	91
4	4	48
5A, 5B	5, 12	93 and 54 respectively
6A, 6B	6,9	85-100
7A, 7B	7	85
8A, 8B	8	90
9	11	86

*Srinivasachary et al. (2007)

Primer designing, PCR amplification and sequencing

The selection of appropriate genes is followed by designing of primers for the whole length of the genes to amplify the entire loci of the blast genes in such a way that the forward primer targets the upstream region (500 bp before the transcription start site) and the reverse primer targets the 3' UTR region. Proper primer designing and optimization of the reaction condition for PCR plays crucial role in allele mining followed by cloning of the amplicons using suitable cloning vector for the purpose of sequencing. Otherwise selected markers which can differentiate the susceptible and resistant genotypes can be directly sequenced. The high quality sequences (Phred score of more than 20 per base) can be compared with the reported gene sequences using ClustalW software (www.ebi.ac.uk/Tools/clustalw/).

Identification of superior alleles

Superior alleles responsible for blast resistance, caused by the fungus *Magnaporthae grisea* can be identified by comparing the phenotypic data of the selected core germplasm with the sequence data. The structural features of the gene and amino acid sequence variation can be compared with the reported *PiK*^{h}, *Pita* and NBS-LRR genes. Babu et al. (2014) recently used comparative genomics approach in identifying the QTLs linked to blast resistance in finger millet through association mapping approaches. In their study, 58 SSRs were designed from 82 GenBank accessions representing the different genes influencing the blast resistance in rice and finger millet from CDS, 5'UTR, 3'UTR and intron regions of the sequences. They sequenced the alleles from both resistant and susceptible genotypes. The sequences from resistant

genotypes found high similarity with the rice blast *Pi* genes and NBS-LRR regions while the sequence obtained from the susceptible genotypes did not show any similarity with any of the NBS-LRR region. The amino acid sequence of finger millet resistant genotype was further compared with the previously cloned plant disease resistance genes which showed the characteristic NBS motifs of kinase-2 and kinase 3a (Fig. 1) of plant R-genes, confirming that the

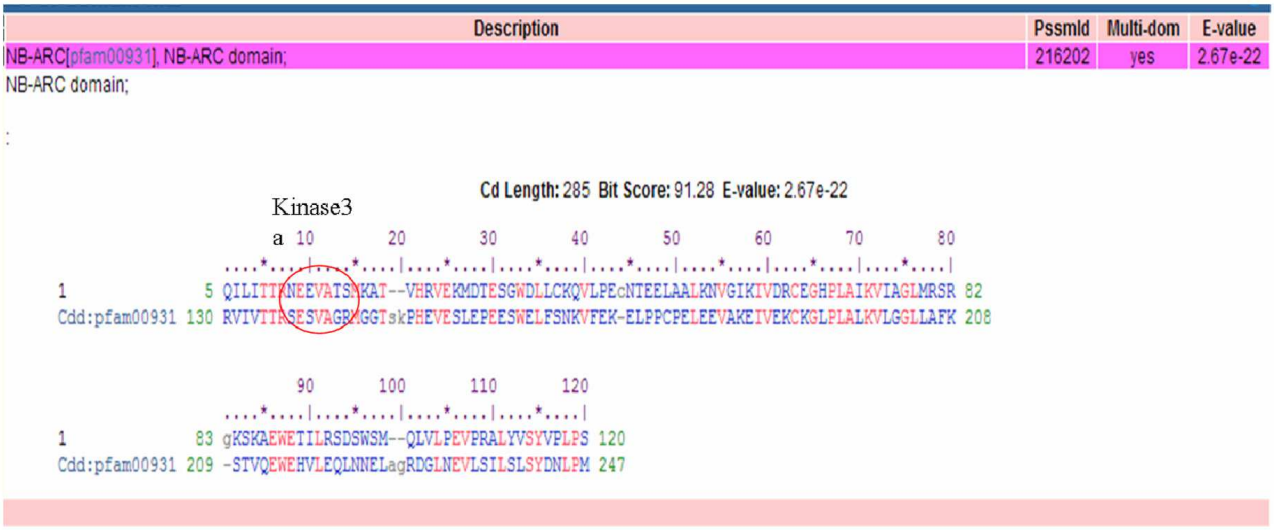

Fig. 1. The kinase3a motif of NB-ARC domain present in the sequence of finger millet genotype as obtained from CD domain search (obtained from Babu et al. (2014))

sequences characterized in their study belonged to the NBS-LRR gene super family. The rice genomic marker RM262 (*Pi d(t)*) and genic marker FMBLEST32 (*Pi 5*) were found to be linked to finger blast disease through association mapping. The study established genetic relationships among the closely related genotypes and thus help build a more complete picture of the molecular characterization of finger millet genotypes. Such a characterization of genetic variation within natural populations and among breeding lines is crucial for the effective blast management in finger millet or any other target crops.

Bioinformatics for allele mining

Selection of right bioinformatics tools for analyzing the available data is an important step for successful allele mining studies. Several software tools are available for analyzing the complex nucleotide data, prediction of putative structural or functional components of macromolecules, transcription factor binding sites, and sequence polymorphisms and to predict the amino acid changes which are responsible for changes in encoded protein structure and/or function. Different software like PLACE (plant cis acting regulatory DNA elements) (Higo et al. 1999), TRANSFAC (Matys et al. 2003), JASPAR (Bryne et al. 2008), MATinspector (Cartharius et al. 2005) are databases for transcription factor binding site and to identify the TF motifs, while the tools like W-AlignACE (Chen et al. 2008) and MEME (Multiple EM for Motif Elicitation) (Bailey et al. 2006) are used for motif discovery. The software BioEdit is used for nucleotide sequence analysis and ClustalW for sequence alignment. For primer designing different software are available like FastPCR (Kalendar 2009) for nucleotide sequence analysis and primer designing and, primer 3 (Rozen and Skaletsky 2000) for primer designing.

Conclusion

Due to its tremendous potential, allele mining can be visualized as a link between effective utilization of genetic resources and genomics driven molecular plant breeding. Screening of core germplasm and phenotyping are the two most important tasks that decide the effectiveness of allele mining along with efficient identification of core sets. Identification of sequence variation will pave the way to identify superior alleles and their use in MAS and crop improvement. As allele mining is an emerging field of molecular breeding, it will be very useful for those crops which have full/partial sequence information. However, in

case of neglected crops like millets, especially finger millet, there is a need to establish synteny relationships with similar species or genera for identifying similar genes responsible for agronomically important traits through comparative genomics which has proven to be a successful approach in crops like barley and some grasses. The proposed methodology of allele mining in this review will be highly useful for identification of superior/novel alleles responsible for blast resistance, and other important traits in finger millet.

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