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ORIGINAL ARTICLE

In vitro induction of auto-allotetraploid in a newly developed wild rice line from *Oryza alta* Swallen

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Abstract

Oryza alta Swallen is an important germplasm for rice resistance breeding; however, its CCDD genome (2n = 48) resulted in low crossability when the wild rice was crossed with *O. sativa* and restricted the success of transferring the desirable traits into cultivated rice. Induction of polyploidy is an efficient way for overcoming the low crossability among different species. A new *O. alta* line, Huaye 5, was developed by our group in 2016, which had high fertility (64.93%) and photoperiod-insensitive. Huaye 5 was used to induce auto-allotetraploidy using tissue culture in the present study. The tissue culture system was established by comparing five basic media (N6, B5, MS, NB and MB), two hormones (2,4-D and 6-BA) for induction and two differentiation media (MS and NB), and then induced auto-allotetraploid in the wild rice line by colchicine. The medium and hormone combinations of NB + 2,4-D (2.5 mg/L) + 6-BA (1.0 mg/L) produced the induction rate of 20%, and MS medium was found to be a suitable medium for callus induction with a differentiation rate of 10.15%, and the treatment of 600 mg/L colchicine for 24 h was the best protocol for inducing auto-allotetraploid. Subsequently, auto-allotetraploid plants (2n = 96) were obtained in the present study and their ploidy levels were detected by using flow cytometry, stomata size and chromosomes count methods. Many inclusions in the parenchyma cells surrounding vascular bundle were observed in auto-allotetraploid rice compared to the parent. We developed a new germplasm from *O. alta*, and established a protocol of in vitro induction of auto-allotetraploid, which can be used for crossing with autotetraploid rice.

Key message

We have successfully established a protocol for the in vitro induction of auto-allotetraploid in *Oryza alta*, which can be used to cross with autotetraploid or neo-tetraploid rice.

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Keywords Rice (*Oryza sativa*) · *O. alta* Swallen · Allotetraploid · Huaye 5 · Tissue culture

Introduction

Oryza alta Swallen is an allotetraploid perennial wild rice belonging to the *Officinalis* complex and contains a CCDD genome with 2n = 48 chromosomes and it is closely related to other two species of the *Officinalis* complex, namely *O. latifolia* and *O. grandiglumis* as they have same genome and chromosome numbers (Wang et al. 2010; Gireesh 2018). It is endemic to Central and South America, and native to the Amazon River basin in South America (Vaughan 1994; Veasey et al. 2004). The wild rice is resistant to striped stem borer, bacterial grain rot disease (Mizobuchi et al. 2016) and resistant to zigzag stripe leafhopper (Mao et al. 1995), tolerant to drought, cold and withstand heat stress (Jin et al. 2013; Qin et al. 2007). *Oryza alta* contained higher nitrate contents, higher levels of starch, glucose and fructose, and higher levels of organic acids than *O. sativa* (Sung et al. 2017), and these unique physiological and primary metabolism characteristics can be exploited to improve growth and productivity of cultivated rice (Zhao et al. 2008). However, the chromosome group of *O. alta*, CCDD, is distantly related to cultivated rice of AA group (Mao et al. 1995; Yamaki et al. 2013), which makes it difficult to transfer elite genes to *O. sativa* by direct crossing.

Although hybridization between cultivated and distantly related wild species are very difficult (Fu et al. 2007; Jena et al. 2016), and many researchers have demonstrated that it is possible to overcome the difficulties of wide cross, such as *O. alta* crossing with *O. sativa*, and to transfer elite genes to cultivated rice by crossing combined with embryo rescue culture (Mao et al. 1995; Yan et al. 1996; Yi et al. 2008; Zhang et al. 2014). By rescuing hybrid embryos, in vitro F_1 plantlets were obtained in 2x × 2x combinations between *O. sativa* and *O. officinalis* (Fu et al. 2011). Wang et al. (2005) obtained hetero-hexaploid AACCDD hybrids through hybridization and embryo rescue techniques by colchicine treatment. Cytological mechanism of interspecific reproductive isolation was investigated in the hybrid of *O. alta* and *O. sativa*, which was developed by embryo rescue culture, and demonstrated that endosperm defect resulted in the death of young embryo (Fu et al. 2007). Interspecific hybrids between cultivated rice (*O. sativa*) and wild rice species (*O. latifolia*) were generated by young embryo rescue (Mao et al. 1995; Yi et al. 2008). A tissue culture optimization system was established for *O. alta* with high regeneration efficiency through callus induction (Liang et al. 2014). In fruits, embryo rescue technique was successfully used to produce interspecific hybrids (*Prunus persica* crossed with *P. armeniaca* and *P. salicina*) (Liu et al. 2006). Several intra-varietal crosses were generated between transgenic Alamo and the switch grass varieties through in situ immature embryo rescue (Kausch et al. 2016). Wei et al. (2018) concluded that the use of interspecific hybridization can enrich the genetic background of cultivars with ideal agronomic traits.

Induction of polyploidy is an efficient technique for improving agronomic traits and developing new varieties of many plants. In angiosperm plants, polyploidy has been induced to obtain new traits (Cheng and Korban 2011), which improved plant quality and increased its yield (Qiao et al. 1989; Li et al. 2012). Autotetraploid rice showed larger grain seeds, panicles, leaf area, but low seed set is a major hindrance in its utilization (Shahid et al. 2010, 2013). Intersubspecific polyploid rice hybrids showed significant heterozygosity and hybrid vigor compared to diploid rice hybrids (Shahid et al. 2011, 2012; Yang et al. 2014; Wu et al. 2017). Polyploidy enhanced allelic interactions at pollen sterility loci, including *Sa*, *Sb*, and *Sc*, which cause abnormal chromosome behavior and lead to low pollen fertility in autotetraploid rice hybrids (He et al. 2011; Wu et al. 2014, 2015; Li et al. 2016). Low fertility of autotetraploid rice is not only caused by the differential expression of genes involved in pollen development, but also by sequence variation and epigenetics (Li et al. 2017, 2018; Chen et al. 2018). Recently, some autotetraploid rice lines showed stability during meiosis and produced high seed set (Xiong et al. 2019). Neo-tetraploid rice with more than 70% seed set was developed by our group, which produced higher seed set (> 80%) and high heterosis when crossed with different autotetraploid rice lines having low seed set (Guo et al. 2017; Chen et al. 2019).

The in vitro induction of polyploidy with colchicine has been frequently reported in many crops, such as tomato, *Citrus sinensis*, shrubs and herbs (Bouvier et al. 2002; Zhang et al. 2007; Xing et al. 2011; Huang et al. 2014). Ma et al. (2016) generated autotetraploid plants through in vitro chromosome doubling of the 'Hanfu' apple cultivar (Malus × domestica) using colchicine treatment and recently in flowering Chinese cabbage, heterologous haploid offspring were obtained by embryo rescue and heterologous diploids were obtained by colchicine-induced chromosomal doubling (Wei et al. 2018). RFLP analysis showed heterozygous genotypes at most of the loci identified from sterile plants between *O. sativa* and *O. alta*, and pollen mother cells and root-tip cells exhibited 36 univalent chromosomes for most the F_1 plants, and one fertile plant showed a seed set of 14.11% (Mao et al. 1995). However, little information is available about the establishment of tissue culture system for *O. alta* with high seed set by combining seed culture with colchicine techniques to develop auto-allotetraploid wild rice. In the present study, we used a newly developed wild

rice line with high seed set, Huaye 5, which was developed from *Oryza alta* Swallen, to establish *O. alta* tissue culture system. First, we used different basic media, hormones and differentiation media, and then induced auto-allotetraploid in the new wild rice by colchicine to compare the morphological characteristics of auto-allotetraploid of *O. alta*, which will provide a new germplasm for polyploid rice breeding.

Materials and methods

A new wild rice line, Huaye 5 (2n = 48), was developed by our research group from *Oryza alta* Swallen in 2016. *Oryza alta* Swallen was kindly donated by IRRI (International Rice Research Institute). The seeds of Huaye 5, which were grown at the *Oryza* Genus Germplasm Resources Conservation Base of South China Agricultural University (SCAU), Guangzhou (23°16N, 113°8E), were used for callus induction and subculture, and the calli were employed in chromosome doubling experiments by colchicine.

Callus induction, proliferation and differentiation

The callus induction and proliferation were done according to Liang et al. (2014) with some modifications. Five basic medias, namely N6, B5, MS, NB and MB, and two phytohormones, i.e. 2,4-D and 6-BA, were used to establish a suitable combination and concentration of phytohormones for inducing callus with good quality for subculture and chromosome doubling through colchicine.

The seeds of Huaye 5 were surface-sterilized with 75% ethanol for 90 s, and then disinfected twice with 0.1% mercuric chloride for 10 min. Then, seeds were rinsed with sterile distilled water for 5 times, and transferred to petri dishes with sterile filter paper to remove excess water. The sterilized seeds were inoculated in a medium for inducing callus, in which a total of 300 seeds were placed on each medium. Each medium had 30 petri dishes, and ten seeds were inoculated on each petri dish. The inoculated petri dishes were incubated in a dark chamber at 25 °C for 50 days.

To get more and better callus, the medium, NB + 2,4-D (2.0 mg/L) + activated carbon (0.2 g/L), was employed in the subculture of callus, which were inoculated in the dark chamber with 25 °C for 15 days. After subculture of calli, the calli were inoculated on MS + KT (2.0 mg/L) + NAA (0.2 mg/L) medium at 27 °C for 40 days for differentiation.

Table 1 Agronomic traits of *Oryza alta* Swallen and Huaye 5

Traits	*O. alta* (Mean ± S.D.)	Huaye 5 (Mean ± S.D.)
No. of panicles per plant	8.48 ± 0.02	4.19 ± 0.11
Panicle length (cm)	74.70 ± 0.09	86.91 ± 1.08
Flag leaf length (cm)	41.80 ± 0.03	50.01 ± 0.78
Flag leaf width (cm)	4.60 ± 0.01	4.90 ± 0.58
Filled grains per plant	178.00 ± 1.6	227.40 ± 15.80
Total grains per plant	1033.00 ± 2.66	350.22 ± 16.37
Seed setting (%)	12.77 ± 0.07	64.93 ± 0.92
Length of ten grains (cm)	7.71 ± 0.01	7.71 ± 0.42
Width of ten grains (cm)	2.53 ± 0.01	2.61 ± 0.28

Auto-allotetraploid induction

Two methods were employed for auto-allotetraploid induction: (1) Calli were treated with colchicine using four different concentrations: 200, 400, 600, and 1000 mg/L for colchicine-soaking, combined with 24 and 48 h incubation times, in the dark at 25 °C with shaking at 150 rpm. (2) Calli or buds were co-cultured in a liquid medium containing 200 mg/L colchicine for 120 h, in the dark at 25 °C with shaking at 150 rpm. After the treatment with colchicine, the calli were placed on MS + KT (2.0 mg/L) + NAA (0.2 mg/L) medium for differentiation. Differentiated seedlings were further cultured in MS + KT 2.0 mg/L + 6-BA 2.0 mg/L + NAA 0.5 mg/L + IAA 0.5 mg/L + 0.5 g/L of activated carbon medium. Seedlings of about 15 cm were transplanted in green house for further investigations and detection of polyploidy.

Analysis of auto-allotetraploid

The leaves of differentiated seedlings and Huaye 5 (control) were collected for the analysis of polyploidy. Three methods were employed to analyze the polyploidy levels of the samples, which included: (1) Stomata observation by following the procedures of Speckmann et al. (1965). The upper epidermis of the leaves was peeled off with a sharp tweezer, and placed on a glass slide to observe the

Table 2 The rate of callus induction in different media and hormones (%)

Names	A1(2.5/0)[a]	A2(2.5/0.5)	A3(2.5/1.0)	A4(5.0/1.0)	Mean
N6	2.14 ± 0.94	9.67 ± 1.62	9.29 ± 1.78	12.14 ± 1.95	8.31
B5	1.36 ± 0.99	8.00 ± 1.39	10.67 ± 1.90	6.67 ± 1.60	6.68
MS	4.33 ± 1.14	12.96 ± 2.05	16.97 ± 2.23	15.33 ± 1.96	12.40
NB	2.50 ± 1.38	12.86 ± 1.98	20.00 ± 1.48	14.29 ± 2.59	12.41
MB	3.75 ± 1.18	9.61 ± 1.52	15.63 ± 1.74	8.40 ± 2.06	9.35
Mean	2.82	10.62	14.51	11.37	9.83

[a]"(2.5/0)" indicated 2,4-D (2.5 mg/L) + 6-BA(0 mg/L); "(2.5/0.5)" indicated 2,4-D (2.5 mg/L) + 6-BA(0.5 mg/L); "(2.5/1.0)" indicated 2,4-D (2.5 mg/L) + 6-BA (1.0 mg/L); "(5.0/1.0)" indicated 2,4-D (2.5 mg/L) + 6-BA (1.0 mg/L)

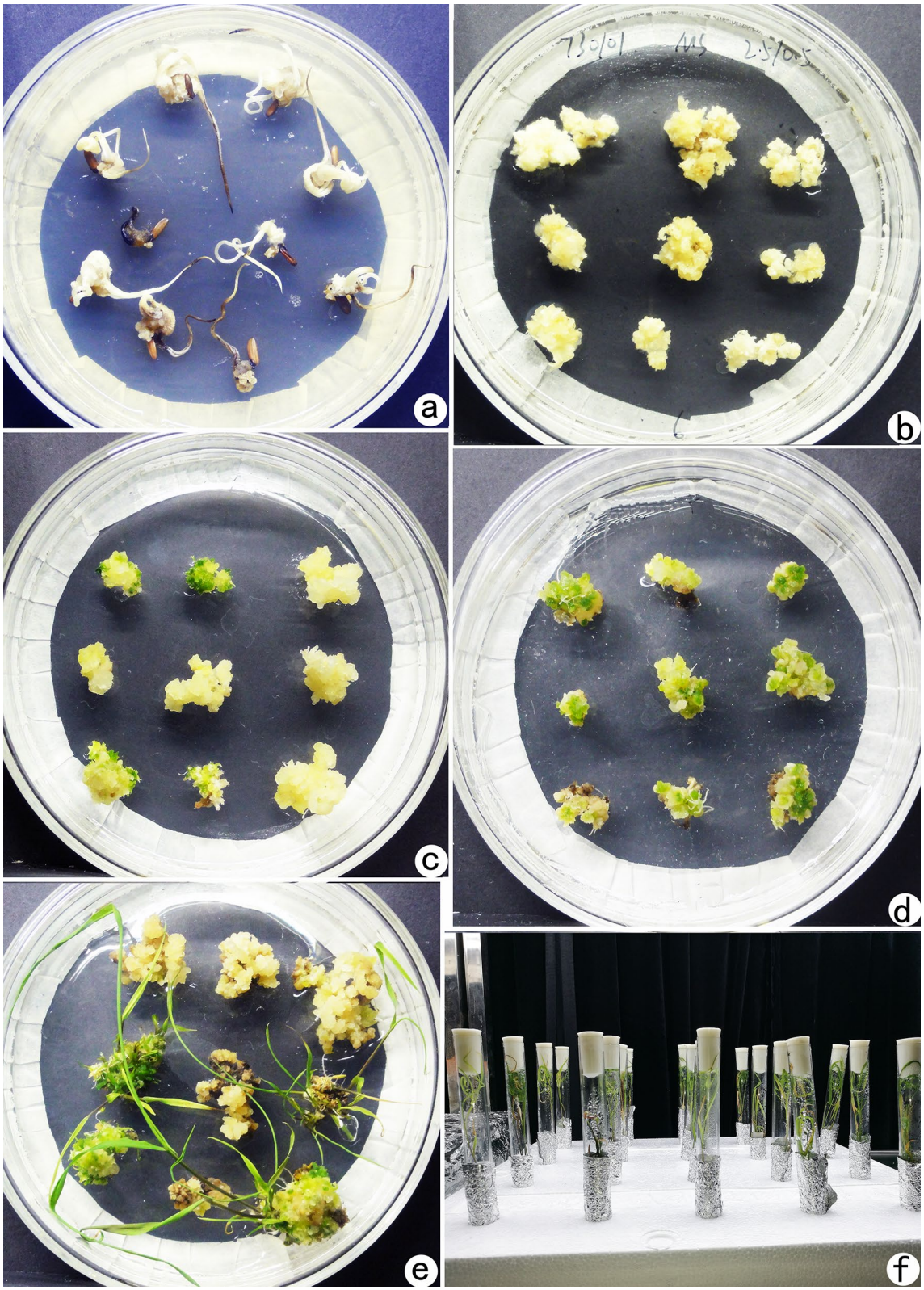


Fig. 1 Callus induction and differentiation in different medium. **a** Callus produced from seeds under NB media. **b** Callus sub cultured for 15 days on NB media. **c**, **d** and **e** Callus differentiated for 7 days and 15 days and 42 days, respectively. **f** Differentiated seedlings

Table 3 Effects of different culture media on differentiation in *Oryza alta Swallen*

Basic medium	Inoculated number	Number of calli regenerated	Differentiation rate (mean ± SD) (%)
NB	560	26	4.74 ± 1.23
MS	564	56	10.15 ± 1.47

Table 5 Comparison of co-culture treatment of callus and buds

Material	Colchicine (mg/L)	No. of treated samples	No. of seedlings	No. of abnormal seedlings	Abnormality rate (%)
Calli	200	60	3	0	0.00
Buds	200	20	6	4	66.67

Table 4 Effects of different concentrations of colchicine on differentiation in Huaye 5

Colchicine (mg/L)	Time of treatment (h)	No. of treatments	No. of seedlings survived	Survival rate (%)
0 (CK)	24	49	4	8.16
	48	50	1	2.00
200	24	63	2	3.17
	48	54	0	0.00
400	24	55	2	3.64
	48	67	0	0.00
600	24	54	7	12.96
	48	63	0	0.00
1000	24	65	5	7.69
	48	56	0	0.00

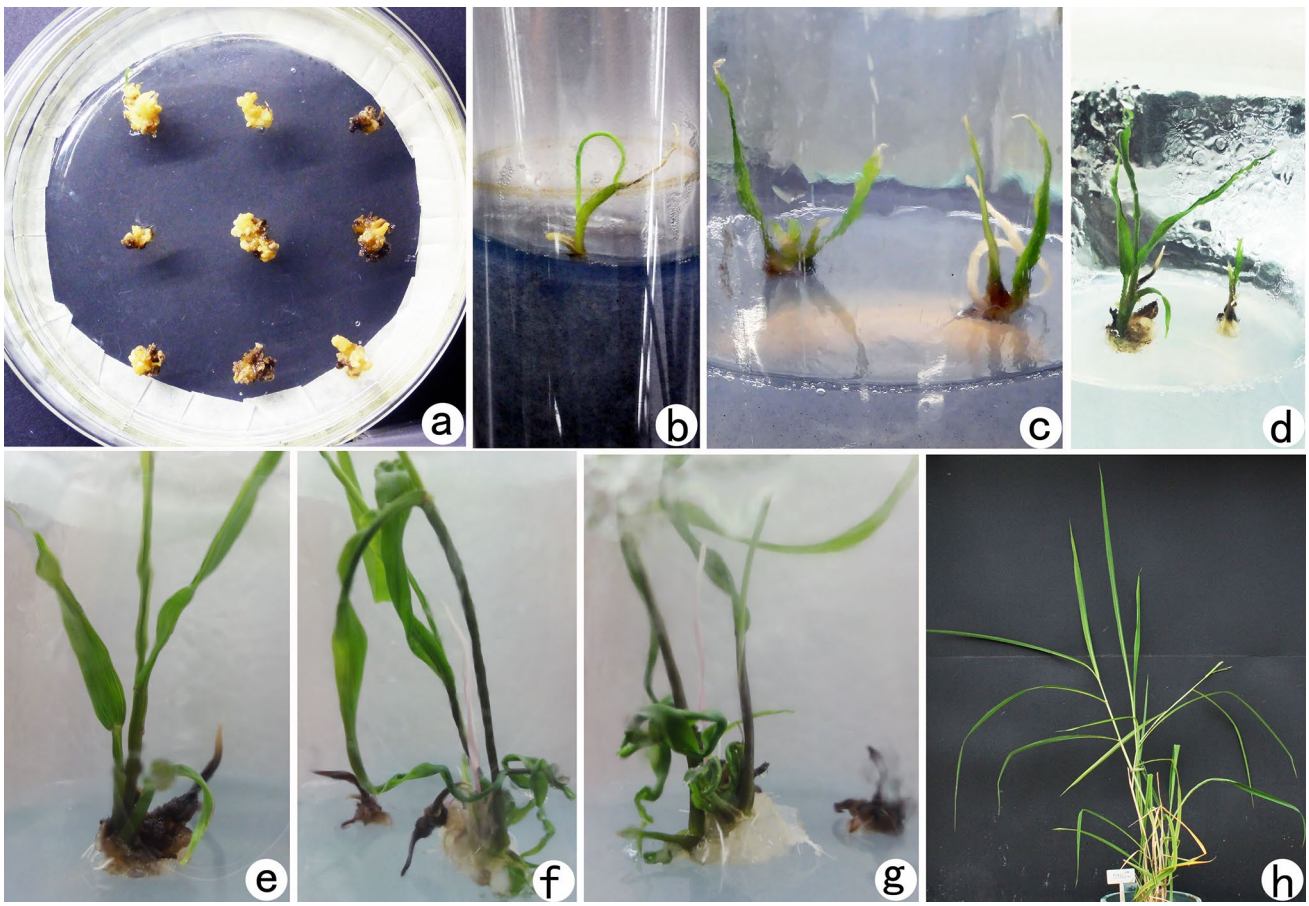
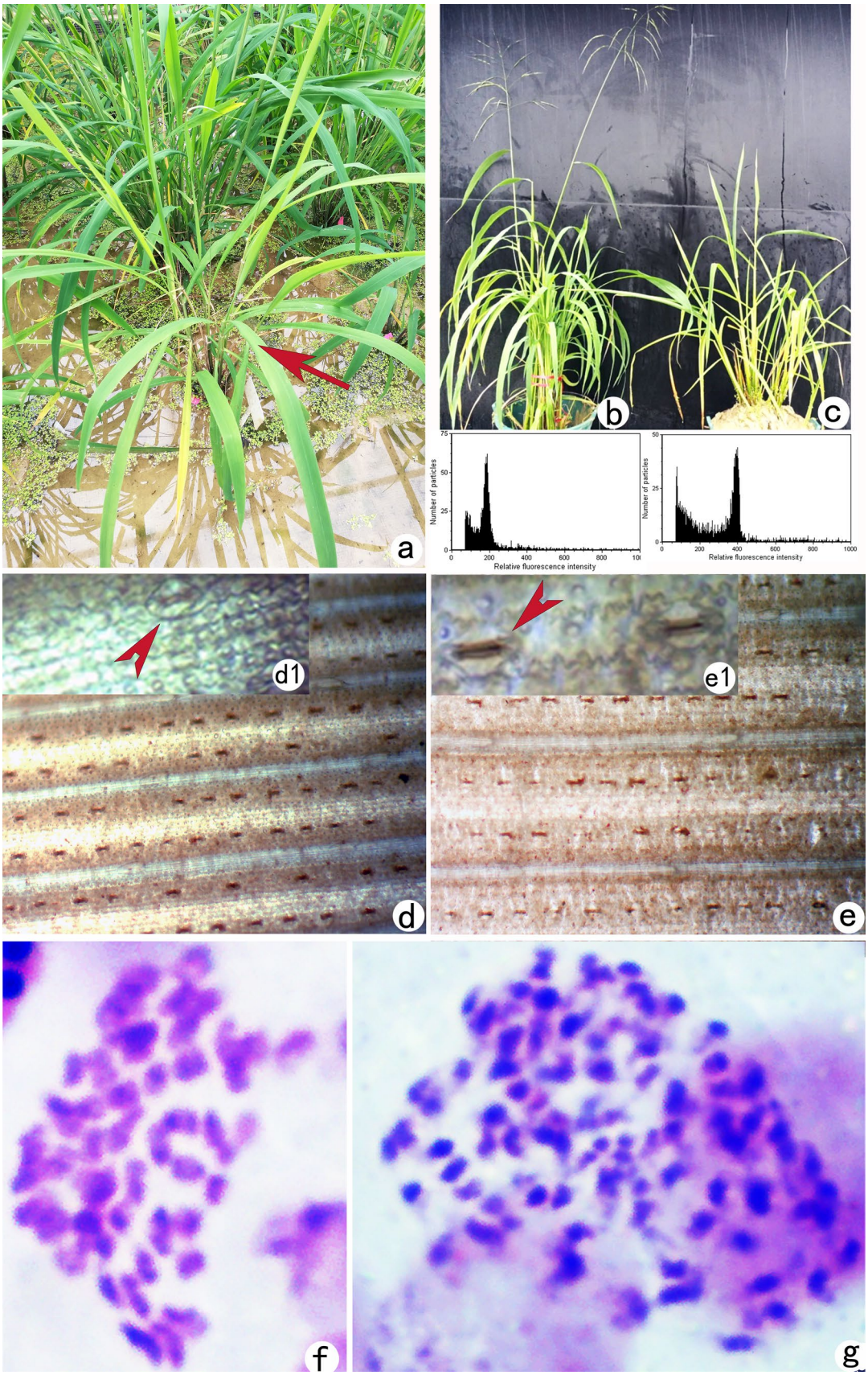


Fig. 2 Callus or buds of Huaye 5 and its auto-allotetraploid co-cultured in a liquid medium containing colchicine. **a** and **b** are calli co-cultured in liquid medium containing colchicine for 5 days and 15 days, respectively. **c** and **d** are buds co-cultured in a liquid medium containing colchicine for 9 days and 27 days, respectively. **e**, **f** and **g** are the different abnormal seedlings from the buds co-cultured in liquid medium containing colchicine. **h** Auto-allotetraploid plant


a
b
c
Number of particles
Relative fluorescence intensity
Number of particles
Relative fluorescence intensity
d1
d
e1
e
f
g

◂**Fig. 3** Seedlings, stomata and chromosome counts comparison between Huaye 5 and its auto-allotetraploid. **a** Huaye 5 (control) and auto-allotetraploid seedling (arrow). **b** Huaye 5 plants and its flow cytometric analysis results. **c** Auto-allotetraploid plant and its flow cytometric analysis results. **d** Stomata of Huaye 5; d1 indicated the amplification of stomata in Huaye 5 (arrow head). **e** Stomata of auto-allotetraploid; e1 indicated the amplification of stomata in auto-allotetraploid (arrow head). **f** Chromosomes of Huaye 5 (2n = 48). **g** Chromosomes of auto-allotetraploid (2n = 96)

stomata under microscope (Motic) using × 40 objective. (2) The flow cytometry analysis. About 20–30 mg of young leaf tissue of each sample was ground on a Petri dish containing 1 mL of icy marry buffer for obtaining the nuclear suspension. 400 µL of extraction buffer was added and the leaf pieces were cleaved with a sharp razor blade, and then added 1600 µL of staining buffer and the cell debris was filtered out with a 500-mesh filter and transferred the filtered nuclei into the glass detection tube. The samples were analyzed after 5 min of incubation in flow cytometer and the results were captured (Dolezel 1997). (3) Chromosome counting was done according to Parera and Dahanayake (2016). Young root tips of the samples (about 0.5 cm) were taken and dipped in 0.002 mol/L 8-hydroxyquinolin for 2 h. Then, they were washed with distilled water and kept for 18 h in ethanol (95%) and acetic acid with a ratio of 3:1 (V/V), respectively. Again, root tips were washed with distilled water and put into 1 N HCl solution. They were kept in water bath for 15 min at 65 °C temperature. After hydrolysis, root tips were rinsed with distilled water for 10 min and cut to obtain shorter root tips about 1.5 mm. The slides were prepared according to the squash technique and stained with 2% Giemsa. The number of chromosomes were observed and counted under microscope (Motic BA200).

Young stems of differentiated seedlings and Huaye 5 (control) were collected to observe transverse sections of stems using WE-CLSM (whole-mount eosin B-staining confocal laser scanning microscopy) according to Li et al. (2017). After fixation with FAA followed by a series of dehydration, the stem samples were cut by hand in about 1 mm diameter. The sections were transferred to a microscopic slide, stained with 10 mg/L eosin B ($C_{20}H_6N_2O_9Br_2Na_2$, FW 624.1, a tissue stain for cell granules and nucleoli) solution (dissolved in 4% sucrose) for 5 min at room temperature and covered with a slide cover and then observed using the Leica SPE laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany).

Results

Breeding procedure of Huaye 5 from *O. alta*

About 500 g seeds of *O. alta* were harvested during 2007 from our farm at South China Agricultural University, Guangzhou (Supplementary Fig. 1a). These seeds were divided into two parts, and radiated by Co^{60} using a dose of 320 Gy and 350 Gy in 2009. All seeds radiated by 350 Gy were died, and about half of the seeds germinated that were treated by 320 Gy, and about 300 seedlings were transplanted into the field (Supplementary Fig. 1b). Fortunately, one mutant plant was found in M_2 in 2010, which flowered during September in Guangzhou, Guangdong. The plant was self-crossed for more than ten generations, which displayed significant differences in heading date and seed setting compared to its parent line, *O. alta*, and was designated as "Huaye 5" in 2016 (Supplementary Fig. 1c–e). The heading date and seed setting of Huaye 5 were 125 days and 64.93%, while 220 days and 12.77% in *O. alta*, respectively (Table 1). Huaye 5 is a new line, which was developed from *O. alta*, and could be planted two times during a year, i.e. early and late seasons, in Guangzhou (23°16′N, 113°8′E), China. However, its parent line, *O. alta* is sensitive to photoperiod, and could be planted once in a year in Guangzhou, i.e. if planted on 1st March, it would flower during October.

Callus induction, proliferations and regeneration

We compared five basic media and two hormones, and investigated the callus induction rate in different media and hormones combinations. The results showed that induction rate without 6-BA hormone was the lowest with mean induction rate of 2.82%, while the mean induction rate of hormone combination 2,4-D (2.5 mg/L) + 6-BA (1.0 mg/L) was the highest (14.51%). B5 basic medium produced the lowest among other media with a mean induction rate of 6.68%, while NB basic medium had the highest, with a mean induction rate of 12.41%. Medium and hormone combination of NB + 2,4-D (2.5 mg/L) + 6-BA (1.0 mg/L) had the highest induction rate of 20% (Table 2, Fig. 1a), suggesting that it was a suitable combination for callus induction of mature embryos of Huaye 5.

Using the medium NB + 2,4-D (2.0 mg/L) + 6-BA (0.2 g/L) for subculture, the callus proliferation improved and the number of calli increased significantly (Fig. 1b). The proliferation of callus was followed by differentiation using MS medium + KT (2.0 mg/L) + NAA (0.2 mg/L) and NB + KT (2.0 mg/L) + NAA (0.2 mg/L), respectively. Two media were compared with the same concentration of hormones, and the results showed that callus differentiation rate

of Huaye 5 was high on MS medium with mean differentiation rate of 10.15% as compared to NB medium (Table 3, Fig. 1c–f), suggesting that MS medium is more suitable for callus differentiation of Huaye 5.

Induction of auto-allotetraploid in Huaye 5 by colchicine

Calli of Huaye 5 were immersed in different colchicine concentrations, including 200 mg/L, 400 mg/L, 600 mg/L and 1000 mg/L, for 24 and 48 h and then the differentiation rate was investigated (Table 4; Fig. 2a, b). Based on the survival rate, the treatment of 600 mg/L colchicine for 24 h displayed the highest differentiation rate (12.96%), while 400 mg/L, 600 mg/L and 1000 mg/L over 48 h had zero differentiation rate. The treatment of 600 mg/L colchicine for 24 h is the best protocol for inducing polyploidy in Huaye 5 by colchicine.

Three seedlings were obtained from calli that were co-cultured in a liquid medium containing colchicine, and six seedlings were developed from buds. High abnormality was observed in buds treatment with a rate of 66.67% compared to callus treatment with zero abnormal rate (Table 5; Fig. 2c–g).

Identification of ploidy levels of plants

The DNA quantity of 27 plants was detected by flow cytometry for polyploidy analysis, and six plants displayed autoallopolyploid like characters i.e., significant variations including larger stomata than Huaye 5 (Figs. 2h, 3b–e; Supplementary Fig. 2a–e). The chromosomes of one plant were counted as 96, while 48 in Huaye 5 (Fig. 3f, g), therefore that plant was identified as auto-allotetraploid. Moreover, we observed the transverse section of the stems of Huaye 5 and its auto-allotetraploid plant using WE-CLSM, and found significant differences between them, such as many inclusions in the parenchyma cells surrounding vascular bundle of the auto-allotetraploid rice but few in Huaye 5 (Fig. 4).

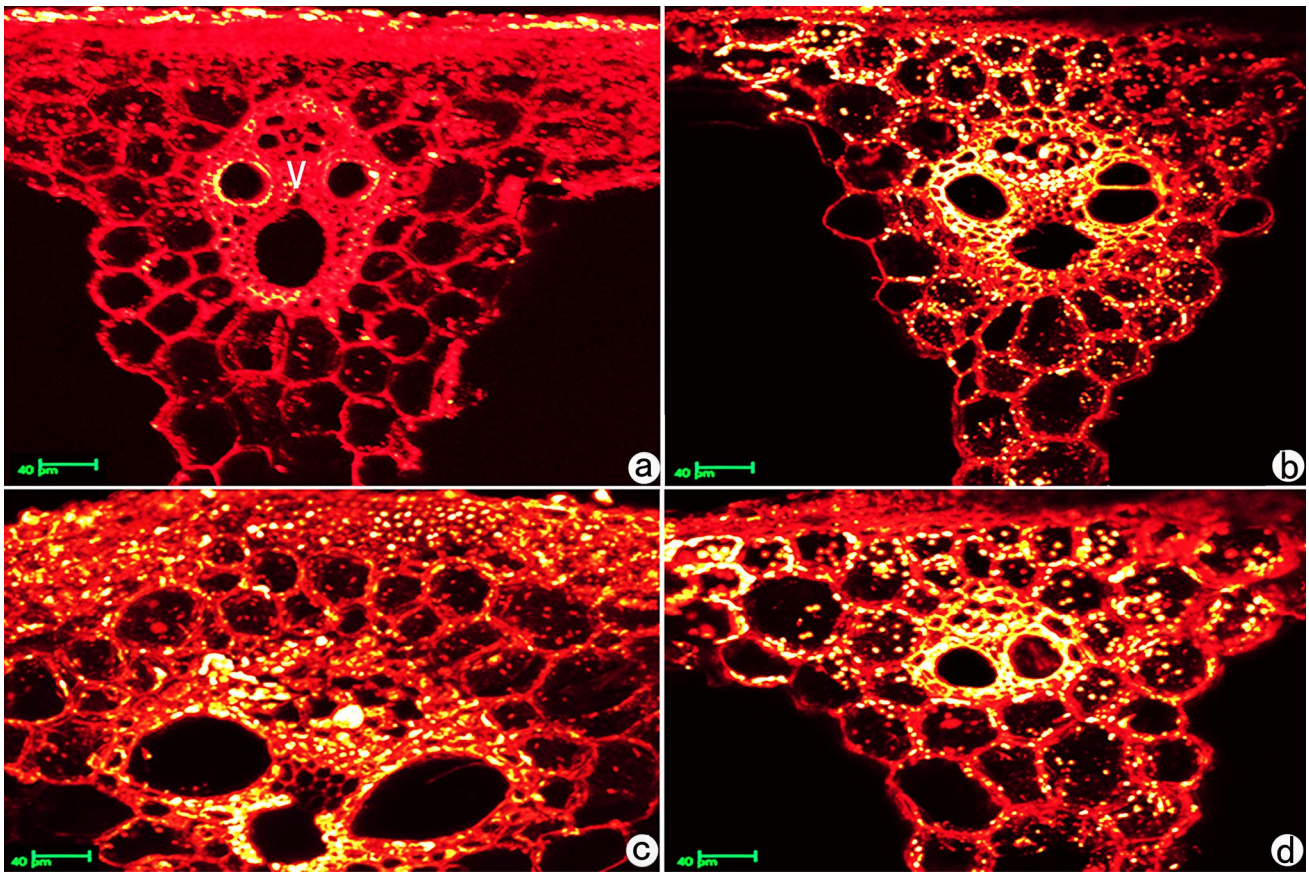


Fig. 4 Transverse section of the stem of Huaye 5 and its auto-allotetraploid plant. **a** Huaye 5. **b–d** auto-allotetraploid plant. Many inclusions were observed in the parenchyma cells surrounding vascular bundle in auto-allotetraploid germplasm but little in Huaye 5. "V" indicates vascular bundle

Discussion

Factors affecting tissue culture in the newly developed wild rice line from *Oryza alta* Swallen

Genotype, induction medium and hormones have a great effect on tissue culture system of different plant species (Ni et al. 2007; Liu et al. 2010; Zhang et al. 2010; Huang et al. 2019). Genotype showed a significant effect on the callus induction and plant regeneration in *japonica* and *indica* rice varieties (Abe and Futsuhara 1986). Similarly, this phenomenon also happened in *O. alta* and its new line Huaye 5. Callus induction and differentiation rate was lower in the new line of *O. alta* than that of *O. alta* reported by Liang et al. (2014). We considered that the low induction rate might be related to the genotype of *O. alta*. To establish a suitable tissue culture system for *O. alta*, different culture media and hormones were used and the results showed that different media and ratio of hormones have great influence on the callus induction and differentiation. MS medium with NB + 2,4-D (2.0 mg/L) + 6-BA (0.2 mg/L) performed the best compared to other media. Our results were consistent with the findings of Ai et al. (2012), who found that MS medium with 2.0 mg/L 2,4-D and 0.5 mg/L 6-BA was most suitable for induction and differentiation. MS supplemented with 2.0 mg/L NAA and 0.5 mg/L IAA was the best for the embryoid formation and the plantlet regeneration (Ni et al. 2007). Liu et al. (2010) revealed that MS containing 0.4 mg/L KT, or 1/2, MS containing 1.0 mg/L KT and 1.0 mg/L NAA was able to promote the differentiation of embryogenic callus effectively. This study showed that 2,4-D hormone is a necessary factor for callus initiation and both 2,4-D and 6-BA hormones had significant effects on callus induction in *O. alta*. Addition of 6-BA promoted the callus induction from mature embryos, which was in line with the results of Ni et al. (2007) when leaves of *O. alta* were used as explants to induce calli without the addition of cytokinin. Li et al. (2005) also revealed that addition of 6-BA in culture media produced the highest regeneration rate.

Factors affecting the induction of auto-allotetraploid in the newly developed wild rice line from *Oryza alta* Swallen

Colchicine-induced tetraploids have proven to be fertile and can be crossed sexually with another diploid of different species to produce hybrid-triploid plants (Aleza et al. 2010; Urwin 2014), and has been successfully employed to induce chromosome doubling in many plants. In this study, we developed a new germplasm with high fertility from *O. alta*, and found that colchicine is effective to induce the auto-allotetraploid in new line. Plant survival rate decreased significantly with the increase in colchicine concentration as well as prolonged treatment duration. Many studies showed that treatments with higher concentrations of colchicine for longer periods of time sharply reduced the survival rate (Beck et al. 2003; Xu et al. 2011; Chen et al. 2011; Parera and Dahanayake 2016; Mustafa et al. 2017; Zhou et al. 2017). Furthermore, similar effects of high doses of colchicine on growth inhibition were reported by Rauf et al. (2006). Higher concentrations of colchicine take long time for callus regeneration in rice (Nilanthi et al. 2009). Colchicine produce large amount of toxins which could cause the irreversible death of the callus cells (Xu et al. 2011). Here, 600 mg/L colchicine treatment for 24 h was found to be suitable for *O. alta* differentiation. We observed different phenotypic abnormalities in some germinated seedlings. Therefore, it is worth mentioning that not all the seedlings obtained by colchicine induction survived, some seedling died and some sustained different degrees of phenotypic abnormalities. We concluded that the occurrence of abnormalities in cultured cells has been attributed to a level of 2,4-D in culture medium. High levels of 2,4-D in cultured plant cells cause meiotic abnormalities (Bayliss 1973). Shoot regeneration was slow in colchicine treated media compared to non-treated regeneration media. Our findings were consistent with the study of Parera and Dahanayake (2016) who concluded that high level of 2,4-D affects shoot regeneration ability especially with higher concentrations and longer durations that inhibited more seriously. Interestingly, we found many inclusions in the parenchyma cells surrounding vascular bundle in auto-allotetraploid rice but few in Huaye 5, suggesting a different metabolizing system in the auto-allotetraploid plant, which is worthy of further studies.

Conclusion

Here, we developed a new wild rice line with high fertility, Huaye 5, from *Oryza alta* Swallen, and established a tissue culture system for new line. The best protocol for Huaye 5 was found to be NB + 2,4-D (2.5 mg/L) + 6-BA (1.0 mg/L) for induction, with MS as a medium for differentiation and the callus treatment with 600 mg/L colchicine for 24 h to obtain auto-allotetraploid of Huaye 5. The present study provided a new germplasm (Huaye 5) of *O. alta* to develop auto-allotetraploid for crossing with autotetraploid or neotetraploid rice lines.

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Author contributions XDL conceived and designed the experiments. FNS, XDL, MQS, LSZ and NK wrote the paper. LSZ, FNS, MQS and JWW performed the experiment and analyzed the data. XDL and JWW developed Huaye 5. All authors read and approved the final version of manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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