Journal of Plant Biochemistry and Biotechnology (April–June 2022) 31(2):370–379
https://doi.org/10.1007/s13562-021-00689-7

ORIGINAL ARTICLE

Production and characterization of somatic hybrids between rice (*Oryza sativa* L.) and reed (*Phragmites communis* Trin.) obtained by protoplast fusion

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Received: 29 September 2020 / Accepted: 21 June 2021 / Published online: 2 July 2021

Abstract

Protoplasts were isolated from cell suspension cultures of rice (*Oryza sativa* L.) and reed (*Phragmites communis* Trin.), fused by a PEG–high pH-high Ca^{2+} method and cultured on Ry-2 medium to obtain minicalli. Hybrids were selected using lack of dividing ability of reed protoplast and plant regeneration ability of rice protoplast. Regenerated plantlets showed intermediate traits of both or either of parents and the leaves showed variant traits in the shape. Especially, mature plants showed intermediate traits of parents or variant traits in reproductive organs. Selected hybrid plants did not have fertility because pollen and egg cells developed abnormally. Chromosome counting of hybrid plants revealed their cells were tetraploid (2n = 4x = 72) and aneuploidy. The numbers of the chromosome were different not only between the hybrid plants but also within the individual one. However, the peroxidase, the random amplified polymorphic DNA and the simple sequence repeat analysis indicated that regenerated plants were somatic hybrids. The somatic hybrids obtained in this study suggested the useful information for the production of intergeneric hybrids of rice and other plants, and for the characterization of the traits in hybrids.

Keywords Somatic hybrids · SSR · *Oryza sativa* · *Phragmites communis* · Intermediate traits

Abbreviations

2, 4-D	2, 4-Dichlorophenoxyacetic acid
NAA	α-Naphthaleneacetic acid
BA	6-Benzyladenine
PEG	Polyethylene glycol
MS	Murashige and Skoog (1962)
B5	Gamborg et al. (1968)
Ry-2	Yamada and Zhi-qi (1986)
RAPD	Random amplified polymorphic DNA
SSR	Simple sequence repeat

Introduction

Protoplast fusion permits the hybridization of sexually incompatible species of the higher plants and the transfer of cytoplasmic genomes between such species. Thus, the introduction of the desirable genes from other species would lead to an increase of the plant gene pool (Smyda-Dajmund et al. 2016; Tomiczak et al. 2017; Wang et al. 2001).

Since the first interspecific hybrid was obtained by protoplast fusion (Carlson et al. 1972), many somatic hybrids have been produced. Not only interspecific but also intergeneric somatic hybrids have been produced in various genera, such as *Arabidopsis* (Gleba and Hoffmann 1989), *Brassica* (Kirti et al. 1992; Ishikawa et al. 2003), *Nicotiana* (Laiqur et al. 1998), *Solanum* (Guo et al. 2010; Iwamoto 2007), *Cucumis* (Jarl et al. 1995), *Citrus* (Dambier et al. 2011; Teresa, 2018), *Lycopersicon* (Guri et al. 1988; O'Connel and Hanson 1987) and *Gentiana* (Tomiczak et al. 2015; Wójcik and Rybczyński 2015). Protoplast fusion has been used for crop improvement such as breeding of stress-resistance lines (Collonnier 2001; Luthra et al. 2019) and combination of cytoplasmic genes (Aleza

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et al. 2016; Trabace et al. 1996) in various species, particularly in *Solanaceae* and *Brassicaceae*. However, a few hybrid cereals, a particularly important group of plants, have been obtained by this technique due to the lack of procedures available for the efficient plant regeneration from protoplasts. Successful reports of somatic hybrids in *Gramineae* have been limited to interspecific hybrids in *triticum* (Aixia and Guangmin 2004; Fengning et al. 2003), intergeneric hybrid of wheat and maize (Chunhui et al. 2003), intergeneric hybrid of rice and mangrove grass (Kumar et al. 2018) and interspecific hybrids in rice (Hayashi, 1988, 1989).

The methods for plant regeneration from protoplast-derived calli, were developed in many plants. Also different protoplast fusion experiments were undertaken where calli (Guan et al. 2010; Jarl et al. 1995) and plants (Fiuk and Rybczyński 2008; Yamaguchi and Shiga 1993) were obtained. The asymmetric method by which protoplast of one parent was inactivated before fusion has been widely used for selection of the somatic hybrids (Forsberg, 1998; Wei et al. 2001). The characterization of calli and plants was based on morphological traits (Waara and Glimelius 1995), isoenzymes (Fengning et al. 2003), GISH (Collonnier et al. 2003; Fu et al. 2004), DNA marker (Fu et al. 2004; Mizuhiro et al. 2001), cytoplasmic hybrid (Cappelle et al. 2007; Trabace et al. 1996) and genetic analysis of the progeny (Rakosy-Tican et al. 2015). Developing the efficient methods for selection of the hybrid and for their characterization would improve the chances of obtaining desirable interspecific somatic hybrids.

Progress in regenerating plants from protoplasts of rice (*Oryza sativa* L.) (Feng et al. 2006; Hayashi et al. 1989) has made the application of this technique available to cereals. Mature hybrid plants between rice (*Oryza sativa* L.) and wild *Oryza* species were obtained to incorporate useful traits of the wild species into rice (Hayashi et al. 1989). Hybrid calli between rice and mangrove grass (*Myriostachya wightiana*) showed better tolerance to salt stress than control (Kumar et al. 2018). Plantlets were regenerated from somatic hybrids of rice and barnyard grass (*Echinochloa oryzicola* Vasing) but mature plants were not obtained, presumably due to the genetic distance between these members of different subfamilies of the *Gramineae* (Terada, 1987). The reed (*Phragmites communis* Trin.) possesses agronomically useful traits such as abiotic stress tolerance. However, the use of reed for rice improvement has been impeded by the sexual incompatibility (Wang et al. 2001).

Therefore, we attempted to use protoplast fusion by using the lack of dividing ability of protoplast in reed and regeneration ability of green plants in rice as a method to incorporate useful traits of reed into cultivated rice. In this study, we successfully produced for the first time intergeneric hybrid plants between rice (*Oryza sativa* L.) and reed (*Phragmites communis* Trin.) by protoplast fusion, and characterized their hybrid nature through morphological, cytological and molecular analysis.

Materials and methods

Plant materials and Cell suspension culture

Mature seeds of *O. sativa* L. '554' (2n = 24) and *Phragmites communis* Trin. 'B-26' (2n = 48) were used in this study. The calli were induced from seeds of these plants on Murashige-Skoog (MS) medium containing 2 mg/L 2, 4-D and subcultured on Gamborg (B5) solid medium containing 2 mg/L 2, 4-D. The calli were transferred onto B5 liquid medium and suspension culture was performed for 2–3 months. Subculture was maintained by adding fresh medium every 2–3 d for more than 3 months at 25°C.

Protoplast isolation and fusion by PEG

The minicalli consisting of vigorous spherical cells were collected by centrifugation at 500 × *g* for 3 min, and treated with solution containing 0.1% Pectolyase Y–23, 1% cellulase Onozuka RS, 0.5% $CaCl_2 \cdot 2H_2O$ and 0.5 mol/L glucose for 4–5 h. The protoplasts of both species suspended at the density of 1×10^6 protoplasts/mL were mixed at the ratio of 1:1.

Protoplast fusion was carried out according to the polyethylene glycol (PEG)–high pH-high Ca^{2+} method on the Petri dishes 6 cm in diameter. The equal volume of 33% PEG solution (molecular weight 1540) was added to the protoplast suspension. 90 min later, it was washed 5 times with high pH-high Ca^{2+} solution. Finally, it was washed with culture medium. The density of the protoplast was adjusted to 1×10^6 protoplasts/mL by the addition of the culture medium. Then, it was incubated in the dark at 25°C. The number of the fused cells was determined as the number of cells having 2 or 3 nuclei after staining with acetocarmine.

Fused protoplast culture and plant regeneration

While the fused protoplasts between rice and reed were cultured in Ry-2 medium (Yamada and Zhi-qi 1986), fresh medium with decreased osmotic concentration was supplemented every 7–10 days. When minicalli were formed, they were cultured on the same solid medium and visible colonies were obtained. The calli 2–3 mm in diameter were transferred onto MS medium containing 2 mg/L 6-BA and 0.5 mg/L α-NAA to regenerate plant. Regeneration

efficiency (%) of calli was calculated as: (No.of calli showing plant regeneration/No.of calli tested) × 100.

Morphological observation

All the plants regenerated from a callus were considered belonged to the single hybrid line (ex.H-116).

Morphological traits of regenerated plantlets such as root shape, the number of buds per callus, leaf color, rhizome and the number of buds per coleoptile were examined. Mature plants of each genotype were evaluated for leaf shape, structure of glume, pistil and stamen shape, and pistil and stamen numbers per spikelet.

Chromosome number

Chromosome preparations in root tip cells of parents and hybrid plants were made by the enzymatic maceration/air drying method (Fukui and Iijima 1991). They were spread onto glass slides, stained with Giemsa solution and then chromosome numbers were examined.

Isozyme, RAPD and SSR analysis

The electrophoretic patterns of the isozyme peroxidase of parents and hybrid plants were analysed using 200 mg of young leaves. After homogenization of the tissues in 1 mL of extraction buffer and centrifugation, the supernatant was used for polyacrylamide gel electrophoresis and the gels were stained according to Laiqur et al. (1998).

Total DNA was extracted using the method described by Hashizume et al. (1996). In total, 36 primers (OPA, OPB and OPR, Operon Technologies, Alameda, CA, USA) were used to reveal the RAPD between the parents. Five selected primers (OPA-07, OPB-05, OPR-06, OPR-11 and OPR-14) were used to identify hybrids. A thermal cycler (C1000 Touch TM thermal cycler (BIORAD)) was used for DNA amplification. DNA templates were amplified according to Kaneko et al. (2000). The PCR products were electrophoresed on a 1.0% agarose gel.

SSR analysis of the nuclear genome extracted from four hybrid lines was conducted using five selected primers (RM319, RM207, RM101, RM31 and RM246) from twenty four primers. SSR reaction conditions were according to Guo et al. (2007). The PCR products were electrophoresed on 8.0% polyacrylamide gel, and then stained by ethidium bromide. The gels were photographed under UV light.

Results and Discussion

Regeneration of somatic hybrids from fused cells

The minicalli composed of vigorous cells were treated with the enzymes 2 days after subculture. There were few undigested single cells. The color and the shape of cytoplasmic particles in rice protoplasts were different from reed protoplasts (Fig. 1a, b). The fusion efficiency by PEG treatment was 16–20%.

Many of the fused cells initiated the cell division after 5–15 days as the rice protoplast but a few fused cells showed the first cell division 28 d after the culture (Fig. 1c). This shows that the cell division was delayed by the intergeneric cellular recombination. In this culture condition, reed protoplasts didn't divide but remained alive during 30 d. Some minicalli of rice and fused cells showed slight contraction of protoplasm about 14 d after culture. When 0.3 mol/L osmotic medium was added, the minicalli failed to grow. This treatment was repeated twice. When the low-osmotic medium was added at 7 d interval, 3 times as many minicalli as control were obtained (Fig. 1d, e, f). This showed that early minicalli originated from rice protoplast, and the addition of the medium could increase the minicalli formation efficiency of hybrid because the deficiency of the nutrients in the medium limited cell growth.

Minicalli were cultured on solid medium (2 mg/L 2, 4-D) and transferred onto the regeneration medium to regenerate plantlets. As shown in Table 1, rice protoplast generated no green plantlets (3.5% of white plants) and the regeneration efficiency of reed was over 90%, while the regeneration efficiency of hybrid was 32.3%, out of which 9.8% regenerated normal green plants.

When there was no selection marker, the asymmetric fusion method for hybrid selection was used widely (Aixia and Guangmin 2004; Chunhui et al. 2003). We used the genetic complementation with the difference in the division ability of protoplast and the regeneration ability of calli in rice and reed for hybrid selection, showing that the efficiency of fused cells could be increased by controlling the addition period of medium and somatic hybrids could be selected effectively using differences of regeneration ability.

Morphological characteristics of somatic hybrids

Morphological traits of regenerated somatic hybrids were examined (Table 2). Line 1, 34 and 56 had the rhizomes. Line 16 and 116 had no lateral root. Number of buds per callus varied from 5 to 18 in different lines. This was consistent with the other results that in vitro plants had the

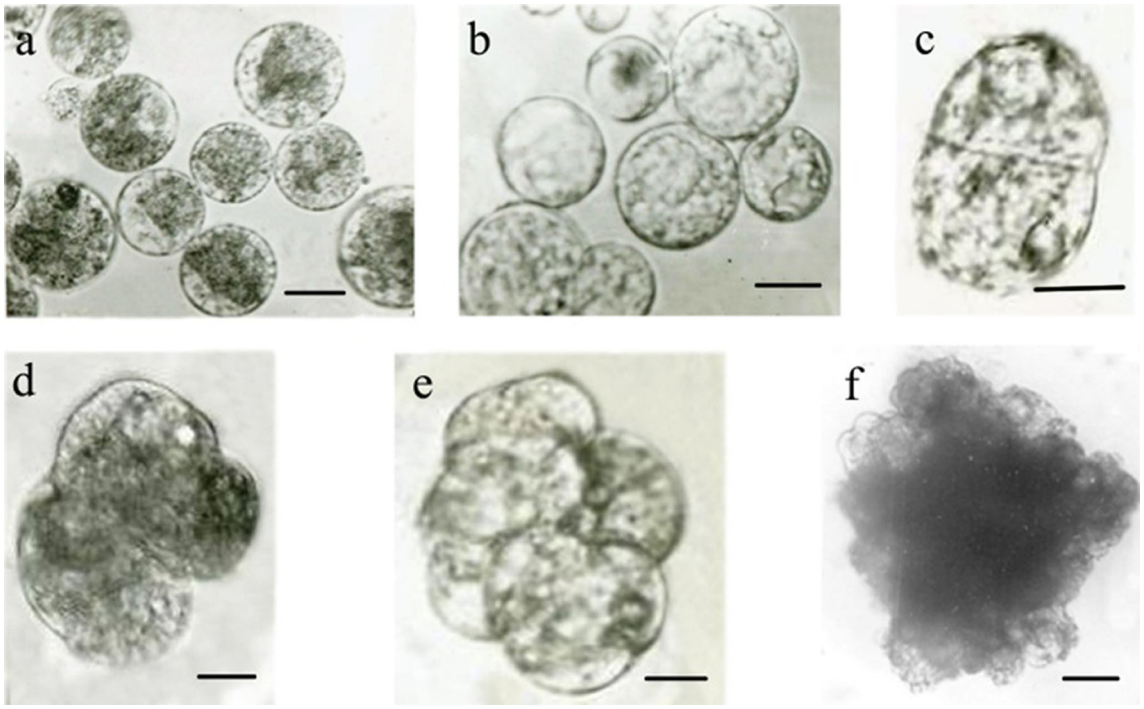

Fig. 1 A sequence of images showing the formation of a minicalli from a fused protoplasts between rice and reed: **a** Rice protoplasts, **b** Reed protoplasts, **c** First cell division, **d** Second cell division, **e** Cell colony formation after 3–4 weeks of culture, **f** Appearance of minicalli that can be seen with naked eye. Bar a–e is 10μm and f is 100μm

Table 1 Regeneration efficiency of minicalli

Fusion partners	Number of calli transferred	Number of calli showing regeneration				Regeneration efficiency (%)
		Total	Green	White	Only root	
Rice*	57	2	0	2	4	3.5
Reed*	32	29	29	3**	-	90.6
Rice + Reed	133	43	13	30	5	32.3

*indicates the callus sub-cultured for 20 months. **indicates some white plants were among green ones

Table 2 Morphology of somatic hybrids between rice and reed

Line	Lateral root[a]	Number of buds/callus	Number of bud/ coleoptile	Shape of glume (length/width)	Length of pedicel (mm)
Rice	++	1–4	1	2.3 ± 0.3	2–5
Reed	–	20–40	1(exceptionally 2–4)	9.8 ± 1.9	5–15
1	+	18	1	3.7 ± 0.6*	4–20
13	+	13	1	3.3 ± 0.4*	2–6
16	–	12	1	3.8 ± 0.8*	3–16
34	+	16	1	3.5 ± 0.4*	3–20
56	+	11	3	3.7 ± 0.8*	2–12
116	–	9	2	3.3 ± 0.6*	3–10

[a] + + and + indicate the many lateral roots and a few lateral roots, respectively

*$P<0.05$

intermediate traits of parents or the traits of one parent (Fork 2001).

Somatic hybrids in pot stage had variant traits or parental traits for leaf margin (Fig. 2a, b, c) and leaf surface (Fig. 2d, e, f). Leaf margin of somatic hybrids was different from both rice and reed. The dentation of rice and reed was erect and prostrate respectively, however the margin of hybrids was obtuse dentate. Somatic hybrids had no hairs on the leaf surface like reed and their leaves were thicker than parents.

Characteristics of reproductive organs were notable. As shown in Table 2, evident differences in the appearance of glume were observed between different hybrid plants. The length/width ratio of glume of rice and reed were 2.3 and 9.8, respectively. However, the length/width ratio of the hybrids was over 3.3, the intermediate trait between the parents. (Fig. 3i) Rice has no awn in glume, whereas reed has. Somatic hybrids showed awns of varying length. The surface of glume is rough in rice and smooth in reed. Hybrids showed intermediate characteristics of the parents. Length of pedicel varied from the line to the line: line 1, 16 and 34 had longer pedicel than rice, line 53 and 116 had intermediate size pedicel and line 13 had similar pedicel to rice. The glume of hybrids was thicker than rice. In addition, variants were observed in the structure and the shape of glume. Line 1 and 16 had glumes with more than 3 sepals, some glumes of these plants had only lemma or palea.

Next, the stamen numbers per spikelet were observed (Table 3). Rice and reed have 6 and 3 stamens in one spikelet respectively, whereas lines showed many variations. Line 16, #34 and 116 showed 30%, 12.5% and 30% variants, respectively. Most variants had mid stamen numbers of parents, but some lines including lines 34 showed 7 different stamens in length. However, the lengths of stamens in a spikelet were different. Among them, some were longer than pistil, others were same as pistil and there were even ones 1 mm shorter than pistil. The structure of stigma varied severely. As shown in Fig. 3a, b, rice and reed had two branched feathery stigmas. However, 30–40% of flowers of the somatic hybrids had more stigmas compared to parents. Especially, line 1 and 116 had 5 stigmas and line 34 had 7 stigmas. Then, there were many variations including different sizes and hair of stigmas. The pistils with three stigmas were different in sizes and positions of stigmas.

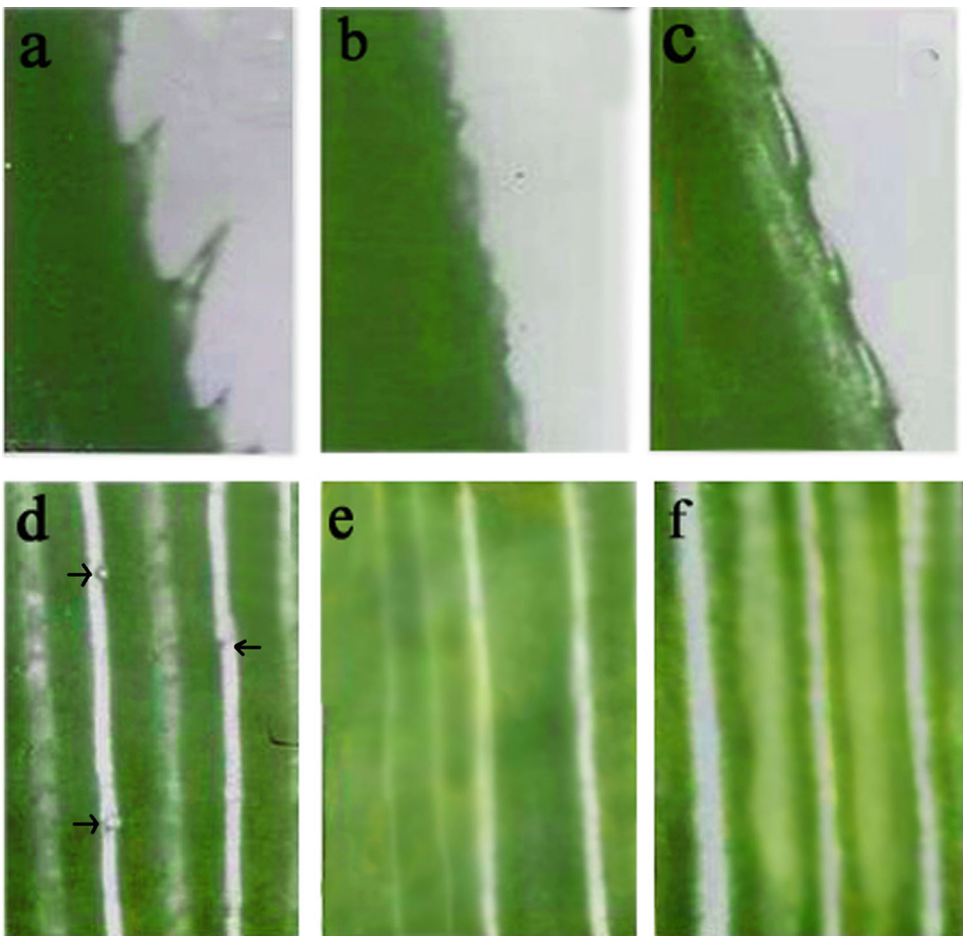

Fig. 2 Characteristics of leaf shape of pot plant: **a, b, c** Leaf margin (a Rice, b Hybrid, c Reed), **d, e, f** Upper leaf surface (d Rice, e Hybrid, f Reed). Rice-specific hairs on the leaf surface are indicated by arrowhead

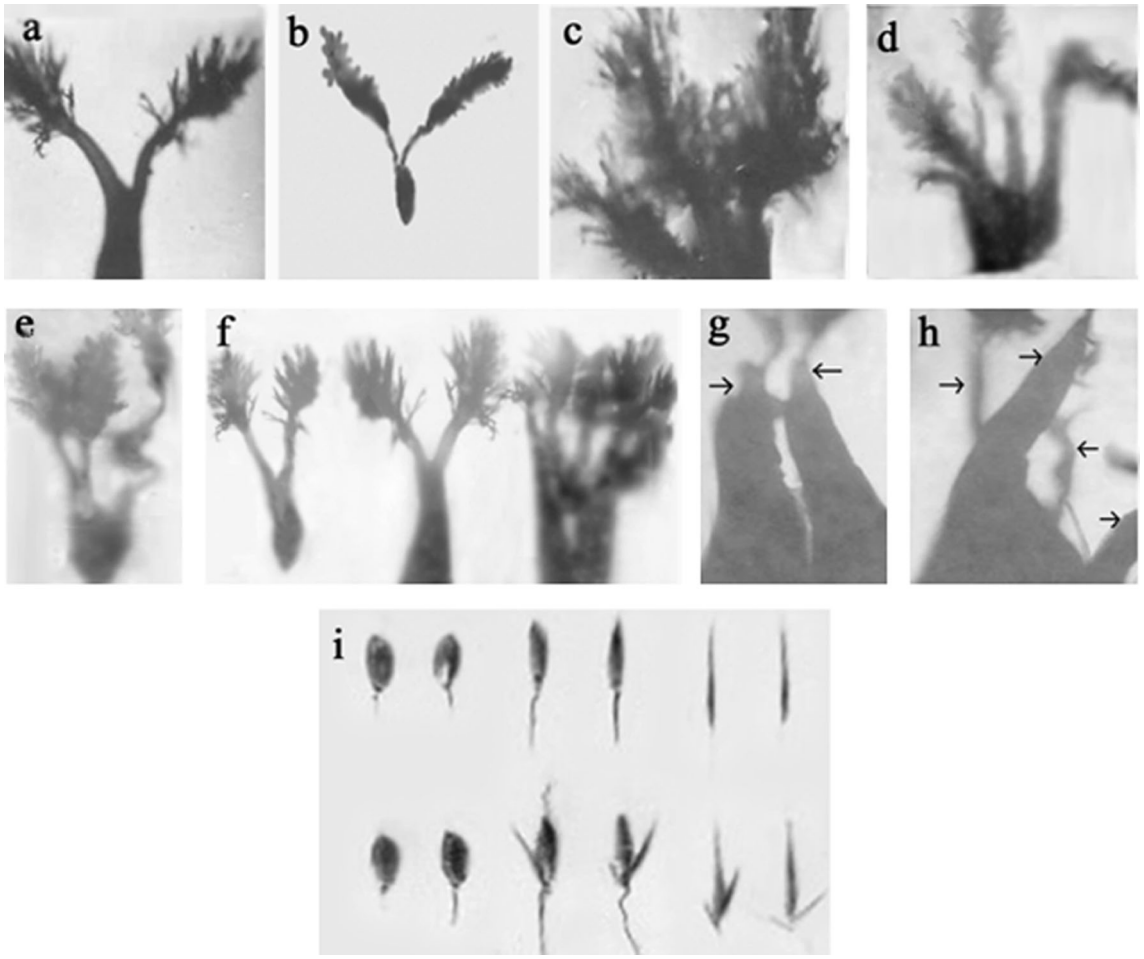

Fig. 3 Phenotypic characteristics of reproductive organs: **a** Pistil of rice, **b** Pistil of reed, **c, d** Various pistil shapes of hybrids, **e** Two pistils of one glume, **f** Three different pistils of one glume, **g** Two ovaries developed in a glume, **h** Four ovaries developed in a glume. The ovaries developed are indicated by arrowhead, **i** Shape of glume. Left is rice glume, middle is hybrid glume and right is reed glume

Table 3 Stamen, stigma and pistil number per glume

Line	Number of plants observed	Stamen number					Variation *(%)	Number of plants observed	Stigma number					Variation * (%)	Number of plants observed	Pistil number				Percentage of plants showing more than one pistil per glume (%)
		3	4	5	6	7			2	3	4	5	7			1	2	3	4	
Rice	54	36	8	3	54	1	0.0	63	63	9	2	1	1	0.0	43	43	19	32	13	0.0
Reed	36		6	2	22		0.0	47	47	13	3	1		0.0	66	2	4	1	3	97.0
1	33		6	7	18		33.3	41	29	10	2			29.3	39	35	7	7		12.8
16	26		12		42		30.8	46	29	21	7			38.0	42	35	5			16.7
34	48				45		12.5	29	17					41.4	32	27	17			15.6
116	65						29.2	96	67					30.2	106	79				25.5

*indicates the ratio of individuals with stamen and stigma number different from parents

Number of pistil per spikelet was examined. The rice has only one pistil per glume and 97.0% flowers of reed have 2–4 pistils. 12.8–25.5% of flowers of hybrids had more than one pistil (Table 3). Pistils were also different,

Table 4 Chromosome number of hybrids

Line	No. of cells examined	Chromosome number	Tissue
Rice	29	24	Root*, callus
Reed	43	48	Root*, callus
1	27	62–70	Root*, callus
13	12	55–61	Callus
16	24	44–52	Root*, callus
22	18	41	Callus
34	22	56	Callus
53	21	63–72	Callus
56	17	64–68	Root*, callus
116	26	50–57	Root*

*indicates the root of regenerated plants

for example, 3 pistils in a glume had two or more branched stigmas (Fig. 3c, d, e, f). Somatic hybrids failed to produce normal pollen and glumes remained open. When pollinated with normal rice, hybrids didn't develop seed. However, some ovaries of these somatic hybrids developed without pollination. When 2 ovaries developed in a glume, development of two ovaries were similar, but when 4 ovaries in a glume developed, development of ovaries were different (Fig. 3g, h). In addition, there were glumes in which 2 ovaries were merged or ovaries were longer 1.2–1.3 times than glume.

Chromosome number of somatic hybrids

As shown in Table 4, rice and reed had 24 and 48 chromosomes, respectively (Fig. 4a, b). The chromosome number showed the difference between hybrid plants and within individual hybrid plants. White plant lines possessed 24 chromosomes like rice, whereas green plant lines possessed 41 to 72 chromosomes. The differences in chromosome numbers were found within individual hybrid plants of line 1 and 56. Especially, line 1, 16, 53 and 56 showed nearly 72 chromosomes while line 13, 22 and 34 had less than 72, meaning aneuploidy (Fig. 4c, d, e, f), suggesting that all the green plants were somatic hybrids. It was already found that the chromosomes were deleted during the culture of somatic hybrids (Fengning et al. 2003; Suiyun et al. 2004; Tomiczak et al. 2017).

Molecular analysis

To confirm hybrid nature of the plants regenerated from the fusion product, peroxidase in leaves were analyzed electrophoretically. All the analysed five lines showed the hybrid patterns. Figure 5 only shows the results of the

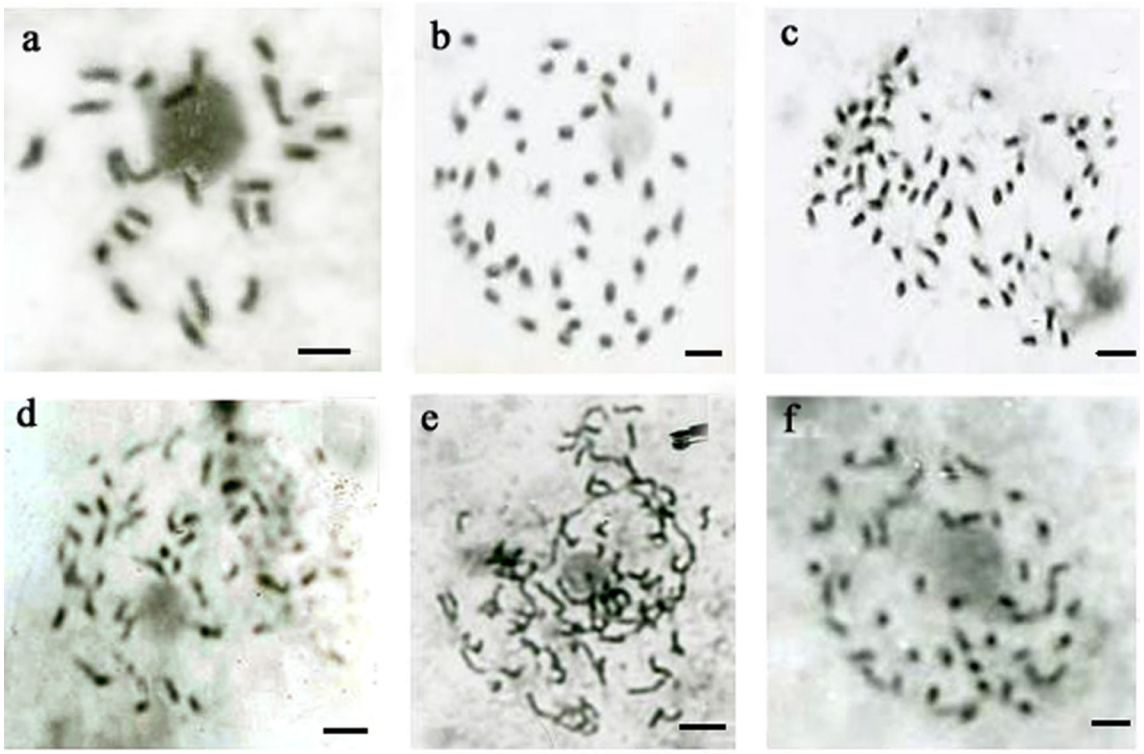

Fig. 4 Chromosome number of rice, reed and somatic hybrids: **a** Rice (2n = 24), **b** Reed (2n = 48), **c** H-1 (2n = 68), **d** H-116 (2n = 52), **e** H-56 (2n = 64,), **f** H-16 (2n = 46). Bar 2 μm. Chromosome samples were made from the roots of regenerated plants

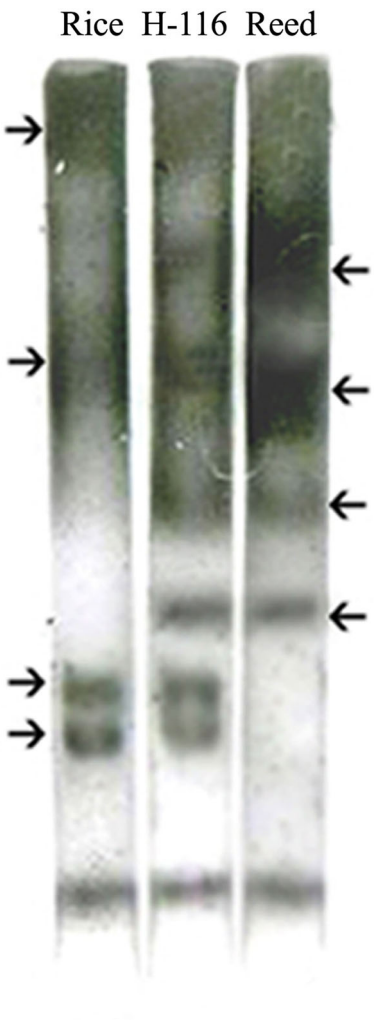

Fig. 5 Peroxidase banding patterns of rice, reed and the somatic hybrids. Parent-specific fragments are indicated by arrowhead

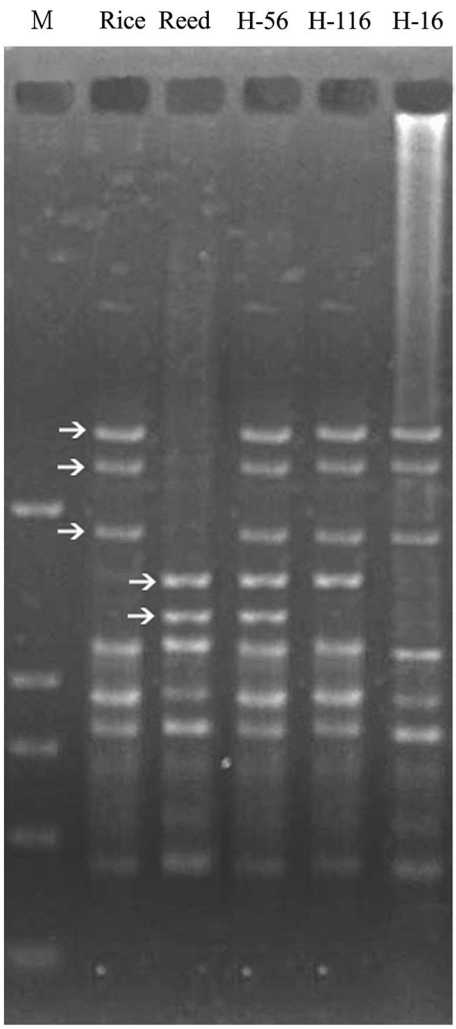

Fig. 6 RAPD analysis of somatic hybrids and parent species by primer OPR-06: Lane M is molecular weight DNA Ladder 2 kb. Parent-specific polymorphic fragments are indicated by arrowhead

H-116 line. While both rice and reed had 4 bands, hybrid had 8 bands of their parents. This suggested that analyzed line is a somatic hybrid which shows the bands of both rice and reed.

Next, for RAPD analysis, 36 random primers were used to survey the polymorphisms between the parents. Among them, five polymorphic primers were used for analysis of the hybrid plants, and one of them is shown in Fig. 6. All or most of the RAPD markers from the parents were found in 3 hybrids, suggesting that the majority of two genomes were retained in somatic hybrids. SSR analysis with five primer pairs (RM319, RM207, RM101, RM31 and RM246) was conducted to determine the nuclear origin of these hybrids, and two of them are shown in Fig. 7. All the analysed plants had specific fragments from rice, while with RM319 two specific fragments from reed were lost in somatic hybrid 16 and with RM207 two specific fragments from reed were lost in somatic hybrid 1 and 16, suggesting that the rice genome were retained in the somatic hybrids while part of the reed genome were deleted in the somatic hybrids.

To date a number of studies for protoplast fusion have been performed, however, only a few studies have reported the somatic hybrids in rice. The mature hybrid plants between rice (*Oryza sativa* L.) and wild *Oryza* species, were obtained to incorporate useful traits of the wild species into rice (Hayashi Y et al. 1988), and *Oryza sativa* L. (AA) and *Oryza punctata* Kotschy ex Steud. (BBCC) to reconstruct new interspecific rice genomes (Feng et al. 2006). The plantlets were regenerated from somatic hybrids of rice and barnyard grass (*Echinochloa oryzicola* Vasing). However, mature plants were not obtained, presumably due to the genetic distance between these members of different subfamilies of the *Gramineae* (Terada

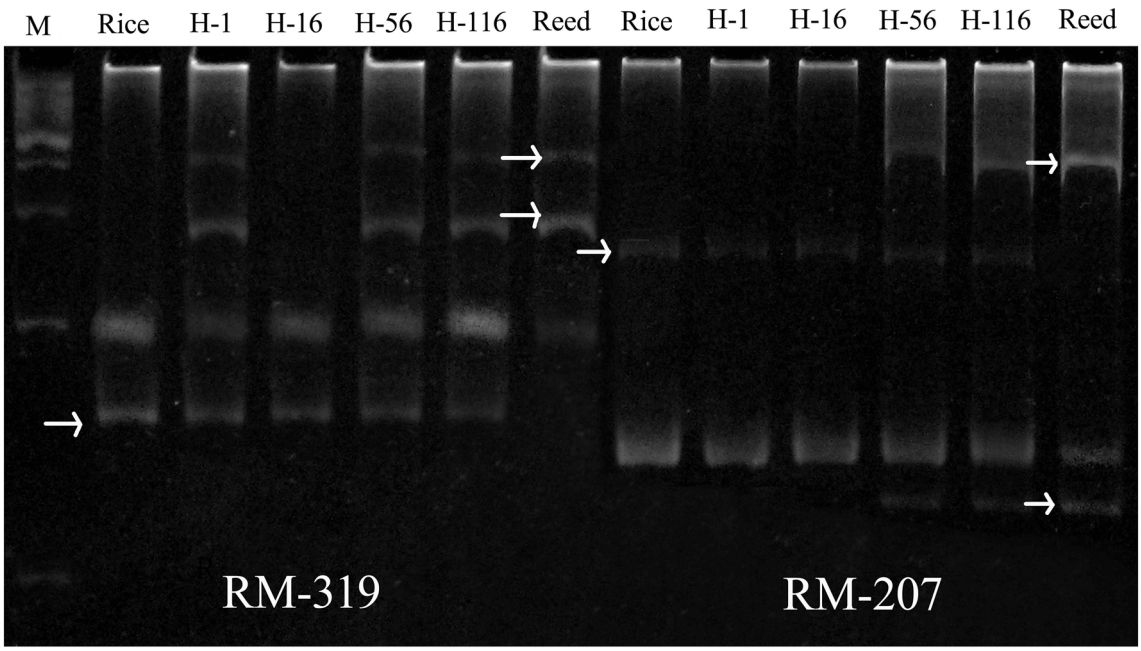

Fig. 7 SSR analysis of somatic hybrids and parent species by primer RM319 and RM207: Lane M is molecular weight DNA Ladder 100 bp. Parent-specific polymorphic fragments are indicated by arrowhead

et al. 1987). Some of present hybrid plants flowered and did not produce a progeny. The somatic hybrids presented in this study provide a useful example for the production of the intergeneric hybrid between rice and other plants, and for the increase of genetic variability in the rice.

Acknowledgements We appreciate Prof. Hyong Bom Pak, Faculty of Life Sciences, Kim Il Sung University, DPR Korea for invaluable advice. This study was supported by the DPR Korea State Commission of Science and Technology.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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