Production and characterization of alien chromosome addition lines in *Allium fistulosum* carrying extra chromosomes of *Allium roylei* using molecular and cytogenetic analyses

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Abstract

Allium roylei was employed for the production of alien chromosome addition lines in *A. fistulosum.* Interspecific hybridization between *A. fistulosum* and *A. roylei* successfully produced F_1 hybrids. Chromosome doubling of an F_1 hybrid was carried out to produce amphidiploids. After two times backcrossing of the amphidiploids with *A. fistulosum*, a BC_2 generation was obtained with chromosome numbers (2*n*) ranging from 16 to 23. Alien monosomic addition lines (AMAL, FF+nR, 2*n* = 17) appeared with the highest frequency. Furthermore, multiple addition lines (MAL, 2*n* = 18 – 23) were also observed with lower frequencies. Five AMALs (FF+1R, +3R, +4R, +5R, and +8R) and ten MALs (2*n* = 18 – 23) were characterized using isozyme and DNA markers. The extra chromosomes from *A. roylei* clearly altered the biochemical characteristics of the MALs. Variations in sugar, cysteine sulfoxide, and flavonoid contents were observed among the MALs in various amounts. *Allium fistulosum–A. roylei* allotriploids (2*n* = 24, FFR) showed significantly higher saponin content and antifungal activities of saponin extracts against isolates of *Fusarium oxysporum* f. sp. *cepae* in comparison with *A. fistulosum*. This first report of *A. fistulosum–A. roylei* addition lines opens the possibility of developing novel *A. fistulosum* cultivars with enhanced nutritional value and disease resistance.

Keywords: *Allium fistulosum*, *Allium roylei*, alien addition lines, antifungal activity, biochemical variation

Introduction

The Japanese bunching onion (JBO) (*Allium fistulosum* L., $2n = 2x = 16$, genomes FF) is an important *Allium* species in East Asia (Inden and Asahira 1990). It has been cultivated by both open-pollinated and F_1 hybrid seeds. In Japan, there are approximately 120 registered JBO cultivars with improved quality, heat tolerance, and bolting resistance (Inden and Asahira 1990). *A. fistulosum* has been reported as a good source of disease resistance which may be of interest for breeding (Kik 2002). However, it still suffers from some serious diseases, such as *Fusarium* wilt (Dissanayake et al. 2009) and downy mildew (Maude 1990). Disease resistance and high consumer quality including taste and flavor, are the main breeding objectives for the JBO.

In the breeding of cultivated *Allium* species, wild relatives are important sources for introducing new desirable traits via interspecific hybridization (Kik 2002). *Allium roylei*, a wild species originating in India, has attracted considerable attention in onion breeding for downy mildew resistance (Scholten et al. 2007) and alloplasmic male

sterility (Vu et al. 2011). This wild species also possesses other useful characteristics such as partial resistance to leaf blight (De Vries et al. 1992) and moderate resistance to *Fusarium* basal rot (Galvan et al. 2008). Therefore, exploitation of *A. roylei* for the breeding of *A. fistulosum* would be valuable. Recently Khrustaleva and Kik (1998, 2000) reported the successful uses of *A. roylei* as the bridging species in order to transfer some important genes from *A. fistulosum* to *A. cepa*. Long before, McCollum (1982) reported successful crosses of A. roylei with A. fistulosum. However, no further backcrossing generation has been reported since then. Meiotic irregularities, which were moderately frequent in the *A. roylei–A. fistulosum* hybrid (McCollum 1982), may hamper the introgression process of genes from *A. roylei* to *A. fistulosum* via backcrossing. Doubling of the sterile F_1 hybrid is one way to overcome these barriers (Singh 2003). In a previous study, a high number of alien addition lines of *A. cepa* carrying extra chromosomes from *A. roylei* were produced by backcrossing the doubled F_1 hybrid (Vu et al. 2012). Alien addition lines, which carry the extra chromosomes of wild species and the normal chromosome complement of recipient species, would speed up the introgression process of the wild species by producing chromosome substitution and translocation lines (Singh 2003). In this study, we first report the use of *A. roylei* for the production of alien addition lines in *A. fistulosum*. A preliminary study on the

variation of the biochemical content and antifungal activities against four isolates of *Fusarium oxysporum* f. sp. *cepae* was also conducted on the alien addition lines.

Materials and methods

Crossing procedure for the production of *A. fistulosum–A. roylei* chromosome addition lines

Figure 1 describes the crossing procedure for the production of *A. fistulosum* addition lines with extra chromosomes from *A. roylei*. *Allium fistulosum* 'Kujyo-kodaikei' (genomes FF, $2n = 2x = 16$, seed parent) was crossed with *A. roylei* '97175' (genomes RR, $2n = 2x = 16$, pollen parent) to produce F_1 hybrids (genomes FR, $2n = 2x = 16$). The chromosomes of an F_1 hybrid were doubled using colchicine to produce amphidiploids (genomes FFRR, $2n = 4x = 32$). The colchicine was applied by culturing a primordial stem in the Linsmaier and Skoog (LS) media containing 0.1 % colchicine in a dark condition for 4 days before being transferred to LS free hormone media and cultured for 2 months. After that, the amphidiploids were backcrossed with three different *A. fistulosum* cultivars ('Kujyo-Hoso,' 'Banchusei-Hanegi-Keitou,' and 'Nebuka-Negi-Keitou') to produce BC_1 progenies. The BC_1 plants were then backcrossed with the three *A. fistulosum* cultivars to produce BC_2 progenies. Crosses

were carried out by hand pollination in a screen-covered isolation greenhouse in Yamaguchi, Japan (N34°11', E131°28'). One month after pollination, the ovules of the BC_2 were cultured and generated on an MS solid medium (Murashige and Skoog 1962) containing 3.0 % (w/v) sucrose and 2.0 % (w/v) agar at 25 ºC in dark conditions until germinated, between May and August. After germination, the cultures were treated with 8 hours day length and 50 % humidity. Healthy seedlings were then planted in sand in plastic trays and transplanted to pots from November to December. The BC_2 plants were grown in a greenhouse and fertilized each week with a nutrient solution containing 15: 8: 17 (N: P_2O_5: K_2O, w/w/w) (OK-F-1; Otsuka Chemical Co., Osaka, Japan) or 6.5: 6: 19 (w/w/w) (Hyponex; Hyponex Co., Marysville, OH, USA). The chromosome numbers of the BC_2 plants were counted using Feulgen nuclear staining followed by the squash method. The karyotype analyses were undertaken according to the standard nomenclature system for the chromosomes of *Allium* (Kalkman 1984), which was agreed upon at the Eucarpia 4th *Allium* Symposium (De Vries 1990).

Characterization of alien chromosomes using isozyme and DNA markers

The BC_2 plants with $2n$ = 17 to 23 were further characterized using five isozymes and five DNA markers. The chromosomal locations of the five DNA markers were reported

in *A. cepa* or *A. fistulosum* as shown in Table 3. Chromosomal locations of the two isozymes and five DNA markers in *A. roylei* were determined from those that had been assigned in *A. cepa* and *A. fistulosum* because of the close genetic relationship between the species. Extraction of enzymes, electrophoresis, and staining were carried out following the method of Shigyo et al. (1995) and Van Heusden et al. (2000b). For DNA marker analyses of *A. fistulosum*–*A. roylei* addition lines, the total genomic DNA of the parental and BC_2 plants was isolated from fresh leaf tissue using a miniprep DNA-isolation method (Van Heusden et al. 2000a). The polymerase chain reaction (PCR) amplifications of the markers F3H, CHS-B, and AMS12 were evaluated as described previously (Masuzaki et al. 2006a, b). For amplification of the marker ACM024, the reaction mixture (20 µL) contained 100 ng of DNA, 2 mM 10×PCR buffer, 0.2 mM dNTP mixture, 0.8 µM each of forward and reverse primers, 1.5 mM $MgCl_2$, and 0.5 units of r *Taq* polymerase. Touchdown PCR was performed to amplify the marker ACM024 as follows: initial denaturation at 94 ^{o}C for 2 min, followed by 10 cycles at 94 ^{o}C for 0.5 min, 65 ^{o}C for 0.5 min, and 72 ^{o}C for 0.5 min, where the annealing temperature is reduced by 1 ^{o}C per cycle; then 35 cycles at 94 ^{o}C for 0.5 min, 55 ^{o}C for 0.5 min, and 72 ^{o}C for 0.5 min, and a final extension at 72 ^{o}C for 4 min on a program thermal cycler iCycler^{TM} (Bio-Rad, Hercules, CA, USA). To amplify the

marker SiR-1, the reaction mixture (25 μL) contained 50 ng of DNA, 2 mM 10×ExPCR buffer, 0.2 mM dNTPs, 0.5 μM each of forward and reverse primers, and 0.625 units of Ex *Taq* polymerase. The PCR condition for SiR-1 was as follows: initial denaturation for 3 min at 94 ºC and 40 cycles of PCR amplification (1 min denaturation at 94 ºC, 1 min annealing at 70 ºC, and 1 min primer extension at 72 ºC). The PCR products were separated on 2 % agarose or 5 % polyacrylamide gel electrophoresis according to the method of Yaguchi et al. (2009).

Determination of the sugar content in *A. fistulosum–A. roylei* chromosome addition lines

Plant materials used for the preliminary analysis included *A. fistulosum*, *A. roylei*, the F_1 hybrid, the amphidiploid, and different *A. fistulosum–A. roylei* multiple addition lines. The preliminary analysis was done to analyze the sugar content, including fructose, sucrose, and glucose. The multiple addition lines was cultivated for a year so the number of new plants multiplied from vegetative propagation were very limited. Only one sample for each line was collected in December of the next year. The leaf blades were cut into small pieces and mixed thoroughly. Two grams of the leaf-blade tissues were extracted using hot 70 % ethanol as described by Hang et al. (2004). Every extract

was stored at -20 ºC until analysis. The 70 % hot-ethanol extract was filtered through a Sep-Pak C18 cartridge column followed by a 0.5 µm filter (Katayama Chemical, Osaka, Japan) to remove pigments prior to HPLC analysis. Sugars in each filtrate were analyzed three times using an HPLC system (Hitachi LaChrom Elite) equipped with a refractive index detector (Hitachi L-7490). An aliquot of the filtrate (20 µL) was injected into the HPLC apparatus fitted with a LiChrospher 100 NH_2 (Merck) column of 4×250 mm with a column temperature of 35 ºC. The mobile phase was acetonitrile: water (80 : 20, v/v) at a flow rate of 0.8 mL/min with a retention time of 30 min. The internal standards were prepared by dissolving glucose, fructose, and sucrose at a concentration of 0.5 % in 70 % aqueous ethanol.

HPLC analysis of flavonoids and S-alk(en)yl-*L*-cysteine sulfoxides (ACSOs) in *A. fistulosum*–*A. roylei* chromosome addition lines

The plant materials for analyses of flavonoids and ACSOs were the same as those for the sugar analysis. Five grams of leaf-sheath tissues from each plant were extracted with hot 70 % ethanol as described by Hang et al. (2004). The 70 % hot-ethanol extractions were then used for flavonoid analysis. To analyze the ACSOs, two grams of the leaf-blade tissues were microwaved for two minutes to denature the alliinase and

extracted with distilled water. The flavonoid and ACSO contents were determined using HPLC according to the method described by Vu et al. (2013).

Extraction of saponins and evaluation of *in vitro* antifungal activities of saponins

Roots of *A. fistulosum*, *A. roylei*, an amphidiploid (FFRR), and an allotriploid (FFR) were collected a year after the chemical content analysis and used for saponin extraction. Freeze-dried root tissues (0.2–0.4 grams) were ground thoroughly using a blender and then extracted three times with 100 mL of *n*-hexane. The remaining root materials were extracted three times with 100 mL of 70 % methanol and filtered. The filtrate was vacuum dried and dissolved in 100 mL of water. After that, *n*-butanol with the same volume of water (100 mL) was added. The *n*-butanol fraction was separated three times using a separation funnel. The *n*-butanol fractions were vacuum dried to give crude saponins. The saponins were visualized by spotting the butanol fraction on a thin layer chromatography (TLC) and then developed using a system of chloroform: methanol: water (6: 3: 1). The TLC plates were sprayed with *p*-anisaldehyde reagents and heated at 100 ^{o}C for 10 min. The saponin contents were determined using a spectrophotometer in accordance with Ebrahimzadeh and Niknam (1998). Diosgenin (purity: approx. 95 %, Sigma, USA) was used as a standard for establishing a calibration curve. The ANOVA for saponin data was conducted with the General Linear Model of SPSS statistical

software version18.0 with advanced models (SPSS Japan Inc., Tokyo, Japan). Differences between means were located using Tukey's multiple range test.

The antifungal activities of the crude saponins were tested on four *Fusarium oxysporum* f. sp. *cepae* pathogens (Takii and AC214 isolated from bulb onions; AF60 and AF22 isolated from *A. fistulosum*). Pathogens were obtained from the Laboratory of Molecular Plant Pathology, Faculty of Agriculture, Yamaguchi University, Japan. The antifungal activity was evaluated by an agar-plate diffusion method, using 3.2 cm diameter Perspex plates of potato dextrose agar (PDA). Crude saponin was added to obtain a final concentration of 1000 ppm. The plates were inoculated with a 5 mm plug containing the fungi grown on a PDA for five days. Plates were incubated at 25 ºC, and the fungal radical growth was measured after one week by measuring the diameter of the fungal hypha that was grown on the plate. Each experiment was performed in triplicate with the water treatment as a control. Dunnett's multiple test was used for comparison of antifungal activities between *A. fistulosum* and the amphidiploid and allotriploid.

GISH analysis

To confirm the existence of an *A. roylei* chromosome in the *A. fistulosum* genetic background, GISH analysis was performed. GISH analysis was carried out with a monosomic and a double-monosomic addition line according to the method of Khrustaleva and Kik (2000) with minor modifications.

Selfing and backcrossing of the addition lines

One monosomic (FF+3R) and one double-monosomic (FF+3R+8R) addition line were used for selfing and backcrossing, respectively. The two plants were grown in pots in the green house at Yamaguchi University. All umbels were bagged (selfing) and hand-pollinated (backcrossing). In backcrossing, the stamens were removed to avoid selfing.

Results

Production of *A. fistulosum–A. roylei* chromosome addition lines

A. fistulosum 'Kujyo-kodaikei' set germinable F_1 hybrid seeds when crossed with *A. roylei* '97175' as the pollen parent. After doubling the chromosomes of the F_1 hybrid, amphidiploid plants were obtained. In the backcrossing between the amphidiploids and three different cultivars of *A. fistulosum*, 31 BC_1 plants were produced (Table 1). The

chromosome numbers (2*n*) of the BC_1 plants were 24 (29 plants) and 32 (two plants) (Table 2). Subsequently, 29 BC_2 plants were produced from backcrossing between allotriploid BC_1 plants and *A. fistulosum* (Table 1). The chromosome numbers (2*n*) of the BC_2 plants ranged from 16 to 23 (Table 2). The plants with 2*n* = 17 appeared with the highest frequency (eight plants). Lower frequencies (one to six plants) were observed in plants with 2*n* = 16, 18, 19, 20, 21, 22, and 23.

Characterization of extra chromosomes from *A. roylei* via molecular markers

Van Heusden et al. (2000b) reported that isozyme loci *Lap-1*, *6-Pgdh*, and *Pgi-1* are located on chromosomes 1, 2, and 5, respectively, in *A. roylei*. Furthermore, the two isozyme loci, *Got-2* and *Gdh-1*, were allocated on chromosomes 6 and 8 of *A. cepa* (Shigyo et al. 1994, 1995). *Allium fistulosum* and *A. cepa* had different band patterns of the five isozymes *Lap-1*, *6-Pgdh*, *Pgi-1*, *Got-2*, and *Gdh-1*. The introgression of gene encoding for *Lap-1* from *A. roylei* in the BC_2 plants was determined by the presence of bands from both *A. fistulosum* and *A. roylei* (Figure 2). Meanwhile, the BC_2 plants that possessed encoding genes of *6-Pgdh*, *Pgi-1*, and *Got-2* in *A. roylei* showed bands from the parental bands with additional bands of intermediate mobility between the two parents. The presence of gene encoding for *Gdh-1* from *A. roylei* in the BC_2 plants was

confirmed by bands at intermediate positions between the parental bands. There are two pattern types of intermediate mobility (Figure 3). The results of isozyme analysis in the BC_2 progenies are included in Table 4. With the five isozyme markers, the three AMALs (FF+1R, FF+5R, and FF+8R) were characterized, and the presence of extra chromosomes 1R, 2R, 5R, 6R, and 8R was detected in a double-monosomic addition line ($2n$ = 18) and other MALs ($2n$ = 20, 21, 22, and 23).

All of the DNA markers used in this study were able to show polymorphism between *A. fistulosum* and *A. roylei*. The DNA fragments derived from *A. roylei* were used to confirm the presence of *A. roylei* respective chromosomes. Two AMALs, FF+3R and FF+4R, were identified by one EST and one SCAR marker (Si-R and CHS-B, respectively) (Table 4). Furthermore, extra chromosomes of *A. roylei* (2R, 3R, 4R, and 7R) were also detected in the double-monosomic addition line and the other MALs via DNA markers.

In summary, with the use of five isozyme and five DNA markers, five AMALs ($2n$ = 17), one double-monosomic addition line ($2n$ = 18), and nine MALs ($2n$ = 20, 21, 22, and 23) were characterized.

GISH analyses were carried out with one AMAL ($2n$ = 17, FF+3R) and a double-monosomic addition line ($2n$ = 18, FF+3R+8R) for further confirmation of the

chromosome constitutions of these lines (Figure. 4). FF+3R showed an intact chromosome 3 of *A. roylei*, one recombinant *A. roylei*–*A. fistulosum* chromosome, and other intact chromosomes of *A. fistulosum*. The double-monosomic line FF+3R+8R had two intact chromosomes of *A. roylei*, in addition to a complete set of 16 chromosomes from *A. fistulosum*, without any translocation.

Selfing and backcrossing of the addition lines

Selfing and backcrossing were carried out in the AMAL (FF+3R) and the double-monosomic addition line (FF+3R+8R), respectively (Table 5). A high number of plants in the next generation after selfing and backcrossing had chromosome number 2*n* = 16. However, addition lines with 2*n* = 17 and 18 were also obtained with a lower number of plants.

Biochemical characteristics of the alien addition lines

The contents of some chemical compounds (sugars, ACSOs, flavonoids, and saponins) were preliminarily investigated in multiple addition lines together with the parental, allotriploid, and amphidiploid lines. Preliminary investigation was done because only one plant survived. Consequently, only one replication could be done for the analysis.

However, variations of the chemical contents were observed among the investigated lines (Figure 5).

All three kinds of ACSOs were detected in the three cultivars of *A. fistulosum*. In *A. roylei*, PeCSO had the highest proportion, followed by AlCSO, while MeCSO was not detected. The amphidiploid FFRR and one of the allotriploids FFR showed very low MeCSO content. The MeCSO contents in some MALs, for example H8, H10, H11, and H6 were moderate. Total ACSO content was limited in the hypo-allotriploid FFR-4R (H10 and H11).

In *A. fistulosum*, quercetin and kaempferol were totally absent. Meanwhile, these two compounds appeared at relatively high levels in *A. roylei*. The two compounds were also detected in the amphidiploid, allotriploids, and multiple addition lines that possessed chromosome 5R of *A. roylei*. In the multiple addition lines that lacked chromosome 5R, the two compounds were undetectable. In terms of morphology, the multiple addition lines with chromosome 5R had red leaf sheaths, while those without chromosome 5R showed had white leaf sheaths. A large increase in kaempferol content was observed in a hypo-allotriploid FFR-4R (H10).

Differences were observed between *A. fistulosum*, *A. roylei*, the allotriploid (FFR), and the amphidiploid (FFRR) in the total amount of saponins extracted from the roots

(Figure 6). Significantly higher saponin content was observed in the allotriploid plant in comparison with the *A. fistulosum* and the amphidiploid plant.

Saponins of *A. fistulosum* showed higher antifungal activities than those of *A. roylei* against all four fungal isolates (Figure 7). Meanwhile, saponins of *A. fistulosum* and the amphidiploid had the same levels of fungal inhibition against the four isolates. Saponins of the allotriploid had significantly higher antifungal activities against the two isolates AC Takii and AF22 in comparison with those of *A. fistulosum*.

Discussion

This study reports, for the first time, the successful production of *A. fistulosum–A. roylei* chromosome addition lines. In crossings between the amphidiploids (FFRR) and the diploids *A. fistulosum* (FF), the seed set was high, ranging from 25 to 40 %. In the case of crossings between the allotriploids (FFR) and the diploids *A. fistulosum* (FF), the seed set was extremely low (0.48–2.6 %). This phenomenon might be due to the high proportion of non-functional female gametes produced by the allotriploids. A similar result was also described in the backcrossings of *A. cepa–A. fistulosum* allotriploids (Hang et al. 2004). In contrast, backcrossings of *A. cepa–A. roylei* allotriploids had relatively high rates of germinated seeds (Vu et al. 2012). We did not succeed in

completing the eight possible types of AMALs, but we found that AMALs with $2n = 17$ appeared with the highest frequency among the BC_2 plants. The MALs of *A. fistulosum* with extra chromosomes from *A. roylei* also appeared with lower frequencies. Therefore, we think the addition of chromosomes from *A. roylei* does not decrease the survival ability of the female gametes produced from the *A. fistulosum–A. roylei* allotriploids. These results differed from those of Vu et al. (2012), who found a high number of plants with $2n = 16$, followed by $2n = 17$ in the BC_2 generation.

Employing isozyme and DNA markers in this study enabled us to successfully identify the presence of *A. roylei* in the BC_2 plants in most cases. However, seven BC_2 plants showed differences between the cytogenetic and molecular data. These plants might be derived from chromosome substitution or recombination during meiosis of the *A. fistulosum–A. roylei* allotriploids. This result is in agreement with a previous study, which reported frequent chromosome parings and moderately frequent meiotic irregularities (e.g., univalents) in pollen mother cells of the *A. fistulosum–A. roylei* hybrid (McCollum 1982). As an example, the GISH result of our study showed a recombination in an AMAL (FF+3R) and a true double-monosomic addition line without recombination (FF+3R+8R). The recombination probably resulted from the chiasma formation during the meiosis of the allotriploid as also reported by Vu et al.

(2012). Further GISH analyses are required to reveal the genomic constitutions and recombination frequencies of all of the BC_2 plants.

This study demonstrated that *A. roylei* chromosomes in an *A. fistulosum* genetic background resulted in modifications of the content and composition of chemical compounds compared to *A. fistulosum*. The additions of all eight chromosomes from *A. roylei* may contribute to the increase of sugar content in the leaf blades of *A. fistulosum*. The hypo-allotriploid with an absence of chromosome 8 of *A. roylei* also showed a higher total sugar content as compared with *A. fistulosum*. From this result, it seems that chromosome 8 of *A. roylei* may not carry important factors for promoting sugar synthesis in *A. fistulosum*. This result was different from that of previous studies, which suggested that chromosomes 8 of *A. cepa* and *A. fistulosum* carry anonymous factors related to an increase of sugar content in *A. fistulosum*–*A. cepa* and the *A. cepa*–*A. fistulosum* addition lines, respectively (Yaguchi et al. 2008, 2009). Further investigations into sugar content together with chromosomal locations and expression of the major enzyme genes related to sugar synthesis at different plant development stages are needed to clarify the effects of additional chromosomes from *A. roylei* on the production of sugars in *A. fistulosum*. Regarding ACSOs, proportions of different types and total content in *A. fistulosum* were shown to be modified by extra

chromosomes from *A. roylei*. Due to the absence of MeCSO in *A. roylei*, it might be that the chromosomes derived from *A. roylei* in the diploid background of *A. fistulosum* carry anonymous factors that inhibit the synthesis and/or promote the degradation of MeCSO in *A. fistulosum*. The overall flavor of *Allium*-derived plants is determined by the ratios and amounts of ACSOs (Block 2010). Therefore, additional chromosomes from *A. roylei* would actually alter the flavor of *A. fistulosum*. Some addition lines with very low ACSO content could be mildly pungent. These lines would be very good breeding material for developing low-pungency cultivars of *A. fistulosum*. Shigyo et al. (1997a) reported that only one *A. fistulosum*–shallot monosomic addition line FF+5A showed a reddish-yellow leaf sheath and suggested that chromosome 5 of the shallot possesses important genes for controlling pigment production. Furthermore, the authors found a large number of peaks attributable to flavonoids in the FF+5A (Shigyo et al. 1997b). This study reported a similar result, that only the *A. fistulosum*–*A. roylei* addition lines that carry the extra chromosome 5R of *A. roylei* show a red leaf sheath. Chromosome 5R of *A. roylei* would also carry important genes related to flavonoid synthesis in *A. roylei*. Further determination of chromosomal locations of structural enzyme-coding genes and regulatory genes in the pigment biosynthetic pathways of *A. roylei* is needed to confirm this result. The

saponin content in leaves of *A. roylei* was higher than in those of *A. fistulosum* (Vu et al. 2013). Thin-layer chromatography also showed qualitative differences in saponins between these two species. The present study found that the FFR triploids had significantly higher saponin content and saponin antifungal activities than did *A. fistulosum*. The additional saponin content of the FFR allotriploids would be derived from *A. roylei* saponin biosynthesis controlled by the introgressed genes located on the extra chromosomes of *A. roylei*. The observations of chemical modifications in the *A. fistulosum–A. roylei* addition lines would bring helpful information regarding chromosome manipulation to improve the consumer quality as well as the disease resistance of *A. fistulosum*. The introgression of desirable traits, such as *Fusarium* or downy mildew resistance, from *A. roylei* to *A. fistulosum* is promising for the future, as a BC_3 generation could be produced via initial trials of selfing and backcrossing of the *A. fistulosum–A. roylei* addition lines.

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Figure captions

Fig. 1 Method for producing alien addition lines of *Allium fistulosum* with extra chromosomes of *A. roylei*

Fig. 2 *Lap-1* zymograms and the schematic illustration of *A. fistulosum* 'Kujyo-Hoso' (FF), *A. roylei* '97175' (RR), MALs (H8, H11, H10, H6, and H5), double-monosomic addition line (H9) and BC_2 FF (H7)

Fig. 3 *Gdh-1* zymograms and the schematic illustration of *A. fistulosum* 'Banchusei-Hanegi-Keitou' (F^1F^1), *A. fistulosum* 'Nebuka-Negi-Keitou' (F^2F^2), *A. roylei* '97175' (RR), amphidiploid (FFRR), and allotriploid (FF^1R). BC_2 plants showed 2 patterns (a and b)

Fig. 4 Somatic metaphase cells of a monosomic addition line ($2n$ = 17, genomes FF+3R) (**A**) and a double-monosomic addition line ($2n$ = 18, genomes FF+3R+8R) (**B**) in the BC_2 generation after genomic *in situ* hybridization

Fig. 5 Sugar, cysteine sulfoxide, and flavonoid contents of the three cultivars of *A. fistulosum*: 'Kujyo-Hoso' (FF1), 'Banchusei-Hanegi-Keitou' (FF2), 'Nebuka-Negi-Keitou' (FF3); *A. roylei* '97175' (RR); F_1 hybrid (FR); amphidiploid (FFRR); allotriploids (FFR1, FFR2); and *A. fistulosum* - *A. roylei* chromosome addition lines (H8 - H7). [a] Not carried out. [b] Not detected

Fig. 6 Saponin contents in the roots of *A. roylei* '97175' (RR), *A. fistulosum* 'Kujyo-Hoso' (FF), amphidiploid (FFRR) and allotriploid (FFR). Vertical bars indicate + and – standard error. Different letters indicate a significant difference among the lines according to Tukey's multiple range test

Fig. 7 Antifungal activities of saponins in the roots of *A. fistulosum* 'Kujyo-Hoso', *A. roylei* '97175', amphidiploid (FFRR), and allotriploid (FFR) against the four isolates of *F. oxysporum* f. sp. *cepae*. Dunnett's multiple test was used for comparison of antifungal activities between *A. fistulosum* and each of the amphidiploid and the allotriploid. * indicates significant higher antifungal activity than *A. fistulosum* at $p < 0.05$

Figure 4
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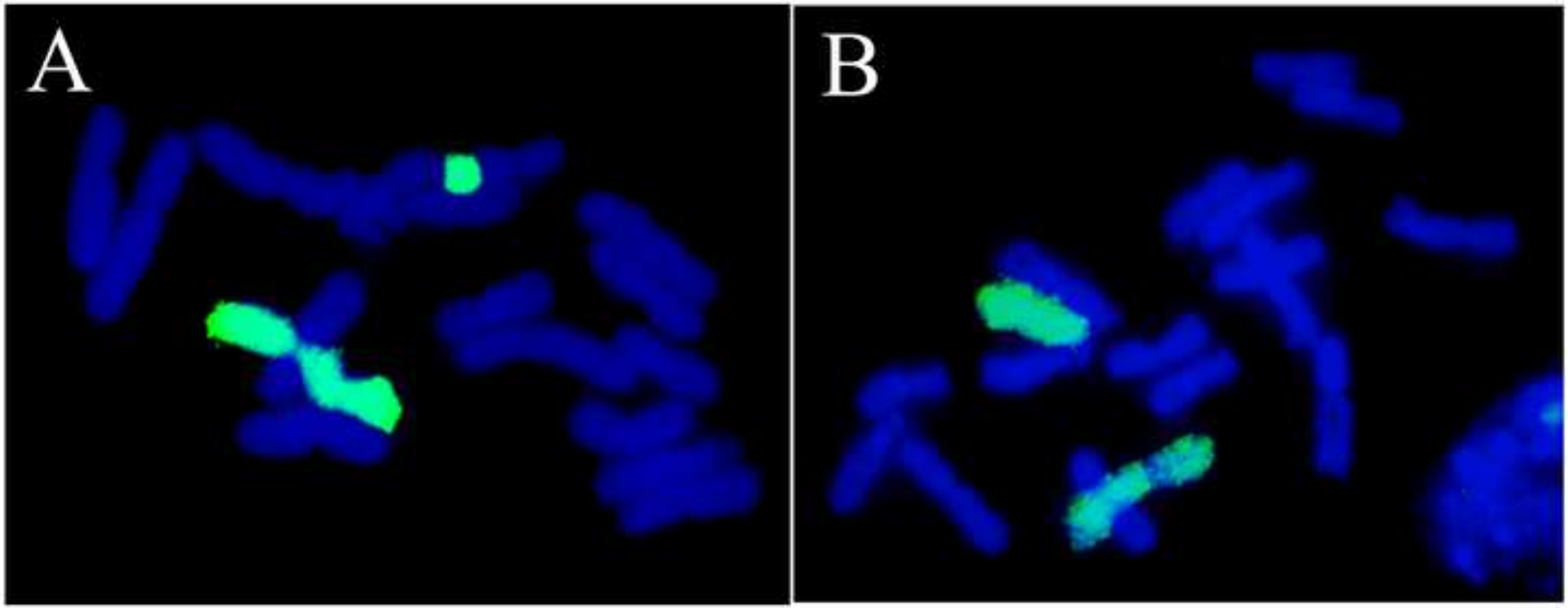

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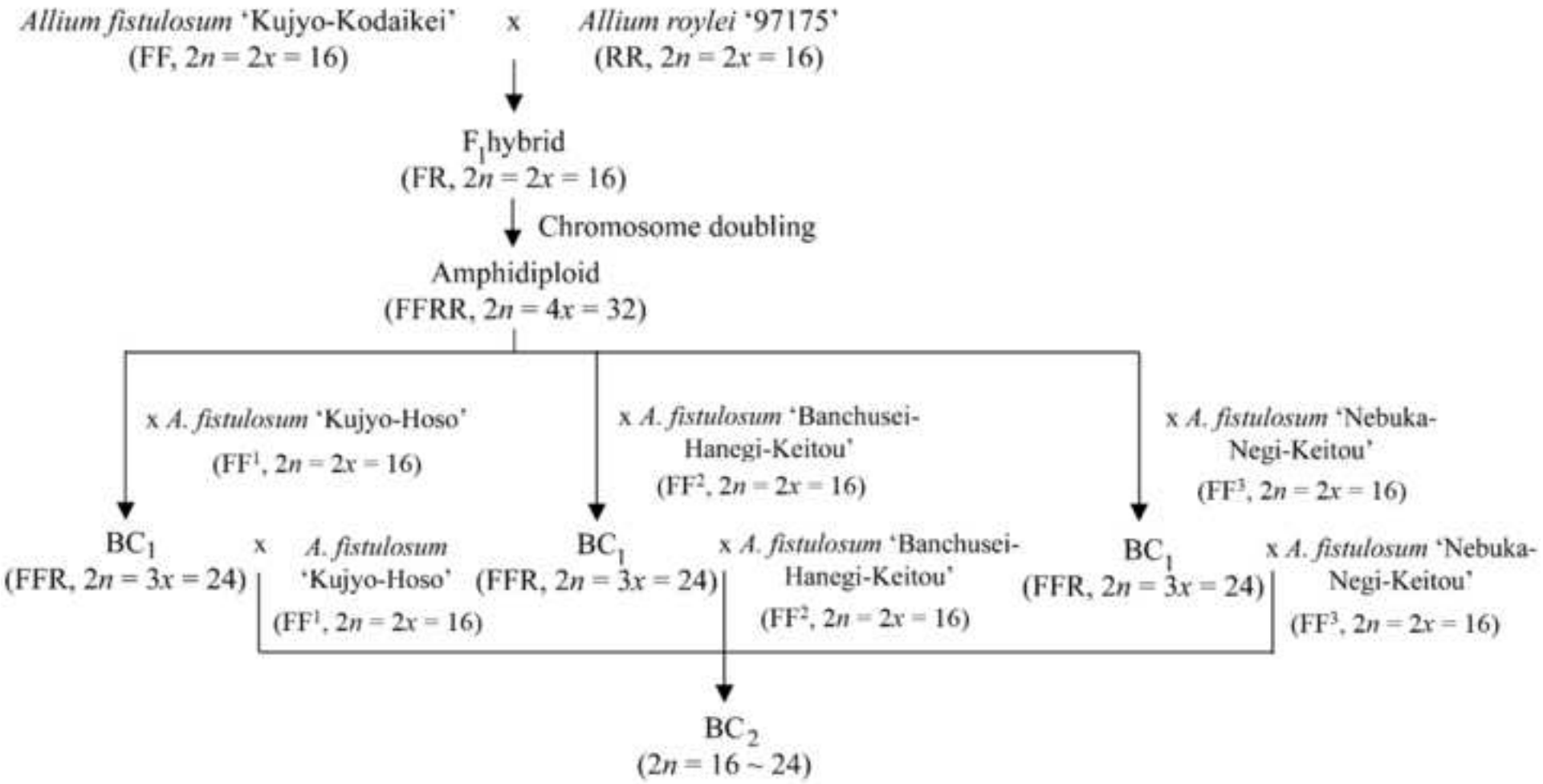

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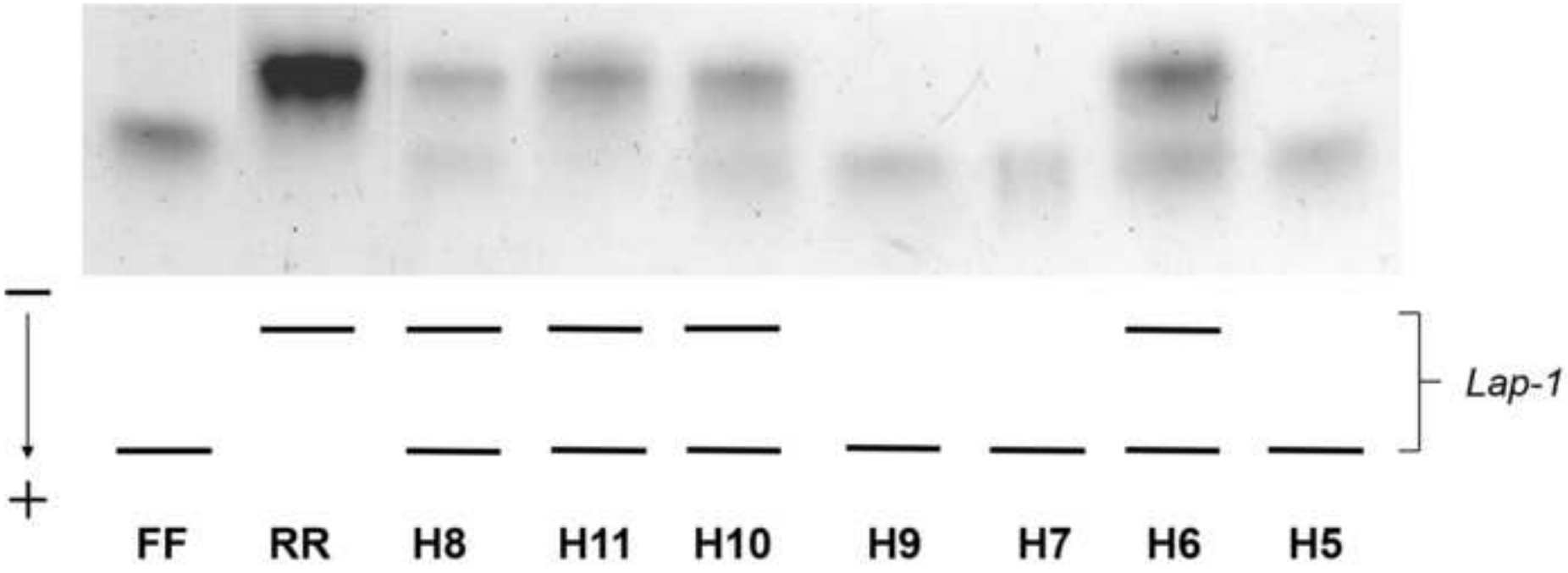

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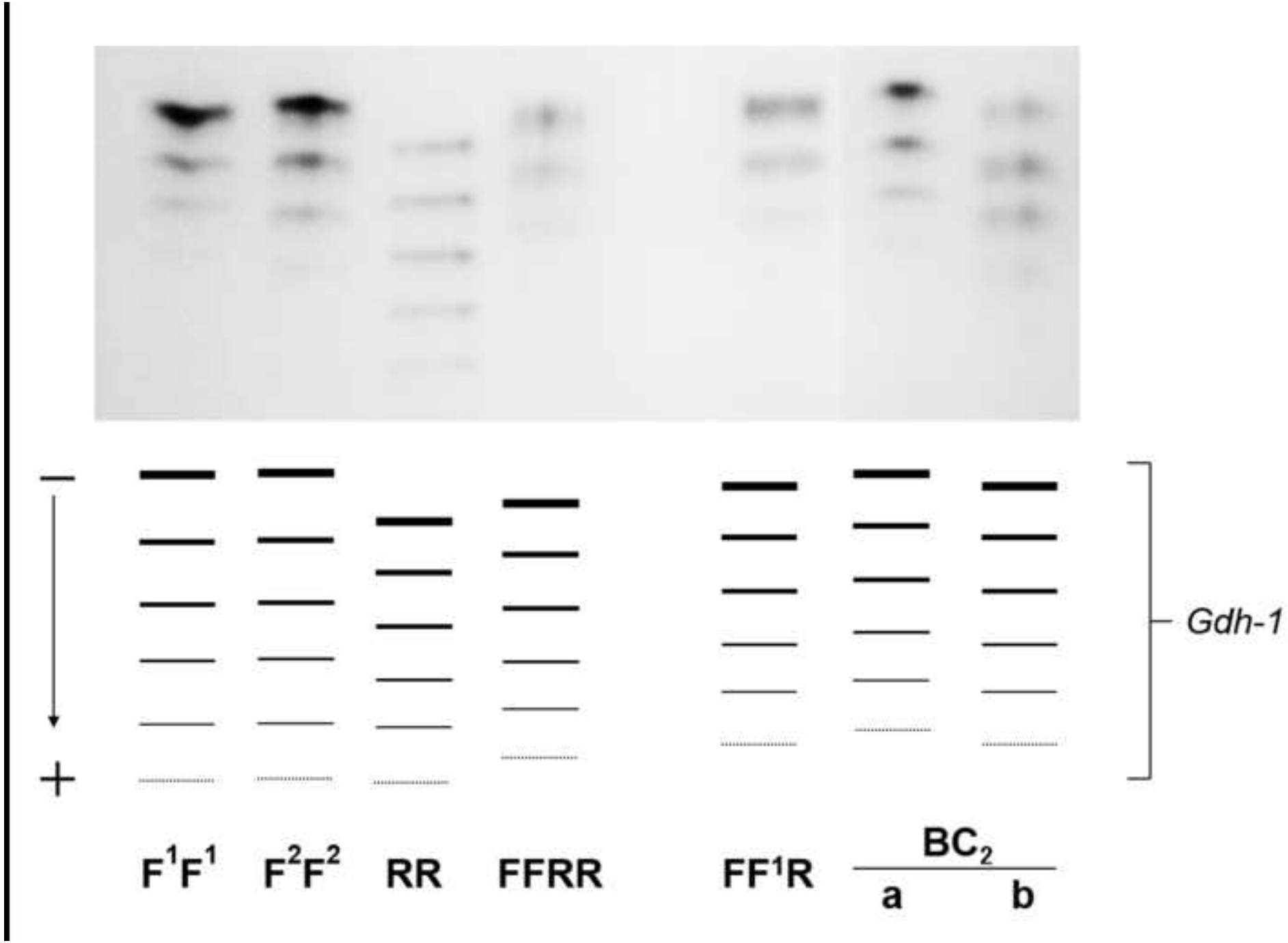

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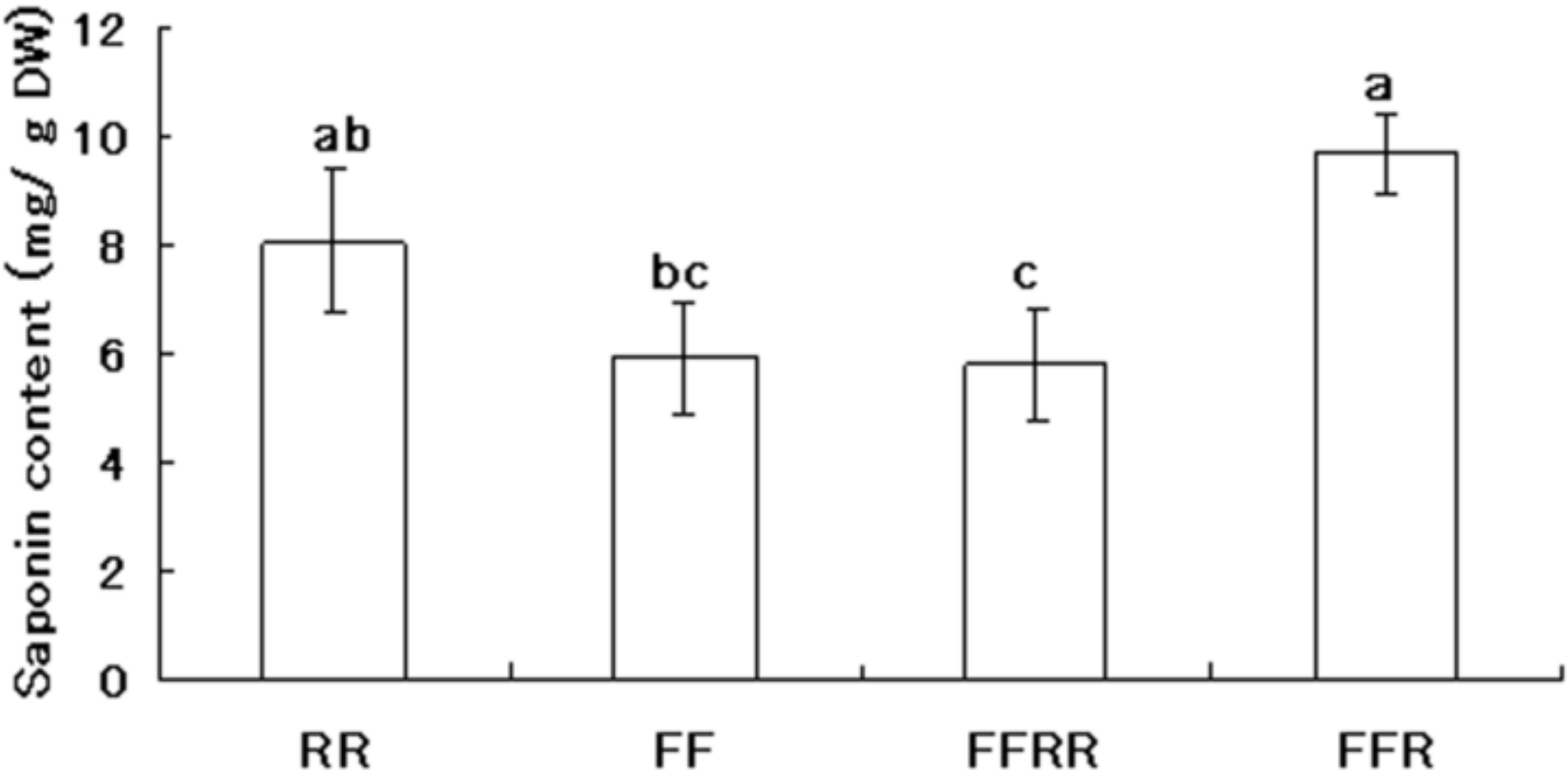

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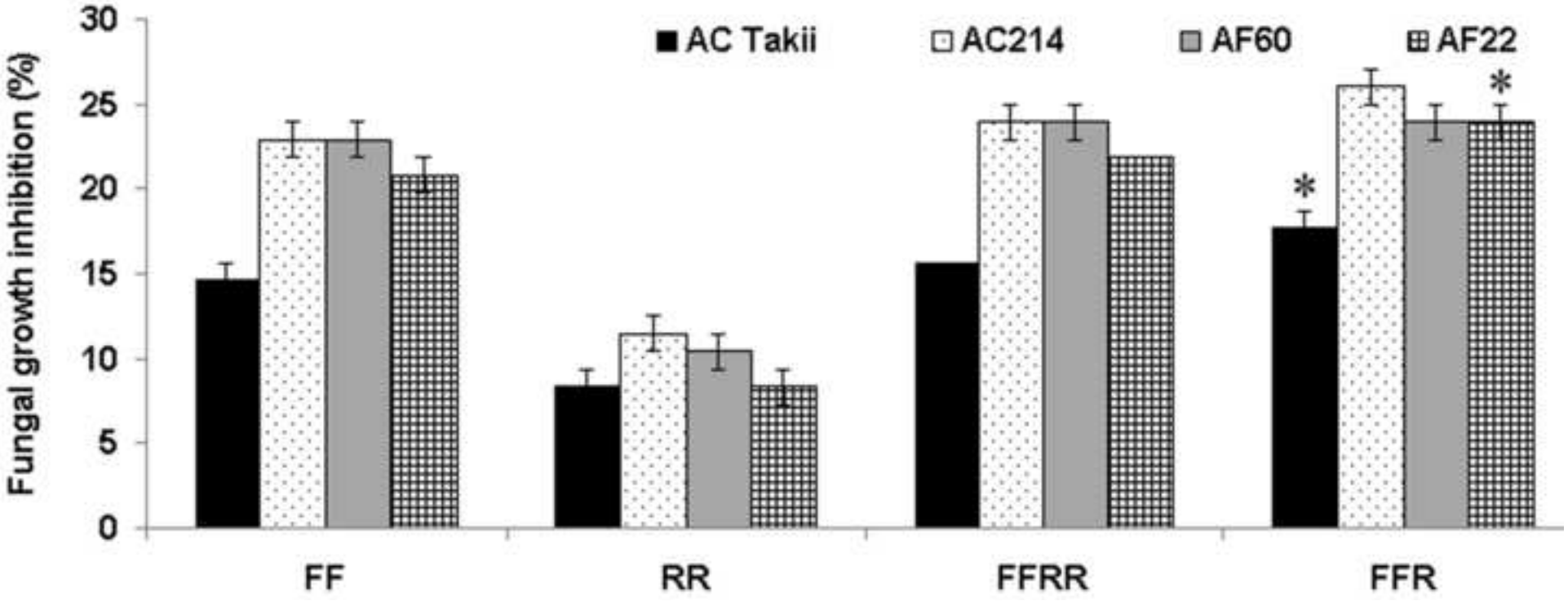

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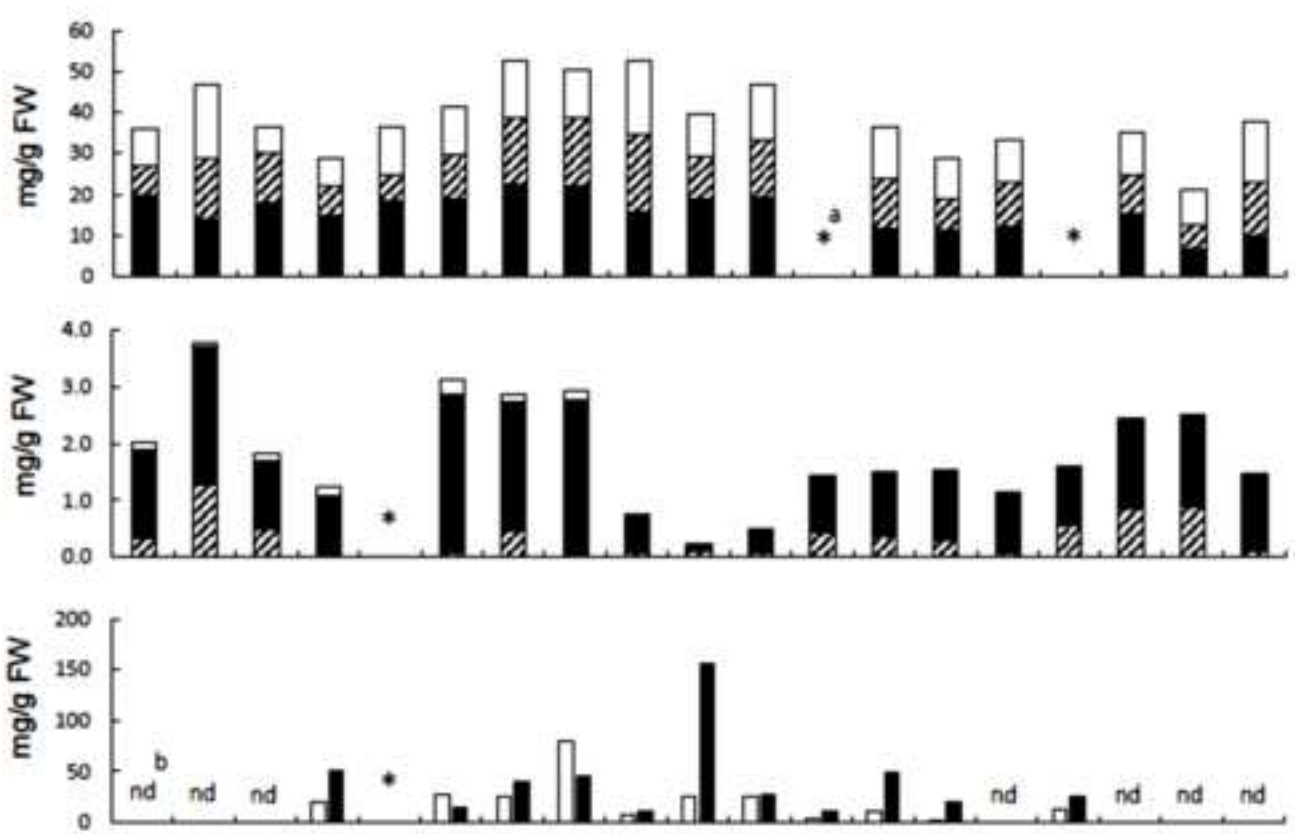

	FF1	FF2	FF3	RR	FR	FFRR	FFR1	FFR2	H8	H10	H11	H3	H4	H2	H6	H5	H1	H9	H7
Extrachromosome from *A. roylei* determined by molecular markers	-	-	-	1R	1R	1R	1R	1R	1R	1R	1R	1R	1R	1R	1R	-	1R	-	-
	-	-	-	2R	2R	2R	2R	2R	2R	2R	2R	2R	-	-	2R	2R	-	-	-
	-	-	-	3R	3R	3R	3R	3R	3R	3R	3R	3R	3R	3R	3R	-	-	3R	-
	-	-	-	4R	4R	4R	4R	4R	4R	-	-	4R	4R	-	-	-	-	-	-
	-	-	-	5R	5R	5R	5R	5R	5R	5R	5R	5R	5R	5R	-	5R	-	-	-
	-	-	-	6R	6R	6R	6R	6R	6R	6R	6R	6R	6R	6R	6R	6R	-	-	-
	-	-	-	7R	7R	7R	7R	7R	7R	7R	7R	7R	7R	7R	7R	7R	-	-	-
	-	-	-	8R	8R	8R	8R	8R	-	8R	8R	-	-	8R	-	-	8R	8R	-
Chromosome number (2n)	16					32	24		23			22		21		20	18		16

table
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Table 1. Seed set, seed germination, and number of seedlings survival in the backcrossings of amphidiploids (2*n* = 32, genomes FFRR) and triploids (2*n* = 24, genomes FFR) to three cultivars of *A. fistulosum*: 'Kujyo-Hoso' (F^1F^1), 'Banchusei-Hananegi-Kei-Tou' (F^2F^2), and 'Nebuka-Negi-Keitou' (F^3F^3)

Cross combination	Backcrossed generation	Number of flowers pollinated	Number of seeds produced	Number of seeds that germinated	Number of seedlings that survived
FFRR x F^1F^1	BC_1	5	12	2	1
FFRR x F^2F^2	BC_1	149	223	80	14
FFRR x F^3F^3	BC_1	173	378	160	16
FFR x F^1F^1	BC_2	3972	115	26	6
FFR x F^2F^2	BC_2	5116	295	21	10
FFR x F^3F^3	BC_2	2107	333	25	13

Table 2. Variation of chromosome numbers in BC_1 and BC_2 progenies

Backcrossed generation	Number of plants in observation	Frequency of plants									
		Chromosome number ($2n$)									
		16	17	18	19	20	21	22	23	24	32
BC_1	31	0	0	0	0	0	0	0	0	29	2
BC_2	29	1	8	2	1	2	5	4	6	0	0

Table 3. DNA markers for identification of extra chromosomes from *A. roylei* in BC_2 progenies

Primer set	Genbank accession no. or microsatellite motif	Forward and reverse primers	Type of marker	Chromosome	Reported
ACM024	CF435407	5’-CCCCATTTTCTTCATTTTCTCA-3’ 5’-TGCTGTTGCTGTTGTTGTTG-3’	EST	2	Tsukazaki et al.2008
F3H	AY221246	First 5’-AGAGAGGGGAAATATGTAGG-3’ 5’-GGCTCCTCTAATATCGGTT-3’ Second 5’-TGGAAAGAAGGGCGGTTTC-3’ 5’-TAATGGCCATGGTCACCAAG-3’	SCAR	3	Masuzaki et al. 2006a
SiR-1	CF434863	5’-TGCAGCTCTTTCTCAAGTTGG-3’ 5’-CAGAGCAGGACATGCCATAG-3’	EST	3	McCallum et al. 2007
CHS-B	AY221245	First 5’-CACCTGTCCGAAGACATCC-3’ 5’-CCCTCCTTACTTGAGTTCTTCC-3’ Second 5’-GTGAAGCGCTTCATGATGTACC-3’ 5’-GGATGCGCTATCCAAAACACC-3’	SCAR	4	Masuzaki et al. 2006a
AMS12	$(CA)_{25}$	5’-AATGTTGCTTTCTTTAGATGTTG-3’ 5’-TGCAAAATTACAAGCAAACTG-3’	SSR	7	Masuzaki et al. 2006b

Table 4. Identification of extra chromosomes in *A. cepa-A. roylei* addition lines via chromosome-specific isozyme and DNA markers

Chromosome number	Group	Frequency of plants	Chromosome specific markers											Extra chromosome
			1R	2R		3R		4R	5R	6R		7R	8R	
			Lap	*6-Pgdh*	ACM024	F3H	Si-R	CHS-B	*Pgi-1*	*Got-2*	Karyotype	AMS-12	*Gdh-1*	
17	1	1	+[a]	o[c]	-	-	-	-	-	-	-	-	-	1R
	2	3	-[b]	o	-	o	+	-	-	-	-	-	-	3R
	3	1	-	o	-	o	-	+	-	-	-	-	-	4R
	4	1	-	o	-	o	-	-	+	-	-	-	-	5R
	5	2	-	o	-	o	-	-	-	-	-	-	+	8R
18	1	1	-	-	o	+	o	-	-	-	-	-	+	3R, 8R
	2	1	+	-	o	-	-	-	-	-	-	-	-	unidentified
19	1	1	-	o	-	o	+	-	-	-	+	-	+	unidentified
20	1	1	-	+	o	-	o	-	+	+	o	+	-	2R, 5R, 6R, 7R
	2	1	+	o	-	o	+	+	-	+	+	+	-	unidentified
21	1	1	+	+	o	+	+	-	-	+	o	+	-	1R, 2R, 3R, 6R, 7R
	2	1	-	o	+	o	+	-	+	+	+	+	-	2R, 3R, 5R, 6R, 7R
	3	1	+	-	o	+	o	-	+	+	+	+	+	unidentified
	4	1	+	o	+	o	+	+	+	+	+	+	-	unidentified
	5	1	+	o	+	o	+	-	+	+	+	+	-	unidentified
22	1	1	+	o	+	o	+	+	+	-	-	-	+	1R, 2R, 3R, 4R, 5R, 8R
	2	1	-	o	-	o	+	+	+	+	+	+	+	3R, 4R, 5R, 6R, 7R, 8R
	3	1	+	-	o	+	o	+	+	+	o	+	-	1R, 3R, 4R, 5R, 6R, 7R
	4	1	+	+	o	+	o	+	+	+	o	+	-	unidentified
23	1	1	-	o	+	o	+	+	+	+	+	+	+	2R, 3R, 4R, 5R, 6R, 7R, 8R
	2	2	+	+	o	+	o	-	+	+	o	+	+	1R, 2R, 3R, 5R, 6R, 7R, 8R
	3	1	+	+	o	+	o	+	+	+	o	+	-	1R, 2R, 3R, 4R, 5R, 6R, 7R
	4	1	+	o	+	o	+	+	+	+	+	+	-	1R, 2R, 3R, 4R, 5R, 6R, 7R

[a] Presence
[b] Absence
[c] Not carried out

Table 5. Seed set, seedlings survival, and chromosome number of seedlings in backcrossing of a double-monosomic addition line (2*n* = 18, genomes FF+3R+8R) to *A. fistulosum* 'Senbon-Negi' (F^4F^4) and selfing of a monosomic addition line (2*n* = 17, genomes FF+3R)

Cross combination	Number of flowers pollinated	Number of seeds produced	Number of seeds that germinated	Number of seedlings that survived	Number of seedlings in observation of chromosome number	Number of seedlings		
						Chromosome number (2*n*)		
						16	17	18
(FF+3R+8R) x F^4F^4	159	52	37	29	25	19	5	1
(FF+3R) selfed	88	58	32	17	17	13	4	0