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ORIGINAL PAPER

Alien genes introgression and development of alien monosomic addition lines from a threatened species, *Allium roylei* Stearn, to *Allium cepa* L.

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Abstract To produce alien monosomic addition lines (AMALs) of *Allium cepa* (genomes CC, $2n = 2x = 16$) carrying extrachromosomes from *Allium roylei* (RR, $2n = 2x = 16$), reciprocal backcrossing of allotriploids ($2n = 24$, CCR) with diploids ($2n = 16$, CC) and selfing of a single allotriploid were carried out. The chromosome numbers in the BC_2F_1 and BC_1F_2 progenies ranged from 16 to 32. Forty-eight plants were recorded to possess $2n = 17$ among a total of 169 plants in observation. Through the analyses of isozymes, expressed sequence tag (EST) markers, and karyotypes, all eight possible types of *A. cepa*–*A. roylei* monosomic addition lines (CC+1R–CC+8R) could be identified. Seven types of representative AMALs (without CC+2R) were used for the GISH analysis of somatic chromosomes. Except for CC+6R, all AMALs showed an entire (unrecombined) extrachromosome from *A. roylei* in the integral diploid background of *A. cepa*. A single recombination between *A. cepa* and *A. roylei* was observed on the extrachromosome in the remaining type. All alloplasmic AMALs possessing *A. roylei* cytoplasm showed high or complete pollen sterility. Only the autoplasmic CC+4R with *A. cepa* cytoplasm possessed relatively high pollen fertility. The bulbs of CC+4R displayed the distinct ovoid shape that discriminates them from spherical or oval ones in other AMALs. Downy mildew screening in the field showed higher resistance in *A. roylei*, a hypo-allotriploid (CCR-nR, $2n = 23$), and an allotriploid (CCR, $2n = 24$). Meanwhile, no complete resistance was found in some AMALs examined. This was the first trial toward the establishment of a complete set of *A. cepa*–*A. roylei* monosomic additions.

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Introduction

Allium cepa L. is one of the most economically important species in the section *Cepa* of the genus *Allium* (Brewster 2008). It contains two main groups: the Common onion group and the Aggregatum group. The Common onion group includes the bulb onion, which is the most important *Allium* vegetable crop grown worldwide, with a long cultivation history dating back to approximately 3200 B.C. (McCollum 1976). Meanwhile, the shallot is the most important vegetable and spice crop of the Aggregatum group (Fritsch and Friesen 2002) and is cultivated mainly in low-latitude areas (Siemonsma and Piluek 1993). Up to now, onion breeding has focused on the F_1 hybrid seed production in most parts of the world because of its many favorable characteristics, such as male sterility, resistance to bolting and bulb splitting, high yield, and multiple types of bulb shapes and skin colors (Dowker 1990). On the contrary, crossbreeding of the shallot has been poorly examined because of its main propagation system based on division. Most probably, *A. cepa* itself does not carry resistance genes against modern diseases and pests or other

interesting traits, e.g., a vigorous rooting system (Shigyo and Kik 2008). The exploitation of wild species through interspecific hybridization has, therefore, been recommended for introducing novel characteristics, especially those that are limited in the *A. cepa* gene pool (Kik 2002).

Researchers are now interested in *Allium roylei* with a potential gene reservoir, as this wild species proved to be completely resistant to downy mildew (*Peronospora destructor*) (Kofoet et al. 1990) and partially resistant to leaf blight (*Botrytis squamosa*) (De Vries et al. 1992b) and *Fusarium* basal rot (Galván et al. 2008). This species is considered as a species from the primary (Shigyo and Kik 2008) or secondary (Shigyo 2007) gene pool of *A. cepa* based on the gene pool concept proposed by Harlan and De Wet (1971). Interspecific hybrids between *A. roylei* and *A. cepa* and its backcrosses to *A. cepa* were first recorded by Van der Meer and De Vries (1990). In a study involving a crossing experiment, only a slight isolation barrier was found between *A. cepa* and *A. roylei* (Van Raamsdonk et al. 1992). A close relationship between these two species was also demonstrated by meiotic observations of their F_1 hybrid (De Vries et al. 1992a). Furthermore, *A. roylei* could be used as the bridging species to introduce the genes from *A. fistulosum* into *A. cepa* genomes (Khrustaleva and Kik 2000). Using the tri-hybrid population derived from this bridge-cross, Galván et al. (2011) recently found two quantitative trait loci (QTLs) from *A. roylei* located on chromosomes 2 and 3 contributing to mycorrhizal response. Vu et al. (2011) reported the substitution of *A. roylei* cytoplasm for the production of novel alloplasmic male sterile lines in *A. cepa* via the use of their amphidiploid.

An alien monosomic addition line (AMAL), which is a line with an extrachromosome from a related species, can improve the process of genetic introgression from donor species into recipient species through the production of chromosome substitution and translocation lines (Singh 2003). In *Allium*, a combination of *A. fistulosum* and *A. cepa* has been reported for AMAL production (Peffley et al. 1985; Shigyo et al. 1996; Hang et al. 2004a). The AMALs of *Allium* crops could be used as the tools for the chromosomal assignment of several morphological characteristics (Shigyo et al. 1997b) and phytochemical phenotypes (Shigyo et al. 1997a; Hang et al. 2004b; Dissanayake et al. 2008; Yaguchi et al. 2008, 2009a, b; Masamura et al. 2011) as well as the assignment of linkage groups in the donor species on its specific chromosomes (Van Heusden et al. 2000b; Martin et al. 2005; Tsukazaki et al. 2008, 2011). On the other hand, an increment of the combination number for AMAL development seems to provide effective alternatives to extend the genetic variability of cultivated *Allium* species. In a previous study (Vu et al. 2011), a successful result was reported in the backcrossing of *A. cepa* diploid (genomes CC) with *A. cepa–A. roylei* allotriploid (CCR, $2n = 3x = 24$). Plenty of hyper-diploid plants with $2n = 17$, which may possess an alien extrachromosome from *A. roylei*, could be observed in the BC_2 progeny derived from this cross combination. The aims of the present study were to select a complete set of *A. cepa–A. roylei* monosomic addition lines from the BC_2 progeny via the use of molecular marker and cytogenetic techniques and to find some favorable agronomic traits such as male sterility or downy mildew resistance in this set.

Materials and methods

Crossing procedure for production of monosomic addition lines

The plant materials were *A. roylei* (RR, $2n = 2x = 16$) and two groups of *A. cepa* (CC, $2n = 2x = 16$): shallot (Aggregatum group) and bulb onion (Common onion group). In a previous study, a capital letter "A" was used to indicate the shallot chromosome (Shigyo et al. 1996). However, the "C" used for the onion chromosomes can also be used for those of shallot (De Vries 1990). Therefore, a capital "C" was used for both shallot and bulb onion chromosomes in this study. To produce *A. cepa–A. roylei* monosomic addition lines, an interspecific F_1 hybrid was obtained from a cross between *A. roylei* '97175' and shallot '86208' at first (Fig. 1). After doubling the chromosomes of the clones derived from this single F_1 hybrid, two amphidiploids (plant codes: '28-1' and '33', CCRR, $2n = 4x = 32$) were obtained. These two amphidiploids (as seed parents) were backcrossed with a late bolting bulb onion unknown variety, 'Banchusei', and an asexually propagated shallot clone, 'Chiang Mai' (as pollen parents), to produce BC_1 progenies as allotriploids (CCR, $2n = 3x = 24$), i.e., 'CM26' and 'CM23', respectively. Backcrosses with *A. cepa* or selfings were made to obtain BC_2 or BC_1F_2 populations that were named as BR, SR, RB, and SY (see Fig. 1). The parts surrounded by the frame in this figure were carried out in this study. All processes of the crossings and raising seedlings were carried out according to Vu et al. (2011).

Chromosome observation and karyotype analysis

All plants of BR, SR, RB, and SY lines were used to determine chromosome number. Somatic chromosomes of root tips were observed by Feulgen nuclear staining followed by the squash method. The karyotype analyses were according to the standard nomenclature system for the chromosome of *Allium* (Kalkman 1984), which was

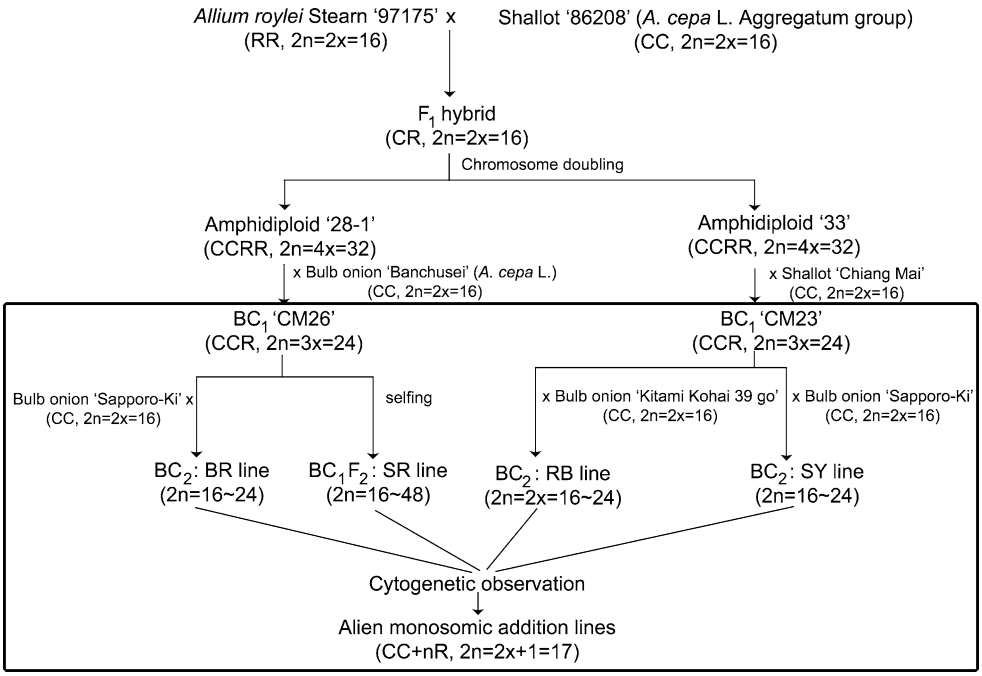

Fig. 1 Method for producing alien monosomic addition lines of *A. cepa* with extrachromosomes from *A. roylei*

generally agreed upon at the Eucarpia 4th *Allium* Symposium (De Vries 1990).

Characterization of alien chromosome based on isozyme and EST markers

Chromosome constitutions of the plants in BC_2 and BC_1F_2 progenies, which possess the chromosome number $2n = 17$, namely alien monosomic addition lines (AMALs), were further characterized using isozyme and EST markers. Van Heusden et al. (2000b) reported that isozyme loci *Lap-1*, *Pgm-1*, and *Pgi-1* are respectively located on 1R, 4R, and 5R in *A. roylei*. The characterization of extrachromosomes from *A. roylei* was, therefore, based on the analyses of these three isozyme loci. The extraction of enzymes, electrophoresis, and staining were carried out following the method of Shigyo et al. (1995a, b) and Van Heusden et al. (2000b).

Because there is a close genetic relationship between *A. roylei* and shallot, we assumed the chromosomal locations of ten EST markers in *A. roylei* from a homoeologous chromosome of the shallot chromosome to which the shallot allele corresponding to each of the markers had been assigned (Table 1). To find the chromosomal locations in *A. cepa* of the three specific markers CA4H, TC2418, and TC1088, the analyses of these markers were carried out in a complete set of *A. fistulosum*–shallot monosomic addition lines produced by Shigyo et al. (1996). For EST marker analyses of the *A. cepa*–*A. roylei* monosomic additions as well as the complete set of *A. fistulosum*–shallot monosomic additions, the total genomic DNA of the parental plants and AMALs was isolated from fresh leaf tissue using a mini-prep DNA-isolation method (Van Heusden et al. 2000a). The reaction mixture contains 20 ng template DNA, 1× Ex buffer, 0.2 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, and 0.25 units Ex *Taq* in a total volume of 10 µl. All PCRs were performed as follows: initial denaturation for 3 min at 94°C and 35–40 cycles of PCR amplification (1 min denaturation at 94°C, 1 min annealing at 60–79°C, and 1 min primer extension at 72°C) on a Program Thermal Cycler iCycler™ (Bio-Rad, Hercules, CA, USA). The number of cycles in PCR amplification and the annealing temperature were optimized for each EST marker. The ramp times were carried out under default conditions with adjusted temperatures at the maximum ramp rate with minimum ramp time. The PCR products were separated by 2% agarose electrophoresis or 5% polyacrylamide gel electrophoresis (PAGE) according to the method of Yaguchi et al. (2009a). If they were monomorphic after denaturing PAGE, they were subjected to restriction analysis. Five microliters of PCR products was incubated for 24 h at 37°C in a total volume of 10 µl using 2 U of restriction enzyme and subsequently resolved by 2% agarose gel electrophoresis or PAGE. Restriction enzymes *Alu*I (for the markers TC131, ACAHN07, and ACAHM36), *Afa*I (for CA4H and TC1088), *Mbo*I (for MSPS), *Hin*fI (for TC2418 and DFR), and *Taq*I (for FLS) (Toyobo, Osaka, Japan) were used in an attempt to generate polymorphism between *A. cepa* and *A. roylei*. Three restriction enzymes *Xsp*I (for CA4H), *Mbo*I (for TC2418), *Kpn*I (for TC1088) were used to reveal polymorphism between shallot and *A. fistulosum*.

Table 1 EST markers for identification of extrachromosomes from *A. roylei*

Primer set	GenBank accession No.	Forward and reverse primers	Chromosome	Reported	Restriction enzyme
CA4H	AY541032	5′-TCGGCAACTGGCTCCAAGTC-3′	1	This study	*Afa*I
		5′-TGATTGACCAGTTCCGCTATGCC-3′			
TC131	AY541031	5′-CCTGCAGAATGAGCTCATC-3′	2	Yaguchi et al. (2009b)	*Alu*I
		5′-GAAGTTTCCGCCATGCAC-3′			
ACAHN07	CF443350	Outside 5′-TTGATAACTCCAATGGGTGTA AATGTCC-3′	3	Kuhl et al. (2004)	*Alu*I
		5′-ATCCTGCTTGGTATCAAGCGGCATGT-3′			
		Inside 5′-GCAAAGAAAGGACTGCTTGTCAA TGCT-3′			
		5′-ATTGCTCGGATCTCTGAGTCCATGTAG-3′			
SiR	CF434863	5′-TGCAGCTCTTTCTCAAGTTGG-3′	3	McCallum et al. (2007)	–[a]
		5′-CAGAGCAGGACATGCCATAG-3′			
FLS	AY221247	5′-TTAAGGACGACCACTGGTT-3′	4	Masuzaki et al. (2006)	*Taq*I
		5′-CCACGACATCCGTGACT-3′			
TC2418	CF438524, CF438854	5′-CATCAGGAGAAGATATCAATGCTC-3′	5	This study	*Hin*fI
		5′-GCCTGTCTTCACTCCTAGTC-3′			
TC1088	CF451692, CF448264	5′-TAACGCCGTCAAACTCTACC-3′	6	This study	*Afa*I
		5′-CTCGGTGGACAACCTTACC-3′			
ACAHM36	CF443252	Outside 5′-TTCCTTGCTCAAGGATACG AGGATG-3′	7	Martin et al. (2005)	*Alu*I
		5′-GATCATTTCATCGTCATTTGCCTCG-3′			
		Inside 5′-GAAGAGAAGATGCTGGGGAATC TCG-3′			
		5′-GAGCAGATAGCTCTGCAGCACTCTG-3′			
DFR	AY221250	5′-ACAGATGTACTTTGTGTCCA-3′	7	Masuzaki et al. (2006)	*Hin*fI
		5′-GCTTCATCGAACATACTTTCC-3′			
MSPS	EU164758	5′-GAAGGCTGATATTGTTGGTGAAG-3′	8	Yaguchi et al. (2008)	*Mbo*I
		5′-TGTGTCGTAGGAGCCTGATG-3′			

[a] No restriction enzyme was used

Bulb morphology assessment

After harvesting, the bulb morphologies of some lines in each of eight AMALs were evaluated. The maximum horizontal and vertical diameters of the bulbs were measured, bulb weights were recorded, and bulb colors were noted.

GISH analysis

The procedure of GISH analysis was according to the method of Khrustaleva and Kik (2000) with minor modifications.

Seven individual plants of *A. cepa–A. roylei* AMALs (CC+1R, +3R, +4R, +5R, +6R, +7R, and +8R) were used for GISH analysis. Total genomic DNA was extracted from 4 g of young leaves from shallot 'Chiang Mai' and *A. roylei* '97175'. The extracted DNA of *A. roylei* was labeled with digoxigenin-11-dUTP by a standard nick-translation protocol (Roche Diagnostics GmbH, Mannheim, Germany). The extracted DNA of shallot was used as a blocking DNA.

The hybridization mixture contained 50% (v/v) deionized formamide, 10% (w/v) sodium dextran sulfate, 10% (v/v) 20× SSC, 0.25% (w/v) SDS, 1 ng/μl digoxigenin-labeled DNA of *A. roylei*, and 0.06–0.08 μg/μl blocking DNA of shallot. Digoxigenin-labeled DNA was detected with anti-digoxigenin-FITC raised in sheep (Roche, Mannheim, Germany), then amplified with anti-sheep-FITC raised in rabbit (Vector Laboratories, Burlingame, USA), and finally amplified with anti-rabbit-FITC raised in goat (Vector Laboratories, Burlingame, USA).

Meiotic observation and pollen fertility test

Acetocarmine smears of pollen mother cells (PMCs) were used for meiotic studies. Fresh pollen grains were stained with 1% acetocarmine to observe their viability. The

Table 2 Seed set, seed germination, and number of seedlings survival of allotriploids CM23 and CM26 (CCR) in backcrosses with bulb onion and selfing

Cross combination	Line	Number of seeds produced	Number of seeds that germinated	Number of seedlings that survived
CM23 × 'Kitami Kohai 39 go'	RB	178	130	127
CM23 × 'Sapporo-Ki'	SY	–[a]	52	52
'Sapporo-Ki' × CM26	BR	3	3	3
CM26 selfed	SR	11	6	6

[a] Not counted

Table 3 Frequency of chromosome numbers in BC_2 (BR, RB, and SY) and BC_1F_2 (SR) progenies

Line	Number of plants observed	Frequency of plants								
		Chromosome number								
		16	17	18	19	22	23	24	28	32
RB	108	68	38	–[a]	–	–	1	1	–	–
BR	3	2	1	–	–	–	–	–	–	–
SR	6	2	1	–	–	1	–	–	1	1
SY	52	38	8	1	1	1	1	2	–	–
Total	169	110	48	1	1	2	2	3	1	1

[a] Not determined

evaluation of pollen fertility was performed according to Shigyo et al. (1999). The pollen fertility was checked during the first or second week after blooming of the first floret in each flower umbel.

Downy mildew screening in field

A test on downy mildew resistance for some AMALs and two multiple alien addition lines from RB lines was carried out in the experimental field of Takii & CO., LTD., in Konan City, Shiga Prefecture, Japan (N35°00′, E136°05′). The bulbs were sown in pots, and germinated plants were then transplanted to the field in the beginning of October 2009. Spores were collected from the surface leaves of artificially inoculated plants with a fine paintbrush and suspended in distilled water with a final concentration of 10^3 spores/ml. A spore suspension was sprayed over the plants for six times in 2010 (on 5, 17, and 23 March and on 5, 8, and 20 April). The first day of sporulation for each inoculated plant was recorded. Symptoms on plant leaves were observed in the field during the growing period.

Results

Production of alien monosomic addition lines

In backcrossing and selfing of allotriploids (CCR, $2n = 24$) to produce BC_2 (RB, SY, and BR lines) and BC_1F_2 (SR line), seed setting could be obtained and most of the seedlings survived (Table 2). All plants of BC_2 and BC_1F_2 progenies were used for the chromosome count. Their chromosome numbers are shown in Table 3. The chromosome number of BC_2 plants varied from $2n = 16$ to 24. On the other hand, in the BC_1F_2 progeny, the chromosome number ranged from $2n = 16$ to 32. In total, the highest frequency of plants was found with chromosome number $2n = 16$ (110 plants), followed by $2n = 17$ (48 plants). Other chromosome numbers, $2n = 18$, 19, 22, 23, 24, 28, and 32, were recorded with a low number of plants (one to three in each case). Finally, all 48 plants recorded as $2n = 17$ were used for further characterization of their chromosome constitution.

Identification of alien monosomic additions

The analyses of karyotypes, isozymes, and EST genetic markers were carried out to identify the extrachromosomes of *A. roylei* in plants with $2n = 17$ that had been produced. As the chromosome 6 of *A. roylei* showed the most distinctive sub-telocentric shape in its basikarotype (Sharma and Gohil 2008), the AMALs possessing this chromosome could easily be recognized by conventional karyotype analysis. However, the identification of the other AMALs by karyotyping was not always easy. Therefore, the chromosome constitutions of all AMALs were confirmed further by molecular markers. Every plant of 48 AMALs was analyzed with at least one chromosome-specific marker (isozyme or EST markers) for each of eight different chromosomes derived from *A. roylei* (1R–8R).

The three isozymes could show the clear polymorphism between the parental lines [*A. cepa* (shallot and bulb onion)

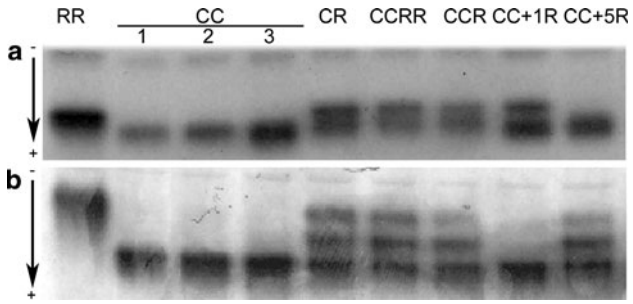

Fig. 2 *Lap-1* (**a**) and *Pgi-1* (**b**) zymograms in *A. roylei* '97175' (RR), shallot '86208' (CC1), shallot 'Chiang Mai' (CC2), bulb onion 'Kitami Kohai 39 go'(CC3), F_1 hybrid (CR), amphidiploid '33' (CCRR), allotriploid 'CM23' (CCR), AMAL 'RB34' (CC+1R) and AMAL 'RB109' (CC+5R)

and *A. roylei*]. Therefore, they could be adopted for determining the presence of specific chromosomes of *A. roylei* in the BC_2 and BC_1F_2 progenies. The F_1 hybrid, amphidiploid, and allotriploid plants showed both bands of *A. roylei* and *A. cepa* for the locus *Lap-1* (Fig. 2a). An additional band at intermediate mobility between the parental bands was found for the locus *Pgi-1* (Fig. 2b). The summary of isozyme analysis in AMALs was included in Table 4. With the isozyme markers *Lap-1* and *Pgi-1*, the AMALs CC+1R and +5R were characterized.

The three EST markers CA4H, TC2418, and TC1088 were, respectively, determined to be located on the chromosomes 1, 5, and 6 of *A. cepa* via the use of the complete set of *A. fistulosum–A. cepa* monosomic additions (Fig. 3). These three markers were, therefore, included in EST marker analyses for identification of extrachromosome in *A. cepa–A. roylei* monosomic additions in this study.

All EST markers, except for the SiR marker on chromosome 3, appeared as one single PCR product on 2% agarose gel in both *A. roylei* and *A. cepa*, and no polymorphism was detected. For the SiR marker, two PCR products were observed in *A. roylei*, from which the size of one product is distinguishable from one product of *A. cepa*. For the monomorphic markers, an interspecific polymorphism was generated using restriction enzymes to show polymorphisms between *A. cepa* and *A. roylei*. The DNA fragments derived from *A. roylei* were used to confirm the presence of *A. roylei* respective chromosomes (Fig. 4). The results of EST marker analyses in AMALs were summarized in Table 4. All EST markers, except for TC1088, were present in one or more AMALs. Seven AMALs (CC+1R–5R and CC+7R–8R) were identified by EST markers. Although the marker TC1088 for chromosome 6 was not detected in any AMAL, two AMALs, RB82 and RB120, were determined to possess three sub-telocentric chromosomes (homoeologous chromosome group 6) by karyotype analysis. RB82, however, also showed the presence of the marker for chromosome 7 of *A. roylei*, and its chromosome constitution was determined as unidentified. RB120 was confirmed by the following GISH analysis. Figure 5 shows representative somatic metaphase chromosomes of a complete set of AMALs.

Finally, this study demonstrated that the production of a complete set of AMALs was not impossible in the species combination of *A. cepa* (recipient) and *A. roylei* (donor). The chromosome constitutions of 13 plants could not be identified due to the absence of all markers (11 plants) or the presence of more than one different chromosome-specific markers (two plants). Among AMALs of both RB and SY lines, the highest frequency of plants was found in CC+8R (RB: 34%, SY: 38%), followed by CC+7R (13%, 25%) and CC+3R (8%, 13%). The other AMALs, CC+1R, +2R, +5R, and +6R, appeared with lower percentages. All eight possible AMALs, except for CC+4R, were obtained in RB and SY lines. However, one plant of CC+4R appeared in BR lines when the allotriploid was used as the pollen parent.

GISH analysis of alien monosomic additions

The GISH analyses included seven individual plants, RB34, SY45, BR1, SY7, RB120, SY24, and RB66, which were determined previously through karyotype and molecular markers as AMALs CC+1R, +3R, +4R, +5R, +6R, +7R, and +8R, respectively (Fig. 6a). All types of the examined AMALs, except for CC+6R, showed an intact chromosome from *A. roylei* as a single chromosome addition with a complete set of 16 chromosomes from *A. cepa* without any translocation. This result confirmed the integrity of the additional chromosome from *A. roylei* in each of these six AMALs. One exception, RB120, showed a single recombinant chromosome 6R′ with segments derived from both *A. roylei* and *A. cepa* in an integral diploid back ground of *A. cepa*. The green fluorescence segment derived from *A. roylei* occupied the terminal half of the long arm of the chromosome 6R′.

Meiosis in alien monosomic additions

Five different AMALs derived from five individual plants comprising four plants of SY7 (CC+5R), RB120 (CC+6R′), SY24 (CC+7R), and RB66 (CC+8R) that were previously used for GISH analysis and plant SY42 (CC+1R) were further investigated for their meiotic behavior during flowering time of 2011 (Fig. 6b). Meiosis of the AMALs CC+2R, +3R, and +4R was not observed because some plants of these lines did not develop flower stalks in this year, and the collection time of flower buds from the other plants was late to obtain PMCs at metaphase-I(MI). In the four AMALs, CC+1R,

Table 4 Identification of extrachromosomes in *A. cepa–A. roylei* monosomic addition lines via chromosome-specific isozyme markers (*Lap-1*, *Pgm-1*, and *Pgi-1*) and EST markers (the others)

Line	Group	Frequency of plants	*A. roylei* chromosome-specific genetic markers														Extrachromosome
			(1R)[a]		(2R)	(3R)		(4R)		(5R)		(6R)[b]		(7R)		(8R)	
			Lap-1	CA4H	TC131	ACAHN07	SiR	*Pgm-1*	FLS	*Pgi-1*	TC2418	Karyotype	TC1088	ACAHM36	DFR	MSPS	
RB	1	1	1[c]	–[e]	0	0	–	0	–	0	–	0	0	0	–	0	1R
	2	1	0[d]	–	1	0	–	0	–	0	–	0	0	0	–	0	2R
	3	3	0	–	0	1	–	0	–	0	–	0	0	0	–	0	3R
	4	1	0	–	0	0	–	0	–	1	–	0	0	0	–	0	5R
	5	1	0	–	0	0	–	0	–	0	–	1	0	0	–	0	6R
	6	5	0	–	0	0	–	0	–	0	–	0	0	1	–	0	7R
	7	13	0	–	0	0	–	0	–	0	–	0	0	0	–	1	8R
	8	1	0	–	0	0	–	0	–	1	–	0	0	0	–	1	Unidentified
	9	1	0	–	0	0	–	0	–	0	–	1	0	1	–	0	Unidentified
	10	11	0	–	0	0	–	0	–	0	–	0	0	0	–	0	Unidentified
SY	1	1	–	1	0	–	0	–	0	–	0	–	0	–	0	0	1R
	2	1	–	0	0	–	1	–	0	–	0	–	0	–	0	0	3R
	3	1	0	0	0	–	0	0	0	1	1	–	0	–	0	0	5R
	4	2	0	0	0	–	0	0	0	0	0	–	0	–	1	0	7R
	5	1	–	0	0	–	0	–	0	–	0	–	0	–	0	1	8R
	6	2	0	0	0	–	0	0	0	0	0	–	0	–	0	1	8R
BR	1	1	–	0	0	–	0	–	1	–	0	–	0	–	0	0	4R
SR	1	1	–	0	0	–	0	–	0	–	1	–	0	–	0	0	5R

[a] The symbols inside parentheses show chromosome numbers of *A. roylei* on which the genetic markers are located

[b] Chromosome 6 was characterized by karyotype analysis

[c] Presence

[d] Absence

[e] Not conducted

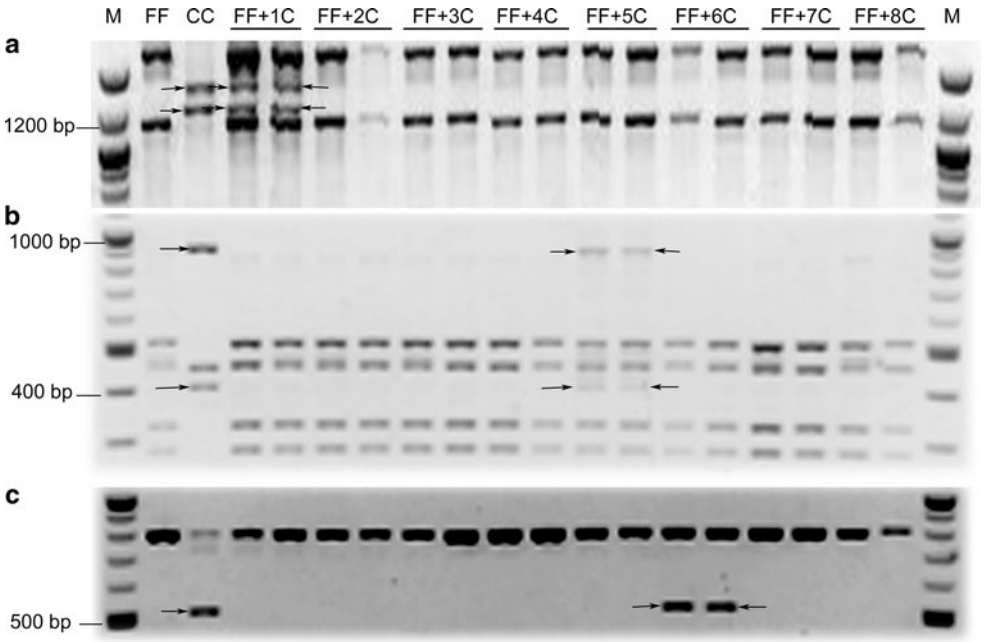

Fig. 3 Representative PCR amplification profiles of EST markers CA4H (**a**), TC2418 (**b**) and TC1088 (**c**) after digestion by restriction enzymes on 2% agarose in *A. fistulosum* (FF), shallot 'Chiang Mai' (CC) and a complete set of *A. fistulosum*–shallot monosomic addition lines (FF+1C–FF+8C). *Arrows* indicate chromosome-specific markers of shallot. *M* molecular size marker (100 bp ladder)

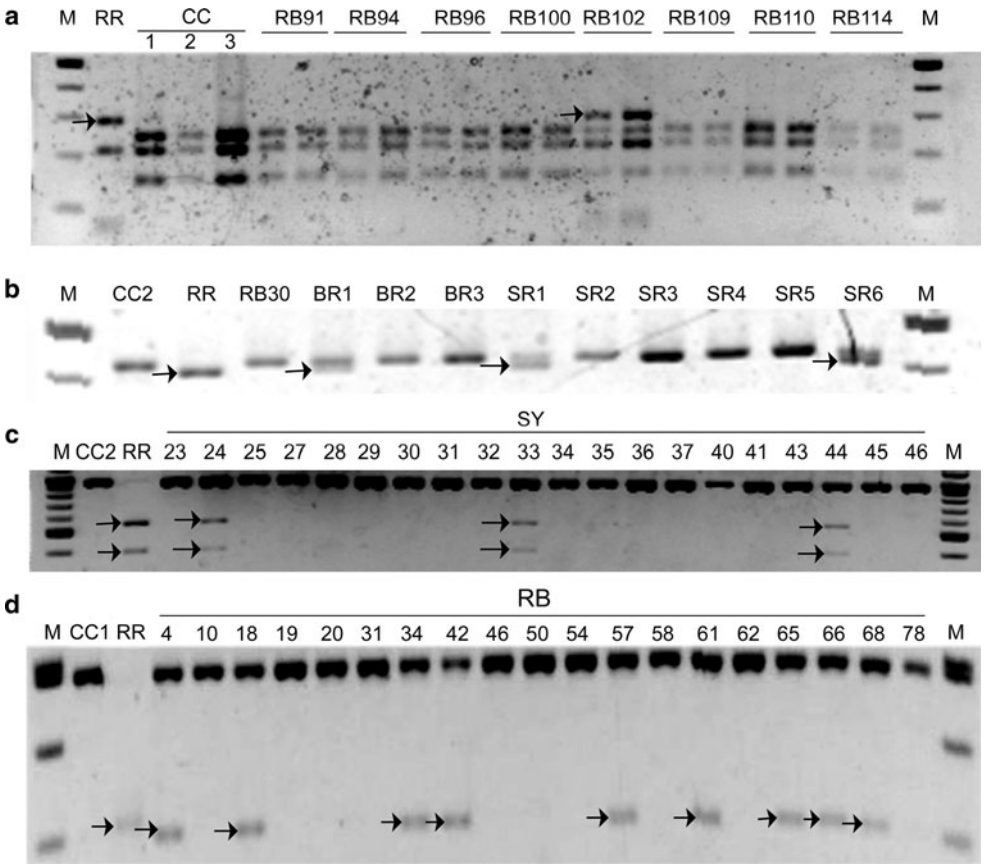

Fig. 4 Representative PCR amplification profiles of the chromosome-specific EST markers ACAHN07 (3R) (**a**), FLS (4R) (**b**), DFR (7R) (**c**) and MSPS (8R) (**d**) after digestion by restriction enzymes on 2% agarose (**a**–**c**) and 5% polyacrylamide (**d**) in *A. roylei* '97175' (RR), shallot '86208' (CC1), shallot 'Chiang Mai' (CC2), bulb onion 'Kitami Kohai 39 go'(CC3), BC_2 (RB, SY and BR), and BC_1F_2 (SR). *Arrows* indicate chromosome-specific markers of *A. roylei*. *M* molecular size marker (100 bp ladder)

+5R, +7R, and +8R, a high percentage of PMCs at the MI of meiosis formed eight bivalents and one univalent; and the cells with seven bivalents and one trivalent also appeared, but with limited numbers ranging from 6.7 to 24.3% (Table 5). However, CC+6R′ showed eight bivalents and one univalent or seven bivalents and one trivalent of chromosome 6 (Fig. 6b), with the possibility of around 50% for each case (Table 5).

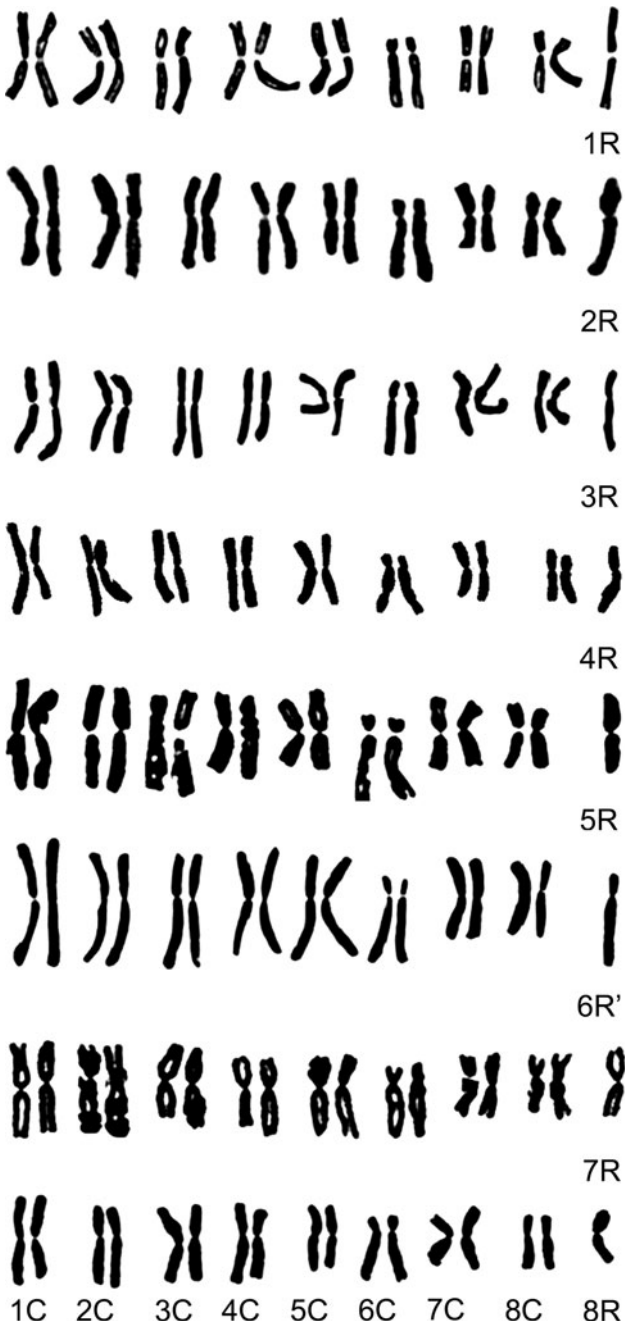

Fig. 5 Somatic metaphase chromosomes of eight different *A. cepa*–*A. roylei* monosomic addition lines (CC+1R, RB34; CC+2R, RB54; CC+3R, RB62; CC+4R, BR1; CC+5R, RB109; CC+6R′, RB120; CC+7R, RB81; CC+8R, RB68)

Pollen fertility of alien monosomic additions

A total of the 24 plants that came into bloom in the spring of 2007–2010 were checked for their pollen fertility (Table 6). All plants except BR1 (CC+4R) showed very low pollen fertility or completely sterility (0–4.3%). The CC+4R showed a 42.6% of the pollen fertility. This plant is an autoplasmic line derived from the cross when a bulb onion was used as a seed parent. Meanwhile, all other plants are alloplasmic lines carrying cytoplasm from *A. roylei*.

Bulb morphology of alien monosomic additions

Bulb characteristics including shape, color, and weight were investigated with several types of AMALs. Shallot showed spherical bulbs with purple outer scale, while *A. roylei* displayed ovoid bulbs and brown outer scale. Therefore, the introgression of single chromosome from *A. roylei* might actually alter the morphological features on bulbs in the recipient species. The bulb of BR1 (CC+4R) was distinct from the others and had an ovoid shape and brown skin color (Table 7, Fig. 6c). The bulbs of CC+2R, +3R, +5R, and +8R were spherical, whereas those of CC+1R, +6R′, and +7R, were oval (Fig. 6c). The red purple color of the outer bulb skin was observed in CC+2R, +3R, +5R, and +7R. The other three AMALs, CC+1R, +6R′, and +8R, were light purple. The heaviest bulb was found in CC+4R (Table 7).

Screening for downy mildew resistance

Fourteen plants of six AMALs [CC+1R (one plant), +3R (two), +4R (one), +5R (one), +7R (four), and +8R (five)], one plant of hypo-allotriploid, RB35 ($2n = 3x - 1 = 23$, CCR-8R), and one plant of allotriploid, RB51 ($2n = 3x = 24$, CCR), were used to screen for resistance to downy mildew in the field (Table 8). The screening test for CC+2R and +6R was not carried out due to insufficient number of plant materials. Seven plants (one from CC+3R, four from CC+7R, and two from CC+8R) showed an early sporulation after the first inoculation of spores. Six other plants (one plant from CC+4R, one from CC+5R, two from CC+8R, and two from the allotriploids) showed a late sporulation after four cycles of inoculation. Furthermore, late sporulation (after six inoculation cycles) was observed in three remaining plants, each from the CC+1R, +3R, and +8R. However, after six cycles of inoculation, all plants of the AMALs had severe disease symptoms and died at the end of the growing period. On the other hand, light and moderate symptoms appeared in the hypo-allotriploid, RB35, and the allotriploid, RB51. These two lines could survive until a final phase.

Discussion

In diploid recipient species, AMALs had thus far been obtained in 20 different species combinations, including that of the present study (Singh 2003). However, all the possible types of AMALs were found in only eight cases,

while, in others, a complete set was lacking. The largest holders were *Beta* with three cases, *Allium* with two, and the remaining three species (*Lycopersicon*, *Nicotiana*, and *Oryza*) with one.

In *Allium*, Peffley et al. (1985) reported four types out of the *A. cepa–A. fistulosum* monosomic additions derived from a cross between allotriploid (seed parent) and bulb onion. They assumed that hyperploid zygotes or seedlings may possess a low survival rate, which resulted in few progeny plants with extrachromosomes. It was also difficult to complete eight possible shallot (*A. cepa* Aggregatum group)–*A. fistulosum* monosomic additions (Hang et al. 2004a). They suggested that the possible existence of deleterious alleles that are maintained in the heterozygous state remaining in shallot genomes may reduce the survival rate of progeny plants homozygous for these alleles. In this study, we produced *A. cepa–A. roylei* monosomic additions through three different cross combinations. In 2005, a pilot crossing, allotriploid CCR (♀) × bulb onion, had been carried out at first in an experimental field at Yamaguchi University (lat. 34°N, long. 131°E), being less than successful due to an insufficient number of the obtained progeny. A sufficient number of the BC_2 progenies (RB and SY) could be surprisingly obtained in the same way for crossing at Hokkaido (44°N, 142°E) in 2006. This could be interpreted assuming that the seed and pollen fertilities of *A. roylei* and its derivatives vary considerably with latitude. Furthermore, AMALs appeared with obviously high frequency (28.8%) in these progenies. This result shows a good agreement with that of Peffley et al. (1985). Multiple crossings at high latitude regions seem to be a valid and feasible way to produce monosomic addition lines of *A. cepa–A. roylei* although those of *A. fistulosum*–shallot were not the case (Shigyo et al. 1996). On the other hand, all types of AMALs, except CC+4R, were developed from RB and SY. One possibility for the absence of CC+4R is that a relatively low number of AMALs (46 plants) in RB and SY lines were produced. Actually, we could find the presence of chromosome 4R via *Pgm-1* marker in some multiple addition lines ($2n = 19, 22, 23, 24$) of BC_2 plants in RB and SY (data not shown). In addition, it is well possible that the appearance frequency differs between chromosomes. Shigyo et al. (1996) reported that the appearance frequencies varied largely among eight types of *A. fistulosum*–shallot monosomic additions. The authors also suggested that the extrachromosomes might affect the survival of AMALs. While, one plant of CC+4R was obtained via a special cross combination between the bulb onion 'Sapporo-Ki' (seed parent) and the allotriploid. Therefore, the other possibility might be that the transmission rate of chromosome 4R through male gametes was higher than that through female gametes. The maternal transmission of alien chromosomes from a donor into a recipient is generally higher than the paternal one in many crops (Multani et al. 1994; Singh et al. 1998; Akaba et al. 2009). However, in some special cases, the preferential transmission through male gametes has been reported in some specific chromosomes of wild species, such as chromosome $5H^tL$ in the transmission from *Elymus trachycaulus* to common wheat (Jiang and Gill 1998) or chromosome 5R from rye to wheat (Efremova et al. 1996). To explain the phenomenon in our study, it is necessary to investigate the occurrence rate of CC+4R in a larger number of BC_2 plants from reciprocal backcrosses.

The modifications of morphological characteristics in AMALs are mostly due to the interaction between genes of the recipient and donor parents (Singh 2003). It has been reported that the traits of color, shape, and size of bulbs in *A. cepa* are quantitatively inherited (El-Shafie and Davis 1967; McCollum 1971). McCollum (1966) suggested the genetic determiners of the bulb height and shape index (the ratio of bulb height to bulb diameter), which might be viewed as two measures of the same genetic traits. On the other hand, bulb diameter and bulb weight were affected by

Fig. 6 **a** Somatic metaphase cells of seven types of *A. cepa–A. roylei* monosomic addition lines (1R, 3R, 4R, 5R, 6R′, 7R, and 8R) after genomic in situ hybridization. *Arrow* indicates recombinant chromosome 6. **b** Chromosome pairings at MI in PMCs of five types of monosomic addition lines (1R, 5R, 6R′, 7R, and 8R). *Black* and *white arrows* indicate univalent and trivalent, respectively. **c** Dormant bulbs of shallot (P1), *A. roylei* (P2) and a set of monosomic addition lines (1–5R, 6R′, 7–8R)

Table 5 Chromosome configurations in PMCs at MI of *A. cepa–A. roylei* monosomic addition lines

AMALs	Plant code	No. of PMCs observed	Frequency of PMCs	
			Chromosome pairing	
			8II + 1I	7II + 1III
CC+1R	SY42	40	33 (82.5)[a]	7 (17.5)
CC+5R	SY7	37	28 (75.7)	9 (24.3)
CC+6R′	RB120	41	21 (51.2)	20 (48.8)
CC+7R	SY24	30	28 (93.3)	2 (6.7)
CC+8R	RB66	30	26 (86.7)	4 (13.3)

[a] Numbers in parentheses indicate percentages

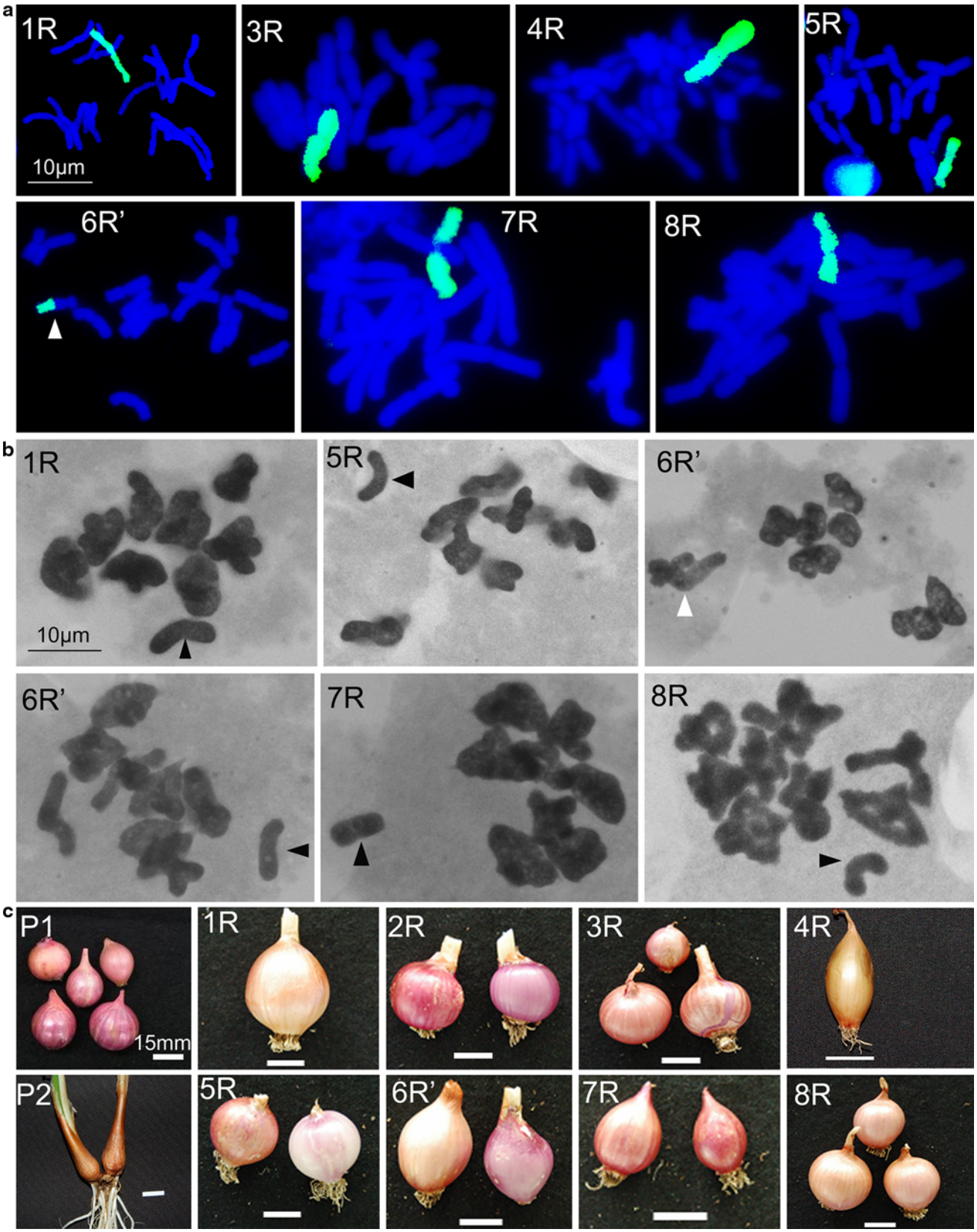
a
1R
3R
4R
5R
10μm
6R'
7R
8R
b
1R
5R
6R'
10μm
6R'
7R
8R
c
P1
1R
2R
3R
4R
15mm
P2
5R
6R'
7R
8R

Table 6 Pollen fertility in *A. cepa–A. roylei* monosomic addition lines

Plant material	CC + extrachromosome	Plant code	Pollen fertility (%)								Average
			2007		2008		2009		2010		
			1st	2nd	1st	2nd	1st	2nd	1st	2nd	
AMALs	CC+1R	SY42	–[a]	–	–	–	–	–	0.0	0.0	0.0 ± 0.0
	CC+2R	RB54	–	–	2.6	2.6	0.0	–	–	–	1.7 ± 0.9
	CC+3R	RB62	–	–	0.0	0.0	–	–	–	–	0.0 ± 0.0
		RB86	–	–	0.0	–	–	–	–	–	0.0 ± 0.0
	CC+4R	BR1	–	–	42.6	–	–	–	–	–	42.6
	CC+5R	RB109	–	–	0.0	0.0	–	–	–	–	0.0 ± 0.0
	CC+6R′	RB120	–	–	0.0	0.0	–	–	–	–	0.0 ± 0.0
	CC+7R	RB20	–	–	0.0	0.0	0.0	0.0	–	–	0.0 ± 0.0
		RB58	–	–	15.8	1.0	0.1	0.3	–	–	4.3 ± 3.8
		RB78	–	–	0.0	0.0	0.0	–	–	–	0.0 ± 0.0
		RB81	6.2	–	0.4	4.3	0.0	0.0	–	–	2.2 ± 1.3
		RB94	–	–	0.0	0.0	–	–	–	–	0.0 ± 0.0
	CC+8R	RB4	–	–	0.2	0.0	0.3	0.0	–	–	0.1 ± 0.1
		RB18	–	–	–	–	0.8	0.1	–	–	0.5 ± 0.4
		RB42	–	–	0.0	0.0	0.5	0.0	–	–	0.1 ± 0.1
		RB61	–	–	–	–	0.0	–	–	–	0.0 ± 0.0
		RB65	–	–	–	–	0.0	0.0	–	–	0.0 ± 0.0
		RB66	–	–	0.0	0.0	2.7	0.0	–	–	0.7 ± 0.7
		RB68	–	–	0.0	1.5	0.5	0.0	–	–	0.5 ± 0.3
		RB85	–	–	0.0	0.0	0.0	–	–	–	0.0 ± 0.0
		RB96	–	–	–	–	0.0	0.0	–	–	0.0 ± 0.0
		RB100	–	–	0.0	0.0	–	–	–	–	0.0 ± 0.0
		RB114	–	–	0.0	0.3	0.0	–	–	–	0.1 ± 0.1
		RB117	1.3	3.5	0.0	0.0	0.0	–	–	–	1.0 ± 0.7
A. roylei '97175'			–	–	23.0	–	–	–	–	–	23.0
Shallot 'Chiang Mai'			–	–	93.7	–	–	–	–	–	93.7
F_1 hybrid			–	–	0.2	–	–	–	–	–	0.2
Allotriploid 'CM23'			–	–	0.3	–	–	–	–	–	0.3
Allotriploid 'CM26'			–	–	11.5	–	–	–	–	–	11.5

[a] Not observed

the environmental conditions. The distinct ovoid bulbs of CC+4R, resembling those of *A. roylei*, may suggest that some major genes related to this bulb feature are located on the chromosome 4 of *A. roylei* and interact with the genes in *A. cepa* genomes to define the final trait of this AMAL. Either way, it is possible to identify at least CC+4R morphologically. In this study, isozyme and EST markers, the chromosomal locations of which had been reported in *A. cepa* (Shigyo et al. 1995a, b; Martin et al. 2005; Masuzaki et al. 2006; McCallum et al. 2007; Yaguchi et al. 2008, 2009b) and *A. fistulosum* (Yaguchi et al. 2009a), were applied for the characterization of extrachromosomes from *A. roylei* in the AMALs. The result of marker analyses corresponded well with that of the karyotypic observation, except for TC1088 which was not present in the translocation line CC+6R′. Three of the seven isozyme markers employed for the correspondence of paternal (*A. roylei*) and maternal (*A. cepa*) linkage groups in the previous study (Van Heusden et al. 2000b) were presently used to identify *A. roylei* alien chromosomes in an *A. cepa* diploid background. The universal chromosome-specific markers in *Allium* could accurately detect the homoeologous chromosomes of *A. roylei* as well as the two cultivated species in alien addition lines. The six lines possessing each extrachromosome of 1R, 3R, 4R, 5R, 7R, and 8R were proven to be true AMALs through GISH. In these AMALs, the majority of PMCs showed preferential parings between homologous chromosomes of *A. cepa*, leaving the chromosomes of *A. roylei* as univalent. On the other hand, the presence of trivalents between chromosomes

Table 7 Morphological characteristics of bulbs in *A. cepa–A. roylei* monosomic addition lines

AMALs	Number of lines observed	Number of plants	Bulb diameter (mm)	Bulb height (mm)	Bulb weight (grams)	Bulb diameter/ bulb height	Bulb shape	Outer scale color
CC+1R	1	2	21.6	33.0	7.3	0.65	Oval	Light purple
CC+2R	1	2	34.9	33.0	20.9	1.05	Sphere	Red purple
CC+3R	3	9	29.2 ± 3.8	28.4 ± 1.5	5.3 ± 0.5	1.04 ± 0.03	Sphere	Red purple
CC+4R	1	2	38.6	60.8	43.4	0.63	Ovoid	Brown
CC+5R	1	4	29.7 ± 1.9	28.2 ± 0.9	13.0 ± 2.3	1.05 ± 0.04	Sphere	Red purple
CC+6R′	1	2	34.1	31.6	17.4	1.08	Sphere	Light purple
CC+7R	5	25	17.4 ± 0.5	25.5 ± 0.6	3.4 ± 0.2	0.69 ± 0.02	Oval	Red purple
CC+8R	10	36	27.0 ± 0.8	29.5 ± 0.7	9.5 ± 0.6	0.92 ± 0.03	Sphere	Light purple

Table 8 Downy mildew screening of *A. cepa–A. roylei* monosomic addition lines, hypo-allotriploid, allotriploid, and *A. roylei*

Plant materials	Chromosome constitution	Plant code	Days of first appearance of sporulation after first inoculation	No. of inoculation cycles before sporulation	Degree of symptom on leaves	Final evaluation
AMALs	CC+1R	RB34	47	6	++[a]	S[d]
	CC+3R	RB62	11	1	++	S
		RB86	47	6	++	S
	CC+4R	BR1	32	4	++	S
	CC+5R	RB109	32	4	++	S
	CC+7R	RB58	11	1	++	S
		RB78	4	1	++	S
		RB81	11	1	++	S
		RB94	11	1	++	S
	CC+8R	RB42	32	4	++	S
		RB65 (2)[f]	32	4	++	S
		RB68	11	1	++	S
		RB85	47	6	++	S
		RB96	4	1	++	S
Hypo-allotriploid	CCR-8R	RB35 (2)	32	4	+[b]	R[e]
Allotriploid	CCR	RB51	32	4	+−[c]	R
A. roylei	RR	95001-3 (5)	47	6	+− or +	R
		95001-6 (5)				
		95001-10 (5)				
		95001-21 (5)				
		95001-24 (5)				
		97175-1 (2)				

[a] Severe symptom

[b] Moderate symptom

[c] Light symptom

[d] Susceptible

[e] Resistant

[f] Number of bulbs used

of the two species in these lines showed a different phenomenon from *A. fistulosum–A. cepa* monosomic additions reported by Shigyo (1997c), in which only bivalents and univalents were observed. This result suggested that *A. roylei* possesses a higher degree of homoeology with *A. cepa* than *A. fistulosum*. The translocation was observed in only one AMAL with a 6C-6R recombinant chromosome in the integral diploid background of *A. cepa*. The recombination point might have been located at the interstitial region. This translocation line probably resulted from chiasma formation

among chromosome 6 of *A. cepa* and *A. roylei* during meiosis of the allotriploid 'CM23' used in backcrossing. This line showed nearly an equal rate of PMCs with the univalent and trivalent, suggesting that the recombinant chromosome 6C-6R has high homology with the chromosome 6C of *A. cepa*. In the present study, the integrated applications of the three different methodologies enabled us to clarify chromosomal constitutions of our AMALs.

In our previous study, a complete male sterility was recorded in diploid *A. cepa* harboring cytoplasm from *A. roylei* or alloplasmic lines (Vu et al. 2011). It was suggested that the sterility would be caused by incompatibility between the nuclear genomes of *A. cepa* and the cytoplasm of *A. roylei*. The data of this study would, therefore, indicate the effect of *A. roylei* cytoplasm on the pollen fertility of the AMALs. Tsutsui et al. (2011) newly found stable male sterility in alloplasmic AMALs of *Brassica rapa* carrying the cytoplasm and an extrachromosome from *Moricandia arvenis*. The probable presence of its fertility-restoring gene could not be proved since all of the alloplasmic AMALs carrying cytoplasm from *A. roylei* showed an adequate level of male sterility. The restoring genes of pollen fertility were found in some specific types of alloplasmic *Moricandia arvenis–Raphanus sativus* monosomic additions (Bang et al. 2002) or in the syntenic group 6 of alloplasmic *Brassica campestris–B. oxyrrhina* monosomic additions (Srinivasan et al. 1998). Relatively high pollen fertility was only found in the autoplasmic CC+4R, likely because of the lack of effect of the *A. roylei* cytoplasm. The direct introduction of the 4R chromosome to the alloplasmic line might be possible through a crossing with the CC+4R as a pollen parent. If a fertility-restoring gene existed on this chromosome, the restoration of pollen fertility would be clearly observed in the alloplasmic CC+4R.

The resistance of *A. roylei* to downy mildew (*P. destructor*) in greenhouse and field conditions is well-known (De Vries et al. 1992a). Recently, its resistance gene allocated on the chromosome 3 was transferred successfully into a bulb onion, and a disease-resistance test was carried out in a trial field in Europe (Scholten et al. 2007). In this study, we carried out the disease-resistance test under field conditions in Japan with *A. roylei* and the *A. cepa–A. roylei* chromosome addition lines. From the results of downy mildew screening, no complete resistance was found in any of the tested plants. In this study, even *A. roylei* showed the symptoms with sporulation on the leaves. This finding was irreconcilable with the results of the above two articles which reported a complete resistance in *A. roylei*. It has been reported that disease resistance in various plants is affected by environmental conditions as well as pathogen populations. In maize, the resistance inheritance to downy mildew (*Peronospora sorghi*) was affected not only by certain conditions (Williams 1984) but also by regions and pathogenic differences (Singburaudom and Renfro 1982). As mentioned in a previous report (Schwartz 2008), a high-humidity environment, as in Japan, seems to be more favorable for downy mildew development than a relatively dry environment, as in Europe. One possible hypothesis for this phenomenon is that the susceptibility was due to the severe condition of the disease-resistance test under high humidity at the field in Japan. In addition, Japanese fungal strains might be different from European ones. All the AMALs including those of CC+3R were completely susceptible. This result is in contrast to the result of Scholten et al. (2007) who used GISH to show that a single introgression of *A. roylei* on chromosome 3 was enough for resistance to downy mildew. However, the light-to-moderate symptoms and the survival of *A. roylei*, the single deletion, and the multiple addition lines indicated that they had higher levels of resistance than those of all the tested AMALs. Among the AMALs, the plants of CC+1R (RB34), CC+3R (RB86), and CC+8R (RB85) may show higher resistance than those of the others, because they showed the latest sporulation. This result may indicate the existence of some genes responsible for disease resistance located on some chromosomes of *A. roylei*, which is in the contrast to the conclusion of previous studies that the resistance is based on one, dominant gene. Sabry et al. (2006) found that one locus on chromosome 2 of maize had a major effect and was associated with downy mildew resistance in all test environments. Moreover, they found two additional QTLs on chromosomes 3 and 9 of maize showing minor additive effects on resistance, but only in a specific environment. The present study would raise the question about whether the other genes exist on the other chromosomes of *A. roylei*, which might show additive effects with the resistance gene previously found on chromosome 3 under the field condition of Japan. Another issue that needs to be considered is the plants of the same AMAL type, such as CC+3R or CC+8R, showed different appearance of sporulation. One possibility might be derived from the discrete segregations of the several genetic factors related to their resistibility in the diploid background of these two AMAL types.

In this study, a complete set of seven *A. roylei* whole chromosomes and one recombinant chromosome, 6C-6R, were added to *A. cepa*. Even when the completion of possible eight AMALs proves impossible, assignment of DNA markers to *A. roylei* chromosomes can be done using the DNA fingerprints of our incomplete set. This set would be helpful in the construction of a genetic map in *A. roylei*, which serves as the potential tool for its genetic studies. Furthermore, the complete set of AMALs is an invaluable material that makes it easier to localize loci responsible for the interested traits in breeding program, as the whole

donor genome is divided into individual extrachromosomes adding to the genetic background of the recipient species. It, therefore, would enable rapid introgression of desired alien genes from *A. roylei*, a currently threatened species, into *A. cepa*.

At present, the set of AMALs has been maintained vegetatively in our laboratory. To avoid high risk of losing materials via vegetative propagation, it is essential to find a way of the permanent maintenance of our AMALs for future verification in several other institutes. Shigyo et al. (1998, 2003) reported that another useful method for maintaining a complete set of *A. fistulosum*–shallot monosomic additions was seed propagation via a combination of selfings and backcrossings AMALs (seed parent) x *A. fistulosum*. While, the transmission rates of extrachromosomes of *A. cepa*–*A. roylei* monosomic additions will show different tends from those of *A. fistulosum*–shallot monosomic additions. The detailed analyses of the transmission rates are underway to find the best maintenance procedure of the novel *Allium* AMALs. In the near future, it will be possible for us to provide the seed of the AMALs for scientists as well as breeders all around the world.

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