Production of novel alloplasmic male sterile lines in *Allium cepa* harbouring the cytoplasm from *Allium roylei*

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With 2 figures and 5 tables

Abstract

To develop alloplasmic lines of *Allium cepa*, cytoplasmic substitution by continuous backcrossing was performed using *A. roylei* as a cytoplasm donor. The chromosomes of a single F_1 plant between *A. roylei* (♀) and shallot were doubled, and then backcrossing with shallot was performed to produce BC_1 as allotriploids. One allotriploid plant was used for backcrossing with bulb onion to produce BC_2 with 2n = 16, 17, 23, and 24. The BC_2 plants possessing 16 chromosomes were checked for pollen fertility in Yamaguchi, Japan (N34°11'), and then backcrossed with bulb onion again to evaluate their seed-setting characteristics. The pollen fertility of BC_2 ranged from 0% to more than 10%. A large number of plants showed no pollen fertility (0%). In Kagawa and Hokkaido, most of the plants were completely pollen sterile, while the percentages of seed sets in BC_2 were high enough to reproduce BC_3 seeds. The results of this study revealed that an introgression of *A. roylei* cytoplasm into *A. cepa* would be useful for the development of a novel CMS line in *Allium*.

Key words: *Allium* ------- alloplasmic line ------- CMS ------- shallot ------- bulb onion ------- *Allium roylei* ------- SSR marker ------- male sterile

Bulb onion (*Allium cepa* L., genomes CC, 2n = 16) and shallot (*Allium cepa* L. Aggregatum group, genomes AA, 2n = 16), two closely related groups, belong to the *Cepa* section of the genus *Allium* (Tashiro et al. 1982). Bulb onion is the most important *Allium* crop, with large numbers of cultivars developed and grown in many parts of the world (Shigyo 2007), while shallot is an economically important crop cultivated in low-latitude areas such as Southeast Asia. The breeding systems of the two groups have diverged considerably. Bulb onion breeding is based completely on the production of hybrid seed because of its seed-propagation capacity (Peterka et al. 1997). Shallots have been propagated vegetatively for a long time through the development of scale buds followed by division (Rabinowitch and Kamenetsky 2002). However, shallot cultivars grown from seeds have recently been introduced from the Netherlands and Israel (Brewster 2008).

Male sterility is known to be the most important characteristic in the breeding of *Allium* crops (Brewster 2008). In onion, cytoplasmic male sterility (CMS) was discovered in 'Italian Red' with cytoplasm S and two homologous recessive genes by Jones and Clark in 1943 (Havey 2002). In 1965, Berninger found the second source of CMS carrying cytoplasm T in the cultivar 'Jaune paille des Vertus' (Havey 2002). Male sterility in this case was restored by a dominant allele at locus (A-) or dominant alleles at two complementary loci (B-

C-), found by Schweisguth in 1973 (Shigyo and Kik 2008). Both types of CMS are used in commercial F_1 hybrid seed production, although CMS-S is used most frequently worldwide (Havey 2000).

When the genetic basis of CMS sources is too narrow, plants are extremely vulnerable to an impending disaster; the disease susceptibility of the Texas cytoplasm of maize is an example (Peterson et al. 1975; Levings III 1990). The cytoplasm gene pool for the breeding of onion and shallot would be widened by interspecific hybridisation (Chuda and Adamus 2009). Genetic diversity within species is an important factor in obtaining diverse types of CMS (Seiler 1992). Although the cytoplasms of the wild species have proved to be useful for breeding in *Allium* (Yamashita and Tashiro 1999a), the exploitation of these species for new CMS resources has remained limited.

Allium roylei is known as a potential gene reservoir for bulb onion (Kik 2002). The resistant genes of *Botrytis squamosa* (de Vries et al. 1992a) and *Peronospora destructor* (de Vries et al. 1992b) of this wild species were successfully introgressed into bulb onion. However, no study has reported the use of *A. roylei* for the production of novel CMS in cultivated *Allium* species. We carried out this study to develop alloplasmic male sterile lines of *A. cepa* with cytoplasm from *A. roylei* via interspecific hybridisation and backcrossings.

Materials and Methods

Procedure for the production of cytoplasmic substitution lines: The crossing procedure is described in Figure 1. A strain of *Allium roylei* Stearn '97175' (genomes rRR, 2n = 2x = 16, seed parent) was crossed with a strain of shallot '86208' (aAA, 2n = 2x = 16, pollen parent) to obtain interspecific F_1 hybrids (rAR, 2n = 16). The chromosomes of an F_1 hybrid were doubled by colchichine to get an amphidiploid plant (rAARR, 2n = 32). A single amphidiploid plant was then crossed with a strain of shallot from Thailand 'Chiang Mai' as a pollinator to produce an allotriploid BC_1 progeny (rAAR, 2n = 24). One plant of bulb onion 'Kitami Kohai 39 go' (cCC, 2n = 2x = 16) was used as a pollinator to cross with an allotriploid plant (plant code: CM23). The chromosome numbers of the BC_2 progeny were confirmed by counting the somatic chromosomes of root tips using Feulgen nuclear staining followed by the squash method. The BC_2 plants possessing 16 chromosomes were selected for the evaluation of pollen and seed fertility. The crossings of the allotriploid plant with bulb onion were carried out by hand pollination in a screen-covered isolation greenhouse in Hokkaido (N43^{o}54', E142^{o}29') in 2006. Healthy seeds were sown in cell trays. The surviving BC_2 plants were then transplanted into pots and grown from November to December in a greenhouse. They were fed each week with a nutrient solution containing 15: 8: 17 (w/w/w) (OK-F-1, N:

P_2O_5: K_2O; Otsuka Chemical Co., Osaka, Japan) or 6.5: 6: 19 (w/w/w) (Hyponex; Hyponex Co., Marysville, OH, USA). All BC_2 plants were grown in an experimental field in Yamaguchi, Japan (N34°11', E131°28'), in 2007.

Isozyme analysis: Plant materials were *A. roylei* '97175', shallot 'Chiang Mai', shallot '86208', bulb onion 'Kitami Kohai 39 go', the F_1 hybrid, the amphidiploid, the allotriploid, and all of the diploid BC_2 plants. Four enzymes, leucine aminopeptidase (LAP; EC 3.4.11.1.), glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1.), phosphoglucomutase (PGM; EC 5.4.2.2.), and phosphoglucoisomerase (PGI; EC 5.3.1.9.), were examined. Young expanding leaves were used for enzyme extraction. Extraction, polyacrylamide gel electrophoresis (PAGE), and enzyme activity staining were carried out according to Shigyo et al. (1995a, b) and van Heusden et al. (2000b).

Analysis of chloroplast DNA: The identification of cytoplasms of BC_2 progeny was based on chloroplast DNA (cpDNA) analysis of six cultivated species [shallot 'Chiang Mai', bulb onion 'Kitami Kohai 39 go' (cytoplasm S), Chinese chive 'Hirohaba Nira', rakkyo (unknown), Japanese bunching onion 'Kujyo Hoso', and *A. x wakegi* 'Kanshirazu'], four wild species [*A. roylei*

'97175', *A. galanthum* '97205-22', *A. altaicum* '97205-25', and *A. vavilovii* '97203-8'], and the diploid BC_2 plants.

Total genomic DNA was isolated from young fresh leaf tissue using a mini-prep DNA isolation method (van Heusden et al. 2000a). In the study reported by Araki et al. (2010), the DNA polymorphism in various *Allium* species was detected by using a set of consensus SSR primer pairs developed by Chung and Staub (2003). The analyses based on the two-primer sets ccSSR11 and ccSSR17 were used to distinguish chloroplast genomes in *A. roylei* from those in shallot 'Chiang Mai' and bulb onion 'Kitami Kohai 39 go'. Therefore, the cpDNA was analyzed by PCR amplification of these two primer sets in our study. Polymerase chain reactions (PCR) were performed with the two primer sets in a Program Thermal Cycler iCycler™ (Bio-Rad, Hercules, CA, USA). The reaction mixture in a total volume of 25 µl contained 0.3 µM of each primer, 0.12 mM dNTPs, 0.6 x Gold Buffer, 1.25 U of Ampli Taq Gold polymerase, 50 ng of total DNA, and 1.5 mM $MgCl_2$ (Takara, Shiga, Japan). The thermal cycling profile was programmed as follows: initial denaturation for 11 min at 95 ^{o}C, followed by 35 cycles of 30 s at 94 ^{o}C, 30 s at 55 ^{o}C, and 30 s at 72 ^{o}C, with a final extension at 72 ^{o}C for 10 min. The PCR products were electrophoresed in 2% agarose containing 0.001% Ethidium bromide in 1 x TBE buffer. If the products were monomorphic in the agarose gel, they were

subjected to 5% denaturing polyacrylamide gel electrophoresis (PAGE) with silver staining according to the procedure of Martin et al. (2005).

Capillary electrophoresis of PCR products was performed with one 5' fluorescent-labelled forward primer (NED for ccSSR11 and HEX for ccSSR17) using 15-µl volumes. Each reaction mixture contained 0.5 ng of total DNA, 0.8 x PCR buffer, 0.16 mM of dNTPs, 4 mM $MgCl_2$, 10 pmol each of forward and reverse primers, and 1.25 U of Ampli Taq Gold polymerase. The thermal cycling profile was programmed as follows: an initial denaturation for 11 min at 95 ^{o}C followed by 28 cycles of 1 min at 94 ^{o}C, 1 min at 50 ^{o}C, 1 min at 72 ^{o}C, and a final extension at 72 ^{o}C for 45 min. The analysis with the ABI PRISM 310 and subsequent analyses were conducted using a modification of the procedure of Araki et al. (2003).

Pollen fertility test: In 2007, the diploid BC_2, *A. roylei* '97175', shallot 'Chiang Mai', the amphidiploid, and the allotriploid were used for pollen fertility tests in Yamaguchi. Then the pollen fertility of the BC_2 grown in this city was examined again in 2008. In the autumn of 2007, nine BC_2 plants of low pollen fertility (1% or lower) in Yamaguchi were transferred to greenhouses in Kagawa (N34^{o}20', E133^{o}50'), and five were transferred to Hokkaido. These plants were checked for pollen fertility during the flowering

time in 2008. Each year, the pollen fertility of the BC_2 was examined two times: when flowers started to bloom and one week later. The assessment of pollen fertility was carried out according to the procedure of Shigyo et al. (1999). About 1,500 pollen grains, collected in equal numbers from three florets at full bloom per plant, were observed with no replication. Fresh pollen grains were stained with 1% acetocarmine. The number and shape of the nuclei of the pollen grains were observed. Each pollen grain was classified into one of four classes: 0, no nucleus; 1, one nucleus; 2, one vegetative nucleus and one round sperm nucleus; and 3, one vegetative nucleus and one crescent-shaped sperm nucleus. Class 3 pollen grains were considered fertile. The percentage of fertile grains in all observed pollen grains was used to indicate pollen fertility.

In vitro pollen germination: Anthers from each BC_2 plant possessing pollen fertility lower than 10% in Yamaguchi in 2007 (33 plants) and 2008 (57 plants) were used as the materials. Pollen from each anther was incubated on a medium containing 10% sucrose and 1% agar at 25 ^{o}C for 2 h. Pollen grains were considered as germinated when the pollen tube length exceeded the pollen diameter. Fifty pollen grains per anther were examined.

Seed fertility test: The seed fertility of the diploid BC_2 plants was estimated by selfing and backcrossing with the pollen of bulb onion. The seed fertility was estimated by the percentage of ovules that developed into seeds (see Table 5) according to Shigyo et al. (1999). Nine plants in Kagawa were used for both selfing and backcrossing. In Hokkaido, all five plants were used for selfing, and two of them were used for backcrossing. All plants were grown in pots in greenhouses. All umbels were bagged and hand-pollinated except for those that underwent open pollination. In backcrossing, the stamens were removed to avoid selfing.

Results

Production of alloplasmic lines in *A. cepa* harbouring the cytoplasm from *A. roylei*

After backcrossing with bulb onion, a few clones of a single allotriploid produced a total of 178 BC_2 seeds, most probably from three to four umbels (Table 1). The germination rate of the seeds (73.0%) and the survival rate of the seedlings (97.7%) were relatively high. Finally, 127 BC_2 seedlings were obtained. The chromosome count using all these BC_2 plants revealed that the chromosome numbers of the plants were 2n = 16, 17, 23, and 24 (Table 2). Eighty-one diploid plants with chromosome number 2n = 16 were recorded

with the highest frequency (63.8%). The alloplasmic *A. cepa* plants possessing 16 chromosomes were selected for further analyses of isozymes, cpDNA, pollen fertility, and seed fertility.

Isozyme analysis

Polymorphisms were detected between *A. roylei* and *A. cepa* for four isozyme loci: LAP-1, GOT-1, PGM-1, and PGI-1. The F_1 hybrid, the amphidiploid, and the allotriploid showed both bands of *A. roylei* and *A. cepa* for the LAP-1 locus. The other three-isozyme loci showed the presence of parental bands and additional bands of intermediate mobility between the two parents. However, the diploid BC_2 plants had only bands of *A. cepa* and no band of *A. roylei* for all four-isozyme loci. The chromosomal locations of the four-isozyme loci in *A. cepa* were reported previously: LAP-1 on C1, GOT-1 on C2, PGM-1 on C4, and PGI-1 on C5 (Shigyo et al. 1995a, b; van Heusden et al. 2000b). The results of isozyme analysis revealed no recombination between *A. roylei* and *A. cepa* at the four-isozyme loci in the diploid BC_2.

Chloroplast DNA analysis

Two ccSSR pairs (ccSSR11 and ccSSR17) allowed for the detection of bp-length polymorphisms between the four wild species, the six cultivated species,

and the diploid BC_2 plants (Fig. 2). The primer pair ccSSR11 helped to detect polymorphisms between *A. roylei* and shallot, but not bulb onion. All BC_2 plants showed the same band pattern as that of *A. roylei*. Likewise, the primer pair ccSSR17 allowed the visualisation of the bp-length polymorphism between *A. roylei* and bulb onion. In this case, the BC_2 had a band size identical to that of *A. roylei*. The results demonstrated that the cytoplasm from *A. roylei* was successfully transferred to *A. cepa*. The detected polymorphisms of genetic fragments revealed variations in cytoplasmic genomes between the two species.

Pollen and seed fertility in alloplasmic lines

In 2007, the parental plants and most of the alloplasmic plants were checked for pollen fertility (Tables 3 and 5). The amphidiploid plant had an increase in pollen fertility (96.0%) compared with its parents (Table 5). On the other hand, the pollen fertility drastically decreased in the allotriploid hybrid (0.1%). The alloplasmic plants started to develop flower stalks in the last 10 days of April and bloomed from the last 10 days of May to the middle of June. The pollen fertility of 52 plants was determined in the first time when the flowers started to bloom; and that of 49 plants was determined in the second time (one week later). The pollen fertility varied from 0% to more than 10%. In the first and the second times, complete pollen sterility was observed in 11 and 8 plants,

respectively. In the second time, the average pollen fertility was higher than it was in the first time.

In 2008, sixty-five alloplasmic plants were checked for pollen fertility at Yamaguchi for both times of observation (Table 3). Most of the plants flowered earlier than they had in 2007. Flower stalks were seen from the last 10 days of March. The blooming period was from the beginning of May to the middle of May. Completely pollen-sterile plants were observed with a higher frequency than they were in 2007. The average pollen fertility in 2008 was lower than that in 2007. The assessment of pollen fertility during the first time gave a higher value than that during the second time. The same phenomenon had been observed in 2007.

In summary, the data of pollen fertility showed a variation between the two times of observation in each year and also between the two years. Generally, sterile plants with 0% pollen fertility were observed during all observation times, and the frequency of sterile plants was especially high in 2008.

In both 2007 and 2008, the pollen germination test was carried out with most of the alloplasmic plants that possessed less than 10% pollen fertility (Table 4). A large number of plants showed no pollen germination. The mean values of pollen germination throughout the two years were extremely low.

All 14 alloplasmic plants that were grown in Kagawa and Hokkaido in 2008 had almost 0% pollen fertility except for two alloplasmic plants (RB 24 and RB 84) that possessed much lower pollen fertility compared with their parental plants, as shown in Table 5. Meanwhile, self-pollination of the alloplasmic plants was carried out in both places. The low seed set of the self-pollinated plants agreed with the extremely low pollen fertility. In Kagawa, three (RB 55, RB 77, and RB 115) out of nine self-pollinated plants showed very low seed. Six other plants had seed sets ranging between 1% and 20%. All five plants in Hokkaido were unable to produce seeds when self-pollinated. Furthermore, backcrossings with bulb onion were simultaneously carried out with all nine plants in Kagawa and two plants in Hokkaido. All plants produced BC_3 seeds, and the seed set varied from 3% to a high value of 85%.

Discussion

Alloplasmic male sterility has been established in bulb onion, shallot, and *A. fistulosum* by transferring the cytoplasm from *A. galanthum* (Havey 1999; Yamashita and Tashiro 1999a; Yamashita et al. 1999b). In these studies, the F_1 hybrid was used for many backcrosses to substitute the cytoplasm of *A. galanthum*. Here, we report a new procedure for the cytoplasm introgression of *A. roylei* into *A. cepa* by backcrossing the doubled F_1 hybrid. This method shortened the time to obtain the alloplamic male sterile lines, which were

observed in the second backcross generation. An interspecific hybrid between *A. roylei* and *A. cepa* and its backcross to *A. cepa* was reported in a previous study (van der Meer and de Vries 1990). According to their report, the interspecific hybrid (RC) was obtained from *A. roylei* as the seed parent when *A. roylei* was crossed in the open with *A. cepa*. However, in the backcross to a male fertile onion, it was difficult to get seedlings when the F_1 hybrid was a seed parent. Meanwhile, a number of seedlings were produced from a male sterile onion in combination with functional pollen of the F_1 hybrid. In later studies, the same type of interspecific hybrid was also used as the pollen parent for the production of BC_1 progenies (Kofoet et al. 1990; de Vries et al. 1992a, b). These results suggested the presence of lethal genes on the nuclear genome of *A. cepa* against the cytoplasm of *A. roylei.*

In the breeding scheme for the development of new onion F_1 hybrid varieties, it takes 10 years to establish the A line (male sterile line), B line (male sterile maintainer), and C line (restorer) (Shigyo and Kik 2008). The advantage of our alloplasmic lines is that the onion breeder can reduce the burden of time without choosing an appropriate B line. The CMS system would be conditioned by the incompatibility between the cytoplasm of *A. roylei* and the nucleus of *A. cepa*. Therefore, any population of *A. cepa* might be used as a maintainer for male sterility.

The formation of seeds in all alloplasmic plants in backcrossings proved that cytoplasm from *A. roylei* did not strongly affect seed fertility in *A. cepa*. In the previous studies, it was found that the cytoplasm of *A. galanthum* did not intensely influence the seed fertility of shallot and *A. fistulosum* (Yamashita and Tashiro 1999a; Yamashita et al. 1999b). The seed productivity of CMS lines of *A. fistulosum* was equal to that of the cultivar 'Kujyo' under field conditions (Yamashita and Tashiro 2004). On the other hand, the cytoplasm from *A. fistulosum* caused seed sterility in common onion (Ulloa-G et al. 1995).

In conclusion, cytoplasm from *A. roylei* proved to be a vital source of novel CMS lines in *A. cepa*, including bulb onion and shallot. From the population of alloplasmic *A. cepa* lines, we found plants with complete pollen sterility, a low seed set in selfing, and a high seed set in backcrossing. The plants will be used for further production of the next backcrossed generations. Future tests of pollen fertility and seed set will be carried out in field studies. Elite plants with complete pollen sterility and a high percentage of seed sets have potential for practical use in *A. cepa* breeding.

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Table 1 Seed set, seed germination, number of seedling survival in the backcrossing of a triploid hybrid shallot - *A. roylei* (CM23, 2n=24, genomes AAR) to bulb onion 'Kitami Kohai 39 go' (CC)

Number of seeds produced	Number of seeds that germinated	Percentage of seeds that germinated	Number of seedlings that survived	Percentage of seedlings that survived
178	130	73.0	127	97.7

Table 2 Variation of chromosome numbers in BC_2 progeny

Number of plants observed	Frequency of plants										
	Chromosome numbers (2n)										
	16	17	18	19	20	21	22	23	24	28	32
127	81	44	-[z]	-	-	-	-	1	1	-	-

[z] Not observed

Table 3 Variation of pollen fertility in alloplasmic lines in Yamaguchi in 2007 and 2008

Year	Number of plants observed	Number of times	Frequency of plants				Mean ± SE (%)
			Pollen fertility (%)				
			0	>0 - 1	>1 -10	>10	
2007	52	1st	11	14	21	6	3.91±1.20
	49	2nd	8	8	22	11	9.64±2.15
2008	65	1st	34	19	12	0	0.63±0.16
	65	2nd	28	16	16	5	2.39±0.68

Table 4 Variation of pollen germination rate in alloplasmic lines in Yamaguchi in 2007 and 2008

Year	Number of plants observed	Frequency of plants			Mean ± SE (%)
		Rate of pollen germination (%)			
		0	>0 - 10	>10	
2007	33	20	10	3	2.84±0.78
2008	57	39	16	2	1.44±0.40

Table 5 Pollen fertility and seed set in self-pollinated and backcrossed alloplasmic lines and their parental plants

Plant material	Pollen fertility (%)						Seed set[z] in self-pollinated plant (%)		Seed set in backcrossed plant (%)[y]	
	Yamaguchi[x]		Kagawa[w]		Hokkaido[w]		Kagawa[w]	Hokkaido[w]	Kagawa[w]	Hokkaido[w]
	1st	2nd	1st	2nd	1st	2nd				
Allium roylei '97175'	91.73	-[v]	-	-	-	-	-	-	-	-
Shallot '86208'	28.80	-	-	-	-	-	-	-	-	-
Shallot '18-5'	92.57	-	-	-	-	-	-	-	-	-
Amphidiploid AARR	96.00	-	-	-	-	-	-	-	-	-
Triploid AAR	0.11	-	-	-	-	-	-	-	-	-
RB 7	0.10	ND	ND	ND	-	-	1.24	-	16.68	-
RB 21	ND[u]	ND	ND	ND	-	-	16.69	-	13.51	-
RB 41	0.05	0.44	ND	ND	-	-	20.11	-	8.83	-
RB 55	ND	ND	ND	ND	-	-	0.04	-	15.93	-
RB 60	-	-	ND	ND	-	-	20.26	-	3.21	-
RB 77	0.12	0.12	ND	ND	-	-	0.02	-	24.98	-
RB 84	-	-	0.78	0.9	-	-	7.92	-	11.30	-
RB 105	0.12	ND	ND	ND	-	-	6.05	-	23.45	-
RB 115	ND	0.63	ND	ND	-	-	ND	-	9.27	-
RB 11	ND	0.06	-	-	ND	ND	-	ND	-	-
RB 24	ND	ND	-	-	0.31	ND	-	ND	-	-
RB 95	-	-	-	-	ND	ND	-	ND	-	-
RB 101	0.13	0.19	-	-	ND	ND	-	ND	-	85.00
RB 111	0.32	ND	-	-	ND	ND	-	ND	-	29.63

[z] Seed set (Percentage of ovules that developed into seeds) = $\frac{\text{Number of seeds produced}}{\text{Number of flowers pollinated x Number of ovules per flowers (6)}} \times 100$

[y] Bulb onion was used as the pollen parent for backcrossing

[x] Data obtained in 2007

[w] Data obtained in 2008

[v] Not conducted

[u] Not detected

Figure legends

Figure 1. Procedure for producing alloplasmic lines in *Allium cepa* harbouring the cytoplasm from *A. roylei.*

Figure 2. Schematic illustration from PCR amplification profiles of two-primer sets ccSSR-11 (a) and ccSSR-17 (b) in 1, *A. roylei*; 2, *A. galanthum*; 3, *A. altaicum*; 4, *A. vavilovii*; 5, shallot; 6, bulb onion (S cytoplasm); 7, rakkyo; 8, Chinese chive; 9, Japanese bunching onion; 10, *A. x wakegi*; 11-17, diploid BC_2 plants.

Figure 1, Vu et al.

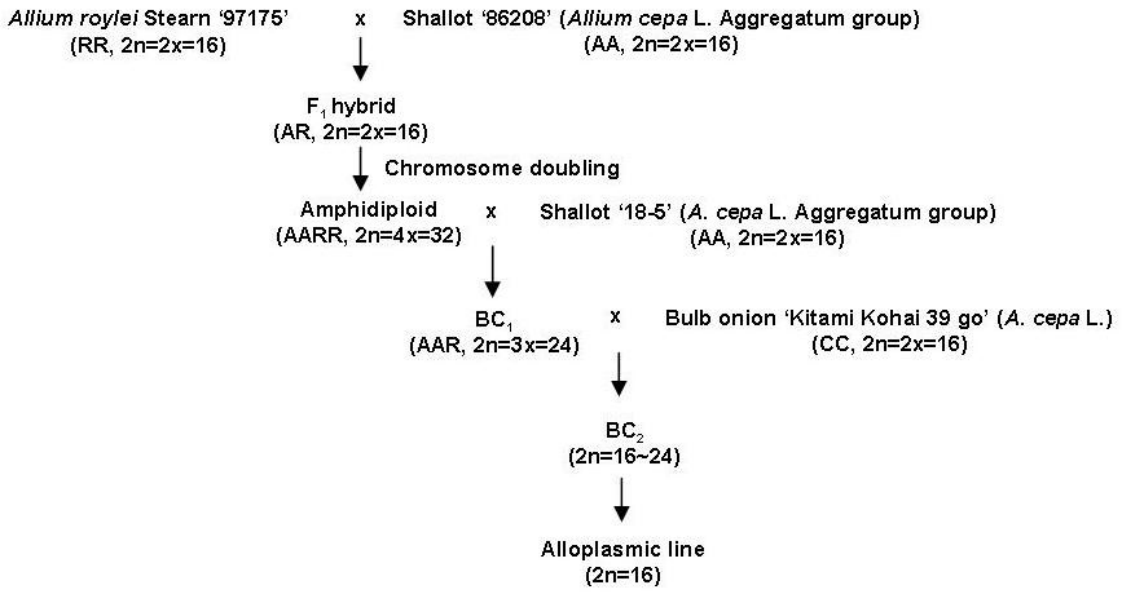

Figure 2, Vu et al.