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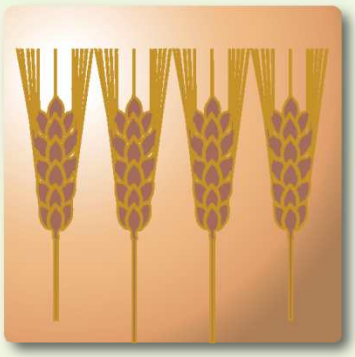

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Poorna Bhog, a high yielding mutant variety of rice (see page 44).

To Our Readers

Mutation induction as a tool in plant breeding and for genetics or genomics research is of continuous and increasing interest. Since the onset of the sequencing revolution the ability to target specific genes and to detect mutations in them has brought about a renaissance in plant mutation breeding. We are seeing increased interest in plant mutation induction which in addition to plant breeding is being used as a major tool in determining gene/allele function. This renaissance is also being fuelled by climate change as there is increasing urgency to develop crops that are more resilient to the effects of climate change and plant mutation breeding offers a fast response.

As a consequence the Plant Mutation Reports (PMR) are in competition with a growing number of indexed journals with various impact factors that offer high quality standards and wide distribution through online publications. These are attractive and authors prefer to submit their data to these journals as they grant greater visibility and scientific merit. In response to this situation, and the necessity for the best possible application of the limited resources available, we have decided to discontinue PMR as a regular journal. However, in order to allow for the publishing of short notes, e.g. on the release of new mutant varieties or success stories in plant mutation breeding, we will strengthen our regular publication of the Plant Breeding & Genetics Newsletter (PBGN), which is published twice a year. The PBGN has sections on “success stories” and reports on the progress of Agency programmes in Technical Cooperation (TC) and in Coordinated Research Projects (CRP). We are particularly interested in short stories on the impact that the release of improved mutant varieties have on farmers, users and consumers in your respective countries. Please contact us if you have a success story and would like to have it published in the newsletters. Also, I would like to use this opportunity to encourage your submissions of officially released mutant varieties to our Mutant Varieties Database (MVD). This database is unique and functions as a witness for the useful applicati-

on of nuclear technology in food and agriculture. Currently the database lists more than 3200 released mutant varieties in more than 200 crop species and the number of plant species subject to mutagenesis also continues to rise. The MVD is currently being improved to facilitate submission of information and to provide more advanced search and data analysis tools.

As to the current and final regular volume of PMR, I am happy that we can present an interesting mixture of two short notes on the development of new mutant varieties of rice in India, a review on the current status and trends in cassava mutation breeding and four research articles dealing with various topics in plant mutation. One of the articles addresses the characterization of various types of mutations in wheat as a resource for functional genomics, thus giving an example of the trend in broadening the use of mutation induction. Another article reports on the nature of molecular variation induced by gamma irradiation of barley as analysed by Amplified Fragment Length Polymorphisms (AFLPs) and Single Sequence Repeats (SSRs). This issue of PMR also gives a forum for results produced by a recently completed CRP on "Molecular Tools for Quality Improvement in Vegetatively Propagated Crops Including Banana and Cassava". Reports are included on the production of haploid tissue of the diploid *Musa* species *M. acuminata* cv. 'Matti' and on the analysis of carotenoid-protein content variation in pigmented cassava storage roots and its implication for traditional breeding strategies and use of induced mutations.

Stephan Nielen

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Research Article

Molecular Variability in Barley Structural Mutants Produced by Gamma Irradiation

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Abstract

Single Sequence Repeat (SSR) and Amplified Fragment Length Polymorphism (AFLP) markers were used to survey gamma ray induced genetic variation in a set of 13 originally produced structural barley (*H. vulgare* L.) mutants from cv. Freya; including 8 single translocation lines, 3 double translocation lines and 2 multiple reconstructed karyotypes. Both marker systems contributed to the evaluation of the radiation induced DNA alterations and revealed in general 0.49% polymorphisms in the studied genotypes. AFLPs were observed with 3 out of 10 PstI/MseI primer combinations. Transmissible microsatellite instability at loci with perfect (AT)n repeats located in the introns of the *rubisco activase* and *waxy* was documented in three mutant lines. The results emphasize that in addition to point mutations, small indels (2bp) form the major group of the gamma induced DNA alterations.

Keywords: radiation mutagenesis, AFLPs, SSRs, *Hordeum vulgare* L.

Introduction

Mutagenic agents are known to produce a range of mutant types. Two main types of genetic alterations are: gene or point mutations and structural mutations or chromosomal rearrangements. There are clear indications that mutagens are able to induce a spectrum of mutation types. Chemicals like 8-ethoxycaffeine were found to produce a high frequency of chromosomal rearrangements without affecting the rate of the gene mutations [1]. On the other hand, sodium azide under specific treatment conditions is capable of inducing a high frequency of point mutations which do not affect chromosome integrity [2].

The development of marker detection systems in recent years allowed the estimation of genetic diversity at the DNA level. Simultaneous application of different DNA marker systems is expected to facilitate the effective evaluation of both natural and induced genetic variability as well as their rational use in breeding programmes. DNA markers can also be used to study the molecular nature of induced mutations.

The eukaryotic genome contains a large number of repeated sequences, such as mini- and micro-satellites, as well as expanded simple tandem repeats which are inherently unstable and may promote increased frequency of induced mutations. The intrinsic instability of these sequences in mammals (reviewed in [3]), as well as in plants [4, 5], manifests in length change mutations and can be further enhanced by radiation. Several studies [6, 7, 8] have documented an increased frequency of induced mutations in these sequences and confirmed their possible use as markers to study the genetic effects of ionizing radiation in both animals and plants. The increased frequency of mutation induction at these small genomic loci cannot be attributed to the direct effect of radiation induced DNA double strand breaks and hence seems to be "untargeted" [7]. In addition, Niwa [3] reported that the radiation induced genomic instability exerts another feature of "delayed mutations" with postponed generation after the radiation exposure. The processes underlying the effects mentioned above are still not entirely determined but it is supposed that radiation induced events may trigger the repeated sequences to become genetically less stable [9]. Meanwhile, the use of such sequences as reporters of induced mutation was demonstrated in a number of studies [8, 10, 11].

The objective of this study is to survey the occurrence of the transmitted to progeny DNA polymorphisms in a number of originally produced by gamma radiation barley structural mutants. AFLPs and SSRs were used to elucidate the nature of the induced molecular variation.

Materials and methods

Plant material

Thirteen barley structural mutants and their parental line (two rowed spring cv. Freya) were used as experimental material. Mutant forms T-1586, T-20, T-26, T-48, T-59, T-63, T-67 and T-68 are single reciprocal chromosome translocations, and T-16, T-58 and T-66 contain double translocations. All these mutant lines were precisely characterized with respect to the chromosomal localization of the translocation breakpoints [12]. In addition, two multiple reconstructed karyotypes, namely PK-169 [13] and PK-88-4 [14], which carry reciprocal translocations and pericentric inversions were used in the molecular analyses. All chromosomal rearrangements were induced by gamma radiation of dry seeds of cv. Freya (doses of 150 Gy or 180 Gy).

Genomic DNA extraction

DNA was isolated from 2-3 weeks old leaf samples bulked from 5 plants per genotype as well as from 5 randomly chosen individual plants of two subsequent progenies (20 plants per genotype in total) using the CTAB method according to the procedure of Murray and Thompson [15] with minor modifications.

AFLP analysis

AFLP analysis was performed as originally proposed in [16] with minor modifications starting with 250 ng bulked DNA samples of each genotype. DNA was digested with 10 U PstI and 5 U TruI (MseI isoschizomer) (Fermentas) in buffer recommended by the manufacturer. Following ligation and pre-selective amplification, selective amplification was carried out with 10 primer combinations using Cy5 labelled Pst I primer + 2 and 3 selective nucleotides (GCA or GAC) combined with one of the 5 Mse I primers used (M13, M15, M20, M37, M39) (Table 1). The amplification products were resolved in 6% polyacrylamide gel using an ALF express II DNA analyser (Amersham Pharmacia).

TABLE 1. AFLP PRIMERS USED IN PRE-SELECTIVE AND SELECTIVE AMPLIFICATION

Primer	5'-3' Sequence
M 13	GAT GAG TCC TGA GTA A + AG
M 15	GAT GAG TCC TGA GTA A + CA
M 20	GAT GAG TCC TGA GTA A + GC
M 37	GAT GAG TCC TGA GTA A + ACG
M 39	GAT GAG TCC TGA GTA A + AGA
P 64	GAC TGC GTA CAT GCA GG + AC
P 67	GAC TGC GTA CAT GCA GG + CA

SSR analysis

Twenty-five genomic SSR markers (HVM03, HVM04, HVM07, HVM20, HVM27, HVM36, HVM40, HVM49, HVM60, HVM62, HVM65, HVM67, HVM74, Bmac0032, Bmac0213, Bmac0399, Bmag0125, Bmag0135, Bmag0140, Bmag0211, Bmag0378, Bmag0518, EBmac0565a, HvLox, HVCMA) and 1 EST-SSR (GBM1042) derived from the published barley genetic maps were chosen. The PCR conditions applied for amplification of the studied microsatellite loci were as described in [17-19]. In addition 9 EST-SSR markers (TC45216, TC46739, TC48823, TC51192, TC55352, TC57684, TC57516, BG367984, BU986714) were selected from the http://wheat.pw.usda.gov./ITMI/EST-SSR/LaRota/ and tested in the analysed lines. PCR amplification was performed in 20 µl reaction mixture containing 100 ng DNA, 1 µM of each primer, 200 µM of each dNTPs, 1.5 mM $MgCl_2$ and 0.4 units of Taq polymerase. Amplification conditions were an initial denaturation at 94ºC for 3 min, and 30 cycles at 94ºC for 1 min, 60ºC for 1 min, and 72ºC for 1 min. A final extension of 5 min at 72ºC was performed. In cases where the forward primer was labelled with a Cy-5 fluorescence label, the fragment analysis was carried out on the automated laser fluorescence sequencer ALF Express II (Amersham Biosciences). Alleles were sized with software Allele locator 1.03. All PCR amplification reactions were performed in a PCR system 9700 (Applied Biosystem).

Results and discussion

Two widely utilized DNA marker systems (AFLPs and SSRs) were used to study the nature and the level of gamma ray induced genetic variation in a set of 13 originally produced structural barley mutants. The comparative analyses of the surveyed mutant lines and the parental genotype cv. Freya revealed gamma ray induced mutations at several chromosomal loci.

For AFLP analysis a total of 10 primer combinations were used as follows: PstI (P64 and P67) as labelled forward primers and 5 MseI (M13, M15, M20, M37, M39) as reverse primers. The AFLP assay showed that almost all tested primer combinations produced identical patterns for both the mutant barley lines and the initial parent genotype, cv. Freya. Primer combinations P64/M13 and P64/M39 generated polymorphic fragments of 325 bp in T-26 (Fig. 1) and 550 bp in the mutant line T-48, respectively. The observed polymorphisms are due to gamma ray induced point mutations.

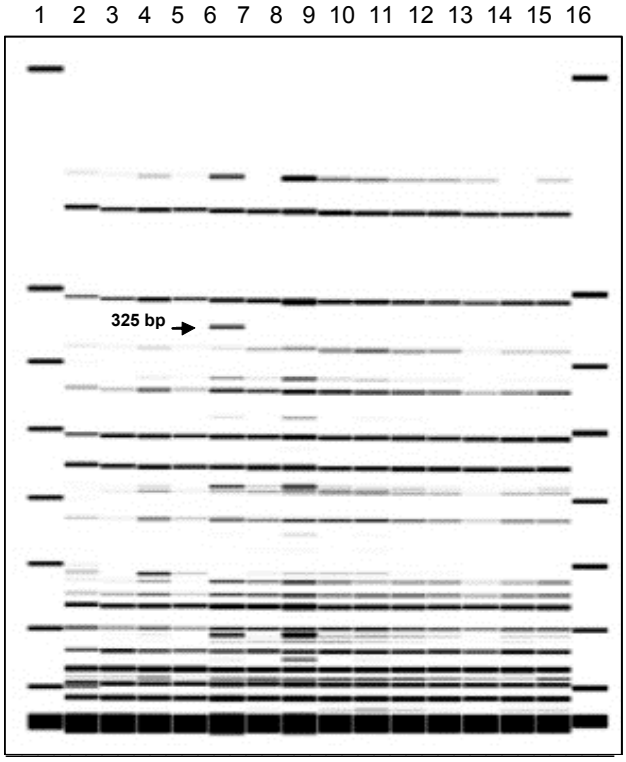

Fig. 1. AFLP profiles of the initial parent genotype and its mutant lines obtained by the primer combination P64/M13. (1) Ladder 50-500 bp, (2) cv. Freya, (3) T-1586, (4) T-16, (5) T-20, (6) T26, (7) T-48, (8) T-58, (9) T-59, (10) T-63, (11) T-66, (12) T-67, (13) T-68, (14) PK-169, (15) PK-88-4, (16) Ladder 50-500 bp.

Among the remaining 34 SSR markers, only two (HVM03 and HVM04) detected mutation induced allele variation in the mutant lines. The level of radiation induced polymorphisms accounted for 0.63%. The SSR analysis showed a new allele variant (243 bp) in two

mutant lines T-20 and T-68 compared to the initial geno-genotype, cv. Freya, and other tested mutant lines (241 bp) at HVM04 locus (Fig. 2).

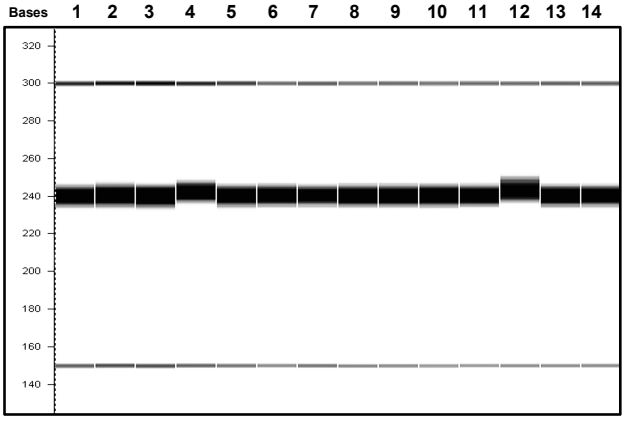

Fig. 2. Allele variation at HVM 04 - 7HS locus in mutant lines and the initial genotype – cv. Freya using bulked DNA (progeny 2005). (1) cv. Freya, (2) T-1586, (3) T-16, (4) T-20, (5) T-26, (6) T-48, (7) T-58, (8) T-59, (9) T-63, (10) T-66, (11) T-67, (12) T-68, (13) PK-169, (14) PK-88-4.

A new allele variant (198 bp) in the structural mutant line PK-88-4 was observed at the HVM03 locus. Both SSR loci are located in the introns of the genes for starch synthase (*waxy)* localized in the short arm of chromosome 7H and rubisco activase residing in the long arm of chromosome 4H. No length variations were observed in the other SSR loci.

As a quantitative measure, the number of polymorphisms obtained by SSR markers in this study is higher (1.4x) in comparison to the applied AFLP enzyme/primer combinations. Both analyses (AFLP and SSR markers based) revealed in general 0.49% polymorphisms in the studied barley mutant lines. Josi-Saha and Gopalakrishna [11] analysed the radiation induced polymorphisms in *Sesbania rostrata* mutants using RAPD, ISSR and AFLP marker systems. The authors observed that the hypervariable region based ISSR markers are superior in detecting the radiation induced variability.

The stability of the radiation induced SSR alleles over generations was analysed in the next progeny using randomly chosen individual plants and bulk DNA from the same mutants and the parental genotype cv. Freya. The analysis showed stable inheritance variation in the repeat length (including gains and losses of repeats) induced by γ-rays at both SSR loci in the mutant lines T-20, T-68 and PK-88-4, respectively (d+
ata not shown).

A stable transmission of short (1-4 bp) deletions to the progeny has also been observed by Naito et al. [21] in *A. thaliana* after treatment with γ rays or carbon ions in comparison to the extremely large ones (up to >6 Mbp). The authors proposed that the non-transmissibility of the large deletions may be due to the deletion of a particular region that contains a gene or genes required for gamete development or viability.

This study revealed that amongst the screened set of SSR markers with different composition and lengths of the repeats the observed polymorphisms in the structural mutant lines were found only in loci with perfect (AT)n repeats such as HVM03 and HVM04 located in the introns of both *rubisco activase* and *waxy* genes, respectively. Polymorphisms were not observed in the perfect (GA)n, (AC)n, (AG)n SSRs and those with compound repeats containing also (AT)n repeats (Ebmac0565 and Bmac0032). Absence of SSR mutation induction was also observed in the other selected EST-SSR loci such as HvLox, HVM07 and those from an EST data base (http://wheat.pw.usda.gov) composed of different (AT)n repeats. The data may suggest that γ irradiation (probably) affects the structure of some SSR loci with perfect (AT)n repeats located in the non-coding DNA sequences by adding or subtracting repeat units. However, only a small number of SSR loci were investigated. Based on an in vitro assay it was found by Schlotterer and Tautz [27] that the base composition of the repeat units affects the mutation rate, i.e. sequences with high AT content mutate more frequently than those with high GC content. This indicates that the template stability may affect the mutation rate, perhaps by reducing the frequency of strand-slippage events. The templates at (AT)n SSR loci in barley may be unstable, leading to an increased frequency of mutation. Among the SSRs with perfect (AT)n repeats used in this study, those with shorter repeat length such as HVM04 ($(AT)_9$) mutated at higher rate than the longer ones, HVM03 ($(AT)_{29}$). Our results disagree with other studies, postulating that longer repeats show more mutations due to increased chances of error by single strand slippage during DNA replication [28-30].

As a further and different extension of this model Kovalchuk et al. [8] suggested another model for replication slippage which accounts for more complex mutations, gains and losses of multiple repeat units, as well as losses of loci and insertions of DNA of unknown origin. Their experiment was based on a very complicated pattern of SSR mutations in a germline of wheat exposed to post-Chernobyl radioactive contamination.

Conclusion

This survey established that the frequency of both transmitted point mutations and small indels (insertions/deletions of 2 bp) in the DNA sequences analysed is higher in comparison to the limited rate of the radiation-produced stable structural chromosomal changes in the respective barley (*H. vulgare* L.) mutant lines. One plausible explanation of this phenomenon might be the variation in the survival capacity of the visualized types of genetic alterations in the process of diplontic selection.

Acknowledgements
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Research Article

Some ENU Induced Mutations: A Resource for Functional Genomics in Bread Wheat

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Abstract

Induced mutations have been extensively used in the past for crop improvement. More recently a renewed interest in induced mutations has been witnessed due to their utility in functional genomics research. In the present study eight different types of mutations that were induced in common wheat (*Triticumaestivum*) due to ENU (N-ethyl-N-nitrosourea) treatment were grouped into four classes (stem mutants, reproductive mutants, leaf mutant and spike mutants). Two of the stem mutants, namely axillary branching and reduced node (1-2), were novel and interesting. *In silico* studies were conducted using candidate genes that were reported to produce similar mutant phenotypes in other species. We identified six wheat genomic sequences that were considered orthologous to the sequences for branching genes from rice and maize. Similarly, using EST database we identified 11 unigenes which matched a gene responsible for reduction in number of nodes in maize. These sequences that are involved in axillary branching and reduced number of nodes may be used as candidates for further studies of above mutants in bread wheat.

Key words: Axillary branching, chemical mutagenesis, ENU, reduced node

Introduction

Induced mutations have been extensively used in crop plants both for basic and applied research. In the past, these mutations were utilized for crop improvement leading to the release of more than 3200 mutant varieties (http://mvgs.iaea.org/), which included 274 varieties of wheat (*Triticumaestivum* L.). There are many other reports of interesting and useful induced mutations in wheat. Some of these mutants include the following: tolerance to drought [1], tolerance to yellow and brown rust [2], maintained vegetative phase [3, 4], monoculm phenotype [5] and semi-dwarfness [6]. These available mutants make an important source of genetic variation in common wheat, which can be utilized not only for wheat breeding but also for basic research at the molecular level.During the last four decades, however, mutation research has witnessed a renewed interest due to the availability of a variety of new technologies, which made it possible to either induce novel and targeted mutations or to utilize induced mutations for the study of the mechanisms involved in a variety of developmental and physiological processes [7]. Mutations have also been utilized for the discovery of targeted genes and/or to study their functions [8]. Some of the examples of using mutations for developmental/physiological studies include the following: (i) homeotic flower mutants in *Antirrhinum majus* [9]; (ii) leaf and early senescence mutants in rice [10, 11]; (iii) stem/shoot, root and reproductive mutants in *Arabidopsis thaliana* [12-14] and (iv) pigment mutants in *Petunia hybrida* [15]. Similarly, starting in 1970s, genetic engineering made it possible to either insert new genes, or disrupt and alter pre-existing genes in a targeted manner [16]. For instance, approaches of insertional mutagenesis involving T-DNA and transposable elements (TEs) and gene targeting involving use of nucleases like ZFN (zinc finger nucleases) and TALE (transcription activator like effector) proteins have become available for mutation research [17-20]. Another technology which influenced mutation research, was the availability of DNA based molecular markers during 1980s, which made it possible to identify and map/clone specific genes [5, 21]. Some of the more recent approaches for mapping genes/QTLs include MAGIC (Mutant-Assisted Gene Identification and Characterization) and BSR-Seq (Bulked Segregant RNA Sequencing) [22, 23]. Finally, the emergence of genomics research during 1990s made it possible to study mutations at the molecular level using gene/genome sequences. Techniques like RNAi and VIGS [24, 25] allowed the discovery of genes for a variety of traits, and those of TILLING and Eco-TILLING allowed allele mining at the molecular level [20]. Availability of high throughput and cost effective next generation sequencing (NGS) technologies also facilitated the use of mutations for gene/allele discovery [23]. Therefore, induced mutants have become an important resource to study structure function relationships of genome sequences with unknown functions utilizing approaches of forward and reverse genetics (for a review see Ref. [20]).

The above mentioned renewed interest in mutations and the emergence of new areas of mutation research also demanded that the physical and chemical mutagens used in the past for inducing mutations be revisited and that if necessary, newer mutagens be used. Among physical mu-

tagens, alpha particles and fast neutrons are being used to generate mutations that are largely due to deletions (e.g. DEALING, Deleteagene). Similarly, among chemical mutagens newer mutagens, like methyl nitroso urea (MNU), hydroxyl amine, nitrous acid and N-ethyl-N-nitrosourea (ENU) are being recommended, since they induce mutations involving base substitutions, including transitions and/or transversions (for a review, see [20]). Keeping this in view, during the present study in common wheat ENU was used for inducing novel and interesting mutations in bread wheat that may be utilized for understanding the underlying genetic basis of the mutant traits, and may later be followed by genetic mapping and TILLING using molecular approaches. In this communication we describe our preliminary results related to the efficacy of ENU for the induction of mutations and also describe characteristic features (including inheritance) of some of the interesting new mutants obtained during the present study. The possibility of mapping and cloning the genes responsible for these mutant phenotypes in wheat using both wet lab and *in silico* approaches are also discussed.

Materials and methods

Seed material

Seed of bread wheat cv.PBW550 available in the wheat germplasm of the Department of Genetics and Plant Breeding, CCS University, Meerut, India, was utilized for treatment with ENU for induction of mutations.

Mutagen treatment and raising of M_1 population

Two thousand and five hundred (2500) dry seeds (11% moisture content) of bread wheat cv. PBW550 were presoaked in water for 4 h at room temperature. Presoaked seeds were treated at room temperature with 2mM solution of ENU for 12 h, followed by washing under running tap water for 4 h. The treated seeds were sown at the Research Farm of the Department of Genetics and Plant Breeding, CCS University, Meerut, India, during the crop season 2008-2009. Each of the 850 surviving M_1 plants were selfed by bagging the main spike. At the time of maturity, M_2 seed from individual bagged spikes were harvested and stored for raising a M_2 population next year.

Raising of M_2 population and recording of data

During crop season 2009-2010, the above M_2 seed was used to raise spike-to-row progenies in rows of 1.5 m with row-to-row distance of 30 cm and plant to plant distance of 15 cm. Wheat cv. PBW550 was used as the control and was planted as single rows along with the M_2 progenies in each block of 45 rows. Data on the number of normal and mutant plants in each row was recorded only on 503 progenies, each having more than four plants (rows having less than four plants were rejected). However, in segregating rows, the number of wild type and mutant plants was often not adequate to study segregation ratios and to apply chi-square test for goodness of fit (this was done in M_3 generation). Different types of mutants available in the segregating progenies were identified and characterized. Spikes of M_2 plants were bagged to obtain selfed seed to be used for raising M_3 progenies.

Raising of M_3 population and recording of data

During the crop season 2010-11, M_3 progenies of individual normal and mutant plants belonging to each of the segregating M_2 progenies were raised along with the control to study segregation and to identify/confirm the true mutants. In each progeny, 25-30 plants were raised, each in plots of three rows of 1.5 m length with a row-to-row distance of 25 cm, and plant-to-plant distance of 15 cm. In progenies showing segregation, data were recorded on the number of wild type and mutant plants. In progenies showing no segregation, 5 plants per mutant progeny and 10 plants per control progeny were used for recording data on plant height, tillers per plant, spike length and number of spikelets per spike.

Statistical analysis

To test the data for 3 (normal): 1 (mutant) segregation ratio, chi-square test for goodness of fit was used at 5% level of significance

Search for candidate genes for the mutant traits in wheat

Sequences of possible candidate genes (for each trait of interest) from related species were utilized as query sequences, for blastN and TblastX tools in search of orthologous sequences in wheat genome. For this purpose, NCBI (http://www.ncbi.nlm.nih.gov/), TIGR (http://plantta.jcvi.org/) and DFCI (http://compbio.dfci.harvard.edu/tgi/plant.html) databases and the 5x wheat sequences produced in U.K. (http://www.cerealsdb.uk.net/) were searched. The search involved the following activities: (i) Orthologues for axillary branching gene were searched by using the sequences of the genes responsible for axillary branching trait in rice (*d14, d3, d10/OsCCD8, htd1/CCD7*) [26, 27]; two other maize sequences (*CCD7* and *CCD8*) that are orthologous to branching genes in pea, *Arabidposis,* petuniaand rice [28] were also used as query sequences. Orthologous wheat sequences were also utilized for identification of conserved domains through conserved domains search service of NCBI. TblastX was also performed using a sequence *Ostb1* [29] of rice that is orthologous to *tb1* [30] of maize, and was earlier reported to be responsible for branching in rice. (ii) For mutants exhibiting reduced number of nodes, wheat sequences orthologous to *ZmMADS3* (AF112150.1) of maize [31] were searched in the above three databases; *ZmMADS3* was earlier reported to be responsible for reduction in number of nodes. (iii) For senescence mutants a search was made using *Osl85* and *Osh36* sequences of rice [32] that are responsible for early senescence.

Results and discussion

M_1 generation, identification of mutants in M_2 generation and their validation in M_3 generation

Out of 2500 seeds treated with 2mM ENU, 850 (34%) plants survived in M_1 generation. In M_2 generation, out the 850 M_1 spike-to-row progenies, 503 progenies had more than four plants each; 40 of these M_2 progenies segregated for 16 apparently different types of viable mutations and two progenies segregated for lethal (albino) mutations (Table 1). Ten (25%) of the 40 progenies carrying viable mutants segregated for dwarf mutants (Table 1), which thus represented the most frequent mutant type. M_3 progenies from individual plants belonging to M_2 rows segregating for different types of mutants were examined for validation of mutants (Table 2 for more details see supplementary Table 2S). The 40 M_2 segregating rows apparently had 16 mutations and were advanced to M_3 generation, using 2-4 plants from each M_2 progeny. However, for 28 of these 40 progenies, no mutants were observed in M_3 generation indicating that the phenotypic variants recorded in M_2 generation in these cases were not true mutants. In the remaining M_3 progenies derived from 12 M_2 progenies only 8 of the 16 mutants (observed in M_2) could be validated. Individual M_3 mutant progenies either had only mutant plants or exhibited segregation, giving a good fit to 3 (normal): 1 (mutant) ratio.

TABLE 1. DIFFERENT TYPES OF MUTANTS ALONG WITH THE NUMBER OF PROGENIES IN M_2 GENERATION OF BREAD WHEAT CV. PBW550 RAISED FOLLOWING ENU TREATMENT

S. No.	Mutant	Type of mutation: Lethal(L)/ Viable(V)	Number of segregating progenies
I	**Lethal mutant**		
(i)	Albino	L	2
II	**Viable mutants**		
1	*Stem mutants*		
(i)	Axillary branching	V	1
(ii)	Reduced node (1-2)	V	1
(iii)	Dwarf	V	10
(iv)	Monoculm habit	V	3
(v)	High tillering	V	1
(vi)	Partially lethal	V	2
2	*Leaf mutants*		
(i)	Early leaf senescence	V	1
(ii)	Yellow leaves with Necrotic spots	V	1
(iii)	Broad leaves	V	1
3	*Spike mutants*		
(i)	Fusiform ear	V	3
(ii)	Clubbed shape ear	V	1
(iii)	Crooked penduncle	V	1
(iv)	Bent penduncle	V	1
4	*Reproductive mutants*		
(i)	Non-flowering	V	5
(ii)	Late flowering	V	4
(iii)	Stay green and lax ear	V	4
	Total		42

Morphology and genetics of mutants

Eight true breeding mutants recovered in this study were grouped in four classes and were used for further studies. These mutants are described in the following text.

1. Stem mutants

Stem mutants included three different mutants related to alteration in shoot morphology i.e. axillary branching, reduced node (1-2 nodes only) and dwarf mutants.

(i) An axillary branching mutant

In bread wheat, tillers arise from the basal nodes during vegetative growth (Fig. 1(A) and 2(A)). The axillary branches differ from tillers in their position and timing of emergence. They arise from axillary buds in the axils of leaves higher up in the culms, where stem internodes have elongated, rather than from the basal nodes (Fig. 1(B) and 2(B)). Out of three M_3 progenies for this mutation, two progenies segregated for the mutant phenotype, each giving a good fit to 3 (normal): 1 (mutant) ratio, while the remaining third progeny had all mutant plants. The mutant phenotype appeared after nearly 130 days of sowing, when flowering was already over in control plants.

The extent of branching in the mutant plants also differed and appeared to have an effect on plant height, although the negative correlation observed between plant height (mean 53.5; range 46-70cm) and number of tillers with branches (mean 8.6; range 1-35) was not significant (r = -0.39). The number of nodes per tiller in the mutants was higher (mean = 8.00 nodes per tiller) than in the control plants (3.6 nodes per tiller; Fig. 1(D)). The number of nodes in the axillary branches varied from 1 to 3 with a mean of 1.9 nodes per axillary branch.Also, the mean number of nodes per tiller in individual plants (data recorded only on five main tillers) had a negative correlation with plant height (r = -0.64), spike length (r = -0.81) and spikelets per spike (r = -0.87), and had a positive correlation with number of tillers having axillary branching (r = 0.59). The spikelets were also narrower in the mutant plants than in the control plants (Fig. 1(F)). The grain number per ear was also reduced in mutant plants and there was a significant negative correlation (r= -0.53) between number of tillers with branches (mean 8.6; range 1-35) and total grain number per mutant plant (mean 211; range 6-610).

As mentioned above, a more pronounced expression of axillary branching in the mutant plants had a negative impact on plant height. Similar behaviour was reported in branching mutants reported in other plant systems including rice [26, 29, 33] and petunia [34, 35]. This may be due to the distribution of resources to the axillary branches that was otherwise meant to be utilized for attaining normal plant height. The mutant phenotype (axillary branches) appears only after the formation of primary inflorescence. In mutant plants that germinated late the expression of mutant phenotype was relatively poor, perhaps as a result of rise in temperature during the latter part of the crop season. Therefore the effect of tempera-

ture on the expression of the mutant phenotype may be investigated further.

TABLE 2. SEGREGATION PATTERN OF EIGHT DIFFERENT TYPES OF MUTANTS IN M_3 GENERATION OF BREAD WHEAT CV. PBW550 RAISED FOLLOWING ENU TREATMENT

S. No.	Mutant	Total number of progenies	Number of progenies showing good fit to 3 (normal):1 (mutant) segregation	Number of homozygous mutant progenies
1.	*Stem mutants*			
(i)	Axillary branching	3	2	1
(ii)	Reduced node (1-2)	1	-	1
(iii)	Dwarf	11	9	2
2.	*Leaf mutant*			
	Early senescence of leaves	3	2	1
3.	*Spike mutants*			
(i)	Fusiform ear	2	-	2
(ii)	Clubbed shape ear	1	-	1
(iii)	Bent penduncle	3	2	1
4.	*Reproductive mutant* Non- flowering mutant	2	2	-

Fig. 1. Whole plants and different parts of wild type and axillary branching mutant in wheat (A-G: in each case 1 is normal, and 2 is mutant). (A) diagrammatic representation of whole plant, showing no axillary branching (1), and a plant with axillary branching (2). (B) images of actual plants; (C) tillers showing increase in the number of nodes (with leaves at each node) in the mutant; (D) tillers with leaves removed; (E) spikes showing reduced length and presence of supernumerary spikelets in the mutant; (F) spikelets showing narrow spikelet in the mutant; (G) florets from a single spikelet showing increase in number of florets in the mutant.

Fig. 2. Whole plants and different parts of wild type and reduced node mutant in wheat (A-G: in each case 1 is normal, and 2 is mutant). (A) diagrammatic representation of whole plant showing a normal plant (1), and a plant with reduced number of nodes (2). (B) images of actual plants ; (C) tillers showing decrease in the number of nodes (with leaf at solitary node) in the mutant; (D) tillers with leaves removed; (E) spikes showing reduced length in the mutant; (F) spikelets showing reduced length of awns in the mutant; (G) florets from a single spikelet showing increase in number of florets in the mutant.

Since the axillary branch mutant is being reported for the first time in wheat, no information is available on its genetics. However, genetic analyses of axillary branching in terms of QTL mapping, identification and isolation of genes has been reported in other species such as *Setariaitalica*[36],*Oryza sativa* [29]and*Zea mays* [30]. In *Setariaitalica,* 4 QTLs for axillary branching have been reported, one each on chromosomes 6 and 9 and two on chromosome 5 [36]. In rice, genes for axillary branching are located on rice chromosomes 1, 3, 4, 6 and 11 (*d10* [33]*, Ostb1* [29]*, OsCCD7* [27]*, d3* [26]*, d27* [37]), and in maize a gene for branching is located on chromosome 1(*tb1* [30]).

The formation and subsequent growth of axillary buds in the axils of the leaves is controlled by multifunctional pathways of hormones such as auxin, cytokinin and strigolactone as shown in *Arabidopsis* [38], pea [39], rice [40] and petunia [34]. These studies revealed that axillary branching mutants are either deficient in the synthesis of a novel hormone strigolactone or have defects in a signaling pathway for this hormone. The mutants that are defective in hormone production include *max1, 3* and *4* of *Arabidopsis*, *rms1* and *5* of pea*, htd1, d10, d17* of rice and *dad1* of petunia (for references see above). The mutants such as *max2* of *Arabidopsis*, *rms4* of pea and *d3* of rice havebeen shown to be due to defects in signalling pathways [39- 41].

Most of these earlier reported axillary branching genes were used for *in silico* blast similarity search studies with an objective to find wheat orthologues, but similarity was found only with known rice genes (*Ostb1*, *d14, d3, d10/OsCCD8, htd1/CCD7*) and maize sequences that were orthologous to branching genes in pea , *Arabiopsis,* petunia and rice (*CCD7* and *CCD8*). The results of analysis showed that one of the wheat ESTs, BJ314006.1 exhibited significant (>70%) similarity with the sequence of rice branching gene *Ostb1*. Six wheat consensus sequences (obtained using 5x wheat database) that were orthologous to sequences of the branching genes of rice (*d14, d3, d10/OsCCD8, htd1/CCD7*) and maize (*CCD7* and *CCD8*) were also identified (information is available in supplementary Table 1S). The wheat EST sequence that was identified carried a TCP conserved domain, which seems to be involved in plant development and is also present in several genes responsible for branching, including *tb1* of maize, *Ostb1* of rice and *brc1* and *2* genes of *Arabidopsis* [42, 43]. Also, five of the above six wheat consensus sequences contained three other common domains including α-β hydrolase, RPE65 and COG3670, which have also been reported in rice and maize genes for branching [26-29]. The above solitary wheat EST and the six genomic wheat orthologues, identified during the present study may include parts of the putative gene responsible for axillary branching in wheat mutant recovered during the present study. This is a resource for further detailed study.

(ii) Reduced node (1-2) mutant

Reduced node mutant phenotype was observed both in M_2 and M_3 generations, although mutants were observed only in a solitary progeny in each case. In the solitary M_2 progeny, 6 plants were wild-type and only 2 were mutants (showing segregation). M_3 progenies were raised from 3 wild and 1 mutant, but only the progeny from mutant gave mutants and no segregation was observed in the remaining 3 M_3 progenies raised from wild type plants. However, the mutant nature was further confirmed in M_4 generation. The mutants had a mean of 1.6 nodes per tiller as against a mean of 3.6 nodes per tiller in the control (Fig. 2). Similarly, the mean length of internode in mutant was 7.5 cm against a mean of 11.6 cm in the control. Thus the plant height (average 19 cm) of the mutants was reduced to 65 to 70% that in the control plants. The mutants had higher average number of tillers per plant (up to 13) than in the control cv. PBW550 (up to 8). The spike length (mean 4.5 cm) of the mutant plants was only ~50% that in control plants, and was associated with reduced number of spikelets/spike (average 14 spikelets) but with a higher number of florets per spikelet (generally 4 in the mutant and 3 in the control).

In an earlier study of maize, based on genetic analysis, the reduction in number of nodes was attributed to alteration in expression of gene *ZmMADS3* coding for a MADS box transcription factor [31]. Therefore, blast similarity search was conducted using the sequence of the above gene *ZmMADS3* as query sequence against wheat transcript database; 11 unigenes each showing >90% similarity were identified. These 11 wheat unigenes may represent putative candidate genes for reduced node number in the wheat mutant reported during the present study. Using the sequences of the above wheat unigenes, suitable molecular markers are being developed, and will be used in our ongoing study on genetic mapping of the mutant gene/QTL responsible for reduced node number in the wheat mutant. The information generated may be utilized for isolation and cloning of the genes responsible for node number in wheat.

(iii) Dwarf mutants

As many as 10 M_2 progenies apparently segregated for plant height. From each of these M_2 progenies, 3-4 M_3 progenies were raised, but reduced plant height or semi-dwarf mutants segregated only in 4 M_2 derived M_3 progenies (these were further confirmed in M_4 generation). These mutants showed true segregation for the mutant phenotype in M_3 generation, satisfying 3 (normal): 1 (mutant) ratio. It is obvious that the remaining M_2 progenies did not carry true mutants. The dwarf mutants showed a significant reduction (20 to 65%) in plant height, with average height ranging from 19 to 61 cm, as against mean height of 84 cm in the control cv. PBW550.

A number of reduced plant height mutants in wheat were also reported in the past [44- 48]. Earlier inheritance studies of such mutants suggested either partially dominant or complete recessive control of plant height [48, 49]. More than 20 reduced height (*Rht*) genes have been reported in wheat [50]. Allelism test can now be conduct-

ed to confirm whether the new mutations for reduced plant height are allelic to the presently known dwarfing genes or the dwarf mutants recovered during the present study are due to mutations at one or more novel loci controlling plant height.

2. A leaf mutant (early senescence of leaf mutant)

The mutant exhibiting early senescence was observed in a solitary M_2 progeny. Four M_3 progenies were raised from this segregating M_2 progeny. Of these two M_3 progenies segregated for the mutant phenotype, one of these M_3 progeny had all mutant plants and the remaining M_3 progeny had only wild type plants. The mutants were phenotypically weaker than the control plants and were characterized by visible yellowing/necrosis of leaf margins well before the senescence of leaves of the control plants at maturity. The mutant plants were also smaller in height (mean 60 cm) than control plants (84 cm) and had a reduced number of grains per spike (mean, 11 grains per spike); control plants had a mean of 36 grains per spike. In the mutant plants, only the spikelets from the middle region of the ear could set grains, and these grains had also shrunk. This mutant also showed monogenic recessive control, giving a good fit to 3 (normal): 1 (mutant) ratio in segregating M_3 progenies. The above phenotype, particularly the shrunken seed, may be directly attributed to early senescence of leaf [51- 53].

It is known that senescence is the last stage of leaf development and is a type of programmed cell death in plants. At the molecular level the senescence programme is believed to be regulated by specific senescence genes [54-59]. During leaf senescence many genes associated with photosynthetic activity and other anabolic processes get down-regulated [60], whereas dozens of genes designated as senescence associated genes (SAGs) are up-regulated. SAGs are predicted to encode transcription factors, receptors for senescence perception and components of intercellular protein trafficking [61]. Among the prominent SAGs are genes responsible for the execution of senescence syndrome encoding degradative enzymes, proteinases [62-64], lipases [63, 64], nucleases [65, 66], chlorophyllases [67] and enzymes for nutrient recycling such as glutamate synthase [68]. Genomic approaches in *Arabidopsis* and rice together with the use of mutants allowed identification of many senescence regulating genes that participate in a complex molecular network and regulate senescence. Transcription factors of WRKY family are involved in the regulation of various physiological programmes that are unique to plant processes, including plant defense, senescence and trichome development, as shown in *Arabidopsis* [69]. In wheat, nine senescence associated genes (SAGs) have been identified by using rice SAG sequences [70]. Besides the above, transcription factors of WRKY family have also been reported to be involved in leaf senescence in wheat [71]. Some of these gene sequences may be candidates for the early leaf senescence mutant reported during the present study.

3. Spike mutants (fusiform and club shaped spike and bent peduncle mutants)

This class of mutants included mutants having an altered spike morphology, which differed from that of the control plants. Four spike variants (fusiform spikes, club shape spikes, spikes with crooked peduncle and spike with bent peduncle) were observed in M_2 generation; only three of these four spike mutants (except crooked penduncle) were recovered and validated in M_3 generation, where progenies either segregated showing a good fit to 3 (normal):1 (mutant) ratio or contained only mutant plants (no segregation). Wheat mutants with altered spike morphologies were also induced following EMS mutagenesis [72].

4. Reproductive mutants (non-flowering mutants)

'Non-flowering' mutants were recovered in five M_2 progenies. In M_3 progenies derived from two M_2 progenies, segregation for mutant and wild type plants was observed thus validating the mutant. In the remaining M_3 progenies derived from rest of the three M_2 progenies, no segregation was observed. The two segregating progenies gave a good fit to 3 (normal): 1 (mutant) ratio showing monogenic recessive control for the mutant phenotype. These mutant plants did not flower and remained vegetative during their entire life span. The mutant plants were characterized by bushy appearance with dwarf plant type, and the plants remained green until senescence. The mutants could be maintained in heterozygous condition and were recovered in the selfed M_3 progenies of two heterozygous M_2 plants, suggesting their true mutant nature.

'Non-flowering' mutants (also called ever vegetative mutants) were earlier obtained following EMS mutagenesis in wheat and were attributed to deletion in *Vrn1* gene that is responsible for vernalization insensitivity in einkorn wheat [3, 4]. Another gene *Wap1* (wheat APETALA1), which is a key gene in the regulatory pathway that controls phase transition from vegetative to reproductive growth in common wheat, is an ortholog of the *Vrn1* gene. During the present study, ENU mutagenesis might have caused deletion or inactivation of *Wap1* or *Vrn1* gene leading to flowerless phenotype.

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Research Article

Production of Haploid Tissues and SNP Analysis of the Genome in *Musa acuminata* cv.'Matti'(AA)

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Abstract

Haploid and doubled haploid plants are of considerable value in genetic studies, genomics and plant breeding, allowing characterization and exploitation of genes where only one allele is present. Inbred banana lines have not been developed and with very few haploids or homozygotes reported, we aimed to develop new materials for genetic and genomic studies and to see if protocols could be improved using a range of genetic material not previously investigated. Anther culture was carried out using four *Musa acuminata* (AA) cultivars grown in homestead cultivation in Kerala, southern India. Protocols based on using modified MS medium supplemented with 2,4-D and BA (0.1 mg l^{-1} each) were used. Embryogenic callus was regenerated from 4% of anthers of cv. Matti. The callus produced somatic embryosthat developed small leaves but haploid plant regeneration was not achieved. Regenerated leaf tissues had a haploid set of chromosome complements. The haploid tissuesprovided a source of DNA which may be suitable for whole genome shotgun approaches to re-sequencing, where the high levels of heterozygosity in *Musa* would impair assembly.

Key words: *Musa acuminata* (AA) cultivars, haploid tissues, heterozygosity, SNP analysis.

Introduction

Genetic improvement of bananas and plantains is challenging because of their extremely low seed set rate in conventional cross breeding schemes [1]. Most cultivated bananas are triploid (2n = 3x = 33) and are female and male sterile, although there may be small amounts of residual fertility in some genotypes such as Gros Michel [2]. Seed set in conventional crosses varies with both genotype and time[3]. The initial steps in cross breeding programmes in banana include hybridization and selection of recombinants at the diploid level [4]. Pollen fertile diploids with improved disease resistance are crossed with triploids (3x) to produce tetraploid (4x) selections. The tetraploids are then crossed back to improved diploids to produce secondary triploids [1]. The heterozygosity of the diploids makes the outcome of crosses unpredictable with many generations needed to obtain inbred lines. By contrast, homozygous lines can be produced in a single year by chromosome doubling of haploid plants [5]. Since the first successful regeneration of haploid plants from developing microspores in *Datura* anthers [6], efforts have been directed towards improving techniques for commercially important species [7]. Haploids can arise naturally through parthenogenesis (development of haploid plants from unfertilized eggs) or androgenesis (development of haploid sporophytes from pollen). They can also be artificially induced through the culture of anthers [8] and microspores [9] which has been found to be the most efficient technique for obtaining large numbers of haploid plants [10]. Development of reliable culture protocols is prerequisite to initiating an effective doubled haploid production system in applied breeding programmes. Doubled haploids are valuable for genetics because all loci are homozygous so the phenotype can be related to gene alleles.

A reference sequence of the genome of banana is now being generated from a doubled haploid accession of the *Musa acuminate* ssp *malaccensis* 'Pahang' (http://www.intl-pag.org/19/abstracts/W10_PAGXIX_069.html). Given the level of heterozygosity found within diploid *Musa* accessions, assembly of shotgun sequence reads would be very difficult, even once a reference genome is established (http://www.intl-pag.org/16/abstracts/PAG16_W05_45.html). It is therefore important to be able to generate additional haploid or double haploid *Musa* lines which can be used for re sequencing and comparisons of the genes between different accessions. The present study reports development of haploid tissue from anther culture of one of four diploid *Musa acuminata* cultivars tested.

Materials and methods

Four diploid cultivars with distinct and contrasting morphological characters based on *Musa* descriptors (IPGRI/INIBAP, 1996) were selected for anther culture (Table 1). The terminal floral head was collected from the plants after the development of the fruit bunch and used as the donor material for anther isolation. This structure contains in all developmental stages. The bracts with flowers were removed sequentially until they became too small to remove by hand.

TABLE 1.MORPHOLOGICAL CHARACTERS OF THE FOUR DIPLOID CULTIVARS OF MUSA ACUMINATA SELECTED FOR ANTHER CULTURE

Qualitative characters		*Musa acuminata* cultivars			
		Matti (AA)	**Sannachenkadali (AA)**	**Njalipoovan (AB)**	**Adukkan (AB)**
Habit	Pseudostem colour	Green	Red	Medium green	Medium green
	Wax on leaf sheath	Not waxy	A little waxy	A little waxy	Moderately waxy
	Blotches at petiole base	Large blotches	Small blotches	Sparse blotches	Sparse blotches
	Blotch colour	Dark brown	Brown/black	Brown/black	Brown/black
	Petiolar canal	Curved outwards	Wide, erect margins	Curved inwards	Curved inwards
	Petiole margin colour	Pink	Pink/purple	Pink/purple	Pink/purple
	Colour of midrib dorsal surface	Light green	Red/purple	Light pink	Light green
	Blotches on the leaves of water sucker	Absent	Narrow blotches	Absent	Absent
Inflorescence	Bract curling	Rolls back	Rolls back	Bract lifts	Rolls back
	Bract shape	Lacelolate	Lacelolate	Narrowly ovate	Narrowly ovate
	Bract apex	Slightly obtuse	Acute	Obtuse	Slightly pointed
	Outer bract colour	Purple/brown	Deep maroon	Purple/brown	Pink/purple
	Inner bract colour	Red	Red	Red	Orange/red
	Bract scar	Prominent	Prominent	Medium	Medium
	Bract colour fading	Continuous	Fades to yellow towards base	Continuous	Continuous
	Wax on the bract	Very waxy	Not waxy	Very waxy	Very waxy
	Peduncle colour	Light green	Red/purple	Dark green	Dark green
	Peduncle hairiness	Hairy, long hairs	Hairy, long hairs	Hairless	Hairless
Flower	Free tepal of male flower	Several folding	Several folding	Simple folding	Simple folding
	Lobe colour	Yellow	Orange	Yellow	Yellow
	Free tepal colour	Translucent	Translucent	Translucent	Pink
	Filament colour	White	Cream	Cream	White
	Anther colour	White	Cream	Cream	White
	Style basic colour	White	Cream	White	White
	Style pigmentation	White	Pink/purple	Pink	Pink
	Stigma colour	Orange	Orange	Cream	Yellow
	Male flower colour	Cream	Cream	Pink	Pink
	Ovary colour	Cream	Cream with pink	Pink	White
	Ovary shape	Straight	Arched	Arched	Arched
Fruit	Bunch position	Slightly angled	Slightly angled	Hanging at 45°C	Slightly angled
	Bunch shape	Cylindrical	Cylindrical	Truncated cone shaped	Cylindrical
	Fruit position	Curved upwards	Curved upwards	Curved upwards	Curved towards stalk
	Fruit shape	Slightly curved	Straight in distal part	Straight in distal part	Straight
	Fruit apex	Lengthy pointed	Lengthy pointed	Pointed	Bottle neck
	Fruit colour (unripened)	Green	Red/purple	Light green	Green
	Fruit colour (ripened)	Bright yellow	Red/purple	Yellow	Yellow
	Pedicle	Short	Long	Short	Long
	Pedicle surface	Hairy	Hairy	Hairless	Hairles

The flower heads were washed in 1% laboline (liquid detergent, Qualigens, India) for 5-10 minutes, rinsed in tap water and then rinsed 2 or 3 rinses in autoclaved double distilled water. Male flower buds 10–15 cm in length were collected from the bunch, quickly dipped in 95% (v/v) ethanol, and then flamed. This procedure to surface sterilize the buds was repeated three times. Further manipulations of the plant tissues were carried out using aseptic conditions. Immature male flower (5-6 cm), containing five stamens with fully developed anthers were isolated and transferred into a Petri dish containing filter paper that had been moistened with sterile water. One anther from each flower was squashed in 1% aceto-carmine and microspore developmental stage was determined by microscopic examination. Ten anthers (each approx. 1 cm in length) containing microspores at the uninucleate stage were placed in a Petri dish (9.5 cm diameter) containing 30 ml of solid medium. The modified MS medium contained macro- and micro-nutrients [11], vitamins of Morel [12], casein hydrolysate (500 mg l^{-1}) and 20 g l^{-1} sucrose. Auxins used were 2, 4- D (2, 4 dichlorophenoxy acetic acid, 0.1-0.45 mg l^{-1}), IAA (indole -3- acetic acid, 0.1-0.5 mg l^{-1}), and picloram (0.1-0.5 mg l^{-1}). The 2,4-D and IAA was used in combination with the cytokinins BA (benzyl adenine, 0.1-0.4 mg l^{-1}) and kinetin (0.1-0.4 mg l^{-1}). The medium was solidified with 6 mg l^{-1}agarose. The pH of the medium was adjusted to 5.7 before autoclaving. Cultures were kept at 27°C in darkness. After initiation of cultures anthers were maintained on the same culture medium without subculture until callus formation. Androgenic embryos were transferred onto MS medium containing 30 gl^{1}sucrose, 0.1, 0.5 or 1 mg l^{-1}2,4-D with 1-5 mg l^{-1} BA. The cultures were kept at 27°C under photoperiod of 16 h. The photographs were taken by stereomicroscope 10x magnification.

For chromosome preparation, the *in vitro* leaves were fixed in acetic ethanol overnight, washed in a solution of 75 mM KCl and 7.5mM EDTA (pH 4.0) and digested in cellulose (2%) pectinase (2%) mixture for 1-1.5 hat 37^0C. The suspension was filtered through a 150 µm nylon mesh, pelleted, re-suspended in KCl:EDTA solution and incubated for 5 min at room temperature. The protoplast pellets were washed three times with 70% ethanol and 5 µl of the suspension was dropped on pre-chilled slides. Protoplasts were burst with the application of 5 µl 1:3 acetic ethanol fixative, and slides were rinsed in 100% ethanol and air dried. The slides were stained with DAPI and observed under UV microscope.

To investigate the homozygosity of the DNA from the putative haploids, DNA was extracted using the CTAB method from the *in vitro* leaves and parental material. *Musa acuminate* Calcutta 4 provided as reference DNA. A set of 10 PCR primers was used to amplify the DNA from the parents and regenerants using the annealing temperatures indicated (Table 2). PCR products were analysed by gel electrophoresis or by sequencing the PCR products. The sequences were analysed using BioEdit7.1.3 version.

TABLE 2. PRIMERS USED FOR HETEROZYGOSITY ANALYSIS IN ANTHER TISSUE CULTURE DERIVED MATERIALS

No	PRIMERS	Details of Primers	Sequences	Ann. Temp	Product size in Calcutta 4
1	MA82I11MS1 F MA82I11MS1 R	Microsatellites from BAC	CACCAAAAGCCAACACTTTG CATCCCTCGAGAAAAGAAGG	54°C	759
2	MA54N07MS1-F MA54N07MS1-R	Microsatellites from BAC	TCAAATGCAAAGCGACATTAG TTTCCTGCATGTCATCCTTAAC	54°C	794
3	MA64C22UA1-F MA64C22UA1-R	Genes from BACs	CCATAGGGTTGAAGCTCCTG ACATGTGCGCAATGATTTG	55°C	751
4	MA25J11UA1-F MA25J11UA1-R	Genes from BACs	TTGTTTTCGTGCGATTAGGG GGGCACAATATGAGACACTGG	55°C	756
5	1LHSP-F 1LHSP-R	Class1 LMW heat shock proteins	GCAGAGCAACCTGTTCGAC CAACCAGAGATTTCGATGGAC	64°C	550
6	MA4_52NO2-F MA4_52NO2-R	Low temp induced protein	TGCTTTTATGGGAGCAGTGAC TGGATTTGAATTGGGAGATTG	60°C	450
7	LTIP-F LTIP-R	Low temp induced protein	AAATGCGGCACTTTTCATTC GAACAAGGCTCGCATCTCTC	64°C	590
8	STP-F STP-R	Salt tolerant proteins	TCTGTGTCCTCACCAAAACG CGGGACTGTGATGAACCTG	60°C	640
9	70 HSP-F 70 HSP-R	70 kDa heat shock proteins	GGGATCACCATTACCAATGAC GGCCGAACTAAATCCACCTC	64°C	500
10	DRPF-R DRPF-F	Drought responsive family proteins	GATGCGTGGAGCCGATTC TCATCGATGTGGCAGCAG	60°C	340

Results

The white powdery callus was regenerated from the tip of 6% of anthers inoculated on MS medium supplemented with 2,4-D (Fig. 1(a)). The response of anthers varied with genotype and the plant growth regulators used. In cv. Matti only 2% of the explants produced callus on the medium supplemented with 0.1 mg l^{-1} 2,4-D whereas 6% explants initiated callus on the medium supplemented with 0.4 mg l-1 2,4-D. Addition of IAA or Picloram in the medium was not efficient for callus initiation (Table 3). Callus was observed 75-90 days after initiation of culture.

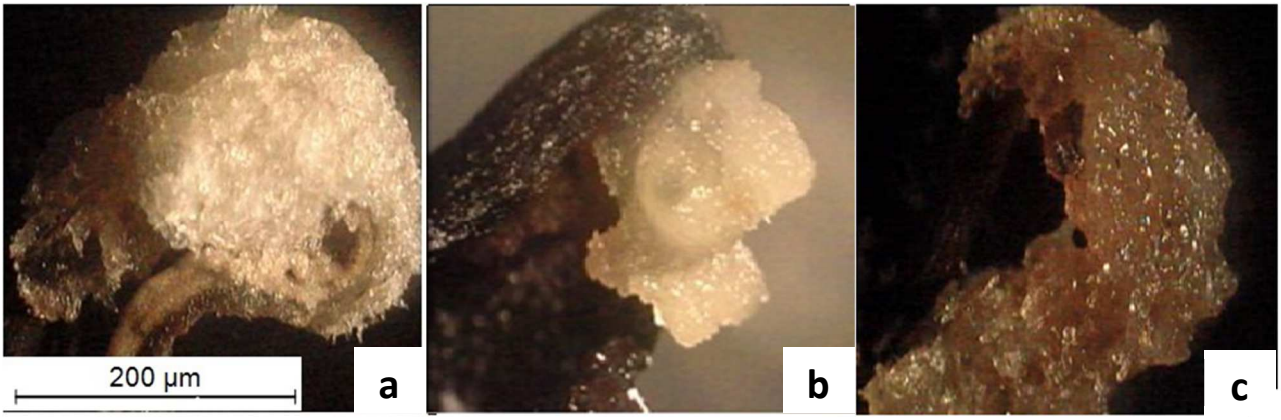

Fig 1(a). White fragile callus initiated from the tip of anthers from Musa acuminata cv. Matti (10X scale 200 µm); 1(b): White solid callus initiated from the tip of anthers from Musa acuminata cv. Matti (10X scale 200µm); 1(c): Light brown callus from the entire surface of anthers from Musa acuminata cv.Matti (10X scale 200 µm).

TABLE 3. RESPONSE OF ANTHERS FROM FOUR*MUSAACUMINATA* CULTIVARS ON MS MEDIUM SUPPLEMENTED WITH AUXINS AND CYTOKININS

Auxin Type	Auxin Conc. (mgl^{-1})	Cyto Type	Cyto Conc. (mgl^{-1})	Percentage response (%)			
				Matti (AA)	Sannachenkadali (AA)	Njalipoovan (AB)	Adukkan (AB)
2,4-D	0.1	BA	0.1	4.36±0.43^{d}	2.00±0.57^{b}	2.78±0.54^{ab}	1.70±0.49^{d}
	1		0.2	4.76±0.74^{d}	3.83±0.56^{a}	1.6±0.46^{bc}	0
	2		0.3	6.19±0.45^{c}	2.22±0.89^{b}	0.95±0.54^{cd}	0
	2.5		0.4	3.69±0.52d^{e}	0.90±0.52^{bc}	0	0
	0.1	Kin	0.1	0	0	0	3.60±0.51^{bc}
	1		0.2	0	0	1.88±0.54^{bc}	1.68±0.48^{d}
	2		0.3	0	0	0.91±0.52^{cd}	0
	2.5		0.4	0.876±0.50^{gh}	0	2.80±0.53 ^{ab}	0
IAA	0.1	BA	0.1	9.406±0.54^{a}	1.00±0.57^{bc}	0.93±0.53^{cd}	2.65±0.50^{cd}
	1		0.2	7.823±0.50^{b}	1.92±0.55^{b}	1.81±0.54^{bc}	3.89±0.56^{ab}
	2		0.3	8.103±0.52^{b}	0	0	4.72±0.45^{a}
	2.5		0.4	1.736±0.50^{ef}	0.99±0.57^{bc}	0	4.90±0.56^{a}
	0.1	Kin	0.1	0	0	1.92±0.54^{bc}	0
	1		0.2	0	0	3.44±0.49 ^{a}	0
	2		0.3	2.626±0.51e	0	0	0
	2.5		0.4	0	0	0	2.41±0.46^{d}

The anthers inoculated on MS medium supplemented with 2,4-D and BA showed callus initiation from the tip of the anthers (Fig.1(b). The white solid callus showed potential for embryogenesis. Maximum callus regeneration was observed on the medium supplemented with IAA and BA (Table 3). The callus regenerated from the entire surface of the callus and was light brown in colour (Fig. 1c). The white callus initiated from 2,4-D:BA (0.1 mg l^{-1} each) medium was transferred on MS medium supplemented with 0.1,0.5 or 1.0 mg l^{-1} 2,4-D and 1-5 mg l^{-1} BA. A few globular embryo development was observed from the callus inoculated on MS medium supplemented with 0.1 mg l^{-1} 2,4-D and 4 mg l^{-1} BA (data not given) but further development of these embryos was not obtained. Instead green small shoot like structure developed on the medium (Fig. 2). Further subculture produced more callus and leaf development. Cytological analysis of leaf meristem showed 11 chromosomes(Fig. 3).

Ten primer pairs were used for PCR amplification of the parental diploid banana cultivar and the haploid DNA. The reference DNA was *Musa acuminata* Calcutta 4. The microsatellites region showed 17 CAT repeats in Matti (parental line), 11 CAT repeats in haploid line and 27 CAT repeats in Calcutta 4 (reference DNA). Gene specific primers from the BAC library amplified similar regions with no SNPs with very few deletions of one or three base pairs. Protein specific primers produced

homozygous regions with a few SNPs in all the samples (Table 4). The STP primer produced 100% homozygous sequences in parental and haploid lines with only 5 SNPs in reference DNA (Fig. 4).

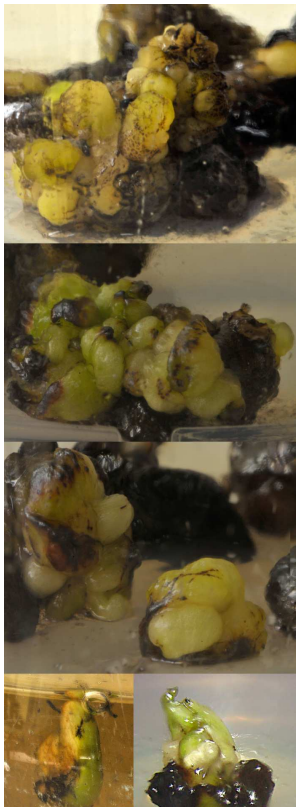

Fig. 2. Small shoots emerged from the callus on MS medium supplemented with 0.1 mg l^{-1} 2,4-D and 4 mg l^{-1} BA.

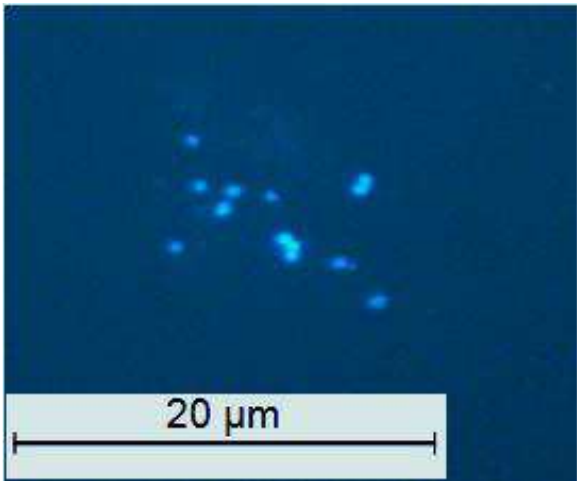

Fig. 3. Haploid set of chromosomes (n=11) from the leaf meristem stained with DAPI under fluorescence microscope (scale 20 µm).

TABLE 4. HOMOZYGOSITY IN DIPLOID PARENTAL LINES AND HAPLOID ANTHER-DERIVED LINE. INDELS ARE INDICATED AS DELETIONS OF *X*BP (e.g. 33 BP) OR *X* INSERTIONS OF *N*BP (e.g. 3(2BP).

Marker	**Matti/AA (Parental line)**				**Haploid anther-derived from Matti A**				**Calcuta-4 (Ref)**			
	Bases	Indels	SNPs	No of repeats\ Mutations	Bases	Indels	SNPs	No of repeats\ Mutations	Bases	Indels	SNPs	No of repeats\ Mutations
MA82I 11MS1	687 homozygous	33bp, 2(2bp) 1(1bp)	16	17CAT	678 mixed	52bp, 5(1bp), 2(2bp)	8	11CAT	482 homozygous	2bp, 2(1bp)	3	27CAT
MA54 N07MS1	759 homozygous	8bp, 3(1bp)	5	15CT	199 mixed	2bp, 3bp, 12(1bp)	2	0	741 homozygous	8bp, 2(1bp)	6	13CT
MA64 C22UA1	727 mixed	1(1bp)	8	0	546 mixed	5(1bp), 1(2bp), 1(3bp)	0	nil	607 homozygous	2(1bp), 1(3bp)	nil	nil
MA25J 11UA1	642 homozygous	2(1bp)	0	0	559 homozygous	2(1bp)	0	4	730 homozygous	3(1bp)	0	3
Class1 LMWHSP	422 homozygous	3bp	0	3	425 homozygous	1bp	2	2	422 homozygous	0	6	2
70 HSP	415 homozygous	3bp, 1(1bp)	0	0	416 homozygous	1(1bp)	1	1	414 homozygous	3bp, 1(1bp)	2	2
MA4_52 N02	395 mixed	2(1bp), 3(2bp)	0	26CT	400 homozygous	14bp,3bp ,2bp	0	15CT	415 mixed	18bp, 1bp, 2(2bp)	4	39CT
LTIP	588 homozygous	1(1bp)	0	3	587 homozygous	0	0	0	591 homozygous	0	0	1
STP	584 homozygous	2bp	2	0	581 homozygous	2bp	2	0	589 homozygous	2(1bp)	0	2
DRFP	291 homozygous	5(1bp)	3	5	290 homozygous	5(1bp)	3	2	277 homozygous	2bp,6(1bp)	0	4

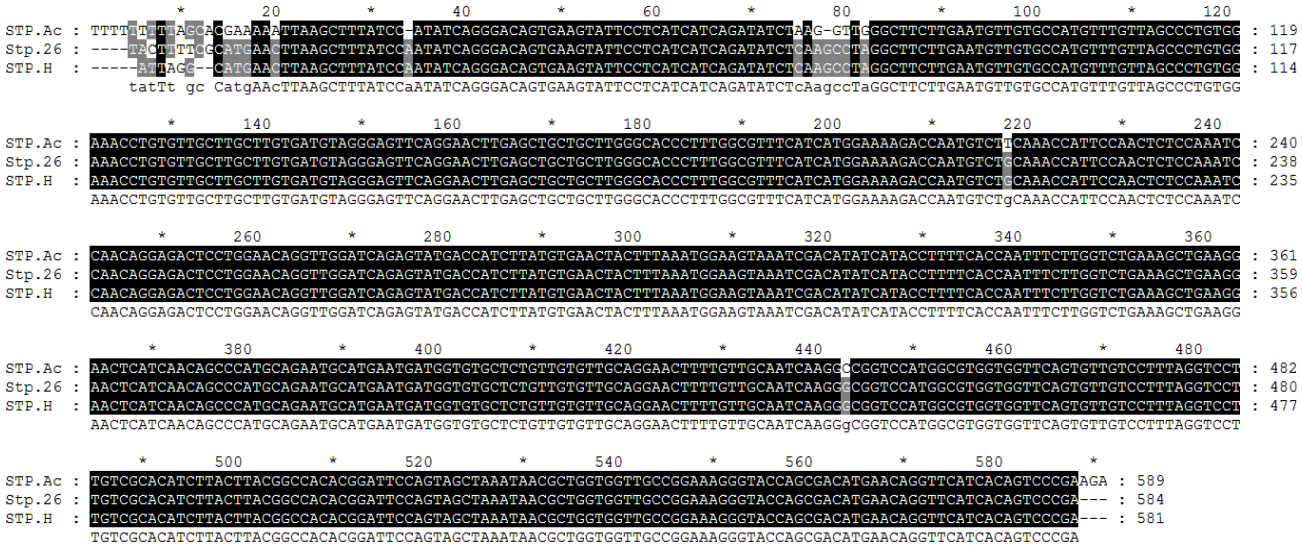

Fig 4. ClustalW multiple alignments of DNA sequences from Musa acuminate cv. Matti, cv. Calcutta 4 and Haploid line amplified with STP primers. STP-Ac (cv. Calcutta 4 (Reference genome); STP-26 cv. Matti (Parental Line); STP-H (Haploid genome).

Discussion

The results here show that haploid callus could be regenerated from anther cultures of *Musa acuminata* cv. 'Matti'. In banana, haploid plant regeneration from anther culture was reported in *Musa balbisiana* [5]. They reported that callus phase usually preceded embryo formation, and the frequency of haploid plant regeneration was genotype dependent. In diploid *Musa acuminata* cultivars anther culture produced non-embryogenic callus except in a few anthers inoculated on MS medium supplemented with 2,4-D and BA. All the cultivars used in the present study were domesticated diploid varieties and all of them produced parthenocarpic fruits without any seeds. All the inoculated anthers were in uninucleate stage and non-embryogenic callus regeneration was observed from the surface. Contrary to the results in *Musa balbisiana* cultures, very little *in vitro* response was observed in the domesticated diploid *Musa acuminata* cultivars. In the results here, even the regenerated somatic embryos did not develop into plantlets. There was no diploidization in the cells as the leaf meristem showed haploid set of chromosomes. The homozygosity was confirmed by analysis of the DNA from the regenerant material. All markers were homozygous and derived from the parent except for one microsatellite. As might be expected from the high levels of polymorphism seen in microsatellites, the new allele has presumably developed from a mutation during tissue culture or perhaps in the plant used for regeneration. A double haploid of the *M. acuminata* ssp. *malaccensis* cv. 'Pahang' has also been developed [13], and is being used for whole genome shotgun sequencing [14]. The low number of reported A genome doubled haploids ever reported supports the difficulty found in the present work in regenerating material from different genotypes. Although knowledge on the evolution of clonally propagated crops under domestication remains poor, De Langhe et al. suggested a hypothesis of an evolution of edible AA types by hybridization between subspecies of *M. acuminate* [15]. Individual hybrids may carry different recombinant chromosomes. The haploid chromosome set of *M. acuminata* and *M. balbisiana* has a single site of the 45S rDNA [16]. Nucleotide structure and diversity analysis of ITS-5.8S-ITS2 region in *Musaceae* revealed presence of more than one type of ITS sequences within some *Musa* species and clones. The RAPD and ISSR analysis of the domesticated South Indian banana cultivars also classified the AA genome cultivars in two classes [17]. Because of the genome heterozygosity, the haploid genome may not be able to develop into plantlets. Further studies are needed for the development of haploid plants from these cultivars.

In vitro response of the anthers from the culture in the domesticated diploid *Musa acuminate* cultivars from Kerala, South India, was very poor. Only 4% of the anther produced embryogenic callus and the somatic embryos developed only small dwarf leaves. The leaf meristem showed haploid set of chromosomes. Further studies are needed to standardize the protocols for haploid regeneration for application in banana breeding programmes and for gene identification or genomic analysis.

Acknowledgement

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Research Article

Studies on Variation of Carotenoid-Proteins Content in Cassava (*Manihot esculenta* Crantz) Storage Root Reveal Implications for Breeding and the Use of Induced Mutations

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Abstract

Carotenoid-Protein content in cassava storage root (CSR) is low but variable, and characterization of this variability is lacking. Accumulation of carotenoids occurs in chromoplast and depends on a broad class of proteins named carotenoid associated proteins (CAP), lipids and the biosynthesis of carotenoids. Twenty-nine landraces and progeny of 200 individuals were accessed for CAP and carotenoid content varied in two ways. First, related to landrace diversity, total buffer extractable proteins (TBEP), buffer insoluble proteins (BIP) and total carotenoid and β-carotene content were assessed. Significant differences were observed in the tested genotypes. Secondly, analyses related to storage root tissue age were assessed by TBEP. This showed protein content decreased and total carotenoid content increased as secondary growth proceeds. Further carotenoid-proteins complex (CPC) identified in carotenoid contrasting landraces showed different proteins profile in SDS-PAGE with proteins size of 18 and 33 kDa in low carotenoid (IAC12.829) and 18-20-30-33 kDa in a high total carotenoid landrace (Cas74.1). Progeny analysis for TBEP and total carotenoid content confirmed the interdependence of carotenoid-proteins association by correlation analysis, estimated heritability of individual traits and grouping clones for carotenoid-proteins content. Results allow us to conclude that: natural carotenoid-protein content varies due to differential genetic background and storage root tissue age; carotenoid-protein complex showed variation in protein and carotenoid types; estimated heritability of proteins and carotenoids traits showed different values. The establishment of a genetic component allows future strategies including traditional breeding and the use of induced mutations to create novel variation for the nutritional improvement of cassava tubers.

Introduction

In tropical regions of the globe cassava storage root (CSR) represents 20% of the world crop production (FAO 2010). Nutritionally, dietary intake provided by CSR is carbohydrate (85% starch), pro-Vitamin A carotenoids [1-3] and low protein content [3, 4]. Progress in conventional breeding programs to boost carotenoid-protein content in CSR depends largely on natural occurrence of variation in germplasm collection [3, 5].
Detailed characterization of carotenoid-protein content variation is missing mainly due to the lack of critical information especially on the sink capacity of CSR. Chromoplast is a relevant target sink because it is stable and requires a significant amount of functional proteins to fulfil its cellular function during carotenoid accumulation [6-10]. Natural massive accumulation of intermediary carotenoids in the membranous system of chromoplasts of storage organs depends on a broad set of proteins named carotenoid-associated proteins (CAP) and lipids [11], and enzymes for the biosynthesis of carotenoids [12]. We hypothesize that CAP and carotenoids content variation observed in the diversity of pigmented CSR landraces may lead to the formation of a relevant carotenoid-protein sink.
The present study focused on searching for carotenoid-protein content variation in pigmented CSR in landrace diversity, and estimation of genetic parameters from crosses of selected progenitors in a 200 offspring population.

Materials and methods

Plant material and tissue preparation

Twenty-nine accessions of pigmented cassava landraces from a GENEBANK collection and 200 individuals from cross of selected parentals cultivated in field plots at Embrapa Genetic Resources and Biotechnology or Embrapa Cerrados in Brasília were used in the present study. Material from storage roots was extracted in three ways and used for analysis as follows: (1) the bulk of the

root tissue from the central cylinder was freeze-dried and extracts were used for quantification of proteins, total carotenoid, β-carotene and observation of the protein profile in SDS-PAGE; (2) root tissue layers dissected as illustrated in de Souza et al. [13], were freeze-dried and extracts were used for quantification of proteins and total carotenoids; (3) fresh root tissues were immediately processed and used for preparing chromoplast enriched suspension.

Proteins and carotenoid extraction

Extracts from tissue powder (100-500 mg powder) of storage roots of 29 cassava landraces were buffer fractionated as follows: for Total Buffer Extractable Proteins (TBEP) the tissue powder was added to 2 mL of EB (Tris 80 mM pH 6.8, NaCl 0.2 M, Pefabloc SC 0.1% and DTT 25 mM) and after addition of 4 volumes of acetone the material was vortexed, sonicated, incubated at -20°C, and centrifuged (30000 rpm/4°C/20 min). Total protein was solubilized from the pellet with 2 mL of Protein Suspension Buffer – PSB (Tris 10 mM, NaCl 0,2 M, EDTA 50 mM, Chaps 5 mM, DTT 10 mM, Pefabloc 50 mM) for protein quantification. A new set of tissue powder was treated with 2 mL of EB and the material (without acetone treatment) was vortexed, left at room temperature for less than 1 hour, and subsequently centrifuged (30000 rpm/4°C/20 min). The procedure was repeated twice and the pooled buffer soluble proteins (BSP) were used for protein quantification. The remaining pellet was mixed with 2 mL PSB, vortexed, heated at 60°C, cooled at room temperature, and incubated at -20° for 1 hour, before centrifugation (30000 rpm/4°C/20 min). The Buffer Insoluble Proteins (BIP) was used for protein quantification using Bradford assay according to the manufacturer's instructions (BioRad micro assay).

Tissue powder (100-500 mg powder) from storage roots of 200 individuals from an F1 population was rehydrated with 2 mL extraction buffer (EB) (Tris 80 mM pH 6.8, NaCl 0.2M, Pefabloc SC 0.1% and DTT 25 mM). The solution was vigorously vortexed and sonicated for extraction of total protein and carotenoids using phase separation with the addition of 4 volumes acetone, incubation at -20°C for 1 hour, and centrifugation (30000 rpm/4°C/20 min). The supernatant was collected and pooled and the pellet was washed twice with pure acetone and centrifuged (30000 rpm/4°C/20 min). Acetone was evaporated under N_2 flush, and the formed yellow colour phase was solubilized in anhydrous ether for total carotenoid quantification. The remaining pellet was vortexed in 3mL PSB, sonicated and heated at 60° C for one hour. After centrifugation (30 000 rpm/4°C/20 min), total buffer extractable proteins (TBEP) were quantified as described above.

Total carotenoid extraction and quantification

About 5-20 g each of powder from CSRs of 29 landraces was transferred to a 50 mL 'Falcon' tube, hydrated with an equal volume of ddH_2O for 10 min and mixed with a scoop of Hyflo Super Cel (SIGMA brand) followed by vigorous vortexing with acetone and subsequent centrifugation (5000 rpm/RT/15 min). A pooled supernatant, after three washes with acetone, was sequentially transferred with 1/5 of the acetone volume into 50 ml petroleum ether with centrifugation (2000 rpm/RT/15 min). After 5x washes with ddH_2O, the collected solvent phase passed through anhydrous sodium sulfate before using it to generate the HPLC spectrum and to quantify specific carotenoids using a standard curve for β-carotene as reported in Kimura et al .[14]. In an alternative extraction procedure samples were separated for quantification by spectrophotometry. In brief, samples (100 to 2000 mg powder) were hydrated with ddH_2O, and after adding petroleum ether mixed with politron pulses, sonicated and centrifuged (4000 rpm/4ºC/20 min). The solvent phase was collected, filtrated through anhydrous sodium sulfate, and the volume was adjusted with petroleum ether for OD spectrum readings at 300 to 600 nm and λ_{max}.

Spectrophotometric quantification of total carotenoid

Total carotenoid was estimated using the formula µg/g = OD * 104*V / Acm%11 * DWt, where OD = Absorbance in the higher peak (obtained by light absorption spectrum in petroleum ether), Acm%11= 2592 - carotenoid extinction coefficient in petroleum ether, V = volume of extraction (mL) and DWt = dry weight of powder (g). Optical densities readings were taken in a spectrophotometer SPECTRA_Max (Model 384 PLUS).

Specific carotenoid identification and quantification by HPLC

Carotenoid standard preparation, identification, and sample quantification were carried out as described by Kimura et al. [14]. Specific carotenoid quantification in the storage root calculation considered integration of the peak area in the HPLC chromatogram. A correction factor for a specific carotenoid extinction coefficient [15] was calculated in relation to the extinction coefficient of β-carotene using the standard curve for β-carotene. For those carotenoids with an unknown extinction coefficient, the correction factor was 1. A standard curve prepared with β-carotene extracted from carrot [14] was used for quantification of the corrected areas and the results were expressed as µg/g DW.

Chromoplast enriched suspension preparation

Fresh storage roots from cv. IAC 12-829 and landraces CAS031 and CAS74.1 were sliced and treated with homogenate buffer (Tris-HCl 100 mM (pH 8.2), EDTA 8 mM, KCl 10 mM, $MgCl_2$ 2 mM, sucrose 400 mM, and PMSF 1 mM) for 2 hours in a cold room, and ground with a household blender to obtain a paste

that was filter through three layers of cheesecloth. The filtrate was centrifuged (400 rpm/4°C/20 min), and the supernatant from this was collected and re-centrifuged (20000 rpm/4°C/40 min). The resulting pellet, enriched with chromoplasts, was suspended in 100 mL of homogenate buffer (HB), centrifuged (20000 rpm/4°C/40 min) and washed once with HB. The pellet containing the Chromoplast Enriched Suspension (CES) was collected in 100 ml of suspension buffer (Tris-HCl 100 mM (pH6.8), 250 mM NaCl) and used for carotenoid-protein complex (CPC) separation.

Size exclusion chromatography of chromoplast enriched suspension

A 5 ml aliquot of CES was loaded on to a 1 m x 1.5 cm internal diameter column containing Sepharose CL 6B-200 in suspension buffer (SB). Fractions of 1 ml were collected at a flow rate of 1.5 ml/min and an aliquot of 200 µL used to OD readings at 280 nm and 461 nm using a spectrophotometer (SPECTRA-Max; Model 384 PLUS). Fractions profile revealed three peaks; two of them were pooled for further analysis.

SDS-PAGE analysis

Total buffer extractable proteins (TBEP), chromoplast enriched suspension proteins (CES) and pooled protein fractions from carotenoid-protein complex and non-carotenoid-protein complex were separated by one dimensional SDS-PAGE as previously described (de Souza et al. 2008).

Carotenoid identification in CPC by HPLC

Pooled fractions from size exclusion chromotography (SEC) peak 1 were added with equivalent acetone volume and sonicated for one hour. After centrifugation (20000 rpm/4°C/40 min), the supernatant was collected and the acetone partially evaporated and further dried by filtering through anhydrous sodium sulfate. After passing through a 22 µm Millipore syringe filter, the preparation was thoroughly dried in N_2 and suspended with Methanol:MTBE:ddH_2O (81:15:4). Carotenoid composition was identified by HPLC using a WATERS C18 column.

Data analysis

Statistical analyses were performed using R Statistics (http://www.r-project.org/), SISA (http://www.quantitativeskills.com/sisa/) and XLSTAT package (http://www.xlstat.com/en/my-xlstat.html). Parameters such as heritability (calculated as h^2=Var (A)/Var (P)), data distribution pattern (normality test), and individuals' frequencies (frequency distribution) were calculated. Principal component analysis (PCA) was done using silhouette width technique to establish cluster divergences (genetic structure of the data) and variation within clusters (random genetic variability). Numbers of K-clusters were recognized under width silhouette (*sj*) test that varies from 0 to 1, where values close to 0 refer to not well established clusters and values closest to 1 to well established clusters.

Results

Carotenoid–protein content variation

Protein content variation was assessed based on buffer solubility in stepwise fractionation as aqueous buffer soluble proteins (BSP), buffer insoluble proteins (BIP) and total buffer extractable proteins (TBEP) combining both fractions in a direct single extraction step. Total buffer extracted proteins (TBEP) across landraces (Fig.1(a)) varied from 0.27 (cv. IAC 12.829) to 8.0 mg/g DW (landrace CAS006), while buffer soluble proteins (BSP) and buffer insoluble proteins (BIP) varied from 0.8 to 4.7 mg/g DW and 0.2 to 3.3 mg/g DW respectively (Fig.1(b)). This variation corresponded to 69-73% presence of proteins in BSP and 36-41% in BIP (Fig. 1B). Values for TBEP and BIP correspond to 8 and 22 times more protein in the high compared to low carotenoid landraces. Total carotenoid and β-carotene content variation ranged from 0.26 to 61.66 µg/g DW and 0.07 to 49.91 µg/g DW respectively (Table 1).

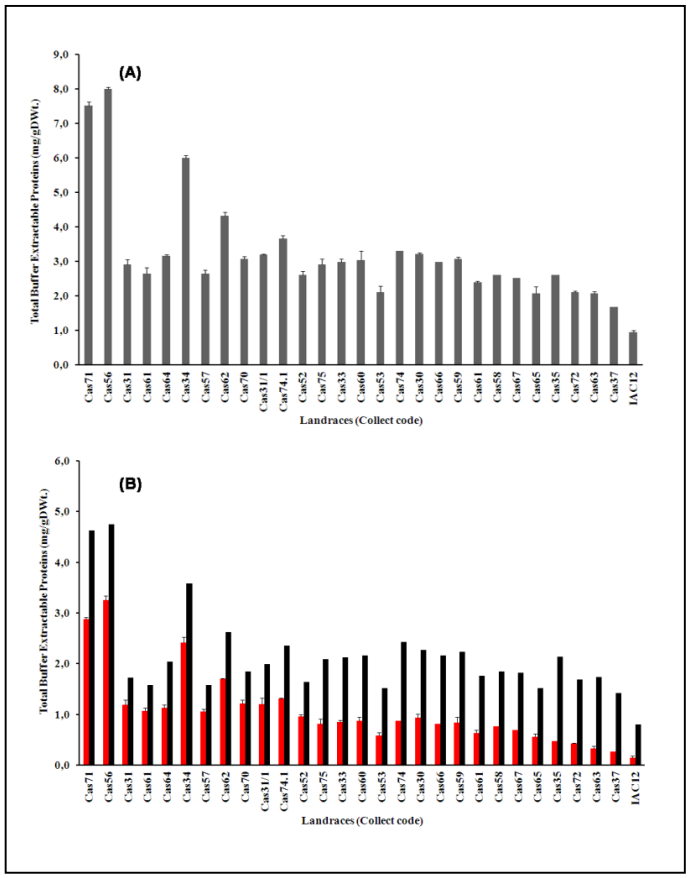

Fig. 1. Protein content variation in bulk tissue of cassava storage root for 29 landraces. (A) Total buffer extractable proteins (TBEP). (B) Buffer soluble proteins (BSP) in black bars, and insoluble proteins (BIP) in red bars. Proteins fractionation and quantification using Bradford assay procedure is described in Materials and methods.

Protein distribution in root tissue layers

The effect of tissue age on protein and total carotenoid content was performed based on dissecting tissue layers as described by de Souza et al. [13]. The distribution of TBEP and carotenoids contents followed a particular pattern with variable magnitude, depending on tissue type (layers 1 and 2), and tissue age (layers 3, 4 and 5) across landraces (Fig. 2). Overall, while values for TBEP where always higher in tissue system I (layer 1, corresponding to phelogen/pheloderm) and tissue system II (layer 2, corresponding to cambium/phloem), in the case of tissue system III (corresponding to secondary parenchyma), these values showed decreasing content from layers 3 to 5 across all landraces studied. Total buffer extractable protein content decreased as the tissue got older, layers 3-4-5, in tissue system III across all the landraces studied (Fig. 2(a)). For the case of total carotenoid content, an opposite pattern of variation was observed (Fig. 2(b)). Taken together, these results indicated a strong influence of tissue type (tissue system I and II) and age (layers in tissue system III) on the accumulation of TBEP and total carotenoids as secondary growth proceeds.

TABLE 1. VARIATION IN TOTAL CAROTENOID (SPECTROPHOTOMETRIC ASSAY) AND TOTAL β-CAROTENE (HPLC PROCEDURE) CONTENT OBSERVED IN STORAGE ROOT OF 29 CASSAVA LANDRACES. NR REFERS TO PEAK WITHOUT RESOLUTION FOR ESTIMATION THE AMOUNT OF B-CAROTENE IN THE HPLC PROFILE.

Colleting Code	Total Carotenoid Content (µg/g DW)	Total β-carotene Content (µg/g DW)	Proportion of total β-carotene over total carotenoid (%)
CAS063	0.26	NR	-
CAS037	0.26	0.07	27
CAS072	0.34	NR	-
CAS035	0.39	NR	-
CAS058	0.52	NR	-
CAS065	0.56	NR	-
IAC12.829	0.65	0.95	68
CAS059	0.88	0.44	50
CAS066	1.68	0.37	22
CAS067	1.82	NR	-
CAS061	4.40	2.93	67
CAS033	6.62	6.44	97
CAS075	10.74	4.71	44
CAS068	10.76	3.85	36
CAS074	11.71	6.30	54
CAS060	13.30	8.08	61
CAS031	13.97	5.49	39
CAS030	14.5	3.85	27
CAS064	14.84	11.43	77
CAS070	14.84	13.54	91
CAS053	17.18	11.89	69
CAS061	19.40	1.66	9
CAS052	19.45	3.10	16
CAS057	24.41	14.87	61
CAS071	24.57	20.68	84
CAS032	30.77	8.32	27
CAS034	30.77	13.96	45
CAS074.1	33.54	10.65	32
CAS062	61.66	49.91	81

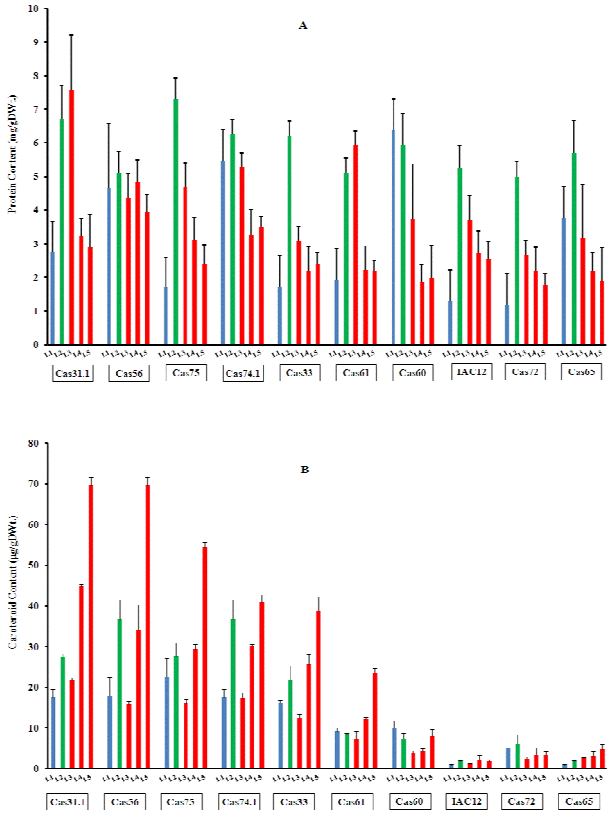

Fig. 2. Total buffer extractable proteins content (A) and total carotenoid content (B) distribution in tissue layers according to advanced secondary growth model in 10 representative landraces. Concept and sampling procedures for layers [13].

Carotenoid-protein correlation

Pearson's matrix correlation analysis showed that coefficients of correlations between carotenoid and protein content were statistically significant high for data sets from landraces and F1 population (Table 2). These results provide the first reported information for cassava storage root and a guide for further characterization of particular individuals.

Carotenoid-protein association identification and characterization

One of the possibilities to explain the correlation between carotenoid and proteins was to understand the carotenoid-protein association. Here we provide results for isolation, identification and characterization of stable carotenoid-proteins association in cassava storage root by using size exclusion chromatography (SEC) of chromoplast enriched suspension. The SEC elution profile for carotenoid-proteins association was performed in three genotypes of contrasting carotenoid content - cultivar IAC 12-829 (Fig. 3(a)) with low carotenoid content, landrace-Cas31 (Fig. 3(b)) of intermediary total carotenoid content, and landrace CAS074.1 (Fig. 3(c)) of intermediary total carotenoid content – showed two major peaks. Peak 1 (common to all cultivars) was eluted within the void volume of the column as determined with blue dextran and showed carotenoids (OD readings at 461 nm) eluting together with proteins (OD readings at 280nm). The other peaks showed no carotenoid elution together with proteins. Similar results have been reported with carrot roots [8, 16, 17]. These results indicated that stable molecular associations between proteins and carotenoids occur in chromoplast at different magnitude, depending on the genotype.

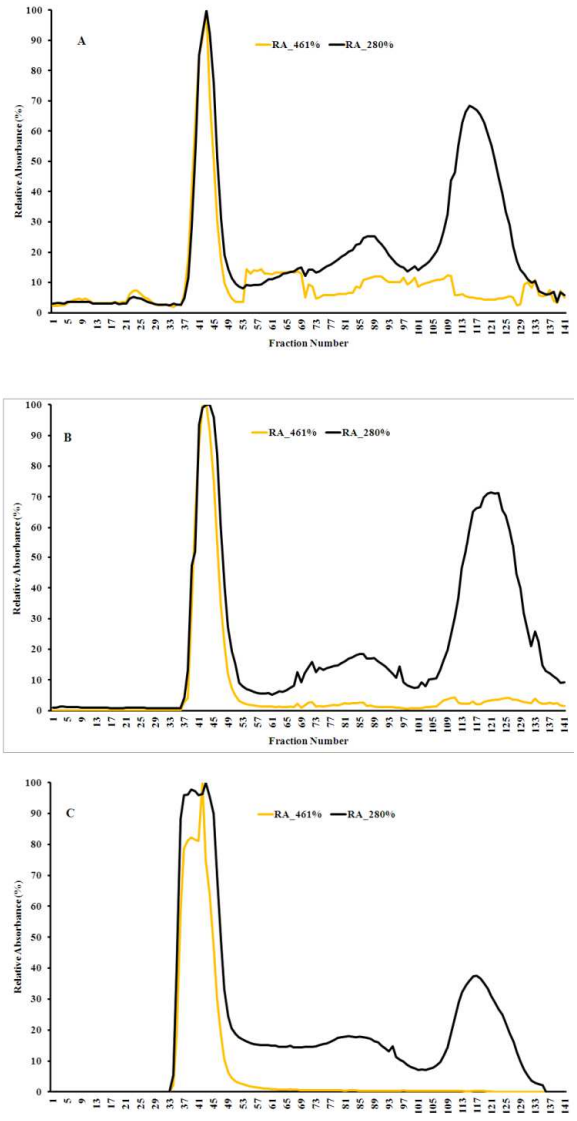

Fig. 3. Size exclusion chromatography (SEC) profile of chromoplast enriched suspension from cultivar IAC 12-829 (A), landrace CAS31 (B), and CAS074.1 (C). Fractions were monitored at both 280nm and 460nm corresponding to protein and total carotenoid. For open chromatography running specification and conditions see Material and methods. Fractions 35 to 49 were pooled and named Carotenoid-Protein Complex (CPC).

The types of carotenoids and proteins were further characterized by their HPLC profile and SDS-PAGE profile for carotenoids and proteins respectively. A specific carotenoid type was observed in the HPLC carotenoid profile (Fig. 4) that is similar to the one reported by others [14], however, with different abundance depending on the genotype. Proteins band pattern showed (Fig. 5) differences in protein band size and numbers, depending on the genotype. An 18 kDa band abundant in commercial cultivar IAC 12-829, 20 and 33 kDa bands common and abundant in landrace Cas031 and Cas74.1, and finally a 42 kDa band unique to landrace Cas31 were observed. Carotenoid-protein complexes have been documented in several plants systems at SDS-PAGE level [6, 8, 10, 16]. All of them showed similar protein profile in SDS-PAGE in terms of protein size variation ranging from 15 to 70 kDa.

Carotenoid-protein content variation in a progeny population

A progeny of 200 F1 individuals, derived from a cross of a high protein and carotenoid content landrace (CAS074.1) and a commercial variety (CPAC 753) with low content of protein and carotenoids was used to access the genetic parameters associated with those traits. Both traits (carotenoid and proteins content) showed a similar pattern of frequency of occurrence (Fig. 6) but different statistical significance level for normality distribution test (Table 3). Principal component analysis (PCA) revealed that the first principal component (carotenoid content) explained 60% (Fig. 7(a)) of the total variability, and its weight values showed a positive correlation with proteins and discriminated individuals based on this component. Two groups were established. Group I was composed of four individuals (11/12, 12/12, 14/12, and 21/12) showing high protein and high carotenoid content (Fig 7(b) and Table 4). Group II formed twenty sub-groups after identification and removal of Group I from the analysis (Fig. 8**),** where the width silhouette test (sj) value was highly significant at the level of 0.60. The Pearson correlation matrix showed high levels of R value as 0.68 (p-value=0.008) for correlation between total carotenoid and TBEP (Table 2). One-way ANOVA allowed us to infer on the estimated heritability of both traits indicating an h^2-value of 0,010 for TBEP and 0,339 for total carotenoid.

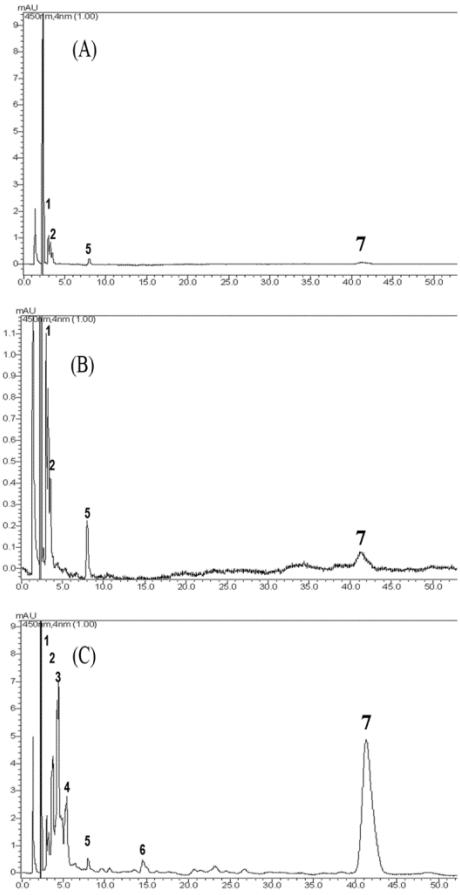

Fig. 4. HPLC profile of carotenoid present in peak 1 (carotenoid associated to protein complex (peak I) from SEC profile as in Figure 3. (A) Commercial cultivar IAC 12-829 with showing low total carotenoid content. (B) Landrace CAS3156 with presenting intermediary amount of total carotenoid content. (C) Landrace CAS74.1 with presenting high amount of total carotenoid content. HPLC running conditions used a monomeric 3 µm C18 waters column with a solvent mobile phase of acetonitrile:ethyl acetate:methanol (80:10:10). Peak numbers refer to 1. Neoxanthin; 2. Violaxanthin; 3. Lutein; 4. Unkown; 5. Unkown; 6. Zeinoxantin or ß-Cryptoxanthin; 7. Trans ß-Carotene.

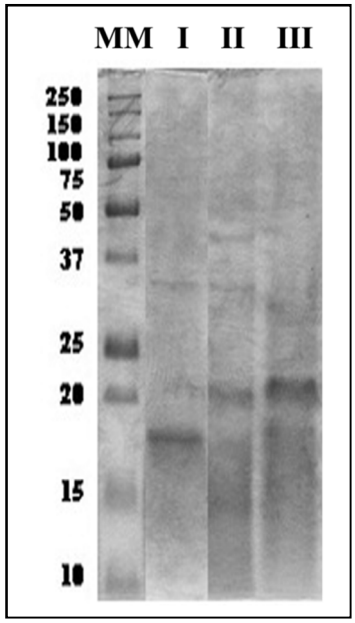

Fig. 5. SDS-PAGE proteins profile associated to carotenoid (peak I) separated in SEC as in Figure 3. Lane MM contains protein molecular markers. (I) proteins from peak 1 in cultivar IAC12.829; (II) proteins from peak 1 in landrace CAS31; (III) proteins from peak 1 of landrace CAS74.1. An 18 kDa band abundant in commercial cultivar IAC 12-829, 20 and 33 kDa bands common and abundant in landrace Cas 31 and Cas74.1, and finally a 42 kDa band unique to landrace Cas31 were observed.

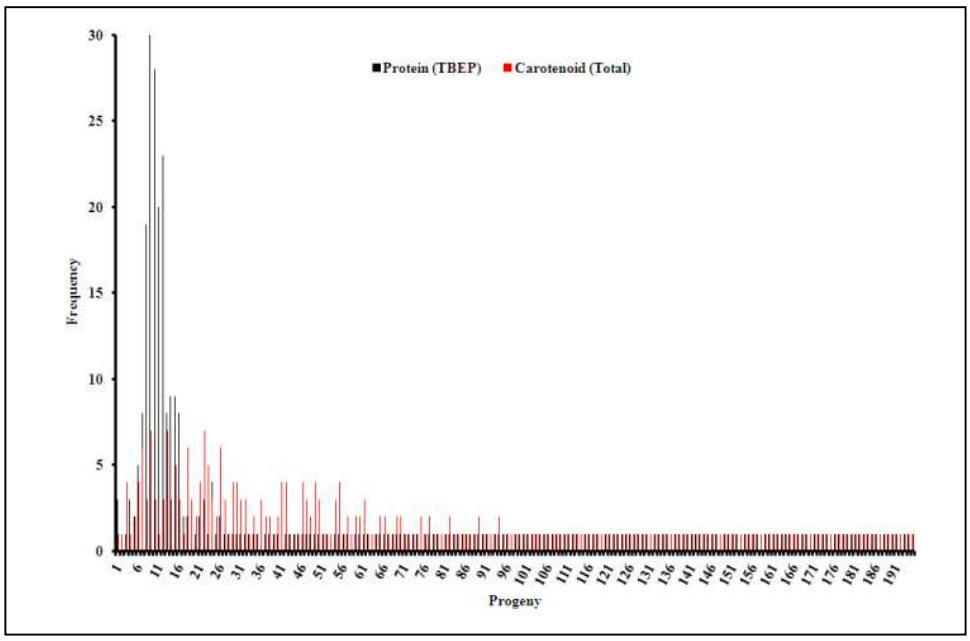

Fig. 6. Frequency (%) distribution for TBEP and total carotenoid content across 200 individuals in the progeny test. The biochemical phenotype defines a continuous distribution in this segregation population that is caused by the combined effects of allelic variation at several segregating loci (multigenic) and the environment.

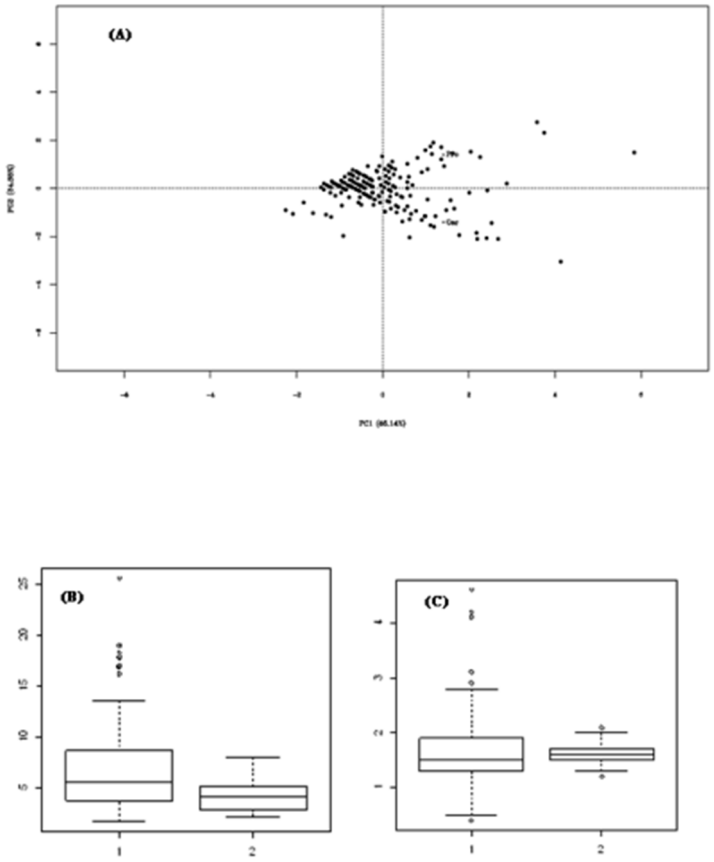

Fig. 7. Scatter plot (A) of a rank-1 matrix showing entries for TBEP and total carotenoids values measured across 200 individuals from the F1 progeny and box plot for carotenoid (B) and proteins (C) group distribution.

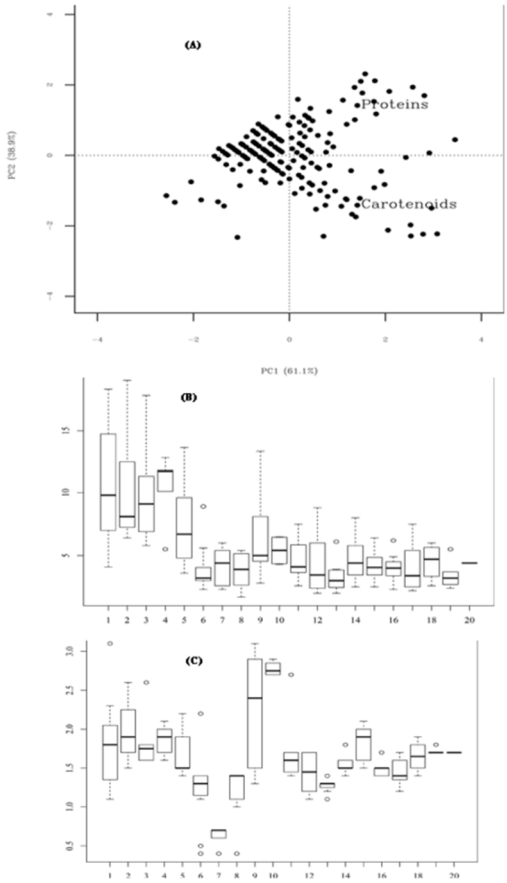

Fig. 8. Scatter plot (A) of a rank-2 matrix showing entries for TBEP and total carotenoids values measured across 196 individuals from the F1 progeny and box plot for proteins (B) and carotenoid (C) group distribution.

TABLE 2. TEST FOR CORRELATION BETWEEN BUFFER FRACTIONATED PROTEIN EXTRACT (TOTAL BUFFER EXTRACTABLE PROTEIN – TBEP, BUFFER SOLUBLE PROTEIN – BSP, AND BUFFER INSOLUBLE PROTEINS – BIP) AND TOTAL CAROTENOID CONTENT.

Proteins Ext.	R-value	R^2-value	p-value
Landraces (29)			
TBEP	0.680	0.462	0.008
BSP	0.416	0.173	0.068
BIP	0.463	0.205	0.045
Progeny (196)			
TBEP	0.299	0.089	0.0001

Note: R-value is correlation coefficient; R^2-value is the coefficient of determination and p-value level of probability of significance for R-value in the landraces and progeny study. (Pearson correlation matrix with p-values different from 0 with a significance level alpha=0.05.)

TABLE 3. TEST FOR DATA NORMALITY DISTRIBUTION FOR A PROGENY OF 200 INDIVIDUALS TESTED.

Statistics tests	Protein	Carotenoid
	Progeny	
Skewness	0.04126	0.49645
s.e. Skewness	0.1226	0.0709
Kurtosis	-0.6057	-1.04936
s.e. Kurtosis	0.2447	0.1418
CI (95%)	44.19>50.4647>56.75	74.5 >77.8141> 81.13

TABLE 4. GROUPS OF INDIVIDUALS, AS DISCRIMINATED BY PRINCIPAL COMPONENT ANALYSIS FOR TBEP AND TOTAL CAROTENOID CONTENT.

Groupings	Sub-groups (#)	Group ID	Individuals ID	Protein (mg/DWt.)	Carotenoid (µg/gDWt.)
Group I	g (4)	g	11/12	4.1	9.9
		g	12/12	4.6	17.6
		g	14/12	4.2	8.3
		g	21/12	4.1	25.6
Group II	1 (n=16)	a	1/12	1.4	6.7
		a	5/12	1.3	7.3
		a	38/12	2.1	11.7
		a	42/12	1.5	5.9
		a	50/12	1.4	5.6
		a	107/12	1.4	4.7
		a	113/12	1.7	7.5
		a	141/12	1.5	4.1
		a	142/12	1.4	5.9
		a	158/12	2.1	4.6
		a	160/12	2.0	3.3
		a	167/12	1.5	3.0
		a	175/12	1.4	3.4
		a	180/12	1.6	4.8
		a	182/12	1.3	2.4
		a	193/12	1.7	3.7
	2 (n=6)	b	2/12	1.7	12.5

Groupings	Sub-groups (#)	Group ID	Individuals ID	Protein (mg/DWt.)	Carotenoid (µg/gDWt.)
		b	40/12	2.0	12.8
		b	86/12	1.3	2.8
		b	89/12	1.5	10.7
		b	93/12	2.9	13.3
		b	96/12	2.5	4.7
	3 (n=8)	c	3/12	16	16.2
		c	14/12	4.2	8.3
		c	24/12	1.7	6.8
		c	34/12	1.7	17.8
		c	36/12	1.6	11.8
		c	87/12	1.5	8.8
		c	92/12	2.9	5.0
		c	94/12	2.4	6.7
	4 (n=5)	d	4/12	1.8	18.3
		d	11/12	4.1	9.9
		d	16/12	1.9	7.5
		d	17/12	1.9	12.5
		d	22/12	1.5	7.7
	9 (n=11)	e	10/12	2.1	4.1
		e	27/12	2.4	12.0
		e	37/12	1.7	5.5
		e	39/12	1.9	10.1
		e	56/12	1.9	4.8
		e	57/12	1.8	4.7
		e	82/12	1.4	4.9
		e	108/12	1.7	6.5
		e	150/12	1.5	4.2
		e	153/12	1.5	4.4
		e	157/12	1.9	3.6
	10 (n=4)	f	15/12	2.0	7.5
		f	23/12	1.7	8.1
		f	30/12	1.6	10.7
		f	85/12	1.4	3.9

Discussion

The results from the present study showed that carotenoid-proteins content varied significantly among 29 cassava landraces indicating the existence of a notable genetic diversity. Overall, 8x more TBEP (mg/g DW) and 17x more BIP observed in the high compared to the low carotenoid landrace correspond up to 237x total carotenoid (µg/g DW.) and 713x more β-carotene respectively. Correlation coefficient values of R=0,680 for TBEP and 0,463 for BIP support the identification of carotenoid-protein complex and variation in protein content by comparing high (landrace Cas74.1) and low (cv IAC12.829) carotenoid content. Therefore, this dual concomitant accumulation of total carotenoid, β-carotene and proteins could lead to an important sink for carotenoid-protein in CSR. Detailed analysis of proteins from the carotenoid-protein complex indicated different sets of proteins related to carotenoid content present in the diverse landraces. While in the commercial variety IAC 12.829 with low total carotenoid content two protein bands of 18 and 33 kDa were detected, the high carotenoid content landrace CAS074.1 exhibited four proteins with size of 18-20-30-33 kDa. A protein band of 42 kDa was uniquely present in landrace CAS31. This diversity in carotenoid-protein content has also been observed in several non-photosynthetic tissues of many plant systems. Massive accumulation of carotenoids correlates directly to: (1) biosynthesis of components of the structures [6, 7, 8, 10, 16, 18]; (2) proliferation of

carotenoid sequestering structures rather than with changes in gene expression or enzyme abundance of the carotenoid biosynthesis pathway [19]; (3) dependence on their sequestration in specialized supramolecular structures [11, 20, 21]. Together, these studies lead to the recognition of a broad class of proteins named carotenoid associated proteins (CAP) [21]. CAP relates to cell functions such as pro-plastid and plastid differentiation and interconversion [6, 10, 21], chromoplast formation [8, 9, 22], chromoplast differentiation [11, 18], and carotenoid sequestration [10, 23]. Recently we showed that the major proteins classes present in carotenoid-protein complex of cassava belong to the small heat shock protein family and a different isoform of HSP21 may be responsible for the difference between low and high carotenoid content landrace [17]. At the same time, the carotenoid species present in carotenoid-protein complex varied according to the genotype sources (either cultivar or landrace). While in cultivar IAC 12-829 three carotenoid species were detected, landrace Cas31 and CAS74.1 showed four and seven carotenoid types respectively. Studies with carrot [8, 16], non-photosynthetic chromoplasts [8, 24, 25], algae [26] and cyanobacteria [27] showed similar results. This variability may account for the diversity of protein size observed across genotypes in the present study as well as across species in different studies.

Genetic transfer of the high carotenoid-protein character was studied in an F1 population with 200 individuals. The results suggest a high heritability of carotenoid content and low heritability for protein content. The high heritability for carotenoid content (0.33) indicates preponderance of additive gene action in the expression of this trait; therefore selection might be effective through this character in a segregating generation. The case of protein content, which showed low heritability (0.01), indicates non-additive gene action and a possible strong genotype x environment interaction playing a crucial role in the expression of this trait. Another possible explanation for the protein content diversity could be related to starch content as it has been shown to be negatively correlated [28]. Parenchyma cells in cassava storage root are packed with 85% starch (based on dry weight base), thus, for an impact of increasing carotenoid-protein content, a significant amount of starches have to be reduced. Therefore, selection for high proteins content for future breeding may not be recommended as direct measurement, but as an indirect measurement using values of carotenoid content.

Besides carotenoid-protein variation being genotype associated as demonstrated above, it also varied with root tissue type and age. The distribution of TBEP and carotenoids in five tissue layers followed a distinct pattern with variable magnitude depending on the landrace. While TBEP content decreased within tissue layers in tissue system III, total carotenoid content tended to increase. Taken together, these results indicated a strong influence of tissue age (layers in tissue system III) on the accumulation of total carotenoids and TBEP as secondary growth proceeds. The results presented in this study, represent the first reporting of a storage organ like the one in cassava storage root (CRS). Together, the genetic diversity observed in the landraces and progeny studies as well as the high throughput screening (HTS) for a direct (landraces access) and indirect (progeny access) quantitative assay for both traits revealed a possibility of using this concept for selection of cassava storage root enriched with carotenoid and proteins in a conventional breeding programme, or using induced mutations for further trait enhancement.

Conclusions and forward look

Results from this study allow us to conclude that: natural carotenoid-protein content varies due to genetic background of landraces and storage root tissue age; carotenoid-protein association is stable across landraces and clones, and showed variation in protein and carotenoid types; estimated heritability of proteins and carotenoids traits showed different values. The role of proteins associated with massive carotenoid accumulation and the mechanisms of carotenoid sequestration in CSR are on going research activities in our laboratory.

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Review

Cassava Mutation Breeding: Current Status and Trends

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Abstract

Cassava (*Manihot esculenta* Crantz) is an important energy source in the diets of millions of people in the tropical and subtropical regions of the world, especially the poor. Also its industrial uses are steadily growing for starch, animal feed and bio-ethanol. Although it has high economic and social relevance, few major scientific efforts have been made to improve the crop until the 1970s. With the goals and objectives of cassava improvement through breeding, different strategies have been developed during the last several decades, such as evaluation and selection of the local landraces, introduced germplasm (as clones or segregating F1 population), hybridization (including inbreeding by both recurrent backcross schemes and double haploids (DH)), interspecific hybridization, polyploidy breeding, genetic transformation, use of molecular markers and mutation breeding. Induced mutation breeding on cassava has been explored in the last several decades with few published papers. Yet, the production of novel genotypes, such as high amylose and small granule mutants and mutants with tolerance to post harvest physiological deterioration (PPD), has been reported. These results suggest that mutagenesis could be an effective alternative for cassava breeding. However, many drawbacks still exist in cassava mutation breeding, such as the occurrence of chimeras. Validated and developing protocols for different biotechnologies, such as TILLING protocol, cassava genome sequencing and cassava somatic embryogenesis, will significantly ameliorate the drawbacks to traditional mutation breeding, and consequently aid the routine application of induced mutation in both cassava improvement and in gene discovery and elucidation.

Introduction

Cassava (*Manihot esculenta* Crantz) is a member of the genus *Manihot*, and the family Euphorbiaceae. Along with maize, sugarcane and rice, cassava constitutes the most important sources of energy in the diet of most tropical countries of the world. The species originated in South America [1], and was domesticated less than 10 000 years ago. Early European sailors soon recognized the advantages of the crop and carried it to Africa. From there, traders later introduced it to Asia. Currently, cassava is an important crop in regions at latitudes between 30^0 N and 30^0 S, and from sea level up to 1800 meters above sea level. Cassava is also a very rustic crop that grows well under marginal conditions where few other crops could survive, and subject to a wide variation of environmental factors. A recent scientific report mentioned that "Cassava offers climate change hope for Africa. When other staples can suffer from heat and other problems of climate change, cassava thrives". [2]. Cassava owes part of its popularity to the wide diversity of uses of the roots: fresh or processed for human food and animal feed, and in various industrial products including starch and starch-derived products, alcohol and high fructose-glucose syrups.

Despite its wide use in food and industries, cassava, occasionally called an "orphan" crop, rarely attracts the eyes of breeders and few major scientific efforts have been made to improve the crop until the 1970s. With the creation of the International Center for Tropical Agriculture (CIAT) in Colombia and the International Institute of Tropical Agriculture (IITA) in Nigeria in the early 1970s, a new era has begun for cassava with the implementation of successful breeding projects. National research centres in Brazil, Colombia, India Thailand, China and other countries have conducted successful research on cassava as well.

Plant breeding has one of the highest rates of return among the investments in agricultural research. It has been reported that the remarkable increase in the productivity of many crops during the twentieth century was due to genetic gains achieved through crop breeding. Cassava has also benefited from technological inputs in the area of breeding [3]. With the goals and objectives of cassava

improvement, different breeding strategies have been developed in these years, such as evaluation and selection the local landraces, introduced germplasm (as clones or segregating F1 population), hybridization (including inbreeding by both recurrent backcross schemes and double haploids (DH)), inter-specific hybridization, polyploidy breeding, genetic transformation, use of molecular markers and mutation breeding. For the past 50 years, mutation induction has been a routine tool for the generation of genetic variation in crop germplasm, and played a major role in the development of superior plant varieties all over the world [4]. Cassava mutation breeding has been explored in recent decades with few published papers. But with the biotechnology development and genomics discovery of cassava, especially with the development of TILLING technology, mutagenesis could become a productive strategy to complement other technologies in cassava breeding. In this review we discuss the progress and trends in cassava mutation breeding and the cassava breeding strategies are also reviewed.

Cassava breeding strategies

1. Evaluation and selection of the local land races; introduced germplasm (as clones or segregating F1 population)

The simplest and most common method in cassava breeding is selection and release of superior existing landrace varieties, which may be locally selected clones or introductions from another region or countries. For example, most cassava cultivars released in Asia were selected from introduced varieties or F1 seeds, such as in Thailand and China. In recent years, a large batch of both accessions from the cassava genebank in CIAT and hybrid F1 seeds from CIAT breeding groups have been shipped to the Americas and African and Asian countries every year. An aggressive and systematic screening of local landraces, introduced varieties or F1 population will still be the predominant and promising cassava breeding strategy in most countries. Further, wide genetic variability in existing cassava germplasm has not been fully explored and screened [5], [6], [7]. As an example, a new class of cassava (named sugary cassava) from cassava landraces of the Amazon basin was reported, with high free sugar content. There are also several variants in the starch type and composition [8].

2. Hybridization

By far the most common means of creating new gene combinations in cassava is by crossing among distinct clones within the species. Breeding scheme for cassava hybridization is typically either in the mass phenotypic selection or in recurrent selection. Superior individuals are selected over several seasons with mass continual evaluation and selection based on phenotype. Recurrent selection is a common population improvement method used to accumulate favorable alleles in a population during a series of recombination and selection cycles. The introduction of inbreeding offers several advantages: it facilitates the gradual and consistent assembly of favorable gene combinations that (in the mass phenotypic selection system) occurs just by chance; it facilitates the reduction of the genetic load of cassava; and it involves the identification of natural or induced recessive mutations. For example, the amylose-free starch mutant ("waxy") in cassava was discovered via self-pollination [9]. Inbreeding cassava to full homozygous genotypes through traditional self-pollination, however, requires 12-15 years because the time required for each self-pollination cycle is at least two years [10]. The production of double haploids through different strategies (such as anther or microspore culture) is an interesting approach that would reduce the time required to obtain homozygous genotypes. So several institutions, such as CIAT, are currently involved in a project to develop a protocol for the production of DH in cassava. Ongoing work is aimed at producing doubled haploids through anther, microspore or ovule culture, and wild crossed with castor (*Ricinus communis*).

3. Interspecific hybridization

The potential worth of wild *manihot* species is a subject that elicits a wide range of viewpoints. Wild *Manihot* species can potentially be used as a source of useful traits. Use of the wild parent *M. oligantha* was reported to produce a hybrid with increased root protein content [11]; but this protocol did not appear to be stable and highly efficient. Apomixis was also transferred successfully from the wild species *M. neusana*. High yielding cassava clones ensued from interspecific hybridization with wild species such as *M. glaziovii*, *M. pseudoglaz* and *M. cearulescens* [12]. As an example, UnB 110 was selected from a progeny of cassava interspecific hybrid with *M. glaziovii*. It showed high root productivity reaching up to 18 kg/plant after two years in growth under the Brasilia's conditions, with the additional characteristics of tolerance to drought and resistance to mealybug [13].

A necessary precursor to effective utilization of wild *manihot* species is to establish more comprehensive collections of wild *manihot* species, thoroughly evaluate their characteristics, and improve the efficiency of the techniques for transferring traits.

4. Polyploidy breeding

Polyploidy is an intriguing phenomenon in plants that has provided an important pathway for evolution and speciation. It also provides a valuable tool for plant breeding. Polyploidy breeding showed unique advantages in cassava. Garner [14] and Abraham et al. [15] described colchicine induced tetraploids of cassava. The triploids (2n=54) cassava variety ("Sree Harsha") was produced by crossing the cultivated diploids (2n=36) with induced tetraploids (2n=72). The triploids variety was characterized by vigorous growth, erect plant type, broad leaves and stout stem, with significantly higher yields (4.2 kg/plant) than the diploids [16]. UnB 201 was selected among indigenous cassava cultivars, followed by artificial chromosome duplication by colchicine. It is characterized by its high protein (reaching 5.5%), very low HCN contents (around 10-12 mg/kg) and high beta-carotene content reaching 27 mg/kg [17]. UnB 310, a triploids cassava, originated from crossing cassava with *M. oligantha* via the production of unreduced gametes. It is characterized by its extremely vigorous growth, very high productivity (reaching up to 25 kg/plant) after three years in growth and very low HCN contents (around 13 mg/kg) [18].

5. Genetic transformation

After several years of development, the cassava genetic transformation technology has gradually matured, and substantial progress has been made in validating gene function and transgenic breeding since the first simultaneously reported pioneering achievements in cassava genetic transformation by two research groups in 1996 [19], [20], [21]. Cyanogen free cassava [22], provitamin A accumulation cassava [23] and waxy cassava [24] were produced by genetic transformation. Because of public concern about the biosafety of using traditional selectable marker gene include antibiotic resistant and herbicide resistant genes, it is necessary to develop better accepted marker genes or marker free technology in cassava [21].

6. Use of molecular markers

Molecular markers have not accelerated cassava breeding as it has in other crops. The first and only example in cassava for the successful use of marker assisted selection (MAS) was the official release of CR 41-10 in Nigeria in 2010. This genotype was originally selected using a set of molecular markers linked to resistance to CMD in Colombia (where CMD is not present), and evaluated in Africa for general adaptation and agronomic performance [10]. Cassava genetic improvement can be made more efficient through the use of easily assayable molecular genetic markers that enable the precise identification of genotypes without the confounding effect of the environment, increasing heritability. MAS can also greatly enhance the efficiency of phenotypic mass selection by the efficient reduction of large breeding populations at the seedling stage based upon a "minimum selection criteria". Another application of MAS in cassava breeding is reducing the length of time required for the introgression of traits from wild relatives [25].

7. Development of new cassava genotypes through induced mutation

The routine application of mutagenesis for crop improvement requires the production, handling and assaying of large mutant population. This can be expensive, laborious and time consuming. Moreover, compared to more directed genome manipulation, mutagenesis can be random, resulting in unpredictable outcomes, sometimes favourable, othertimes not. As cassava is primarily vegetativele propagated, cassava mutation breeding is further confronted with the problems of the occurrence of chimeras and the absence of meiosis to remove deleterious alleles and create the recessive genes [26]. Till now, only few reports on cassava mutagenesis (the polyploids induced by colchicine not covered here) were published as reviews (Table 1). Moreover two cassava varieties bred by mutation breeding were registered in IAEA database (reference to the website: http://mvgs.iaea.org/). Taken the *in vivo* stakes of five cultivars (such as "ISU") as explants treated with 25-30 Gy, the mutant (ISU-W) features large sized starch granules in the tubers, improved cooking quality and tolerance to African cassava mosaic virus disease (ACMVD), discovered and released as the new variety "Tebankye" in Ghana in 1997 [27]; http://www.iaea.org/Publications/Reports/Anrep97/fooda gri.pdf). By taking the variety "SC124" as parent material, another elite cassava clone was selected from the mutation population, and released as a new variety "Fux-

uan 01" in China in 2005 (http://www.gxseed.com.cn/Bred/Show_breedAUD.asp?Id=5252 (in Chinese)).

To correct specific genetic defects in cassava popular cultivars, mutation breeding was initiated in India in the 1950s and later; preliminary studies resulted in the generation of lines with special morphological characteristics and chlorophyll mutations (reviewed by Nayar et al. [28]. Subsequently a mutant with short petioles, isolated by Nayar and Rajendran [29] from H-165 was reported to have practical application. Incidentally, the chlorophyll mutant reported by Nayar and Rajendran [29], showed conspicuously high HCN content, both in the leaves and roots; and it appears that HCN content in cassava is controlled by recessive genes. Although few published papers focus on cassava induced mutation, mutagenesis has played a significant role in cassava breeding to create novel cassava genotypes. It is worth mentioning that four distinctive root phenotypes were identified at CIAT in an M2 population derived from 1400 irradiated botanical seeds [30]: (a) with small granule and high-amylose starch, (b) with tolerance to PPD, (c) with "hollow" starch granule, (d) starchless. The high amylose starches in different crops offer advantages for the production of sweets, adhesives and corrugated boards, the paper industry and reduce the uptake of fat in certain fried products; and the reduced granule size and the obvious irregularities in their surface would lead to a facilitated hydrolysis [25]. The two mutant genotypes (2G15-1 and 5G108-4) showed excellent levels of tolerance to PPD after harvest [31]. TILLING, a very promising reverse genetics molecular approach, has been applied to identify all the mutants by the cooperation between IAEA and CIAT [32, 33].

TABLE 1. INDUCED MUTAGENESIS IN GENETIC IMPROVEMENT OF CASSAVA OVERVIEW

Genotypes and explants	Treatments	Specific objectives	Research units (the first)	Major results	Reference
"H-165", *in vivo* stakes	Irradiated with Gy	Tentatively to explore mutagenesis	Central Tuber Crops Research Institute (CTCRI), India	A mutant with short petioles was isolated; incidentally, the chlorophyll mutant showed conspicuously high HCN content, both in the leaves and roots.	[28]
"Bosom nsia", *in vivo* stakes and *in vitro* shoot tips	Irradiated with 25, 30 and 35 Gy	To obtain clones resistant to the ACMV disease	Biotechnology and Nuclear Agricultural Research Institute, Ghana	Radiosensitivity tests showed the doses of 25, 30 and 35 Gy were suitable for mutagenesis. Four variants tolerant to ACMV, suggested by inoculation and ELISA tests.	[42]
Five cultivars, such as "ISU", *in vivo* stakes	Irradiated with 25 and 30 Gy	To improve the cooking quality	University of Science and Technology, Ghana	The mutant (ISU-W) features large sized starch granules in the tubers, improved cooking quality and tolerance to ACMVD, and released as the new variety "Tebankye" in Ghana.	[27]
"MCOL22" and "MCOL1505", young leaf lobes and somatic embryos	Irradiated with Gy from 0 to 38 incrementally	To determinate the radiosensitivity of different explants	International Atomic Energy Agency (IAEA), Austria	LD50 for embryogenic response of leaf explants was at around 20 Gy, while that for somatic embryo germination was around 10 Gy.	[43]
Three cultivars, 10 cm stakes with 5 nodes	Irradiated with 20, 25 and 30 Gy	To reduce the cyanogenic levels	National Root Crops Research Institute, Nigeria	There was a wide variation in HCN, dry matter and starch content of the screened MV2 plants, among which 14 variant lines with low HCN content.	[44]

"PRC 60a", young leaf lobes, different stage somatic embryos and cotyledonary segments	Irradiated with 50, 100, 200 or 300 Gy	To obtain novel mutants	National University of Singapore, Singapore	Globular-stage somatic embryos were selected as suitable experimental materials, and 50 Gy was the optimal dose for inducing mutations. A batch of mutants were obtained, among which line S14 and S15 showed large morphological variations, with 17 and 60 fold root yields respectively, compared to the original parent.	[45]
17 elite IITA-derived varieties, *in vitro* stem with two nodes	Irradiated with 5, 10, 15, 20, 25 and 30 Gy	To determinate the radiosensitivity for cassava accessions	International Atomic Energy Agency (IAEA), Austria	The optimal doses of Gy irradiation varied from 12 Gy to 25 Gy.	[46]
1400 botanical seeds from different full or half-sib families	Irradiated with 200 Gy	To obtain novel mutants	International Center for Tropical Agriculture (CIAT), Colombia	Four distinctive root-phenotypes were identified: a) with small granule and high-amylose starch, b) with tolerance to PPD, c) with "hollow" starch granule, and d) starchless	[30]
Four landraces, stakes	Irradiated with 35 Gy	To obtain novel starch mutants	University of the Free State, South Africa	four mutants with high amylose contents (26.8%-32.7), four mutants with low amylose contents (11.7%-14.0%), unexpected mutants such as putative free-sugar mutants were identified respectively.	[47]

While random mutagenesis may reduce the efficiency of mutation breeding in cassava, molecular biology strategies offer mechanisms for increasing efficiency via direct querying the target genes for changes. Reverse genetics strategies, especially TILLING, have shown to be efficient tools for identification of physical or chemical mutation events [34]. TILLING has been used in the identification of induced mutations in different crops, such as Arabidopsis, rice, maize, wheat; and TILLING public platforms and commercial services for different plant species have been established [35]. Mutation based approaches for cassava improvement are being supported through IAEA coordinated research projects (CRP). The project "Molecular tools for quality improvement in vegetatively propagated crops including banana and cassava" (CRP No. D23027) was recently completed and the ongoing "Enhancing the Efficiency of Mutagenesis through an integrated biotechnology pipeline" (CRP No. D24012) focuses on barley, rice, banana and cassava. Further with the public release of the first cassava (AM560-2) genome sequence in 2009, the genome sequencing of more cassava genotypes are underway. The draft genome sequences of Ku50 (high starch content cassava), W14 (Asian landrace) and CAS36 (sweet cassava) were finished in October 2012, being edited and will be online in two months (Prof. Wenquan WANG,). The Beijing Genomics Institute (BGI) reported that it will work with CIAT to sequence 5 000 cassava genotypes, which will hold great promise for cassava mutation breeding (http://bgiamericas.com/collaboration-for-large-scale-genome-sequencing-of-cassava-%E2%80%93-fourth-major-food-crop-in-developing-world/). Some cassava materials are currently being sequenced as part of Arizona/Berkeley Cassava Genomics BMGF grant (http://www.cassavabase.org/forum/posts.pl?topic_id=1). With other useful data, these efforts will provide gene annotations and information on alleleic diversity that are useful for targeted mutagenesis approaches such as TILLING.

Another drawback to the induced mutation in cassava, like other vegetatively propagated crops, is the incidence of chimeras. However the strategies for mitigating this drawback have been developed through a strategic use of biotechnologies. In banana, also as a typical vegetatively propagated crop, mutation breeding has been developed well. Meristem cultures traditionally used in mutagenesis

experiments in banana lead to chimerism, due to their multicelluar origin. However the multi-apexing technique was shown to be effective in dissociating chimerism, the average percentage of cytochimeras reduced from 100% to 8% after three subcultures, although it did not eliminate chimerism completely [36]. It had achieved fruitful results on banana mutation breeding by combining TILLING protocol, chimerism elimination via multi-apexing technique, and traditional induced technique; and careful genotypic evaluation suggests that chimeras are rapidly dissolved after treatment of meristems with the chemical mutagen EMS [37]. Instead of meristem cultures, embryogenic cell suspensions (ECS) can be used as explants for induced mutation. This should avoid chimerism with any mutagens owning to the unicellular origin of somatic embryos [38, 39]. Similarly the research on dissociating chimerism in cassava has also been explored; and polyploidy mutants were obtained by taking strategic techniques to identify and isolate the chimeric mutants [28, 40]. The protocol of chimerism elimination via multi-apexing technique could also be explored in cassava mutation breeding with *in vivo* stakes or *in vitro* stems as explants. Protocols for irradiation of nodal segments followed by subculturing have been published for cassava [41]. Simultaneously the protocol of cassava somatic embryogenesis, friable embryogenic calli (FEC) induction and suspension culture has been developed well with the development of cassava genetic transformation technology in recent years [21], which will provide valuable tools for cassava mutation breeding. One severe drawback for banana mutation breeding is that the recessive loss of function phenotypes is difficult to detect owning sterility in triploid banana. But as for cassava, this problem could be solved by self-hybridization. Because genetic changes can be detected by TILLING in the M_1 generation, propagation, field planting and self-hybridization can all be performed before phenotypes are evaluated. The expectation therefore is, that TILLING approaches will fruitful in fertile species such as cassava.

Concluding remarks and future

Continually, the cassava scientific community in the world is recognizing the critical roles of breeding strategies in cassava improvement. Thus, "designed cassava varieties", such as herbicide-resistant genotype, are needed to address the demand of diversified markets and the expected increase in global food insecurity. There is a compelling need to invest significant efforts in the development of strategies for an efficient use of induced mutation technologies. Although, identified bottlenecks in routine induction, isolation and deployment of mutations in vegetatively propagated crops (such as cassava) exist, which could be ameliorated through a strategic use of biotechnologies. For example TILLING protocols hold great promise for the use of molecular biology techniques to rapidly whittle down the size of the putative mutant population before field trials or other assays, which will greatly save cost, labor and time. It could be a major contribution to enhance the efficiency of using induced mutations in crop breeding.

Validated and developing protocols for induced mutation breeding exist, but a need remains for the assemblage, adaptation and interlacing of these novel cell and molecular techniques into components of the induced mutations process, as well as the adaptation to cassava.

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Short note

Poorna Bhog, a High Yielding Mutant of Rice

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Abstract

Development of non-lodging, semi dwarf varieties is a major challenge for the improvement of the aromatic rices as they command a high price in the international markets. With an objective to improve Pusa Basmati-1, a popular, semi dwarf basmati variety further, mutation approach was employed to isolate mutants with higher yield potential while retaining the good grain quality. Of the several promising mutants evaluated, CRM2203-4, a semi dwarf mutant, recorded consistently higher yield over the parent in the multi-location trials. Though this mutant cannot be classified as basmati, this mutant with its superior yield and good grain quality ('A' grade) which was released as Poorna Bhog provides the farmers a viable option for higher economic returns.

Key words: Mutation, Poorna Bhog, basmati, semi dwarf, grain quality

Introduction

Rice, the world's most important food crop, is the primary source of food for more than half of the world's population. It is being grown in diverse agro-ecologies and consumed as a staple food and also as a delicacy. Rice is unique among the cereals as it is generally consumed in its kernel form and its properties are ideal components for manufacturing several food products. The quality and delicacy of rice and rice products have been the basis of selection over thousands of years by farmers, which has resulted in the development of aromatic/speciality rice having unique taste characteristic traits in the grain that are not normally present in the common rice. High value rice developed for specific palate, taste, aroma and high quality rice based products offer a wide range of eating quality to millions of consumers. The grain yields of these genotypes are low in comparison to non-aromatic rice but their high sale value and easy marketability not only compensates the demerits but ensures high returns.

Major gains in both productivity and production have enabled India to attain and sustain self-sufficiency in rice and from being a net importer of rice prior to the green revolution; the country has progressed to become a major exporter of rice. With dramatic increase in production and availability of sufficient reserves of buffer stocks of rice, the market has become quality conscious. The farmers, to augment the returns significantly from rice cultivation, view rice as a cash crop and look for varieties having value added characteristics to fetch higher returns.

In the 1990s, aromatic rice began to explode on the international markets and currently around 30 million tons of rice is being traded annually at international level. The high volume of trade and increasing consumer demand for aromatic rice led to the development of numerous specific cultivars with unique aroma, flavour and taste of their own.. The two traits of quality improvement that are crucial for the market at the international level are high head rice recovery (> 60%) and intermediate amylose content (20-25%) while aroma, a special characteristic of some traditional varieties, usually commands a price premium (http://www.knowledgebank.irri.org/).

Though the estimated returns to rice quality improvement are not as large as returns attributed to improvements in yield, they can be still quite high. Basmati rice, having premium grain quality, constitute the major part of rice exports from India and the efforts to augment the Indian basmati exports further led to the development of Pusa Basmati-l, a semi dwarf, high yielding basmati type which occupies a major share of the area under basmati cultivation. This development was of significance as the possibility of success in developing a semi dwarf, non-lodging, high yielding basmati type was limited due to environmental factors [1, 2], inter group sterility barriers [3, 4], complicated polygenic mode of inheritance, complex breeding behaviour of quality parameters [5], and lack of infrastructure for quick method of estimation of grain quality and aroma in varietal improvement programs. In addition, as most of the traditional basmati

varieties were poor combiners, breeding/selection methodologies especially convergent and back cross strategies were adopted to break the undesirable genetic blocks and to increase the frequency of favourable recombinants. Despite its yield superiority, Pusa Basmati-1 grains have long awns, a drawback for both farmers and millers point of view. In an effort to improve Basmati rice further, while keeping the grain quality traits intact, mutation approach was attempted.

Materials and methods

Three popular Basmati genotypes i.e. Basmati 370, Pusa Basmati-1 and Pakistan Basmati were employed in the study using gamma rays for irradiation purpose and the breeding chronology was described earlier [6].

Results and discussion

The study has resulted in isolation of several promising mutants and a mutant from Basmati 370, which possess all the quality traits of Basmati, was released as 'Geetanjali' in 2005 [6]. Of the four promising mutants isolated from Pusa Basmati1 from the same study, CRM 2203-4, a semi dwarf, non-lodging, photo insensitive, mutant yielded consistently higher than Pusa Basmati-1 (Fig. 1).

Fig 1. Poorna Bhog (a). crop (b). grains (c). kernels.

The mutant has outyielded the parent in different trials conducted over years. In the multi-location trials conducted by the All India Coordinated Rice Improvement Programme (AICRIP), it recorded 21.25% yield increase over Pusa Basmati 1 (yield control) and 82.57% over Taroari Basmati (quality control) [6] (Table 1(a)). In the station trials conducted at the Central Rice Research Institute, the mutant has demonstrated its yield superiority (27.95%) over Pusa Basmati-1, its parent (Table 1(b)). In the adaptive trials also, conducted over several locations, it has demonstrated its superiority over the parent and also Geetanjali, another control (Table 1(c)). The analysis of yield and yield attributing traits has revealed that the mutant's higher yield over the parent can be attributed to the presence of higher number of spikelets per panicle while its grain weight is slightly lower than that of parent (Table 2).

TABLE 1. PERFORMANCE OF CRM 2203-4 IN DIFFERENT YIELD EVALUATION TRIALS [KG/HA]. (FIGURES IN PARENTHESIS INDICATE THE YIELD ADVANTAGE (%) OF THE MUTANT OVER THE CONTROL

a) Multi-location trials in Basmati zone (38 entries, 5 locations)

	CRM2203-4	Pusa Basmati-1 (Yield control)	Taroari Basmati (Quality control)
Mean	5718	4716 (21.25%)	3132 (82.57%)

b) Station trials

Year/Season	CRM2203-4	Pusa Basmati-1	Geetanjali
2001/Wet	6287	-	5038 (24.8)
2003/Dry	7394	4128 (79.1)	4878 (51.6)
2004/Dry	4857	4425 (13.3)	4401 (10.4)
2005/Dry	4722	4167 (24.8)	4279 (10.4)
2005/Wet	5520	3560 (55.1)	-
2006/Dry	5087	4697 (8.3)	4593 (10.8)
2007/Dry	5004	4816 (3.9)	4774 (4.8)
2009/Dry	5175	4325 (19.7)	4239 (22.1)
Mean	5506	4303 (27.95)	4527(21.62)

c) Adaptive trials

Year/Season	Location	Yield (kg/ha)		
		CRM 2203-4	Control	Yield advantage%
2003/Dry	Bhanjanagar	3764	2452 (Pusa Basmati-1)	53.5
2003/Wet	Bhanjanagar	4130	2780 (Pusa Basmati-1)	48.6
2004/Wet	Bhanjanagar	3620	2800 (Pusa Basmati-1)	29.3
2004/Wet	Bhadrak	3878	3293 (Geetanjali)	17.8
	Puri	3220	2920 (Geetanjali)	10.3
2005/Wet	Bhanjanagar	3920	2820 (Pusa Basmati-1)	39.0
	Puri	3940	-	
	Bhadrak	2971	2009 (Pusa Basmati-1)	47.9
2006/Wet	Puri	3325	2440 (Geetanjali)	36.3
	Bhanjanagar	2950	1540 (Pusa Basmati-1)	91.6
2007/Wet	Jajpur	3780	-	
	Bolangir	5971	5342	
	Keonjhar	4596	-	
2010/Wet	Cuttack	3512	-	
	Bolangir	5050	4642	
	Puri	2195	-	
	Keonjhar	3728	-	

TABLE 2. THE YIELD CONTRIBUTING CHARACTERS OF CRM 2203-4

Genotype	PL(cm)	Spikelet No./ p	1000 gr.wt (g)
CRM 2203-4	30.5	196	19.7
Pusa Basmati 1	29.4	137	21.8

The prominent grain quality traits of CRM 2203-4 include a high kernel length (6.3 mm) i.e. 'A' grade length, white, translucent kernels, very high head rice recovery (69.6%), absence of grain chalkiness, intermediate amylose content (23.32%) and pleasant aroma (Table 2). Though its kernel elongation ratio after cooking was high (1.7), the kernel length after cooking (KLAC) was moderate (10.6). Despite possessing good grain quality, CRM 2203-4 could not be classified as a Basmati because its values for grain quality traits like KL and KLAC do not meet the prescribed Basmati standards. However, the 'A' grade grain quality of CRM 2203-4 can ensure high returns from the market and its yield advantage (>20%) over the parent can lead to significant economic gains.

Since the mutant can obtain high price from the market, it was evaluated for its suitability and adaptability in different agro climatic zones of Odisha over several years. The mutant's high yield potential (4.5-5.0 t/ha) and easy marketability has made it highly popular with the farmers, millers and traders. The station trials and the adaptive trials have clearly established its yield superiority of the mutant over its parent Pusa Basmati 1 and also over Geetanjali, a Basmati type variety developed under the same programme. The mutant has resistance against neck blast

and biotype 3 of gall midge and moderate levels of resistance against yellow stam borer and sheath rot.
As its cultivation contributed significantly to higher income generation to the farmers, the mutant was released as Poorna Bhog in 2012 for the state of Odisha and was recommended for growing in both shallow low lands and irrigated areas. The grain quality of varieties like Porna Bhog may pave way for export of long grain non-basmati types as another class of export quality rice.

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Short Note

Development CR Boro Dhan-2, a High Yielding Mutant of Rice for Boro Situation

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Abstract

Boro rice is an ancient system of rice cultivation practiced in Eastern India and Bangladesh during winter (Oct.-Nov. to May-June) in low lying areas taking advantage of the residual water in the field after the harvest of wet season rice crop. Boro rice has contributed significantly to the rice production scenario of the region and its immense potential provides vast scope for expansion. The desirable traits for improved cultivars for boro situation include photo-insensitivity, cold tolerance at the seedling and early crop establishment stages, resistance against major diseases and insects and high yield. This report presents the development of CR Boro Dhan-2, a high yielding mutant of China 45, may help in further enhancement of production and productivity of boro rice in the years to come.

Introduction

Boro rice has been an age old traditional system of rice cultivation practiced in Eastern India and Bangladesh, and a similar system is known in Nepal by the name Hiunde Dhan. It is a winter season, photo-insensitive, transplanted rice cultivated in waterlogged, low-lying and medium areas during the period between Oct.-Nov. to May-June. It takes advantage of the residual moisture left after the harvest of wet season rice in the deeply flooded low lands where water stagnation prevails during the winter. Productivity of boro rice is much higher compared to wet season rice and the average yield of boro is about 80% higher than the combined yield of aus and aman crops grown in the same fields [1]. As boro rice varieties yield as high as 5-7 t. ha-1, boro rice cultivation has been found to be quite profitable.

The yield potential of rice, the major cereal crop of India, has to be significantly increased to combat the worsening food security situation. There is immense potential for increasing rice production in Eastern India through expansion of boro rice in the nine million ha of flood prone, waterlogged and deep water areas. Boro rice has high potential for increased production because of abundant sunlight, control of water level in field and lesser pest and disease problems. The main impediment for boro rice was the low temperature during winter and unavailability of varieties with cold tolerance at seedling stage [2]. Some of the attributes for the plant type conceived for boro rice are cold tolerance at seedling and early crop establishment stages, synchronized tillers, dwarf to semi-dwarf plant stature, genetic resistance to biotic stresses (stem borer and blast), high yield potential (10t/ha), grain dormancy and intermediate amylose content [3, 4].

Mutation breeding has proved to be useful in improving specific characteristics of well adapted genotypes and mutagenesis has been successfully used to improve various quantitative characters including yield. The induced mutants with desirable traits have been directly or indirectly used in developing a large number of mutant plant varieties that have contributed significantly to increased agricultural production over the past half century worldwide. Though relatively large populations are required for selecting a desirable trait and only a very minor fraction of inducted mutants will be useful [5], mutation breeding can address specific problems in crops like rice. Mutation approach was followed in China 45 with an objective to induce mutants with improved yield which can replace the best performing improved varieties like Gautam and Joymati in boro situation and this report presents the successful development of CR Boro Dhan-2, a high yielding cultivar suitable for boro situation.

Materials and methods

The variety selected for improvement was China 45, a selection made at the Central Rice Research Institute, India, the earliest rice cultivar released in Nepal in 1959 for Chaite season and the first rice crop in the double rice cropped areas of the Tarai and inner Tarai regions. It is an early maturing variety having a lodging prone, semi-tall plant stature, thin culms, shy tillering ability and is a low yielder. However, China 45 possesses adequate levels of tolerance to low temperature at both the seedling and early crop establishment stages, and is highly adapted to boro situation. The seeds of China 45 were irradiated with 30 Kr gamma rays and the selections were made from M2 generation onwards. The selection criteria include non-lodging plant stature (short culm), higher tiller number, long panicle and higher number of spikelets/ panicle in the mutants.

Results and discussion

More than 50 000 plants were grown in the M2 generation and selections were based on the plant height, panicle traits and single plant yield. The selections continued till M6 generation and the three mutant selections were evaluated for their yield and other desired characters. One of the promising mutants, CR 898, has consistently

showed high promise in the station trials conducted over years (Table 1).

TABLE 1. PERFORMANCE OF CR898 IN STATION TRIALS

Year	Grain yield (t/ha)	
	CR 898	Lalat (Control)
2001	5.9	4.5
2002	6.9	5.2
2003	7.4	5.9
2004	6.4	4.8
2005	6.3	5.7
2006	6.3	5.8
Mean	**6.6**	**5.3**

Fig 1. CR Boro Dhan -2 (CR 898), a variety released for boro situation.

The mutant is of medium early duration (125 d in wet season while the duration may vary from 150-170d in boro situation depending upon the sowing time) with a non-lodging, short plant stature (90 cms) (Fig. 1). It has good tillering ability and the panicles are dense (250-260 grains/panicle) and long (26.0cm). The grains are of medium slender type with seed dormancy of about a week after maturity. The mutant possess high level of tolerance to cold at both seedling and establishment stages, tolerance to insects like yellow stem borer (YSB), and has resistance to diseases like blast and moderate resistance to bacterial blight and sheath blight as compared to controls, i.e. IR 64, Gautam and Krishna Hamsa.

The mutant line CR898 was nominated for multi-location evaluation trials conducted by the All India Co-ordinated Rice Improvement programme (AICRIP) and was evaluated for three years in several boro locations in India with the designation IET 17612. The mutant has consistently displayed yield superiority (~ 15%) over the experimental controls i.e. Gautam (a widely grown HYV in boro areas), IR64 and Krishna Hamsa and was ranked first among all the inbreed varieties evaluated (Table 2) [6-8] during the trials.

The mutant was also evaluated in the farmer fields by the State Agriculture Department of Odisha in the boro season at 7 and 26 locations in 2006 and 2007 respectively, and the yield levels were in the range of 3.8 - 5.6 t/ha. The yields were higher by 13% over Lalat and 26.3% over Naveen, the yield controls. The mutant (IET 17612) was screened for its level of cold tolerance during the boro season (2004-05) at Gerua, Assam. The crop was raised with three different sowing dates viz.15.11.04 (D1) 30.11.04 (D2) and 15.12.04 (D3) during the peak winter period with the minimum temperature ranging between 10-12° C. Based on its survival percentage and seedling damage at the seedling stage and the crop establishment during the early vegetative phase, the mutant was found to possess adequate level (score-3 in a scale of 1-9) of tolerance to low temperature which is critical for its high performance.

Due to its excellent performance in the three years of evaluation at the national level by the nodal agency (AICRIP), IET 17612 was identified for release and was recommended for the boro areas of Assam and Odisha. In 2008 it was released as Chandan for the state of Odisha, and later in 2010, it was released at national level as CR Boro Dhan-2. This mutant has high yield potential and can withstand the cold stress at the seedling as well as the crop establishment stages, and the introduction of this mutant may raise the rice production levels in the target area.

Conclusion

The development and release of CR Boro Dhan-2 demonstrates the utility of mutation approach to alter few traits in a well-adapted variety to a specific environment.

TABLE 2. PERFORMANCE OF IET 17612 (CR 898) IN MULTI-LOCATION TRIALS CONDUCTED UNDER BORO SITUATION

Year of testing	No. of locations	IET 17612	Control 1 (Gautam)	Control 2 (IR 64)	Control 3 (Krishna Hamsa)
I year, 2002	10	6984	6208 (+12.5)	5884 (+18.69)	6089 (+14.70)
II year, 2004	11	5752	5136 (+12.0)	5019 (+14.60)	5260 (+9.40)
III year, 2006	7	5564	4524 (+22.99)	5099 (+9.12)	4648 (+19.70)
Mean		**6145**	**5289 (+15.33)**	**5334 (+14.36)**	**5332 (+14.40)**

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