Genetic studies of disease resistance and bolting time based on genomic analysis in Japanese bunching onion (*Allium fistulosum* L.)

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Chapter I: General introduction

Cultivated and wild species of alliums belong to the genus *Allium* in the Allioideae subfamily of the Amaryllidaceae (The Angiosperm Phylogeny Group, 2009). The genus comprises more than 800 species and includes the cultivated onion, garlic, scallions, shallots, and leeks, as well as chives and hundreds of other wild species (Li et al., 2010). *Allium cepa* L. ($2n = 2x = 16$), which includes bulb onion (*A. cepa* L. Common onion group, genomes CC) and shallot (*A. cepa* L. Aggregatum group, genomes AA), is among the most important cultivated species in the world. The bulb onion ranks second in value after tomatoes on the list of cultivated vegetable crops worldwide and the annual production of bulb onions is around 85 million tons (FAO, 2013). Although only 1.2% of the world bulb onion crop is produced in Japan, the annual value of this crop is more than a hundred billion yen.

The bunching onion (*A. fistulosum* L., $2n = 2x = 16$, genome FF), also referred to as the Japanese bunching onion or Welsh onion, originated in northwestern China and is mainly cultivated in East Asian countries, in particular in Japan, China, and Korea (Kumazawa and Katsumata, 1965; Ford-Lloyd and Armstrong, 1993). Annual production of bunching onion is one of the highest among fruit and vegetables grown in Japan, following production of tomato, strawberry, and cucumber (Ministry of Agriculture, Forestry and Fisheries, 2014). Many local varieties are adapted to various climatic conditions, and are classified into four groups: ‘Kaga’, ‘Senju’, ‘Kujo’, and ‘Yagura-Negi’, according to morphological and agronomic adaptation traits (Inden and Asahira, 1990). Traits that are major targets for improvement in bunching onion are disease resistance, yield, late bolting, improved consumption qualities (e.g., low pungency or high sugar content), and suitability for mechanized farming, although the genetic system of these traits is unclear. The breeding methods for bunching onions are relatively unsophisticated in comparison to those used for other major crops. Bunching onion is a typical allogamous crop that arose through protandry, resulting in a high rate of outcrossing (Ford-Lloyd and Armstrong, 1993). It is self-compatible, but suffers from severe in breeding depression when self-pollinated. Open-pollinated varieties of bunching

onion exhibit very high degrees of genetic heterogeneity (Haishima et al., 1993) and even parental lines of F_1 hybrids maintain a certain degree of genetic heterogeneity (Tsukazaki et al., 2006).

Many hybrid cultivars of bunching onion adapted to different climatic conditions and growing seasons have been developed by private breeding companies. However, there are sometimes serious impediments to their cultivation, such as various diseases and insect pests. For example, rust caused by *Puccinia allii* (DC.) F. Rudolphi is a fungal foliar disease in bunching onions that causes substantial deterioration of quality and yield. Uredospores released from uredinia by the pathogen are the main inocula for secondary infections throughout the year. Epiphytotics of this disease occur frequently in relatively cool and moist seasons (mainly in the autumn and spring) (Takeuchi, 1990). Because no commercial bunching onion cultivar possesses sufficient rust resistance, large amounts of fungicides are used to control this disease. It is difficult to apply fungicides at the time of disease outbreak, because the growing period of bunching onion is very long. Also, the overuse of fungicides may have adverse effects on the environment and human health, in addition to a financial burden on farmers. Thus, breeding for rust resistance would be an effective, economical, and ecologically compatible measure to prevent epiphytotics.

A. cepa was reported to be less susceptible to *P. allii* (Morinaka, 1985; Jennings et al., 1990a). Although *A. fistulosum* and *A. cepa* are in the section *Cepa* of the genus *Allium* (Hanelt, 1990), the genetic basis of resistance to rust in *A. cepa* remains unknown. Introgression of resistance genes from related species can be effective for development of novel cultivars. Monosomic alien addition lines (MAALs) are invaluable materials for localizing genes that control traits of interest, as an entire donor genome is subdivided into individual extra chromosomes added into the genetic background of a recipient species. A complete set of bunching onion – shallot MAALs ($2n = 2x + 1 = 17$, FF+1A through FF+8A) were developed (Shigyo et al., 1996) for mapping in *A. cepa* (Shigyo et al., 1997; Masuzaki et al., 2006b; 2006c; Yaguchi et al., 2009; Vu et al., 2012) and will help to determine which shallot chromosomes carry rust resistance.

A recurrent selection program for rust resistance in bunching onion was previously conducted, and a rust-resistant parental line ‘Negi Chuukanbohon Nou 1’ was developed

(Yamashita et al., 2005; Wako et al., 2012). This line shows a high level of field resistance to rust, so molecular markers linked to genes controlling rust resistance are needed for efficient selection. Because many agricultural traits are probably controlled by quantitative trait loci (QTLs), breeders seek DNA markers closely linked to QTLs to use for marker-assisted selection. Approximately 2000 genomic simple sequence repeats (gSSRs) have been identified in the bunching onion genome (Song et al., 2004; Tsukazaki et al., 2007) and genetic linkage maps have been constructed using these SSR markers (Ohara et al., 2005a; Tsukazaki et al., 2008; 2011; 2012). A major QTL for pungency was identified using the population SaT03, which was derived from a cross between a rust-resistant line and a susceptible line (Tsukazaki et al., 2012). This mapping population will be useful for investigating QTL for rust resistance.

Bolting, the premature formation of flowering stems before harvest, has a severe impact on the yield and quality of bunching onion products during spring and early summer because the flower stalks are so tough that they are inedible. Therefore, the late-bolting trait is indispensable in cultivars for spring and summer production. Bunching onion, which is biennial like bulb onion, vernalizes for flower induction in response to low temperature and short photoperiod (Brewster, 2008). Genetic variation in bolting time in bunching onion is based on seedling age and low-temperature requirements (Inden and Asahira, 1990). However, other genetic studies of flowering are very limited in bunching onion, so it will be important to investigate QTL for bolting time as a first step toward understanding the genetics of flower initiation.

In *Allium* vegetable crops, molecular genetic studies are less advanced than in other major crops. RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) analyses have also been performed in *Allium* (Wilkie et al., 1993; Bradeen and Havey, 1995; van Heusden et al., 2000a; 200b; Ohara et al., 2005a; 2005b; Ipek et al., 2005). King et al. (1998) reported a linkage map of bulb onion based on RAPDs and RFLPs (restriction fragment length polymorphisms). Fischer and Bachmann (2000) reported the first development of 30 SSR markers in bulb onion. In contrast, hundreds of expressed sequence tag (EST)-derived SSRs, EST-derived single nucleotide polymorphisms (SNPs), and insertion–deletion (InDel) markers have been

developed from medium-scale sequencing of ESTs in bulb onion (Kuhl et al., 2004; Martin et al., 2005). Some of these markers have been effectively used to construct linkage maps not only in bulb onion (Martin et al., 2005; McCallum et al., 2006; 2007; 2012), but also in bunching onion (Tsukazaki et al., 2008; 2011). Recently, transcriptome sequencing using next-generation sequencing (NGS) technology has been conducted and used to detect single nucleotide polymorphisms (SNPs) and insertion–deletions (InDels) between cultivars or individual plant lines, thereby facilitating the construction of high-resolution genetic maps (Baldwin et al., 2012; Duangjit et al., 2013). As for bunching onion, more than 50,000 unigenes were recently obtained from transcriptome shotgun assembly of next-generation sequencing data, and numerous DNA markers based on SSRs, SNPs and InDels have been developed (Tsukazaki et al., 2015). However, comparative mapping between *A. fistulosum* and *A. cepa* has not yet been conducted because the number of markers common to both species is limited. Development of a linkage map with markers common to both bunching onion and bulb onion will facilitate comparative mapping and analysis of orthologous genes in both species.

The present studies were conducted with the following objectives: 1) to investigate the susceptibility of *A. fistulosum* and *A. cepa* cultivars to rust and identify the *A. cepa* chromosome(s) related to rust resistance, 2) to identify QTL for rust resistance in *A. fistulosum*, 3) to identify QTL for bolting time in *A. fistulosum*, and 4) to construct an *A. cepa* linkage map using a DH population.

This dissertation is composed of six chapters. Chapter 2 addresses Objective 1). Chapter 3 discusses Objective 2). Chapter 4 refers to Objective 3). Chapter 5 focuses on Objective 4). And finally, Chapter 6 provides a general discussion. This dissertation compiles the results of studies conducted by the author at the Laboratory of Vegetable Crop Science, Division of Agrobiology, Department of Biological and Environmental Science, Faculty of Agriculture, Yamaguchi University and at the Vegetable Breeding and Genome Division, Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization, Japan, with the above-mentioned objectives from 2007 to 2016 (Wako et al., 2015; 2016).

Chapter II: Screening and incorporation of rust resistance from *Allium cepa* into bunching onion (*Allium fistulosum*) via alien chromosome addition

Introduction

Rust caused by *Puccinia allii* (DC.) F. Rudolphi is a serious foliar disease in bunching onions and causes substantial deterioration of quality and yield. Because no current commercial bunching onion cultivar possesses sufficient rust resistance, large amounts of fungicides are used annually to prevent and control this disease. Recurrent selection for rust resistance in bunching onion has been previously conducted and a parental line with rust resistance has been successfully developed (Yamashita et al., 2005; Wako et al., 2012). Although this line shows a moderate level of field resistance to rust, it does not completely control the disease. Therefore, it would be desirable to identify stronger sources of rust resistance that could be incorporated into cultivars.

Also, information regarding the pathogenicity of a range of *P. allii* isolates on *Allium* germplasm is limited. Jennings et al. (1990a) reported on screening experiments in the UK in which *A. cepa* showed higher resistance than *A. fistulosum* or *A. ampeloprasum* ssp. *porrum* (leek) after inoculation with *P. allii* isolates from leek. *A. cepa* is less susceptible to *P. allii* isolated from *A. fistulosum* in Japan (Morinaka, 1985). *A. fistulosum* and *A. cepa* have been classified into the section *Cepa* in the genus *Allium* (Hanelt, 1990), but the genetic basis of resistance to rust in *A. cepa* remains unknown. Introgression of resistance genes from related species can be effective for the development of novel resistant cultivars.

MAALs contain an extra chromosome from a related species. A complete set of MAALs is invaluable material for facilitating localization of genes responsible for traits of interest, as an entire donor genome is subdivided into individual extra chromosomes added to the genetic background of a recipient species. Shigyo et al. (1996) developed a complete set of bunching onion – shallot (*A. cepa* Aggregatum group) MAALs ($2n = 2x + 1 = 17$, FF+1A through FF+8A). Each MAAL thus displays distinct phenotypic and physiological characteristics (Shigyo et al., 1997), and can be used to identify which shallot chromosomes are responsible for its rust resistance. Using MAALs, Vu et al.

(2012) demonstrated that shallot chromosome 2A carried genes for *Fusarium* wilt resistance as well as others encoding enzymes that produce antifungal metabolites against *F. oxysporum*. These complete addition lines have also been effectively used to determine the chromosomal locations of genes involved in flavonoid biosynthesis (Masuzaki et al., 2006b; 2006c), sulfur assimilation (McCallum et al., 2007), and sucrose metabolism (Yaguchi et al., 2008).

The objectives of the present study were to investigate the susceptibility of *A. fistulosum* and *A. cepa* cultivars to rust isolates from bunching onion, and to identify which *A. cepa* chromosome is responsible for rust resistance using alien chromosome addition lines.

Materials and methods

Plant materials and *P. allii* isolate

Ten cultivars of bunching onion, four cultivars of bulb onion (described in Table 1), shallot 'Chiang Mai' from Thailand, and selfed progenies of a complete set of bunching onion − shallot MAALs (Shigyo et al., 1996) were used for seedling tests. Seedlings from each MAAL were screened for shallot-derived chromosomes using isozyme or Sequenced Characterized Amplified Region (SCAR) markers. Chromosomes 1A, 2A, and 6A were identified using the *Lap-1*, *Got-1*, and *Got-2* isozymes, respectively (Shigyo et al., 1994; 1995). Chromosomes 3A, 4A, 5A, 7A, and 8A were identified using the SCAR markers API40, *Allinase*, AJK265, 5S rDNA, and API73-2, respectively (van Heusden et al., 2000b). The addition of chromosomes in these lines was confirmed using microscopic observation or flow cytometry (Shigyo et al., 2003). One hundred seeds from selfed progenies of each MAAL were sown, and more than eight plants carring each monosomic addition were identified. For tests using adult plants, the MAALs, multi-chromosome addition lines, and hypoallotriploids (2n = 2x + 2–7 = 18–23) (Hang et al., 2004; Yaguchi et al., 2008) were grown in pots for over one year. Uredospore inoculum of *P. allii* was collected from a field in Seiro, Niigata Prefecture, Japan, multiplied on bunching onion plants, and then stored at -30 °C until just before use. Inoculum for use

Table 1. Number of uredinia on leaves of *Allium cepa* and *A. fistulosum* cultivars at 14 days after inoculation with *Puccinia allii*.

Cultivar	Species	Origin	No. of uredinia per cm leaf length*			
Senshu-chuko-ki	*A. cepa*	Takii Seed Co. Ltd., Kyoto, Japan	0.10	±	0.02	a
Kaiduka-wase	*A. cepa*	Sakata Seed Co., Yokohama, Japan	0.12	±	0.05	a
Imai-wase	*A. cepa*	Sakata Seed Co., Yokohama, Japan	0.21	±	0.04	a
Shonan-red	*A. cepa*	Sakata Seed Co., Yokohama, Japan	0.24	±	0.10	a
Shimonita	*A. fistulosum*	Kaneko Seed Co., Maebashi, Japan	1.01	±	0.15	b
Hikawa	*A. fistulosum*	Nihon Norin Seed Co., Tokyo, Japan	1.16	±	0.15	bc
Kasho-ipponfuto	*A. fistulosum*	Sakata Seed Co., Yokohama, Japan	1.24	±	0.16	bc
Shimotae	*A. fistulosum*	Mikado Kyowa Seed Co. Ltd., Chiba, Japan	1.27	±	0.15	bc
Choetsu	*A. fistulosum*	Mikado Kyowa Seed Co. Ltd., Chiba, Japan	1.27	±	0.16	bc
Choju	*A. fistulosum*	Mikado Kyowa Seed Co. Ltd., Chiba, Japan	1.47	±	0.13	bc
Kincho	*A. fistulosum*	Mikado Kyowa Seed Co. Ltd., Chiba, Japan	1.52	±	0.17	bc
Yoshikura	*A. fistulosum*	Musashino Seed Co. Ltd., Tokyo, Japan	1.53	±	0.21	bc
Kujo-futo	*A. fistulosum*	Takii Seed Co. Ltd., Kyoto, Japan	1.80	±	0.23	c
Ishikura-nebuka-fuyufuto	*A. fistulosum*	Kaneko Seed Co., Maebashi, Japan	1.82	±	0.21	c

*Mean ± SE (n = 20), Significant difference detected using Tukey-Kramer HSD test (P = 0.05). Values followed by the same letter are not significantly different.

on multi-chromosome addition lines and hypoallotriploids was obtained from uredinia on bunching onion leaves collected from a field located in Sodegaura, Chiba Prefecture, Japan.

Inoculation of seedlings

Seedling tests were conducted to compare rust resistance among cultivars of *A. fistulosum*, *A. cepa* and the MAALs. Seeds for bunching onion, bulb onion, shallot, and MAALs were sown into plastic pots (6 cm in diameter) and grown in a greenhouse for approximately 90 days until leaves were 30 cm in length. In our preliminary tests, seedlings less than 60 days old were not suitable because their leaves senesced easily after inoculation. To inoculate each plant, a mixture of 1 mL of 5×10^4 uredospore suspension with 0.1 % polyoxyethylene sorbitan monolaurate (Tween® 20, Nacalai Tesque, Inc., Japan) and 0.5 % talc (Nacalai Tesque, Inc., Japan) was sprayed onto the leaves. Inoculated plants were placed in a growth chamber (KPSH-30, Ozawa Seisakusho Co. Ltd., Japan) maintaining a temperature of 18 °C and a 12 h light/dark cycle. To maintain damp conditions for the germination of uredospores, the atmosphere was kept saturated with water using an ultrasonic humidifier (FT-10N-14, UCAN Co. Ltd., Japan) for 24 h after inoculation. After incubation for 14 days, the number of uredinia produced on the youngest full-grown leaf of each plant was counted. For inoculated seedlings from the MAALs and inoculated adult plants from the multi-chromosome addition lines and hypoallotriploids, the quantities of uredinia were scored using a 0–3 scale as follows: 0, no uredinia pustule; 1, 1–20 uredinia per leaf (slight symptoms); 2, 21–100 uredinia per leaf (moderate symptoms); 3, more than 101 uredinia per leaf (severe symptoms).

Inoculation of adult MAAL plants

Inoculation tests were conducted in the field to examine the rust resistance of full-grown shallot and MAAL plants. Plants of approximately 30 cm in height that had been grown in plastic pots were transplanted into plots in a plastic greenhouse in a randomized block design with three replicates and were grown for 2wo months. Before inoculation, 0.006% polyoxyethylene nonylphenyl ether or KUMITEN (Kumiai Chemical Industry

Co., Ltd., Japan) was sprayed onto plants to stimulate germination of the subsequently inoculated spores. Each plant was sprayed with a mixture of 1 mL 5 × 10^3 uredospore suspension with 0.01% Tween 20 and 0.5 % talc. Beginning 14 days after inoculation, the severity of rust symptoms was scored on full-grown leaves of each plant at 2-week intervals. Plant responses to inoculation were ranked using a 0–5 scale as follows: 0, no uredinia; 1, 1–3 uredinia per leaf; 2, 4–10 uredinia per leaf; 3, more than 11 uredinia per leaf; 4, uredinia distributed over the entire leaf; and 5, uredinia distributed densely over the entire leaf. To evaluate disease intensity, the area under the disease progress curve (AUDPC) was calculated according to Shaner and Finney (1977), using the following equation:

$$\text{AUDPC} = \sum_{i=1}^{Ni-1} (t_{(i+1)} - t_i)(DS_{(i+1)} + DS_i)/2$$

where Ni = number of observations; t_i = days at the i^{th} observation ($t_0 = 0$); and DS_i = rust severity at the i^{th} observation ($DS_0 = 0$). After inoculation, the temperature inside the plastic greenhouse ranged from 10 to 23 °C.

Inoculation of adult plants from multi-chromosome addition lines and hypoallotriploid lines

A total of 34 individuals from multi-chromosome addition lines and hypoallotriploids possessing between 1 and 7 shallot chromosomes were used. A solution of 0.1% Tween 20 was sprayed onto the plants to completely remove wax layer of leaves at 1 day before inoculation. Each plant was sprayed with a mixture of 5 mL of 1 × 10^5 uredospore suspension with 0.01% Tween 20 and placed in a moist chamber under high humidity for 48 h. Inoculated plants were then incubated in a greenhouse regulated at 18–20°C, and rust severity was scored 14 days after inoculation. The scales for rust severity were the same as those used for seedling tests. Inoculation experiments were replicated twice in different years.

Results

Comparison of resistance between *A. fistulosum* and *A. cepa* cultivars

In both *A. fistulosum* and *A. cepa*, small white spots first appeared on each leaf 8–9 days after inoculation. After 2–3 days, the spots developed into orange pustules (i.e., uredinia). The mean number of uredinia per cm of leaf length at 14 days after inoculation differed between *A. fistulosum* and *A. cepa*. Numbers of uredinia on plants of all of the tested *A. fistulosum* cultivars were significantly greater than in *A. cepa* (Table 1). These data shows that *A. cepa* is highly resistant to rust isolated from *A. fistulosum.* Significant differences in uredinia numbers among *A. fistulosum* cultivars were observed. The cultivars 'Kujo-futo' and 'Ishikura-nebuka-fuyufuto' were highly susceptible to rust.

Rust resistance of shallot and bunching onion – shallot MAALs in seedling tests

Similar to observations on bunching onions and bulb onions, small white spots first appeared on the leaves of shallot and MAAL plants at 8–9 days after inoculation. The number of white spots on shallot leaves was greater than that on bulb onion leaves. Most of the spots remained white and few developed into orange uredinia (Fig. 1). By 14 days after inoculation, no uredinia were produced (scored as 0 on the rust symptom scale) on nearly 40% of shallot plants and the number of uredinia per leaf was fewer than 20 (scored as 1) on 90% of plants (Table 2). In contrast, more than 101 uredinia developed per leaf in 70% of *A. fistulosum* 'Kujo-hoso' plants (scored as 3). Within a complete set of *A. fistulosum* – shallot MAALs, half of the FF+1A plants were scored as 1 on the rust symptom scale and the remaining half of the plants were scored as 2 or 3. The mean rust symptom severity for FF+1A (1.6) was significantly lower than that of FF (2.7). In FF+1A plants, either white spots did not develop into uredinia, or pale green haloes appeared around the few uredinia that did develop. All plants from the lines FF+2A through FF+8A were scored as 2 or 3 on the rust symptom severity scale, with rust severities similar to those of FF.

Evaluation of adult plant resistance of bunching onion – shallot MAALs in the field test

In *A. fistulosum* 'Yoshikura', a few uredinia were observed 14 days after inoculation, and rust symptoms increased with days after inoculation. Uredospores released from

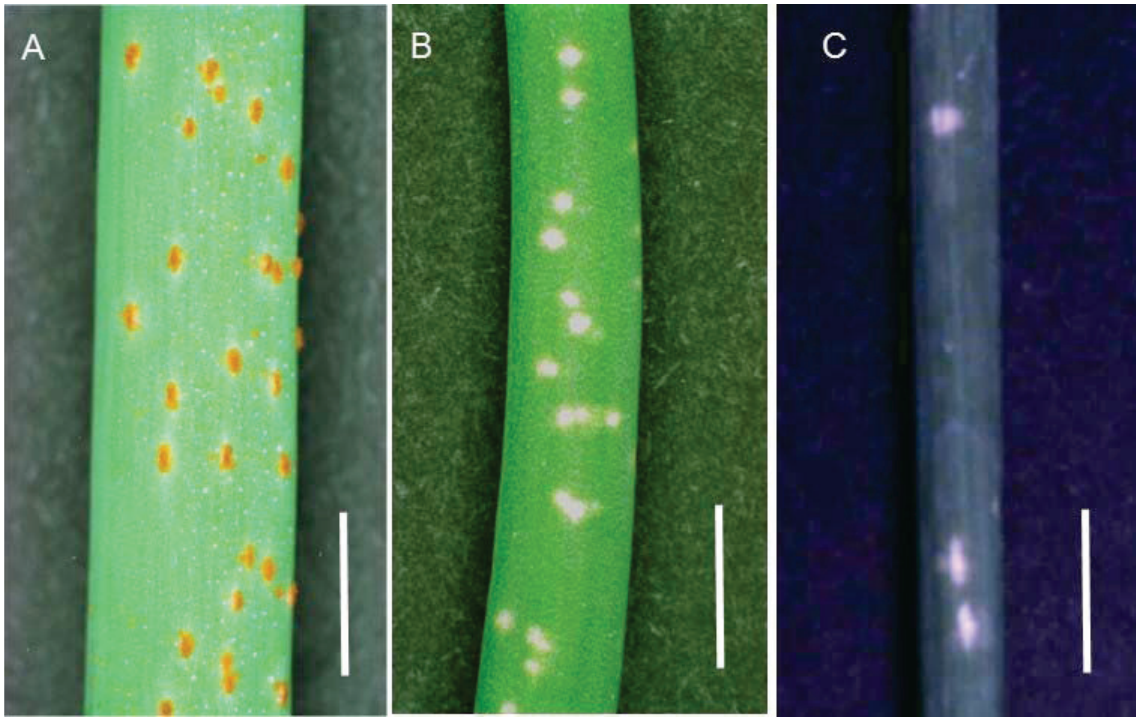

Fig. 1. Symptoms on leaves of *Allium fistulosum*, shallot, and FF+1A seedlings 14 days after inoculation with *Puccinia allii*. A, *A. fistulosum* 'Kujo-hoso'; B, shallot 'Chiang Mai'; C, FF+1A. Scale bar = 1 cm.

Table 2. Rust severity scores on *Allium fistulosum* (FF), shallot (AA), and eight *A. fistulosum* – shallot MAALs (FF+*n*A) at 14 days after inoculation with *Puccinia allii.*

Cultivar or line	No. of plants	Frequency distribution of rust severity[a]					
		0	1	2	3	Mean	
FF (Kujo-hoso)	30			9	21	2.7	
AA (Chiang Mai)	47	18	24	5		0.7	**
FF+1A	20		10	8	2	1.6	**
FF+2A	8			5	3	2.4	
FF+3A	27			5	22	2.8	
FF+4A	12			7	5	2.4	
FF+5A	8			2	6	2.8	
FF+6A	10			2	8	2.8	
FF+7A	19			1	18	2.9	
FF+8A	14			3	11	2.8	

[a] Rust severities were ranked as follows: 0, no uredinia; 1, 1–20 uredinia per leaf; 2, 21–100 uredinia per leaf; 3,more than 101 uredinia per leaf.

** Significant differences from FF were detected using Wilcoxon rank-sum test (P = 0.01)

uredinia appeared to infect newly expanded leaves, so rust severity reached scores 4 to 5 at 6 weeks after inoculation. Significantly fewer uredinia were observed developing on shallots (Fig. 2). The AUDPCs of FF+1A, FF+4A, FF+5A, FF+6A, FF+7A, and FF+8A were similar to that of *A. fistulosum*, suggesting these MAALs were all susceptible to rust. The AUDPCs of FF+2A and FF+3A were relatively lower than those of *A. fistulosum* and other MAALs, however the differences between them were not significant.

Evaluation of adult plant rust resistance in bunching onion – shallot multi-chromosome addition lines

Shallot was scored as 1 on the rust severity scale (< 20 uredinia per leaf) in each replicate (Table 3). The rust severity scores of plants from multi-chromosome addition lines and hypoallotriploids were generally higher than those of shallot, regardless of the particular additional chromosomes. In the first experiment, nine plants with shallot chromosome addition were scored as 1 on the rust severity scale. However, in the second experiment, only one plant possessing all of the shallot chromosomes except for 4A, and 2 plants possessing 1A and 5A were scored as 1 on the disease severity scale. Although two FF+1A+5A individuals showed low disease severity comparable to that of shallot in both experiments, some individuals from addition lines containing shallot chromosomes 1A and 5A exhibited high disease severity.

Discussion

In the present study, *A. cepa* cultivars including bulb onions and shallots showed high resistance at different ages to a *P. allii* isolate derived from *A. fistulosum*. These results were consistent with those of previous studies (Morinaka, 1985; Jennings et al., 1990a). Morinaka (1985) investigated the pathogenicity to *Allium* crops of five isolates of *P. allii* collected from *A. fistulosum* grown in different regions in Japan. All of the isolates showed no pathogenicity to Chinese chive (*A. ramosum*) or Japanese scallion (*A. chinense*). These isolates produced white flecks on leaves only on bulb onion, leeks, and chives (*A. schoenoprasum*), and some isolates did not form sporulating uredinia. Jennings et al. (1990a) reported that *P. allii* isolates derived from *A. porrum* (leeks) produced pale

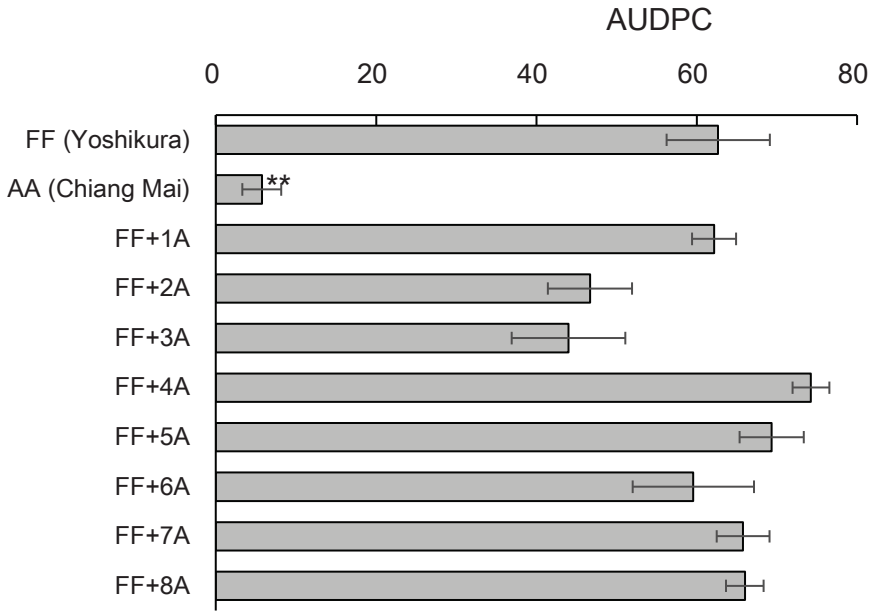

Fig. 2. Area under the disease progress curves (AUDPCs) for *Allium fistulosum* (FF), shallot (AA), and eight *A. fistulosum* – shallot MAALs (FF + *n*A) 6 weeks after inoculation with *Puccinia allii*. ** indicates significant difference from *A. fistulosum* detected by Student's *t* test ($P = 0.01$). Bars indicate ± SE ($n = 3$).

Table 3. Rust severity on *Allium fistulosum*, shallot, and *A. fistulosum* – shallot hypoallotriploids at 14 days after inoculation with *Puccinia allii.*

No. of chromosomes	Chromosomal composition									Rust severity score[a]	
										1st ex.	2nd ex.
16	FF (Kujo-hoso)									1–2[b]	2[b]
16	AA (Chiang Mai)									1	1
23	FF +	1A	2A	3A		5A	6A	7A	8A	1–2[b]	1–2[b]
23	FF +	1A	2A	3A	4A	5A	6A		8A	3	3
22	FF +	1A	2A		4A		6A	7A	8A	1	2
22	FF +	1A	2A	3A	4A		6A	7A		1	2
22	FF +	1A		3A		5A	6A	7A	8A	1	2
22	FF +	1A	2A	3A		5A	6A	7A		2	2
22	FF +	1A	2A		4A	5A	6A	7A		3	2
22	FF +	1A	2A	3A	4A	5A	6A			3	2
22	FF +	1A	2A	3A		5A	6A		8A	3	3
22	FF +	1A	2A	3A		5A	6A	7A		3	3
21	FF +	1A	2A		4A	5A	6A			2	3
21	FF +	1A	2A	3A	4A	5A				2	3
21	FF +	1A	2A	3A				7A	8A	2	3
21	FF +	1A	2A	3A		5A	6A			2	2
21	FF +	1A	2A	3A		5A			8A	2	3
21	FF +	1A	2A			5A	6A		8A	3	NT[d]
20	FF +	1A	2A			5A		7A		1	2
20	FF +	1A			4A		6A		8A	2	2
20	FF +	1A		3A		5A	6A			2	2
20	FF +	1A	2A	3A		5A				3	2
20	FF +	1A		3A	4A		6A			3	NT[d]
19	FF +	1A				5A			8A	1–2[b]	2b
19	FF +	1A		3A				7A		3	2
18	FF +	1A				5A				1–2[c]	1[b]
18	FF +		2A		4A					2	3
18	FF +	1A			4A					3	3
17	FF +					5A				1	2
17	FF +			3A						1[b]	2[b]
17	FF +								8A	2	2

[a] The scales for rust severity scores are noted in the footnote for Table 2. [b] Two plants were tested.
[c] Three plants were tested. [d] Not tested.

green flecks and small pustules with haloes on leaves of *A. cepa*, and the quantity of pustules on plants of this species was lower than that on *A. fistulosum*. In our results, white flecks were found on leaves of *A. cepa* after inoculation, and few uredinia formed. Thus, while *P. allii* can presumably infect and colonize *A. cepa,* it does not appear able to form sporulating uredinia (Jennings et al., 1990a). Although the quantitative differences in uredinia formation between *A. cepa* and *A. fistulosum* were significant, variation in uredinia formation within each species was low. In *A. fistulosum*, there were significant differences between cultivars in field resistance of full-grown plants, which indicated that recurrent selection for rust resistance was successful (Yamashita et al., 2005). In seedling tests in the present study, no significant differences were observed among cultivars except for 'Shimonita', 'Kujo-futo', and 'Ishikura-nebuka-fuyufuto'.

Allium ramosum, *A. chinense*, and *A. schoenoprasum* have been less useful for breeding rust resistance in bunching onion because they belong to subgenera and sections that are genetically distant from *A. fistulosum*. However, *A. cepa* and *A. fistulosum*, which have been classified into a single section *Cepa,* are able to cross-pollinate (Emsweller and Jones, 1935). Therefore, *A. cepa* should be focused as an important genetic resource for breeding in *A. fistulosum*. No other rust-resistant species within the section *Cepa* has been reported. Rust resistance genes from *A. cepa* will likely be introduced into *A. fistulosum via* introgressive hybridization. *A. cepa* and *A. fistulosum* have the same chromosome numbers ($2n = 2x = 16$) and similar karyotypes (Albini and Jones, 1988). However the DNA content of the *A. cepa* genome is 28% greater than that of *A. fistulosum* (Labani and Elkington, 1987), and *A. cepa* chromosomes are an average of 12% larger at somatic metaphase than those of *A. fistulosum* (Jones and Rees, 1968). To date, attempts to introgress genes from *A. fistulosum* into *A. cepa* by crossing *A. cepa* and *A. fistulosum* followed by backcrossing to *A. cepa* have not been successful, except in only one report (Peffley and Hou, 2000). Sterility in backcrossed generations is thought to be due to an imbalance between the nuclear and cytoplasmic genomes (Ulloa et al., 1995). On the other hand, van der Meer and de Vries (1990) showed that *A. roylei* ($2n = 2x = 16$) crosses readily with either *A. cepa* or *A. fistulosum*. Khrustaleva and Kik (1998) showed that the three parental genomes in the first generation bridge cross *A. cepa* × (*A. fistulosum* × *A.*

roylei) could be distinguished from each other by means of genomic *in situ* hybridization. Recombination between the three genomes was frequently observed in meiotic anaphase 1 and prophase 2 chromosomes of the first-generation bridge cross and in mitotic chromosomes of the second-generation bridge cross (Khrustaleva and Kik, 2000).

Within a set of *A. fistulosum* - shallot MAALs, only FF+1A showed significantly lower rust disease symptoms during the seedling stage (3 months after sowing). This result suggests that the gene(s) related to rust resistance are located on chromosome 1A of shallot and could perform in the genetic background of *A. fistulosum*. Because the resistance level of FF+1A was comparatively lower than that of shallot, other genes involved in rust resistance might exist on chromosomes other than 1A. It is also possible that alleles on *A. fistulosum* chromosomes could counteract or weaken the function of resistance gene(s) from shallot. The degree of rust resistance exhibited by MAALs, multi-chromosome addition lines, and hypoallotriploids was unclear in full-grown plants under both controlled-environment and field conditions, even though shallot showed distinct resistance. Some individuals of FF+1A+5A repeatedly showed low severity of symptoms comparable to that of shallot. However, other individuals with chromosome additions did not show resistance regardless of whether they carried chromosomes 1A and 5A from shallot. Thus, consistent results regarding the relationship between rust resistance and shallot chromosomes have not been obtained. These results implicate possible interactions between genes from shallot and *A. fistulosum*. In field tests, plants were continuously attacked by rust for a long period of time. As the growth and differentiation of the pathogen is retarded in *A. cepa* (Jennings et al., 1990a), uredinia formation likely varies depending on host and environmental conditions. Results in the present study indicate that *A. cepa* possesses resistance to rust at the seedling stage, but not at the adult plant stage. Jennings et al. (1990b) discussed the effects of plant age, leaf position, and leaf segment on infection of leek by rust. They proposed that evaluation of resistance should be carried out on several leaves per plant on replicate adult plants and that several components of the disease, including the latent period, pustule density, and pustule length, should be measured. Resistance to rust and other diseases often changes as plants mature (de Jong, 1995).

Vu et al. (2012) reported that shallot expresses chemical compounds that show antifungal effects against *F. oxysporum*. In the present study, FF+2A showed the highest level of resistance to *Fusarium* wilt among the eight *A. fistulosum* – shallot MAALs. The FF+2A line also showed a specific saponin band derived from shallot. Lanzotti et al. (2012) reported the inhibition of some phytopathogenic fungus by saponins extracted from white onion. However, the addition of chromosome 2A had no effect on the rust resistance of lines carrying it in the present study.

A. fistulosum – shallot MAALs are highly fertile (Shigyo et al., 1999b) and the extra chromosomes can be transmitted via both male and female gametes (Shigyo et al., 1999a). Recombination between *A. fistulosum* and *A. cepa* can be induced during homoeologous chromosome pairing and crossing over during meiosis. Many similar studies using MAALs in other crops have been described previously. For example, Savitsky (1978) developed *Beta vulgaris – B. procumbens* MAALs for nematode (*Heterodera schachtii*) resistance and selected resistant diploid plants from among their progenies that were assumed to have translocations between homoeologous chromosomes. Resistance genes for the foliar disease *Cercospora beticola* and the soil-borne fungus *Polymyxa betae*, the vector of Beet Necrotic Yellow Vein virus, were found in *Beta vulgaris – B. procumbens* MAALs and *B. vulgaris – B. patellaris* MAALs (Paul et al., 1992, Mesbah et al., 1997). Kaneko et al. (1996) identified a Turnip Mosaic Virus resistance gene in kale – radish MAALs. Akaba et al. (2009) reported club root resistance originating from a radish chromosome in a *Brassica napus – Raphanus sativus* MAAL. Peterka et al. (2004) reported a monosomic chromosome addition for transferring resistance to beet cyst nematode from radish (*R. sativus*) to rape (*B. napus*). Thus MAALs will be useful materials for stable introgression of beneficial genes from extra chromosomes into recipient chromosomes.

In conclusion, the present study reported resistance of *A. cepa* cultivars including bulb onion and shallot against *P. allii* isolated from *A. fistulosum* in controlled environment and field tests. The gene(s) for rust resistance located on chromosome 1A act mainly during the seedling stage. The information presented here will be helpful in breeding programs for the development of rust-resistant bunching onion varieties.

Chapter III: Mapping of quantitative trait loci for rust resistance in bunching onion

Introduction

Bulb onion (*A. cepa*) is highly resistant to rust (Wako et al., 2015), therefore, so resistance genes from A. *cepa* will be introduced into *A. fistulosum via* introgressive hybridization using alien chromosome addition lines. On the other hand, considerable variation has been observed in the degree of disease severity, namely the area under the disease progress curve (AUDPC) (Wako et al., 2012). Bunching onion cultivar types such as 'Senju-Aigara', 'Kaga', and 'Kujo' exhibit high susceptibility to the disease, whereas cultivars like 'Senju-Aiguro' and 'Senju-Kurogara' were found to be more resistant than the others. This indicated that the rust resistance present in bunching onion was likely a quantitative trait. Among the 133 cultivars tested, 'Seito Ippon', 'Iwai 2', 'Choju', 'Senami', 'Fuyuogi-Ippon', and 'Toyokawa-Futo' showed the lowest values of AUDPC (Yamashita et al., 2005). Using these relatively resistant cultivars, a recurrent selection program was conducted to achieve a high level of field resistance to rust, because this breeding method has been successful in improving quantitatively inherited traits in other outcrossing crops. As a results of two cycles of recurrent selection for rust resistance followed by several generations of continuous selection within selfed-lines, a parental line 'Negi Chuukanbohon Nou 1' with the highest rust resistance found so far in bunching onion was developed (Wako et al., 2012).

Quantitative trait loci (QTL) analysis based on a genetic linkage map can effectively reveal the mode of inheritance of agronomic traits. However, despite the economic importance of bunching onion, its genetic characteristics have been poorly studied and molecular approaches were needed to help clarify the genetic control of many traits of interest. Approximately 2000 gSSRs have been identified from bunching onion (Song et al., 2004; Tsukazaki et al., 2007) and used to construct genetic linkage maps (Ohara et al., 2005a; Tsukazaki et al., 2008; 2011; 2012). In a previous study, a major QTL for pseudostem pungency was identified using the SaT03 population derived from a cross between a rust-resistant line Sa03 and a susceptible line T03 (Tsukazaki et al., 2012).

Sa03 is a selfed line derived from ‘Negi Chuukanbohon Nou 1’.

In the present study, QTL analyses of the rust resistance of ‘Negi Chuukanbohon Nou 1’ were conducted in order to determine its genetic basis and to develop DNA markers for efficient selection of rust resistance in bunching onion.

Materials and Methods

Plant materials and pathogen

The mapping population SaT03 (119 individuals) was used in the present study. SaT03 is the segregating F_2 derived from a cross between Sa48-10s-7ic-3s-2ic-3s-1s-9s (Sa03) and T26-4s-2s-2s (T03). Sa03 is an inbred line derived from the cultivar ‘Chuukanbohon Nou 1’ (Wako et al., 2012) and is highly resistant to rust under field conditions. T03, a susceptible type, is an inbred line derived from the cultivar ‘Fuyuwarabe’ (Wako et al., 2010).

The *P. allii* isolate used in the present study was same as that described in Chapter II. Because *P. allii* is an obligate biotrophic pathogen, it requires live host tissue to reproduce. Therefore, *P. allii* uredospores were collected from bunching onion leaves and stored at -30 °C until just before use as inoculum.

Evaluation of rust resistance

Triple inoculation tests were conducted in the greenhouse at the NARO Institute of Vegetable and Tea Science to examine rust resistance in the mapping population SaT03 (Table 4). Seeds of F_2-derived F_3 ($F_{2:3}$) lines of the SaT03 population were sown in 200-cell plug trays and transplanted into the plastic greenhouse in a randomized design with five replications and 10 plants per replication. The plants were grown for several months until reaching the adult plant stage. Inoculation was performed by spraying with a mixture of 1 mL 5×10^3 uredospore suspension with 0.01% Tween 20 and 0.5 % talc as described in detail in Chapter II. The disease index (DI) was calculated according to the severity of rust symptoms scored from one to four times after inoculation. DIs were defined using a 0–5 scale as follows: 0, no uredinia; 1, 1–3 uredinia per leaf; 2, 4–10 uredinia per leaf; 3, more than 11 uredinia per leaf; 4, uredinia distributed over the entire leaf; and 5, uredinia

Table 4. Sowing, planting, inoculation and scoring dates for evaluation of rust resistance in $F_{2:3}$ population.

Trial	Number of $F_{2:3}$ lines	Sowing date	Transplanting date	Inoculation date	Scoring date
2009	100	15 July 2008	3 Sep. 2008	6 Mar. 2009	17 Apr. 2009
2013	80	26 June 2012	28 Aug. 2012	31 Jan. 2013	28 Feb., 6, 13 and 27 Mar. 2013
2014	88	5 July 2013	3 Sep. 2013	3 Apr. 2014	21 Apr., 6 May 2014

distributed densely over the entire leaf. AUDPCs were calculated according to methods described in Chapter II.

QTL analysis

QTL analysis was performed using the composite interval mapping method with the computer program QTL Cartographer version 2.5 (Wang et al. 2007). The log likelihood (LOD) threshold was determined with 1000 permutations at $P = 0.05$. Statistical analysis of the associations between the genotypes of plants in the F_2 generation and the average DI or AUDPC of $F_{2:3}$ plants of the same genotype was conducted at each QTL-linked SSR locus in each trial using the Tukey–Kramer Honestly Significant Difference (HSD) test ($P = 0.05$) with JMP version 4.0 (SAS Institute Inc., NC, USA).

Results

Variation in rust resistance in the SaT03 population

In the 2009 trial, DI was determined only one time at 42 days after inoculation (Table 4). The average DI of Sa03 was 1.4, while the DI of T03 was 3.6 (Fig. 3), and the DI of F_1 hybrids was 3.3. DIs of the $F_{2:3}$ lines ranged continuously from 1.3 to 3.9. In the 2013 trial, DIs were scored four times at 31 to 59 days after inoculation. In the 2014 trials, DIs were scored two times, at 18 to 33 days after inoculation. AUDPCs were used for evaluation of rust resistance in both trials. AUDPCs of Sa03 were 84 in the 2013 trial and 27 in the 2014 trial, while those of T03 were 172 and 52, respectively (Fig. 4). AUDPCs of the $F_{2:3}$ lines ranged continuously from 68 to 162 in the 2013 trial and from 28 to 59 in the 2014 trial. The correlation coefficient between DIs of $F_{2:3}$ lines in the 2009 trial and AUDPCs in the trial 2013 was 0.62 (Fig. 3). The correlation coefficient between AUDPCs of $F_{2:3}$ lines in the 2013 trial and the 2014 trial was also 0.62 (Fig. 4). Transgressive segregation for resistance was observed in the 2013 trial and also for susceptibility in the 2014 trial.

QTL analysis for rust resistance

In the 2009 trial, two QTLs were detected on the linkage groups 1a on chromosome 1

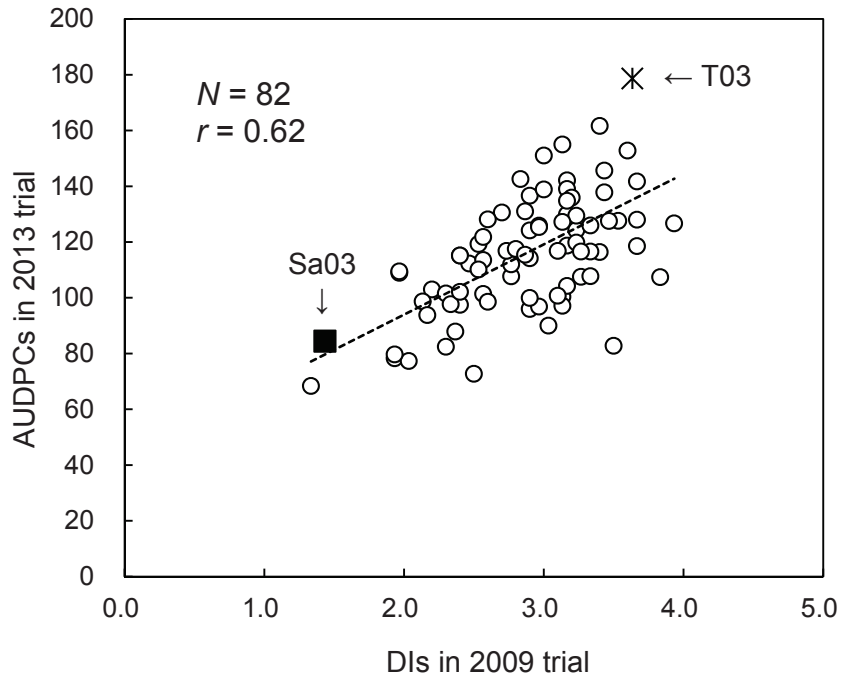

Fig. 3. Distribution and correlation coefficient between disease indices (DIs) in 2009 trial and AUDPCs in 2013 trial with 82 $F_{2:3}$ lines of SaT03 population.

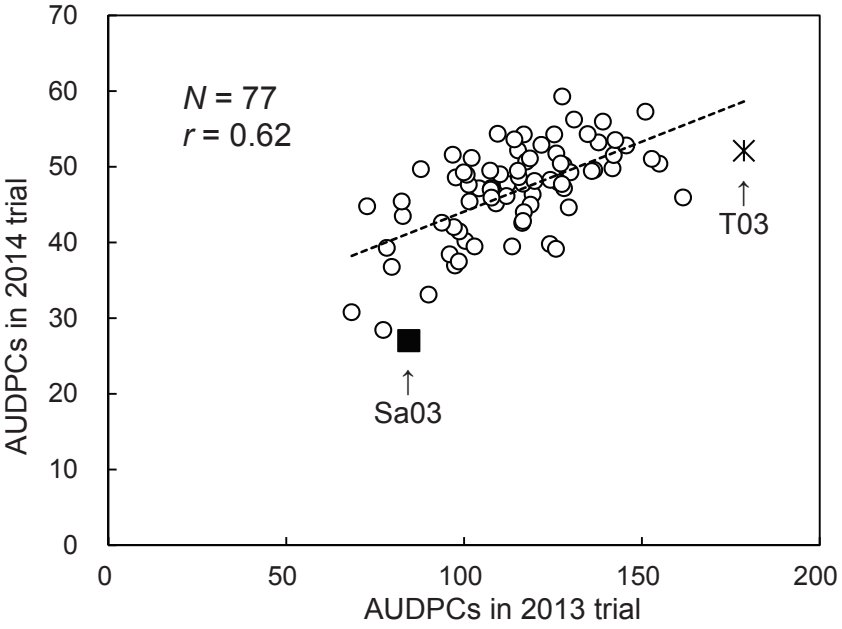

Fig. 4. Distribution and correlation coefficient between AUDPCs in 2013 trial and 2014 trial with 77 $F_{2:3}$ lines of SaT03 population.

(Chr. 1a) and 8a on chromosome 8 (Chr. 8a) and were designated as *qRst1a* and *qRst8a*, respectively (Fig. 5). The LOD peak for *qRst8a* (5.7) was higher than that for *qRst1a* (4.9), and the proportion of phenotypic variation explained by *qRst8a* (20.4%) was also higher than that explained by *qRst1a* (14.2%) (Table 5). To examine the effects of these two QTLs, the F_2 progeny were classified according to the genotype at each marker and the bolting time of $F_{2:3}$ lines was correlated with the nine observed genotypes (Table 6). F_2 progenies homozygous and heterozygous for the linked AFA02F07 and CF436630 alleles derived from the Sa03 parent showed significant differences in rust severity from the progeny carrying the homozygous genotypes derived from the T03 parent (Table 6).

A QTL was detected in a region on linkage group Chr. 3a in the 2013 trial (*qRst3a*). The maximum LOD score (3.6) and the observed variation explained (12.7%) for *qRst3a* were both lower than for *qRst1a* and *qRst8a* detected in the 2009 trial (Table 5). When the $F_{2:3}$ lines were categorized according to the genotype of their preceding F_2 generation at the linked locus ACE320, F_2 progenies homozygous and heterozygous for the Sa03 allele showed significant differences in AUDPC from the progeny carrying the homozygous genotypes derived from the T03 parent (Table 7). No significant QTL was detected in the 2014 trial.

Discussion

There have been several linkage mapping studies for traits in *Allium* crops (Baldwin et al., 2014; McCallum et al., 2006; 2007; Tsukazaki et al., 2012). However, no approach had yet been reported for disease resistance in *A. fistulosum*. Susceptibility to rust in the $F_{2:3}$ individuals showed a continuous distribution from susceptible to resistant, suggesting that rust resistance is controlled by quantitative trait loci. In the present study, three QTLs related to rust resistance were identified in two inoculation trials in the greenhouse. Despite the considerably high correlation of rust severity between trial years, different QTLs for rust resistance were observed. Variances explained by those QTLs were relatively low, suggesting that there are other loci involved in rust resistance in Sa03. In addition, improvement of the current linkage map will make it more informative for future genetic studies in bunching onion.

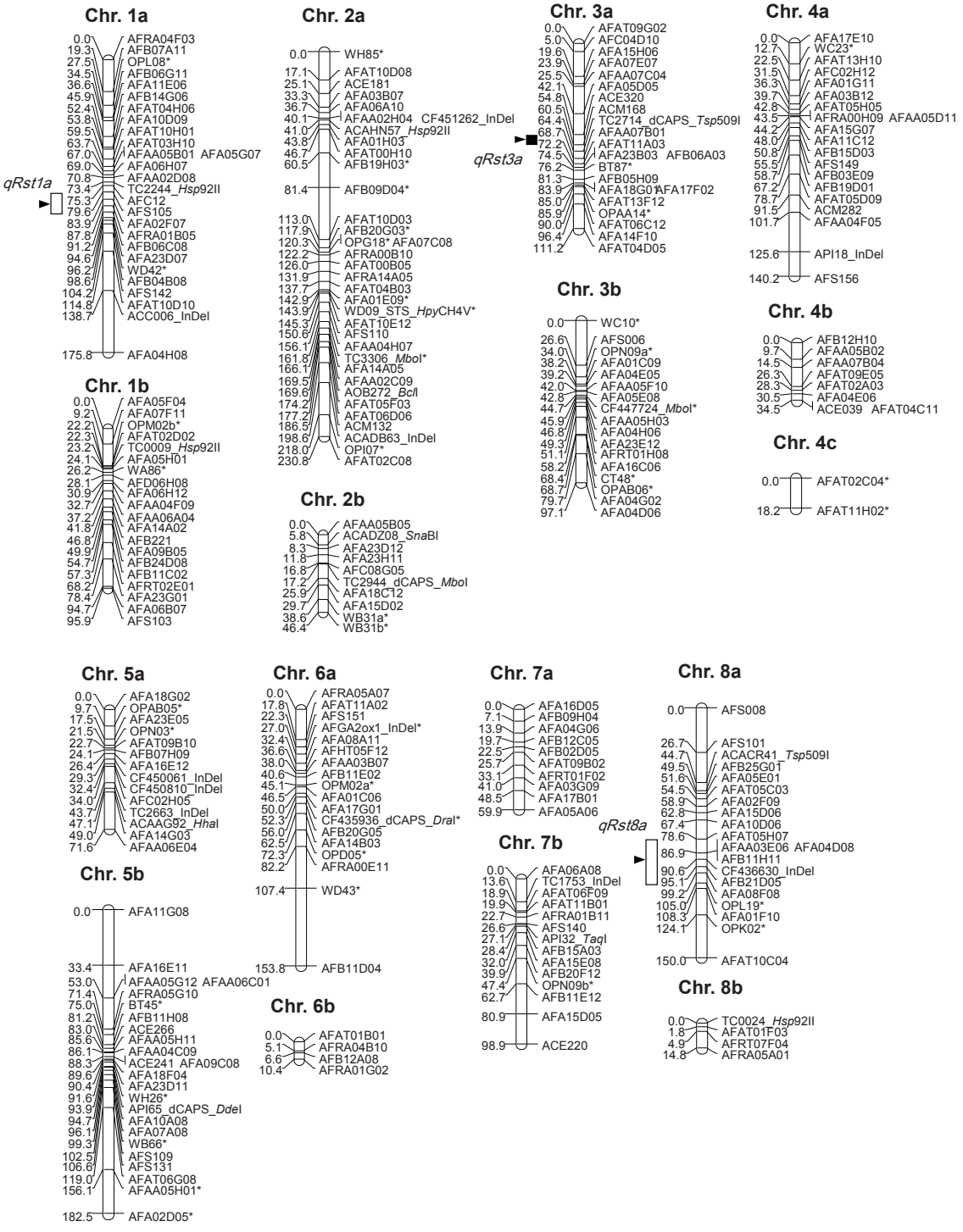

Fig. 5. Linkage map and QTLs detected in SaT03 population in 2009 trials and 2013 trials. QTLs are indicated by the white boxs (2009 trials) and the black box (2013 trials) to the left sides of chromosomes and the positions of LOD peaks are shown by arrows.

Table 5. QTLs for rust resistance in SaT03 $F_{2:3}$ population.

Trial	QTL	Linkage group	Closest marker of peak LOD score	LOD peak	Additive effect[a]	Dominant effect[a]	R^2 (%)[b]
2009	*qRst1a*	Chr. 1a	AFA02F07	4.9	0.3	-0.1	14.2
2009	*qRst8a*	Chr. 8a	CF436630	5.7	0.3	-0.1	20.4
2013	*qRst3a*	Chr. 3a	ACE320	3.6	5.0	-4.0	12.7

[a]Additive or dominant effect of 'Sa03' allele.

[b]Percentage of variance explained at the peak of QTL.

Table 6. Rust severity in $F_{2:3}$ individuals categorized by the F_2 genotypes at AFA02F07 and CF436630 in SaT03 population (Trial 2009).

Population	Marker genotype[a]		Number of $F_{2:3}$ lines	Rust severity[b]	Significant difference[c]	
	AFA02F07 (*qRst1a*)	CF436630 (*qRst8a*)			AA	BB
F_2 (Sa03 x T03)	A	A	7	2.81		*
	A	H	7	3.05		
	A	B	8	3.11		
	H	A	9	2.51		*
	H	H	32	3.00		*
	H	B	8	3.32		
	B	A	4	3.28		
	B	H	9	3.26		
	B	B	7	3.72	*	
Sa03	A	A		1.43		
F_1 (Sa03 x T03)	H	H		3.27		
T03	B	B		3.63		

[a] A, genotypes of homozygous for Sa03 allele (rust resistant); H, heterozygous; B, homozygous for T03 allele (rust susceptible).

[b] Average number of rust severities of $F_{2:3}$ lines.

[c] When significant difference between each genotype and AA or BB is detected by Tukey-Kramer HSD test ($P = 0.05$), asterisk is denoted.

Table 7. Rust severity in $F_{2:3}$ individuals categorized by the F_2 genotype at ACE320 (Trial 2013).

Population	Genotype	Number of $F_{2:3}$ lines	AUDPC[a]	
F_2 (Sa03 x T03)	T03 homo	18	128	a
	Hetero	39	110	b
	Sa03 homo	14	107	b
T03	T03 homo		179	
F_1 (Sa03 x T03)	Hetero		185	
Sa	Sa03 homo		84	

[a] AUDPC of $F_{2:3}$ lines. Different letters indicate significance in Tukey-Kramer HSD test ($P = 0.05$).

Although no significant differences were observed among cultivars of *A. fistulosum* in seedling tests, there were significant differences in field resistance to rust in full-grown plants (Wako et al., 2015). The resistance of Sa03 is derived from the cultivar 'Chuukanbohon Nou 1' (Wako et al., 2012), which was developed by recurrent selection. This parental cultivar likely possesses multiple genes relating to rust resistance.

In a previous study, it was reported that *A. cepa* showed rust resistance and possess genes relating to resistance on chromosome 1A and 5A (Wako et al., 2015). Resistance genes from *A. cepa* will likely be introgressed into *A. fistulosum* using MAALs. However, no recombinant containing a crossover between the genomes of two species both carrying rust resistance has been obtained thus far. In contrast, recurrent selection would help accumulate a number of rust resistance genes in *A. fistulosum*, therefore, this method would be a practical and reliable breeding approach. For example, in vegetable crops, the fruit yield of cucumber has been improved by 10 cycles of recurrent selection (Wehner and Cramer, 1996). Populations with improved resistance to *Verticillium dahliae* were developed by two cycles of recurrent selection in pepper (*Capsicum annuum*) (Palloix et al., 1990).

Rust resistance genes have been studied in other plant species. In particular, extensive analyses have been performed in wheat (Dedryver et al., 1996; Huang and Gill, 2001; Mago et al., 2002; Raupp et al., 1983; Stein et al., 2000) and barley (Dreiseitl and Steffenson, 2000; Feuerstein et al., 1990; Jagathpriya et al., 2003). To date, a number of qualitative and quantitative rust resistance alleles have been found in wheat and barley. The wheat leaf rust resistance genes, *Lr10* (Feuillet et al., 2003) and *Lr21* (Huang et al., 2003), and the barley stem rust resistance gene, *Rpg1* (Brueggeman et al., 2002), were successfully cloned by map-based isolation strategies. In bunching onion, however, rust resistance genes have not yet been identified. The rust resistance developed in this study is not true resistance, but rather what is known as field resistance. This type of resistance is under polygenic control, and is therefore probably more effective against a broad range of pathogenic races and more durable than monogenic resistance, which may break down under severe epiphytotics (Brewbaker, 1983).

The QTLs identified here were identified using STS (sequence-tagged site) markers,

most of which are co-dominant and near the top of the LOD peaks for each QTL. Therefore, they would be useful for selection of bunching onions with a high level of rust resistance.

Chapter IV: Mapping of quantitative trait loci for bolting time in bunching onion

Introduction

Because bunching onions cannot be stored for long periods, adaptation to different growing locations throughout the country and year-round production are very important in Japan. The late-bolting trait is essential in bunching onion cultivars for spring and summer production. Genetic variation in bolting time in bunching onion is based on seedling age and low-temperature requirements (Inden and Asahira, 1990). Low temperature (3–15 °C) is required for flower formation, and the optimal temperatures and exposure periods required for flowering differ according to cultivar (Yamasaki et al., 2000b; Abe and Nakazumi, 2004; Dong et al., 2013). Additionally, a short photoperiod also promotes flower bud initiation and a long photoperiod promotes elongation of the flower stalk after flower initiation (Yamasaki et al., 2000a). However, genetic and molecular studies of bolting time in bunching onion have not been reported thus far.

In *Brassica rapa*, several quantitative trait loci (QTL) controlling flowering time were identified and found to colocalize with the ortholog of *FLOWERING LOCUS C* (*FLC*) from *Arabidopsis* (Schranz et al., 2002; Kakizaki et al., 2011; Kitamoto et al., 2014). Several candidate *BrFLC* genes have also been discussed in terms of their functions in flowering time and the vernalization response in *B. rapa* (Kim et al., 2007; Kitamoto et al., 2014).

In bulb onion, the *FLOWERING LOCUS T* (*FT*)-like genes have been investigated for functional characterization. Up-regulation of the expression of the *AcFT2* gene appears to be involved in the vernalization-dependent initiation of flowering, whereas the expression of two other *FT* genes, *AcFT1* and *AcFT4*, has been correlated to bulb formation (Lee et al., 2013). Additionally, a QTL designated as *AcBlt1* that conditions bolting has been mapped to chromosome 1 (Baldwin et al., 2014), but the association between this QTL and the flowering candidate genes is unclear. Genetic studies of flowering are very limited in bunching onion. Therefore, it is important to investigate the QTL for bolting time as a first step toward understanding the genetics of flower initiation in bunching onion.

In the current study, bolting time was evaluated in two bunching onion populations under different environmental conditions. Major QTLs detected on the same linkage groups in the two populations were compared using the same set of markers. The Taiwanese ever-flowering cultivar 'Bei-cong' was used as the genetic source of the early-bolting trait. Most Taiwanese cultivars have minimal requirements for low-temperature to induce flower initiation (Inden and Asahina, 1990). Yamasaki et al. (2012) reported that 'Bei-cong' showed seed vernalization-like response for flower formation. A linkage map was constructed using a population derived from a cross between the ever-flowering line Ki and the late-bolting line C, and QTL analysis for bolting time was conducted under vernalized and unvernalized conditions. Additionally, QTL for bolting time was identified using another SaT03 map constructed in a previous study, and the resulting maps for these two populations were compared.

Materials and methods

Plant materials

Two mapping populations, KiC (134 individuals) and SaT03 (119 individuals) were used in the present study. KiC is the segregating F_2 derived from a cross between two bunching onion inbred lines, K1s-5s-2s-2s-4s-5 (Ki) and Cho1s-1s-2s-2s-8s (C). Ki is an inbred line derived from the Taiwanese ever-flowering cultivar 'Bei-cong' (named 'Kitanegi' in Japanese, JP138785). C is an inbred line from the late-bolting cultivar 'Cho-etsu' (JP133892). SaT03 is the segregating F_2 derived from a cross between Sa48-10s-7ic-3s-2ic-3s-1s-9s (Sa03) and T26-4s-2s-2s (T03). Sa03, an early-bolting type, is an inbred line derived from the cultivar 'Chuukanbohon Nou 1' (Wako et al., 2010), and T03, a late-bolting type, is an inbred line derived from the cultivar 'Fuyuwarabe' (Wako et al., 2012). The mapping population SaT03 is identical to that previously used for QTL analysis of pseudostem pungency (Tsukazaki et al., 2012).

Construction of a linkage map

Mainly genomic SSRs from bunching onion and EST-SSRs from bulb onion were used in the present study (Tsukazaki et al., 2011). Polymerase chain reaction (PCR) conditions

and genotyping of SSR and EST markers were performed either as described in Tsukazaki et al. (2011) or according to the protocols published at the Vegmarks database (http://vegmarks.nivot.affrc.go.jp). Linkage analysis was performed using the computer program MAPMAKER/EXP 3.0b (Lander et al., 1987), using the Kosambi function to obtain cM values (Kosambi, 1944). A log of the odds (LODs) threshold of 4.0 was used to group and order the markers. Each linkage group was assigned to chromosomes by comparison to the previously constructed maps (Tsukazaki et al., 2008, 2011, 2015).

Evaluation of bolting time

Bolting time was evaluated during field trials in two years for the populations KiC and SaT03. Additionally, bolting time for the KiC population was also evaluated under heated greenhouse conditions in the winter of 2007–2008 (the 2008 greenhouse trial, Table 8).

Firstly, the KiC population was evaluated for bolting time under non-vernalized conditions. A total of 121 F_2-derived F_3 ($F_{2:3}$) lines from the KiC population were sown in 200-cell plug trays (two seeds per plug) on 18 July 2007, then 20 plants per line were transplanted into plastic boxes (65 cm × 18 cm × 22 cm) on 29 August 2007 and grown in the greenhouse kept at a minimum temperature of 10 °C (2008 trial). The KiC population was then grown in the open field at the NARO Institute of Vegetable and Tea Science (34.8°N, 136.4°E) through the winter in 2008–2009 and 2009–2010 (2009 field trial and 2010 field trial) to measure bolting times (Table 8). Bolting time of the SaT03 population was measured in the 2013 field trial and the 2014 field trial (Table 8). Standard crop management for bunching onion was used and the bolting date of each plant was recorded. Bolting dates of F_2 individuals were used to estimate the average date of bolting of all plants in the $F_{2:3}$ lines. A plant was scored as bolting when the top of the flower scape bud emerged. The bolting time of the KiC population was defined as the number of days between the average bolting date of Ki and the bolting date of each $F_{2:3}$ line. In the same manner, the bolting time of the SaT03 population was defined as the number of days between the average bolting date of Sa03 and the bolting date of each $F_{2:3}$ line.

Table 8. Sowing and planting dates for evaluating bolting time in $F_{2:3}$ populations.

Population	Trial	Number of $F_{2:3}$ lines	Number of plants in each lines	Condition	Sowing date	Transplanting date
KiC	2008	121	20	Heated greenhouse[a]	18 July 2007	30 Aug. 2007
KiC	2009	108	60	Open field	11 July 2008	8 Sep. 2008
KiC	2010	108	45	Open field	4 June 2009	10 Aug. 2009
SaT03	2013	80	42	Open field	26 June 2012	28 Aug. 2012
SaT03	2014	87	42	Open field	5 July 2013	3 Sep. 2013

[a] Minimum temperature in the greenhouse was 10 ºC during winter.

QTL analysis

QTL analysis was performed by the composite interval mapping method using the computer program QTL Cartographer version 2.5 (Wang et al., 2007). The log likelihood (LOD) threshold was determined with 1000 permutations at $P = 0.05$. Statistical analysis of the associations between the genotypes of plants in the F_2 generation and the average bolting date of $F_{2:3}$ plants of the same genotype was conducted at each QTL-linked SSR locus in each trial using the Tukey–Kramer Honestly Significant Difference (HSD) test ($P = 0.05$) with JMP version 4.0 (SAS Institute Inc., NC, USA).

Results

Evaluation of bolting time

In the 2008 greenhouse trial, the bolting date of Ki was 12 January 2008, 136 days after transplanting, while bolting in C was not observed before 30 June 2008. The bolting date of the $F_{2:3}$ lines ranged continuously from 49 to 180 days later than that of Ki, and 10 of the $F_{2:3}$ lines did not bolt.

In the field trials in 2009 and 2010, the bolting dates of Ki were 19 November 2008 and 20 November 2009, respectively (Fig. 6). The differences in the bolting dates of C relative to Ki were 159 days in the 2009 field trial and 170 days in the 2010 field trial. The bolting dates of the $F_{2:3}$ lines ranged from 64 to 153 days and from 25 to 152 days later than that of Ki in the 2009 and 2010 field trials, respectively. The bolting times of the $F_{2:3}$ lines in the field trials in 2009 and 2010 were highly correlated ($r = 0.93$). Transgressive segregation was not observed in these three trials.

The differences in the bolting dates of the $F_{2:3}$ lines of the SaT03 population were smaller than those of the $F_{2:3}$ lines of the KiC population. The bolting dates for Sa03 were 26 March 2013 in the 2013 field trial and 28 March 2014 in the 2014 field trial (Fig. 7). The differences in the bolting dates between T03 and Sa03 were 49 days in the 2013 field trial and 51 days in the 2014 field trial. The bolting dates of the $F_{2:3}$ lines were 19 to 60 days later than that of Sa03 in the 2013 field trial and 17 to 65 days later than that of Sa03 in the 2014 field trial. The bolting times of the $F_{2:3}$ lines in field trials in 2013 and 2014 were also highly correlated ($r = 0.81$). Transgressive segregation for late bolting was

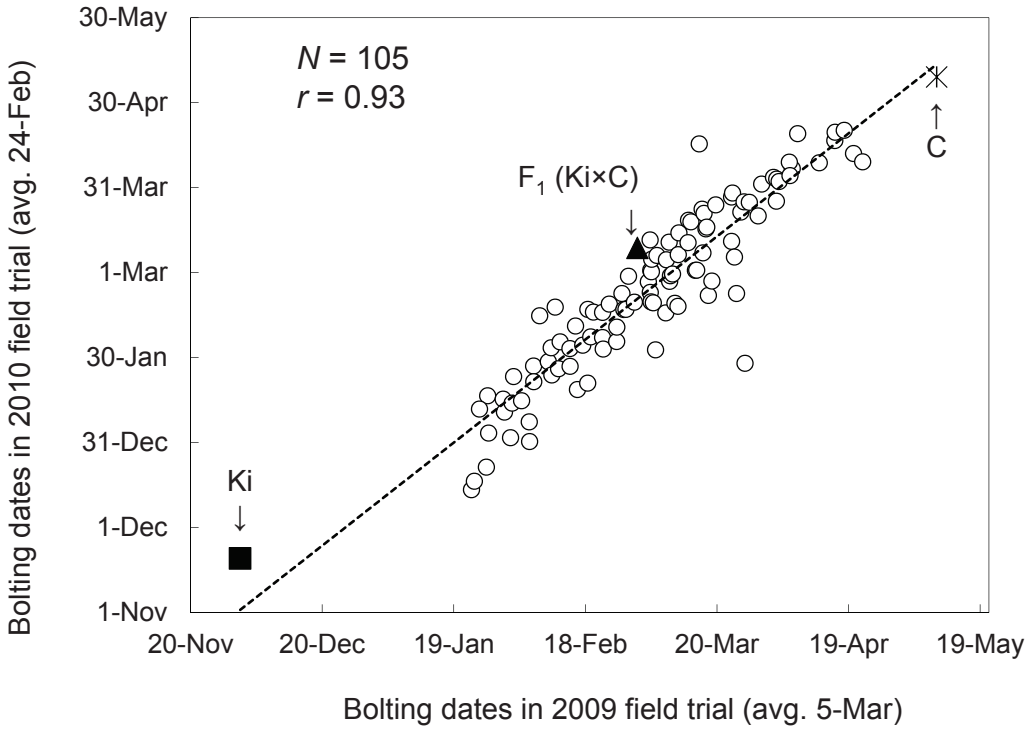

Fig. 6. Distribution of the bolting date in 105 $F_{2:3}$ lines of KiC population and correlation coefficient between trials.

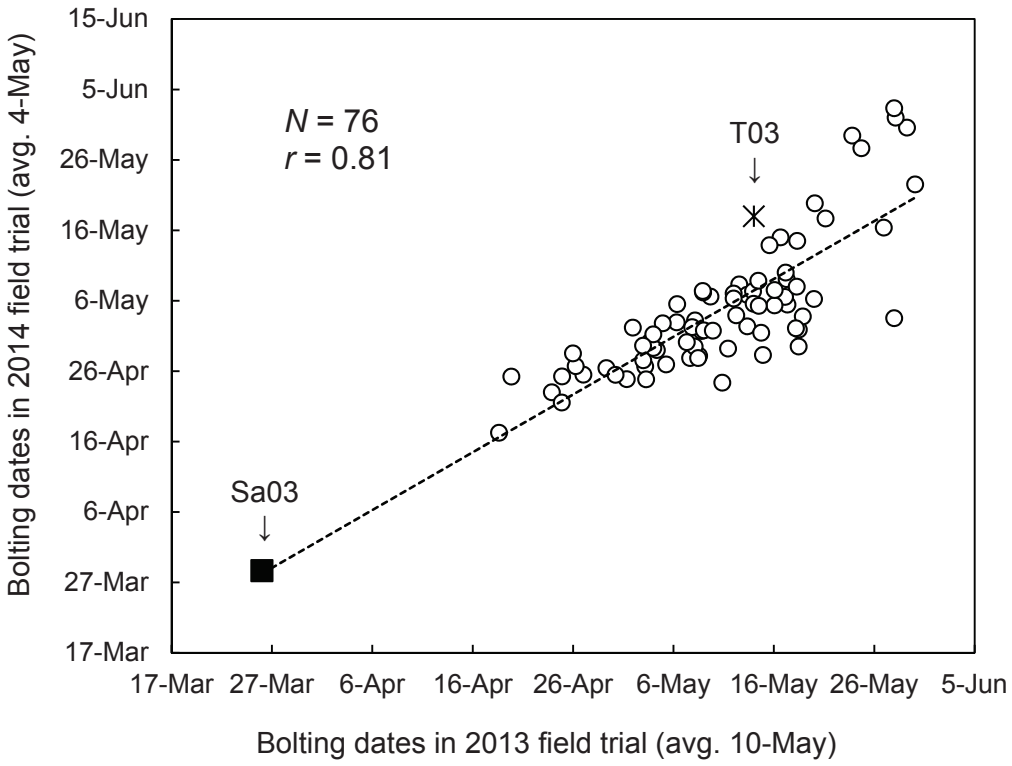

Fig. 7. Distribution of the bolting date in 76 $F_{2:3}$ lines of SaT03 population and correlation coefficient between trials.

observed in both the 2013 and 2014 field trials of the SaT03 population.

Construction of a linkage map in the KiC population

A linkage map consisting of 16 linkage groups was constructed with a total of 266 markers, including 249 SSRs and one gene from bunching onion, and with two SSRs, four InDels (insertion-deletion), and 10 CAPS designed from bulb onion ESTs (Table 9 and Table 10). The total map length was 2802 cM and the average marker interval was 10.5 cM. All of the linkage groups were assigned to eight chromosomes on the previously constructed map using the same markers.

QTL analysis of bolting time in the KiC population

In the 2008 greenhouse trial, two QTLs were detected on the linkage groups 2a on chromosome 2 (Chr. 2a) and 6a on chromosome 6 (Chr. 6a) and were designated as *qBlt2a* and *qBlt6a*, respectively. The LOD peak for *qBlt2a* was higher than that for *qBlt6a*, and the proportion of phenotypic variation explained by *qBlt2a* was also higher than that explained by *qBlt6a* (Table 11).

QTLs were detected on the linkage groups 1a on chromosome 1 (Chr. 1a; *qBlt1a*) and 2a on Chr. 2a (*qBlt2a*) (Fig. 8 and Table 11) using data from the field trials conducted in 2009 and 2010. The LOD peaks for these two QTLs were located in close proximity in both trials. The LOD peaks for *qBlt2a* in the 2009 and 2010 field trials were located close to the QTL detected in the 2008 greenhouse trial. The maximum LOD scores for *qBlt1a* and *qBlt2a* were 10.2 and 8.8 in the 2009 field trial, and 10.3 and 10.7 in the 2010 field trial, respectively (Table 11). The QTL *qBlt1a* and *qBlt2a* accounted for 15.4% and 13.1% of the observed variation, respectively, in the 2009 field trial, and 14.7% and 16.3% of the observed variation, respectively, in the 2010 field trial. Two additional QTLs were found on the linkage groups 1b on chromosome 1 (Chr. 1b; *qBlt1b*) and 3b on chromosome 3 (Chr. 3b; *qBlt3b*) in the 2010 field trial (Fig. 8), with effects lower than those of *qBlt1a* or *qBlt2a*. The maximum LOD scores for *qBlt1b* and *qBlt3b* were 8.8 and 4.7, and the proportion of variance explained by these QTLs were 12.0% and 6.1%, respectively (Table 11).

Table 9. Primer sequences of 2 bulb onion EST-derived SSRs and 249 bunching onion SSR markers located on KiC map.

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
Bulb onion SSR									
ACE112	CF444011	(TCA)6	ccttctgctttgtttattcttcacac	acgagttttgtttgtgagctcttgg	Chr.5b	152.0	127	139	Tsukazaki et al. 2011
ACM006	BQ580184	(CTC)7	gcagttctccctttgtaaaatca	gtgatggatgagtggatgga	Chr.3b	0.0	199	193	Kuhl et al. 2004
Bunching onion SSR									
AFA01C06	AB564753	(TG)7tata(TG)6	tatgtataagaaattgtacggggttgg	aacattaaagggagggtcaatagtatttt	Chr.6a	332.6	244	242	Tsukazaki et al. 2007
AFA01C07	AB499342	(TG)14	cattccagacgtttgttgtgacag	tggtctccaatacgagcatcaaaa	Chr.1b	86.8	284	280	Tsukazaki et al. 2007
AFA01E06	AB499345	(AT)3ggaggctgctagatttccata aca(AC)14(AT)5	caattctggtcaaaccgagactca	tctgttccatctattcatgttcctcc	Chr.4a	102.6	251	266	Tsukazaki et al. 2008
AFA01E09	AB499346	(AT)3ctatatgt(AC)13	cccaacctacttgagggattgcta	gttctgtgtatgcaggcaatttgg	Chr.2a	130.0	166	178	Tsukazaki et al. 2008
AFA01F04	AB795037	(TG)16	gcggtaggcagttttctgttgtgt	tgagtagttgaacattggtggcgt	Chr.1b	0.0	212	196	Tsukazaki et al. 2015
AFA01F12	AB499348	(AC)12(AT)3	tgaaggggacaaaataagaagca	tctcccccacttaaaaagaatttcg	Chr.5a	60.4	164	160	Tsukazaki et al. 2015
AFA01G11	AB564758	(AC)15	tcacactgctggtagccaggttag	ggacctttaaatgtgtagatacgggtg	Chr.4a	34.4	178	180	Tsukazaki et al. 2011
AFA01H08	AB795037	(AC)9at(AC)5atac(AT)5	cacgtttcattaggttggggaaaa	cacaaaaatccacgagtcgaactg	Chr.4a	45.7	257	263	Tsukazaki et al. 2015
AFA02B05	AB795039	(TG)3aaca(TA)8(TG)17aagtgaaacca gagg(caa)3	tgcaacggtagagagatgatggag	tgaaatcccctgcgagattgtagt	Chr.5a	30.5	249	257	Tsukazaki et al. 2015
AFA02B06	AB564763	(AC)12(AT)4atatgtgtatgg(AT)3	atggggagctcaaagttgtgaatc	gggaaggagttccataaacgatcc	Chr.7b	13.0	279	281	Tsukazaki et al. 2011
AFA02C10	AB795040	(TG)3tatgttctaga(AG)3(TG)5ta(TG)3 ta(TG)7	gggtaggtttcccttgtttggaag	tgagggatgcatttgctattttgag	Chr.2a	45.9	252	244	this study
AFA02D02	AB795041	(TA)4aacgttag(TG)13	acgtacatatcgaaccatttcccg	gcctcctccccattcaagaactat	Chr.5b	116.9	142	140	this study
AFA02D03	AB795042	(AC)16a(TA)8	ggatgcatgcttggagtcttgtta	tgaaactgcctcaacacaaaggaa	Chr.2a	238.3	220	224	this study
AFA02D05	AB499354	(TA)3cta(AT)3(AC)11	ttaggcgtcacataccaaagctacaa	tggaatttcaaaaagacaccgagtaa	Chr.5b	12.8	232	234	Tsukazaki et al. 2008
AFA02F09	AB499356	(at)3(ac)14a(ta)3tg(ta)3	cctaggttcattaggggactatagga	ccaagcaccagtatctgcctttct	Chr.8a	61.0	296	290	Tsukazaki et al. 2007
AFA02H08	AB499357	(TA)3cat(AC)4aa(AC)12	agatcttggatagttattaaagtagttccagtaga	gggctgaaatattatgtgggtttg	Chr.6a	180.9	181	187	Tsukazaki et al. 2007
AFA03B12	AB564772	(AC)12	catcattaggcgctacaagggttc	tccagtgggttgtaacaaggaagg	Chr.4a	107.4	220	228	Tsukazaki et al. 2011
AFA03D07	AB499363	(TG)3(CA)18	tgtcctcgaaaatgagatgcttca	tgaaatgtaacccgactgtccaaa	Chr.6a	378.0	301	282	Tsukazaki et al. 2008
AFA03F08	AB499365	(TA)4(TG)7ta(TG)7	ttaggtaaagggacgaaacgacca	tgcctccaggactgaacaatacaa	Chr.1a	110.4	302	286	Tsukazaki et al. 2008
AFA04D08	AB564782	(AC)4gc(AT)3gg(AC)4tcatacaaa(C A)12(TA)6g(AC)4	ttcaatcgcactggtggtatgaac	tgcatgttccatctctacctaacgtg	Chr.8a	0.0	260	262	Tsukazaki et al. 2011
AFA04E02	AB564784	(GC)3(AC)3ttat(AC)5aa(AC)15	ggaaccagttgaggatgtttaaggg	gagcacttttaggtgcctgtgct	Chr.6a	0.0	234	224	Tsukazaki et al. 2011
AFA04E06	AB564786	(TA)7(TG)18	ccctacacattttcatccgctctt	agcttcttgaaggtggatgtgctc	Chr.4b	133.0	234	232	Tsukazaki et al. 2011
AFA04H06	AB564789	(AC)6at(AC)8(AT)3	cagaggagcaatgtcatgcctaga	ggcatgataaaatgagtgaaggtga	Chr.3b	142.2	241	252	Tsukazaki et al. 2011
AFA05A06	AB564791	(AC)12tac(TA)3t(TA)3	cgcatacggtctcatccgctataa	taggatcggcagtgttggaatttt	Chr.1a	0.0	271	280	Tsukazaki et al. 2011
AFA05D05	AB564793	(TA)6cactac(CA)3tacat(AC)4ag(AC)12(AT)3	cttaatgtccccaatgatgtgactt	cgaaagtgtgacggaaattcgttt	Chr.3a	393.9	250	264	Tsukazaki et al. 2011

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFA05E01	AB564794	(AC)13(AT)7	gcgttaaggcattacctatatccacc	cctttggtttgggagtatgtctcg	Chr.8a	50.4	239	237	Tsukazaki et al. 2011
AFA05F04	AB564797	(AC)7aa(AC)11(AT)4catatcct(TA)3	gcatgtatctcgtcttggcaca	tcctttgatggtatttgtaactagatgttt	Chr.1b	101.9	203	211	Tsukazaki et al. 2011
AFA05G05	AB564799	(AC)12ag(AT)5	tgcagtgacaaccagtacaaagca	tccttcactgcatgccatattatca	Chr.7b	92.3	318	296	Tsukazaki et al. 2011
AFA05H01	AB499374	(GT)9(TG)7ttt(TG)12ttttccgtgttgcgt gt(TG)5	acaccttccttgcacatttgctta	tcttcagagaaagatacagaaggggga	Chr.1b	108.3	230	228	Tsukazaki et al. 2008
AFA06A08	AB499376	(CA)6ta(TACA)2t(AC)10atatac(AT) 4	cctcaggagaggggtattttggtt	cttgggaaaggcttctcttgaggt	Chr.7b	138.6	188	190	Tsukazaki et al. 2007
AFA06A10	AB499377	(CAA)3(TA)8(TG)14	gttgccaggtttctttggcttaaa	tgctagcaccaccatttttcatgt	Chr.2a	15.4	290	278	Tsukazaki et al. 2007
AFA06B07	AB564804	(TG)3tata(TG)10	ccttaatgcaaactatgatgaggtatgt	ttgcgtccttcgtgggtatt	Chr.1b	17.1	222	224	Tsukazaki et al. 2011
AFA06E05	AB564806	(CA)5gag(AC)5tatactttaagttcttaagtc ccta(AC)5at(AC)10	gggcataggtgagacatccaaa	aacaaaattaacaacttcgctaccaga	Chr.4a	61.7	255	253	Tsukazaki et al. 2011
AFA06F05	AB795044	(TA)9(TG)7tata(TG)5ta(TG)5ta(TG)5	acgatctgccctacctacttgagg	acgggaagatccttagtgtacgca	Chr.1a	54.0	238	240	this study
AFA06H12	AB499380	(GT)14tttattgtctccatgtatg(TA)4	tggacattgacttttggatgtttagc	gaacacatagaaacagaacatagcgaaga	Chr.1b	88.5	274	266	Tsukazaki et al. 2007
AFA07A08	AB499381	(TG)8tt(TG)5	tctggaaggcggtttaaggctatt	gcatccatatccatatcccacgat	Chr.5b	158.9	251	243	Tsukazaki et al. 2007
AFA07A10	AB499382	(AC)11(AT)4tgtatg(TA)7	actactgggatgaatgcccaaaga	atccagttgcacatgactttg	Chr.4a	174.7	228+230	228	Tsukazaki et al. 2007
AFA07B06	AB795046	(AT)3tca(TA)3gcacaa(AC)11atatac attcatcc(AT)3	atggcttatagacgcatttccgtg	tggttcgagcatttttccttaggt	Chr.7b	0.0	275	273	Tsukazaki et al. 2015
AFA07C08	AB499383	(TG)4(TA)8tctc(TG)9	tgtgctaaagccaggatcattgag	tgctttcaaaagtaaaggggacca	Chr.2a	109.2	244	242	Tsukazaki et al. 2008
AFA07E07	AB564811	(TG)10	gaagatgcctccattttgacatcc	ggtcaatagcgaaaatccaacgtc	Chr.3a	196.2	269	265	Tsukazaki et al. 2011
AFA07F11	AB564813	(TA)4(TG)13	gtgtgggtaacaatttttgaatggg	tttgatgttgtgccacttttgg	Chr.1b	90.4	216	239	Tsukazaki et al. 2011
AFA07G04	AB795047	(AC)11	atctccataaccgcttccaaaaca	acccctagggaggactcattggta	Chr.7b	54.9	271	265	this study
AFA07H04	AB795048	(AC)8	gcaaaaatcaagaatgggtcaagc	gtttcaagtcatgcacgtgtttcc	Chr.7a	86.9	127	127	this study
AFA08E07	AB499391	(TG)13	ataccatggcattccattgaacct	cccctatccactttcaagatgacg	Chr.3b	124.9	223	233	Tsukazaki et al. 2008
AFA08E10	AB499392	(TA)3atagc(TA)3c(GT)11	gtacgatgcgcccaacctactcta	aggaggcaacgctaaataacccat	Chr.3a	88.3	277	281	Tsukazaki et al. 2008
AFA08F05	AB795049	(GT)7ataca(TA)5(TG)9	gattcaaacgcaacttccagatcc	cacagacatgcacaccaatctttg	Chr.6a	412.9	278	284	Tsukazaki et al. 2015
AFA09A11	AB499396	(AC)3(AT)3acat(AC)17at (AC)3g(TACA)2(TA)3	agaactctctgttcgctcattcca	gcgagacttatatcgcctcgctta	Chr.6a	254.1	180	184	Tsukazaki et al. 2008
AFA09C08	AB499397	(TA)4c(AT)4(GT)10a(TG)4c(GT)6at a(TG)9	gaaaccgataatgacttaattgcttgaaaa	ccttatcttaatctgcttccagtctctcaa	Chr.5b	202.8	190	194	Tsukazaki et al. 2007
AFA09F08	AB499400	(AC)3g(CA)12t(AC)3aa(AC)8	gggaacatgtgcaataagacattg	aataatgtgcaacaaaaattaacggtatta	Chr.3a	360.9	248	258	Tsukazaki et al. 2008
AFA10B05	AB795050	(CG)5(TG)12	ggaaaaagccgtcgaaaataaagc	tagatggtcgctcctatcctgctc	Chr.3a	217.7	259	261	this study
AFA10B06	AB499402	(AC)15(TC)6	ccgcaaactggactaactgtacaaa	ggcagaatagcccaagtgttttca	Chr.1a	281.5	281	302	Tsukazaki et al. 2007

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFA10C07	AB499403	(AC)22(AT)7	caacattgcaaacatgtcacaca	gcactgagtttgcgttgagcta	Chr.5b	27.5	255	247	Tsukazaki et al. 2007
AFA10G05	AB795051	(AC)12(AT)9	ccagactaaaaggaacccaactgc	tggtttgaatgtggtcagtggtct	Chr.5a	121.5	186	194	this study
AFA11B05	AB499408	(TA)6(TG)16ttgtggattggc(AT)3	ctcggtccaactttggagttatcg	tatggatataacggggactggctg	Chr.3a	234.4	250	254	Tsukazaki et al. 2008
AFA11C02	AB795052	(TC)3cttctcaataataagctatctgctta(CA)3(TG)6(GT)7	aataggcacttgtccaggaagcag	gcattcaatcatgtgcttgtctca	Chr.2a	172.3	225	223	this study
AFA11C09	AB564822	(TG)11tttgcgtgcgcg(TG)3catgcgtgcc(TA)3ca(TA)3	cagaacaacgttttggtgactgga	gctgttagatctgccttctccctg	Chr.7a	73.8	283+291	285	Tsukazaki et al. 2011
AFA11D02	AB564823	(AT)4gcatgtatg(TA)6cacatatc(TG)9tt(TG)4tcc(GT)3	cggcaaacctgttaaatcc	tttatacataaacacaaacacacacg	Chr.1b	84.5	165	169	Tsukazaki et al. 2007
AFA11E06	AB564825	(AT)3t(AC)6at(AC)4ttataccctatttccttga(AC)13	gaaaaggtttttcaaaggagattttca	gacttccacgggcaaggat	Chr.1a	59.3	246	242	Tsukazaki et al. 2011
AFA11E12	AB499410	(AC)5tcttc(AC)12	gctggacggacttctgtatgcttt	cgaccttaagtcataaacgtggtaa	Chr.8a	130.6	220	208	Tsukazaki et al. 2008
AFA11G05	AB795053	(TA)5(TG)11	ggtcaggagcaacccaaacattac	tcctttaccaccatacctgattttg	Chr.5a	0.0	237	239	this study
AFA13E04	AB795054	(TA)6tgtggg(TG)12	agccaggtcaaaatgtcggataac	gggcaaacttctgttgtgtaaccc	Chr.3b	20.7	220	208	this study
AFA13G12	AB795055	(AC)12	ggatttccttaggcgatttgatga	agtcgttacaactccatcgctgtg	Chr.3a	285.4	259	261	Tsukazaki et al. 2015
AFA14A02	AB499412	(TG)12(TA)3	aaagtggacgtaggcacattctgg	tagccgtgggttgaagagaaaaa	Chr.1b	69.7	244	246	Tsukazaki et al. 2007
AFA14A11	AB795056	(AC)10	ctcgacggggtgctagtaaagaga	ttgactccacatccacgtacgaat	Chr.2a	167.6	145	143	this study
AFA14B03	AB564831	(AT)3(AC)17	actgcatctttccaggttttgctc	tacatcgcacgtgccaaaatactc	Chr.6a	305.6	227	215	Tsukazaki et al. 2011
AFA14E04	AB795057	(AC)16	gaattttcgccatatcaaccatc	cgtatgagagcatacttgttgtatttga	Chr.8a	102.8	208	218	this study
AFA14E07	AB795058	(TG)7ta(TG)7(TA)6	gaagcaacacaacttcaaaacgga	gacgaaatttccttcttcatccga	Chr.8a	103.4	126	123	this study
AFA15D05	AB564835	(TG)10	gcatttggagtagtgggcttaaagg	acagtcagaatcgcatcgataaca	Chr.7b	65.0	213	217	Tsukazaki et al. 2011
AFA15E08	AB499418	(GT)8attg(TA)3	tgagaagtgtgtgtaaggcaaggc	gccccaaagtcatactgctggtag	Chr.7b	152.2	241	245	Tsukazaki et al. 2008
AFA15G01	AB795060	(TG)13	ctagtgagaccatgccgcttagtg	aaccagggatggaaaatccaagat	Chr.6a	272.8	202	210	Tsukazaki et al. 2015
AFA15H06	AB564837	(TG)12	ccaatactgattaagcaacttgtgttatgaa	tcgtcatcaagtaggcatttctcaaa	Chr.3a	176.5	173	170	Tsukazaki et al. 2011
AFA16B11	AB795061	(TG)13	ctttgtttggaatggtggtgatga	gccgtgattttctcctatcaatcg	Chr.1b	133.9	293	286	Tsukazaki et al. 2015
AFA16C06	AB499420	(TG)18	ggagctctggaacgttcgaactaa	attgtcaaaatcaagccacggaag	Chr.3b	73.9	254	256	Tsukazaki et al. 2008
AFA16D05	AB499421	(AC)12(AT)5	aattcacccataacttccgctacg	gtgcaacactactgacctcgcatt	Chr.7a	60.1	204	188	Tsukazaki et al. 2008
AFA16E07	AB564840	(GT)3(ATGT)2a(TG)6tatg(TATATG)2(TG)4tc(TA)4tccg(TG)3caga(TG)4	tatgcgtagacatgagggcacaat	cacaaacatgagaaatacgttggca	Chr.3a	343.9	215	213	Tsukazaki et al. 2007
AFA16E11	AB564840	(TG)3tatgtatgta(TG)6tatgtatatgtata(TG)5tc(TA)4tccg(TG)3caga(TG)4	tatgcgtagacatgagggcacaat	cacaaacatgagaaatacgttggca	Chr.5b	66.0	203	205	Tsukazaki et al. 2007
AFA17A05	AB499422	(TG)22	ttccatcaattacactgccagcac	tgctgaacttggtatgttgatactcca	Chr.8b	40.3	181	161	Tsukazaki et al. 2007
AFA17A12	AB564843	(GT)3atatacatg(GTTT)2gtgttgca(TA)7(TG)9(CG)3tgcg(TG)9tt(TG)4	ggcagcagtgttctggggttatac	agcctgagcacatcaaatacacga	Chr.8a	19.0	264	268	Tsukazaki et al. 2011

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFA17B01	AB564844	(TG)3ccatgg(T)6gtatggtgggcttaat(AC)6ag(AC)14tc(AC)3	tgtatggtccaggtttcattgtgc	caagcactgcagagagagttcagc	Chr.7a	105.3	175	177	Tsukazaki et al. 2011
AFA18A02	AB564850	(AC)10(AT)3	aaggctacgcaaagaaatcaccaa	acctgcacctgtttgcttgagtta	Chr.7b	79.6	186	190	Tsukazaki et al. 2011
AFA18A08	AB795062	(AC)9	ggttgtattggcaaattgcactga	ttgaatttctaccaacctgtatccc	Chr.2b	51.0	206	204	this study
AFA18C12	AB564851	(AC)15	gaacgattccaaaggcacgataag	catggctgagcacttgagaggtta	Chr.2b	0.0	222	214	Tsukazaki et al. 2011
AFA18F04	AB564852	(TA)5(TG)12	atccaaggtttgtgaccaattt	acaccaaccgagcatataatcaa	Chr.5b	188.0	249	237	Tsukazaki et al. 2011
AFA18G02	AB564853	(TA)3gtgcatatcaaagtgcagacatgaatat(AC)11	tgaaaacaacagttgcaaaccagg	tttttccctctatgacttcggcac	Chr.5a	28.4	292	294	Tsukazaki et al. 2011
AFA18H08	AB795063	(TG)13	tcgtgtctgtatctgtgtccgtgt	gtttatccttctggtgaaatgccc	Chr.2a	0.0	290	286	this study
AFA23A08	AB564855	(TG)11	agaatcagagtcttggttggtgcc	attctaaaccatggtctgtcggga	Chr.7b	94.7	252	248	Tsukazaki et al. 2011
AFA23C12	AB499427	(AC)20	aaactgatcaaaatgtgccccact	gagaatccgattaaaaacgagcaag	Chr.1a	97.9	154	134	Tsukazaki et al. 2008
AFA23D11	AB564858	(TA)6(TG)13	caatcacacacagcattgagcatc	ctgatttgttcagttgatgattgcg	Chr.5b	175.3	291	279	Tsukazaki et al. 2011
AFA23G01	AB564861	(TA)8(TG)14cgc(AT)3gtagatcatatgtatg(TA)3tggatgtttatt(TG)4	catcatttccatcacctccatgtc	aagggctaaggtttctcttcctaca	Chr.1b	33.8	254	244	Tsukazaki et al. 2011
AFA23H11	AB564862	(TA)3catatgtatgtacgtgta(TG)3tatgtaa(TA)6(TG)17	catgaccacactttgtcgcatttt	tctcgttctgaattcaaccccaat	Chr.2b	66.9	237	231	Tsukazaki et al. 2007
AFAA00B01	AB795064	(AC)13	atggtgtaagggcattttgtttgg	gatgggaaaagatgtcaagaagca	Chr.3a	161.8	172	174	this study
AFAA01A08	AB795065	(CA)7(TA)5cacaa(AC)4	cattgcaacttttgtttatatgtcttgtgc	ccccttgtccacctctcaatgt	Chr.2b	39.4	260	256	this study
AFAA01G08	AB795066	(TG)4tctatg(CA)7t(AC)6aaa(TA)8t(AC)7	ttgtgtcaccggttttgttttatg	agccaaccccgtattcgtaatgt	Chr.4b	87.5	238	226	Tsukazaki et al. 2015
AFAA02D08	AB499432	(TG)10(TA)6tgtatgtatgcatgta(TG)7	ctatggccaagttccattttgagc	tatttttacagcaacggagcagca	Chr.1a	86.7	181	169	Tsukazaki et al. 2007
AFAA03B07	AB564866	(ATT)3gttgtt(TA)7(TG)12	aaagaagagaaaacaattgctacaaccgta	ttgaatttcccaacagattaatatcattacaa	Chr.6a	162.0	186	204	Tsukazaki et al. 2011
AFAA03C05	AB564867	(AG)12	ttgggaaatttgggaattagggtc	caccataaatcgttttgcttacacc	Chr.4b	31.5	201	189	Tsukazaki et al. 2011
AFAA04D06	AB499435	(AC)4(A)5tattaatacaaacc(AC)4agat(AC)4gcatgc(AC)4tcat(AC)14(AT)11	ttattagccatgatcaacacacacaaaaa	agattcaagcagagtcaaggtattcaatg	Chr.3a	65.9	289	275	Tsukazaki et al. 2008
AFAA04F04	AB795067	(AC)16(AT)9	tgggcttgtgaagtaagttgagca	tggggatgaaattcttttaagggg	Chr.4b	0.0	282	278	this study
AFAA04F05	AB564870	(AC)3(GC)3(AC)4at(AC)9	gtgaccgtaaaacattttgccgaa	agtgaagatttgccttggttttgg	Chr.4a	69.4	259	254	Tsukazaki et al. 2011
AFAA04F12	AB795068	(AT)3gattat(AC)5aaaaa(AC)7cctc(TG)8tttgta(TG)3attgtgca(TG)5cgtgtt(TG)5attgtgcgtatgcgta(TG)4	tctgccttgaatattgagtaactgcc	tgacttcacaaatgacacgaatgtg	Chr.3a	263.2	280	284	this study
AFAA04H07	AB564872	(TG)20	tgcacctctacaagtcaactgggt	atgttgtccaagcactttgatgga	Chr.2a	182.8	269	261	Tsukazaki et al. 2011
AFAA05B02	AB499436	(AC)10(AT)11(AC)8(AT)3acacaa(AC)9(AT)9acg(TA)5agag(TG)4	cgtaaagttatccatacaatgccca	ggaccagttatccatacgtacaca	Chr.4b	73.9	296	288	Tsukazaki et al. 2008

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFAA05D11	AB564874	(AT)3gcatacaaat(AC)4aa(AC)9ttata tgta(TG)3	gcaaacccaacatatacatgaacac	tttactatgcatggccgattaccc	Chr.4a	39.6	178	210	Tsukazaki et al. 2011
AFAA05F07	AB795069	(AT)3gta(TG)7tt(TA)3aa(TG)5(TA) 5tgtatatgtata(TG)5	aataaatcatccaccttccatgcg	tttctgcttcggtgctaactgttt	Chr.3a	127.0	242	244	this study
AFAA05G12	AB564877	(AT)4(AC)12	caacgtttcttgtatgtcagctcattc	gtgacatcgccttagacttgatcg	Chr.5b	42.8	216	218	Tsukazaki et al. 2011
AFAA05H03	AB564878	(TA)3c(AT)3gta(TG)10(TA)8(TG)4t a(TG)3ta(TG)6(TA)7	gaacacatcctattgaacctgcatc	gtgattgttttagcaatggtaaggg	Chr.3b	74.5	225	243	Tsukazaki et al. 2011
AFAA05H11	AB564879	(TA)3c(GT)4tagtatgc(AT)9(AC)12	gaaacgttgtggcctctttagcat	tcatgttctcccttcatttcgtga	Chr.5b	107.9	238	236	Tsukazaki et al. 2011
AFAA06A01	AB795070	(AT)3gtgtatatct(AC)13(AT)10tgaga tttatatctat(TG)3tatc(TA)3	ttcataacataacgtgataacatattgaaa	taaacatgggcatataattaggcatt	Chr.2a	214.8	225	229	Tsukazaki et al. 2015
AFAA06C01	AB564881	(AC)5aaaaacatacat(AC)11aa(AC)3(AT)3	tggtactcactgatttaatggcataat	ctttaatgatgaatctgttgcatacg	Chr.5a	18.8	276	280	Tsukazaki et al. 2011
AFAA07B01	AB564883	(AC)11	gggcccaactgaacagagtcatag	cccaactcactccagaacactacaa	Chr.3a	292.4	224	232	Tsukazaki et al. 2011
AFAA07B04	AB564884	(TC)6(CA)6at(AC)4attatacaccc(AC) 7(AT)7(AC)8at(AC)3atgcgtgca(TA) 4gatgtatatgtgta(TG)3tatgt(AC)3atat aca(TA)3cacccag(AC)4	caacttgaacatgatttggaagtattta	tgtgtatgtgtgtgtctgggtgt	Chr.4b	99.9	253	255	Tsukazaki et al. 2011
AFAA07C04	AB564885	(CA)9aaacacagaagacacag(AC)3aaa cacagaag(AC)3ag(AC)5atgta(TG)7	ttttgcgagattctgcttcttt	ctgaaaaagtgttatgaacttgaggtatt	Chr.3a	331.6	245	237	Tsukazaki et al. 2011
AFAA07E06	AB564887	(TG)6tt(TG)13	atgtatcattgactgtatttgcaccttctaa	atcaacatcagtctttttaaattgcatcagta	Chr.6a	352.0	216	239	Tsukazaki et al. 2011
AFAA07H05	AB795071	(TA)3tgtgtctcagaatt(TA)3tgtgggtga a(TG)7tt(TG)3	tggcagtgtttagtgttgggttga	gcacgtaagaaaaaccacacccat	Chr.6a	316.6	174	172	this study
AFAA07H07	AB795072	(AC)14	cccacaacatacacacacatcttca	tcttcctcctcctgcttatcctcc	Chr.2b	43.9	180	182	this study
AFAT00B05	AB499440	(AT)3ctatgctaaccttctctaaagtacaac(T A)6tttcgtgtatc(TG)12	tgtccactattcacggcttactca	gatgggtctgcctcctccttattt	Chr.2a	117.8	202	206	Tsukazaki et al. 2007
AFAT00B07	AB795073	(AC)7gc(AC)6(ATAC)4ct(AC)9a(T A)8	cctatacttgtcatccaatagtgaacc	attagtaatggacgggcctaggga	Chr.2a	197.8	260	252	Tsukazaki et al. 2015
AFAT00B08	AB795074	(TG)6	tgttggtatgtgttcatgcaaggtt	gacacccacccacacactatcact	Chr.8b	0.0	252	250	this study
AFAT00H10	AB564890	(GT)8(T)7aaaatgagtga(GT)9(AT)4c(TG)3(TA)3tgta(TGTGTA)2(TG)6	agcacacaaatactaagagaaaaatcagca	agaaaatttcatctttagcaacactttga	Chr.2a	35.1	255	257	Tsukazaki et al. 2011
AFAT01F03	AB499443	(AC)11at(AG)9aatt(AC)3gcg(CA)3t gcatgcacg(CA)3att(AC)6	tgacatagacccttttgtaggaggaaa	tgcatacatacatacatacgcatatacatacaa	Chr.8b	26.7	210	200	Tsukazaki et al. 2008
AFAT01H05	AB564893	(AC)4aa(AC)7atatgcttatattg(GT)3	tagcagaagccttgtagcgaggatag	gaatgcaataaaatgacacatcaagca	Chr.2a	139.3	154	150	Tsukazaki et al. 2007
AFAT02A03	AB564893	(AC)4aa(AC)7atatgcttatattg(GT)3	tagcagaagccttgtagcgaggatag	gaatgcaataaaatgacacatcaagca	Chr.4b	116.1	238	236	Tsukazaki et al. 2007
AFAT02B11	AB795393	(AC)8atctacacgtgtatgtatgt(AC)7	gcatcgggtcagacgctagtattt	caagaagacaaaaggatgactgaag	Chr.3b	64.2	221	217	Tsukazaki et al. 2007

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFAT02C04	AB696898	(AC)8ttc(T)5aagc(TG)3tattatttcttcatgtgaccat(TC)3	cttgccttttcttcgttttt	ttgcatgattggctaaagg	Chr.4a	20.8	187	184	Tsukazaki et al. 2012
AFAT02C08	AB564894	(TC)13	ctaacaaccaatcaaaactaagataaca	acataatgcgctgacttgga	Chr.1a	30.0	225	239	Tsukazaki et al. 2011
AFAT02F09	AB795075	(TG)4cata(TG)11(TA)4ga(TA)3gata(TG)7	tgaaacatgaagccttccaggata	ggttgtatgtcaggaattgtctggg	Chr.1a	247.5	135	137	this study
AFAT02H05	AB499445	(AC)12tg(AT)4	tctcgtaccagtttctccttttcc	gattcggatcaatacagggaatgc	Chr.4a	95.0	292	294	Tsukazaki et al. 2008
AFAT03F10	AB795077	(TG)3tatctatgtatgta(TG)9	tctcaaattccctcctagatccca	catggaggagtagcagatcgaaca	Chr.2b	45.9	227	229	this study
AFAT03H10	AB564899	(AT)4ttatgagagtctt(AC)5atgcat(AC)4atgcgta(TG)3tatg(TA)3cag(AC)11(AT)7	gcacttcctacttgattcacacaagc	ttgatttcatggtgtataaactggttaatctt	Chr.1a	118.5	194	170	Tsukazaki et al. 2007
AFAT04B03	AB499448	(TA)7(TG)22	tttttcatttcagcataaagtaattttcta	ataacaacggtaaccaacgctctaa	Chr.2a	147.3	245	231	Tsukazaki et al. 2008
AFAT04D05	AB564903	(TG)15atattgcattactgatatgcaaa(TA)3agttctc(TA)3	gttgctgatttctttctcattttccagt	tggtgcaaattctaaattttcctttgat	Chr.3a	313.6	195	193	Tsukazaki et al. 2011
AFAT04F03	AB564904	(CA)8tattat(AC)5gc(AC)3t(CA)3tgcg(CA)3tacatt(CATA)3gat(AG)3actgttgccgaactaacaatt(TC)4	caaaaggtgagaaagggctacatga	gagagagaaattgttagttcggcaaca	Chr.1a	80.2	161	165	Tsukazaki et al. 2007
AFAT04H04	AB564906	(AC)10(AT)5	cagaattgagccacggaagttt	ctcttgctagggtcaaactaacctct	Chr.4b	55.2	193	185	Tsukazaki et al. 2011
AFAT05B10	AB795078	(AC)3caatgttgatctatctactcaaat(AC)10at(AC)12	cctcattttgtcctattgctcattc	ggggccaactcattagagataggg	Chr.3b	35.2	258	256	Tsukazaki et al. 2015
AFAT05H05	AB499458	(AC)11(AT)6(AG)3aaatga(T)6aaaatatag(AT)3	ttgttccgcaaatagttaaactcaa	tgttcgaatcttacgtaacgtgatt	Chr.4a	103.4	117+131	131	Tsukazaki et al. 2008
AFAT06C11	AB564912	(AC)23(AT)4	ccacaaactacagcctgaaatgga	gatacaaagacgtgagacgtggga	Chr.5b	143.1	138	146	Tsukazaki et al. 2011
AFAT06C12	AB564913	(TA)6(TG)15tt(TG)6	gcgcttaagcaccattacacaaga	cccatgatcttccaaaatctagaac	Chr.3a	377.9	260	266	Tsukazaki et al. 2011
AFAT09B02	AB564918	(AC)8gtgtaca(TA)3	caaagggcaatccaatggtagaga	gcgatagttcttcgttattttgggc	Chr.7a	78.1	268	272	Tsukazaki et al. 2011
AFAT09B05	AB564919	(GT)3acacata(TG)3acta(AG)13gcgtttaaaacc(AT)3	aggcggggttatttgactacatga	aatcacaataggaaaccaaccgga	Chr.5b	132.1	214	210	Tsukazaki et al. 2011
AFAT09B10	AB564920	(AT)7(AC)10	gctaaagtatagggcgcgactcaa	tcccattagacttcatcaacattgc	Chr.5a	24.9	292	304	Tsukazaki et al. 2011
AFAT09E05	AB499461	(TG)11	aactgcgagtaatttctcaacaactgc	ggaggaaaatctctactcccagcatc	Chr.4b	108.4	255	257	Tsukazaki et al. 2008
AFAT09F07	AB795079	(AC)8	gcacaaatttcctcatccctgttc	tgtgcagcaagagcgtaatctagg	Chr.3a	358.2	267	265	this study
AFAT09G02	AB564921	(AG)22tgtt(TA)4tgtatgcatacgtgc(AT)3gtatgga(TG)4	cgccattttcttcttctccctgta	aaaattggtccaagccaaggaact	Chr.3a	34.0	260	266	Tsukazaki et al. 2011
AFAT09G03	AB795080	(CA)7taaatacat(AC)8at(AC)7(AT)3	ctgccatccctctttctctttcaa	cttaccaatctatgccagcaagcc	Chr.6a	88.2	247	249	this study
AFAT10D10	AB564924	(GT)7attta(TG)3	agtgattgaaagtcctagggaggt	ttcatttacaaaccatgccacacg	Chr.1a	162.4	220	218	Tsukazaki et al. 2011
AFAT11A02	AB564928	(TA)3tc(TG)3tatgc(AT)3(GC)6(AC)4agagatacgtatgttgacatgtg(AT)4cta(TG)4tt(TG)8	gcgcgcacacacacagagatac	tccgctcttttatccttatatttctaattggtt	Chr.6a	59.4	165	158	Tsukazaki et al. 2011

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFAT11A03	AB499464	(AT)3gtatgtaaacttgttatt(TA)3aatatcaaaaactatgtaaaat(TA)4(TG)9tcttacatgtaaaaatatcagcgatt(TA)5	cttgtcctatatgtattgaatgttgtccaaa	cggaatattttggtgtgcatgtgt	Chr.3a	302.2	216	218	Tsukazaki et al. 2008
AFAT11B03	AB564930	(AC)3agat(AC)9	taatgctcgatggctgcaaa	tttgagatggtgatttaataaaatatgtgac	Chr.6a	281.2	220	216	Tsukazaki et al. 2011
AFAT13C12	AB795081	(AG)3taagagtattatctgtatgttaactca(TA)6tgcata(TG)7ggtgcttgctcta(TG)3tagtgttgtaaacaaact(TG)5	ggggtatcaaacccttcattcaaa	tgatttcccatacacactatgcactg	Chr.3a	0.0	283	285	this study
AFB01B03	AB499467	(AG)11	gattgcattcgatttcgttgagtg	cagcacacttcgtttggatttcag	Chr.1a	185.5	194	220	Tsukazaki et al. 2007
AFB01E09	AB795082	(TC)12	gcgggtaggtgaacgacaagtaag	ttctgtgaaggtctcaccacttcg	Chr.2a	177.0	275	295	Tsukazaki et al. 2015
AFB02D05	AB564937	(CA)3(AG)3aaaac(GA)14	tatgtttgaatcacgctacgcaagt	tgacttttatgtatatcgttgggttagca	Chr.7a	45.9	251	253	Tsukazaki et al. 2011
AFB03B05	AB564941	(TC)10cc(TC)11ggtata(ATTT)2ataatgttt(AAC)3	gctgatatggatgaaagcaagagg	ctgcttcgagatgattgcaaaaag	Chr.7a	0.0	269	275	Tsukazaki et al. 2011
AFB03E02	AB795083	(AG)4at(AG)5gg(AG)5aaagagaa(AG)6	ggctcaagaaagttttcacaggga	tcaacttgtcgtgctttcgtttgt	Chr.3a	276.1	278	276	this study
AFB04A09	AB499475	(GA)3aagagaa(AG)13	actagagtcattgcgagacggagg	tattttgttgagcggaagtcagca	Chr.3a	368.7	252	268	Tsukazaki et al. 2007
AFB04B12	AB499477	(AC)6(TC)15	ctagacttgaggggaggatgggtt	ggagagggcaacatgcatttttag	Chr.3b	101.4	235	233	Tsukazaki et al. 2007
AFB04F07	AB795395	(TC)10	agcaatatgaaggagacacatgg	cccaaactaatttagggttacaattc	Chr.2a	164.1	152	154	Tsukazaki et al. 2007
AFB04H04	AB499479	(TC)3ctcc(CT)3t(TC)7	cgcaacacaaaagataacgtctcg	agacttcgacgtagagtccgatgg	Chr.7a	93.9	293	289	Tsukazaki et al. 2008
AFB05D11	AB499480	(TC)3cttatcccttctcaacgctgctt(TC)3cttcttacatgacgtcgcc(TC)13ttctcacatgtatgctttg(CT)3	actttgataacccacccagtcca	ggttccagcgatgaccaattttt	Chr.6a	200.7	294	290	Tsukazaki et al. 2008
AFB05H09	AB499483	(AG)17(CG)5	gcgagcgtatcccctaagaagaat	ttggtccctagatttcactgcaca	Chr.3a	385.4	270	268	Tsukazaki et al. 2007
AFB06C08	AB499484	(AG)3gg(AG)18	cgcacaaaacatatagggagaggg	tctccgtaagaagacctgcactca	Chr.1a	151.7	170	144	Tsukazaki et al. 2007
AFB06E05	AB499485	(TC)13	tttgaattggcagagacaatggtg	tgtatggaggaagggctgaaagag	Chr.1b	59.1	149	127	Tsukazaki et al. 2007
AFB07E06	AB564953	(GAG)3aaagag(GA)3	tgcattgaagaagaggaagaggaga	ggcaaatatcacgcttagaccagg	Chr.4a	0.0	267	247	Tsukazaki et al. 2011
AFB07F07	AB795084	(AG)8ggatgatgctctca(AG)5	atcgtgacgtggagattgagagg	ttcatctctgaatatgagcgcagc	Chr.4a	131.2	137	132	this study
AFB08D06	AB564956	(AG)18	gggtgtatgtgtacggctatgggt	ctccgtctcctacctcctccaaat	Chr.6a	245.7	242	246	Tsukazaki et al. 2011
AFB08G07	AB564958	(TC)7tt(TC)5	ctcttcctttgacttcaacccca	attcttcgggatgaaatggaggat	Chr.4a	81.4	230	232	Tsukazaki et al. 2011
AFB09D04	AB696900	(AG)8	tgagatggtgtggtttggtaggaa	aatcttgattgccaacgctgatct	Chr.2a	41.0	169	171	Tsukazaki et al. 2007
AFB09G09	AB564960	(CT)13tatatgcatc(TAT)3	ccaagacaaaagattcctatacaccc	aatcagacatcgaggatgcaaaca	Chr.6a	212.5	205	203	Tsukazaki et al. 2007
AFB09H04	AB564961	(CA)8taa(TC)12	atatcacaagtacgcactccctca	agttatattgggcggcagtgttgt	Chr.7a	58.5	198	208	Tsukazaki et al. 2011
AFB10D09	AB795396	(TC)9	ggttccttttggttctgtgtttgc	ccatacagatttccttaagagacga	Chr.5b	122.8	301	303	Tsukazaki et al. 2007
AFB11E02	AB564965	(AG)10	cattgagggaaaggtgcgaattac	tcatgcgtcatcccacttctgtat	Chr.6a	291.5	129	149	Tsukazaki et al. 2011
AFB11E05	AB564966	(TC)3caactca(TA)3tcgatccc(TC)7ggcactataccgaaccc(TC)3caagtca(TA)3ttgatccc(TC)7aacactacactgacc(TC)3	aaataccgacccctttctccttcct	ggacaggtttggagatgacgaagt	Chr.1a	92.3	301	297	Tsukazaki et al. 2011

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFB11H08	AB564968	(TC)8cc(TC)19	gttgatcaaacacccatgataacag	tgtctttaagtgattcgtcggcaa	Chr.5b	94.5	261	257	Tsukazaki et al. 2011
AFB12A08	AB564970	(AG)15	caacactctaattcgaacaaaaatgaga	catccgaaataaaattgatagtggaca	Chr.6b	0.0	154	174	Tsukazaki et al. 2011
AFB12H10	AB564974	(AC)5(GA)10gcacattcgt(TA)3	gatagggtaagagttgtcgccttaga	cgtgtgcaaagtgacttatatattacttaaact	Chr.4b	55.2	151	143	Tsukazaki et al. 2011
AFB13G10	AB499488	(AG)17	aaaaggtaaaggaggcgtgtaggc	atctgccgggattgatttcctact	Chr.2b	59.2	240	236	Tsukazaki et al. 2008
AFB13H10	AB795085	(TC)9	catgttcattcacactttcgttcg	aagaaggcaggagttggaaggagt	Chr.2a	24.3	200	194	Tsukazaki et al. 2015
AFB14D09	AB795086	(TC)8	ccggtcgtcattagttaagccttg	attctaactggagctgctgcgact	Chr.3a	319.2	281	283	this study
AFB15D03	AB564977	(AG)8	ggtcctgaactctctccaaatcca	ctccaattccctttcagtccattg	Chr.4a	74.0	226	222	Tsukazaki et al. 2011
AFB15G10	AB795089	(TC)3aaacgacaacttctttcctagccgtca(TC)7	aagatgtctgatcgccgttgtgta	aggatctccccaacacagtatgga	Chr.3a	256.0	197	199	Tsukazaki et al. 2015
AFB17B09	AB564980	(AG)8	ctagtggaagaagtcgcaggcac	caagtaagcctctgccatctcctc	Chr.1a	73.8	127	125	Tsukazaki et al. 2007
AFB17G01	AB795397	(TC)7	gattcaagtgcccacttttcaaca	gcagaagaagaatgaatgtgagcc	Chr.7b	114.9	255	251	Tsukazaki et al. 2007
AFB18A09	AB795090	(AG)8	gcttaagcactgcatatcatccgc	tccacctttcagatgtctccctc	Chr.3b	79.2	291	299	this study
AFB18H05	AB795091	(AG)6	gttaaattggagagttggcgttcg	atcttgcttgcctccctctattcc	Chr.2b	21.1	227	229	this study
AFB19A01	AB564982	(AG)6(G)5(AG)7gtgtatagttaactgta(AG)4	atgtgtgtgtgggtgtacgaggtt	cccttgttttaatttacccacccc	Chr.8a	37.4	257	261	Tsukazaki et al. 2011
AFB19D01	AB564983	(GA)6agagaacgaaagggatggagattatg(GA)3	ttcaaggtaagcgctgcatcaata	cttttcctttccaaatttccagcc	Chr.4a	55.9	260	262	Tsukazaki et al. 2011
AFB19H03	AB696901	(TC)3ttt(CTC)3c(TC)11	caaggatcatgaccatttggaagg	gccgtgacagatacaaggctcttt	Chr.2a	50.2	285	287	Tsukazaki et al. 2012
AFB20E09	AB795092	(TC)14	aacacataccggaggcaacctaga	atgtatgcttcggattgctgatga	Chr.2a	84.2	280	260	Tsukazaki et al. 2015
AFB20F12	AB564984	(AG)11(TG)3	agtatccacccatttggttgttgg	atgtttcaatacatccactccggc	Chr.7b	46.9	228	226	Tsukazaki et al. 2007
AFB20G03	AB696902	(AG)25(TG)5	tgcttcttgtcactggttaggtatgc	ccagaagtcagattcacaaacaga	Chr.2a	56.4	202	219	Tsukazaki et al. 2012
AFB21D05	AB564987	(AG)7	ttccattcaaatccatcaagca	ctcccaccacacgccatt	Chr.8a	46.2	183	187	Tsukazaki et al. 2011
AFB228	AB564988	(CT)17	cgaatctaaccccaaacgag	acgacgatggatggaagg	Chr.6a	194.0	206	208	Tsukazaki et al. 2011
AFB22B04	AB795093	(AG)6	tcttcgaggagagtcatggtaggg	aaattgtgccacgtcctcaaaagt	Chr.2b	34.0	269	271	Tsukazaki et al. 2015
AFB22C11	AB564990	(AG)7	tgaggtaaaggatggatgctgaca	tccaacattttctttctcctccca	Chr.4a	65.2	251	247	Tsukazaki et al. 2011
AFB22C12	AB795094	(TA)3caa(AT)3tatatgcaatc(AC)3agcatatcccttactatcttat(TC)14	cgcagaagctcaaaaagtgtgaaa	cctctccacaagttcatcggatct	Chr.6a	261.2	227	241	this study
AFB237	AB499495	(ca)3tgt(ga)16	aggcgtagcataagggtg	gtgtgcgtgagtgactgtg	Chr.7a	25.8	144	120	Tsukazaki et al. 2008
AFB23C03	AB795095	(TC)3ctgcatctatcc(TC)10	aagatggatagcggcttaaacaag	tcaagcaaatatcgcgtctgaaac	Chr.6a	116.0	131	143	this study
AFB23G02	AB499496	(AG)13aa(AC)3	aagcaaagatcatagcccgagatg	gttgcaaaggtaatcggtaacaaag	Chr.8a	42.5	219	225	Tsukazaki et al. 2008
AFB25D02	AB795096	(AG)15ggac(AG)4	ttcactactaaaaataaaataaaaatccgaataga	gccgcagctacaaacctatctc	Chr.2a	160.0	230	240	this study
AFB25G01	AB564995	(TC)20	tgagatgttgagtggagagtcgct	tgcaatgtcaagtccatgttcact	Chr.8a	72.5	219	209	Tsukazaki et al. 2007
AFB277	AB499498	(CT)15	aggtggatgggttatgtcc	tccttttcacactctttcttcc	Chr.4b	117.5	103	109	Tsukazaki et al. 2008
AFC01E09	AB499499	(TCT)7	ccctttgttcgacaagatttcca	caagacagcgatgacaaggaagaa	Chr.7a	53.7	140	143	Tsukazaki et al. 2007
AFC01F02	AB795097	(AG)3g(GAA)8	atggaaatgatccatgaggaggaa	ttggagggatcgcctcagttacta	Chr.3a	206.7	249	239	this study
AFC02H05	AB564998	(GAT)4ttggatttg(GAT)3(GAA)8	gtcctctatcaaggattgggagca	gctctttctcaatagcatcaaatgcc	Chr.5a	37.8	108	111	Tsukazaki et al. 2011

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFC02H08	AB564999	(TC)3(TTC)3c(TCT)3ttcatctgt(TCT)6ctc	cttcgtagtcacacctccttcggt	gtgaaattccgagagaatgggatg	Chr.1a	67.0	154	160	Tsukazaki et al. 2011
AFC03A04	AB795098	(GAA)7ga(AG)3	aataaaccaaacatgtccaagccg	tttcgctgcgattgtgagattcta	Chr.7b	88.5	145	141	this study
AFC03B02	AB795099	(TCT)6atttt(CTC)3	atcgtggtcaccatctcttccttc	gatgagggagtggaaatgaaaacg	Chr.3b	87.0	130	136	this study
AFC04D10	AB565002	(GA)3aggaaaagaggagataagggaagtaaaa(GAT)3(GAA)4at(AGA)4	gatgcaaacgatcctggaagaaag	gactcatctccctccgaactagca	Chr.3a	42.0	165	162	Tsukazaki et al. 2011
AFC08G05	AB499503	(GA)3a(GAA)11aa(AGA)3	gttaaaggcccattgggtatgaca	gaagttcgatgccttcatgtctca	Chr.2b	85.2	293	290	Tsukazaki et al. 2007
AFC09E09	AB795100	(TCT)4tcatcc(TCT)3	caaaagttccttttggttcgacg	gagaagcaaagcagtgtgcaaaaa	Chr.2a	165.8	246	243	this study
AFC10G12	AB795101	(GAA)7	acacgttgctagtgaagtgcaagc	ccataagactcgtacacccttggc	Chr.4a	50.9	190	200	this study
AFC12	AB499505	(TTC)8	tgcgattgtgagattttgt	ccagcgtttggatagttg	Chr.1a	137.8	239	236	Tsukazaki et al. 2007
AFD01C08	AB565006	(GTT)3(GCT)8	tgagactaggatagcttggagctt	tcctttaccttcgagatcgacaca	Chr.1b	41.9	251	257	Tsukazaki et al. 2011
AFHA00C17	AB795102	(AC)4(TC)4t(AC)5gtgca(TG)3	tattgaagtttgaatgcccgtcg	tgtatgaggcagcattctactgtgg	Chr.5b	0.0	190	195	this study
AFHT02B03	AB565010	(TG)3gatggatcgcttga(GT)9	ggatattgatggatcggtggtgat	aaccggtccctaccttagcacaat	Chr.8a	24.4	201	199	Tsukazaki et al. 2011
AFHT05F12	AB565012	(TG)3(TA)8	tcagtttgggaaggtctaggggt	gattggtaggagatggaaacacgc	Chr.6a	232.2	237	245	Tsukazaki et al. 2011
AFRA00E11	AB565014	(TA)8	cccatacacacacacacatctaggc	cctcatatatctgcacatgcgtcg	Chr.6a	341.2	268	282	Tsukazaki et al. 2011
AFRA04B10	AB499515	(TG)5cagttgta(TG)8cgcgtgcat(GCGT)2gtgcatgc(GT)3atgtgctac(TG)3ca(TG)4cagttgtgta(TG)5catagttgta(TG)7	gtgagtgtgcagttttgtgagggt	gtgcgcaggtccatctgtaaaaa	Chr.6b	10.1	296	298	Tsukazaki et al. 2007
AFRA04D06	AB795103	(TG)12tct(AC)3	atcagtgatcggacatagctgctg	caatcacctctccatttttaacgcc	Chr.5b	102.1	175	179	this study
AFRA04D09	AB499516	(TG)3tatgtgtatctaca(TG)3ca(TG)7tatatttatatgtatg(TA)3	ctagcggggaatttatcctggttc	cgatacgcccaacttactcgactt	Chr.4b	44.0	147	149	Tsukazaki et al. 2007
AFRA04F03	AB499517	(AC)4(GC)3(AC)3atacat(AC)7(AT)6	accgacttttcacatttgctccat	ccattacggaaggcaataaagaacc	Chr.1a	126.8	230	243	Tsukazaki et al. 2008
AFRA05F04	AB795104	(TG)15	ccccttacacatttccaattgctc	tcaaatcatggctgtcttcctcaa	Chr.7a	68.8	249	255	this study
AFRA05G10	AB565023	(AC)10(TC)5t(TA)4	ttaggcactaatgggaggttccct	caaccgaccatgagctgtgaaata	Chr.5b	82.9	207	209	Tsukazaki et al. 2011
AFRA07E10	AB499519	(TC)9	cagaactagacggtttgggaggaa	agctcatattttgaagctcgcagg	Chr.4a	99.5	170	172	Tsukazaki et al. 2007
AFRA11F11	AB499520	(CT)3atggtttttaagcatcatatacat(AC)5(AT)4(AC)4ata(TACA)2(TA)5t(AC)9	ccgccattagtgagagactacgtg	gggcaaacggttacatatagccaa	Chr.2a	154.5	213	217	Tsukazaki et al. 2008
AFRA12C02	AB565027	(TG)3cgtatgca(TG)8(CG)3tgcgtgcgc(GT)5	aatagagacccccctgcacttgtcc	tgcaaaaacaaaggaaagaggacc	Chr.1a	103.8	295	297	Tsukazaki et al. 2011
AFRA12C11	AB565028	(AC)11	catctttggtccagtgcataacga	cgattcctgtcactactaggtttggc	Chr.7a	79.5	256	254	Tsukazaki et al. 2011
AFRA12E05	AB499522	(TA)6(TG)15	aaaagtcaatgccaagcccatcta	gatttccttgtgatattcgacgtg	Chr.3b	69.8	303	305	Tsukazaki et al. 2008
AFRA12G10	AB795105	(AC)18	gcaggggtggctatttggataagt	gctcgctgactcttggtaggaatc	Chr.5b	112.4	247	245	this study
AFRA13A04	AB795106	(TG)3atgattggttgttatttaaag(TG)9tttatg(TA)3(TGT)3	aggctgttgacgatgcaaatgata	actcgtggctgtctatcagctcct	Chr.3a	247.3	178	176	this study

Table 9. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Reported in
					Linkage group	position (cM)	Ki	C	
AFRA13E10	AB795107	(GA)3agttctaagaaaattatgataa(AC)7aa(AC)7	agtcacggccataaagtgatggag	ggagttgttttggtttgcttttgg	Chr.3a	416.3	277	273	this study
AFRT01B06	AB795108	(TG)8	aaatatcagaattgagccacggga	aaaaacaaggcgggtcgacgg	Chr.1a	115.2			this study
AFRT03H01	AB795109	(CG)5c(AC)8	acccatattttgcagcactcctgt	agtaaggggttgtggatcctagc	Chr.4a	59.9	229	227	this study
AFRT07F04	AB565034	(CT)3atagatacattttat(AC)4atatacaat(AC)4atacaatatgcatgcat(AC)4atgcatat(AC)4atgcatat(AC)4gcatat(AC)5gcacat(AC)5gcacatacat(AC)8atatacat(AC)4atatacat(AC)3(AT)3(AC)3gcacatatacatat(AC)4tca(TA)3	cacacatatacaatacacacacatacaatatgc	ccgacaccaaaaatgaagaagtagc	Chr.8b	18.9	220	254	Tsukazaki et al. 2011
AFRT08B03	AB565035	(GT)3ataggtattgcgtgcatatagatac(AT)4(GT)8	taggggtctgcatactccacagt	aataacttcacacgcgcttgcata	Chr.8a	14.1	271	269	Tsukazaki et al. 2011
AFS006	AB499314	(AC)13(AT)6	gtgaccttatgtaggggttaggatt	tcgctccattcaaattaaaaa	Chr.3b	50.9	282	294	Tsukazaki et al. 2008
AFS008	AB499315	(AC)6at(AC)6	ggttatgaatactagcggggatag	atcagaacaacattctgctgacta	Chr.8a	62.6	254	256	Ohara et al. 2005
AFS017	AB499318	(TC)5catctctttac(TC)15	tgaacttttatttatgcttcttcctc	atggaggacgaaggtgggtt	Chr.8a	118.4	237	235	Ohara et al. 2005
AFS039	AB499319	(AT)8	cgggtaataacggatatcataaaca	cagttgttacatgtggtatcagagc	Chr.6a	347.8	286	290	Ohara et al. 2005
AFS058	AB499322	(AC)8(AT)6	tggactgaggtgaaaggatgg	acctttccattgcgtttgg	Chr.7b	106.0	199	203	Tsukazaki et al. 2008
AFS072	AB795110	(TG)8	ctttgttttggtggggtaaactcg	ccactcaacagaccaatcatcaca	Chr.8b	11.8	148	160	this study
AFS088	AB499323	(TG)10	tatcttcgagcacggttcttcttgt	atggcttcgatgatggatagttgta	Chr.8a	87.1	174	160	Song et al. 2004
AFS099	AB499325	(AC)13(AT)8cacacttata(AT)3	tgcccctcattaataacaacatgac	ttaatcgcattgacaaagtttattt	Chr.1b	81.2	235	245	Song et al. 2004
AFS103	AB696903	(TA)9(TG)9(TA)3tatatactt(AT)3	ttttacctagatattttcgaatttca	catctttctttttcactagcttcctg	Chr.1b	15.9	255	227	Ohara et al. 2005
AFS109	AB499329	(TG)3tt(TG)7	cctatgtctttacctatccaaccaaca	ccgaatttcaagtgtgtcaagtttt	Chr.5b	134.5	193	197	Song et al. 2004
AFS111	AB499330	(AT)3gtgtctcta(TG)8	tgtttaatggacttttcaatgcctgt	gcattaaaatgaagaaatcccgaag	Chr.2b	89.8	230	228	Song et al. 2004
AFS131	AB499333	(AC)8	caacaaatcagagagaaacagatga	actgtatatttatgtatcactccatgtaaa	Chr.5b	137.5	159	161	Ohara et al. 2005
AFS142	AB499335	(AC)3aa(AC)11(AT)5gt(TA)8	tgagagaattaatattattggaggcctat	ataaaatgacaaccaacccatgtta	Chr.1a	153.7	248	252	Ohara et al. 2005
AFS149	AB499337	(CA)12(TA)7tcacttgtac(AT)4	aaccaattgattacctctcatctgc	tgcggaccttccatagtctgtataa	Chr.4a	97.6	199	193	Tsukazaki et al. 2006

Table 10. Primer sequences of 13 bulb onion EST and 1 bunching onion-derived non-SSR markers located on KiC map.

Marker name	GenBank Accn.	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	KiC map		Fragment sizes (bp) in		Restruction enzyme	Reported in
				Linkage group	position (cM)	Ki	C		
ACFLS2_MseI	AY647262	ttaaggacgaccactggtt	ccacgacatccgtgact	Chr.4a	115.8	197+105+46	125+105+72+46	*Mse* I	this study
API27_MspI	AA451547	gacaagaccatcactaagctct	agtactcctcagtcctgcct	Chr.2b	81.7	420+98	518	*Msp* I	this study
API32_TaqI	AA451548	aaaaccaaatcgtttgtgcc	aaaccaaagcaggcaattaac	Chr.7b	126.5	376	312+64	*Taq* I	this study
CF450061_InDel	CF450061	tgaagtcagatgaaaattggaaaa	tgcgaggtttttccatttg	Chr.5a	39.4	219	220	InDel	Tsukazaki et al. 2011
CF441057_MseI	CF441057	atgcaaatttaaccatccagaact	tgatatggtggaagcacaactg	Chr.1a	297.8	200	150+50	*Mse* I	Tsukazaki et al. 2011
CF451262_InDel	CF451262	gattgcactgacagctcacttc	cactcctcttatcggtttgtgg	Chr.2a	30.4	150	153	InDel	Tsukazaki et al. 2011
TC0009_Hsp92II	TC3870/TC5647	taaaagagaccgatgccttcat	agaaccggcacgataacagata	Chr.1b	92.4	139+105+94+78+54+24+9	149+105+84+78+54+24+9	*Hsp* 92II	this study
TC0024_Hsp92II	TC3945	ccattaaatacctccattatcatttccc	caccaagtacacccacttatcatcca	Chr.8b	31.8	182+116+106+54	222+182+106+54	*Hsp* 92II	this study
TC0408_Hsp92II	TC6957	gatacatatgcgagggacaagg	ctgttaatggttggttcagcact	Chr.1a	210.5	90+70	160	*Hsp* 92II	this study
TC0411_Hsp92II	TC411	ctgggtttacggaagaaagcggaa	agcaccattgacttcctttccttg	Chr.5b	129.9	228+163+141+31	258+163+141+31+30	*Hsp* 92II	this study
TC1753_InDel	TC6173	tggcaaaggaatttttcttacaa	tttgagctttgagaatgcaaca	Chr.7b	31.9	104	102	InDel	Tsukazaki et al. 2011
TC2062_Tsp509I	TC2062	tcctccgtcttctccatctactacca	atttcacccatttcctccttcgtc	Chr.4b	122.9	435	250+185	*Tsp* 509I	this study
TC2678_NsiI	TC2671	caacgtggcatcaccaatg	ccttgcaatggtgaaagactg	Chr.3a	406.3	400+16	211+189+16	*Nsi* I	this study
AFGA2oxl_InDel	-	cacttccttatgtccctaacata	cctgaaggtgcaatgtatagtc	Chr.6a	71.0	600	1200	InDel	Shiraiwa et al., personal communication

Table 11. QTLs for bolting time in KiC $F_{2:3}$ population.

Trial	QTL	Condition	Linkage group	Closest marker of peak LOD score	LOD peak	Additive effect[a]	Dominant effect[a]	R^2 (%)[b]
2008	*qBlt2a*	Greenhouse	Chr. 2a	AFA01E09	9.2	-19.5	-2.0	30.5
2008	*qBlt6a*	Greenhouse	Chr. 6a	AFB23C03	4.3	-13.8	-9.4	22.1
2009	*qBlt1a*	Open field	Chr. 1a	AFB11E05	10.2	-13.7	-1.7	15.4
2009	*qBlt2a*	Open field	Chr. 2a	AFAT04B03	8.8	-14.1	-1.6	13.1
2010	*qBlt1a*	Open field	Chr. 1a	AFB11E05	10.3	-18.7	-5.7	14.7
2010	*qBlt1b*	Open field	Chr. 1b	AFA05F04	8.8	-16.3	-5.8	12.0
2010	*qBlt2a*	Open field	Chr. 2a	AFAT01H05	10.7	-20.2	-4.1	16.3
2010	*qBlt3a*	Open field	Chr. 3b	AFAT02B11	4.7	-11.3	0.2	6.1

[a] Additive or dominant effect of Ki allele.

[b] Percentage of variance explained at the peak of the QTL.

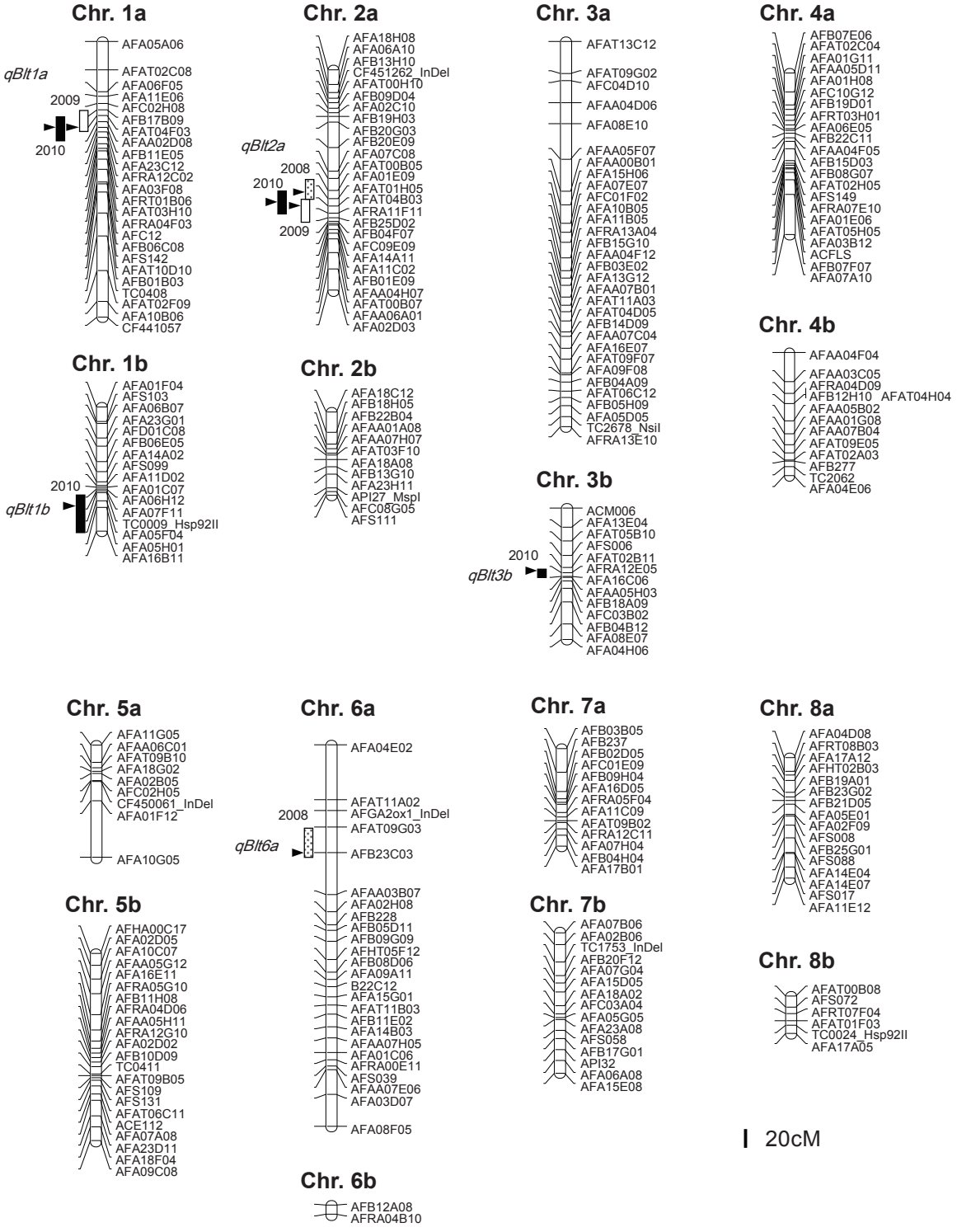

Fig. 8. Linkage map and QTLs detected in KiC population in different trials. QTLs are indicated by the boxes to the left sides of chromosomes and the positions of LOD peaks are shown by arrows.

The LOD peak for *qBlt1a* was located in close proximity to the marker AFB11E05 in the field trials in 2009 and 2010 (Fig. 8). The LOD peaks for *qBlt2a* were located close to the marker AFAT04B03 in the 2009 field trial and were located near the marker AFAT01H05 in the 2010 field trial. To examine the effects of the two QTLs, the F_2 progenies were classified according to their genotypes at each marker, the bolting time of $F_{2:3}$ lines were correlated with the nine observed genotypes (Table 12). The F_2 progeny homozygous for the linked AFB11E05 and AFAT01H05 alleles derived from the Ki parent showed significant differences in bolting time from the progeny carrying the homozygous genotypes derived from the C parent (Table 12).

QTL analysis of bolting time in the SaT03 population and comparative mapping of *qBlt2a*

A major QTL was detected in a consistent region of linkage group Chr. 2a in both field trials in 2013 and 2014 (Fig. 9 and Table 13). The maximum LOD scores for this individual QTL were 10.2 and 19.7 in the 2013 field trial and the 2014 field trial, respectively. The phenotypic variances explained by this QTL were 33.9 % in the 2013 field trial and 46.8 % in the 2014 field trial. When the $F_{2:3}$ lines were categorized according to the genotype of their preceding F_2 generation at the linked locus AFAT10E12, there were significant differences in bolting time between the genotype categories (Table 14).

Comparative mapping of Chr. 2a between the KiC and SaT03 populations was conducted using the same 10 markers. Two of the markers, AFA01E09 and AFAT04B03, which were closely linked to the LOD peaks for *qBlt2a* in the KiC population, were assigned to Chr. 2a in the SaT03 population. These markers were also linked to QTLs detected in the SaT03 population and were adjacent to AFAT10E12 (7.6 cM), which is close to the LOD peak for these QTLs. Therefore, these QTLs are considered identical.

Discussion

To obtain information for marker-assisted selection of the late-bolting trait in bunching onion, QTLs for bolting time were identified in two populations derived from crosses

Table 12. Bolting time in $F_{2:3}$ individuals categorized by the F_2 genotypes at AFB11E05 and AFAT01H05 in the KiC population in the field trials in 2009 and 2010.

Population	Marker genotype[a]		Number of $F_{2:3}$ lines	2009 field trial			2010 field trial		
	AFAT01H05 (*qBlt2a*)	AFB11E05 (*qBlt1a*)		Bolting time[b]	Significant difference[c] AA	BB	Bolting time[b]	Significant difference[c] AA	BB
F_2 (Ki x C)	A	A	5	74		*	77		*
	A	H	12	72		*	70		*
	A	B	2	99		*	96		
	H	A	20	72		*	59		*
	H	H	23	94	*	*	95		*
	H	B	11	113	*		119	*	
	B	A	7	95	*	*	98		*
	B	H	21	113	*		121	*	
	B	B	6	131	*		142	*	
Ki	A	A		0			0		
F_1 (Ki x C)	H	H		90			109		
C	B	B		159			170		

[a] A, genotypes homozygous for Ki allele (early-bolting); H, heterozygous; B, genotypes homozygous for C allele (late-bolting).

[b] Average number of days between bolting dates of Ki and $F_{2:3}$ lines.

[c] Asterisk indicates significant difference between each genotype and AA or BB detected by Tukey-Kramer HSD test ($P = 0.05$).

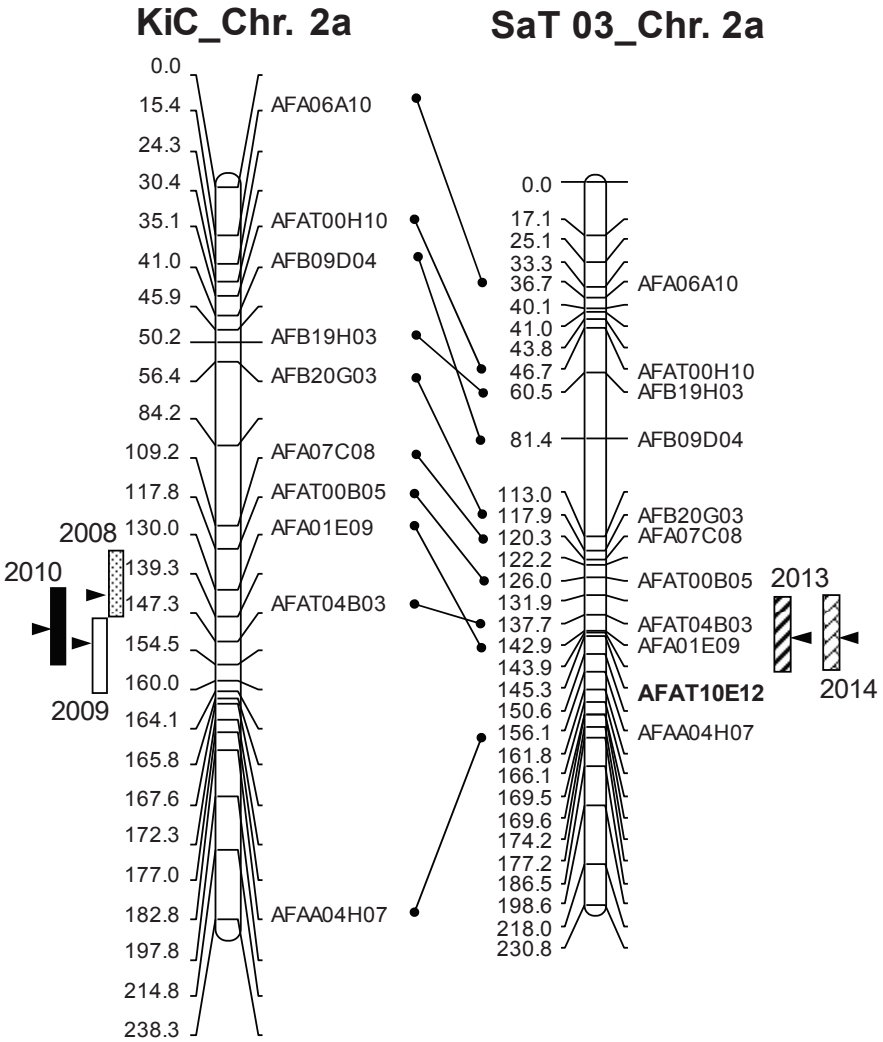

Fig. 9. Linkage map comparison between KiC and SaT03 populations at linkage group Chr. 2a. Common markers are connected with lines.

Table 13. QTL for bolting time in the SaT03 $F_{2:3}$ population in 2013 and 2014 field trials.

Trial	QTL	Linkage group	Marker closest to peak LOD score	LOD peak	Additive effect[a]	Dominant effect[a]	R^2 (%)[b]
2013	*qBlt2a*	Chr. 2a	AFAT10E12	10.2	-10.7	-6.9	33.9
2014	*qBlt2a*	Chr. 2a	AFAT10E12	19.7	-12.3	-10.1	46.8

[a] Additive or dominant effect of 'Sa03' allele.

[b] Percentage of variance explained at the peak of QTL.

Table 14. Bolting time in $F_{2:3}$ individuals categorized by the F_2 genotype at AFAT10E12 (Field trial in 2013).

Population	Genotype	Number of $F_{2:3}$ lines	Bolting time[a]
F_2 (Sa03 x T03)	T03 homozygous	10	56 a
	heterozygous	48	45 b
	Sa03 homozygous	20	40 c
T03	T03 homozygous		45
F_1 (Sa03 x T03)	heterozygous		12
Sa	Sa03 homozygous		0

[a] Average number of days between bolting dates of Sa03 and $F_{2:3}$ lines. Different letters indicate significant difference according to Tukey-Kramer HSD test ($P = 0.05$).

between parental lines differing in bolting time. Because bolting times were highly correlated between trial years in both the KiC and SaT03 populations (Fig. 6 and Fig. 7), bolting time appears to be highly heritable.

In the KiC population, a total of five QTLs could be identified on four chromosomes. Among these, two QTLs, *qBlt1a* and *qBlt2a*, were both detected in the field trials in 2009 and 2010 (Table 9). The closest markers to the LOD peaks for these QTLs were the same for *qBlt1a* (AFB11E05) or proximal for *qBlt2a* (AFAT04B03 in the 2009 field trial and AFAT01H05 in the 2010 field trial) (Fig. 8). In addition, when the F_2 genotypes were classified according to these linked markers, there were significant differences in bolting time between the homozygous Ki and C genotypes (Table 12). Therefore, these two QTLs will be effective for predicting bolting time under field conditions. *qBlt1a* was identified in the field trials in 2009 and 2010, but was not identified in the 2008 greenhouse trial. From these results, *qBlt1a* is likely involved in the low-temperature requirement for vernalization response because plants were grown at temperatures higher than 10 °C in the 2008 greenhouse trial.

In the SaT03 population, only a single QTL was detected during repeated field trials in 2013 and 2014 (Table 13). Comparative mapping of *qBlt2a* on both the KiC and SaT03 linkage maps revealed that *qBlt2a* would be identical in both populations. The parental line T03 that harbors the late-bolting trait requires lower temperatures to induce bolting than does Sa03 (data not shown). These results suggest that *qBlt2a* plays an important role in vernalization leading to bolting and flowering based in bunching onion.

In a previous study, a major QTL for pseudostem pungency was identified on Chr. 2a in the SaT03 population (Tsukazaki et al., 2012). The position of the pungency QTL accorded with *qBlt2a*, the bolting time QTL detected in the present study. Although it is quite unlikely that genes controlling pungency also function in flowering, the genes corresponding to these traits are closely linked in the parental line T03. The T03-type allele at AFAT04B03 closely linked to the QTL for both pungency and bolting time has not been found in Ki or C (data not shown) and has been observed in only a limited number of cultivars belonging to the bunching onion cultivar group ‘Kaga’ (Tsukazaki et al. 2012). These results suggest that the T03 allele of AFAT04B03 is derived from this

genetic resource; however, no effect of this allele on variation in the late bolting trait was found.

The varietal differences in bolting time of bunching onion have been related to the differences in their low temperature requirements for flower-bud formation and in the size of adult plants (Inden and Asahira, 1990). Yamasaki et al. (2000a) reported that the optimum temperature for vernalization in bunching onion was 7 °C; however, some cultivars could initiate flower buds at 15 °C (Yamasaki et al., 2000b). The very early-flowering cultivar 'Bei-cong' has a low requirement for cold treatment to induce flower initiation (Lin and Chang, 1980), and can thus flower without overwintering in Japan. In order to detect QTL for bolting unrelated to a cold requirement, the KiC population was evaluated under a minimum of 10 °C in the 2008 greenhouse trial. One QTL (*qBlt6a*) was detected only in the 2008 trial and might control a flower induction through a mechanism other than green plant vernalization. Although it is not clear whether Ki requires temperatures higher than 10 °C for flowering, this line might use an atypical pathway for induction of flowering similar to that of 'Bei-cong', which possesses a seed vernalization-like response (Yamasaki et al., 2012).

T03, the late-bolting line, has short plant stature as it is derived from the short-statured cultivar 'Fuyuwarabe' (Wako et al., 2010). Plant hormones of the gibberellin family stimulate shoot elongation and bolting (Davies, 2004). Shiraiwa et al. (2011) reported that the gene coding gibberellin 3-oxidase (*AfGA3ox1*) catalyzes the conversion of GA_9 to GA_4 and GA_{20} to GA_1 in bunching onion. *AfGA3ox1* was highly expressed at early and middle developmental stages in flower stalks, and was mapped to chromosome 7A of shallot (*A. cepa* Aggregatum group) (Shiraiwa et al., 2011). Although *AfGA3ox1* has not been mapped in bunching onion, this gene is not predicted to be associated with the QTL for bolting time identified in the present study.

In conclusion, this study found that a major QTL for bolting time was located on Chr. 2a in bunching onion. The T03-derived allele at AFAT10E12, an SSR locus linked to *qBlt2a* would be a useful marker for selecting bunching onions with late bolting traits.

Chapter V: Construction of an *Allium cepa* linkage map using doubled haploid technology

Introduction

A. cepa is subdivided into two groups: Common onion, which is referred to as bulb onion; and Aggregatum, which is known as shallot (Fritsch and Friesen, 2002; Hanelt, 1990). Shallot has a close genetic relationship to bulb onion, although it differs from bulb onion in morphological and ecological characters. Shallot is an important genetic resources for the improvement of bulb onion because it carries useful traits such as resistance to *Fusarium oxysporum* (Vu et al., 2012). Genetic studies in *A. cepa* have been limited due to its outcrossing nature, biennial generation time, and inbreeding depression.

Although several polymorphic isozyme loci in bulb onion have been found (Cryder et al., 1991), these were insufficient for construction of a linkage map. Recently, genetic mapping of *A. cepa* using DNA markers has progressed (Wilkie et al., 1993; Bradeen and Havey, 1995; van Heusden et al., 2000a; 2000b; Ipek et al., 2005; Martin et al., 2005; Baldwin et al., 2012; McCallum et al., 2012; Duangjit et al., 2013) and genetic information on various traits has been amassed. For example, QTLs controlling sucrose and fructan contents (McCallum et al., 2006) and genes encoding lachrymatory factor synthase (Masamura et al., 2012) have been reported. SSR markers are ideal DNA markers owing to their simplicity, reproducibility, and codominant inheritance (Jones et al., 1997). Fischer and Bachmann (2000) reported for the first time the development of 30 SSR markers from bulb onion. Araki et al. (2009) analyzed the genetic relationships among *A. cepa*, *A. fistulosum*, *A. vavilovii*, *A. galanthum*, *A. roylei*, and *A. altaicum* using these SSR markers. Tsukazaki et al. (2008; 2011) developed numerous SSR markers from bunching onion (*A. fistulosum*) and used them to construct linkage maps in bunching onion.

Doubled haploid (DH) techniques that use the chromosomal doubling of haploid plants can shorten the time needed, offer homozygous pure lines, and provide valuable materials for genomic analysis (Alan et al., 2003). Because dominant loci could be treated equally to co-dominant loci in segregating DH population, efficient mapping could be conducted

in wheat (Jia et al., 2005), Chinese cabbage (Ajisaka et al., 2001) and cabbage (Voorrips et al., 1997) etc. However, a linkage map construction using DH lines has not been reported in *Allium*. Campion and Allon (1990) first reported the haploid plant regeneration from female gametes by *in vitro* culture of unpollinated flowers in *A. cepa*. Although chromosome doubling was induced in small percent of regenerated plants (Jakše et al., 2003; Sulistyaningsih et al., 2006), the colchicine treatment was necessary in almost haploid plants to survive.

In the present study, gynogenic individuals derived from F_1 plants crossed between shallot and bulb onion DH lines were produced. Informative markers polymorphic between the parental DH lines were developed and a linkage map was constructed using this gynogenic population. In addition, several markers closely linked to useful traits were mapped.

Materials and methods

Plant materials and unpollinated flower culture

F_1 hybrids from a cross between the shallot DH line DHA as a seed parent and the bulb onion DH line DHC as a pollen parent were used for unpollinated flower culture. DHA was derived from the shallot strain 'Chiang Mai' from Thailand, and DHC was derived from the long-day onion cultivar 'Sapporo-ki' from Japan, as described in Abdelrahman et al. (2015). Unpollinated flower culture of F_1 hybrids was conducted according to the methods of Sulistyaningsih et al. (2006), with some modifications. Flower buds collected 3 days before flowering (6–7 mm in length) were sterilized in 70% ethanol and 0.01% mercury (II) chloride. Twenty-one florets were placed on a Petri dish (9 cm in diameter) containing B5 solid media (pH 5.8) with 4% (w/v) sucrose and 2 mg/l 2,4-dichlorophenoxyacetic acid. Plates were incubated in a growth chamber maintained at 25 °C with a day length of 16 h for 3 months. Shoots emerging from florets were transferred to MS solid media with 4% sucrose and cultured for two months. Thereafter, plantlets were acclimatized and grown in greenhouse. The number of somatic chromosomes in each plantlet was determined by the Feulgen staining and squash method after treating seedling root tips with 0.05 % colchicine at 4 °C for 3 h.

Marker analysis

Genomic DNA was isolated from leaf blades of regenerated plants according to the method described by van Heusden (2000a) and used for marker analysis. To identify female-derived plants, a primer set (see Table 16) was used to amplify a fragment of the GI (GIGANTEA; Gene Bank Accession No. GQ232756) gene that is polymorphic between DHA and DHC. PCR was performed as follows: pre-incubation at 94 °C for 3 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 94 °C for 1 min. PCR products were digested with *Taq*I for 3 h and were separated by electrophoresis through 2% (w/v) agarose gel. To detect polymorphisms between DHA and DHC, SSR markers derived from the *A. cepa* genome-(*A. cepa* gSSRs, Fischer and Bachmann, 2000), SSR markers derived from the *A. fistulosum* genome(*A. fistulosum* gSSRs), SSR markers derived from *A. cepa* ESTs (Kuhl et al., 2004; Martin et al., 2005; Jakše et al., 2005; Tsukazaki et al., 2008; 2011) and other mainly CAPS and SCAR markers (Kuhl et al., 2004; Masuzaki et al., 2006a; 2006b; 2006c; McCallum et al., 2006; Yaguchi et al., 2008) were screened for polymorphisms between parental lines. In addition, eight markers linked to the *Ms* locus (Park et al., 2013; Yang et al., 2013) were also screened.

PCR was performed in a 10-μL reaction mixture containing 20 ng template, 1 μL 10 × PCR buffer, 0.8 μL dNTPs (2.5 mM each), 0.8 μL forward primer (10 μM), 0.8 μL reverse primer (10 μM), and 0.05 μL Ex Taq Polymerase (5 units/μL; Takara Bio Inc.). PCR conditions appropriate for each primer set are described in Table 15, Table 16, and Table 17. PCR products were separated on 3% (w/v) agarose gel or 5% (w/v) denaturing polyacrylamide gel according to the methods of Song et al. (2004), Ohara et al. (2005a), and Tsukazaki et al. (2008). In addition, some forward primers were Xuorescent-labeled with 6-FAM, NED, PET, or VIC dyes (Applied Biosystems, CA, USA) prior to use for PCR. PCR products were loaded onto a capillary DNA sequencer (ABI3730; Applied Biosystems), and analyzed using GeneMapper ver. 3.0 software (Applied Biosystems).

Construction of linkage map

Table 15. Primer sequences and mapping information of 43 *Allium cepa* and 31 *A. fistulosum* SSR markers located on the linlage map.

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condition[a]	Annealing temperature (°C)	DH map		Chromosomal location assigned in previous studies[c]	Reported in
						Linkage group[b]	Position (cM)		
A. cepa EST-derived SSR (32)									
ACE031	CF445265	(CAA)7	ATGATTTTTCGAGACGCCAAAATGAG TTCAAAGAGCGCTACGGGCTATAGTG	i	50.0	Chr.8	0.0	8C	Tsukazaki et al., 2011
ACE052	TC387	(GAA)8	AGTCGTATGAATCCTTCTTCTCCAT TTCTCTTTGCTTTGATTCTTTGATG	i	50.0	Chr.1b	31.7	4C	Tsukazaki et al., 2011
ACE063_2	CF438796	(CAG)8	TTTAGAGTCCAAGAATGGGTAGACAAG AGTTCTCCCTTAAACAGTGCAAAAT	i	55.0	Chr.1a	24.6		This study
ACE068	CF449065	(GCA)9	AGCTACGAGCACGATCTAAGCCATT CTGCCTTCCTTAATCTTCCCAGGT	i	50.0	Chr.4	73.2	4C	Tsukazaki et al,. 2011
ACE069	TC6947	(GCC)3(GCA)10	GTTATTCAACCAACAAAGCACCCC ACTTAGTGCTGTAACTGGCCGGAG	i	55.0	Chr.2	89.0	2C	Martin et al., 2005 Tsukazaki et al., 2011
ACE072	TC6464	(GGA)6	ACCATCCGCTGGTAATGAGACAAT GTCCTGCATAGCTGATCCCAATTT	i	50.0	Chr.2	15.2	2C	Tsukazaki et al., 2011
ACE080	TC1121	(TA)22	AGGATTAAAATGGGAGAAAGAGAT TTGAGAATAAGAAGCCGCTTGA	i	50.0	Chr.1b	0.0	1F	Tsukazaki et al., 2008
ACE092	CF439016	(TA)4tgtacatacgta(TG)5(TA)7g(AC)4	AGATGGATTGTTCCAAAGCCCATAA CTGCACAACGTACCCAATTAAGCA	i	55.0	Chr.6a	15.5		This study
ACE127	TC1106	(TG)4tatg(TA)6tgtgg(TG)3(TA)6	TCGAGACGAGCACGCATGAAAA AGCTCCATTGTCCGTTCCATT	i	55.0	Chr.4	55.9	5F	Tsukazaki et al., 2008
ACE163	CF449247	(AGC)6	GGATGGAGGTTTCAGAGACAAG GCTGCAGTATTTCTTGCTGATG	i	55.0	Chr.6a	2.0		This study
ACE192	CF438037	(TG)8(GT)5	ACACATGCGTCTGTGTGTATGT TAGCTCTCTCGTATCTCCCCAG	i	55.0	Chr.6a	22.8		This study
ACE209	CF442000	(ATAC)7(AT)5	CGTACCAGTGGAAGATTTGTCA CAGGAAGCTGCAAATGATAGTG	i	55.0	Chr.4	70.4		This study
ACE241	CF446529	(CT)5cc(CT)4	ACTCTCTTCTTCGAGCTCCCTC CCGTTTTCCGACATTATTTTCT	i	55.0	Chr.5b	0.0	5F	Tsukazaki et al., 2011
ACE292	TC6067	(A)8c(A)8c(A)10ac(A)10	TGGAAGCATCTGATATTAGAAAAAGA TCGAAGAAGCTTCAGAGAGGAT	i	55.0	Chr.2	130.2		This study
ACE302	TC6971	(A)8g(A)10	CGAACTGCATAAAAGTTAGGGC CTCGAGAAGGCTAAGGCAAATA	i	55.0	Chr.7	158.0		This study

Table 15. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condition[a]	Annealing temperature (°C)	DH map		Chromosomal location assigned in previous studies[c]	Reported in
						Linkage group[b]	Position (cM)		
ACE469	CF441873	(T)10	TTGGCATATATGATCCTATGCAG CGAGAAATAAATCAAGCACAGC	i	55.0	Chr.4	19.7		This study
ACE489	CF444905	(A)10	AAAGATCGATCCCAAGTCTCTG TGGTAGCTCCATGCATAATCAA	i	55.0	Chr.3a	66.1		This study
ACE547	TC4431	(A)8g(A)10g(A)10(GAAA)4	CAAACTTGAATCCCTTCCAAAA GGTATCCAAGTTAGTGCCCAGA	i	55.0	Chr.4	19.7		This study
ACM009_A	CF436678	(AGC)6	GCAACGGTAGAAGAACCTGC AACCTCTTTTGGTGCCTCCT	i	50.0	Chr.1a	50.6	1C	Kuhl et al., 2004 Tsukazaki et al., 2011
ACM009_B	Id.	Id.	Id.	Id.	Id.	Chr.3a	0.0		
ACM024	CF446873	(GCA)10	CCCCATTTTCTTCATTTTCTCA TGCTGTTGCTGTTGTTGTTG	i	55.0	Chr.2	89.1	2F	Kuhl et al., 2004 Tsukazaki et al., 2008
ACM065	CF449328	(TG)24	TAAGCTCTGATGGAGGATGGTT GTCGGTCACATTGAAAACTGAA	i	50.0	Chr.5b	34.4	5C	Kuhl et al., 2004 Tsukazaki et al., 2011
ACM071	CF449595	(AG)10	TCTCATTTCAACTTTCTACCTATCC CTGACATTTGCTCGACTGGA	i	50.0	Chr.5a	15.1	5F	Kuhl et al., 2004 Tsukazaki et al., 2008
ACM132	CF447889	(ATAC)14ac(CATG)4	TGCACACCGTTTCCATTTTA ATGGGGCCTGGTAAGTTTTT	i	55.0	Chr.2	60.3	2F	Martin et al., 2005 Tsukazaki et al., 2011
ACM134	CF449417	(GA)8	CACACACCCACACACATCAA ACACACACAAGAGGGAAGGG	i	58.0	Chr.7	59.1	7C	Martin et al., 2005 Tsukazaki et al., 2011
ACM168_2	CF450904	(TG)6	GGCCATGAGACATACAACTCAA AACTGCAAGAAGAGAAATTGCC	i	50.0	Chr.3b	40.1	3F	Tsukazaki et al., 2011
ACM169_2	CF451226	(T)8cc(AT)4, (TG)4ta(TG)6	AACATTCTCTTCCCCCTTCTTC GAGGAGAGGAGTTACGTGTTGC	i	50.0	Chr.3b	32.0		This study
ACM170	CF437581	(TTC)6	TTCTGCAATGAAAACACATTGA ATCCAACTGAGTCGGCAATC	i	50.0	Chr.8	53.2		Jakše et al., 2005
ACM177	CF440609	(TCA)14	TGAGACTGGTGCAGGAACTG CAAGGAAATACATTTGTGGAAAA	i	50.0	Chr.2	148.0	2C	Martin et al., 2005 Tsukazaki et al., 2011
ACM225	CF444176	(A)12	GTGTTTTATACTTGCGCAATCC TCAAATCAAGCTCGAACAAAAA	i	50.0	Chr.4	81.5		This study
ACM266	CF451915	(TG)6	GGGTTCAACCTCTGAAGTCTTG TAAGTTCACATGAGCATCCCAC	i	50.0	Chr.2	11.9		This study

Table 15. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condition[a]	Annealing temperature (°C)	DH map		Chromosomal location assigned in previous studies[c]	Reported in
						Linkage group[b]	Position (cM)		
ACM304	DQ273270	(CA)7	GAATTTAGGCCCATTTCAAGG TGATTTGCCTAATGTTTTTACG	ii	64.0	Chr.5b	16.0	5C	McCallum et al., 2012
A. cepa gSSR (11)									
AMS02	-	CCACACCACACACA CCACCACACACCAC A	GCATTAACTATCTAAAACATTG CCATCAACTCATAACAGGT	ii	54.0	Chr.7	77.1		Fischer and Bachmann, 2000
AMS03-199	-	(GT)21	TAACCCTAGGATGAGTTGAG GGATTTCCTCTTGAGATGA	iii	56.0	Chr.7	53.7		Fischer and Bachmann, 2000
AMS03-254		Id.	Id.	Id.	Id.	Chr.4	0.0		
AMS03-293		Id.	Id.	Id.	Id.	Chr.2	30.7		
AMS03-490		Id.	Id.	Id.	Id.	Chr.5a	42.0		
AMS03-580		Id.	Id.	Id.	Id.	Chr.8	74.5		
AMS17	-	(CA)7tg(CA)21(TA)3	AGTGGACTCAAGGCAGATG ATCACCATTCACCGTTTACT	ii	58.0	Chr.1b	49.5		Fischer and Bachmann, 2000
AMS21	-	(CA)25	GGTTGTTTCCACTACACTTGAG CGTCCTTGGTATTCTTGTGC	ii	56.0	Chr.1a	63.1		Fischer and Bachmann, 2000
AMS23	-	(AT)5(GT)19	GCTGTTCACTGGTCTATCTGG ATTCGGTGCTGATTTTCG	iii	58.0	Chr.4	78.3		Fischer and Bachmann, 2000
AMS29	-	aag(AAAG)2ggata(GAA)3aagaaagagaagaaagaa(C AA)2(CA)2	CATCAGAAAATCGCATCAC TTGAAACTTGGAAGGTTGTC	ii	54.0	Chr.8	30.8		Fischer and Bachmann, 2000
AMS30	-	(CA)8cg(CA)22(TA)4	CACTAATGGGGTAAATAATGTTCTAC TTGCCTTGAAATCCAGAC	iii	57.4	Chr.2	143.8		Fischer and Bachmann, 2000

Table 15. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condition[a]	Annealing temperature (°C)	DH map		Chromosomal location assigned in previous studies[c]	Reported in
						Linkage group[b]	Position (cM)		
A. fistulosum gSSR (31)									
AFA01E09	AB499346	(TA)3tctatatgt(AC)13	CCCAACCTACTTGAGGGATTGCTA GTTCTGTGTATGCAGGCAATTTGG	i	50.0	Chr.2	107.1	2F	Tsukazaki et al., 2008
AFA01F12	AB499348	(AC)12(AT)3	TGAAGGGGGACAAAATAAGAAGCA TCTCCCCCACTTAAAAAGAATTTCG	i	50.0	Chr.2	0.0	5F	Tsukazaki et al., 2015
AFA01H12	-	(TA)6(TG)18	CAGAACAATCAATAGCCACGACCA TCAGGAGACCGCCTACCTTTTGTA	i	50.0	Chr.2	77.6	3F	Tsukazaki et al., 2008
AFA02C03	-	(TA)5(TG)17	TGCTTCACTGCATCCCATATTGTT TTCAGGTTTGGAGACCACCAAGTT	i	50.0	Chr.2	103.7		This study
AFA02E09	-	(AC)4at(AC)11(AT)6	TGCATACACACACACGCAGACATA TTCCCCCTTTCTAGTCATCATGGA	i	50.0	Chr.6b	0.0	6F	Tsukazaki et al., 2008
AFA03D03	-	(TA)3catgta(TG)8ca(TG)7ta(TG)3	TGTCCAAAGCAGTGTTAGCTGGAA CCAAGCACATGAAAGTGCACAAG	i	50.0	Chr.3a	18.8	3F	Tsukazaki et al., 2008
AFA03D07	AB499363	(TG)3(CA)18	TGTCCTCGAAAATGAGATGCTTCA TGAAATGTAACCCGACTGTCCAAA	i	50.0	Chr.7	11.6	7F	Tsukazaki et al., 2008
AFA03F08	AB499365	(TA)4(TG)7ta(TG)7	TTAGGTAAAGGGACGAAACGACCA TGCCTCCAGGACTGAACAATACAA	i	50.0	Chr.5b	9.7	1F	Tsukazaki et al., 2008
AFA07G06	-	(TG)5tc(TA)5(TG)14	TGAACCGTTCCCATCTACCTTCTG ATGAGATCGTGATACAACAAGGCG	i	50.0	Chr.6a	3.0	8F	Tsukazaki et al., 2008
AFA09C08	AB499397	(TA)4c(AT)4(GT)10a(TG)4c(GT)6ata(TG)9	GAAACCGATAATGACTTAATTGCTTGAAAA CCTTATCTTAATCTGCTTCCAGTCTCTCAA	i	50.0	Chr.2	71.1	5F	Tsukazaki et al., 2007
AFA10A08	AB499401	(TG)11	GTTTAGGGCGTAAAATCTAAACGCT GTGCTTTTGACTAACCTCGCATCC	i	50.0	Chr.6a	0.0	5F	Tsukazaki et al., 2008
AFA10B06	AB499402	(AC)15(TC)6	CCGCAAACTGGACTAACTGTACAAA GGCAGAATAGCCCAAGTGTTTTCA	i	50.0	Chr.6a	49.5	1F	Tsukazaki et al., 2007
AFA11G05	AB795053	(TA)5(TG)11	GGTCAGGAGCAACCCAAACATTAC TCCTTTACCACCATACCTGATTTTG	i	50.0	Chr.8	40.8	5F	Wako et al., 2016
AFA13G12	AB795055	(AC)12	GGATTTCCTTAGGCGATTTGATGAAGTCG TTACAACTCCATCGCTGTG	i	50.0	Chr.6b	2.0	3F	Tsukazaki et al., 2015

Table 15. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condition[a]	Annealing temperature (°C)	DH map		Chromosomal location assigned in previous studies[c]	Reported in
						Linkage group[b]	Position (cM)		
AFA16E07	AB564840	(GT)3(ATGT)2a(TG)6tatg(TATATG)2 (TG)4tc(TA)4tccg(TG)3caga(TG)4	TATGCGTAGACATGAGGGCACAAT CACAAACATGAGAAATACGTTGGCA	i	50.0	Chr.7	0.0	3F	Tsukazaki et al., 2007
AFA23G01	AB564861	(TA)8(TG)14cgc(AT)3gtagatcatatgtatg (TA)3tggatgtttatt(TG)4	CATCATTTCCATCACCTCCATGTC AAGGGCTAAGGTTTCTCTTCCTACA	i	55.0	Chr.7	97.7	1F	Tsukazaki et al., 2011
AFAA03F01	-	(AC)8	CGACTTTGTTTCTCGCTCTTGGTT AAATTGCACAAGGCTCTGCGAGAT	i	50.0	Chr.7	40.9	7F	Tsukazaki et al., 2007
AFAA06C01	AB564881	(AC)5aaaaacatacat(AC)11aa(AC)3(AT)3	TGGTACTCACTGATTTAATGGCATAAT CTTTAATGATGAATCTGTTGCATACG	i	50.0	Chr.5a	61.7	5F	Tsukazaki et al., 2011
AFAT00B05	AB499440	(AT)3ctatgctaaccttctctaaagtacaac (TA)6tttcgtgtatc(TG)12	TGTCCACTATTCACGGCTTACTCA GATGGGTCTGCCTCCTCCTTATTT	i	50.0	Chr.3b	0.0	2F	Tsukazaki et al., 2007
AFAT00B07	AB795073	(AC)7gc(AC)6(ATAC)4ct(AC)9a(TA)8	CCTATACTTGTCATCCAATAGTGAACC ATTAGTAATGGACGGGCCTAGGGA	i	50.0	Chr.3b	53.7	2F	Tsukazaki et al., 2015
AFAT04C08	-	(AG)14(TG)6tatc(TG)5tctgtg(TATG)2ta(TATG)2tag(TG)5	TGGTACCATAAACCTCGAACCAATG CATATTCAAGGGAATGCCACATGC	i	50.0	Chr.7	95.0	7F	Tsukazaki et al., 2008
AFAT05E04	-	(TA)3tg(TA)3tctctgtgtctgtg(TC)3(TG)8gggggtgtg (GT)3(TG)5	TGTTATTTGAGGTGAAGCAGCAAGA CCATTTTTGATGTGGAGCAAAACC	i	50.0	Chr.8	22.2	8F	Tsukazaki et al., 2008
AFB08D06_A	AB564956	(AG)18	GGGTGTATGTGTACGGCTATGGGT CTCCGTCTCCTACCTCCTCCAAAT	i	50.0	Chr.3a	42.4	6F	Tsukazaki et al., 2011
AFB08D06_B	Id.	Id.	Id.	Id.	Id.	Chr.1b	37.3		
AFB20E09	AB795092	(TC)14	AACACATACCGGAGGCAACCTAGA ATGTATGCTTCGGATTGCTGATGA	i	50.0	Chr.2	59.6	2F	Tsukazaki et al., 2015
AFB237	AB499495	(CA)3tgt(GA)16	AGGCGTAGCATAAGGGTG GTGTGCGTGAGTGACTGTG	i	50.0	Chr.2	131.7	7F	Tsukazaki et al., 2008
AFB23C03	AB795095	(TC)3ctgcatctatcc(TC)10	AAGATGGATAGCGGCTTAAACAAG TCAAGCAAATATCGCGTCTGAAAC	i	55.0	Chr.4	14.8	6F	Wako et al., 2016
AFC09D10	-	(GAA)8	GATGGGTGTCAGCGTTGTTAAGTG TCATTTCCCCGAGTCTTTCTCTTG	i	50.0	Chr.3a	11.1	4F	Tsukazaki et al., 2008

Table 15. (continued)

Marker name	GenBank Accn.	Core repeats	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condition[a]	Annealing temperature (°C)	DH map		Chromosomal location assigned in previous studies[c]	Reported in
						Linkage group[b]	Position (cM)		
AFRA03E09	-	(CG)4c(AC)9a(TA)5tctat gtgcg(TG)3	TTGCCAGGATGAGAAGAGGT TTGCCAGGATGAGAAGAGGT	i	50.0	Chr.4	36.3		This study
AFS015	-	(TA)4tgtatg(TA)6tg(TA) 5tgtg(TA)6tg(TA)3	ATCTCACTGTCCTTGTACCTGAAAG CATCTTGACTTTGTGATATTTGTGC	i	50.0	Chr.1b	24.7	1F	Ohara et al., 2005 Tsukazaki et al., 2008
AFS111	AB499330	(AT)3gtgtctcta(TG)8	TGTTTAATGGACTTTCAATGCCTGT GCATTAAAATGAAGAAATCCCGAAG	i	50.0	Chr.3b	24.0	2F	Song et al., 2004 Tsukazaki et al., 2008

[a] i, an initial denaturation step at 94 ºC for 2 min; 10 cycles at 94 ºC for 30 s; at annealing temperature + 10 ºC (reducing by 1 ºC per cycle) for 30 s and 72 ºC for 30 s ; 35 cycles at 94 ºC for 30 s; at annealing temperature for 30 s and 72 ºC for 30 s; and a final 4 min extension at 72 ºC. ii, an initial denaturation step at 94 ºC for 3 min; 40 cycles at 94 ºC for 1 min; at annealing temperature for 1 min and 72 ºC for 1 min; and a final 7 min extension at 72 ºC. iii, an initial denaturation step at 95 ºC for 11 min; 40 cycles at 94 ºC for 30 s; at annealing temperature for 30 s and 72 ºC for 30 s; and a final 10 min extension at 72 ºC.

[b] Linkage groups determined chromosomal identies using monosomic addition lines were indicated in boldface type.

[c] 'C' represents the chromosome number of *A. cepa* and 'F' indicates that of *A. fistulosum*.

Table 16. Primer sequences and mapping information of 28 *Allium cepa* EST-derived non-SSR markers located on the linlage map.

Marker name	Marker type	GenBank Accn.	Annotation	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condi-tion[a]	Annealing temperature (°C)	Restriction enzyme	Map localization		Chromosomal location assigned in previous studies[b]	Reported in
								Linkage group	position (cM)		
ACAAX07	CAPS	CF447127	*Vitis vinifera* Tubulin alpha-1 chain-like	TGAGTTGGTGATTTCAGCCACGGAGA GCATGTGCCCCGTGCTATATTTGTTG	i	55.0	*Taq* I	Chr.5b	23.9		Kuhl et al., 2004
ACABE16	CAPS	CF447747	*Ricinus communis* Serin/threonine protein phosphatase 2a requlatory subynit A	AAGATTCCGTACGCCTGTTAGCTGTTG CTTGACACACGGAAGAATGTGCTGTAT	i	55.0	*Afa* I	Chr.4	76.7		Kuhl et al., 2004
ACABX01	CAPS	CF448816	*Oryza sativa* OsNHX1	CATTCTCACAGTGTTCTTTTGCGG ATTGGATAAGAAAGAAAGAGGGAA	i	55.0	*Afa* I	Chr.1a	0.0	6C, 6F	Kuhl et al., 2004 Tsukazaki et al., 2011
ACAEJ67	CAPS	CF451546	*Ricinus communis* Ayxin response facter	GCAGGTATCAGCGTCAACTAATAAGGAA CATGACTGTCTGTGGACGACTTGCAC	i	55.0	*Afa* I	Chr.5a	0.0		Kuhl et al., 2004
ACE6067	CAPS	AJ006067	*Allium cepa* mRNA for invertase	TTGCCAGGATGAGAAGAGGT TTGCCAGGATGAGAAGAGGT	i	55.0	*Taq* I	Chr.3b	42.4		This study
AF212154	SCAR	AF212154	*Allium cepa* ATP-sulfurylase (ATPS)	CCAAAACCGCCTAATCCTCA GGCACCGACATATTTACAAAGG	ii	63.0	-	Chr.3a	70.2		This study
AF458090	CAPS	AF458090	*Allium cepa* high affinity sulfate transporter (ST)	TACATGGCTGGATGCCAAAC ATTCACAACTCCTGGTACCTTTC	ii	68.0	*Alu* I	Chr.7	8.1		This study
AY221244	SNP	AY221244	*Allium cepa* putative chalcone synthase A (CHS-A)	CGATACATGCACGTAAACGAAC ATGCGCTCGACATATTCCC	ii	68.0	-	Chr.2	97.8	2C	Masuzaki et al., 2006a, 2006b
AY221246	CAPS	AY221246	*Allium cepa* flavonone 3-hydroxylase (F3H)	AGAGAGGGGAAATATGTAGG GGCTCCTCTAATATCGGTT	ii	65.0	*Alu* I	Chr.3a	50.9	3C	Masuzaki et al., 2006a, 2006b
AY221250	SCAR	AY221250	*Allium cepa* dihydroflavonol 4-recuctase (DFR)	CAAAAGCCCGAATACGATG CGGTTCATTTGGATGATGG	ii	65.0	-	Chr.7	106.8	7C	Masuzaki et al., 2006a, 2006b
AY541032	CAPS	AY541032	*Allium cepa* cinammate 4-hydroxylase (CA4H)	TCGGCAACTGGCTCCAAGTC TGATTGACCAGTTCCGCTATGCC	ii	70.0	*Afa* I	Chr.1a	43.2		This study

Table 16. (continued)

Marker name	Marker type	GenBank Accn.	Annotation	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condi-tion[a]	Annealing temperature (°C)	Restriction enzyme	Map localization		Chromosomal location assigned in previous studies[b]	Reported in
								Linkage group	position (cM)		
AY585677	SCAR	AY585677	*Allium cepa* anthocyanidine synthase (ANS)	TTTGCTCGATCGTTTAGCRGAAGAAGA TGAGGATGATGACAAAGTTAGCGGAGC A	ii	68.0	-	Chr.4	23.5		Kim et al., 2005
AY700850	CAPS	AY700850	*Allium cepa* Chalcone isomerase (CHI)	TGCTTTGATTCAGTCATCC AATAATCGACTCCAATACGG	ii	67.0	*Fok* I	Chr.3a	58.8	3C	Masuzaki et al., 2006a, 2006b
CF434892	CAPS	AY434892	*Cinnamomum osmophloem* 4-coumarate-CoA ligase (4CL)	AGTAGCCATGAATCCCAACCTC TTCACGCCCTGTACAGATTCC	ii	67.0	*Taq* I	Chr.1a	9.0		This study
CF437610	SNP	CF437610	*Asparagus officinalis* acid inveltase (AIV)	GGTTCAAAAGACGCATCCAA TAATCCTGCCCATTATCAGAAGT	ii	60.0	-	Chr.2	124.3	2C	Yaguchi et al., 2008
CF443389	CAPS	CF443389	*Chrysanthemum* x *morifolium* geranylgeranyl pyrophosphate synthase (GGPS)	CCTAAACCTGTTCTAACACCTAC CAGTGGATTCAAGATCCACTAC	ii	60.0	*Hae* III	Chr.5a	67.6		This study
EU164758	CAPS	EU164758	*Allium cepa* sucrose-phosphate synthase (SPS)	GAAGGCTGATATTGTTGGTGAAG TGTGTCGTAGGAGCCTGATG	ii	64.4	*Afa* I	Chr.8	60.2	8C	Yaguchi et al., 2008
FT3	SCAR	-	*Allium cepa* flowering locus T-like protein 2 (FT3)	CAATGGTGATGCATGAGCC GGTGCATACACAGTTTGCC	ii	62.0	-	Chr.2	19.7		This study
GQ232751	CAPS	GQ232751	*Allium cepa* CONSTANS-like protein (COL)	GGAGAAGGTGACACGTGG GTCTGCTGTACGGGTTGG	ii	66.0	*Hae* III	Chr.4	8.2		This study
GQ232756	CAPS	GQ232756	*Allium cepa* GIGANTEA (GI)	GATCTTGCATCTAGACTC ACATAGTTTGCCTCAAG	ii	60.0	*Taq* I	Chr.7	57.2		This study
TC141	CAPS	TC4475	*Silene latifolia* endoglucanase	GCAACTGGAAATAACACTTACC GGTAGTGTTTGCCATATCCC	i	65.0	*Mbo* I	Chr.2	120.2		This study
TC1698	CAPS	TC5921	*Desulfitobacterinm hafniense* diguanylate cyclase	CAGATACTGCTCGGCTTACTAAATTCAC TCTATCTGAATTAGGAGGTGCATATCCC	i	57.0	*Taq* I	Chr.1a	68.0		Kuhl et al., 2004

Table 16. (continued)

Marker name	Marker type	GenBank Accn.	Annotation	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condi-tion[a]	Annealing temperature (°C)	Restriction enzyme	Map localization		Chromosomal location assigned in previous studies[b]	Reported in
								Linkage group	position (cM)		
TC1915	CAPS	BQ580175	*Tamarix hispida* cationic peroxidase	GGACAAATTTGCTGCTATGGG ACCCTGGGTACCAGTAAGG	i	57.0	*Afa* I	Chr.4	40.6		This study
TC5837	CAPS	CF436276	*Brachypodium distachyon* chlorode channel protein CLC-c-like	CGTGCTGCTCCTATGGACTAC GCTCAGGAGCCACTGAGAAC	i	65.0	*Hha* I	Chr.1b	41.9		This study
TC6857	CAPS	CF436951	*Triticum aestivum* DNA polynerase alpha catalytic subunit	CGTGAGATCACAGCAAAGCATC GATCATGACTTTCTACGGCAAAGG	i	58.0	*Taq* I	Chr.2	114.4		This study
TC7174	CAPS	CF435297	*Asparagus officinalis* early flowering protein 1 (EF1)	GTGGAGTTGGCAGCATCAGG CGGTAGGATTGGCAGCAAGG	i	70.0	*Fok* I	Chr.8	41.6		This study
TC7745	CAPS	CF445035	*Lycoris longituba* transcription factor putative late elongated hypocotyl (LHY)	TGGACTGATCAAGAGCAC CACGAGACAACAATCTTCC	i	62.0	*Xsp* I	Chr.3b	46.0		This study
Y07838	CAPS	Y07838	*Allium cepa* fructan 6G-fructosyltransferase (6G-FFT)	TCTTTTGCTCGGTTTGGTTC TTGCCCCAATATGCAAAATC	i	50.0	*Taq* I	Chr.6a	2.0	6C	McCallum et al., 2006

[a] i, an initial denaturation step at 94 ºC for 2 min; 35-40 cycles at 94 ºC for 0.5 min; at annealing temperature for 1 min and 72 ºC for 30 s; and a final 4 min extension at 72 ºC. ii, an initial denaturation step at 94 ºC for 3 min; 35-40 cycles at 94 ºC for 1 min; annealing for 1 min and 72 ºC for 1 min and a final 7 min extension at 72 ºC .

[b] Linkage groups determined chromosomal identies using monosomic addition lines were indicated in boldface type.

[c] 'C' represents the chromosome number of *A. cepa* and 'F' indicates that of *A. fistulosum*.

Table 17. Primer sequences and mapping information of five *Allium cepa Ms* linked markers located on the linlage map.

Marker name	Marker type	Forward primer sequence (5'-3') Reverse primer sequence (5'-3')	PCR condition[a]	Annealing temperature (°C)	Restriction enzyme	Map localization		Reported in
						Linkage group	position (cM)	
jnurf05	SCAR	AACAAATCAATCGCCTGAAAA ATTATGGCCGATTTCTCAGC	i	57.0	-	Chr.2	55.5	Park et al., 2013
jnurf17	CAPS	GAGCCCACTGCTATTGAGGA CCTGGAATGTATAGCAAGCCTAA	i	59.0	*Xho* I	Chr.2	60.7	Park et al., 2013
jnurf20	SCAR	GGTGAAGATGGCATGTGGGAGATCAAA TAGATGATGTTCTCAGGCGGCGGAGTT	ii	65.0	-	Chr.2	38.3	Park et al., 2013
DNF-566	SCAR	TACAGATTTGTTTATCTTCTTCTTCTTCT TTCATTTGTTAGGATGTACTCTTACC	iii	58.0	-	Chr.2	58.9	Yang et al., 2013
RNS-357	SCAR	TCAGTATCAATAGAAGGAATCAC GTATACCATTGGTACTTGATGCA	iii	59.0	-	Chr.2	58.9	Yang et al., 2013

[a] i, an initial denaturation step at 95 ºC for 5 min; 10 cycles at 95 ºC for 30 s; 67 ºC (0.8 ºC decrements in each cycle) for 30 s and 72 ºC for 30 s ; 30 cycles at 95 ºC for 30 s; at annealing temperature for 30 s and 72 ºC for 30 s; and a final 7 min extension at 72 ºC. ii, an initial denaturation step at 95 ºC for 5 min; 40 cycles at 94 ºC for 30 s; at annealing temperature for 1 min and 72 ºC for 1 min; and a final 10 min extension at 72 ºC. iii, an initial denaturation step at 95 ºC for 6 min; 35 cycles at 94 ºC for 30 s; at annealing temperature for 45 s and 72 ºC for 45 s; and a final 5 min extension at 72 ºC.

A total of 107 markers polymorphic between the parental lines and an additional phenotypic marker, a locus controlling bulb pigmentation, were used to construct a linkage map. Standard χ^2 analysis was used to test the segregation pattern at each marker locus for deviations from the expected Mendelian ratio of 1:1 in the population. Linkage analysis was performed using the computer program JoinMap ver. 4.0 (van Ooijen, 2006). The Kosambi function was used to obtain cM values (Kosambi, 1944). A log of the odds threshold of 3.0 was used to group and order the markers. The chromosomal locations of each linkage group were determined by comparison to previously developed maps (McCallum et al., 2012; Tsukazaki et al., 2008, 2011, 2015). Additionally, a complete set of bunching onion-shallot MAALs ($2n$ = 17; FF + 1A to +8A, where F stands for the basic chromosome set of *A. fistulosum*, and A represents chromosomes of *A. cepa*) developed by Shigyo et al. (1996) was used to determine the chromosome identity of the detected linkage groups. Shallot 'Chiang Mai' and *A. fistulosum* 'Kujo-hoso' and monosomic addition lines were used as DNA sources. PCR was conducted as described above and amplified products were separated on 2–3% (w/v) agarose gel.

Results

Production of gynogenic mapping population

Florets from F_1 plants flowered 5–7 days after initiating culture. Ovaries enlarged and shoots emerged from them after 2–3 months. A total of 291 shoots were obtained from 10,604 florets and 100 shoots regenerated into plants. Observation of somatic chromosomes indicated that these regenerated plants included 46 haploids ($2n$ = 8), 40 diploids ($2n$ = 16), three triploids ($2n$ = 24), three tetraploids ($2n$ = 32), and eight chimeric plants (Table 18). Their genotypes at the co-dominant marker locus GI were either shallot-type or bulb onion-type, indicating that all of the regenerated plants were derived from female gametes of F_1 plants (Fig. 10).

Linkage map construction

Of the 666 markers tested, polymorphisms between DHA and DHC were detected with 235 markers (35.3%) (Table 19). The efficiency of polymorphism detection was highest

Table 18. Chromosome numbers of plants from gynogenesis in unpollinated flower culture of F_1 hybrids.

No. of plants tested	Frequency distribution of plants that showed different chromosome numbers					
	$2n = 8$	8, 16	16	24	16, 32	32
100	46	7	40	3	1	3

Table 19. Numbers of polymorphic markers between DHA and DHC.

Origin	Marker type	No. of tested markers	No. of polymorphic markers	Percentage of polymorphic markers
Allium cepa genome[a]	SSR	32	9	28.1
Allium cepa EST	SSR	196	96	49.0
Allium cepa genome[b]	SSR	22	5	22.7
Allium cepa EST	Non SSR	102	32	31.4
Allium cepa genome[c]	SCAR	8	5	62.5
Allium fistulosum genome	SSR	306	88	28.8
Total		666	235	35.3

[a] Fischer and Bachmann (2000).

[b] BAC library

[c] Yang et al. (2012) and Park et al. (2012)

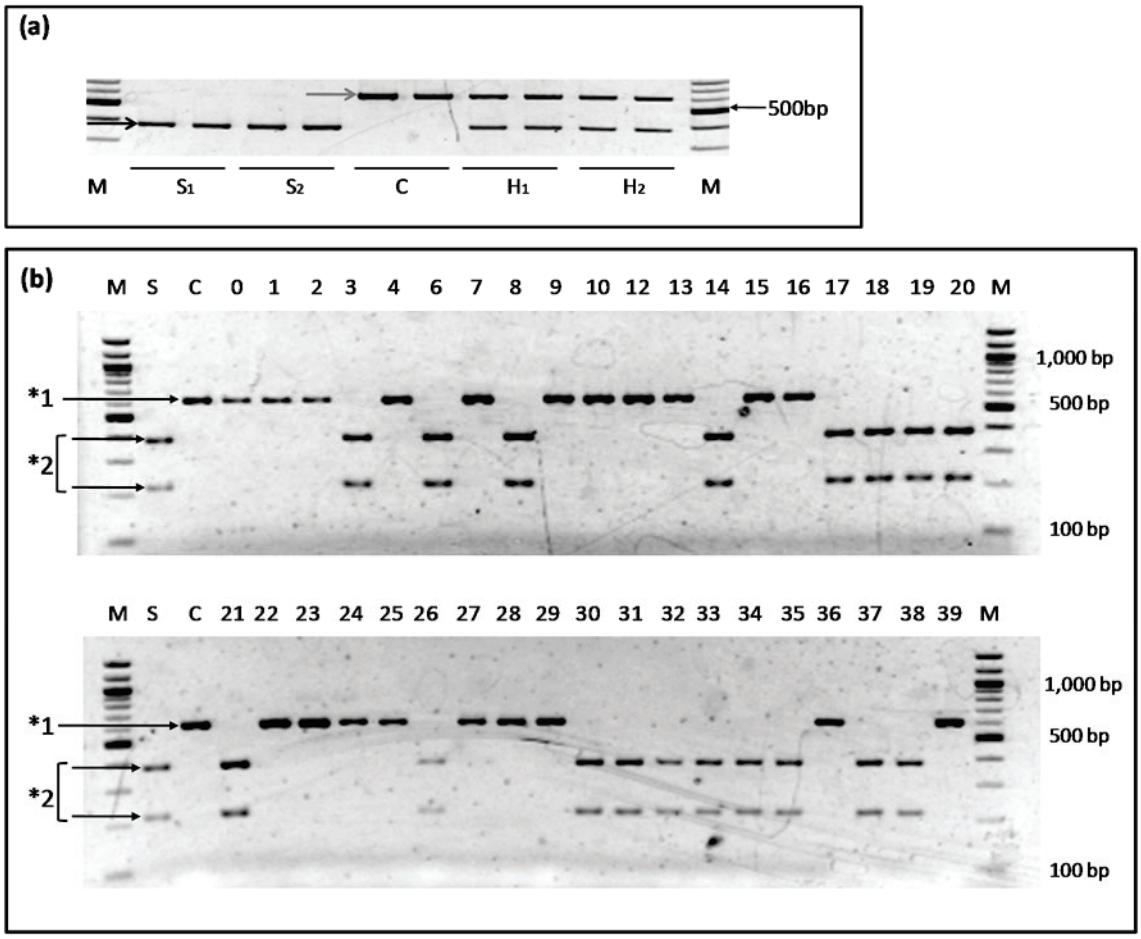

Fig. 10. DNA polymorphisms of CAPS (GI) markers between genotypes.

(a) M, 100bp ladder; S1 and S2, DHA; C, DHC; H_1 and H_2; F_1 crossed between DHA and DHC. **(b)** S, DHA; C: DHC; 0–39, regenerated plants from unpollinated flower culture of F_1 hybrids.

for *A. cepa* EST-derived SSRs (49.0%), followed by *A. cepa* EST-derived InDels or SNPs (31.4%). *A. fistulosum* gSSRs showed polymorphisms at the same frequency as *A. cepa* gSSRs. A linkage map was constructed with 108 markers (32 *A. cepa* EST-SSRs, 31 *A. fistulosum* gSSRs, 28 *A. cepa* EST non-SSRs, 11 *A. cepa* gSSRs, five *A. cepa* SCARs, and the phenotypic bulb color marker) (Fig. 11). The resulting map contains 12 linkage groups spanning 799 cM. Of 107 mapped markers, 25 deviated from the expected 1:1 segregation ratio ($P < 0.05$). Eighteen markers were previously assigned to *A. cepa* chromosomes (Masuzaki et al., 2006a, 2006b, 2006c; Yaguchi et al., 2008; Tsukazaki et al., 2011; Table 15 and Table 16). In the present study, the chromosomal locations of 10 markers were newly identified using MAALs (Fig. 12). From these results, all 12 linkage groups were assigned to eight chromosomes. The bulb skin color phenotypic marker was mapped to the terminal part of chromosome 7 and located in close proximity to the AY221250 marker that was derived from a gene coding dihydroflavonol 4-reductase (DFR) (Fig. 11). Twenty-eight *A. fistulosum* gSSR markers mapped on *A. fistulosum* genetic maps (Tsukazaki et al., 2008; 2011; 2015; Wako et al., 2016) were located on an *A. cepa* linkage map in the present study (Table 15). Of these, only 10 markers shared identical chromosomal locations in two species. Five SCAR markers, jnurf05, jnurf07, jnurf20 (Park et al., 2012), RNS-357, and DNF-566 (Yang et al., 2012), which were reported to be closely linked to the *Ms* nuclear male-fertility restoration locus of cytoplasmic male-sterility (CMS) were located on chromosome 2. Four markers except jnurf20 were mapped in close proximity within 5 cM of each other (Fig. 11).

Discussion

This is the first report of linkage map based on a gynogenic segregating population in *A. cepa*. Compared with previously reported bulb onion maps (e.g., the 1907 cM map reported by Martin et al. 2005), the map constructed in the present study covering 799 cM does not cover the entire genome, therefore, gaps in this map need to be filled in by identifying and mapping more markers. Nevertheless, this linkage map contains useful genetic information for agronomic traits because of the close linkage between the bulb pigmentation and the gene encoding DFR, and assignment of several markers relevant to

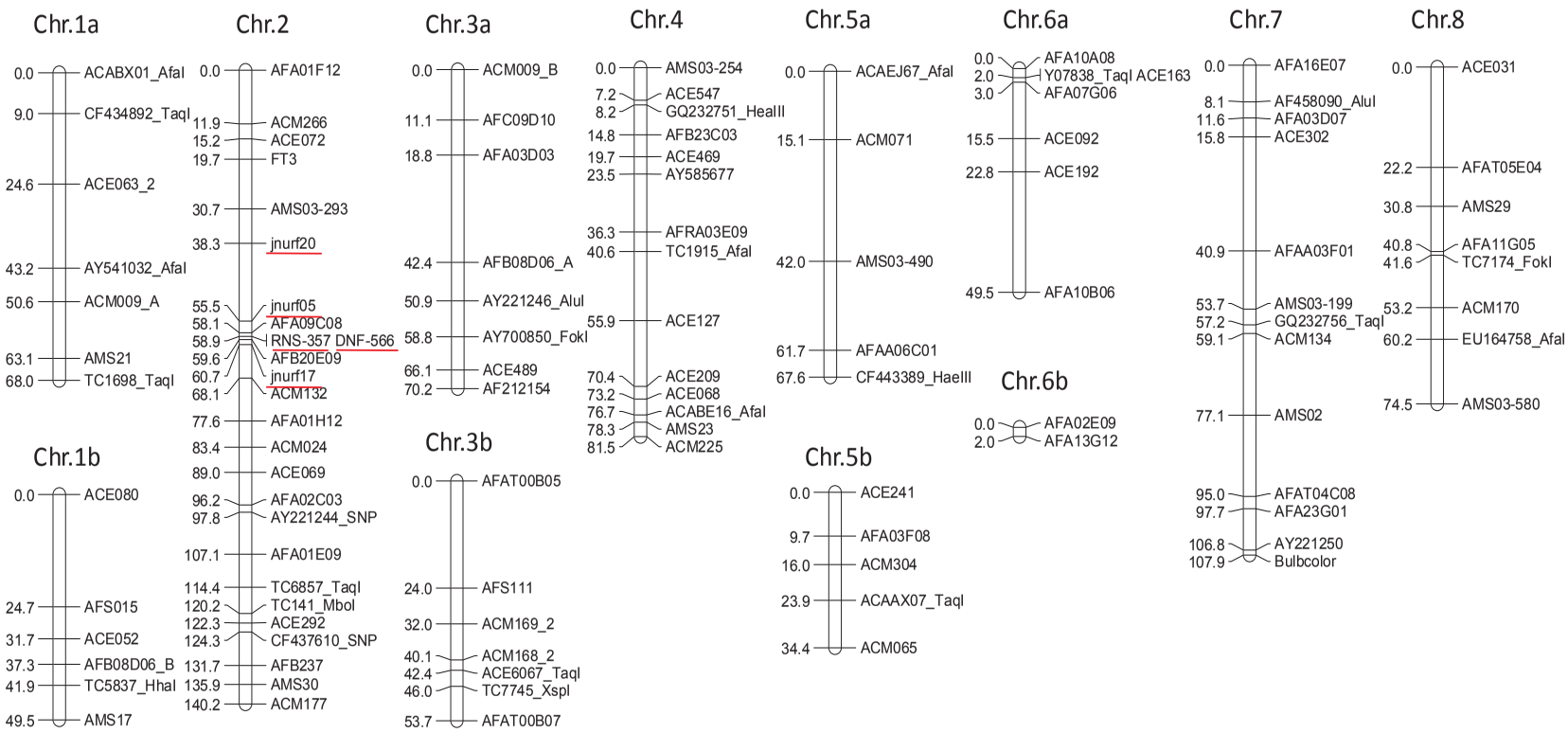

Fig. 11. Linkage map constructed using a gynogenic population derived from F_1 crossed between DHA and DHC. This map consists of 12 linkage groups with 108 markers covering 799 cM. All the linkage groups were assigned to eight chromosomes of *Allium cepa* (Chr. 1–8). Five flanking markers to *Ms* locus located on Chr. 2 developed by Yang et al. (2013) and Park et al (2013) are underlined.

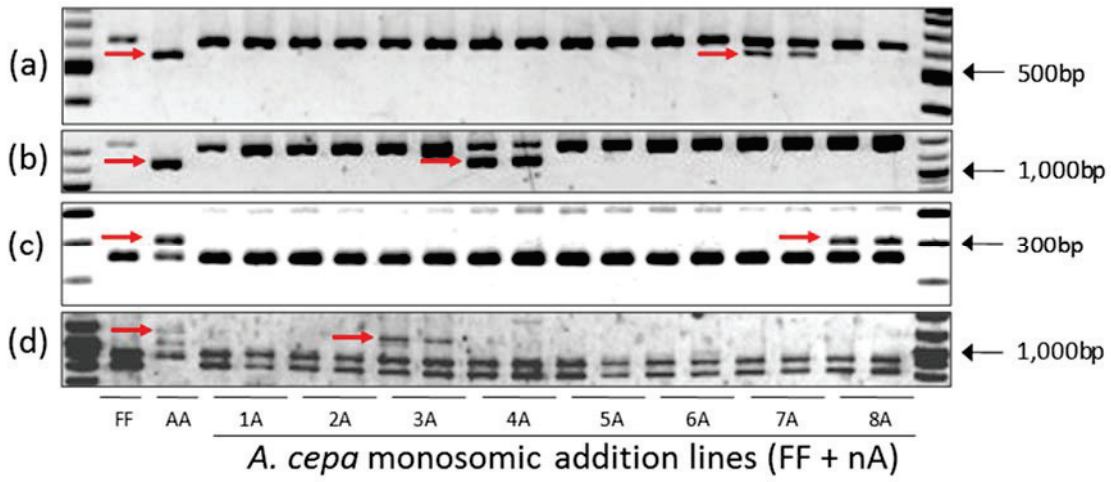

Fig. 12. Identification of relevant *Allium cepa* chromosomes for markers GQ232756 digested with *Fok* I (a), GQ232751 (b), TC7174 digested with *Fok* I (c) and TC7745 digested with *Taq* I (d) by using MAALs. Arrows indicate shallot-specific DNA fragments.

Ms on chromosome 2.

Shallot has a close genetic relationship with bulb onion and is an economically important crop in low-latitude regions (Shigyo and Kik, 2008). Using MAALs, loci affecting contents of a major metabolite that interacts with abiotic stress (Masuzaki et al. 2006c), amino acids, cysteine sulfoxide (Masamura et al. 2011), sucrose, and fructans (Yaguchi et al. 2008) in shallot have been revealed. Specific saponin compounds conferring resistance to *Fusarium* basal rot have been identified in shallot (Vu et al. 2012). These reports demonstrated the potential of shallots as a useful resource for the genetic study and future breeding of bulb onions.

Of the markers used in the present study, *A. cepa* EST-derived SSRs were most informative. SSRs comprised the largest category of polymorphic markers (96) identified here, with a high (49.0%) frequency of marker polymorphism between parental lines. Thirty-two markers were located on the linkage map and the chromosomal assignments of nine of 10 markers were consistent with previous studies (Table 15). Furthermore, seven markers were located on the *A. fistulosum* genetic map and six of these were assigned to the same chromosome (Table 15). Synteny comparisons of the genomes of these species could now be performed using linkage maps with common markers. Many *A. fistulosum*-derived gSSR markers have been useful for mapping in *A. cepa*. Thirty-one *A. fistulosum*-derived gSSR markers were assigned to this linkage map. The frequency of polymorphism in markers derived from *A. fistulosum* gSSRs (28.8%) was equal to those of *A. cepa* derived gSSRs (28.1%). About two-thirds of *A. fistulosum*-derived gSSR markers located on the *A. cepa* map did not show accordant chromosomal assignment. The chromosomal locations of 3 markers (AFA13G12, AFAT00B7, and AFB08D06_A) determined using MAALs differed from those of *A. fistulosum*. Nevertheless, these markers could be useful as common markers in both species, as fewer gSSR markers derived from *A. fistulosum* have been thus far assigned to *A. cepa* genetic maps (Tsukazaki et al., 2011). Additionally, SSR markers allowing high-throughput genotyping using a DNA sequencer can enable efficient and low-cost genetic analyses. Recently, Tsukazaki et al. (2015) isolated more than 2300 di- to penta-nucleotide SSRs from *de novo* assembly of transcripts from *A. fistulosum*. These EST-derived SSRs would be useful for

constructing a linkage map in *A. cepa*.

Molecular markers linked to the male-fertility restoration locus relevant to CMS-S have been developed in onions. Gökçe et al. (2002) identified two RFLP markers flanking the *Ms* locus at distances of 0.9 and 8.6 cM, and Bang et al. (2011) converted these two RFLP markers into PCR markers. Yang et al. (2013) developed SCAR markers (DNF-566 and RNS-357) tightly linked to both alleles at *Ms* locus. Park et al. (2013) developed six SCAR markers linked to the *Ms* locus and mapped these markers onto chromosome 2. In the present study, five of these eight markers were located on linkage group Chr. 2. Two markers, jnurf05 and jnurf17, were closely linked at a distance of 5.2 cM. Park et al. (2013) reported that no recombinant was found between jnurf05 and the *Ms* locus, and jnurf17 also tightly linked to these loci. The genotype of DHA shallot at the *Ms* locus would be *msms* (data not shown). These results suggest that higher resolution mapping of *Ms* locus is possible using a gynogenic segregating population such as the one produced in this study.

These chromosome-specific markers will be useful for intensive mapping of other markers on specific chromosomes to obtain markers closely linked to desirable QTL for agricultural traits, as well as for comparing genomic synteny between *A. cepa* and *A. fistulosum* using linkage maps.

Chapter VI: General discussion

Bunching onion is one of the most important vegetable crops in East Asian countries and represents the highest annual production value of all *Allium* crops in Japan. Since the latter 1980s, F_1 hybrid cultivars of bunching onion have been released quickly to the market. Growers desire cultivars with high yield, quality, and adaptability to a wide range of environmental conditions. However, published studies on the genetics of bunching onion have been very limited. Moue and Uehara (1985) have described the mode of inheritance of cytoplasmic male sterility (CMS) in bunching onion. Yamashita et al. (2010) have discovered male sterile plants from several bunching onion accessions and verified the inheritance mode of the male sterility *via* intraspecific crossings in order to identify CMS resources. CMS is an indispensable trait for F_1 hybrid seed production in bunching onion. Ohara et al. (2004; 2005b; 2009) examined the seedling growth of F_1 hybrids between various inbred lines or cultivars in bunching onion and demonstrated the mode of inheritance and heterosis in seedling growth by diallel analysis and QTL analysis using genetic maps. These results suggest that the breeding of cultivars with vigorous seedling growth is possible by exploiting heterosis, and the resulting cultivars might be suitable for plug nursery systems and machine-assisted transplanting. Tsukazaki et al. (2012) revealed that a major QTL for pungency was located on chromosome 2 in bunching onion. However, genetic studies of disease resistance and bolting time in bunching onion had been lacking despite the importance of these agronomic traits. Firstly, the present study showed the potential of *A. cepa* as a useful resource for breeding rust resistance bunching onion. *A. cepa* cultivars were highly resistant to rust fungus *P. allii* in controlled environment and field tests. The gene(s) for rust resistance located on chromosome 1A were found to act mainly during the seedling stage. Secondly, QTL analysis of rust resistance in the bunching onion parental line developed by recurrent selection was conducted. Three QTLs with minor effects were detected, although no major QTL was detected. Thirdly, bolting time was evaluated in two bunching onion populations under different environmental conditions in order to understand the genetic relationships between late bolting and vernalization. A major QTL was detected on the

same linkage group Chr. 2a in the two populations and their maps were compared using the same set of markers. Fourthly, a linkage map for *A. cepa* was constructed using doubled haploid technology to obtain information for marker-assisted selection in *Allium* crop species. This general discussion refers to the advanced study of breeding for disease resistance and late bolting, and offers suggestions regarding development of marker-assisted selection using genomic information for *Allium* vegetable breeding.

1) Genetic studies of disease resistance in bunching onion

In a previous study, 133 cultivars of bunching onion were evaluated for differences in susceptibility to rust under artificial inoculation in an experimental field; however, no completely resistant cultivar was found in bunching onion (Yamashita et al., 2005). In the present study, *A. cepa* cultivars including bulb onions and shallots showed high levels of resistance at different ages to *P. allii* isolates derived from *A. fistulosum*. These results indicate that *A. cepa* could serve as a useful resource for breeding rust resistance in bunching onions. Screening of a set of bunching onion–shallot monosomic addition lines revealed a high level of resistance only in FF+1A during the seedling stage, suggesting that the gene(s) controlling rust resistance could be located on chromosome 1A of shallot. At the adult plant stage, the degree of rust resistance exhibited by MAALs, multi-chromosome addition lines, and hypoallotriploids was not consistent under controlled-environment and field conditions, even though shallot showed distinct rust resistance. The effects of plant age and position of the inoculated leaf could be considerable, as discussed by Jennings et al. (1990b). Further, environmental conditions influencing infection could be variable, particularly during field tests. From the results of the present study, we can conclude that *A. cepa* possesses much greater rust resistance than does *A. fistulosum* and that multiple genes quantitatively control this trait. Further investigation for more accurate assignment of rust resistance genes to shallot chromosomes is necessary. Introgression of rust resistance of shallot into bunching onion lines could be achieved using MAALs. Recombination between homoeologous chromosomes during meiosis has not been induced by gamma-ray irradiation. However, a minimum of two heteromorphic bivalents has been observed in meiotic analysis of interspecific hybrids between bunching

onion and bulb onion (Peffley, 1986). Complete resistance to downy mildew (*Peronospora destructor*) in onion was found in the wild relative *A. roylei* Stearn, and was introgressed into *A. cepa* using interspecific hybridization and backcross breeding (Kofoet et al., 1990; van der Meer and de Vries, 1990). Although *A. roylei* was taxonomically assigned to the section Schoenoprasum, its nuclear DNA and cpDNA are closely related to *A. cepa* (Havey, 1992; van Raamsdonk et al., 2000; 2003). Successful hybridization between *A. roylei* and *A. cepa* resulted in interspecific hybrid plants and backcross progenies. The downy mildew resistance locus was located on the distal end of chromosome 3 *via* GISH (genomic *in situ* hybridization) (Scholten et al., 2007) and mapping study (van Heusden et al., 2000b). Although a large introgressed *A. roylei* fragment harbors a recessive lethal factor located proximal to the downy mildew resistant gene, a recombinant containing a crossover between the lethal factor and the resistance gene was identified, and a homozygous introgression line was successfully produced (Scholten et al., 2007). *A. roylei* could also serve as a bridging species between *A. cepa* and *A. fistulosum* (Khrustaleva and Kik, 1998; 2000). However, it took about 20 years to introduce the downy mildew resistant gene successfully into the cultivated onion (Scholten et al., 2007), so interspecies introgression of genes is a difficult task.

Among bunching onion cultivars, considerable variation has been observed in the degree of rust severity. This suggests that rust resistance still persists in bunching onion and is a quantitative trait. QTL analysis is effective for revealing the mode of inheritance of the traits and is useful for developing selection markers. In the current study, three QTLs (*qRst1a*, *qRst3a*, and *qRst8a*) were detected in different trials and were validated using a population derived from residual heterozygous lines. Inconsistent results between trials are partly due to differences in evaluation methods despite a relatively high correlation coefficient between two experiments (Fig. 3). Fukino et al. (2013) and Yoshioka et al. (2014) reported a number of QTLs for powdery mildew resistance and downy mildew resistance in cucumber, the identity of which depend on tests conducted under different in temperatures or inoculation methods. The rust resistance in bunching onion is considered to be adult-plant resistance (APR), such as that reported for stripe rust resistance in wheat (Chen, 2005), because few varietal differences in disease severity are

observed at the seedling stage in bunching onion (Table 1). APR is more likely conferred by minor genes that are typically race non-specific, inherited quantitatively, and have greater potential for durability (Chen, 2005). An APR gene usually contributes partial resistance and combinations of 4–5 APR genes can act additively to confer adequate levels of durable resistance (Singh et al., 2011). In wheat, APR genes for stripe rust at 13 loci have been cataloged and more than 160 QTLs that reduce stripe rust severity have been identified (Rosewarne et al., 2013). Combinations of several such QTLs (genes) are required to obtain sufficiently high levels of resistance (Singh et al., 2011). Many studies have shown that selection for resistance can be performed visually in disease nurseries, but clearly such selection is greatly aided by use of molecular markers. Bunching onion lines resistant to rust disease have been selected in the field by inoculation or spontaneous infection. However, disease severity depends on environmental factors and plant conditions. The use of DNA markers enables reliable selection of resistant plants even at the seedling stage, especially because selection for disease resistance controlled by multiple genes requires many plants in a large field, more time, and higher breeding costs than selection for resistance controlled by a single gene.

2) Genetic studies of late bolting in bunching onion

The bolting time of bunching onion cultivars depends on their low-temperature requirements (Inden and Asahira, 1990). In the present study, QTL for bolting time were identified using two populations derived from crosses between parental lines differing in bolting time. In one population KiC, a parental line with the early-bolting trait, was derived from the Taiwanese ever-flowering cultivar ‘Bei-cong’, which has minimal requirements for low-temperature to induce flower initiation. Two QTLs, *qBlt1a* and *qBlt2a* were consistently detected in the two-year field trials. Because markers linked to each QTL have been validated using populations, these two QTLs will be effective for predicting bolting time under field conditions. The late-bolting parental line for the population SaT03 was derived from the recently released cultivar ‘Fuyuwarabe’. ‘Fuyuwarabe’ has novel bunching onion characteristics such as low pungency, short thick leaves, and a pseudostem (Wako et al., 2010). The late-bolting trait is desirable for

extending the growing season of this novel type of bunching onion. A single QTL for late bolting was detected in the SaT03 population during repeated field trials. Comparative mapping showed that *qBlt2a* was identical on the KiC and SaT03 linkage maps. These results suggest that *qBlt2a* plays an important role in vernalization leading to bolting and flowering in bunching onion. In order to remove the effect of a low-temperature requirement, The KiC population was evaluated under a minimum of 10 °C in the heated greenhouse. *qBlt2a* was detected, although another QTL, *qBlt6a*, was observed only in this trial. This unique QTL could control a flower-induction mechanism different from vernalization.

Many genes controlling the induction and timing of flowering have been isolated and characterized in *Arabidopsis* (Crevillén and Dean, 2010; Dennis and Peacock, 2007). In *Arabidopsis*, vernalization results in the epigenetic silencing of the floral repressor *FLOWERING LOCUS C* (*FLC*) (Song et al., 2012). *FLC* encodes a MADS-box transcription factor and has been identified as a repressor of the floral transition. The silencing of *FLC* allows the photoperiodic induction of *FLOWERING LOCUS T* (*FT*), which encodes a mobile signaling protein involved in regulating flowering (Andrés and Coupland, 2012). Lee et al. (2013) reported functional characterization of the *FT*-like family in onion (*A. cepa*) and indicated that *FT-like* genes (*AcFT2*) were involved in the vernalization-responsive initiation of flowering, while other *FT* genes (*AcFT1* and *AcFT4*) were involved in the photoperiodic induction of bulb formation in onion. Baldwin et al. (2014) revealed significant population differentiation in *AcFT2* and *AcSOC1* in bulb onion. They also identified a QTL for bolting, designated as *AcBlt1* on chromosome 1, using the 'Nasik Red' × DH2150 population and discussed the association of these candidate genes with flowering (Baldwin et al., 2014). *AcFT2* was mapped to chromosome 5, and *AcFT1* was mapped to chromosome 1 using the W202A × 'Texas Grano' mapping population. However, upon comparative mapping between two populations, *AcFT1* was not closely linked with *AcBlt1* (Baldwin et al., 2014). Although *AcSOC1*, the homolog of *Suppressor of overexpression of constans 1* (*SOC1*) was revealed on chromosome 1 using a set of MAALs (Shigyo et al., 1996), this gene could not be mapped in either population.

Mapping studies in bunching onion have implied a high degree of genome synteny with bulb onion (Tsukazaki et al., 2008), but comparative mapping has not yet been conducted, because the number of markers common between bunching onion and bulb onion have been limited. Recently, more than 50,000 unigenes for bunching onion were obtained from transcriptome shotgun assembly of next-generation sequencing data, and numerous SSR, SNP, and InDel markers have been developed (Tsukazaki et al., 2015). These unigene collections should be searched for orthologous candidate genes involved in flower formation in the future. These informative studies will facilitate the understanding of genes related to the induction of flowering and comparative mapping of the genomes of bunching onion and bulb onion.

3) Toward application of marker-assisted selection to the breeding of *Allium* vegetables

The efficiency of marker-assisted selection (MAS) using molecular markers closely linked to genes controlling agronomic traits for decreasing breeding costs and time has been demonstrated (Tanksley, 1993; Young, 1999; Kumar, 1999; Ohsawa, 2003). The genetic characteristics of bunching onion and bulb onion had not previously been investigated using molecular approaches. Molecular genetic analyses will allow comparative genomics approaches between bunching onion and bulb onion to facilitate molecular breeding for both crops.

The *A. cepa* linkage map constructed in the present study consists of 12 linkage groups with 108 markers including 32 *A. cepa* EST-SSRs, 31 *A. fistulosum* gSSRs, 28 *A. cepa* non-SSR ESTs, 11 *A. cepa* gSSRs, five *A. cepa* SCARs, and a phenotypic marker for bulb pigmentation, covering 799 cM. This is the first linkage map based on a gynogenic segregating population in *A. cepa*. Although this map has smaller coverage compared with previously reported bulb onion maps (Martin et al., 2005; McCallum et al., 2012; Duangjit et al., 2013), the substantial marker resources accumulated in recent years will enable additional mapping. Many bulb onion-derived markers have been used for genetic linkage mapping in bunching onion (Tsukazaki et al., 2008). In the present study, seven SSR markers derived from *A. cepa* EST that had previously been assigned to *A. fistulosum*

genetic maps (Tsukazaki et al., 2008, 2011) were located on the *A. cepa* linkage map. Additionally, a considerable number of SSR markers derived from bunching onion were allocated to the *A. cepa* linkage map. These results permit comparison of genomes of these species using linkage maps. This map would be useful for molecular mapping and QTL analysis of other agronomic characters of bulb onion. Furthermore, comparative mapping between *A. cepa* and *A. fistulosum* using common markers would facilitate the analysis of the genetics of *Allium* crops.

The availability of genomic information, such as whole-genome sequences, expressed sequence tag sequences, and high-density genetic linkage maps, facilitates transition from traditional to molecular breeding. For example, the whole-genome sequencing and construction of draft genomes have been achieved in tomato, eggplant, and radish (The Tomato Genome Consortium, 2012; Hirakawa et al., 2014; Kitashiba et al., 2014). In bulb onion, Baldwin et al. (2012) performed skim sequencing of 6.6 Mbp genomic DNA using a next-generation sequencer. *De novo* assembly of transcriptome sequence data has been used for construction of high-resolution maps (Duangjit et al., 2013; Kim et al., 2015). Tsukazaki et al. (2015) and Sun et al. (2016) reported more than 50,000 unigenes obtained from transcriptome shotgun assembly, respectively. However, the genus *Allium* includes species with some of the largest nuclear genomes among cultivated plants (12–16 Gbp) with very low gene density of 1 per 168 kb (Jakše et al., 2008). Several molecular markers linked to characteristics have been developed in *A. cepa* (Gökçe and Havey, 2002; Gökçe et al., 2002; McCallum et al., 2006; 2007; Yaguchi et al., 2008; Baldwin et al., 2014) and *A. fistulosum* (Ohara et al., 2009; Tsukazaki et al., 2012). Molecular markers for rust resistance and bolting time QTLs in bunching onion were identified in the present study. The linkage map developed here will enable assembly of genomic sequence data into chromosome models. Such genomic information will accelerate the development of DNA markers linked to important gene functions relevant to agronomic and other traits. It is expected that these research outcomes will facilitate the breeding of *Allium* crops suitable for the needs of growers and consumers in the future.

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SUMMARY

In Japan, bunching onion (*Allium fistulosum* L.) is an indispensable vegetable crop for its economic value, familiarity in cuisines, and health benefits. Little genetic information is available about disease resistance and late bolting, which are essential traits in bunting onion cultivars. Rust caused by *Puccinia allii* Rudolphi is a serious foliar disease of bunching onion. To improve rust resistance of bunching onion, it is necessary to identify genetic resources with high resistance and understand the mode of inheritance of resistance genes. Late bolting is also one of the most important agronomic traits for year-round production in bunching onion because it affects the yield and quality of the harvested products during the spring and early summer. However, genetic and molecular studies of bolting time in bunching onion have not been reported thus far. The combined use of doubled haploid (DH) lines and molecular markers in *Allium* genetic studies can provide essential information for breeding programs.

The present studies were conducted to reveal such useful genetic resources and the genetic basis of rust resistance and late bolting in bunching onion. Further, informative molecular markers were developed and a linkage map was constructed using a DH population of *A. cepa* to improve selection methods for *Allium* vegetable breeding.

(1) Screening and incorporation of rust resistance from *Allium cepa* into bunching onion (*Allium fistulosum*) via alien chromosome addition

Bunching onion (*A. fistulosum* L.; 2*n* = 16; genomes FF), bulb onion (*A. cepa* L. Common onion group), and shallot (*A. cepa* L. Aggregatum group; genomes AA) cultivars were inoculated with rust fungus *Puccinia allii* isolated from bunching onion. Bulb onions and shallots are highly resistant to rust, suggesting that they would serve as useful resources for breeding rust resistant bunching onions. To identify the *A. cepa* chromosome(s) carrying rust resistance, a complete set of eight *A. fistulosum* – shallot monosomic alien addition lines (MAALs) were inoculated with *P. allii*. At the seedling stage, FF+1A showed a high level of resistance in controlled-environment experiments, suggesting that the genes related to rust resistance could be located on shallot chromosome 1A. However, no adult plants from the MAALs, multi-chromosome addition

lines, or hypoallotriploids exhibited strong resistance comparable to shallot. But the addition line FF+1A+5A showed reproducibly high levels of rust resistance.

(2) Mapping of quantitative trait loci for rust resistance in bunching onion

Disease severity was evaluated in an $F_{2:3}$ population derived from crosses between a resistant line Sa03 and a susceptible line T03, and quantitative trait loci (QTL) analysis was conducted using the bunching onion genetic linkage map. In two inoculation trials in the greenhouse, three QTLs related to rust resistance were detected on the linkage groups 1a on chromosome 1 (Chr. 1a), 3a on chromosome 3 (Chr. 3a), and 8a on chromosome 8 (Chr. 8a). Although these QTLs were detected in some trials but not others, they were validated using a population derived from residual heterozygous lines. Variances in rust resistance explained by those QTLs were relatively low, suggesting that other loci are involved in rust resistance in line Sa03.

(3) Mapping of quantitative trait loci for bolting time in bunching onion

The bolting times of two $F_{2:3}$ populations derived from crosses between cultivars differing in bolting time were evaluated and QTL analysis was conducted. When the KiC population, which was derived from a cross between the ever-flowering line Ki and the late-bolting line C, was grown under field conditions, two QTLs associated with bolting time were repeatedly detected on the linkage groups 1a on chromosome 1 (Chr. 1a) and 2a on chromosome 2 (Chr. 2a). However, the QTL on Chr. 1a was not detected when the KiC population was grown in a heated greenhouse under unvernalized conditions. A single QTL with major effect was identified exclusively on the linkage group Chr. 2a in the SaT03 population derived from a cross between early-bolting line Sa03 and late-bolting T03 evaluated under field conditions. QTL located on Chr. 2a in both populations were linked to the same marker loci, suggesting that these regions were strongly related. Simple sequence repeat loci linked to these QTLs had significant effects on bolting time in both populations.

(4) Construction of an *Allium cepa* linkage map using doubled haploid technology

A gynogenic mapping population for constructing the linkage map of *A. cepa* was produced from F_1 plants of a cross between DH shallot and bulb onion lines. This population of 100 plants included 46 haploids ($2n = 8$), 40 diploids ($2n = 16$), and 14

near-polyploids that were obtained from 10,604 unpollinated flower cultures. All of these were confirmed to have been derived from female gametes by analyzing co-dominant markers. Using this population, we constructed a map consisting of 12 linkage groups with 108 markers covering 799 cM. Chromosome assignments of 12 linkage groups were identified using MAALs. Bulb pigmentation, which was used as a phenotypic marker, was closely linked to the DFR gene of on chromosome 7. Five molecular markers were located in close proximity to *Ms*, the fertility restoration locus for cytoplasmic male sterility (CMS) on chromosome 2.

摘 要

ネギ（*Allium fistulosum* L.）は日本人の食によく親しまれ，健康増進に役立ち，わが国における生産額も多い不可欠な野菜である．しかし，品種育成を行う上で有益な病害抵抗性や晩抽性に関する遺伝学的知見の蓄積はなされておらず，これらの形質に着目した育種素材や選抜手法に関する研究開発に支障をきたしている．本研究では，ネギの重要病害であるさび病に抵抗性をもつ品種育成を進展させるため，高度抵抗性を有する遺伝資源の同定とそれらの抵抗性の遺伝様式の解明に取り組んだ．また，晩抽性は春～初夏の生産における収量と品質の安定化に関係する農業形質であり，ネギ抽苔メカニズムの分子遺伝学的解明の端緒として同形質に関与する遺伝領域の解明を試みた．一方で，ネギ属の遺伝学的研究において倍加半数体（DH）系統と分子マーカーを併用することは，育種計画に有益な知見を与える．そこで，ネギ属野菜の育種における効率的な選抜法開発に資するため，*Allium cepa* L.のDH集団を用いて分子マーカーおよび連鎖地図の構築を行った．

（1）*Allium cepa*のさび病抵抗性系統の検索と異種染色体添加による抵抗性のネギへの導入

ネギ（$2n = 16$，ゲノム構成FF）から分離したさび病菌 *Puccinia allii* をネギ，タマネギ（*A. cepa* Common onion group）およびシャロット（*A. cepa* Aggregatum group, AA）に接種したところ，タマネギとシャロットは強い抵抗性を示し，ネギのさび病抵抗性育種に有用な遺伝資源であることが示唆された．抵抗性に関与する *A. cepa* の染色体を明らかにするために，シャロットの8本の染色体を1本ずつ添加した8種類のネギ単一異種染色体添加系統（$2n = 2x+1 = 17$，FF+1A–FF+8A，以下添加系統）にさび病菌を接種した．環境制御装置を用いた幼苗期の接種試験では，FF+1Aが強度抵抗性を示し，シャロットの第1染色体に抵抗性に関与する遺伝子が存在する可能性が示唆された．一方で，単一染色体添加系統，複数染色体添加系統および低三倍体の成植物を用いた接種試験では，シャロットに匹敵する強い抵抗性は発揮されなかったが，FF+1A+5Aにおいて比較的強い抵抗性が再

現良く認められた.

（2）ネギのさび病抵抗性のQTL解析

さび病抵抗性系統‘Sa03’と罹病性系統‘T03’との交雑$F_{2:3}$集団におけるさび病発病程度を調査し，同集団を用いて作成されたネギ連鎖地図を用いて量的遺伝子座の特定（QTL解析）を行った．ビニールハウスを用いた2回の接種検定において，さび病抵抗性に関するQTLが第1，3，8染色体にそれぞれ対応する連鎖群Chr.1a，Chr.3a，Chr.8aに検出された．検定により異なるQTLが検出されたものの，それぞれのQTLに近傍のマーカーの遺伝子型によりそれらの抵抗性に及ぼす効果が立証された．これらのQTLにより説明される分散は比較的小さいことから，‘Sa03’には抵抗性に関与する他の遺伝子座も存在すると考えられた.

（3）ネギの抽苔性に関するQTL解析

抽苔期の異なるネギ系統間交雑による$F_{2:3}$集団について抽苔期を調査し，QTL解析を行った. 台湾から導入した不時抽苔性を有する系統‘Ki’と晩抽性系統‘C’との交雑による KiC集団について，露地圃場で越冬栽培後の抽苔期を調査したところ，2個のQTLが第1および第2染色体にそれぞれ対応する連鎖群Chr.1aおよびChr.2aに再現性よく検出された．春化を抑制させるために，同集団を最低気温10°C以上の加温温室内で栽培したところ，連鎖群Chr.1a上のQTLは検出されなかった. 一方で, 早期抽苔性系統の‘Sa03’と晩抽性系統‘T03’の交雑による SaT03集団について露地越冬栽培で調査した結果，連鎖群Chr.2a上に単一の主要QTLが認められた．両集団の連鎖群Chr.2a上のQTLは同じマーカー座上に位置することから，両者は密接に関連していると考えられた．これらのQTLに連鎖したSSRマーカーは，両集団において抽苔性に関して有意な効果を示した.

（4）倍加半数体技術を用いた *Allium cepa* 連鎖地図の構築

病害抵抗性の程度や抽苔期が異なるシャロットとタマネギのDH系統を交雑したF_1植物から雌性発生分離集団を育成し，これらを用いて *A. cepa* の連鎖地図を作成した．10,604個の未受粉小花を培養して再生した100個体の植物体［うち半数体（$2n=8$）46個体，正二倍体（$2n=16$）40個体，高次倍数体14個体］を用い，12連鎖群，108マーカーからなる全長799cMの連鎖地図が構築された．さら

に添加系統を用いて12連鎖群を8本の染色体に振り分けた．鱗茎部の着色性に関する形態マーカーは，第7染色体連鎖地図のDFR遺伝子と密接に連鎖していた．また，第2染色体連鎖地図上に座乗する細胞質雄性不稔性（CMS）の稔性回復を支配する *Ms* 遺伝子座の近傍に5個の分子マーカーを集中マッピングした．

本研究の結果，ネギのさび病抵抗性および晩抽性に関して有効な育種素材を明らかにするとともに，これらの形質に連鎖するDNAマーカーが開発されたことから，生産者や消費者のニーズに応じた品種育成において効率的な選抜に利用することが可能になる．また，ネギと共通のDNAマーカーをもつ *A. cepa* 連鎖地図が作成されたことにより，今後，両種の比較ゲノム研究を進展させ，ネギ属野菜における有用形質の遺伝系解明に向けた取り組みをより一層加速させる基盤を構築することができた．

List of papers related to the thesis

Screening and incorporation of rust resistance from *Allium cepa* into bunching onion (*Allium fistulosum*) via alien chromosome addition

Tadayuki Wako, Ken-ichiro Yamashita, Hikaru Tsukazaki, Takayoshi Ohara, Akio Kojima, Shigenori Yaguchi, Satoshi Shimazaki, Naoko Midorikawa, Takako Sakai, Naoki Yamauchi, and Masayoshi Shigyo

Genome 58(4): 135–142. 2015

(In relation to Chapter II)

Mapping of quantitative trait loci for bolting time in bunching onion (*Allium fistulosum* L.)

Tadayuki Wako, Hikaru Tsukazaki, Shigenori Yaguchi, Ken-ichiro Yamashita, Shin-ichi Ito, and Masayoshi Shigyo

Euphytica 209(2): 537–546. 2016

(In relation to Chapter IV)