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RESEARCH ARTICLE :

Functional polymorphism for crtRB1 gene loci in tropical maize (*Zea mays* L.) inbred lines

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SUMMARY : Maize (*Zea mays* L.) is an important crop known for its carotenoid diversity among cereals which accumulates significant levels of proA (provitamin A) and non proA carotenoids its kernels. The proA components of maize endosperm promises to solve the major global problem VAD (Vitamin A deficiency). Among several genes involved in 4-carotene biosynthetic pathway, crtRB1 is very important gene associated with three polymorphisms *viz*., 52 TE, InDel4 and 32 TE (Transposable Element) responsible for variation in carotenoid levels in maize endosperm. Due to insertion of TE at 32 UTR (Un Translated Region), crtRB1 again exhibits polymorphism with 3 alleles, however only allele 1(favorable allele; 543bp amplicon) of this crtRB1-32 TE gene will double the â-carotene concentration in maize endosperm and allele 2 and 3 termed as unfavourable. This study was undertaken to find out the allelic difference for crtRB1 gene loci. Totally 228 tropical maize inbred lines were screened for allele 1 of crtRB1 gene using crtRB1-32 TE gene specific markers. Among 228 inbreds, 226 inbreds showed the presence of allele 2 and the two inbreds VL1016247 and VL1016213 possessed both alleles 1 and 2 which are found to be heterozygous for crtRB1 loci. This study indicated the possible use of (VL1016247 and VL1016213) these two inbreds for developing provitamin A (proA) rich maize hybrids using marker assisted selection (MAS).

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BACKGROUND AND OBJECTIVES

Maize (*Zea mays* L.) is the world2 s most widely grown cereal staple crop predominantly serves as a source of energy in human diets and feed for livestock, swine and poultry. According to USDA the global maize production during 2014-15 was 968 mt, with total area of 177 mha. The year by year expansion of maize production is devoted by the growing poultry industry in some of the Asian countries by consuming more than half of the country's maize (Gupta *et al.*, 2015) and if these records continues to grow, by 2050 the demand for maize in the developing world

will be doubled (Rosegrant *et al.,* 2009; Gupta *et al.,* 2015). VAD (Vitamin A deficiency) is currently a global public health problem begins with inflicting morbidity, reduced growth and development, night blindness and ends with loss of vision and lives in the developing world (Azmach *et al.,* 2013). Every year 2.5 to 5 million children become blind and half of them die within a year of losing their vision (World Health Organization, 2009). Thus one of the methods to address the VAD is to breed food crops for increased β-carotene content, to produce crops with enriched proA content (Yan *et al.,* 2010).

Maize germplasm resources exhibit wide genetic variation for carotenoid components in its kernel *viz*., proA (α-carotene, β-carotene, and β-cryptoxanthin) and non-proA (lutein and zeaxanthin) however, only β-carotene has the highest proA activity as compared to other carotenoid compounds (Menkir *et al.,* 2008). Hence maize is referred as a model cereal crop for developing strategies to solve global micronutrient deficiency and shows an assurance for proA biofortification especially through molecular marker-assisted breeding (Vignesh *et al.,* 2012). The carotenoid biosynthesis pathway is well characterized in maize. *LcyE* (lycopene epsilon cyclase) and crtRB1 (β-carotene hydroxylase) are the two genes with naturally existing mutant alleles which have been proposed to play crucial role in the final accumulation of proA carotenoids in the maize endosperm (Babu *et al*., 2013). Through association mapping study, three polymorphisms *viz*., 52 TE (Transposable element; in the 52 - untranslated region), InDel4 (in the coding region) and 32 TE (spanning the sixth exon and 32 -untranslated region) were identified for crtRB1 gene that were significantly associated with the variation for kernel carotenoids in maize (Yan *et al.,* 2010).

The 3'TE polymorphism of the crtRB1 gene creates three alleles *viz*., allele1 (543 bp; without TE insertion), allele 2 (296 bp + 875 bp; with 325 bp TE insertion) and allele 3 (296 bp + 1221 bp + 1250 bp; with 1250 bp TE insertion) (Selvi *et al.,* 2014). Allele1 is termed as favorable since it is correlates with accumulation of higher β-carotene content in maize endosperm by reducing transcript expression of the crtRB1 gene and allele 2 and allele 3 are cause unfavorable effect hence termed them as unfavorable alleles. Babu *et al* (2012) reported that favorable allele of crtRB1-32 TE gene alone could double the β-carotene and total proA content irrespective the genetic constitution of *LcyE5*2 TE and crtRB1-52 TE. Therefore, the current study has been taken up to find out allelic difference for crtRB1-32 TE gene loci only, which ultimately leads to identify β-carotene rich genotypes.

crtRB1 (also known as, *HYD3*) encodes an enzyme carotene hydroxylase was mapped on chromosome 10 (bin 10.06) Sagare *et al*. (2015a). crtRB1 gene involved in the hydroxylation of α-carotene and β-carotene *i.e.* conversion β-carotene (which has 100% proA activity), to β-cryptoxanthin (which has 50% proA activity) and zeaxanthin (which has 0% proA activity) (Yan *et al.,* 2010). Hydroxylation of carotenes reduces the proA carotenoids thereby increasing non-proA xanthophylls (Matthews and Wurtzel, 2007).

Recently PCR-based co-dominant markers associated with proA content in maize kernels were identified for all the three crtRB1 polymorphisms (Yan *et al.,* 2010) and this opened the way for rapid enhancement of proA content in maize kernels through marker-assisted selection (Vignesh *et al.,* 2012). One of the major challenges in maize breeding programme for high proA levels is the quantification of breeding lines. High performance liquid chromatography (HPLC) is the commonly used method for carotenoids analysis because of its accuracy. However it is highly expensive, laborious, relatively low throughput limiting its use for routine breeding within resource-limited plant breeding programs (Vignesh *et al.,* 2015). Hence, screening maize inbreds for favorable of crtRB1-32 TE gene loci using PCR based assay is a best alternative for phenotypic estimation (Sagare *et al.,* 2015b). The present study was undertaken with a final objective to find out allelic difference for crtRB1-32 TE gene loci in tropical germplasms using crtRB1-32 TE gene specific markers.

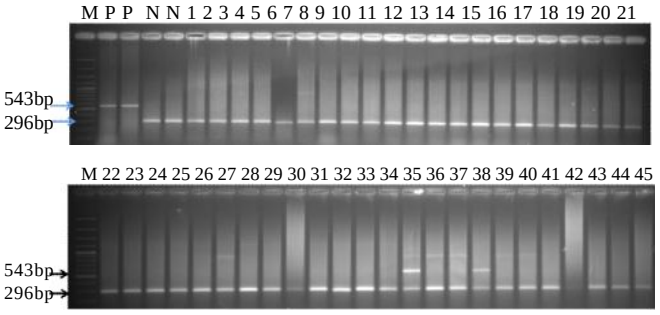
RESOURCES AND METHODS

In the present investigation 228 elite maize inbred lines belong to tropical background were collected from CIMMYT, International Maize and Wheat Improvement Center Asia, Hyderabad (Supplementary Table 1). And two inbreds *viz*., VL121655 and VL121656 derived from Harvest plus program which possesses high β-carotene content and allele 1 of crtRB1-32 TE gene were included in our study as check. Genomic DNA was extracted from seed endosperm using sbeadex maxi plant kit (LGC group cat. no. 41602 and 41620) protocol with few modifications. DNA was quantified using TECAN infinite

M200® PRO spectrophotometer absorbance at 260 nm. PCR was performed by using 3 set of crtRB1-32 TE gene-specific primers (crtRB1 65F: ACACCACATGG ACAAGTTCG, crtRB1 62R:ACACTCTGGCCCATGA ACAC, crtRB1 66R: ACAGCAATACAGGGGAC CAG) as mentioned by Selvi *et al.* (2014). PCR reaction mixtures contained approximately 30 ng DNA as template, 0.5 µl of each primer, 7.0 µl of EmeraldAmpGT® PCR master mix and sterile double-distilled water to a final volume of 15 µl. A touchdown cycling profile (annealing temperature dropping 0.5 °C/ cycle) was used with the conditions: 4 min at 94 °C, followed by 9 cycles of 1 min at 94 °C, 1 min at 64 °C, 1 min at 72 °C, again 19 cycles of 1 min at 95 °C, 1 min at 4 °C, 1 min at 72 °C and 10 min at 72 °C.

OBSERVATIONS AND ANALYSIS

To identify maize lines with the favorable allele, 228 inbreds were characterized for 32 TE polymorphism of crtRB1 using PCR-based co-dominant markers. Out of 228 maize inbreds screened, two inbreds *viz*., VL1016247 and VL1016213 showed the presence of both allele 1 and allele 2 in which they were found to be heterozygous for the concerned gene loci and remaining 226 inbreds showed the presence of only allele 2 (Fig. 1, Table 1).



Gel pictures depicting co-dominant PCR assays for crtRB1-32 TE polymorphism among the maize inbreds (allele type 1 and 2)
M - 100bp marker
P - Positive for favorable allele (Allele 1; 543bp amplicon)
N - Negative for favorable allele (allele 2; 296bp)
Lane 1 to 21, 36, 37 and 39-45 Inbreds with allele 2
Lane 35 and 38 allele 1 and 2 (543bp + 296bp)

Fig. 1 : Gel pictures depicting co-dominant PCR assays for crtRB1-32 TE polymorphism among the maize inbreds

High performance liquid chromatography (HPLC) is the commonly used method for carotenoids estimation because of its accurate results. However HPLC analysis is highly expensive, laborious and relatively low throughput limiting its use for regular breeding within resource-limited plant breeding programs (Azmach *et al.*, 2013). Reports from earlier studies (Yan *et al.,* 2010; Muthusamy *et al.,* 2014; Vignesh *et al.,* 2015 and Sagare *et al.*, 2015b) proved that favourable allele (allele 1) of crtRB1-32 TE gene correlates with higher β-carotene content or functional DNA markers crtRB1-32 TE were consistently and strongly associated with proA (Azmach *et al.*, 2013). Yan *et al.* (2010) confirmed the importance of *Zea mays* crtRB1 gene in final accumulation of β-carotene compounds in maize endosperm and polymorphisms associated with it. However one of the major challenges in maize breeding program for high proA levels is the quantification of breeding lines. Hence screening maize inbreds for favourable allele1 of crtRB1-32 TE based on PCR based assay is a best alternative for phenotypic assay (Sagare *et al.*, 2015b).

Babu *et al* (2012) reported that allele 1 of crtRB1 was significantly associated with higher β-carotene, to support this the results of carotenoids estimation found that the total proA and

β-carotene levels (an average 13-14 µg/g and 15-18µg/g) of genotypes with homozygous favorable allele was 2.4 times and 3.8 times more as compared to the genotypes with unfavorable alleles respectively. Similarly heterozygous crtRB1 genotype showed an average â-carotene 1.9-times (6-8 µg/g vs 3-4 µg/g) and proA 1.5-times (9-11µg/g vs 6-8 µg/g) more than that of homozygous unfavorable. Finally suggested that due to partial recessive gene action, on average the heterozygotes achieved about one-third of the total effect achieved by the homozygous favorable genotype for both β-carotene and proA. Hence from the results of Babu *et al* (2012), we can be say that the two inbreds in our study VL1016247 and VL1016213 which were found heterozygous for allele 1 of crtRB1 gene loci may possess an average β-carotene and total ProA content in the range of 6-8 µg/g and 9-11µg/g, respectively.

Babu *et al.* (2013) reported that the favourable crtRB1 allele is twice efficient in accumulating higher â-carotene when present in homozygous condition than under heterozygous condition, hence favourable allele of crtRB1-32 TE is most preferable if present only in homozygous condition than heterozygous. Thus, to develop hybrids homozygous for crtRB1 favourable allele, both the parents should be introgressed with the

Table 1 : *crtRB1*-3?TE allele distribution in 238 of maize inbred lines

Entry No.	Name	Pedigree
1.	VL102	CML291-1-BB
2.	VL107389	CML326-1-BB
3.	VL103	CML422-2-BB
4.	VL107459	CML470-BB
5.	VL1011	CML472-BBB
6.	VL1014	CML474-BBB-2-BB
7.	VL109449	CML479-2-BB
8.	VL109250	CLQ-RCWQ50-B*7
9.	VL109251	[Ent320:92SEW2-77/[DMRESR-W]EarlySel-#I-2-4-B/CML386]-B-11-3-B-2-#-B*4-1-BBB
10.	VL109463	CL02457-1-B-1
11.	VL109470	([Pop445c1F2-1-1xPop446c1F2]x[Pop446c1F2-358-2xPop445c1F2])-#-38-2-B*4
12.	VL109474	(CA14502/CA14509)-F2-14-1-BBB
13.	VL109480	(CA14502/CA14509)-F2-8-1-B*5
14.	VL109499	(CA14515/CA14502)-F2-10-2-B*4
15.	VL109507	(CML427/CML474)-F2-19-1-BBB
16.	VL109282	(CML474/S92145-2EV-7-3-B*5)-F2-25-1-B*5
17.	VL109287	(CML474/S92145-2EV-7-3-B*5)-F2-58-1-B*5
18.	VL109516	(S92145-2EV-7-3-B*5/CML427)-F2-32-2-B*4
19.	VL109485	[[[K64R/G16SR]-39-1/[K64R/G16SR]-20-2]-5-1-2-B*4/CML390]-B-38-1-B-7-#/[BETASYN]BC1-1-1-1-#-B*5
20.	VL1010766	[[[NAW5867/P30SR]-40-1/[NAW5867/P30SR]-114-2]-16-2-2-B-2-B/CML395-6]-B-20-1-B-3-#/[BETASYN]BC1-3-1-1-#-B*6
21.	VL109524	[DTPYC9-F74-1-1-1-1-BBxDTPYC9-F65-2-2-1-1-BB]-B-3-4-B*5
22.	VL109180	P31C4S5B-23-#-#-4-BBB-B-B-B-B-4-B-B-B-B
23.	VL109181	P45c8-164-1-1-2-8-B*4-2-B*4
24.	VL1066	SO4YLWL-172-B-1-1-B-1-B-B
25	VL109184	DTPYC9-F46-3-1-1-2-3-2-2-B-B-B-B-B
26.	VL1017749	P45c8-164-1-1-2-8-B*4-3-BBB
27.	VL1016210	POB45c9F22-18-3-1-B*4-1-B*4
28.	VL1017256	POB45c9F212-18-2-1-B*10
29.	VL1016214	Messina-03445(S2-Syn)-F1Bulk-22-3-1-B*4
30.	VL105546	[CML329xCML287]F2-38-1-B*8
31..	VL1016212	[CML329xCML20]F2-47-2-B*9
32.	VL1016212	POB45c8-67-1-1-3-B*12
33.	VL1016173	POB45c8-152-1-1-1-2-B*12
34.	VL1016211	(CML20xCML329)-17-3-3-1-B*8
35.	VL1016247	(CML226xCML295)-67-3-4-2-B*8
36.	VL1016242	P446-34-1-4-B*5-1-B*4
37.	VL1017795	Messina-03445(S2-Syn)-F1Bulk-45-3-1-BBB
38.	VL1016213	WLS-F90-2-1-3-B-3-BBB
39.	VL1016178	WLS-F238-2-2-1-B-1-BBB
40.	VL1016179	SO4YLWL-112-B-1-2-B-1-BBB
41.	VL108732	(DT/LN/EM-46-3-1xCML311-2-1-3)-B-F81-1-1-1-BB
42.	VL109186	KSX3601F2-4-4-3-2-1-B*6
43.	VL105617	AMATLCOHS44-5-2-2-1-1-B*6
44.	VL105618	CA03139-6-7-1-BBB
45.	VL105549	CA03130-BB-2-B-1-BBB

favourable allele, or the favourable allele can be introgressed into the parents of already released hybrids through MAS to exploit the established grain heterosis and adaptability (Vignesh *et al.,* 2015). The results from present investigation proved previous findings (Dhyaneswaran *et al.,* 2012; Vignesh *et al.,* 2012; Selvi *et al.,* 2014) by depicting most favourable crtRB1 alleles were rare in frequency and unique to temperate germplasm.

Previous studies reported that favourable allele 1 of crtRB1-32 TE gene correlates with higher β-carotene content (Yan *et al.,* 2010) and were consistently and strongly associated with proA (Azmach *et al.,* 2013). In the present study, except two inbrerds (VL1016247 and VL1016213) remaining 226 inbreds showed the presence of allele 2. The two inbrerds *viz.*, VL1016247 and VL1016213 which are showing the favorable allele (allele 1) and Allele 2 for the crtRB1 gene loci, showed the possible use of inbreds to these inbreds for developing provitamin A (proA) rich maize hybrids using marker assisted selection (MAS).

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