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Introgressing photoperiod/thermo-sensitive genic male sterile gene into Basmati 370 rice

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fgr gene

Gene introgression

Pollen sterility

P/TGMS rice lines

ABSTRACT

The emasculation of male gametes in pollen-recipient parents among self-pollinated crops (rice) is key to producing quality hybrid rice seeds. One of the emasculation tools in rice breeding is the photoperiod-thermo sensitive genic male sterility (P/TGMS) method, which ultimately requires long daylight length and high-temperature growth conditions to induce male gametes sterility. Using the P/TGMS method to produce hybrid *Basmati* rice seeds has been slow because no commercial line has been developed. Crossing the *Basmati* rice line with a non-aromatic rice line produces F_1 with non-*basmati* quality traits. This study aimed to introgress the *p/tgms12-1* gene into *Basmati* 370 by treating P/TGMS lines (IR-7327-2376-157S and IR-75589-31-27833S) with daytime temperatures (>33ºC) under a polythene greenhouse to emasculate pollen and cross-pollinating them with *Basmati* 370. Marker-assisted backcrossing was used to develop the BC_1F_2 *Basmati* breeding lines evaluated for pollen sterility and agro-morphological traits. Pollen sterility was tested by staining with 1% iodine potassium-iodide solution (I_2KI), in which fertile and sterile pollen grains were stained with blue-black and yellow-pink dyes, respectively. The acquisition of near-complete pollen sterility among female parents is a manifestation of the greenhouse temperatures effectively emasculating pollen in P/TGMS parents and BC_1F_2. Analysis of variance on agro-morphological data showed significantly better agro-morphological traits in BC_1F_2 than the parents and significantly higher pollen sterility in P/TGMS lines than *Basmati* 370 ($P \leq 0.05$). The presence of the *fgr* gene in BC_1F_2 lines was confirmed using SSR markers, and the hybrids had both homozygous aromatic and heterozygous non-aromatic traits, the successful development of BC_1F_2 with *p/tgms12-1* and *fgr* genes. The results obtained from this study are a major milestone towards improving *Basmati* rice yields in Kenya using hybrid seeds.

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1 Introduction

Rice (*Oryza sativa* L.) is the world's second most-grown cereal crop after maize and is a staple food for more than half of the world population (Sreedhar and Reddy 2019). It is a strategic food crop in many African countries, where its demand has steadily increased in the past three decades in urban and rural populations (Uyeh et al. 2021). Most global rice is grown and consumed in Asia, constituting more than half of the world's population (Cordero-Lara 2020). Rice was first introduced by European settlers in the coastal region of Kenya from Asia as a cultivated crop in 1907 (Atera et al. 2018). In Kenya, consumer demand for rice has grown over the years and is estimated at 983,000 Mt, exceeding national rice production (181,000 Mt) (Yılmaz and Njora 2021). Kenya's main rice growing areas include Mwea, West Kano, Bunyala and Ahero irrigation schemes, with an average yield of 4.0 t/ha. Mwea Irrigation Scheme is the main rice growing area, accounting for 87% of national rice production (Atera et al. 2018). Potential 9 t/ha rice yields have been reported in Kenya (Atera et al. 2018), highlighting large rice yield gaps.

Basmati rice (locally known as *Pishori)* has good cooking and palatability qualities (Denis et al. 2022). Thus, Kenyan growers and consumers prefer the variety. One of its signature qualities is its characteristic aroma, which increases its demand and, thus, fetches high market prices. In Kenya, *Basmati* 217 and 370 are the key aromatic rice varieties grown, with *Basmati* 370 accounting for over 98% of the total rice grown by farmers at the Mwea Irrigation Scheme (Denis 2020). The main challenge with the commonly grown *Basmati* variety is low yield (about 4.0 t/ha) compared with other high-yielding local rice varieties such as *Komboka*, which yields about 7.0 t/ha (Ng'endo et al. 2022). Consumers' higher demand for Basmati rice is among the factors that have kept prices higher than expected in the Kenya market.

Hybrid rice technology exploits heterosis by pushing the breeding plateau above that of the inbred lines by over 25% (Prasanna et al. 2024). Efforts have been made to improve *Basmati* 370 yield by developing hybrid cultivars, but no significant yield increase has been realized (Akhter and Haider 2020). In hybrid rice technology, a female parent with male gametes completely emasculated is necessary to avoid self-pollination. Two major approaches used in hybrid rice production are cytoplasmic male sterility (CMS) and environment-sensitive genic male sterility (EGMS). In CMS, three lines (CMS, maintainer and restorer lines) are involved (Chang et al. 2016), while EGMS involves two lines, including the maternal parent (EGMS) and the paternal parent with fertile male gametes (restorer). Since rice is predominantly self-pollinated, male sterility systems (MSS) are the most effective methods in hybrid development (Ahmed et al. 2020).

The EGMS involves photoperiod-sensitive genic male sterility (PGMS), first discovered in the *Nongken* 58S rice variety (Zheng et al. 2024) and thermo-sensitive genic male sterility (TGMS) that was discovered in the *Annong* S-1 rice variety (Ali et al. 2021). Collectively referred to as EGMS, the PGMS and TGMS rice lines have sterility-inducing genes in male gametes under long-day light and high-temperature growth conditions. The gametes are, however, viable at the average daylight length of 12 hours and temperatures ranging between 19 and 30°C (Nthakanio and Kariuki 2019). The EGMS system is controlled by recessive nuclear genes, influenced by temperature in TGMS and photoperiod length in PGMS (Amist and Singh 2020). Simple sequence repeats (SSR) markers have been developed and utilized to select p/tgms12-1 genes of chromosome 12 in the rice genome (Zhou et al. 2012). The use of EGMS rice lines in hybrid rice seed production involves two lines, making it cheaper than the CMS method, which uses three lines. Besides, in the EGMS system, the adverse effects of sterility-inducing cytoplasm are not encountered (Abebrese et al. 2018).

Hybrid rice seed technology has improved rice yield by 25-30% above the pure lines (Tongmark et al. 2021). Despite all the achievements, the use of technology is limited in *Basmati* rice because the quality traits such as aroma are under recessive gene control and are thus masked when crossed with non-aromatic rice varieties (Chukwu et al. 2019). Currently, there is a lack of a market-ready *Basmati* breeding line with male sterile genes possessing consumer-preferred quality traits, thus limiting the use of the EGMS method in *Basmati* rice hybrid seeds production. This study aimed to introduce the *p/tgms12-1* gene into *Basmati* 370 to produce breeding lines with consumer-preferred traits for crossing with ordinary *Basmati* 370 in a hybrid rice seed breeding program.

2 Materials and Methods

2.1 Study site

The following research was conducted at the Kenya Agricultural and Livestock Research Organization (KALRO), Mwea, in Kirinyaga County, Kenya. It is within Kenya's main rice growing area, where most Basmati rice is grown (Atera et al. 2018). The site coordinates are Latitude 00º 37'S and Longitude 37º 20'E and receives about 850 mm of rainfall annually. Mwea is approximately 1159 meters above sea level with a temperature range of 15.6°C to 28.6°C and a daily mean temperature of 22°C.

2.2 Plant materials and seeding

The plant materials used for the experiment were two P/TGMS lines (IR-73827-23-76-15-7S and IR-75589-31-27-8-33S) and *Basmati* 370, designated as V1 and V3, respectively. *Basmati* 370 certified seeds were provided by the Mwea Irrigation Agricultural

Development Centre (MIAD), while the Hybrid Rice Project provided the two P/TGMS lines at the University of Embu, Kenya. The seeds were soaked separately in germinating bags and treated by submerging them in 3% hydrogen peroxide (H_2O_2) for 24 hours to break seed dormancy and then incubated for 72 hours at 35°C in an incubator for post-nursery germination. Upon germination, the seedlings were sown in a containerized nursery filled with soil for 21 days in a greenhouse at KALRO, Mwea.

2.3 Evaluation of pollen sterility among rice parents

2.3.1 Experimental design

Two seedlings were transplanted into 10-litre polythene bags filled with farm soil at a spacing of 15 cm between plants. The experimental block comprised 10 bags with two seedlings (totalling 20) of each variety (V1, V3, and *Basmati* 370). The seedlings were placed inside a polyethylene greenhouse, and 10 bags of each variety were placed outside the greenhouse in a completely randomized design (CRD). All agronomic field practices were carried out as per the required standards practice for rice management. At the primordial growth stage (30 days to heading), the greenhouse temperature was maintained above 22ºC at night and 33ºC during the day. The polythene greenhouse was used to effectively realize this temperature because it is affordable for small-scale farmers at the Mwea Irrigation scheme. The greenhouse temperature was first calibrated by lowering the side walls to retain temperature and raising the side walls during the daytime to avoid overheating (Nthakanio and Kariuki 2019). The temperature treatment was maintained to induce male gamete sterility in female parents until the plants attained 50% heading when the treatment was withdrawn. Temperature readings were taken hourly using a maximum-minimum digital thermometer (ISOLAB® Laborgeräte GmbH 059.03.002). Greenhouse temperatures were routinely monitored to retain it below 38°C (to avoid physiological damage) by gradually opening up the greenhouse side walls cover between 9.30 am and 3.00 pm to allow air circulation and avoid overheating and thereafter lowering the walls cover to conserve the temperature till the next day (Nthakanio and Kariuki 2019). This was repeated in the 10 days of the critical fertility point (CSP).

2.3.2 Test for pollen sterility

Pollen sterility was determined using the iodine potassium iodide (I_2KI) staining method following the procedure of Chen et al. (2011). The average temperature in the greenhouse was a mean of 35.1°C. During the first 10 days of heading, 5 plants per line were sampled inside and outside the greenhouse every two days. Three undehised spikelets from the panicle top, middle and base were randomly picked for pollen sterility testing. The samples were preserved in 70% ethanol inside an Eppendorf tube and transported to the University of Embu Research Lab for testing and microscopic observation. Three anthers from each spikelet were stained with a drop of 1% I_2KI onto a glass slide. After staining, the anthers were macerated with forceps to release the pollen and other residues were removed. Pollen observation was done under ×10 objective of the ordinary compound light microscope and categorized into yellow/pink and dark blue stained pollen. The yellow/pink pollen grains were considered sterile, while the dark blue stained pollen was considered fertile as per Chen et al. (2011) procedure. Pollen sterility was expressed as a percentage (Hamad et al. 2022) as shown in the equation below;

$$\%\text{Pollen sterility} = \frac{\text{Total number of sterile pollen}}{\text{Total number of pollen grains}} \text{ X } 100$$

2.4 Introgression of P/TGMS in *Basmati* 370 rice line

2.4.1 Development of F_1, BC_1F_1 and BC_1F_2

The F_1 populations were developed by crossing V1 × *Basmati* 370 and V3 × *Basmati* 370 plant lines, as shown in Figure 1. Pollen from the male *Basmati* 370 that was planted outside the greenhouse was dusted on the female parent glumes of V1 and V3 plants that were treated with the sterility-inducing temperature inside the greenhouse, and thereafter, the panicles were bagged to avoid undesired cross-pollination and sterility-inducing temperature withdrawn. Successful hybrids were confirmed using anthocyanin pigmentation on the base of the stem of F_1 hybrid seedlings. The F_1 population data collected included plant height, panicle length, total number of tillers, productive tillers, days to heading, days to flowering, days to maturity, total number of glumes, and total filled and unfilled glumes of hybrid plants.

To recover the aroma trait, F_1 plants grown outside the greenhouse and manually male-gamete emasculated were backcrossed with *Basmati* 370 as the recurrent parent to obtain BC_1F_1. After that, the BC_1F_1 were grown in a soil-filled concrete trough measuring 16m X 3m in the greenhouse with optimum physiological growth conditions for effective self-pollination to produce BC_1F_2. The obtained BC_1F_2 population was treated with the greenhouse sterility-inducing temperature to segregate the EGMS and non-EGMS lines. Selection of lines with the *fgr* gene was made using simple sequence repeats (SSR) markers: External Sense Primer (ESP), Eternal Antisense Primer (EAP), Internal Fragrant Antisense Primer (IFAP) and Internal Non-Fragrant Sense Primer (INSP) while SSR marker RM12521 was used to select for *p/tgms12-1* gene.

2.4.2 Molecular Analysis

DNA extraction from 21-day-old leaves of parents and BC_1F_2 plants was done following the procedure of Mahuku (2004). Molecular markers (Table 1) were used to select the lines with aroma gene (*fgr)* and p/tgmsgene(*p/tgms12-1*).

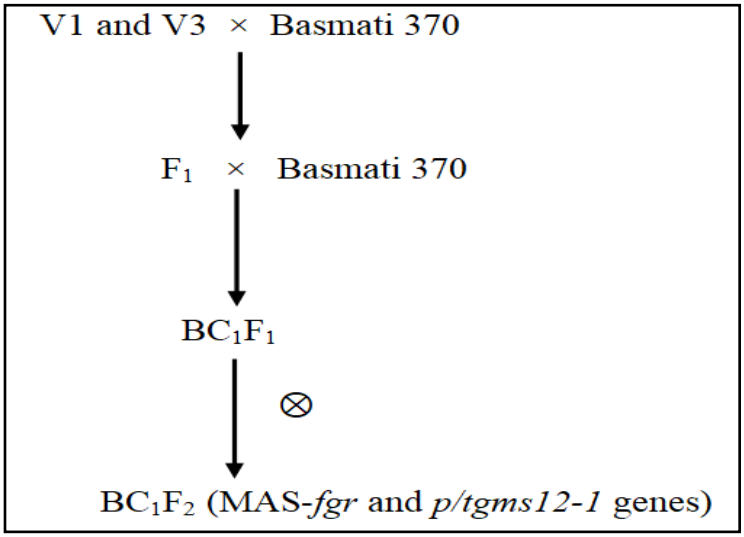

Figure 1 Marker-assisted backcross breeding scheme to introgress *p/tgms12-1* gene into Basmati 370 rice. V1 and V3 are used as female parents, while Basmati 370 is used as a recurrent parent

Table 1 Molecular markers used to screen for *fgr* and *p/tgms12-1* genes

SSR marker	Primer sequences	Tagged gene	References
ESP	TTGTTTGGAGCTTGCTGATG	*fgr*	Bradbury et al. 2005
EAP	AGTGCTTTACAAAGTCCCGC		
IFAP	CATAGGAGCAGCTGAAATATATAC		
INSP	CTGGTAAAAAGATTATGGCTCA		
RM12521	F:5'=CCCTTATCTGCTAGCCTCACACC-3'	*p/tgms12-1*	Zhou et al. 2012
	R:3'=CCACCGGATAATCCTTTAAGTGC-5'		

Note- F is forward primer, and R is reverse primer.

A polymerase chain reaction (PCR) (9800 Fast Thermal Cycler®) was used to amplify the extracted DNA. The PCR reaction mixture of 20 µl was prepared in 0.2 ml Biologix® strip tubes composed of 1.5 µl-50 ng/µl of rice genomic DNA, 1 µl-10 µM of forward and reverse primers, 4 µl Accuris ™ *Taq*-buffer, 12 µl molecular grade water, 1 µl-5 mM dNTPS and 0.2 µlof 5 units Accuris ™ *Taq* polymerase. The PCR amplification profile of the aroma markers (ESP, EAP, IFAP and INSP) was initial denaturation at 95℃ for 2 minutes, followed by 30 cycles at 95 ℃ for 30 seconds, 58 ℃ annealing for 30 seconds, 72℃ elongation for 30 seconds and a final extension at 72 ℃ for 5 minutes, and storage at 4℃. While for the RM12521 primers, initial denaturation occurs at 94 ℃ for 2 minutes, followed by 30 cycles of 94℃ for 30 seconds, 55℃ annealing for 30 seconds, 72 ℃ elongation for 1 minute, a final extension at 72 ℃ for 5 minutes, and storage at 4 ℃. After amplification, the PCR products were resolved in 1.2 % agarose gel electrophoresis, pre-stained with 10 µl-10,000× SafeView™ Classic staining dye and ran in 1× sodium borate buffer at a voltage of 70 V and current of 250mA for 1 hour. Accuris™ Smart Check™ DNA (0.1mg/ml) ladder (3µl) was used to estimate the amplicon sizes of the PCR product. The resolved DNA fragments were observed under a UVP® Benchtop Variable Transilluminator, and the images were captured using a Canon® camera. The individuals were selected based on the expected band sizes.

2.5 Evaluation of agro-morphological traits of BC_1F_2 population

2.5.1 Experimental design

Seeds of BC_1F_2 generation and the parents were prepared by soaking in hydrogen peroxide as described earlier and were sown in a containerized nursery filled with soil for 21 days. Four seedlings of each hybrid and parent line were row-transplanted into three soil-filled concrete troughs measuring 16m X 3m with three replications in a completely randomized block design in the greenhouse at a spacing of 15 cm between plants and 20 cm between rows. All the required agronomic practices were implemented, including watering, weeding and fertilization. High-

temperature treatment at the rice primordial growth stage to induce pollen sterility was also tested on the BC_1F_2 population to validate the successful introgression of the *p/gms12-1* gene. The average daytime temperatures in the greenhouse within the pollen sterility-inducing sensitive phase of the plant growth cycle were effectively sustained at 36.4℃.

2.5.2 Data collection for Agro-morphological traits

Data on the plant height, total number of tillers, and productive tillers was collected at physiological maturity. Evaluation for pollen sterility was conducted, as explained earlier.

2.6 Data Analysis

Data on pollen viability of the parents, plant height, total number of tillers, number of productive tillers, sterile pollen, number of sterile spikelets, days to heading, days to flowering, days to maturity and 1000-grain weight was subjected to analysis of variance (ANOVA) using SAS 9.4 computer software. Tukey's student's test at $P \leq 0.05$ was used for mean separation for all traits, and Pearson's correlation of coefficient analysis of the parameters was implemented.

3 Results

3.1 Effectiveness of greenhouse temperatures on inducing pollen sterility in BC_1F_2 and parental lines

Differences in pollen sterility were noted when the stained pollen was observed under 10× magnification under a light microscope. The pollen grains from the greenhouse stained yellow, while those obtained from outside the greenhouse stained dark blue (Figure 2). Plants grown outside the greenhouse conditions had fertile spikelets (Figure 2D), while spikelets of BC_1F_2 -EGMS under greenhouse growth conditions were completely sterile (Figure 2H).

The analysis of variance results showed significant differences at $p \leq 0.05$ in pollen sterility between the plants grown inside the greenhouse conditions and those grown outside the greenhouse conditions (Table 2). Under the GH conditions, the average percentage of pollen sterility for ten days of pollen collection was 97% (V3), 96% (V1) and 85% (*Basmati* 370). The lines carrying the *p/tgms12-1* gene showed higher sterility in the GH than *Basmat*i 370 despite being grown under similar conditions. The plants that were grown outside the greenhouse showed low pollen sterility of 3.6% (*Basmati* 370), followed by 5.2% (V1) and 6.2%

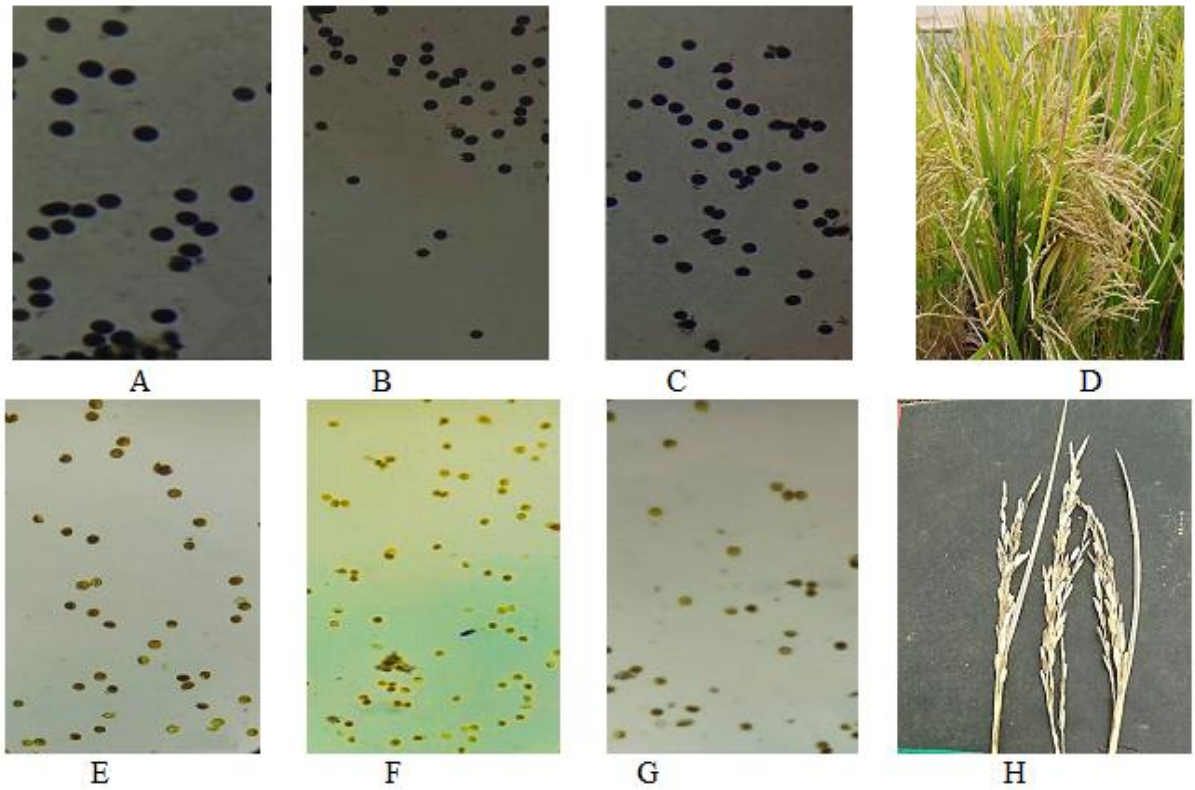

Figure 2 Effects of high temperature on BC_1F_2 pollen and spikelet sterility, figures A, B, and C show dark blue pollen from plants grown outside the greenhouse conditions, while figures E, F and G show yellow-stained pollen from plants grown inside the greenhouse conditions, figure D shows fertile spikelets from plants grown outside the greenhouse while figure H shows sterile spikelets from plants grown inside the greenhouse.

Table 2 Mean pollen sterility percentages of three rice varieties grown under greenhouse conditions (GH) and outside greenhouse (OGH)

Line	GH	OGH
V1	95.6±6.08[a]	5.2±2.04[ab]
V3	96.7±4.16[a]	6.2±1.75[b]
Basmati 370	85.2±3.16[b]	3.6±0.97[a]
Mean	92.5[a]	5.0[b]
CV%	5.0	33.0
P value	<.0001	0.0056

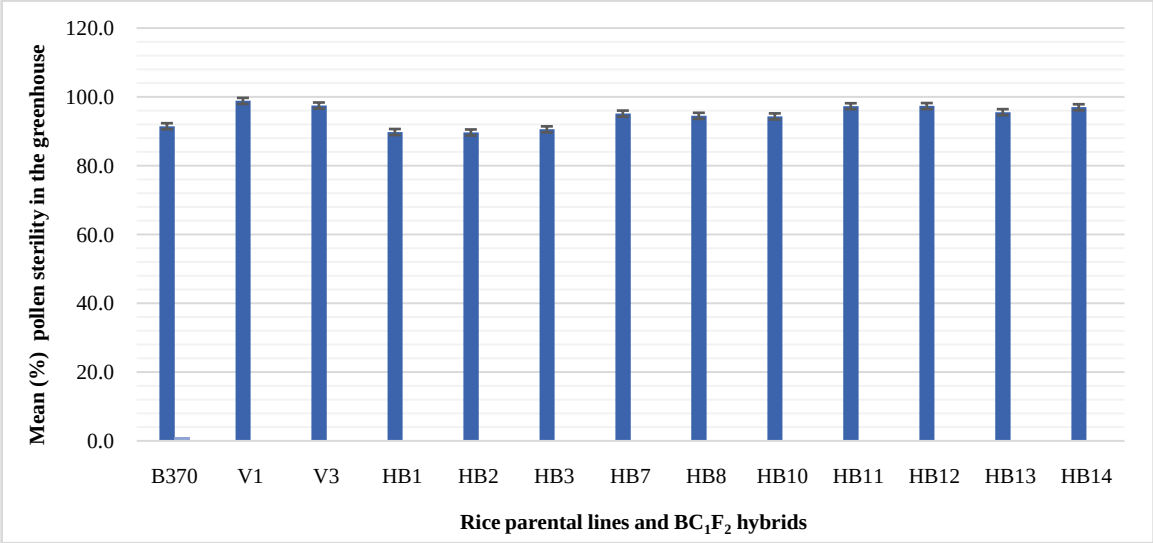

Figure 3 The BC_1F_2 lines sterility levels, SPGH - Sterile pollen in the greenhouse, HB1-HB8- V3 × Basmati 370, HB10 - HB14 =V1 × Basmati 370, B370= Basmati 370, VI and V3 - EGMS.

Table 3 Selection of BC_1F_2 EGMS plants

Crosses	Seeds obtained	F_1 plants selected	BC_1F_1 seeds obtained	Plants selected	BC_1F_2 plants selected using SSR markers
V1×*Basmati* 370	232	96	72	23	5
V3×*Basmati* 370	468	173	66	30	5
Total	700	269	138	53	10

(V3) (Table 2). Although the environment by variety interaction was significant ($p \leq 0.05$), the ranking of the varieties based on pollen sterility did not change. The mean average temperature for the season outside the GH was 23.8°C, which is conducive for pollen fertility (Table 2).

V1 and V3 had the highest percentage of pollen sterility (98%) compared to the BC1F2 hybrids, but there was no significant difference between the parents and the hybrids (Figure 3).

3.2 Introgression of *p/tgms12-1* gene into *Basmati* 370

Successful hybridization between V1× *Basmati* 370 and V3× *Basmati* 370 produced F_1 hybrids that were distinguished from the parents by the presence of anthocyanin pigmentation at the base of the stem and purple tips of the glume (Figure 4). Out of 700 F_1 seeds generated from V1× *Basmati* 370 and V3× *Basmati* 370, 269 plants were selected using anthocyanin pigmentation. These F_1 materials were backcrossed to form a population of 138 BC_1F_1 seeds, which were then selfed and selected to obtain 53 BC_1F_2 progenies (Table 3).

3.3 Agro-morphological analysis of the parents and F_1 hybrids grown outside the greenhouse

Analysis of variance carried out on the agro-morphological traits showed a significant difference ($p < 0.05$) concerning plant height, total tillers, days to heading, days to flowering and days to maturity.

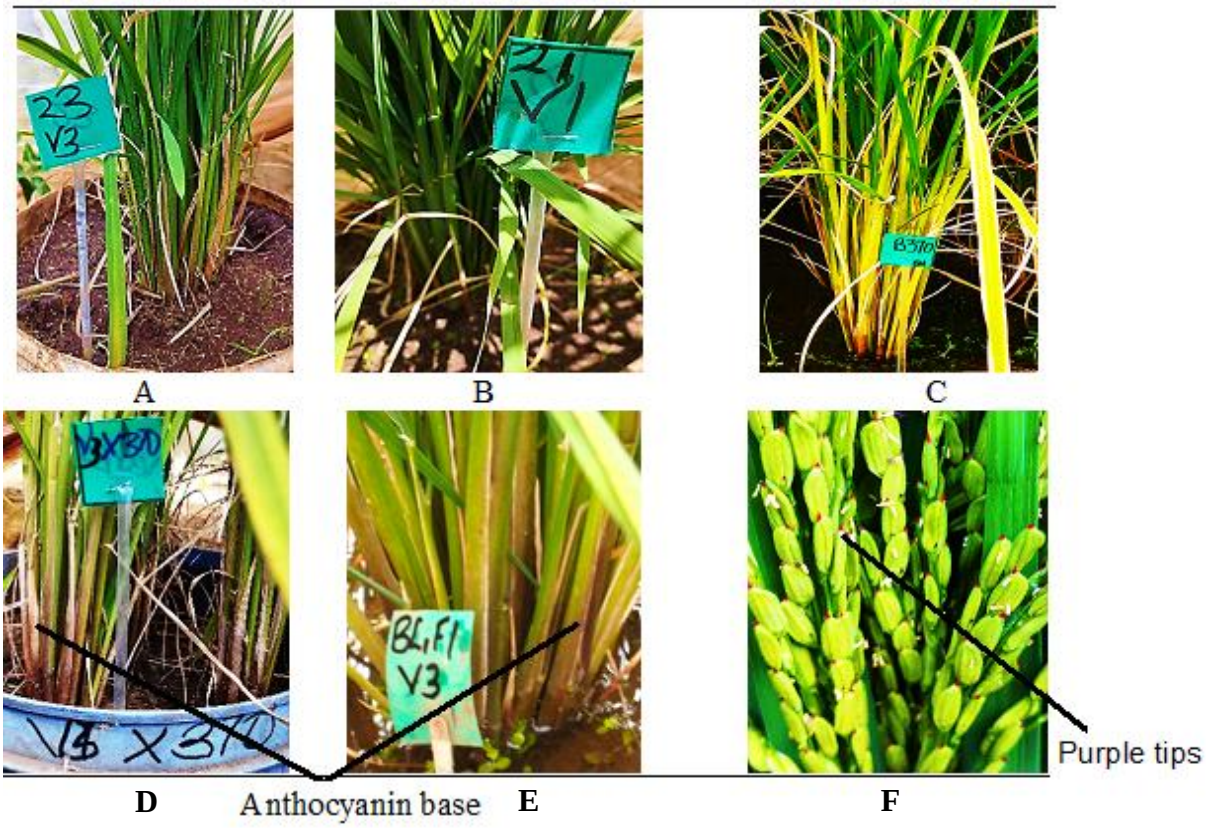

Figure 4 Use of anthocyanin pigmentation as a morphological maker in the selection process of *Basmati* 370 cross breeds, figure 4A, B and C shows rice parents V1, V3 and *Basmati* 370 respectfully, figure 4D shows F_1 hybrid (V3 × *Basmati* 370), figure 4E shows BC_1F_1 and 4F shows purple tips observed F_1 hybrids.

Table 4 Means of agronomic traits of rice parents and F_1 hybrids grown outside the greenhouse

Line	Plant height (cm)	Total number of tillers	Productive tillers	Sterile spikelets	50% Days to heading	50% Days to flowering	50% Days to maturity	1000-grain weight(g)
V1 × *Basmati* 370	112.13±8.02^{b}	20.06±1.20^{b}	16.0±1.70^{b}	89.19±13.75^{a}	98.50±0.65^{b}	100.25±0.63^{bc}	129.25±0.48^{b}	20.5±1.041^{a}
V3 × *Basmati* 370	104.44±2.14^{c}	19.25±1.11^{b}	15.94±0.83^{b}	61.75±7.87^{b}	91.50±6.54^{b}	93.75±6.59^{c}	129.25±1.25^{b}	22.75±0.95^{a}
Basmati 370	132.44±3.85^{a}	29.88±2.08^{a}	26.44±1.92^{a}	11.06±1.70^{e}	105.5±2.72^{b}	108.25±2.96^{b}	130.25±0.63^{b}	19.75±0.25^{a}
V1	85.063±0.78^{d}	20.75±1.92^{b}	17.94±2.04^{b}	47.19±11.58^{c}	123.75±0.48^{a}	126.0±0.71^{a}	139.5±0.65^{a}	21.0±1.23^{a}
V3	84.0±5.19^{d}	21.50±0.76^{b}	19.13±13.64^{b}	31.94±1.85^{d}	128.75±0.85^{a}	130.75±0.85^{a}	141.75±0.25^{a}	19.5±1.19^{a}
Mean	103.61	22.29	19.09	48.23	109.60	111.80	134.00	20.70
Value	<.0001	0.0010	0.0014	0.0002	<.0001	<.0001	<.0001	0.2061

Means with different superscript letters within a column are significantly different ($p \leq 0.05$) according to Tukey's test, the lines V1, V3 and Basmati 370 are rice parent lines.

There was no significant difference with respect to productive tillers and 1000-grain weight. The number of spikelet sterility ranged from 11.06 for *Basmati* 370 to 89.19 for V1 × *Basmati* 370. Among the hybrids, V1 × *Basmati* 370 had a higher panicle sterility rate than V3 × *Basmati* 370. Spikelet sterility percentages were 11.06, 47.19 and 31.94 percent for *Basmati* 370, V1 and V3, respectively. Hybrids had significantly higher spikelet sterility than the parents. Spikelet sterility of the V1 × *Basmati* 370 hybrid was significantly higher than the parental controls. Although V3 × *Basmati* 370 had higher spikelet sterility than the parent, it was only significant to *Basmati* 370, while this was not significant to V1 and V3 (Table 4).

3.4 Correlation coefficient analyses of parent and F_1 hybrids grown outside the greenhouse

A significant and negative correlation was observed between days to heading with plant height (r= 0.575**), days to flowering (r= -0.558**) at P<0.05 and days to maturity (r= -0.724*). Sterile spikelets negatively correlated with total tillers (r= -0.439***) and days to flowering (r=-0.442***). There was a positive correlation between heading days with flowering days (r=0.999***) and days to maturity (r= 0.841***). Days to flowering was positively correlated with days to maturity (r= 0.834***) and negatively with sterile spikelets (r= -0.442***), as shown in Table 5.

3.5 Marker-assisted selection for BC_1F_2 with *fgr* genes

Five BC1F2 lines developed had three bands similar to *Basmati* 370 parent when amplified using 257 bps, 355 bps and 580 bps markers, which identifies them as heterozygous non-aromatic. In contrast, the remaining three hybrids had positive bands for 257 bps and 355 bps markers, associated with heterozygous non-aromatic traits. Two hybrids had one band when amplified with 257 bps marker, a show of homozygous aromatic trait, while V1 and V3 had one band each associated with 355 bps marker, indicating that it is homozygous non-aromatic (Figure 5).

3.6 Selection BC_1F_2 with P/TGMS

The RM12521 marker is a recessive marker linked to a *p/tgms12-1* gene fragment of size 375 and amplified as a monomorphic band. Among 10 BC_1F_2, five had a clear banding pattern similar to V1 and V3, while five hybrids had faint bands inclining toward the *Basmati* 370 banding pattern (Figure 6).

3.7 Agro-morphological performance of the parents and BC_1F_2 lines under high-temperature treatment

Analysis of variance carried out on the agro-morphological traits showed a significant difference (p<0.001) with respect to plant height, total tillers and total number of effective tillers. There were significant differences (p<.001) with respect to the total number of tillers, productive tillers and plant height. The plant height ranged from 55.03 cm for V1 to 102.47 cm for HB14 (V3 × *Basmati* 370). Most of the hybrids were significantly shorter than *Basmati* 370;

Table 5 Correlation coefficients (r) of agronomic traits of parents and F_1 plants

PH	PH							
TT	0.531	TT						
PT	0.123	0.395	PT					
HD	-0.575**	0.072	0.340	HD				
FD	-0.558**	0.073	0.337	0.999***	FD			
MD	-0.724*	-0.183	0.224	0.841***	0.834***	MD		
SS	-0.012	-0.439***	-0.271	-0.427	-0.442***	-0.265	SS	
GW	-0.060	-0.299	-0.265	-0.361	-0.360	-0.197	0.255	GW

Significance codes are *, **and *** representing p<0.05, 0.01 and 0.001 respectively, PH - plant height, TT - total tillers, PT - productive tillers, HD - days to heading, FD - days to flowering, MD - days to maturity, SS - sterile spikelets and GW - 1000 grain weight.

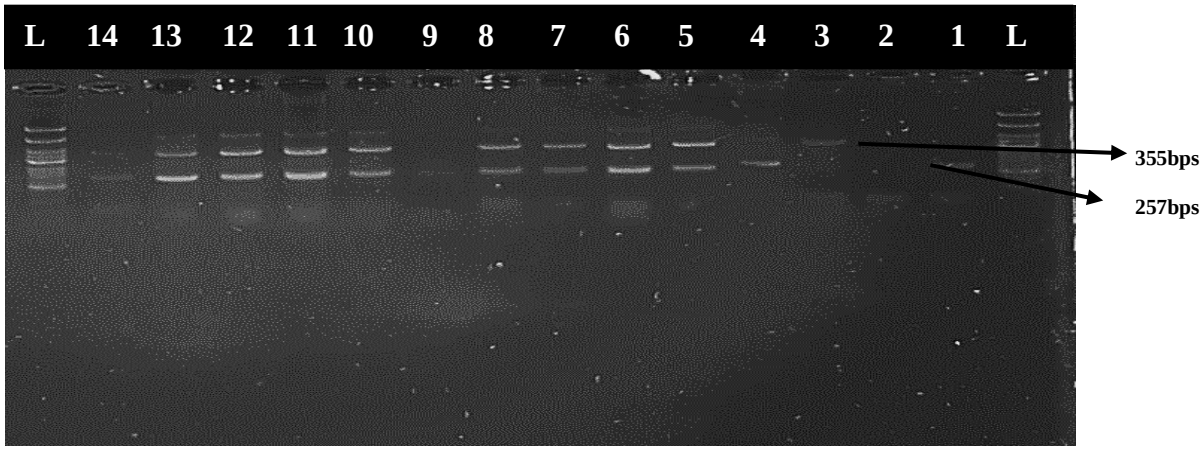

Figure 5 Agarose gel image showing BC_1F_2 breeding population amplified using molecular markers linked to *fgr* gene, L - 1000bps ladder, 2 - V1, 3- V3 while 12- *Basmati* 370 and 1, 4-11, 13 and 14 -BC_1F_2 hybrids.

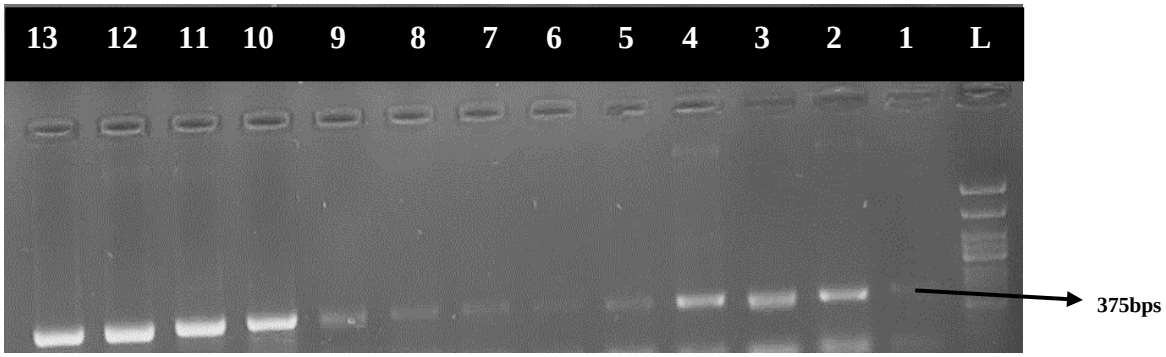

Figure 6 Agarose gel image of BC_1F_2 breeding lines showing RM12521 primer linked to *p/tgms12-1* gene, L - 1000bs ladder, 1 - Basmati 370, 2 - V1, 3 - V3 parents and 1-13 - BC_1F_2 hybrid lines.

Table 6 Means of agronomic traits of rice parents and BC_1F_2 hybrids under high-temperature treatment

Line	Total number of tillers (TT)	Number of productive tillers (PT)	Plant height (cm)
V1	11.33±1.07^{ab}	10.44±0.96^{ab}	55.03±0.63^{d}
V3	7.22±0.63^{c}	6.36±0.53^{c}	63.36±1.46^{d}
Basmati 370	12.06+0.8^{ab}	10.92+0.71^{ab}	100.81+2.66^{a}
V3 × *Basmati 370* (HB1)	15.11±1.05^{a}	13.25±0.91^{a}	90.06±4.10^{abc}
V3 × *Basmati 370* (HB2)	11.33±0.55^{ab}	10.17±0.49^{ab}	78.86±4.29^{c}
V3 × *Basmati 370* (HB3)	10.81±0.83^{bc}	9.28±0.56^{bc}	101.72±2.43^{a}
V3 × *Basmati 370* (HB7)	11.50±0.82^{ab}	10.39±0.75^{ab}	79.94±2.57^{bc}
V3 × *Basmati 370* (HB8)	12.44±0.82^{ab}	11.17±0.70^{ab}	96.19±1.61^{a}
V1 × *Basmati 370* (HB10)	11.69±0.89^{ab}	9.78±0.79^{b}	92.92±2.84^{ab}
V1 × *Basmati 370* (HB11)	10.94±0.71^{bc}	9.75±0.58^{b}	82.28±2.86^{bc}
V1 × *Basmati 370* (HB12)	11.08±0.81^{b}	9.39±0.65^{bc}	92.31±3.22^{ab}
V1 × *Basmati 370* (HB13)	11.33±0.94^{ab}	9.69±0.76^{b}	90.89±4.11^{abc}
V1 × *Basmati 370* (HB14)	13.00±0.62^{ab}	11.19±0.52^{ab}	102.47±1.34^{a}
Pooled Mean	11.53	10.14	86.68
P value	<.0001	<.0001	<.0001

The means with a different superscript letter within a column are significantly different ($P \leq 0.05$) according to Tukey's test, lines V1, V3 and Basmati 370 are rice parent lines and HB1 to HB14 are BC_1F_2 lines.

Table 7 Correlation coefficients (r) of agronomic traits of parents and F_2 plants

Parameter	TT	PT	PH
Total no. of tillers	1.00000		
Productive tillers	0.93670***	1.00000	
Plant height	0.25252***	0.21831***	1.00000

***, significance at p< 0.001, Plant height (PH), total number of tillers (TT), and number of productive tillers (PT).

hence, plant height was significantly decreased. Hybrid 1, 14 and 8 had the highest total number of tillers (15.11, 13.00 and 12.44), respectively. Similarly, hybrids 1, 14 and 8 had the highest productive tillers, including 13.25, 11.19 and 11.17, respectively (Table 6).

3.8 Pearson's correlation coefficient analysis in the BC_1F_2 population

Significant positive correlations were observed between productive tillers, the total number of tillers (r=0.9367***), and plant height

(r= 0.25252***). In addition, productive tillers and plant height were significantly and positively correlated (r= 0.21831***), as shown in Table 7.

4 Discussion

High greenhouse temperatures maintained in the simple greenhouse induced near complete sterility in P/TGMS rice lines that were in contrast to those plants grown under normal temperatures outside the greenhouse. The average temperatures at the study site, Kenya's main rice growing area, ranged from 15.6°C to 28.6°C, which is not conducive to induce complete sterility in P/TGMS rice. In this research, greenhouse intervention could sustain temperatures to above 22ºC at night and above 33ºC during the day, which is ideal for hybrid rice seed production using P/TGMS lines. This type of hybrid seed technology is essential to rice yield improvement since it can raise yield by 25% to 30% above purebred varieties (Tongmark et al. 2021).

The P/TGMS lines form an important component in a two-line hybrid rice seed production system (Li et al. 2024). Male sterility occurrence in P/TGMS is environmentally dependent on temperature and/or photoperiod (Ashraf et al. 2020). The pollen viability in P/TGMS is one of the most important parameters in ensuring hybrid seeds are not contaminated by self-bred seeds (Njau 2017). High temperature (24-32°C) is the key factor contributing to complete sterility in P/TGMS, while low temperatures (below 23°C) contribute to male fertility. Photoperiod also influences the P/TGMS rice lines, where longer photoperiods (>14 hours) enhance male sterility while shorter photoperiods (<12 hours) enhance male fertility (Ashraf et al. 2020). Photoperiod is effective between the critical fertility point (CFP) and critical sterility point (CSP), and this range of temperature is known as the temperature range of photo-sensitivity (Swaminathan 2021). High average temperatures in P/TGMS lines lower the critical photoperiod level required to induce sterility (Peng et al. 2023). When the temperatures are high, they compensate for the reduced photoperiod required to induce complete pollen sterility (Liu et al. 2023). Fertility alteration in P/TGMS lines usually starts from the formation stage of the pollen mother cell to the meiotic division stage. Therefore, the interaction of photoperiod and temperature has been used to realize better induction of pollen sterility, especially in P/TGMS rice lines (El-Mowafi et al. 2021).

In this research, the P/TGMS were treated under greenhouse growth conditions at the primordial stage up to the initiation of heading. This was adequate to induce sterility levels above 90%, comparable to the report of Vishvapriya et al. (2023) and Swaminathan (2021), who observed that complete sterility can be induced when the P/TGMS rice lines at the critical-sterility phase are grown under high-temperature conditions. Pollen sterility correlated positively with high temperature, confirming the ability of greenhouse temperatures to induce sterility significantly in the EGMS lines. Temperatures above 35°C lasting more than one hour during anthesis lead to high sterility levels in rice plants (Hu et al. 2021). Therefore, the greenhouse growth conditions were adequate for complete male gamete sterility in EGMS and significantly reduced hybrid seed adulteration by self-bred seeds.

Breeding efforts using the P/TGMS rice lines have been made by scientists in the last three decades, leading to the release of new varieties using the EGMS breeding method (Swaminathan 2021). The P/TGMS lines have greatly improved the yield potential and grain quality of different rice varieties (Wang et al. 2022). The P/TGMS lines produce fertile hybrid seeds during the sterile phase by crossing them with a fertile male pollinator, thus utilizing heterosis to increase yield. When grown under optimum temperature during their fertile phase, the P/TGMS self-pollinate to propagate themselves for breeding continuity (Ashraf et al. 2020). A single recessive gene controls the P/TGMS traits in rice lines. Since it is a nuclear gene, it is easily transferable through backcrossing to desired varieties (Fang et al. 2023). This provides a broader genetic resource for rice breeding and has helped in the production of rice hybrids with strong hybrid vigour.

Visual scoring for the *p/tgms12-1* gene using RM12521 SSR markers on agarose gel electrophoresis in the BC_1F_2 population was not specific to the target gene since the marker was monomorphic; hence, it was challenging to use markers-assisted selection to identify hybrid from parents in this research. This agreed with Njau (2017), who used the same marker and found that the bands were not polymorphic. The cross between V1 and *Basmati* 370 provided a BC_1F_2 with over 89% pollen sterility grown under male gametes emasculation temperature conditions. Attempts to use heterosis in *Basmati* rice improvement have been reported (Budhlakoti and Baskheti 2021). However, no commercial breeding varieties have been released to the market. The novel BC_1F_2 is a milestone towards producing commercial hybrid rice varieties of *Basmati* origin.

Flavour in rice, made up of taste and aroma, is one of the most important factors in evaluating rice quality (Hu et al. 2020). Scented rice with distinct aroma and good quality traits fetches a premium value in national and international markets (Roy et al. 2020). However, most of these aromatic rice varieties are limited by low yields, poor agronomic performance, and susceptibility to environmental conditions. In addition, they are produced in only a few countries (Dar et al. 2021). The new BC_1F_2 lines, once stabilized and released into the market, will improve *Basmati* yield through heterosis that has been used to break the purebred lines breeding plateau (Njau 2017).

Different volatile aromatic compounds in rice include aldehydes, ketones, organic acids, alcohols, esters, hydrocarbons, phenols,

pyrazines, and pyridine. The 2-acetyl-1-pyrroline (2-AP) is the key compound responsible for the aroma due to low sensory threshold and strong popcorn and sweet flavour (Verma and Srivastav 2022). The 2-AP is a vital characteristic of aromatic rice (Luo et al. 2020). Genetically, aroma in rice is thought to originate from spontaneous recessive mutations in *fgr* (also known as *OsBadh2*/*badh2*/*osbadh2*/*os2-AP* genes) (Dutta et al. 2022). These mutations inhibit the flow of γ-aminobutyraldehyde (GAB-ald) to γ-aminobutyric acid (GABA), which results in the conversion of accumulated GAB-ald to a fragrance component s2-AP through a non-enzymatic reaction process with methylglyoxal (Prodhan and Qingyao 2020).

In *Basmati* 370 rice, the aroma trait is under a recessive gene control (Varatharajan et al. 2021) and has elevated levels of the 2-acetly-1-pyrriline(2AP) in the aerial parts of the rice plant (Bradbury et al. 2005). The betaine aldehyde dehydrogenase (BAD2) enzyme responsible for aroma is due to a deletion in the gene encoding BAD2 on chromosome 8, deactivating the normal BAD2 gene (Bradbury et al. 2005). Rice breeders use a single tube allele-specific PCR to determine the genotypic status of the rice plant if it is either homozygous aromatic, homozygous non-aromatic or heterozygous non-aromatic (Bradbury et al. 2005). Using this method, three heterozygous non-aromatic BC_1F_2 lines and two homozygous aromatic BC_1F_2 were confirmed in this study.

Breeding for recessive genes requires either selection at F_2 segregation or backcrossing F_1 with a recessive gene donor to get homozygous recessive offspring (Hussain et al. 2021). In this research, *Basmati* 370 was used as a donor to produce lines with the *p/tgms12-1* gene. The hybrids produced were not expected to have aroma since the *fgr* gene is a recessive trait. This breeding challenge needs to be solved by producing Basmati with *p/tgms12-1* genes, and if it is achieved, then both male and female parents in the hybrid rice programme will have the aroma and hence the F_1s. This research indicates that the *fgr* and *p/tgms12-1* genes were successfully introgressed into a *Basmati* 370 rice variety validated using molecular marker analysis. Carsono et al. (2023) found that crosses of aromatic and non-aromatic rice verities resulted in non-aromatic hybrids, and since the *fgr* is a recessive gene, backcrossing with a recessive aromatic line is needed to enable the selection of homozygous with aroma trait.

Successful crosses of *Basmati* 370 with V1 and V3 to obtain BC_1F_2 were ascertained using morphological and SSR molecular markers. The SSR molecular markers are commonly used to confirm the hybrids in many plant species, including rice because they are highly polymorphic and codominant (Adiredjo and Ardiarini 2023). In BC_1F_2, the hybrids were isolated with both *fgr* and *p/tgms12-1*, demonstrating the ability to breed a fragrant rice line with *Basmati*-like traits.

Hybrids that involve *Basmati* 370 and the P/TGMS lines (V1 and V3) have conspicuous incidences of anthocyanin, making them distinct from their parents (Njiruh et al. 2013). Therefore, anthocyanin can be used as an ideal morphological marker in selecting F_1 hybrids of *Basmati* 370 and P/TGMS lines (V1 and V3). In this study, the F_1 hybrids were distinguished by the presence of anthocyanin pigmentation in their stem and spikelet tips. These results are similar to previous studies involving *Basmati* 370 hybrids (Njiruh et al. 2013; Nthakanio and Kariuki 2019). The hybrids in this study contained anthocyanin at the base of the stem, spikelet tips and the stigma. Therefore, anthocyanin is a promising and reliable morphological marker for selecting F_1 hybrids involving *Basmati* 370 and P/TGMS lines.

Agro-morphological traits such as plant height (cm), total number of tillers, days to heading, days to flowering and days to maturity showed a positive heterosis between the hybrids and the parents in the F_1 population. The F_1 population planted outside the greenhouse showed some levels of panicle sterility at maturity despite being planted in fertility-inducing conditions. Sterility in F_1 has been reported in hybrids lacking wide compatibility genes (Rao et al. 2021). *Basmati* 370 (cK) had the lowest percentage of panicle sterility because it lacks the p/tgms12-1 gene present in the F_1 hybrids and their parents V1 and V3. Among the F_1 hybrids, V1 hybrids had a higher percentage of panicle sterility than V3 hybrids. In addition, the V1 parent has a higher panicle sterility than the V3 parent. This shows that the V1 has higher heritability than the V3 and is thus more useful in producing an EGMS breeding line. This agrees with Asante et al. (2006), who conducted a study on the inheritance of spikelet fertility from two rice crosses. An increased number of spikelets in the hybrids obtained are an indication of positive heterosis and a yield benefit of hybrid rice lines.

The BC_1F_2 population had more sterile spikelets than *Basmati* 370 under greater than 30 ºC growth conditions. This indicates the presence of the *p/tgms12-1* gene in the hybrids and the absence of the same gene in *Basmati* 370 (cK). This indicates successful introgression of *p/tgms12-1* and *fgr* gene into BC_1F_2, indicating possible production of EGMS of *Basmati* origin. Days to heading, days to flowering and days to maturity were significantly different (shorter) in F_1 populations compared to V1 and V3. Shortening the days to maturity is a desirable trait to help in improving the rice breeding program since a shorter maturing period reduces pest and disease management costs (Heredia et al. 2022). Negative heterosis for traits such as days to heading and plant height is desirable for breeding semi-dwarf and early maturing lines (Gaballah et al. 2022). In the BC_1F_2 population, there was a positive correlation between the number of productive tillers and the total number of tillers. This is a clear indication of positive heterosis, which is a desired trait for breeders to increase yield. Plant height had a

positive correlation with the total number of tillers and the total number of productive tillers, which is agreed upon by Prajapati et al. (2022).

Conclusion

This study revealed that high temperatures above 30˚C in the greenhouse during the day effectively induce the expression of the p/tgms12-1 gene in the EGMS and BC_1F_2 lines, which can potentially expand the space for hybrid seed production. Using polythene greenhouses will accelerate the breeding of novel EGMS lines in medium altitude regions such as Mwea, Kenya, where the normal daylight length and temperature levels are not conducive to applying EGMS system in rice breeding. From this study, it is possible to introgress *p/tgms12-1* genes into *Basmati* 370 rice to develop novel lines with *p/tgms12-1* and *fgr* gene, a breakthrough that will enable the advancement of heterosis in Basmati rice yield improvement. From this study, the BC_1F_2 breeding lines had better agronomical traits than the parents, which will benefit rice breeders more.

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