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Sequencing of S_5 gene in autotetraploid rice *japonica* and *indica* to overcome F_1 hybrids embryo sac sterility

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Autotetraploid rice is a new germplasm developed through diploid chromosome doubling. Hybrids developed by *indica* autotetraploid rice crossed by *japonica* autotetraploid rice has clear biological advantage on F_1 , causing widespread concerns for special research evolution to evaluate and utilize F_1 hybrid vigor. However, the widespread fertility of F_1 *indica* and *japonica* autotetraploid rice is low, which makes it difficult to direct utilization of F_1 vigor. In diploid *indica* and *japonica* rice, the fertility of F_1 hybrids is also proved to be low. Embryo sac infertility is known to be one of the most important reasons for hybrid sterility and many studies has indicated that the primary cause of F_1 sterility was abortion of the embryo sac, which was identified by the genotype of the S_5 gene on the chromosome. Previous studies have cloned S_5 based on common wild rice and cultivated rice and S_5 sequence was obtained and studied. In this paper, typical sequence of different materials of S_5 *japonica* and *indica* rice group of autotetraploid hybrids observed with whole-mount eosin B-staining confocal laser scanning microscopy WE-CLSM was utilized, to overcome F_1 hybrids embryo sac sterility.

Key words: rice (*Oryza sativa* L.); autotetraploid; hybrid sterility; S_5 gene; embryo sac fertility.

INTRODUCTION

Food security, which is the condition of having enough food to provide adequate nutrition for a healthy life, is a critical issue in the world (Chen et al., 1994). About 3 billion people, nearly half the world's population, depend on rice for survival (Kanawapee et al., 2011) and in Asia

as a whole, much of the population consumes rice in every meal (Maclean et al., 2002). The genus *Oryza* originated many years ago and different species got distributed into different continents.

Genus *Oryza sativa*, the Asian cultivated rice is grown

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all over the world (Khush, 1997), while African cultivated rice, *O. glaberrima* is grown on a small scale in West Africa (Mmbando, 2022). The cultivated species originated from a common ancestor with AA genome. Perennial and annual ancestors of *O. sativa* are *O. rufipogon* and *O. nivara* and those of *O. glaberrima* are *O. longistaminata*, *O. breviligulata* and *O. glaberrima* are domesticated in Niger River delta (Sangeetha et al., 2020). Rice is the most important crop to millions of small farmers who grow it on millions of hectares throughout the region, and to the many landless workers who derive income from working on these farms (Ganesan et al., 2022; Shennan et al., 2022). In the future, it is imperative that rice production continue to grow rapidly as the population upsurge, and therefore increased yield became a major concern for rice scientists.

Oryza sativa, commonly known as Asian rice is classified into two main subspecies, *indica* and *japonica*. Interspecific (*indica* × *japonica*) hybrids have great biological superiority (Tong et al., 2011; Zheng et al., 2020; Ouyang et al., 2022) and among all these interspecific hybrids are highly resistant against insect, pest and diseases (Gaikwad et al., 2021; Cordero-Lara, 2020) with enhanced drought tolerance (Kang and Futakuchi, 2019; Jia et al., 2022) as well as high biomass production (Wendel, 2000; Li et al., 2022, 2012). However, these hybrids seed setting rate is low (Zheng et al., 2020; Huang et al., 2021; Kim et al., 2022). In order to create hybrid rice, researchers have been very interested in the significant hybrid vigor between the *indica* (*O. sativa* ssp. *indica*) and *japonica* (*O. sativa* ssp. *japonica*) subspecies of the Asian farmed rice (*O. sativa* L.). The partial sterility that frequently occurs in *indica*-*japonica* crossings is a significant obstacle to the production of such inter-sub specific hybrids (Kato et al., 1928, Sweigart et al., 2019; Zhang, 2020; Ouyang et al., 2022). Similar research revealed that *indica*-*japonica* hybrids' fertility varied greatly, from totally fertile to nearly completely infertile (Fang et al., 2019; Wang et al., 2020; Ouyang et al., 2022) while the majority of inter-sub specific hybrids have markedly decreased fertility (Oka, 1974; Zhang et al., 1997; Li et al., 2020; Kim et al., 2022).

Poor fertility is the main barrier for utilizing heterosis between the two rice (*O. sativa* L.) subspecies, *indica* and *japonica* and the development of autotetraploid hybrids ($2n = 4x = 48$) has been advised as a new method for increasing heterosis in hybrid rice (Chen et al., 2019; Ghaleb et al., 2020; Rao et al., 2022). An autotetraploid *indica/japonica* hybrid combines the advantages of polyploidy and heterosis between *indica* and *japonica* (Hu et al., 2010; Yu et al., 2021a; Huang and Huang, 2022; Rao et al., 2022) and realized to have great potential to increase the yield of rice (He et al., 2011; Ghouri et al., 2019; Chen et al., 2021; Ku et al., 2022). Furthermore, autotetraploid *indica/japonica* hybrids hold more nutrition value than diploid rice (Zhiyong et al., 1987 and He et al., 2011; Chen et al.,

2021, 2022; Ghouri et al., 2023) and therefore said to have stronger potential vigor in rice breeding than diploid rice does (Shahid et al., 2011; Rout et al., 2020; Wu et al., 2020; Chen et al., 2022). Hybrid sterility between two rice subspecies may be overcome by using tetraploid lines followed by intensive selection (Chen et al., 2021; Huang et al., 2022). Also, the gigantic features of the autotetraploid hybrids may establish a plant structure able to support the higher yield (Shen et al., 2022; Zeng et al., 2023). However, the utilization of strong heterosis in the F_1 hybrids between the two subspecies has been difficult because of partial or complete sterility in the hybrids (Shahid et al., 2013; Yu et al., 2021a; Ouyang et al., 2022).

Though, heterosis utilization has been successful in many crops (Liu et al., 1998; Xiao et al., 2021; Landge et al., 2022), not much success on autotetraploid rice as most results were based on diploids crops. According to He et al. (2011) using polyploidy meiosis stability (PMeS) line as a parent improves embryo development and the seed set rate of a tetraploid rice hybrid. Moreover, polyploidy is widely accepted to play an important role in the evolution and breeding of plant species (Udall and Wendel, 2006; Cheng et al., 2022; Mangena, 2023), but nonetheless, a low seed set rate significantly hindered the development of polyploidy rice breeding (He et al., 2011; Xiong et al., 2019; Zhang et al., 2019, Chen et al., 2021; Huang et al., 2022). Consequently, there is an urgent need to overcome sterility of interspecific hybrids of autotetraploid rice in order to utilize its great genetic competence and explore its hybrid vigor.

Embryo sac fertility and pollen fertility are the most important factors which affect the seed setting rate in autotetraploid rice (Shahid et al., 2010; Li et al., 2020; Kamara et al., 2021; Ku et al., 2022, Kamara et al., 2022) and additionally lead to *indica* sterility hybrid *japonicas* (Song et al., 2005; Mi et al., 2019; Rout et al., 2020) since they both significantly affect hybrid fertility.

According to the well-known theory put forth by Ikehashi and Araki (1986), F_1 sterility was mostly brought on by embryo sac abortion, which was determined by the genotype of S_5 located in chromosome 6, and *indica* was $S_5^i S_5^i$ in genotype as well as *japonica* $S_5^j S_5^j$ (Yang et al., 2009; Zhang et al., 2020; Seo et al., 2020; Kallugudi et al., 2022) hence The S_5 locus on chromosome 6 became the primary genetic cause of embryo sac sterility due to several allelic interactions (Lee et al., 2021; Zhang, 2021; Ouyang et al., 2022). Nevertheless, hybrid fertility was decreased as a result of interactions between the *indica* and *japonica* alleles at each of the loci (Xie et al., 2019; Guo et al., 2022; Kallugudi et al., 2022). Yang further explained that because to gene interactions, the *indica* and *japonica* parent types' $S_5^i S_5^j$ hybrids were also infertile S_5^i and S_5^j .

Wide-compatibility varieties (WCVs), a special subset of rice germplasm, have made intersub specific heterosis more useful (Ikehashi and Araki, 1986; Awad-Allah, 2020;

Zhang, 2020; Kallugudi et al., 2022). When crossed to *indica* or *japonica* subspecies, WCVs can overcome obstacles to reproduction and result in fertile hybrids. Hybrids between *indica* and *japonica* parent lines that carry the S_5^n (wide-compatible gene) would have high or normal fertility regardless of whether they have the $S_5^nS_5^i$ or $S_5^nS_5^j$ genotype (Li et al., 2011; Ghaleb et al., 2020; Guo et al., 2022). Wide-compatibility varieties (WCVs) allowed the reproductive barrier between the *indica* and *japonica* subspecies to be breached and gave rice breeding programs the opportunity to create inter-sub specific hybrids.

Progress has been made by previous studies for S_5^n gene sequencing especially in diploid rice and their results indicated that widely compatible kinds ($S_5^nS_5^n$) can produce fruitful hybrids when bred with *indica* or *japonica* varieties (Kinoshita, 1995; Yu et al., 2021; Ghaleb et al., 2020; Vernet et al., 2022). Rice geneticists also cloned S_5^n and found that *the two nucleotide differences between S_5^i and S_5^j resulted in two amino acid substitutions in the relevant protein and intersub specific hybrid sterility* (Chen et al., 2008; Lu et al., 2020; Rout et al., 2020; Yu et al., 2021b).

MATERIALS AND METHODS

Forty-nine autotetraploid rice lines which were planted in the South China Agricultural University's experimental farm was used in this experiment and different hybrids made by *indica* and *japonica* autotetraploid varieties which were planted at the same field were also used, with typical hybrids (*indica* and *japonica*) used as control.

Sequencing of S_5 gene in autotetraploid rice

The results of forty-nine autotetraploid rice lines used in this paper were amplified and sequenced by the Regional Genetic and Breeding Laboratory of South China Agricultural University, College of Agriculture, Guangzhou, Guangdong. The laboratory primers were synthesized by Shanghai Biological Engineering Technology Services Limited Company and these primers were designed on the basis of the sequences of Nipponbare ($S_5^iS_5^j$) and 02428 ($S_5^nS_5^n$) cultivars. This paper used the sequenced results.

Embryo-sac fertility observation

Zeng et al. (2007) used whole-mount eosin B-staining confocal laser scanning microscopy (WE-CLSM) to investigate the embryo-sac structure. 100 to 150 spikelets with developed embryo sacs were taken from each plant after blooming and fixed in FAA (formaldehyde: acetic acid: 50% ethanol = 5:6:89) for at least 24 h. The florets were cleaned in 50% ethanol before being kept at 4°C in 70% ethanol. Under a binocular dissecting microscope, the ovaries were dissected in a Petri dish and successively hydrated in 50% ethanol, 30% ethanol, and distilled water. The samples will undergo a 20 min pretreatment in 2% aluminum potassium sulphate before being stained for 12 h at room temperature with a 10 mg/L solution of eosin B diluted in 4% sucrose. The samples underwent a 20-min post-treatment in 2% aluminum potassium sulphate. The samples

were dehydrated using a series of ethanol solutions (30, 50, 70, 90 and 100%) after being rinsed three times with distilled water. The dehydrated samples were then transferred to a 1:1 mixture of absolute ethanol and methyl salicylate for 1 h, followed by at least 1 h of clearing in pure methyl salicylate solution. Finally, a Leica SPE laser scanning confocal microscope was used to scan the samples.

Seed setting observation

The Shi et al. (2009) approach was used to calculate the seed-set rate. In a nutshell, the average seed dry weight of the harvested individuals was used to calculate seed yield (SY). For each replication sample, 1000 fully formed seeds were used to calculate the seed weight (SW). The average number of well-filled seeds from 100 fully formed pods, which were taken from the principal branch in the centre of the harvested individuals, was tallied to determine the seed number (SN). The number of normally grown pods on each harvested person was known as the pod number (PN). Each harvested individual's plant height (PH) was calculated by measuring it from the stem's base to the tip of the main shoot. The number of operational primary branches (BN) was recorded.

RESULTS

S_5 gene sequence variation

According to the information supplied, forty-nine autotetraploid rice lines sequenced carried S_5 gene sequence. Ten haplotypes carried S_5^n gene while thirty-nine haplotypes carried either S_5^i or S_5^j gene. Figure 1 shows the sequence variations of S_5 loci among 49 autotetraploid rice lines while Figure 2 shows S_5 gene variation analysis among 10 autotetraploid rice varieties carrying S_5^n gene. ■, exon; –intron; *, base position (the first transcription starting point of 02428 is + 1).

Embryo sac fertility and seed setting of *indica* and *japonica* autotetraploid hybrids

As it can be seen from Table 2, a big difference is observed in embryo sac fertility of autotetraploid parents among different materials, embryo sac fertility of 4 materials (T432, T440, T455 and T434) were more than 80%, and low embryo sac fertility rate below 70% were observed in 3 materials (T412, T416 and T424), parent with the highest embryo sac percentage was T432 with 92.25%, with the lowest being T412 with 31.32%. The seed setting results shows that all parents observed were below 70% except T440 which has a seed setting rate of 72.90% and it is the highest among all the observed parents. Embryo sac fertility decreases gradually especially with parent T432 which has embryo sac fertility of 92.25%, but very low seed setting rate of 2.53%, this result may be due to low pollen fertility of these material. Other rice parents like T412 has low embryo sac fertility of only 31.32%, but high seed setting rate of 69.90%, this result may be due to the influences in embryo sac fertility

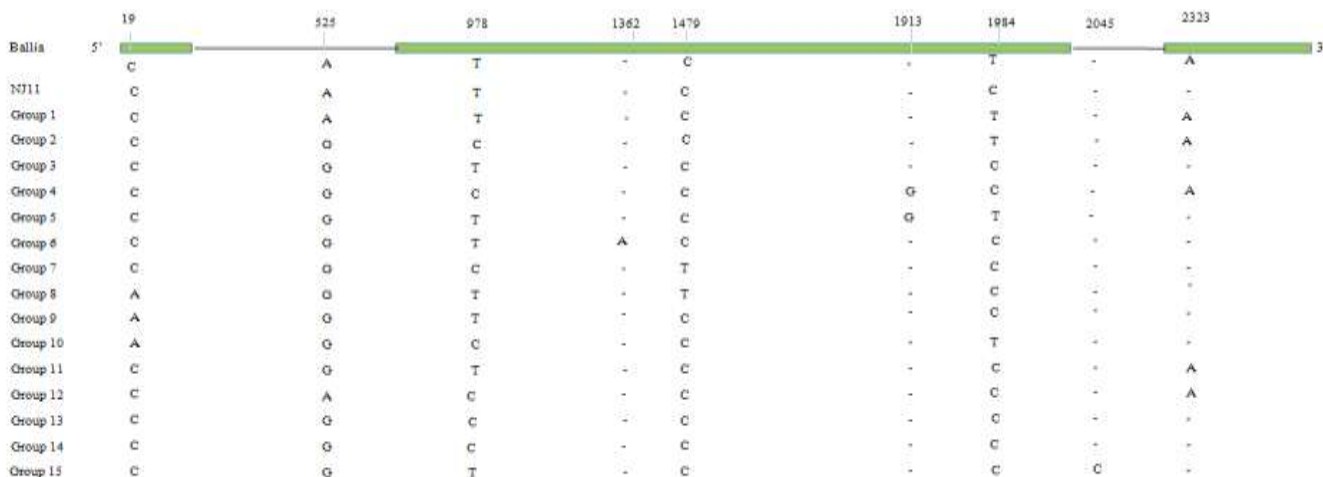


Figure 1. Sequence variations of S_5 loci among 49 autotetraploid rice lines. Source: Supplied by the Genetic and Breeding Laboratory of South China Agricultural University, College of Agriculture, Guangzhou, Guangdong Province, China.



Figure 2. S_5 gene variation analysis among 10 autotetraploid rice varieties carrying S_5^n gene. ■, exon; -intron; *, base position (the first transcription starting point of 02428 is + 1). Source: Supplied by the Genetic and Breeding Laboratory of South China Agricultural University, College of Agriculture, Guangzhou, Guangdong Province, China.

and seed rates from different plant genetic characteristics of this material which may not be stable enough and thus has led offspring separation and differences when it comes to the genetic characteristics and materials instability, which might have caused embryo sac fertility and seed setting deviation. Therefore, in autotetraploid rice genetic research, there is a great need of research expansion especially to the community, as community outreach and research in order to obtain more accurate results.

Moreover, in the total number of 9 hybrids which was observed for embryo sac fertility, only hybrid (T453 x T434) has less embryo sac fertility rate which is less than 80% (70.33%), the remaining 8 hybrids has high embryo sac fertility rate which is higher than 80%, the average embryo sac fertility rate stands at 89.12%. However, this results found it out that there was a significant different between seed setting rate and embryo sac fertility rate of the some materials with the same parent materials like T424 x T440 which has very high embryo sac fertility of 80.23% but very low seed setting rate of 3.49%, this result might also be due to low pollen fertility. Typical *indica-japonica* (without S_5^n) hybrids (T453 x T434) as well as *japonica-japonica* (without *indica* and S_5^n) hybrids

(T424 X T440) generated embryo sacs with a very poor fertility, and many malformed embryo sacs were seen.

Abnormal embryo sacs that either lack an egg apparatus or have polar nuclei that are not properly positioned, normally prevents fertilization to occur, resulting in sterile embryo sacs. As can be seen from Table 3, the total percentage abortion rate of embryo sac rate for both parents and hybrid F_1 generation materials are different from each other with different types of abnormality expressed in different percentages. However, in all types of abnormality, embryo sac degeneration (embryo SAC degeneration) is the most common type, except material T432 (parent) and T432 x T416 (hybrid) which remained with zero percentage embryo sac degeneration. Frequent abnormality appeared in all other materials especially embryo sac without egg apparatus, small embryo sac (Abnormal Small embryo sac) and abnormal polar nuclei (Embryo sac with abnormal polar nuclei). The remained three other types of embryo sac abortion were relatively rare. Among all, only 4 materials (T416, T432, T455 and T424 x T425) 4 materials have a shown relatively small amount of Embryo sac without egg Apparatus. Very small percentage of Embryo sac without female germ unit were shown T412, T424, T455 x T424 6

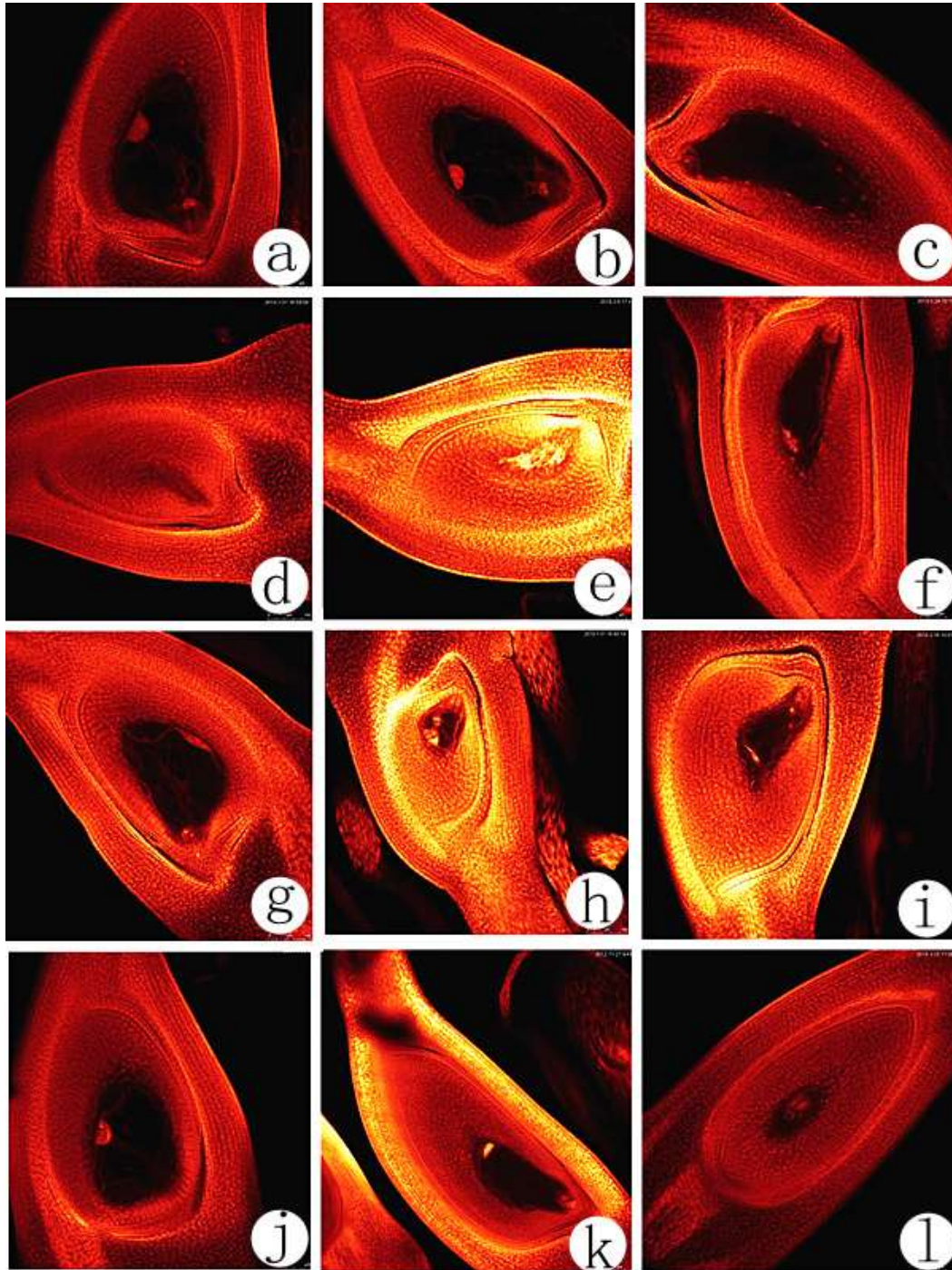


Figure 3. Abnormal embryo sac structures of *indica-japonica* hybrids F_1 . (a) and (b) normal embryo (With all normal egg, polar nuclei and antipodal cells positioned at the right positions) (c) Normal embryo sac after fertilization (d) and (e) Embryo sac degradation, (f) and (g) Embryo sac without an egg apparatus, (h) and (i) Abnormal small embryo sacs, (j) Embryo sac without female germ unit, (k) Embryo sac with abnormal position of polar nuclei and (l) Others.
Source: Authors

while T440, T424 x T43 and T453 x T434 have relatively small percentages of other types of abnormal embryo.

Abnormal embryo sacs included embryo sac

degeneration (Figure 3d and e), embryo sac without egg apparatus (Figure 3e) abnormal small embryo sac (Figure 3g), embryo sac without female germ unit (Figure 3j)

Table 1. Names and types of 49 autotetraploid lines/cultivars.

Material type	Material name
<i>Indica</i>	T41, T42, T44, T410, T413, T417, T419, T420, T421, T425, T426, T428, T429, T430, T442, T442-1, T445, T447, T448, T451, T452, T453, T455, T457, T461, T463, T465, T467, T469, T473 and T479
<i>Japonica</i>	T43, T45, T46, T48, T49, T415, T422, T423, T431, T432, T434, T435, T437, T438, T443, T444, T450 and T456

Thirty-one of these materials are *indica* while eighteen materials are *japonica*.
Source: Authors

Table 2. Embryo sac fertility and seed set of parents and their testcross F₁.

Name of parents and hybrids	Embryo sac fertility (%)	Seed set (%)
T412	31.32	69.90
T416	64.09	18.58
T424	57.36	20.27
T432	92.25	2.53
T440	86.86	72.90
T455	81.39	43.05
T434	90.89	49.78
T424 x T43	95.10	73.76
T455 x T424	88.42	68.81
T424 x T425	82.83	55.88
T432 x T416	96.29	57.71
T416 x T434	97.80	39.72
T435 x T416	98.27	52.66
T432 x T455	92.81	64.20
T424 x T440	80.23	3.49
T453 x T434	70.33	12.87

Source: Authors

and embryo sac with abnormal polar nuclei (Figure 3k). These embryo sacs can't fertilize regularly because they either lack an egg apparatus or have polar nuclei that are positioned incorrectly, leading to sterile embryo sacs. Other abnormal kinds, which were classified as other abnormal types because their frequencies were low, were also discovered in addition to the principal types of embryo sac defects stated above (Figure 3l).

Effects of different sequences of S₅ on embryo sac fertility of intersubspecific hybrids in autotetraploid rice

Table 4 illustrates typical *indica-japonica* (without S_{5n}) hybrids (T453 T434), while hybrids between *japonica* and *japonica* (without *indica* and S_{5n}) (T424 X T440) produced a variety of malformed embryo sacs and had very poor embryo sac fertility. Furthermore, typical *indica-japonica* (without S_{5ⁿ} gene produced the least seed setting percentages (22.02%) as shown in Table 3, while in the same table, varieties with S_{5ⁿ} gene come out with

the highest seed setting percentages (69.32%).

Genetic variations are clearly seen in Table 4. In general, there are many genes that controls seed set and S_{5ⁿ} is one of those gene, which mostly control embryo sac fertility and thus can be the reason why some materials without S_{5ⁿ} have high seed set rate as compared to those with S_{5ⁿ} gene, simply because some other genes were stronger and could be expressed more frequently. The highest F₁ generation seed setting rate S_{5^j} X S_{5ⁿ} were 66.14 and 61.81% respectively, the highest seed set rate percentages of S_{5^j} X S_{5ⁿ} is 69.32% with the least percentage being 22.02% for typical *indica-japonica*. Standard *indica* and *japonica* cultivars' embryo sac fertility and seed set rate considerably increased when they were crossed with S_{5n}-harboring cultivars (Table 1 and 3). *Indica-japonica* cultivars and cultivars with the exon 2 10 bp deletion were crossed to create some testcross F₁ hybrids, whose average embryo sac fertility was similarly quite high (69.32%). When crossed with normal cultivars, all S_{5n}-containing cultivars or accessions showed high embryo sac fertility, as shown in Table 1 (T435 x T416) 98.27%, demonstrating that the

Table 3. Types of abnormal mature embryo sacs in different rice materials (%).

Material	Total abortion rate of embryo sac	Embryo sac degeneration	Abnormal small embryo sac	Embryo sac with abnormal polar nuclei	Embryo sac without egg apparatus	Embryo sac without female germ unit	Other types of abnormal embryo sac
T412	68.68	39.20	24.41	4.67	0.00	0.40	0.00
T416	35.91	18.93	11.39	4.89	0.70	0.00	0.00
T424	42.64	16.00	0.00	24.60	0.00	2.04	0.00
T432	7.75	0.00	5.52	1.21	1.02	0.00	0.00
T440	13.14	9.43	0.00	2.71	0.00	0.00	1.00
T455	18.61	4.12	7.20	4.52	2.77	0.00	0.00
T434	9.11	2.43	3.98	2.70	0.00	0.00	0.00
T424 x T43	4.90	2.48	1.72	0.00	0.00	0.00	0.70
T455 x T424	11.58	6.49	3.12	0.39	0.00	1.58	0.00
T424 x T425	17.17	4.95	0.90	10.32	1.00	0.00	0.00
T432 x T416	3.71	2.10	0.00	1.61	0.00	0.00	0.00
T416 x T434	2.20	0.70	1.10	0.00	0.00	0.40	0.00
T435 x T416	1.73	1.33	0.00	0.40	0.00	0.00	0.00
T432 x T455	7.19	0.00	3.42	2.19	0.00	1.58	0.00
T424 x T440	19.77	11.37	4.65	1.02	0.00	2.73	0.00
T453 x T434	29.67	12.67	5.92	9.38	0.00	0.00	1.70

Source: Authors

Table 4. Genotypic analysis of selected hybrid F₁ generation with seed setting.

Gene type	Hybrids	Seed set (%)
$S_5^j \times S_5^i$	T433 X T469	59.08
	T433 X T458	46.56
	T434 X T452	22.02
	T438 X T413	46.94
	T433 X T443	37.50
$S_5^j \times S_5^n$	T432 X T45	28.32
	T434 X T45	61.81
	T434 X T443	66.14
	T435 X T45	35.55
$S_5^i \times S_5^n$	T44 X T45	69.32

Source: Authors

S5n sequence variation has no effect on S5n's capacity to overcome embryo sac sterility.

DISCUSSION

The reproductive processes, which comprise pollen and embryo sac formation, fertilization, embryogenesis, and endosperm, are significantly responsible for the potential for normal seed set (Brukhin and Albertini, 2021; Underwood et al., 2022; Hu et al., 2023). Plant geneticists and breeders (He et al., 2019; Lu et al., 2020; Mohapatra and Sahu, 2021) examined the ovaries of autotetraploid inter-sub specific hybrids at 1 day and 7 days post-pollination and discovered that some embryo sacs with normal structure did not undergo fertilization, and they also noticed that some ovaries had embryo and endosperm development that was delayed. They also found low seed set, which is consistent with the findings of this article, proving that factors other than embryo sac and pollen fertility also affected seed set. It is advised to conduct more research to identify additional variables that can affect seed planting and embryo sac fertility.

Conclusions

When compared to indica-japonica hybrids without the S5n gene, the embryo sac sterility of these hybrids has improved dramatically. Even though there were no appreciable variations in the materials with opposing sequences, this blatantly demonstrated that S_{5n} can be employed as a tool to overcome embryo sterility in indica-japonica hybrids while tortuously demonstrating that S_{5n} is a non-functional gene.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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