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Study on Analysis of Molecular Diversity and Trait Association for Zinc Deficiency Tolerance in Rice under Submerged Conditions

A.P. Salini, M. Dhandapani[1]

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ABSTRACT

Background: Rice is the most important human food crop in the world and its production is affected by various biotic and abiotic stress including low soil fertility. Zinc deficiency is one of the most important micronutrient deficiencies limiting rice yield. The vast reserve of germplasm of rice grown in different eco systems could be screened to exploit the genotypic differences bfor zinc deficiency tolerance. With the advent of molecular markers, association mapping strategy based on non-random associations between causative loci and phenotype could be employed to resolve the complex traits like zinc deficiency tolerance. With this background, the current study is focused to explore the extent of variability for zinc deficiency tolerance using zinc deficiency score and quantitative traits among 44 germplasm accessions and to identify quantitative trait loci associated with zinc deficiency tolerance using Simple Sequence Repeat (SSR) marker-based association mapping.

Methods: A set of 44 accessions consisting of landraces and improved varieties were selected based on their zinc deficiency score in field (Zn=0.64 ppm) screening experiment conducted in Regional Research Station, Paiyur, Tamil Nadu. Eight accessions from each Zn Def score group along with four other genotypes namely Savulu Samba, Kotta Nel, Paiyur 1 and ADT 39 were chosen for the study. Molecular analysis was carried out among the selected accessions using a total of 40 random Simple Sequence Repeat (SSR) primers.

Result: Our study revealed the existence of diversity at molecular level among the selected accessions. Clustering analysis separated the accessions in two major clusters which were in accordance with the population structure analysis which resulted with two sub-populations. Through our study we identified certain accessions namely, Karuppu Nel, Manipur Local and BAM 440 which recorded high yields along with least zinc deficiency scores implying their ability to act as donors for zinc deficiency tolerance under submerged condition. Subsequent association analysis which resulted in putative association of zinc deficiency score with critical plant traits such as plant height and number of productive tillers, revealed that association mapping could be a viable strategy for understanding genetic mechanism of zinc deficiency tolerance in rice.

Key words: Germplasm, Rice, Screening, Target-environment, Zinc deficiency.

INTRODUCTION

Rice is the most important human food crop in the world and is the source of sustenance and livelihood for about half of the world's population. The rice production is affected by various biotic stresses like pests and diseases and abiotic stresses such as drought and low soil fertility which often prevent crops from reaching their true yield potential (Nanda and Wissuwa, 2016). After nitrogen (N), phosphorus (P) and potassium (K), widespread zinc (Zn) deficiency has been found responsible for yield reduction in rice and it is the most important and most frequently occurring micronutrient deficiency.

Breeding for zinc deficiency tolerance is hampered similar to breeding for other abiotic stress tolerance and nutrient use efficiency due to the poor understanding of the tolerance mechanism which in case is severely affected by the complexity involved in screening. The field screening for zinc deficiency tolerance can be variable based on soil factors like soil heterogeneity and interaction of zinc with other nutrients in the soil. The response of zinc deficiency exhibited by rice plants might also be due to other stress factors like high carbonate- bicarbonate content. Hence, Sadeghzadeh (2013) emphasized on requirement of reliable alternative screening methods.

With the advent of molecular markers, association mapping strategy also known as linkage disequilibrium (LD) mapping, which is based on LD (Linkage disequilibrium) non-

Center for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India.
[1]Department of Basic Engineering and Applied Sciences, Agricultural Engineering College and Research Institute, Kumulur, Tiruchirappalli-621 712, Tamil Nadu, India.

Corresponding Author: A.P. Salini, Center for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India. Email: apstnau@gmail.com

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random associations between causative loci and phenotype could be employed to resolve the complex traits like zinc deficiency tolerance, using natural population. Wissuwa *et al.,* (2006), suggested that by identifying QTLs associated with symptoms of Zn deficiency, it will ultimately be possible to dissect the overall response to Zn deficiency into distinct genetic factors, each associated with a physiological mechanism conferring tolerance. Hence, in this study an attempt had been made to identify quantitative trait loci associated with zinc deficiency tolerance using Simple Sequence Repeat (SSR) markers-based association mapping.

MATERIALS AND METHODS

A set of forty-four accessions were used for molecular analysis using 40 Simple Sequence Repeat (SSR) primers. The accessions were randomly selected based on their zinc deficiency score in field screening experiment which was conducted in Regional Research Station, Paiyur during *Rabi* 2014-15. Eight accessions from each score group of 1(highly tolerant), 3(moderately tolerant), 5 (moderately susceptible), 7 (susceptible) and 9 (highly susceptible) along with four other genotypes namely Savulu Samba, Kotta Nel, Paiyur 1 and ADT 39 constituted 44 accessions used in this study. The details of 44 rice accessions and 40 SSR primers used for the molecular marker analysis are given in Table 1 and Annexure 1 respectively.

Fresh leaf samples were collected from 44 genotypes at three leaf stage was used for isolation of genomic DNA. DNA was extracted following the modified CTAB method developed by Saghai-Maroof *et al*. (1984) with suitable modifications suggested by Hoisington *et al*. (1994). The DNA concentration was quantified and diluted to 30 ng/µl. PCR analysis was carried out using regular protocol for SSR primers in rice. The PCR products were checked for amplification in agarose gel electrophoresis (3 per cent) before loading them on Poly Acrylamide Gel Electrophoresis (PAGE). The PCR products were then run on PAGE at 150 and resolved by ethidium bromide staining procedure and bands were visualized under UV light. The number of alleles for each of the SSR markers across the 44 accessions were identified and used for determining the Polymorphism Information Content (PIC). The PIC value for each SSR markers was calculated based on the formula $Hn = 1 - Sp_i^2$, where p_i is the allele frequency for the i^{th} allele (Nei, 1973). Cluster analysis was done using distance-based approach by calculating pair wise distance matrix to generate a dissimilarity matrix using a shared allele index with DARwin software (Perrier and Collet, 2006).

To understand the population structure, the genotypic data for 40 SSR markers were analyzed by employing a model-based approach available in Structure 2.3.2 (Pritchard *et al.,* 2000). The analysis was carried out using the online version of Structure harvester (http://tayloro.biologyucla.edu/Struct_harvest) developed by Earl and von Holdt, (2012). A value of *K*=2 was selected when the estimate of *delta K* peaked in the range of 1 to 10 sub-populations and inferred ancestry estimates of individuals (Q-matrix) were derived for the selected sub-population (Pritchard *et al.,* 2000). Association between markers and traits was performed using structured association analysis which is implemented in *TASSEL v4.1* as a general linear model (GLM) method (Bradbury *et al.,* 2007). Genotypic data of the 44 rice

Table 1: List of accessions used for molecular analysis.

Accession Name	Zinc deficiency score
Mattai Kar	1
ASD 14	1
BAM 442	1
BAM 271	1
IR 42	1
BAM 440	1
Manipur Local	1
Karuppu Nel	1
CO 38	3
TKM 2	3
Malayalathan Samba	3
Pokkali	3
ASD 1	3
TKM 3	3
Katta Samba	3
Mikuruvai	3
Vellai Chithirai Kar	3
Bharathi	3
BAM 213	5
Saranga	5
CO 46	5
CO 42	5
CR 1009	5
TRY 3	5
T 184	7
ADT 37	7
Karuthakar	7
CO 45	7
CO 13	7
Athira	7
Early Samba	7
Mara Batta	7
Adipu	9
Rasagadam	9
Senkar	9
ADT 49	9
IR 50	9
CO 48	9
IR 64	9
ASD 17	9
Savulu Samba	1
Kotta Nel	3
Paiyur 1	5
ADT 39	9

accessions generated from SSR marker analysis and phenotypic data of the six characters observed in the experiment served as the input data for association mapping. The significant marker-trait associations were declared by $P \leq 0.05$ and the magnitude of the QTL effects were evaluated by R^2 -marker parameter.

RESULTS AND DISCUSSION

The marker assisted breeding for zinc deficiency tolerance is in the primitive stage due to the poor understanding of the underlying mechanisms which vary drastically among the tolerant lines which are identified. Most of the studies to identify QTLs for zinc deficiency tolerance are based on the population developed from cross between single tolerant and susceptible plants and are associated with the traits like zinc content in the grains, tissues and root traits, which are not easy for observations and screening when dealing with huge populations like F_2, RILS, NILs or DHs. In this study we have attempted to understand the association of molecular markers to zinc deficiency scores and few growth and yield characters using association mapping.

A total of 40 SSR markers were evaluated across a subset of 44 genotypes. The levels of polymorphism among the 44 accessions were evaluated by calculating allele

Annexure I: List of SSR primers used for molecular analysis.

Primer	Chromosome/Linkage	Forward sequence	Reverse sequence
RM1	1	GCGAAAACACAATGCAAAA	GCGTTGGTTGGACCTGAC
RM105	9	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGGATCGGGTC
RM125	7	ATCAGCAGCCATGGCAGCGACC	AGGGGATCATGTGCCGAAGGCC
RM221	2	ACATGTCAGCATGCCACATC	TGCAAGAATCTGACCCGG
RM224	11	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
RM231	3	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG
RM152	3	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG
RM250	2	GGTTCAAACCAAGCTGATCA	GATGAAGGCCTTCCACGCAG
RM256	8	GACAGGGAGTGATTGAAGGC	GTTGATTTCGCCAAGGGC
RM287	11	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC
RM11	7	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
RM118	1	ATGAGGATCTGCTTCCGTCTCC	CTCGCGAGCTTTGAGACAAGC
RM135	3	TCCATGCTCTTCAGCTTCTGG	GCTTCTACTGGAGGAGAGCAGAGG
RM 144	7	CATGTTGTGCTTGTCCTACTGC	AGCTAGAGGAGATCAGATGGTAGTGC
RM337	8	GTAGGAAAGGAAGGGCAGAG	CGATAGATAGCTAGATGTGGCC
RM161	5	TGCAGATGAGAAGCGGCGCCTC	TGTGTCATCAGACGGCGCTCCG
RM1664	4	GATCGAACGAACGTGAATGAGC	TAGGGCTAGCTCTTCCACTCAGG
RM205	9	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG
RM21	8	GACAACTCCATATCAACGCAAAGC	GAGGATGGTTGTTCACTTGTTTGG
RM211	2	CCGATCTCATCAACCAACTG	CTTCACGAGGATCTCAAAG
RM215	9	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG
RM234	7	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG
RM235	12	AGAAGCTAGGGCTAACGAAC	TCACCTGGTCAGCCTCTTTC
RM237	1	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC
RM242	9	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG
RM25	8	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC
RM271	10	TCAGATCTACAATTCCATCC	TCGGTGAGACCTAGAGAGCC
RM277	12	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG
RM289	5	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG
RM296	9	CACATGGCACCAACCTCC	GCCAAGTCATTCACTACTCTGG
RM312	1	GTATGCATATTTGATAAGAG	AAGTCACCGAGTTTACCTTC
RM316	9	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC
RM324	2	CTGATTCCACACACTTGTGC	GATTCCACGTCAGGATCTTC
RM335	4	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG
RM401	4	TGGAACAGATAGGGTGTAAGGG	CCGTTCACAACACTATACAAGC
RM407	8	GATTGAGGAGACGAGCCATC	CTTTTTCAGATCTGCGCTCC
RM413	5	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC
RM5	10	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG
RM341	2	CAAGAAACCTCAATCCGAGC	CTCCTCCCGATCCCAATC
RM475	2	CCTCACGATTTTCCTCCAAC	ACGGTGGGATTAGACTGTGC

number and polymorphism information content (PIC) values for each of the 40 SSR loci. The allelic range for a marker across the population is a deciding factor in understanding the genetic diversity of a population. Higher the number of alleles greater is the extent of genetic diversity. The SSR primer pairs used for the analysis, the number of alleles for each SSR locus, gene diversity and PIC values are given in Table 2. The 40 primer pairs detected a total of 143 alleles, with an average of 3.58 alleles per locus. The number of alleles observed at each locus ranged from three to five. Out of the 40 SSR markers, 26 markers were with three alleles, 16 markers were with four alleles and two markers were with five alleles. The average PIC value was 0.56 and it ranged from a minimum of 0.22 (RM 211) to a maximum of 0.75 (RM1, RM413). The PIC value estimated based on the number of alleles is subject to the frequency of individuals under each category across the population. Subsequent to the PIC value estimation, the marker data generated was

Table 2: Measures of genetic diversity based on SSR markers.

Marker	Number of Alleles					PIC Alleles	
	0	1	2	3	4		Value
RM231	10	28	6			4	0.57
RM250	5	35	4			3	0.35
RM224	18	8	18			3	0.63
RM337	14	5	22			3	0.58
RM105	29	8	7			3	0.50
RM1	6	15	8	11	3	5	**0.75**
RM118	17	21	6			3	0.60
RM144	27	6	11			3	0.54
RM341	8	14	16	5		4	0.71
RM11	10	28	5			3	0.51
RM125	10	12	14	7		4	0.73
RM256	4	4	27	7		4	0.54
RM221	3	6	17	16		4	0.67
RM152	2	17	18	7		4	0.66
RM287	8	15	7	14		4	0.72
RM135	26	1	13	4		4	0.55
RM337	3	6	4	31		4	0.47
RM161	1	28	9	6		4	0.53
RM1664	6	10	25	2	1	5	0.60
RM205	5	36	3			3	0.31
RM21	8	15	13	4		4	0.70
RM211	2	3	36			3	0.22
RM215	2	27	15			3	0.51
RM234	7	27	9			3	0.54
RM235	3	32	8			3	0.41
RM237	1	16	19	7		4	0.64
RM242	9	31	1	2		4	0.43
RM25	20	4	14	6		4	0.67
RM271	4	26	2	12		4	0.57
RM277	4	11	25	3		4	0.58
RM289	1	17	16	10		4	0.67
RM296	13	16	10	5		4	0.72
RM312	14	7	23			3	0.60
RM316	30	7	7			3	0.48
RM324	6	31	7			3	0.46
RM335	13	16	15			3	0.66
RM401	8	2	34			3	0.37
RM407	7	29	8			3	0.51
RM413	10	10	10	13		4	**0.75**
RM5	10	28	6			3	0.52
Total						143	22.53
Average						**3.58**	**0.56**

used to assess the extent of genetic diversity adopting cluster analysis.

Clustering analysis based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method using DARwin separated the accessions into two main clusters and three sub clusters in each cluster. Cluster is depicted in Fig 1. The cluster analysis separated the genotypes in to two major clusters indicated the existence of two groups and the possibility of using the population for LD mapping to identify the QTL associated with zinc deficiency tolerance, though the allelic frequencies for the marker loci did not have whole genome coverage, a pre-requisite for the LD mapping in a population. The primers were randomly selected for this study. Structure analysis was carried out to establish the population structure using the allelic frequency of 40 SSR markers employed. The population structure was determined based on the survey of 40 SSR markers across the subset of 44 accessions. The results are presented in Table 3. Optimum number of populations was inferred using the correlated allele frequencies. The analysis resulted with

Table 3: Model based cluster membership coefficients of 44 rice genotype as determined by structure analysis.

Genotype	Q1	Q2	Population
Savulu Samba	0.006	0.994	P2
PYR 1	0.035	0.965	P2
Kotta Nel	0.022	0.978	P2
ADT 39	0.008	0.992	P2
Mattai Kar	0.008	0.992	P2
ASD 14	0.014	0.986	P2
BAM 442	0.075	0.925	P2
BAM 271	0.083	0.917	P2
IR 42	0.021	0.979	P2
BAM 440	0.159	0.841	P2
Manipur Local	0.097	0.903	P2
Karuppu Nel	0.05	0.95	P2
CO 38	0.019	0.981	P2
TKM 2	0.927	0.073	P1
Malayalathan Samba	0.787	0.213	Admixed
Pokkali	0.976	0.024	P1
ASD 1	0.078	0.922	P2
TKM 3	0.068	0.932	P2
Katta Samba	0.167	0.833	P2
Mikuruvai	0.215	0.785	Admixed
Vellai chithirai Kar	0.378	0.622	Admixed
Bharathi	0.99	0.01	P1
BAM 213	0.94	0.06	P1
Saranga	0.094	0.906	P2
CO 46	0.017	0.983	P2
CO 42	0.038	0.962	P2
CR 1009	0.174	0.826	P2
TRY 3	0.988	0.012	P1
T 184	0.839	0.161	P1
ADT 37	0.982	0.018	P1
Karuthakar	0.048	0.952	P2
CO 45	0.857	0.143	P1
CO 13	0.671	0.329	Admixed
Athira	0.993	0.007	P1
Early Samba	0.978	0.022	P1
Mara Batta	0.268	0.732	Admixed
Adipu	0.982	0.018	P1
Rasagadam	0.985	0.015	P1
Senkar	0.991	0.009	P1
ADT 49	0.987	0.013	P1
IR 50	0.053	0.947	P2
CO 48	0.922	0.078	P1
IR 64	0.989	0.011	P1
ASD 17	0.956	0.044	P1

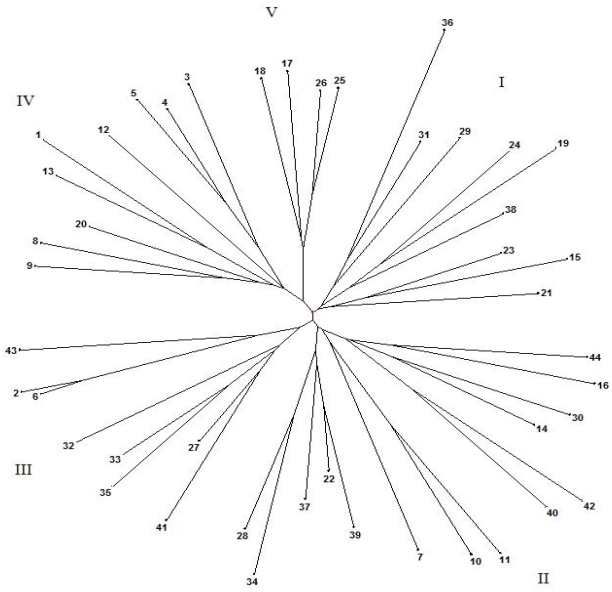

Fig 1: Dendrogram showing the clustering of 44 rice accessions based on 40 SSR markers.

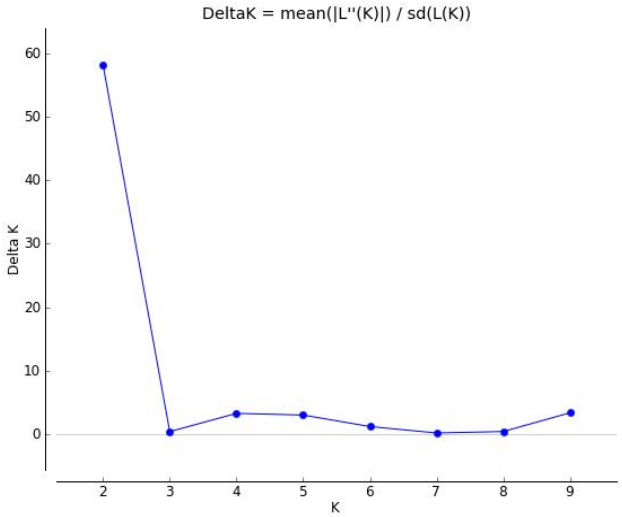

Fig 2a: Determination of number of populations based on secondary statistics.

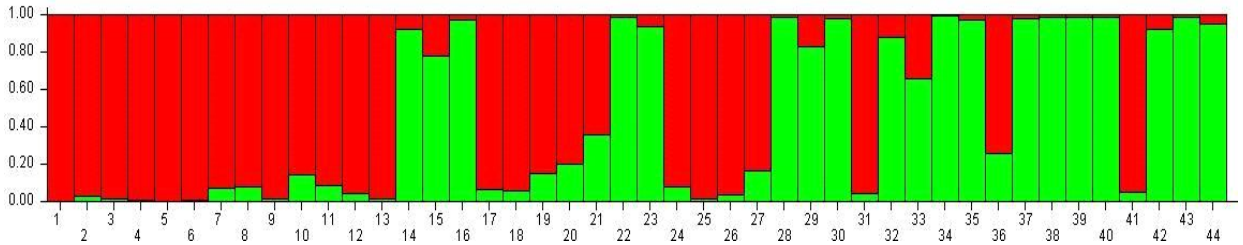

Fig 2b: Population assignment for each accession at K=2 based on STRUCTURE analysis.

Table 4: Putative association of microsatellite marker loci.

Phenotype	Marker	Chromosome	P value	R^2 value
Zinc deficiency score	RM5	1	0.0017	0.3521
Plant height	RM237	1	0.0012	0.2527
Number of productive tillers	RM256	8	0.0009	0.2494
	RM341	12	0.0049	0.1831

optimum K value as two, indicating two possible populations out of 44 accessions (Fig 2a and 2b).

Association analysis using *TASSEL v2.0.1* revealed putative association of four markers *viz.,* RM5, RM237, RM256 and RM341. The associated markers and explained variances are presented in Table 4. RM5 and RM237 were putatively associated with zinc deficiency score and plant height respectively. Wissuwa *et al.,* 2006 reported a QTL Zbz1b at 124 cM in chromosome 1 with the flanking markers RG220–RG109, with the R^2 value of 16.5. Bekele *et al*. (2013) observed that in single marker analysis using 176 RILs of cross Azucena x Moro mutant, the marker RM212 located on chromosome 1 was closely associated with zinc concentration and plant height with adjusted R^2 value of 4.50 and 5.20 respectively. Stangoulis *et al*. (2007) identified QTL for zinc content on chromosome 1 flanked by markers RM34-RM237. Xu *et al.,* 2016 reported an association of RM237 with plant height in chromosome 1 which corresponded to the gene encoding *DGL1*, which is important for cell and organ elongation in rice, this suggests the possibility that zinc deficiency score and plant height may be controlled by similar genomic regions which suggests the close association between the two.

The markers RM 256 at chromosome 8 and RM 341 at chromosome 12 were associated with number of productive tillers. Swamy *et al*., 2014 reported a QTL *nsp12.1* for number of spikelets per plant in a cross between O.*nivara* and Swarna with R^2 value 33.7 at the marker interval of RM341-RM519. Wissuwa *et al*. (2006) reported a QTL *Zmt12* for zinc deficiency induced mortality on chromosome 12 flanked by markers CDO344-1–RG543-1 with adjusted R^2 value of 11.60. This suggests that genes governing number of productive tillers and single plant yield under zinc deficiency could possibly be co-localized with that of zinc deficiency tolerance, which requires further studies for confirmation.

CONCLUSION

In the current study, it is evident that there was exists ample variation at molecular level for zinc deficiency tolerance under submerged conditions. Identification of putative associations RM5 and RM237 with zinc deficiency score and plant height, respectively and that of markers RM256 and RM341 were associated with number of productive tillers suggests that association mapping could be a viable strategy for mapping QTL zinc deficiency tolerance under submerged tolerance.

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