Electronic Journal of Plant Breeding, 1(4): 548-555 (July 2010)

Research Article

Exploitation of natural variability in maize for β - carotene content using HPLC and gene specific markers

Tura Safawo, N. Senthil*, M. Raveendran, S. Vellaikumar, K. N. Ganesan, G. Nallathambi, S. Saranya, V. G. Shobhana, B. Abirami and E. Vijaya Gowri

Abstract

Vitamin A deficiency is a major world health problem, affecting up to 127 million pre-school children and 7 million pregnant women worldwide (West, 2003). Human selection for yellow endosperm has led to diversification of grain carotenoid content and composition. This variation has remained largely untapped in modern breeding programs that have focused nearly exclusively on yield gains. Maize displays considerable natural variation for carotenoid composition, including vitamin A precursors α-carotene, β-carotene, and β -cryptoxanthin. Sixty four maize inbred lines of India were grown and evaluated for whole kernel carotenoids and beta carotene content using high performance liquid chromatography (HPLC). The lines averaged 14 µg/g for total carotenoids (5.58 to 63.9 µg/g) and 1.69 µg/g for β -carotene (0.122 to 4.74 µg/g). High level of β - carotene was observed in UMI 946, UMI 176, UMI 79, UMI 34 and UMI 12 and these would be used in the breeding programs to enhance the β – carotene contents. Previous study showed four natural lcyE polymorphisms explained 58% of the variation in these two branches and a threefold difference in pro-vitamin A compounds. In screening for polymorphisms in key haplotypes, four regions were selected and scored across the entire panel of favourable haplotype using SNP and SSR markers. The polymorphs were obtained for all primer used and the presence of the amplification of for particular marker showed the increase in β - carotene content across the inbreds. Development of LCYE based breeding markers for maize alone will not be effective unless hydroxylation is also controlled, for non pro vitamin A xanthophylls compounds will predominate. So in our future study, we should be aiming at breeding for high β - carotene in maize by emphasizing on characterization of β - carotene hydroxylase genes for enhancing the relative levels of seed β - carotene.

Introduction

Vitamin A deficiency (VAD) affects over 250 million people worldwide and is one of the most prevalent nutritional deficiencies in the developing countries, resulting in significant socio-economic losses. However this can be alleviated through provitamin A carotenoid biofortification of major crop staples such as maize (*Zea mays L*). The dietary habit of many Africans, in which maize is consumed for all three meals a day, indicates that maize is a good target for bio-fortification. Maize is also the dominant subsistence crop in much of Asia, Africa and the Americas, where between 17 and 30% of children under age of 5 are vitamin A deficient. This results in xerophthalmia (progressive blindness), increased infant morbidity and mortality. Poor infrastructure in developing countries has limited widespread use of direct vitamin supplementation. Although bio-fortified foods can potentially be an inexpensive, locally adaptable and long term solution to diet deficiencies, cultural preferences may limit their acceptance. Seed endosperm tissue of maize (*Zea mays L.*) and other grasses (Poaceae) represents 70% of worldwide food production (Chandler and Brendel, 2002), but has limited pro-vitamin A value. Improvement of pro-vitamin A content in staple crops is therefore a critical step toward alleviating vitamin A deficiency worldwide.

Carotenoids are derived from the isoprenoid biosynthetic pathway and are precursors of the plant hormone abscisic acid and of other apo-carotenoids. The first committed step of this pathway is formation of phytoene from geranylgeranyl diphosphate by phytoene synthase (*y1/psy1*). Recent studies in maize suggest that the *psy1* locus has been the target of a selective sweep the following selection for endosperm-accumulating carotenoids and shift from white to yellow kernels. The first branch point of this pathway (Fig. 1) occurs at cyclization of lycopene where action of lycopene beta cyclase (LCYB) at both ends of linear lycopene produces a molecule with two β rings. Alternatively, the coaction of LCYB and lycopene epsilon cyclase (LCYE) generates α, β & £ - carotene that is a precursor to

Dept. of Plant Molecular Biology and Biotechnology[1], Centre for Plant Molecular Biology[1], Tamil Nadu Agricultural University, Coimbatore – 641 003
Email: senthil_natesan@yahoo.com

lutein. Relative activities of LCYB and LCYE are hypothesized to regulate the proportion of carotenes directed to each branch of this pathway. Indeed, transgenic manipulations of LCYE expression in Arabidopsis, potato, and Brassica increase the pool of β ring–containing carotenes and xanthophylls.

Generally, maize exhibits considerable natural variation for kernel carotenoids. The pre - dominant carotenoids in maize kernels, in decreasing order of concentration, are lutein, zeaxanthin, β - carotene, β - cryptoxanthin, and α - carotene. β -carotene contains two pro-vitamin A structures (two non-hydroxylated β - ionone rings) and β -cryptoxanthin and α - carotene one each (single non-hydroxylated β - ionone ring). Among the lines included in our diverse maize panels studied earlier, β - carotene levels reached 13.6 µg/g. However, most yellow maize grown and consumed throughout the world has only 0.5 to 1.5 µg/g of β - carotene. The presence of a wide range of carotenoid content in maize inbreds of Tamil Nadu has been the subject of this study. The exploitation of the natural variation and the induction of mutation in the β – carotene pathway would result in the identification of maize inbreds with higher β – carotene contents, which may then be directly utilised into the bio-fortification programmes.

Materials and methods

Sixty four maize inbred lines were used for this study. The seeds were obtained from the Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore. The seeds were raised in the Eastern block of the Central Farm and were selfed for preserving the seeds in the next season. The dried seeds (moisture 12%) were ground to a fine consistency in the grind mill. The finely ground flour were sieved in a fine mesh and the flour was used for the analysis of β – carotene by means of HPLC and total carotene calorimetrically in the UV/Vis spectrophotometer.

Sample preparation for total and β - carotene

Dry corn is difficult to extract. Rehydration allows efficient penetration of the extraction solvent into the corn tissues. Acetone is used in this method because it is inexpensive and readily available and it penetrates food tissue well. Around 3 g of the ground corn is weighed in a beaker. Sufficient amount of water is added to cover the ground corn (about 10 ml) and let it stand for 30 minutes. About 20 ml of cold acetone is added to the ground flour and keep it stand for 15 minutes. The sintered glass funnel or a Buchner funnel (Whatmann No .4) is used for filtering, and the sample is put in a mortar, ground well with pestle, about 50ml of cold acetone is added (acetone refrigerated for about 2 hrs) and the sample is ground again with the pestle to extract the carotenoids. Filtering through the same funnel was done, collecting the acetone extract in the same suction flask. The mortar and pestle, funnel and residue are washed with small amount of acetone receiving thee washing in the suction flask with the extract. The residue is returned to the mortar and 50ml of fresh cold acetone is added to the macerate and filtered as before. Only two extractions / filtrations are usually enough but the repeat the procedure until the residue becomes colourless.

Colorimetric analysis of total carotene

About 20ml of petroleum ether is placed in a separatory funnel (500ml separatory funnel with Teflon stop cock). One third of the extract is added each time. After each addition, distilled water (~ 300ml) was added slowly, letting it flow along the wall of the funnel. To avoid the formation of an emulsion, do not shake the two separate phases and the lower aqueous acetone phase is discarded. The second portion is added and the operation repeated after the third portion has been transferred to petroleum ether. Around three washings with water (add about 100 ml distilled water let the phase separate and discard the lower phase) is done to remove residual acetone. In the last washing, be sure to discard the lower phase as completely as possible, without discarding any of the upper phase. The upper phase is collected in a 25 ml volumetric flask, passing it through a funnel with anhydrous sodium sulphate to remove residual water. The funnel is washed with a small amount of petroleum ether collecting the washing into the volumetric flask and the volume is made upto 25/50 ml with petroleum ether. The remaining extract was used to measure the absorbance at 450nm. (a total-total carotenoids).

HPLC analysis of β - carotene

High performance liquid chromatography is used to separate components of a mixture by using a variety of chemical interaction between the substance being analyzed (analyte) and the chromatography column (C18). The basic operating principle of HPLC is to force the analyte through a column of the stationary phase (usually a tube packed with small spherical particles with a certain surface chemistry) by pumping a liquid mobile phase (Acetonitrile: Methanol: Ethyl acetate (80:10:10)) at high pressure through the column. The sample to be analyzed was introduced in small volume (10 µl) to the stream of mobile phase and was retarded by specific chemical or physical interaction with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and

mobile phase composition. The time at which a specific analyte elutes come out of the column is called retention time and is considered a reasonably unique identifying characteristic of a given analyte.

DNA isolation and PCR amplification

DNA was isolated from the inbreds as per the modified Dellaporta method (1983). The pellets were dissolved in 100 ul of 0.1X TE buffer and store at 4^0C. The DNA was quantified by using Nanodrop (Nanodrop Spectrophotometer ND - 1000). The absorbance for all samples was measured at 260 nm as double stranded DNA has maximal absorbance at 260 nm. Natural genetic variation in lycopene epsilon cyclase (lcyE) was analyzed in the inbreds with SNP (single nucleotide polymorphism) and gene specific marker that were reported earlier by Harjes *et al*. (*2008*).

a) PCR assay for *lcyE* SNP216 (Tm – 60.6ºC)

SNP 216 L1 – GCGGCAGTGGGCGTGGAT
SNP 216 R1 -
TGAAGTACGGCTGCAGGACAACG

b) **PCR assay for *lcyE* 5'indels / TE (Tm - 55ºC)**

TE103PF F-1 LEFT PRIMER
CGCTAGCAAGCCCATTATTTTTA
TE103PR R-1 RIGHT PRIMER
CGGTATGGTTTTTGGTATACGG

c) **PCR assay for *lcyE* 3'indel (Tm – 59.9ºC)**

3pINDL L2 - GGACCGGAACAGCCAACTG
3pINDL R2 - GGCGAAATGGGTACGGCC

The PCR programme was set up as: Initial denaturation: 94ºC for 5min; Denaturation: 94ºC for 1min; Annealing: TmºC for 1min; Extension: 72ºC for 1min; Final extension: 72ºC for 5min; Go to step 2 for 40 cycles; Hold at 4ºC. Agarose gel (3%) electrophoresis was performed to separate the amplified products.

Results

The estimation of β – carotene using HPLC and total carotene using the spectrophotometer revealed the existence of considerable variation in the carotenoids among the maize inbreds. From the variability curve we have found out ten promising lines with higher β carotene contents (>3 µ g/g) (Figure 8) for the next breeding season. The comparision between shade of yellow and total carotenoid and beta carotene showed that there is poor correlation which is in hormony with Harjes *et al*. (2008). this might be de to the presence of the other carotenoid molecules. The yellow lines averaged 14 µg/g for total carotenoids (5.58 to 63.9 µ) and 1.69 mg/g for β -carotene (0.122 to 4.74 mg/g).

Variability was also observed for kernel carotenoid and β carotene, with some lines accumulating as much as 63 µg/g total carotenoid. Grain color and carotenoid content showed the low correlation between visual grain colour and total carotenoids. The R^2 value was 0.184 for total carotene and 0.033 for β – carotene.

In screening for polymorphisms in key haplotypes, three regions were selected and scored across the entire panel of favorable haplotypes for higher β carotene. The PCR assay is used to survey the high and the low β – carotene lines. The primer LcyE 3'indel (Tm – 59.9ºC) gets amplified at 144 bp. The presence of the band indicates the higher β – carotene levels and its mere absence indicates the lower levels of β – carotene among the maize inbreds. Moreover, the natural mutations like 5' the large promoter insertion and 3'8-bp insertion are expected to increase the β carotene levels. These genomics regions were amplified (144 bp of 3' indels) in the currently surveyed inbred lines with higher β carotene levels. The results are presented in the figure 7.

Discussion

The bio-fortification strategy seeks to take advantage of the consistent daily consumption of large amounts of food staples by all family members, including women and children who are most at risk for micronutrient malnutrition. Bio-fortification provides a feasible means of reaching malnourished populations in relatively remote rural areas, delivering naturally fortified foods to people with limited access to commercially marketed fortified foods, which are more readily available in urban areas. Surveys of a genetically diverse pool of germplasm revealed three significant polymorphisms in the gene encoding LCYε. Strong statistical associations led to the conclusion that genetic variation at this locus significantly altered the ratio of the branches, leading to increased β - carotene. Supporting evidence from an eQTL experiment, carotenoid QTL analysis and a mutagenesis study all pointed to LCYε as the causal factor for the modification of substrate flux. These results allowed the design of PCR-based markers targeted to the three polymorphisms which are currently being used in pro-vitamin A breeding programs (Harjes *et al*., 2008).

Comparisons between b-carotene and total carotenoids with grain colour revealed poor correlations with low R^2 value which indicated that marker-assisted selection (MAS) may prove much more efficient than selection based on colour alone. A simple PCR assay to track and identify LCYε alleles could predict nutritional

content in genetically diverse cultivars found worldwide. Effort in the recent development of LCYε – markers based breeding for maize demonstrated feasibility of a non transgenic, traditional breeding approach to control the pathway branching step and force pathway flux toward β - carotene and its non pro - vitamin A derivatives (the β - branch; Harjes *et al*., 2008).

Variability was also observed for kernel carotenoid and β carotene, with some lines accumulating as much as 63 µg/g total carotenoid. The predominant carotenoids in maize kernels, in decreasing order of concentration, are lutein, zeaxanthin, β carotene, β -cryptoxanthin, and α -carotene. β - Carotene contains two provitamin A structures (two nonhydroxylated β -ionone rings) and β - cryptoxanthin and α -carotene one each in single nonhydroxylated β -ionone ring (Harjes *et al.,* 2008) which led us to emphasize on increasing β -carotene accumulation for maize bio-fortification.

Grain color and carotenoid content showed the low correlation between visual grain colour and total carotenoids R^2= 0.184, β -carotene R^2= 0.033 , in our diverse inbreds explain the difficulty in visual selection for β-carotene content ,so there is a need of other means of quantifying this trait. The HPLC analysis of carotenoids is also much more expensive and inaccessible to developing countries with greatest need for provitamin A. In contrast, polymerase chain reaction (PCR) scoring is found to be more effective in terms of cost, approximately about $1/1000^{th}$ of that of HPLC and its accessibility. For this reason, marker assisted selection (MAS) may prove much more efficient that using colour alone.

Recent study combining information about carotenoid pathways from model organisms with natural variation for carotenoids in maize grain identified several haplotypes of the gene encoding lycopene epsilon cyclase (lcyε; also known as LOC100280448 and *lyce1*) that substantially increased the ratio of β- to α-carotenoids in grain (Yan *et al.,* 2010). Most yellow maize grown and consumed throughout the world has only 0.5 to 1.5 µg/g of β -carotene. Among the lines included in our diverse maize panel, β carotene levels reached as high as 5.83 µg/g. The lowest reading was observed UMI 615, UMI 430, UMI 664 and UMI 27.

Higher levels of β carotene were observed in UMI 946, UMI 176, UMI 79, UMI 34 and UMI 12. In PCR assay the 144-bp insertion allele of 3′TE in LCYE; leading to higher β carotene concentrations, was detected only in the germplasm with higher β carotene but in lower frequency.

A successful story of bio-fortification is the introduction of β carotene rich, orange sweet potato in Mozambique. This study arrives at the acceptance of the orange-coloured staple foods by the maize dieting population. Their regular intake was found to result in improved vitamin A status. These recent results will need to be coordinated with comprehensive breeding and seed distribution efforts to realize the potential of pro-vitamin A bio-fortified maize.

References

Chandler V.L. and Brendel V. 2002. The maize genome sequencing project. ***Plant Physiology*** 130 : 1594 – 1597.

Dellaporta, S.L., Wood, J. and Hicks, J. B. 1983. A plant DNA Minipreparation version 2. Plant ***Mol. Bio, Rep***.1: 19- 22.

Harjes C.E., Rocheford T.R. and Bai L. 2008. Natural genetic variation in lycopene epsilon cyclase for maize biofortification. **Science 319**, 330–333.

Kennedy, G., Nantel G., and Shetty P. 2003. The scourge of "hidden hunger": Global dimensions of micronutrient deficiencies. ***Food Nutr. Agric***. 32:8–16.

West, K.P. Jr. 2003. Vitamin A deficiency disorders in children and women. ***Food Nutr. Bull***. 24, S78–S90.

WHO and FAO. 2003. Joint WHO/FAO expert consultation on diet, nutrition and the prevention of chronic diseases. ***World Health Organ.,*** Geneva, Switzerland.

Yan J., Kandianis C.B., Harjes E.C., Bai L., Kim E.H., Yang X., Skinner D.J., Fu Z., Mitchell S., Li Q., Fernandez M.G.S., Zaharieva M., Babu R., Fu Y., Palacios N., Li J., DellaPenna D., Brutnell T., Buckler E.S., Warburton M.L. and Rocheford T. 2010. Rare genetic variation at *Zea mays* crtRB1 increases β-carotene in maize grain. ***Nature genetics***.

Figure 1. MAIZE KERNEL COLOUR

WHITE

UMI 395 (β-carotene - 0.4257 µg/g)

ORANGE

UMI 69 (β-carotene – 0.78µg/g)

YELLOW

UMI 176 (β-carotene – 5.8 µg/g)

LIGHT ORANGE

UMI 112 (β-carotene – 2.22 µg/g)

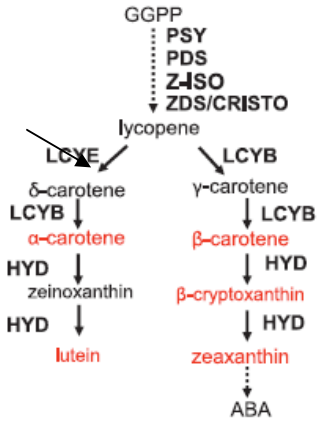

Figure. 1 The biosynthetic pathway of β - carotene

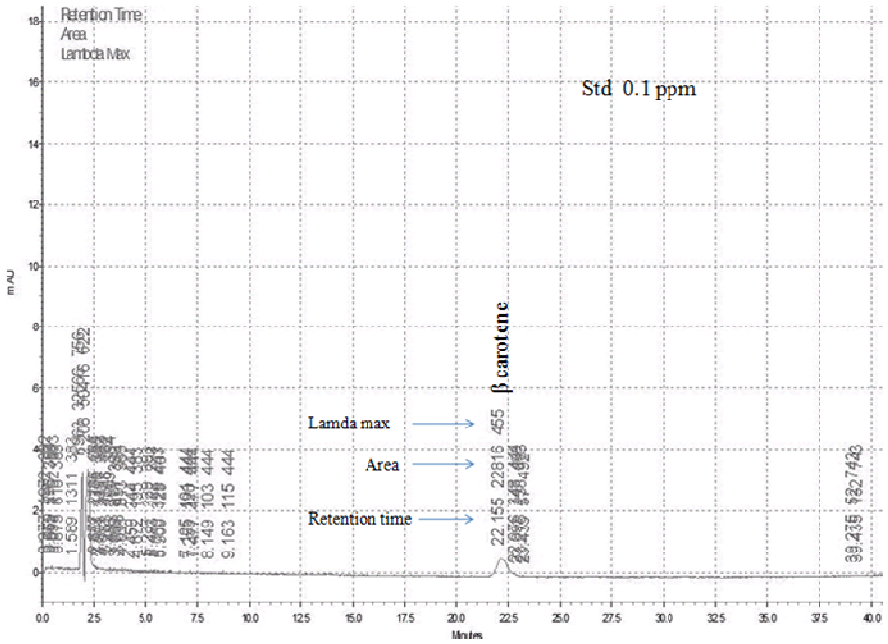

Figure 2. Estimation of β-carotene concentration by using HPLC

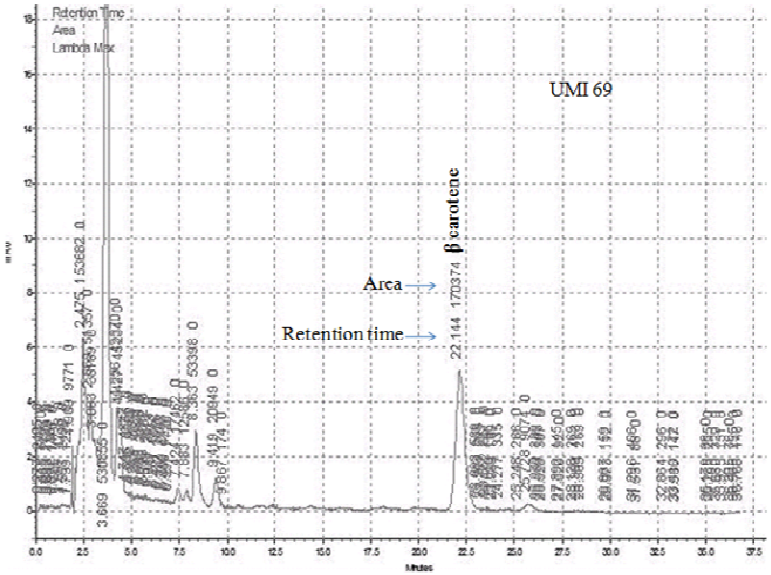

Figure 3. Estimation of β-carotene concentration by using HPLC for UMI 69

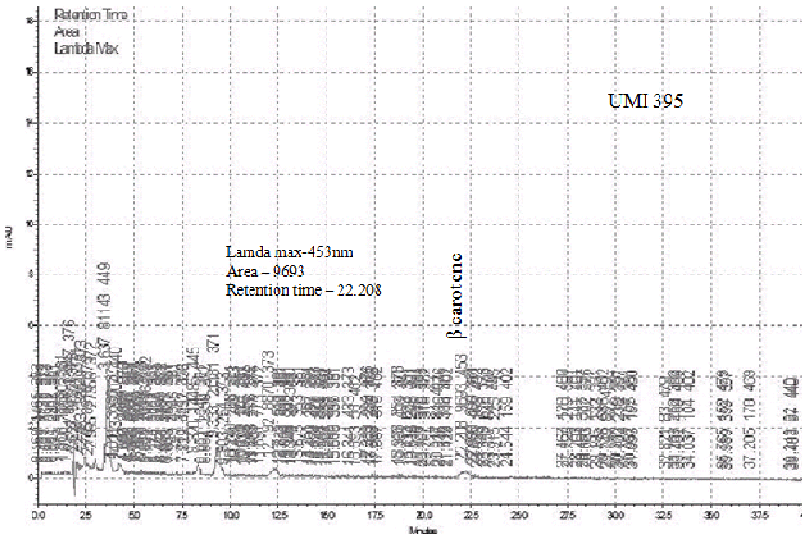

Figure 4. Estimation of β-carotene concentration by using HPLC for UMI 395

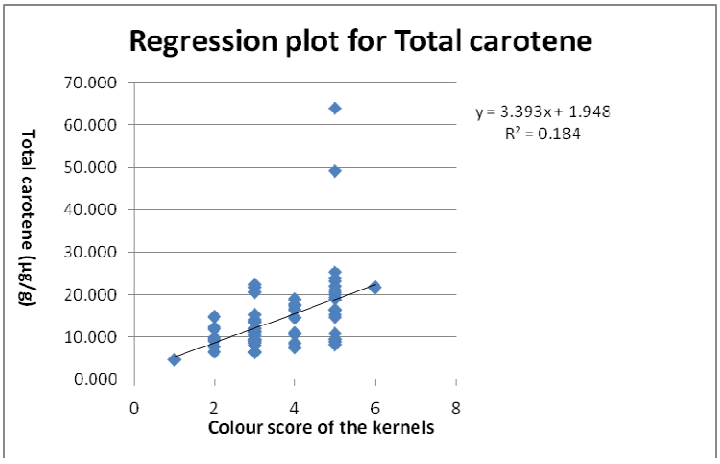

Figure 5. Regression plot for total carotene content (µg/g)

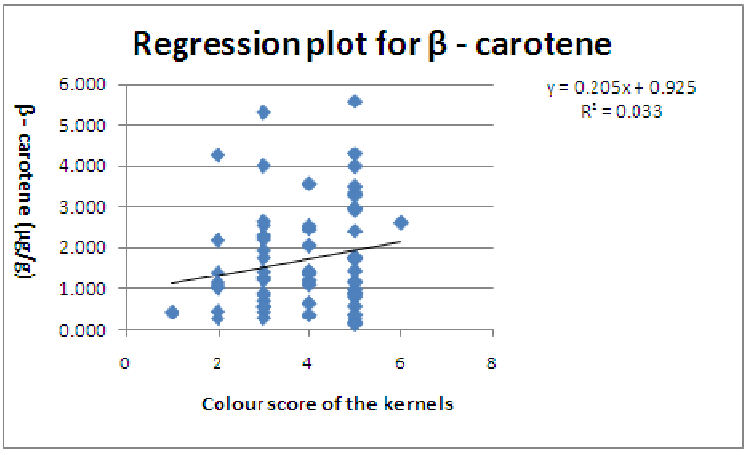

Figure 6. Regression plot for β - carotene content (µg/g)

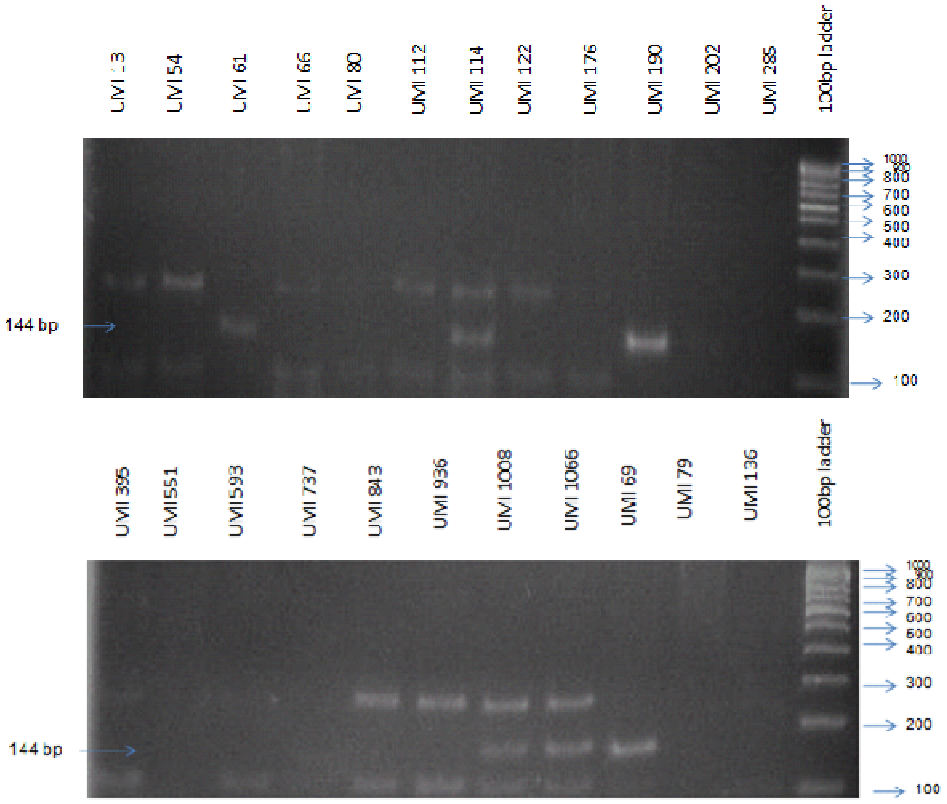

Figure 7. Molecular characterization of the maize inbreds using the primer *lcyE* 3'indel (144 bp)

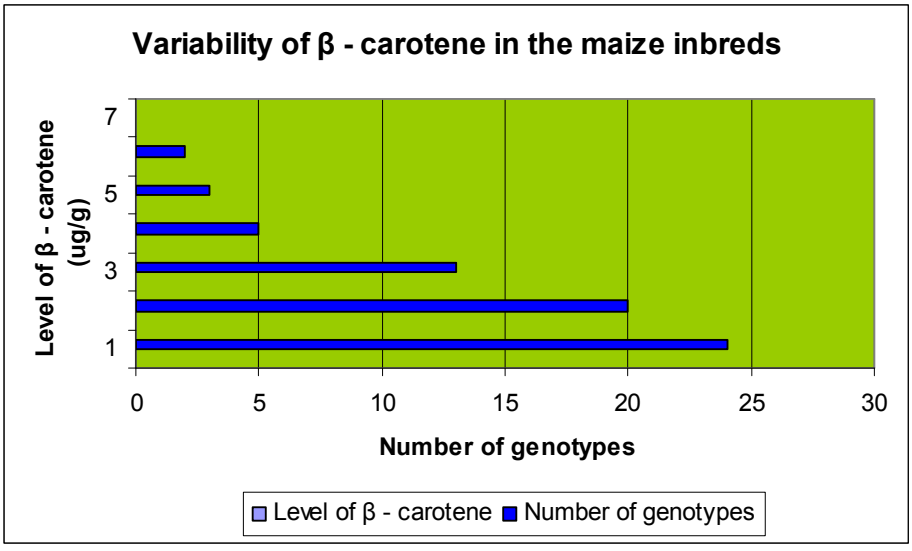

Figure 8. Variability for β carotene among the maize inbreds