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Monomorphic molecular markers are as informative as polymorphic molecular markers

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Abstract

Molecular markers have been assisting breeders in crop improvement. Judicious integration of molecular markers with conventional breeding practices has contributed numerous improved varieties. In the process of MAS, monomorphic markers are most often eliminated from further investigations assuming that they are non-informative. In this study, efforts were made to elucidate information from monomorphic markers. Twenty rice genotypes contrasting for grain zinc content were selected. Specific markers were designed to the already reported genes associated with micronutrient transport from soil to grain. Among designed markers, only OsZIP6c and OsZIP3b were polymorphic and Single-Marker Analysis revealed that OsZIP6c was associated with culm zinc content. Sequencing of amplicons of six markers including four monomorphic markers revealed that nucleotide variations were present in monomorphic markers as well. SMA for nucleotide variations revealed that OsZIP5b, a monomorphic marker on agarose, contained nucleotide changes significantly associated with grain zinc content. This clearly demonstrates that monomorphic markers are as informative as polymorphic markers and should not be eliminated from the study.

Key words: Bioinformatics, MAS, molecular markers, monomorphism, polymorphism, rice, sequencing, SMA, zinc

Introduction

Use of Molecular markers in the field of crop improvement has been a huge success in the recent past. Discovery of tools like PCR, sequencing, improvements in the fields of genomics, transcriptomics and proteomics have given further impetus [1]. As every molecular marker follow mendelian pattern of inheritance, it was very easy for plant scientists to employ them in selecting plants. Hence, the molecular markers tagged with a particular trait are used in selection of elite genotypes eliminating any influence of environment. To ease the selection process, molecular maps are constructed [2]. The first step in molecular map construction, QTL mapping and/or marker-assisted breeding programs is to screen a large number (different kinds) of molecular markers using set of parental lines. Monomorphic markers are usually eliminated from the further study. Most often, using biparental populations, maps are constructed, QTL tagging done and MAS practiced using polymorphic markers [3]. The monomorphic markers, which are eliminated from the study, could be the ones that are associated with the trait of interest. Some of the consequence related to this strategy is discussed in this paper with a practical suggestion to make better use of monomorphic markers and improve the chances of success.

Materials and methods

Research material comprised of ten genotypes with high and ten with low grain Zn content selectedbased on earlier studies [4, 5]using AAS (Atomic Absorption Spectrophotometer) and XRF (X Ray Fluorescence). The selected genotypes were grown during *Summer*-2013 in homogenously mixed soil. The fertilizers applied, irrigation and other management practices were similar for all the genotypes. Estimation of Zn content in the mature grains, culm and roots were done in XRF facility (Oxford Instruments, X-Supreme8000) available at MSSRF (M S Swaminathan Research Foundation, Chennai). For molecular marker analyses, DNA from all the 20 genotypes was isolated by using CTAB method [6].

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The nucleotide sequences of known Zn genes explored were downloaded by Nucleotide database of NCBI. Pick primer tool in NCBI (in collaboration with Primer3) was used to design primers specific to the genes targeted [7]. PCR (Eppendorf, Mastercycler Nexus Gradient) for candidate markers was performed. Single-Marker Analysis (SMA) was done for results at 3% agarose gel, by using SPSS package (Version 16.0).

Sequencing of the amplified products was done at "Chromus Biotech Private Ltd, India". Sequences obtained were aligned using Clustalw(http://www.ebi.ac.uk/Tools/msa/clustalw2)[8]. Jalview tool [9] of the clustalw website was used for the identification of the variations present in these sequences. Sequence of all 22 genotypes (including two checks: Azucena and Moromutant) for six candidate gene chosen was aligned and variations were recorded at each base. Starting loci of the amplicon is considered as position 1 and end point varied with each primer. Any changes in any position of these sequences with any base pair or 'in-dels' were recorded, statistical analysis was done to establish association. Each possible change at any position of the sequence was scored. For sequences having same nucleotide as the consensus sequences was scored as "1", any change was scored as 2, 3 or 4 depending upon the nucleotide substituted. In case of deletion at that locus, it was scored as 9. These scores were then used for the analysis using SPSS (v16.0) for associating these variations in the nucleotide level with the phenotypic variation for Zn content in the root, culm and grain.

Results and discussion

Availability of genetic variability is very important in any crop to understand the genetics of any traits [10]. To understand any gene affecting the trait, it has to be studied in diverse genotypes with a good amount of phenotypic and genotypic variation. The selected genotypes were having variations indeed (Table 1). Highest grain Zn in brown rice was observed in AM-65 (43.95 ppm), lowest in Sarabasti (22.85ppm). Highest culm Zn content was observed in Sarabasti (12.80ppm), lowest in AM-1 (0.40ppm). Highest root Zn content was observed in Satabdhi (6.95ppm), lowest in Nuadhusara (0.20ppm). Although genotypes have been selected based on the grain Zn content, these genotypes also exhibited variations to Zn content in culm and roots.

Molecular marker analysis of selected genotypes

The list of all the Zn transporters obtained by literature survey is shown in Table 2. There are many genes

Table 1. Mean zinc content (mg kg^{-1}) in the selected genotypes of rice

Genotype	Brown rice Zn	Culm Zn	Root Zn
AM-1	42.22	0.40	1.81
AM-143	43.53	1.23	4.18
AM-65	43.92	4.05	3.00
AM-72	42.37	3.60	3.31
AM-81	41.23	4.31	2.92
AM-94B	42.35	2.17	1.90
BJ-18	25.88	10.70	4.22
BJ-24(K)	41.50	4.52	0.20
BPT 5204 /ChittiMutyalu-MS	42.10	4.80	0.60
EdavankudiPokkali	41.93	0.61	0.90
Improved ChittiMutyalu	27.67	1.03	4.59
Kalanamak-Birdapur	27.85	10.30	2.10
KalluRandaikar	24.67	6.89	5.24
KarnaMahsuri	27.67	0.81	1.60
Nuadhusara	28.53	6.90	0.20
R 133-968-2-1	27.78	4.55	3.67
Sarabasti	23.12	12.27	2.30
Satabdhi	28.4	5.30	6.68
Tara	28.87	2.40	5.76
Vandana	42.00	10.63	0.62

Table 2. List of Zn transporters in rice used in the study

S.No.	Transporter	Plant part
1	OsZIP1	Root
2	OsZIP2	Culm
3	OsZIP3	Root
4	OsZIP4	Culm/grain
5	OsZIP5	Culm/grain
6	OsZIP6	Root/culm/flower
7	OsZIP7	Grain/culm
8	OsZIP8	Root/culm/grain
9	OsYSL2	Grain/culm
10	OsNramp	Grain/culm
11	OsIRT1	Culm/root
12	OsNAC	Leaf/root
13	OsNAAT1	Culm/leaf
14	OsNAS3	Culm/leaf

belonging to family of OsZIP, OsYSL, OsIRT, OsNRAMP, OsNAC and OsNAAS. All the genes in these family's needs to be explored in depth to obtain a clear idea about how these transporters are working and how the available knowledge can be employed to enhance the Zn content in rice grains. Improvements in the field of bioinformatics have become a boon for plant breeders, especially for those who are practicing marker-assisted selection [11]. Current study is also one of such examples where bioinformatics tools were applied with marker-assisted selection. Application of bioinformatics will not only make study more focused but it also reduced the cost involved in conducting research.

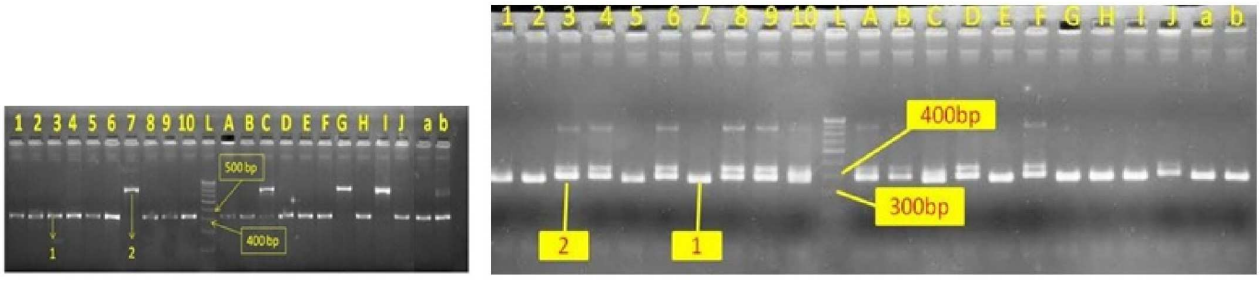
Primers designed in the NCBI database have worked with a size variation for about 50-100bp but, OsZIP7a and OsZIP7b primers did not amplify anything in all the 20 selected genotypes, even after many cycles of repetition and hence, they were eliminated for further analysis. This could be because *japonica* rice sequences were used for primer design and amplification was done in *indica* rice. The genomic variation between the two is well documented. Only OsZIP3b and OsZIP6c were able to detect the polymorphism in the selected genotypes (Fig. 1) and OsZIP6c was associated with culm Zn content (Table 3). Many of the scientific studies conducted till today would have either stopped the experiment right here as the there was a marker discovered to be associated with the trait of interest or even if they have proceeded for sequencing, would have left all those markers which were monomorphic.

Monomorphism vs polymorphism

Main objective of the experiment was to dissect the information which is probably hidden in the monomorphic markers as well. Hence, along with two polymorphic markers namely OsZIP3b and OsZIP6c four monomorphic markers namely OsZIP4a, OsZIP5b, OsYSL2b and OsNramp were also selected for sequencing. These markers are those markers which would have dropped from the further investigation.

Many nucleotide changes were recorded in five out of six amplicons studied. In case of OsYSL2b, there was no variation present but other 5 primers had nucleotide changes in their amplicons. In case of OsZIP6c, as it is polymorphic, there was huge difference in expected (431bp) and observed product size (780bp) in some genotypes. This was due to huge insertion of sequences at two locations. Along with these huge insertions G to A; T to C transitions were also observed. For OsZIP3b major changes were deletions, A to C; G to T or C transversions and T to C transitions. These two were polymorphic markers.

In case of other three makers which were monomorphic in the agarose gel, nucleotide changes were present which was quite interesting and worth observing. In case of OsNramp, major changes include deletions, G to C transversion; G to A transition changes. For OsZIP4a, most of the cases, it was G to A transitions. There was a deletion, A or G to C; T to A or G transversions also recorded. For OsZIP5b, changes were diverse (Fig. 2). G to C; G to T; A to T transversions and A to G; G to A transitions were major along with three insertions. The results of SMA revealed that there is an association between five nucleotide changes in OsZIP5b and grain Zn content; and between eight nucleotide changes in OsZIP6c and culm Zn content (Table 4). Nucleotide changes inthree other primers for OsZIP3b, OsNramp, OsZIP4a manifested no association with trait in this set of genotypes used. The nucleotide changes present in OsZIP5b and OsZIP6c



L: 100bp ladder; 1: AM-65; 2: AM-143; 3: AM-72; 4: AM-94B; 5: AM-1; 6: BPT5204/Chittimutyalu MS; 7: Vandana; 8: Edavankudi Pokkali; 9: BJ-24(K); 10: AM-81; A: Sarabasti; B: Kallu Randaikar; C: BJ-18; D: Improved Chitti Mutyalu; E: Karna Mahsuri; F:R 133-968-2-1; G: Kalanamak-Birdapur; H: Satabdhi; I: Nuadhusara; J: Tara; a: Azucena; b: Moromutant

Fig. 1. Agarose gel profile of OsZIP6c and OsZIP3b

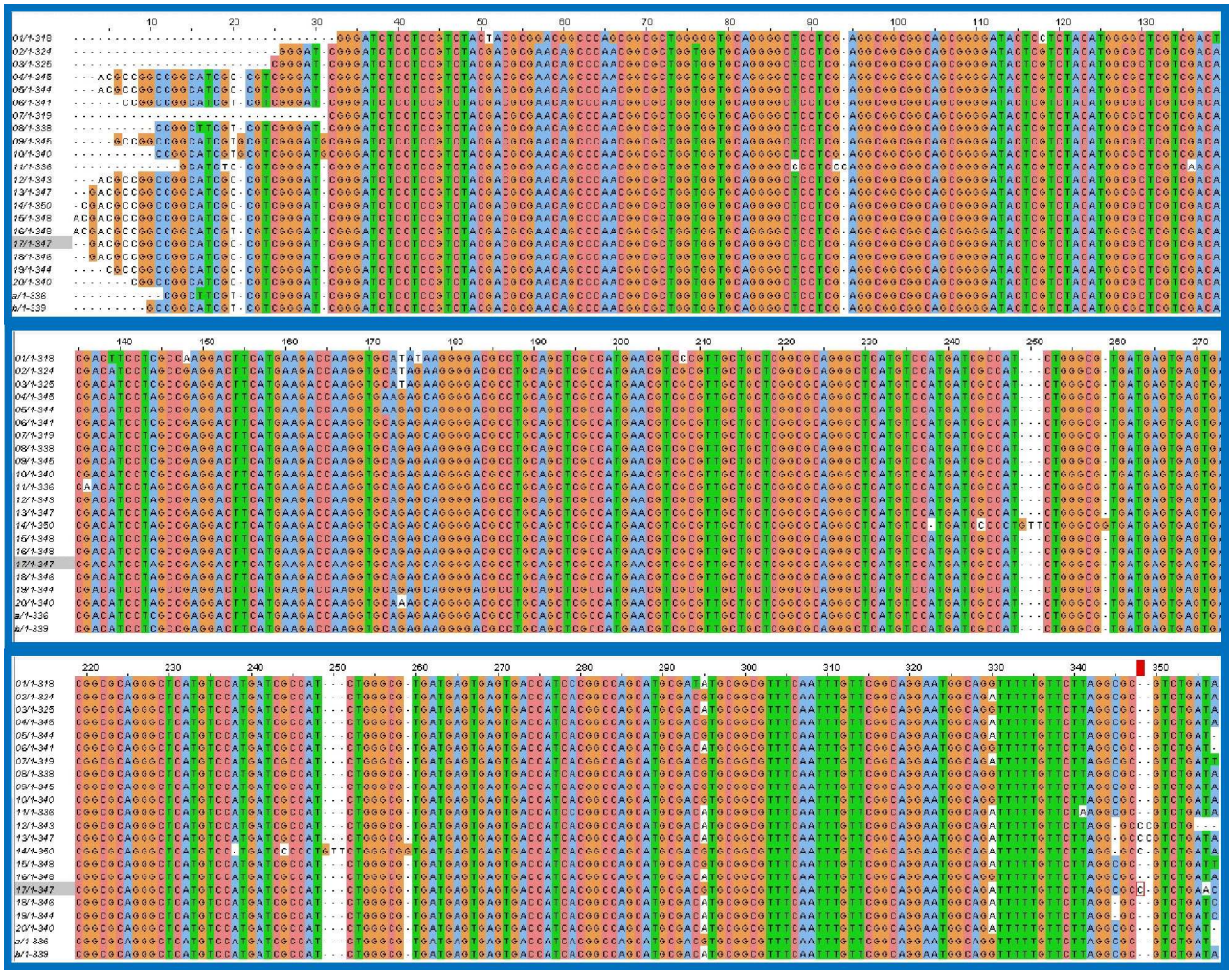


Fig. 2. Multiple sequence alignment of amplicons of OsZIP5b

Table 3. Single-marker analysis of OsZIP3b and OsZIP6c

Primer	Zn content in	P value	R^2 value
OsZIP3b	Grain	0.909	0.001
	Culm	0.446	0.033
	Root	0.874	0.001
OsZIP6c	Grain	0.323	0.054
	Culm	0.001**	0.438
	Root	0.247	0.074

*Significant at 5%; **Significant at 1%

has to be validated in other set of genotypes and can be used as SNPs for improvement of Zn content.

Analysis of sequences of OsZIP6c revealed that there were eight nucleotide changes associated with

Table 4. Single-marker analysis for nucleotide changes in OsZIP3b and OsZIP6c

Primer_position	Zn content in	P value	R^2 value
OsZIP5b_144	Grain	0.020*	0.265
OsZIP5b_177	Grain	0.004**	0.381
OsZIP5b_330	Grain	0.020*	0.265
OsZIP5b_345	Grain	0.009**	0.324
OsZIP6c_217	Culm	0.006**	0.352
OsZIP6c_365	Culm	0.001**	0.438
OsZIP6c_367	Culm	0.015*	0.288
OsZIP6c_369	Culm	0.001**	0.438
OsZIP6c_380	Culm	0.007**	0.444
OsZIP6c_381	Culm	0.001**	0.438
OsZIP6c_441	Culm	0.007**	0.440
OsZIP6c_442	Culm	0.001**	0.438

*Significant at 5%; **Significant at 1%

culm Zn content. It was polymorphic on agarose gel and was significantly associated with culm Zn content. There were two huge insertions between 211-364th position and 369-633rd position. These two inserts were significantly associated with culm Zn content. The phenotypic variance for these inserts was also high in the range of 43.8 to 44.4%. Agarose gel polymorphism among the four genotypes which have high culm Zn content matched with the sequence insertion in these four genotypes with high culm Zn content. Further, variations at two other positions of for OsZIP6c namely, 365th and 367th positions, which were significantly associated with culm Zn content, were outside the insertions. Interestingly, these two positions were exactly in between those two huge insertions, suggesting that these two positions represented two new variations identified by sequencing in addition to those variations identified on agarose gel. Thus, sequencing has been shown to identify more variations in the marker which are polymorphic.

OsZIP5b manifested significant association with grain Zn content at four different positions namely, 144th, 177th, 330th and 345th. This OsZIP5b was completely monomorphic at agarose gel. In usual case, this marker would have dropped from the analysis thinking that there is no scope for further improvement, as variations are not visualized. The phenotypic variance exhibited by nucleotide changes in OsZIP5b for grain Zn content was also high ranging from 26.5% to 38.1%. Two positions were highly associated with a significance of 1%, two others were association with a significance of 5%. If at all this marker was dropped from the experiment, there would have been no explanation for about 26-38% of the variation exhibited by the trait. These results also illustrate that a single nucleotide change in the gene can lead to significant changes in the phenotype, which is very valuable for a breeder to improve the trait.

This clearly demonstrates that monomorphic markers are as meaningful as polymorphic markers. They have a lot of information hidden in them waiting to be dissected and still arelargely being rejected by most of the researchers across the globe. There is a need of understanding such so called “monomorphic markers” so as to understand some of the complex traits like Zn accumulation as we do not have efficient markers discovered tightly associated with the trait. With rapid advances in technology, cost of sequencing is coming down [12, 13]. One more strategy to reduce cost and to get more successful results is, using bioinformatics [14]. There is also availability of large number of bioinformatics resources which can help us in targeting the desired genomic sequences instead of searching all over the genome.

This study also clearly demonstrates usage of bioinformatics resources for targeted dissecting of complex traits and also combined use of bioinformatics and sequencing todissect the hidden meaning in the monomorphic markers. Days are to come where sequencing cost is going to decrease further enabling us to exploit this particular tool to the maximum extent possible. “Focussed search and intelligent sequencing” would dissect the code hidden in the markers which would efficiently assist a breeder in combating some of the global threats like micronutrient deficiency, drought etc., which are lot more complicated to be solved.

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