



## Segregation distortion of anthocyanin morphological marker in $F_2$ population of cross between basmati and environment genic male sterile rice lines

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### Abstract

Basmati rice (*Oryza sativa* L.) is preferred by many Kenyans yet its yield per hectare is low. This challenge led to initiation of Basmati hybrid rice project in Kenya with a goal of leaping yield benefits of heterosis. Since quality traits in Basmati are under recessive gene control, then the two parents in hybrid programme must possess the traits to avoid their masking at  $F_1$  by any other dominant gene. In the process of breeding for suitable parents, Basmati and Environmental genic male sterile lines V1-IR-73827-23-76-15-7S (V1), V2-IR-77271-42-5-4-36S (V2) V3-IR-75589-31-27-8-33S (V3) were crossed to produce  $F_1$ . All the  $F_1$ s plants had strong presence of anthocyanin on the base of their stems but segregated at  $F_2$  population. Thus, the objective of this study work was to evaluate and understand the mode of anthocyanin gene segregation at  $F_2$  population as a potential morphological marker. The  $F_2$  population was analyzed to test hypothesis that anthocyanin marker(s) is under simple Mendelian gene control. However, the results obtained indicated that  $F_2$  did not obey the Mendelian laws of gene segregation. This led to the conclusion that anthocyanin marker experience segregation distortion in  $F_2$  population.

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## Introduction

To produce more rice (*Oryza sativa* L.) to feed the growing population require methods other than the use of traditional high yielding pure-bred lines. The dwarf high yielding varieties have reached yield plateau (Kropff *et al.*, 1994; Yuan, 1994). To increase rice yields above this plateau researchers have adopted the technology duped “super hybrid” rice and “Green super rice” (IRRI, 2010). In rice growing countries like, China, India, Indonesia, Egypt and Vietnam among others, hybrids varieties have shown improved yield above the high yielding inbred varieties. *Hybrid vigour* has been reported to increase rice yield by between 20 to 30 % above the current yields of the dwarf lines (Kush, 1994; IRRI, 2010, Virmani, 1994). To produce hybrid rice, two genetically fixed varieties are cross pollinated to produce filial generation one (F<sub>1</sub>) or hybrid seeds. Plants from such seeds are special in that they express hybrid vigor (Yang *et al.*, 1996; IRRI, 2010). For hybrid seeds, production a completely male sterile female parent is needed that is pollinated with male parent.

Over the years manual removal of male gametes from the plants used as the female parents during cross breeding has been practiced. Unlike cross pollinating plants like maize that's male and female flower parts are separate, in rice (self pollinating crop) the two parts are in one flower making male emasculation difficult. However, with the discovery of cytoplasmic male sterility (cms) large scale production of hybrid rice was possible (Virmani and Sharma, 1992). The use of cms proved to be costly too, since it uses three (sterile, maintainer and restorer) lines, compared to environment sensitive genic **male** sterile (PGMS) that uses two-lines (Mao and Deng, 1993). Besides, the use of cms in hybrid rice seeds production is limited because the commonly used female parent, Wild Abortive (WA) type, (Dalmacio *et al.*, 1995), suffer from cytoplasmic incompatibility with certain male parents and also, for japonica and basmati varieties it has been difficult to get a good restorer line (Virmani and Sharma, 1992). Discovery of photoperiod sensitive male sterile rice (Shi, 1981; Shi

and Deng, 1986.) solved many of the problems associated with cms. In long day-light length and high temperature the PGMS is completely sterile and it is used as female parent in hybrid rice production (Shi, 1981, 1985, Ku *et al.*, 2001) but in short day-light length and low temperatures growth conditions it reverts to fertility, thus it maintains itself (Lopez and Virmani, 2000). Thermosensitive genic male sterile lines (TGMS) (Ali *et al.*, 1995, Virmani *et al.*, 2003), which is sterile in high temperature growth conditions and fertile in low temperature growth conditions (Reddy *et al.*, 2000) can also be used in two-line hybrid rice seed production system. PGMS and TGMS traits are under nucleus gene control (Zhang *et al.*, 1994; Wang *et al.*, 1995), unlike cms that is under cytoplasmic influence (Oka, 1974; Lin *et al.*, 1992). Since the fertility controlling gene is influence by environment they are referred to as Environmental genic male sterile (EGMS) lines. EMGS reduce the problem of genetic degradation because it can be used with many restorer lines (both indica and japonica) to produce hybrid seeds, unlike in cms where they are limited due to maternal line (or cms) and paternal line (restorer line) incompatibility, which lead to F<sub>1</sub> sterility (Lin *et al.*, 1992). PGMS (maternal) lines with diversified background can be produced unlike in cms whereby wild abortive (WA) is the major maternal line used (Virmani, 1996).

To use this technology in Kenya it has been necessary to test for adaptability of EGMS for their utility in hybrid rice seed production. Under long daylight length (prolonged to 14hours in greenhouse) and high temperature growth conditions EGMS have been found to be completely sterile a time when they can be used for crossing, and when grown outside the green house growth condition they are fertile and can maintain themselves (Kanya *et al.*, 2013). The challenge is that, sometime it is difficult to achieve complete sterility in the EGMS. This is experienced where the day-light length is not long enough or temperature is not high enough to reach the critical point required for EGMS gene(s) expressions (Yuan *et al.* 1993). If the sterility genes are not completely expressed, complete male sterility of maternal parent

line is not achieved and there is a correlating problem of contamination of hybrid seeds by self-bred seeds. Therefore, productions of hybrid seeds require method(s) of determining hybrid pure from self-bred seeds. Commonly used methods include conventional ones where plants mature and observation of agronomic traits is done. This is too late because plant will have grown to maturity before separating hybrid from inbred seedlings. Alternative methods include the use of DNA molecular and morphological markers. Use of DNA molecular markers has effectively been used to select for traits such as Bacterial Blight resistance, (Bert *et al.*, 2008; Bagali *et al.*, 2010; Shanti *et al.*, 2010). However, it requires a specialized laboratory and skilled manpower for effective use in breeding. Use of morphological markers like anthocyanin maybe a better method because it is visual and phenotypic. In this research, it was realized that all F<sub>1</sub> seedlings had strong presence of anthocyanin at the base of the stem and thus it can be utilized as a selection marker.

Anthocyanin is a heritable trait (Holton' and Cornish, 1995, Roger, 2000) and has been studied since the days when Mendel studied peas colour. In plants like strawberries red colour is under MYB10 gene control and it is used as a selectable marker (Kortstee *et al.*, 2011). Deliberate breeding of EGMS rice with anthocyanin can done for use as a marker in hybrid seedling selection. Studies in F<sub>2</sub> population revealed that segregation was distorted and non-Mendelian. Elsewhere, cross between intraspecific crosses of rice has revealed a number of incompatibilities that is associated with low F<sub>1</sub> seed rate. Segregation distortion has been observed in F<sub>2</sub> and doubled haploid populations of temperate *japonica* rice and its common in Indica –*japonica* crosses (Yamagishi *et al.*, 2010). Many other plants have shown segregation distortion among them are *Pupulus trichokarpa* which is controlled by a lethal gene *ith* (Bradshaw and Stettler, 1994); maize (Edwards *et al.*, 1987), Lettuce (Landry *et al.*, 1987), rice (McCouch *et al.*, 1988) and barley (Ganner *et al.*, 1991). Specific chromosomal regions are responsible for F<sub>2</sub> segregation distortion due to presence of gametophyte (*ga*) or sterility gene

(S) (Nakagahra 1972; Sano 1990; Xu *et al.* 1997). Production of hybrid basmati seeds is complicated because quality trait like aroma is under control of recessive gene(s) (Ahn *et al.*, 1992), which means that it is masked by dominant trait. It is therefore, necessary to breed basmati rice line with EGMS gene that will be used as a female parent in crosses involving Basmati370 or 217 in hybrid rice seed production. Breeding and analysis of crosses between Basmati and EGMS is therefore, essential so as to select advanced lines with both aroma and EGMS traits. In this process evaluation of anthocyanin realized at F<sub>1</sub> was considered. The objective of this study work was to evaluate mode of anthocyanin gene segregation at F<sub>2</sub> plants as possible morphological marker that can be used in breeding of hybrid seeds. It was hypothesized that anthocyanin is under a normal one or two Mendelian gene segregation. We now report that anthocyanin segregation at F<sub>2</sub> in crosses between each of Basmati 217 and 370 and V1PGMS, V2TGMS and V3PGMS did not tally either with 3:1 nor 9:3:3:1 Mendelian ratios, thus leading to deduction that there is a likelihood of gene segregation distortion.

## Materials and methods

### Materials

The female parental plant materials used for cross breeding were courteously supplied by International rice research institute (IRRI) under the supervision of Kenya Plant Health Inspectorate Service (KEPHIS). These included two PGMS (V1-IR-73827-23-76-15-7S and V3-IR-75589-31-27-8-33S, and one TGMS (V2-IR-77271-42-5-4-36S) varieties that are here code-named as V1PGMS, V3PGMS and V2TGMS respectively. The materials were sown and tested for adaptability at Kenya Agricultural Research Institute (KARI) Mwea-Kimbibi station, and at Mwea Irrigation Agriculture Development (MIAD) which are located in Kirinyaga district in Central province of Kenya on Latitude -0.7°S, and Longitude 37.37E. In this research work unless otherwise stated, long day refer to a 12hours day which was extended to 14hours by illuminating using solar radiation system, and short/normal day refer to normal 12hours of daylight.

On the other hand, high temperature and low temperatures were the green house temperatures, which were minimum of 35°C during the day and 19°C at night respectively. Paternal parents Basmati370 (B370) and Basmati217 (B217) were elite lines grown at KARI Mwea.

#### *Production of hybrid seeds*

All the three female parents (V1PGMS, V2TGMS and V3PGMS) were each crossed with Basmati370 and Basmati217. The female parent were first sown on 19<sup>th</sup> June 2011 in concrete troughs in green house at KARI with a spacing of a 5X15 cm under long day-light length and high temperature growth conditions so as to induce male sterility. All the plants sown in troughs were allowed to grow in greenhouse conditions until panicle morbid stage when they were exposed to solar lamp illumination at 6.00pm up to 8.00pm to ensure long day light-length growth conditions and to induce male sterility. At panicle emergence (heading) the plants were carefully transferred outside the greenhouse in a bucket where each of the three lines were crossed with Basmati370 and Basmati217 and left to grow under natural conditions till maturity. After ripening the F<sub>1</sub> seeds were harvested separately and each stored in a separate bag.

#### *Evaluation of anthocyanin as selective marker for hybrid against selfbred rice lines*

*Evaluation of F<sub>1</sub> plants for anthocyanin:* In January 2012 sowing was done for the F<sub>1</sub> and parental lines. The F<sub>1</sub> and parental seeds were sown at KARI Mwea in January 2012 at spacing of 15cmx15cm. At seedling stage it was discovered that all hybrids had strong presence of Anthocyanin which was missing from all parents apart from V2TGMS. The growing plants were individually monitored for presence of anthocyanin compared to the pure parental lines at three levels. All evaluation was done in green house at KARI Mwea- Kenya in pot (buckets) and in paddy field at MIAD- Mwea.

*Evaluation of F<sub>2</sub> and anthocyanin segregation:* In this phase the F<sub>2</sub> were sown and compared against controls that included parents and F<sub>1</sub> plants. All seeds

were pre-germinated and allowed to grow in nursery for 21 days before transplanting in the rice fields at MIAD-Mwea. Transplanting was done in 17 blocks. Block1-5 was sown with parental lines B370, B217, V1PGMS, V2TGMS and V3TGMS. The F<sub>1</sub>s (HV1B370, HV2B370, HV3B370, HV1B217, HV2B217, HV3B217) were sown in blocks 6-11 while the F<sub>2</sub>s segregating lines from each cross were sown in blocks 12-17. In this sowing phase number of seedlings per block for F<sub>1</sub> varied between 20plants to 50plants. However, 410plants for F<sub>2</sub> segregating seedlings were sown in 41 rows each with 10 plants. A spacing of 20x20cm inter-plant and inter-rows was maintained. Anthocyanin was evaluated by observation for presence of purplish/violet colouration at the base of the plant. Scoring was done by counting the number of segregants with purple stem base against others.

#### *Data analysis*

Emergence of anthocyanin must be due to gene interaction because non of the parents displayed strong noticeable presence of anthocyanin apart from V2TGMS. Since non of parents hand conspicuous anthocyanin other than V2TGMS, it was predicted that the anthocyanin was under interaction of at least two non-allelic gene, at least one from each parent. In this work interaction of two genes will be assumed. Data was recorded in Microsoft excel 2007 version. Segregation variance among the hybrid lines was analyzed using Z-test. The F<sub>2</sub> segregation tendencies were compared among the lines and with conventional Mendelian ratios of 9:3:3:1 for two non allelic and non-linked interacting genes. Since it was difficult to demarcate the four possible phenotypic appearance characteristic of Mendelian gene segregation in a 9:3:3:1 model, it was assumed that segregation model was epistatic with 9 anthocyanin against 7 others or  $9/7 = 1.285714$ .

## **Results**

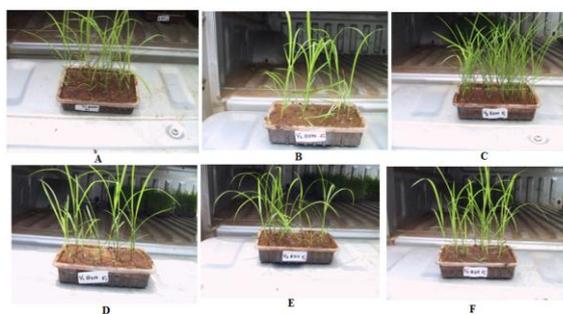
#### *Anthocyanin in F<sub>1</sub> seedlings*

A total of six hybrid lines were produced. These were HB370V1, HB370V2, HB370V3 which were obtained from cross pollination of V1PGMS, V2TGMS and V3PGMS to Basmati370 respectively. The other three,

HB217V1, HB217V2 and HB217V3 were products of V1PGMS x B217, V2TGMS x B217 and V3PGMS x B217 in that order. All the hybrid plants had strong presence of anthocyanin on their stem compared to parents that had no conspicuous anthocyanin, apart from V2TGMS (Fig.1). Strong presence of anthocyanin manifestation was observed in all mature F<sub>1</sub> plants such as presented in Fig.1A-F and in 21day old seedlings (Fig. 2A-F). However, there was no conspicuous presence of anthocyanin in the V1PGMS, V3PGMS, Basmati370 and Basmati217 for both mature parents (Fig 1.G-I) and seedlings (Fig. not included) Although the V2TGMS parents had some anthocyanin observed (Fig 1. J) it was lower than that of the hybrid plants. Besides in the V2TGMS parental materials anthocyanin was non-segregating compared to segregation observed at F<sub>2</sub>. In all the F<sub>1</sub> plants the level of anthocyanin present in the stem and the leaf sheaths were conspicuously observed compared to the control parental plants.



**Fig. 1.** Anthocyanin in F<sub>1</sub> and parent plants. Fig A-C represent cross between V1PGMS, V2TGMS and V3PGMS and Basmati370 while Fig. D-F represent cross between V1PGMS, V3PGMS and V2TGMS and Basmati217. Fig G represents Basmati370 and 217 while H-J represents V1PGMS, V2TGMS and V3PGMS in that order.



**Fig. 2.** Anthocyanin in F<sub>1</sub> hybrid seedlings. Fig A, B,C,D,E, and F represent cross between V1PGMS x370V1, V2TGMS xHB370V2, V3PGMSx HB370V3, HB217V1, HB217V2, V1PGMS x B217, V2TGMS x B217 and V3PGMS x B217 respectively.

#### *F<sub>2</sub> segregation: comparison of segregation ratios among hybrids*

Out of the 410 plants originally sown, each for the six hybrids, some did not survive and the tally was HV1B217 =362plants, HV2B217= 366plants, HV3B217=357 plants, HV1B370=349 plants, HV2B370=341 plants and HV3B370=313plants (Table 1). Variances among the hybrid was done by determining segregation ratios of plants with anthocyanin to non-anthocyanin plants. Among the six hybrids the ratios were highest for HV2B370 and HV2B217 plants with a z-value of 0.212489173 and 0.491663097, and a p-value of 0.1664 and 0.3256 respectively (Table 1). HV1B217 and HV1B217 with z-values of 0.053277025 and 0.040099789 that corresponds to p-values of 0.0398 and 0.032 respectively were the lowest. The anthocyanin to non-anthocyanin plants segregation ratios of HV3B370 were not significantly different from others when

tested at  $\alpha=0.05$ , however HV1B217 and HV2B370 and highest z-values.

*F<sub>2</sub> segregation: comparison of segregation ratios to expected 9:7 dihybrid ratio*

Under z-distribution, the area occupied by each of the hybrids lines were below average or z-value of zero(0)

apart from that of HV1217 and HV2370 which had 0.298678 and 0.818121 respectively. These had p-value of 0.2282 and 0.5878 respectively which was not significant when tested against the Mendelian ratios at  $\alpha= 0.05$  (Table 2).

**Table 1.** Segregation for anthocyanin among the F<sub>2</sub> plants. Comparison of anthocyanin segregation with an assumed 9:3:3:1 ratio. Average observation ratio =of all observed segregation ratios divide by n (n=7).

Line	Observed segregation ratios	Mean of observed ratios	STD $\delta$	z-values	P value
HV1B217	0.732057	0.962876	-0.23082	0.053277025	0.0398
HV2B217	1.423841	0.962876	0.460965	0.212489173	0.1664
HV3B217	0.645161	0.962876	-0.31771	0.10094237	0.0876
HV1B370	0.762626	0.962876	-0.20025	0.040099789	0.032
HV2B370	1.664063	0.962876	0.701187	0.491663097	0.3256
HV3B370	0.549505	0.962876	-0.41337	0.170875277	0.135

**Table 2.** Test of F<sub>2</sub> segregating population against a Mendelian Dihybrid ratios of 9:3:3:1. Expected ratios are the assumed epistatic (9:7) ratios.

Line	Anthocyanin to non-anthocyanin segregation ratios	Expected 9:7 segregation	Mendelian	Mean	Z values	P-value
V1xB217	0.732057	1.285714		0.46246	-1.1972	0.383
V2xB217	1.423841	1.285714		0.46246	0.298678	0.2282
V3XB217	0.645161	1.285714		0.46246	-1.3851	0.4162
V1xB370	0.762626	1.285714		0.46246	-1.1311	0.3708
V2xB370	1.664063	1.285714		0.46246	0.818121	0.5878
V3XB370	0.549505	1.285714		0.46246	-1.59194	0.4441

## Discussion

Breeding for hybrid seeds require a method of selecting hybrid seeds from pure breed seeds. Marker assisted selection (MAS), involving DNA molecular and morphological markers, are some of the available methods. DNA Marker assisted selection require identification of DNA sequence that associate with target phenotype in the desired line (McCouch, *et al.*, 1988) which is laborious. Since morphological markers are phenotypic, they can simplify the MAS. In this study, anthocyanin pigmentation was found to dominate the hybrid seedlings (Fig.1 A-F) more than

the in pure parents (Fig.1-J). Among the parents, anthocyanin was only noticeable among the V2TGMS plants and not segregating. At F<sub>1</sub> anthocyanin was present in all plants to compared segregating F<sub>2</sub> population in which some plants had no noticeable anthocyanin on base of stem. Therefore, anthocyanin can be used as morphological marker to select F<sub>1</sub> seedlings (Fig 1. A-F) especially for crosses involving V1PGMS and V3PGMS. Elsewhere anthocyanin has been reported to be a promising marker in selection of recombinant from non-recombinant plants (De Jong, 2004; Kortstee, 2011). For instance, Strawberry

plants transformed with the MYB10 gene show anthocyanin accumulation in leaves and roots as opposed to non-transformants (Kortstee, 2011). Thus, markers aided selection is becoming important in the field traditionally dominated by conventional phenotypic breeding. Presence of anthocyanin in all the F<sub>1</sub> plants indicated gene dominance. Since, indications were that the two parents contributed to the anthocyanin, a two gene interaction was predicted. However, segregation in F<sub>2</sub> generation behaviour did not tally with the hypothesized 9:3:3:1 nor the 9:7 epistatic ratios. This is an indication that F<sub>2</sub> segregation could have suffered a distortion. Gametic selection has been reported to cause segregation distortion in F<sub>2</sub> progenies and even in anther culture derivatives (i.e., nonmorphogenetic microspore derived calli (NMC) (Guideroni,1989). This segregation distortion is associated with semi-fertility in F<sub>1</sub> hybrids (Ikehashi and Araki, 1984). Cases like this brings about a breeding alert because it may reduce the level of yields expected from hybrids compared to purebred varieties. Evaluation of F<sub>1</sub> obtained from crosses between Basmati and EGMS revealed that F<sub>1</sub> had low seed set rate than parents (Kanya *et al.*, 2013). Low seed set rate observed in F<sub>1</sub> could be a point of non-Mendelian F<sub>2</sub> anthocyanin segregation. However, at F<sub>1</sub> anthocyanin is stable and uniformly expressed. Since anthocyanin is under genetic control (Holton and Cornish, 1995) such as flavonoid biosynthetic gene CHS gene in parsley (Kreuzaler *et al.*, 1983), if the gene can be passed to other varieties it can be used as a good breeding tool to supplement conventional breeding which is slow to select for a target trait (Xu and Crouch, 2008). Effects of low seed set rate at F<sub>1</sub> (incompatibility or distortion) can be reduced by introgressing wide compatibility gene S<sup>5</sup><sup>n</sup> (Ikehashi and Araki, 1984) into cultivars of interests to reduce F<sub>1</sub> spikelet sterility. In situation where a target trait con-segregate with a DNA or a molecular marker then breeding time for such a trait can be drastically reduced. However segregation distortion like the one experienced in this research may slow down the speed of releasing new stable lines for use in breeding. Whichever the case MAS speedup breeding like in the

case of pyramiding four Bacterial Blight resistance genes *Xa4*, *xa5*, *xa13* and *Xa21* into rice lines selection (Shanti *et al.*, 2010). Where else DNA primers/probe markers are expensive and require expertise, morphological markers are simpler to use because they are phenotype (Xu and Crouch 2008). In this research, anthocyanin markers were found to be associated with F<sub>1</sub>s hybrid rice seedlings (Fig. 1). It is only in V2TGMS where anthocyanin was present in both the parental and in F<sub>1</sub> plants. However, anthocyanin in parental plants was lighter compared to F<sub>1</sub> hybrids and did not segregate at F<sub>2</sub>. Since anthocyanin is observable at seedling stage, then it can enable sorting out hybrid from parental seedling as early as 21day seedling stage (Fig. 2).

**Conclusion:** Anthocyanin can be used a morphological marker to select F<sub>1</sub> from pure breed seedling. Segregation of anthocyanin at F<sub>2</sub> between Basmati370 and 217, and V1PGMS, V2TGMS and V3PGMS experience segregation distortion and it is non-Mendelian. This may be an indicator of F<sub>1</sub> spikelet sterility.

### **Recommendation**

Introgression of wide compatibility gene S<sup>5</sup><sup>n</sup> in Basmati rice may be needed for its effectively used in hybrid seed technology.

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