Phytochemical Characterization of Major *Allium* Cultivated Vegetable Crops toward Improving Biotic and Abiotic Stress Adaptations

生物的・非生物的ストレス適応性の改良に向けた主要ネギ属野菜の植物化学的特性評価

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Chapter 1. GENERAL INTRODUCTION

Allium, recognized as the larger genus of monocotyledon plants, has more than 800 species (Friesen et al., 2006; Kamenetsky and Rabinowitch, 2006). They are distributed all over the world, from the frozen zone to the dry subtropics. The species' diversity in a certain area has been influenced by local climatic conditions, which has led to natural selection and, also, local consumer preference regarding taste and flavor. Some varieties are very famous as spices and, also, for their medical benefits, such as onion (*Allium cepa* Common onion group), shallot (*Allium cepa* Aggregatum group), Japanese bunching onion (*Allium fistulosum* L.), and garlic (*Allium sativum* L.). Moreover, Keusgen et al. (2002) mentioned that both *A. sativum* and *A. cepa* extracts have high antibiotic activity against some infections in the human body. Furthermore, Griffiths et al. (2002) reported the two main compounds in bulb onion are flavonoids and *S*-alk(en)yl cysteine sulfoxides (ACSOs), which have perceived benefits for human health, including anticarcinogenic properties, antiplatelet activity, antithrombotic activity, antiasthmatic and antibiotic effects. ACSO compounds themselves act as flavor precursors that will produce a unique flavor and taste when they react with alliinase (Schwimmer and Weston, 1961; Whitaker, 1976). The nutrients that are beneficial to human health have led to the increase in *Allium* consumption. However, from 2012 to 2014, production quantity (± 4 million tons) of bulb onions, Japanese bunching onions, and shallots stagnated, according to figures released by the Food and Agriculture Organization (FAO). Japan contributes 0.007% from the total worldwide production and has become the second largest production country in Asia. This

stagnation may be due to some biotic (pathogen, fungi, bacteria) or abiotic stress (such as heat, cold, and salinity) (Mittler, 2006).

The bulb onion and shallot are two species of the *Allium* genus that have been used as food spices and for their medical properties (Ross et al., 2007). In daily consumption, bulb onions and shallots can be eaten raw or processed with other ingredients to produce an appetizing food. Their specific taste and health qualities come from the interaction between organosulfur compounds and sulfur-containing enzymes that can scavenge oxidizing agents, avoid fatty acid oxidation and bacterial growth, and inhibit the formation of pro-inflammatory messengers (Wilson and Demmig-Adams, 2007). Moreover, shallots also produce higher amounts of phenolic compounds and higher scavenging activity as compared to spring onions (Gayathri et al., 2009). Nevertheless, due to some differences in the production area that lead to physiological and developmental differences between bulb onions and shallots, research on the phytochemical variations between them is very promising for breeding objectives. Various methods were used to determine the phytochemical properties of bulb onions and shallots, including HPLC, LC-MS/MS, and advanced metabolomics. In the near future, by using molecular biology and genetic engineering, either shallots or bulb onions will contribute to improving the others, due to their close relationship (Rabinowitch and Kamenetsky, 2002).

The world consumption of *Allium* species, especially bulb onions, shallots, and Japanese bunching onions, is rising year after year not only because they can enhance food flavor but also for their contribution to human health. For those reasons, it is necessary to improve their disease resistance, abiotic stress tolerance, and taste quality. However, due to their high diversity, including countless varieties and landraces,

selection based on their own characteristics—morphological and phytochemical—is needed. One species could generate more specific good traits than another and vice versa. That characterization process is very important for the breeding mechanism, where the breeder would easily select the most essential trait for a specific cultivation area and time. Furthermore, consumer preferences in one area and or country may differ from those of others as their environment and climatic conditions also differ.

Along with the emergence of global warming, the demand for new cultivars with abiotic stress tolerance is rising. One abiotic stress that contributes to yield losses is heat stress. In countries with four seasons, especially Japan, farmers must deal with high temperatures during summer cultivation. High temperatures can affect JBO production quantity and quality, as the optimum temperature for *A. fistulosum* production is around 15–20°C (Brewster, 2008). A high daytime temperature will cause heat stress and negatively impact plant growth and development (Liu and Huang, 2000; Wang et al., 2003). Heat-tolerant varieties were defined as varieties that could produce a high yield with higher economic quality and greater survival under heat conditions as compared to the standard cultivar (Hall, 1992). As a defense mechanism to heat stress and high UV radiation, the plant will produce an antioxidant compound, such as a phenols, ascorbic acid, or flavonoid (Gill and Tuteja, 2010). Furthermore, production of secondary metabolites, such as flavonoids and ascorbic acid, will increase the nutritional quality of JBOs.

Breeding programs that work with the objectives of improving of *Allium* disease resistance and high consumer quality are needed. In the breeding of cultivated *Allium*, the use of disease-resistant wild relatives is possible and promising (Kik, 2002). *A. fistulosum* is very susceptible to some important diseases, such as *Fusarium* wilt

(Disnayake et al., 2009) and downy mildew (Maude, 1990). Otherwise, there are more than 120 registered Japanese bunching onion (JBO) varieties with some improved tolerance to biotic and abiotic stress (Inden and Asahira, 1990). *Allium roylei* is one of the wild relatives from the genus *Allium* that exhibit downy mildew resistance. Moreover, *A. roylei* is indicated to be an important bridge species that could assist genetic resource introgression from other related species of section Cepa into the onion (Khrustaleva and Kik, 1998, 2000). Long before, McCollum (1982) reported the successful crossing between *A. roylei* and *A. fistulosum* for the first time. Later, Vu et al. (2012) reported the first time that hypo-allotriploid (CCR-nR, 2*n*=23) and allotriploid (CCR, 2*n*=24) of *A. cepa–A. roylei* showed high resistance to downy mildew, whereas some AMALs of *A. cepa–A. roylei* exhibited partial resistance to downy mildew infection.

In this study, bulb onion F_1 varieties and shallot landraces from Indonesia and Vietnam were used 1) to study the variation in chemical properties responsible for taste. Further analyses using the same specimen were done by HPLC and LC-MS methods to characterize 2) the amino acid properties and 3) the *S*-alk(en)yl cysteine sulfoxide biosynthesis differences between bulb onions and shallots. Several F_1 heat-tolerant varieties of *A. fistulosum* were examined 4) to study the effect of summer cultivation on the morphological and phytochemical contents of some JBO summer cultivars. *A. fistulosum* and *A. roylei* were exploited in order 5) to produce alien addition lines of *A. fistulosum* with extra chromosome(s) from *A. roylei*, 6) to characterize extra chromosome(s) from *A. roylei* in *A. fistulosum* addition lines, and 7) to understand the effect of alien chromosome addition(s) on several phenotypic expressions regarding chemical contents and antifungal activity in *A. fistulosum*.

This chapter is the first of a total six chapters in this dissertation. Chapter 2 deals with objectives 1. Chapter 3 deals with objective 2 and 3. Chapter 4 contains information about objectives 4. Chapter 5 refers to objectives 5, 6 and 7. Chapter 6 is a general discussion based on the results provided in chapters 2, 3, 4, and 5. This dissertation is a compilation of the results of studies conducted at the Laboratory of Vegetable Crop Science, Division of Agrobiology, Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Japan, from 2012 to 2017, with the objectives reported above (Ariyanti et al., 2015, 2017).

Chapter 2. COMPARATIVE STUDY ON PHYTOCHEMICAL VARIATIONS IN JAPANESE F_1 VARIETIES OF BULB ONIONS AND SOUTH-EAST ASIAN SHALLOT LANDRACES

Introduction

Bulb onions (*Allium cepa* Common onion group) and shallots (*Allium cepa* Aggregatum group) are two of more than 800 *Allium* species that have been used by humans for their unique characteristics of taste, odor, and health benefits. In the human diet, both bulb onions and shallots are consumed raw or processed as a spice in foods or sauces because of their particular taste and their ability to increase the taste of other foods (Fattorusso et al., 2002; Kopsell and Randle, 1997). Although those two species differ in appearance, color, and taste, they may have similar biochemical, phytochemical, and nutraceutical contents (Benkeblia, 2004). The shallot is commonly used as a condiment in South-east Asian countries, including Indonesia, Malaysia, Vietnam, and the Philippines, whereas the bulb onion is widely used fresh or processed in Europe, America, and East Asia, including China and Japan. In Japan, there are two types of bulb onion cultivars, short day and long day (Shigyo and Kik, 2008), according to their photoperiod requirements for bulb formation, as suggested by Brewster (2008). A Japanese seed company developed three different F_1 varieties—'Okhotsk 222' (early season), 'Kitamomiji 2000' (mid-season), and 'Super Kitamomiji' (late season)—as long-day types suitable for the Hokkaido area. By using these three types through half of the year, farmers can harvest bulb onions from early to late September. In the same way, seven different F_1 varieties were developed as short-day types for the southwestern part of Japan, and can be harvested continuously from late April to early June before the rainy season. These leading varieties can be cultivated predominantly

in the areas of main bulb onion production from north (Hokkaido) to south (Awaji and Saga) in Japan. Even so, no one knows the metabolomic profiles of the two complete sets for long- as well as short-day bulb onion varieties.

Consumer preferences for bulb onions and shallots depend on their eating quality. As mentioned by Kays and Yan (2000), the eating quality is generally considered to be the combination of odor, flavor, and taste. The amounts of some chemical compounds—including sulfur, soluble sugars, amino acids, and flavonoids—will determine the overall taste of the bulb onion or shallot. Sulfur compounds are responsible for the characteristics of flavors, odors, and taste in Allium species, including bulb onions and shallots (Randle and Lancaster, 2002). *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs), which produce volatile compounds, affect the flavor and pungency (Block, 1992). The quality of bulb onions and shallots, especially their sweet or bitter tastes, is affected by their soluble sugar and flavonoid contents (Tamaki et al., 2002). Southeast Asian consumers prefer a pungent taste and soft texture for raw consumption, such as in salads (Sulistyaningsih, personal communication), while sweetness and firmness are vital for quality when processed (Kimura et al., 2014).

Several studies have reported on the potentially important agronomic traits of shallots for plant-breeding purposes. Vu et al. (2012) discovered a novel gene of resistance to *Fusarium oxysporum*, the cause of Fusarium wilt in the Japanese bunching onion (*Allium fistulosum*), located on chromosome 2 of the shallot. Abdelrahman et al. (2015) estimated the responses of doubled haploid shallots (DHAs), doubled haploid onions (DHCs), and F_1 hybrids to some abiotic stresses by means of omics technology. Their results showed that several key genes and metabolites responsible for abiotic stress responses could be up-regulated in DHA and F_1 genotypes, as compared to those

of DHC. Moreover, it is important to gather definite and reliable information on phytochemical contents for a wider range of varieties and/or landraces in order to reinforce the potential usefulness of *A. cepa*.

In this research, we obtained several groups of data sets for different kinds of chemical compounds from bulb onions and shallots in order to study the variation in chemical properties responsible for the taste of F_1 varieties of Japanese bulb onions and Southeast Asian shallot landraces.

Materials and Methods

Plant materials

This study was conducted in two years, 2014 and 2015. Ten F_1 varieties of Japanese bulb onion (*A. cepa* Common onion group), including seven short-day and three long-day bulb onion varieties, were examined. The short-day bulb onions were cultivated in Kagawa Prefecture (34° N, 134° E), and the long-day bulb onions were grown in Hokkaido Prefecture (43° N, 142° E). To maintain the originality of the samples, we collected identical shallot landraces from their original growing areas over two years. Twelve shallot (*A. cepa* Aggregatum group) landraces, divided into nine landraces from Indonesia (Bantul Region 7.9° S, 110.4° E; Probolinggo 7.7° S, 113.2° E; Nganjuk 7.6° S, 111.9° E) (Fig. 1) and three landraces from Vietnam (Ly Son Island 15.3° N, 109.1° E; Quảng Ngãi Province 15.0° N, 108.7° E; Sóc Trăng Province 9.6° N, 105.9° E), were collected from farmers or local markets. The names and origins of

all samples, including cultivation periods, are indicated in Table 1, since a sufficient number of bulbs could be collected in some areas.

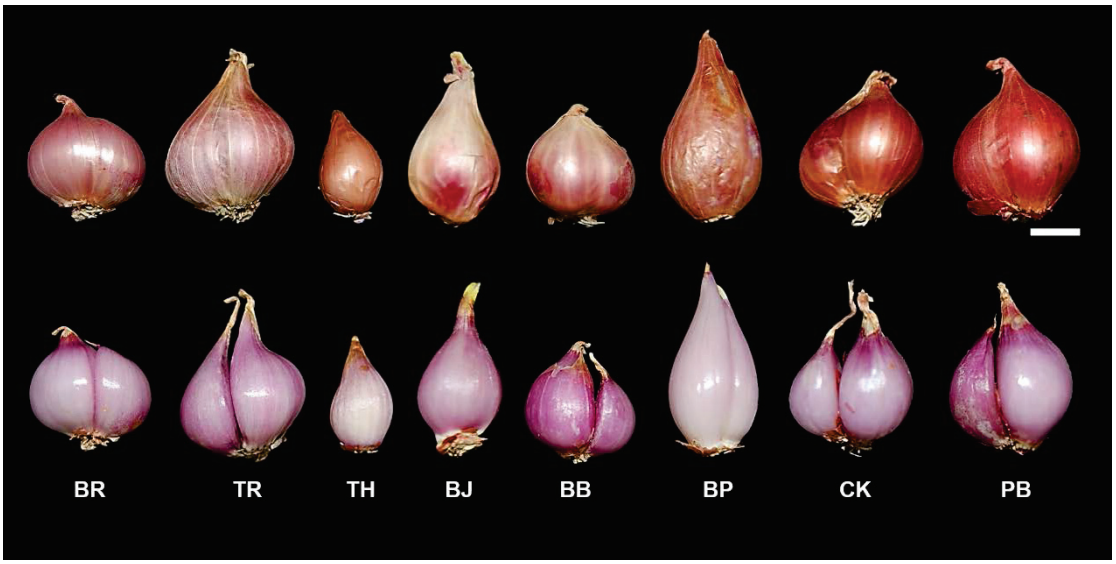


Fig. 1. Unpeeled (upper) and peeled (lower) bulbs of eight Indonesian shallot landraces collected in 2015. Bar indicates 1 cm.

Extraction and determination of *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs)

High-performance liquid chromatography (HPLC) was employed to determine the sulfur compounds of bulb onion varieties and shallot landraces. Three onion bulbs were usually used for each variety as biological replicates. Each onion bulb was cut into quarters from top to bottom. The first part was used for hot ethanol extraction, and the second part was used to determine the ACSO content using water extraction. For shallot samples, however, two to three bulbs of each of the usual three replicates were used for water extraction. The extraction method adopted was the same as that described by Vu et al. (2013).

To analyze *S*-2-propenyl (allyl)-L-cysteine sulfoxide (AlCSO) and *S*-1-propenyl-L-cysteine sulfoxide (PeCSO), a 10-time dilution of the sample was filtrated using a disposable membrane filter (DISMIC®-13 HP ADVANTEC; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). A 25-μL sample was injected into the HPLC system for quantification. Twenty $mg \cdot mL^{-1}$ of 1-propil CSO and a mix of allyl (Al) and propenyl (Pe) CSO, produced by House Foods Corporation, Japan, was used as the standard. The HPLC system included a pump, a degasser, a column oven, a diode array detector set (220 nm, L-2450; HITACHI High-Technologies Corporation, Tokyo, Japan), a data collection system (EZChrom EliteTM; HITACHI High-Technologies Corporation) and an AQUASIL column (4.6 mm Ø × 25 cm long). The solvent, 0.005% trifluoroacetic acid (TFA), flowed for 15 min at a flow rate of 0.6 $ml \cdot min^{-1}$.

The *S*-methyl-L-cysteine sulfoxide (MeCSO) content was determined by using an amino acid analysis method because the method previously used to identify AlCSO and PeCSO produces an overlapping peak of MeCSO, making it difficult to quantify. One hundred μL of the sample and 100 μL of the amino acid standard were dried in a vacuum using Spin Dryer Lite VC-36R (Taitec Co., Ltd., Saitama, Japan). For derivatization, 20 μL of freshly prepared methanol : H_2O : triethylamine : phenylisothiocyanate (7:1:1:1) was added to the dried sample and vortexed. The mixture was incubated for 20 min at room temperature for the reaction process before being dried again in a vacuum condition. The supernatant was dissolved in 100 μL of 5 mM sodium phosphate, pH 7.6, containing 5% acetonitrile. A quantitative analysis method using HPLC apparatus from Nihon Waters (Tokyo, Japan) followed the procedure described by Masamura et al. (2011), with some minor modifications. The injection volume was 50 μL, and the column temperature was 43°C. HPLC analysis was carried out by using

the following solvent system. Solvent A: 19 g of sodium acetate trihydrate and 2 mL of triethylamine were dissolved in 1 L of high purity water. The solution was adjusted to pH 6.08 by the addition of glacial acetic acid. To make up a 10% acetonitrile solution, a 950-mL solution was supplemented by 50 mL of acetonitrile. Solvent B: 60% acetonitrile and 40% high purity water (v/v) were mixed. The transmission of the gradient elution and the flow rate were obtained as described by Masamura et al. (2011).

Extraction and determination of total flavonoid content

The total flavonoid content was determined by the colorimetric method using hot 70% ethanol extract. The method of flavonoid extraction was applied as described by Vu et al. (2013). A 500-µL sample and 500 µL of 100% hexane were mixed in the Eppendorf and then incubated until the mixture separated into two parts. Fifty µL of the down part was taken as a sample to analyze with 50 µL of 70% ethanol and 200 µL of 2% aluminum chloride. The mixture was homogenized thoroughly by pipetting on the microplate and incubated for one hour before analysis. Different concentrations of quercetin—5, 10, 20, and 40 $\mu L \cdot mL^{-1}$—were used as standards. Moreover, a solution of 100 µL of 70% EtOH and 200 µL of 2% aluminum chloride was used as the blank. The total flavonoid content was quantified using the iMark Microplate Reader (Serial number 16548; BIO-RAD Laboratories, Tokyo, Japan) at 420 nm, and the data was read using Microplate Manager 6. To obtain the mean values, all chemical extractions were prepared for three biological replicates. Each extraction was applied to chemical determinations three times.

Extraction and determination of sugar content

The soluble sugar content—including fructose, glucose, and sucrose—was determined by the HPLC method using hot 70% ethanol extract. The method of sugar extraction adopted was the same as that described by Vu et al. (2013). The obtained extract was filtered using a 0.45-μm syringe filter (DISMIC®-13 HP ADVANTEC; Toyo Roshi Kaisha, Ltd.) before being analyzed using a Shimadzu Solusi LC (CBM-20A) HPLC machine equipped with an RI detector (Shimadzu, Kyoto, Japan), an LC-20AD pump (Shimadzu, 3.5 MPa Max 18.0 Min 0.0), and a Shimadzu SIL-20AC autosampler. Sugars from a 20-μL sample were separated on a LiChrospher 100 NH2 250-4.0 column (Kanto Chemical Co., Inc., Tokyo, Japan). Separation was obtained with 80% acetonitrile with a 35°C column temperature and a flow rate of 0.8 $ml \cdot min^{-1}$.

The fructan content was determined using the thiobarbituric acid method (Percheron, 1962) from a 70% ethanol extract. Preparations for fructan analysis were carried out as described by Vu et al. (2013). Fructan quantification was achieved using a spectrophotometer (U-2001; HITACHI High-Technologies Corporation) at a 432-nm wavelength. Chemical extractions were pre- pared for three replications in order to obtain mean values. Each extraction was applied to triplicate chemical determinations.

Qualification of sugar content

Thin-layer chromatography (TLC) was used to separate the sugar fraction of each sample. A hot 70% ethanol extract was spotted on TLC plates before development using a solvent system consisting of 1- buthanol : acetic acid : distilled water (2:1:1) in a glass chamber. Sugar fractions were visualized by applying a coloring reagent for fructooligosaccharides (diphenylamine : aniline : 85% phosphoric acid : acetone

(1:1:10:100), w/v/v/v) using an ink brush and were heated at 115°C, and 0.025% fructose, glucose, sucrose, 1-kestose, and nystose were used as standard chemicals.

Statistical analysis

All of the data sets obtained from content determinations were used to conduct an *F*-test together with Tukey's honestly significant difference (HSD) test. Principal component analysis (PCA) was conducted to obtain the similarities and differences among all bulb onion varieties and shallot landraces. Regression analysis was carried out to clarify the reliability of the data using two years of data from 2014 and 2015 data sets. All statistical analyses were performed using IBM SPSS statistics 19 (IBM, New York, USA).

Table 1. Plant material used in this study and relevant information.

Type	Variety or landrace	Abbreviation	Origin	Cultivation period	
				Sowing[y]	Harvesting
SDO (Short-day type of bulb onion)	'Shippo wase 7 go'	SW	Kagawa prefecture (Japan)	September	April
	'Advance'	AD	Kagawa prefecture (Japan)	September	April
	'Answer'	AN	Kagawa prefecture (Japan)	September	April
	'Shippo Ama 70'	SA	Kagawa prefecture (Japan)	September	May
	'Tarzan'	TZ	Kagawa prefecture (Japan)	September	May
	'Momijinokagayaki'	MK	Kagawa prefecture (Japan)	September	May
	'Momiji 3 go'	MS	Kagawa prefecture (Japan)	September	May
LDO (Long-day type of bulb onion)	'Okhotsk 222'	OK	Hokkaido prefecture (Japan)	March	August
	'Kitamomiji 2000'	KM	Hokkaido prefecture (Japan)	March	September
	'Super Kitamomiji'	SK	Hokkaido prefecture (Japan)	March	September
VNS (Vietnamese landraces of shallot)	'Lyson'[z]	LY	Ly Son island (Vietnam)	November	March
	'Quangai'[z]	QU	Quảng Ngãi province (Vietnam)	November	March
	'Soc trang'[z]	ST	Sóc Trăng province (Vietnam)	November	March
IDS (Indonesian landraces of shallot)	'Biru'	BR	Bantul (Yogyakarta, Indonesia)	June	September
	'Philip'[z]	PH	Nganjuk (East Java, Indonesia)	June	September
	'Tiron'	TR	Bantul (Yogyakarta, Indonesia)	June	September
	'Thailand'	TH	Bantul (Yogyakarta, Indonesia)	June	September
	'Bima Juna'	BJ	Tegal (Central Java, Indonesia)	June	September
	'Bauji Bunder'	BB	Nganjuk (East Java, Indonesia)	June	September
	'Bauji Plompong'	BP	Nganjuk (East Java, Indonesia)	June	September
	'Crok Kuning'	CK	Bantul (Yogyakarta, Indonesia)	June	September
	'Probolinggo'	PB	Probolinggo (Central Java, Indonesia)	June	September

[z] Not analyzed in the second year (2015).

[y] Seed sowing in bulb onions bulbs.

Results

S-alk(en)yl-L-cysteine sulfoxides (ACSOs)

MeCSO in shallot landraces from Indonesia was difficult to identify using the first HPLC method. Overlapping peaks were found in the MeCSO peak area, making it impossible to determine which peak represented the MeCSO content. Therefore, the method of amino acid analysis was used to determine the MeCSO content in Indonesian shallot landraces. All of the data sets of three different ACSOs—MeCSO, AlCSO, and PeCSO—from the samples collected in 2014 and 2015 are shown in Fig. 2 and/or Table 2. The MeCSO of 2014 was not significantly different among short-day and long-day varieties of bulb onion. However, Indonesian shallot landraces produced higher MeCSO than shallot landraces from Vietnam and both bulb onion types. Data from the next year showed a different tendency. Short-day bulb onions produced significantly higher MeCSO than the long-day bulb onions and Indonesian shallot landraces. Results of regression analysis showed that the MeCSO data of 2015 did not correlate with those of 2014 (r = 0.016) (Fig. 3A).

In the 2014 and 2015 trials, the AlCSO contents of bulb onion varieties were lower than those of shallot landraces. There were no significant differences in AlCSO content between bulb onion varieties. However, two shallot landraces from Indonesia, 'Thailand' and 'Bima Juna,' produced significantly higher AlCSO (0.08 $mg \cdot g^{-1}$ FW and 0.09 $mg \cdot g^{-1}$ FW, respectively) than bulb onion varieties in 2015. There was a correlation of AlCSO content between the 2014 and 2015 trials (r = 0.491*) (Fig. 3B). Table 2 shows that the PeCSO contents of bulb onion varieties were not significantly different from those of shallot landraces throughout the two years. As compared to bulb onion PeCSO contents, a relatively higher level of accumulation was observed in one

Indonesian shallot landrace, 'Probolinggo' (1.45 mg·g^{-1} FW in 2014 and 1.64 mg· g^{-1} FW in 2015), as compared with short-day and long-day bulb onions. Moreover, the PeCSO contents of long-day bulb onions were significantly higher than those of short-day bulb onions. Furthermore, the PeCSO data of 2014 were highly correlated with those of 2015 (r = 0.800**) (Fig. 3C).

Flavonoid content

In the 2014 and 2015 trials, a significant difference in flavonoid content was detected between bulb onion varieties and shallot landraces (Fig. 2 and/or Table 2). In shallots, one Indonesian landrace, 'Philip' had a relatively higher level of flavonoid content (0.57 mg·g^{-1} FW) than that of Vietnamese landraces. Although significant differences between varieties were not observed, long-day bulb onion varieties produced a higher amount of flavonoids than short-day varieties. Furthermore, shallots showed higher flavonoid contents than bulb onions. The same tendency was found in both experimental years, as the correlation test showed a highly significant correlation (r = 0.863**) between the flavonoid data of 2014 and 2015 (Fig. 3D).

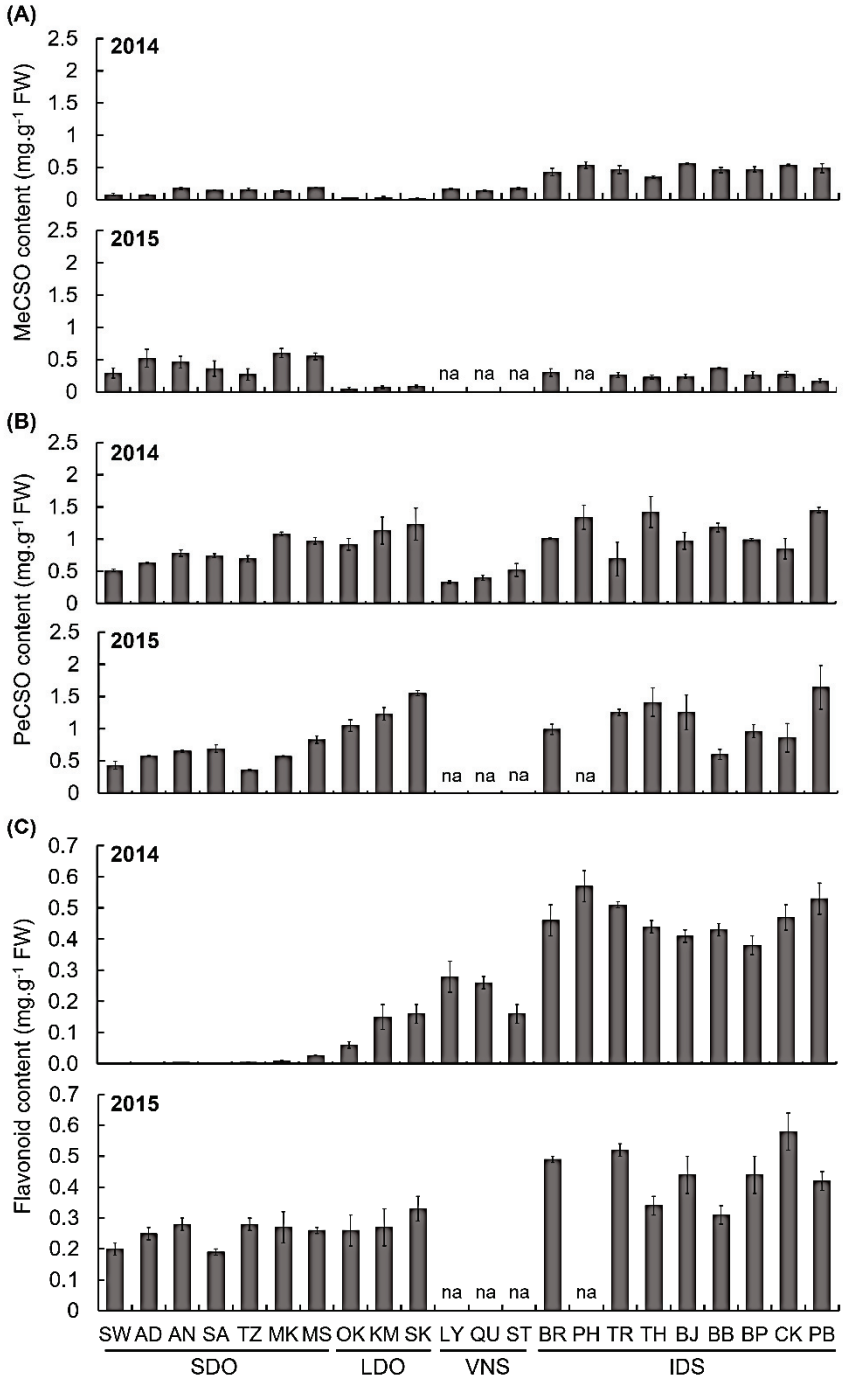


Fig. 2. Quantification of MeCSO (A), PeCSO (B), and flavonoid (C) contents in seven short-day onions (SDO), three long-day onions (LDO), three Vietnamese shallots (VNS), and nine Indonesian shallots (IDS) in 2014 and 2015. Each value is the mean ± SE of three replications. na = not analyzed.

Table 2. Contents of ACSOs and flavonoid (mg.g^{-1} FW) in bulb onions and shallots for 2014 and 2015.

Variety or landrace	2014				2015			
	MeCSO	AlCSO	PeCSO	Flavonoid	MeCSO	AlCSO	PeCSO	Flavonoid
Short-day bulb onion								
'Shippo wase 7 go'	0.08 ± 0.02[y]de[z]	0.00 ± 0.00 d	0.51 ± 0.02 efg	0.00 ± 0.00 ij	0.29 ± 0.08 bcde	0.01 ± 0.00 h	0.43 ± 0.06 d	0.20 ± 0.02 e
'Advance'	0.07 ± 0.01 de	0.01 ± 0.00 cd	0.63 ± 0.01 defg	0.00 ± 0.00 ij	0.52 ± 0.14 ab	0.02 ± 0.00 efgh	0.58 ± 0.01 cd	0.25 ± 0.02 de
'Answer'	0.18 ± 0.02 de	0.00 ± 0.00 d	0.78 ± 0.05 cdefg	0.00 ± 0.00 ij	0.46 ± 0.09 abc	0.01 ± 0.00 gh	0.65 ± 0.02 bcd	0.28 ± 0.02 cde
'Shippo Ama 70'	0.15 ± 0.01 de	0.01 ± 0.00 cd	0.74 ± 0.03 cdefg	0.00 ± 0.00 ij	0.36 ± 0.12 abcd	0.04 ± 0.00 efg	0.69 ± 0.06 bcd	0.19 ± 0.01 e
'Tarzan'	0.16 ± 0.02 de	0.02 ± 0.00 abcd	0.69 ± 0.05 cdefg	0.01 ± 0.00 hij	0.27 ± 0.09 bcde	0.01 ± 0.00 gh	0.36 ± 0.01 d	0.28 ± 0.02 cde
Momijinokagayaki'	0.14 ± 0.02 de	0.01 ± 0.00 cd	1.08 ± 0.03 bcdef	0.00 ± 0.00 ij	0.60 ± 0.07 a	0.02 ± 0.00 fgh	0.57 ± 0.01 cd	0.27 ± 0.05 cde
'Momiji 3 go'	0.19 ± 0.01 cd	0.01 ± 0.00 cd	0.97 ± 0.05 abcde	0.02 ± 0.00 ghij	0.55 ± 0.05 a	0.02 ± 0.00 efgh	0.83 ± 0.06 abcd	0.26 ± 0.01de
Long-day bulb onion								
'Okhotsk 222'	0.03 ± 0.00 de	0.02 ± 0.00 abcd	0.92 ± 0.09 abcdef	0.06 ± 0.01 fghij	0.05 ± 0.08 e	0.03 ± 0.00 efgh	1.05 ± 0.09 abcd	0.26 ± 0.05 de
'Kitamomiji 2000'	0.04 ± 0.01 de	0.04 ± 0.01 abcd	1.13 ± 0.21 abcd	0.15 ± 0.04 efghi	0.07 ± 0.02 e	0.02 ± 0.00 fgh	1.23 ± 0.10 abcd	0.27 ± 0.06 cde
'Super Kitamomiji'	0.02 ± 0.01 e	0.05 ± 0.01 ab	1.23 ± 0.25 abc	0.16 ± 0.03 efgh	0.08 ± 0.21 e	0.03 ± 0.00 efgh	1.55 ± 0.04 ab	0.33 ± 0.04 bcde
Vietnamese shallot								
'Lyson'	0.17 ± 0.01 de	0.03 ± 0.00 abcd	0.33 ± 0.02 g	0.28 ± 0.05 cde	na	na	na	na
'Quangai'	0.14 ± 0.01 de	0.03 ± 0.01 abcd	0.40 ± 0.04 fg	0.26 ± 0.02 de	na	na	na	na
'Soc trang'	0.18 ± 0.02 cde	0.04 ± 0.01 abcd	0.52 ± 0.10 efg	0.16 ± 0.03 efg	na	na	na	na
Indonesian shallot								
'Biru'	0.43 ± 0.06[x] ab	0.03 ± 0.01 abcd	1.01 ± 0.01 abcde	0.46 ± 0.05 ab	0.30 ± 0.06[x] bcde	0.07 ± 0.00 ab	0.99 ± 0.08 abcd	0.49 ± 1.11 abcd
'Philip'	0.54 ± 0.05[x] a	0.04 ± 0.00 abcd	1.34 ± 0.19 ab	0.57 ± 0.05 a	na	na	na	na
'Tiron'	0.47 ± 0.06[x] ab	0.02 ± 0.00 abcd	0.69 ± 0.26 cdefg	0.51 ± 0.01 ab	0.26 ± 0.04[x] cde	0.07 ± 0.01 ab	1.25 ± 0.05 abcd	0.52 ± 0.02 abc
'Thailand'	0.35 ± 0.02[x] bc	0.04 ± 0.01 abcd	1.42 ± 0.24 a	0.44 ± 0.02 ab	0.23 ± 0.03[x] cde	0.08 ± 0.00 a	1.41 ± 0.22 abc	0.34 ± 0.03 abcde
'Bima Juna'	0.56 ± 0.01[x] a	0.05 ± 0.01 abc	0.97 ± 0.13 abcde	0.41 ± 0.02 bc	0.24 ± 0.03[x] cde	0.09 ± 0.01 a	1.25 ± 0.27 abcd	0.44 ± 0.06 abcde
'Bauji Bunder'	0.46 ± 0.04[x] ab	0.05 ± 0.00 abcd	1.18 ± 0.07 abc	0.43 ± 0.02 abc	0.37 ± 0.01[x] abcd	0.05 ± 0.01 b	0.60 ± 0.08 cd	0.31 ± 0.03 cde
'Bauji Plompong'	0.47 ± 0.04[x] ab	0.06 ± 0.01 a	0.99 ± 0.02 abcde	0.38 ± 0.03 bcd	0.26 ± 0.05[x] cde	0.03 ± 0.01 efgh	0.96 ± 0.10 abcd	0.44 ± 0.06 abcde
'Crok Kuning'	0.54 ± 0.01[x] a	0.05 ± 0.01 a	0.85 ± 0.16 bcdefg	0.47 ± 0.04 ab	0.27 ± 0.05[x] bcde	0.04 ± 0.01 cdef	0.86 ± 0.22 abcd	0.58 ± 0.06 ab
'Probolinggo'	0.49 ± 0.07[x] ab	0.02 ± 0.00 abcd	1.45 ± 0.04 a	0.53 ± 0.05 ab	0.17 ± 0.03[x] de	0.06 ± 0.01 ab	1.64 ± 0.54 a	0.42 ± 0.03 abcde

[z] Means with the same letter in a column are not significantly different according to Tukey's test ($P < 0.05$).

[y] Each value is the mean ± SE (n = 3).

[x] Analyzed using amino acid analysis method.

na : not analyzed

Soluble sugar content

The sweetness of bulb onions and shallots seems to be affected by the composition of the soluble sugar content. The total soluble sugar content—including fructose, glucose, and sucrose—was determined in this research using the HPLC method (Fig. 4 and/or Table 3). The statistical analysis data demonstrated that the fructose contents of short-day bulb onions were significantly higher than those of long-day bulb onions and shallots. The same tendency was also observed in the glucose content of bulb onion and shallot samples. No significant differences were found in the sucrose contents of bulb onion varieties and shallot landraces from different areas. However, one Indonesian shallot landrace, 'Thailand' produced a higher sucrose content in the 2014 and 2015 observations. The same tendency was found throughout the two years, as regression analysis showed a significant correlation for fructose (r = 0.866**) (Fig. 3E), glucose (r = 0.959**) (Fig. 3F), and sucrose (r = 0.705**) (Fig. 3G) contents between 2014 and 2015.

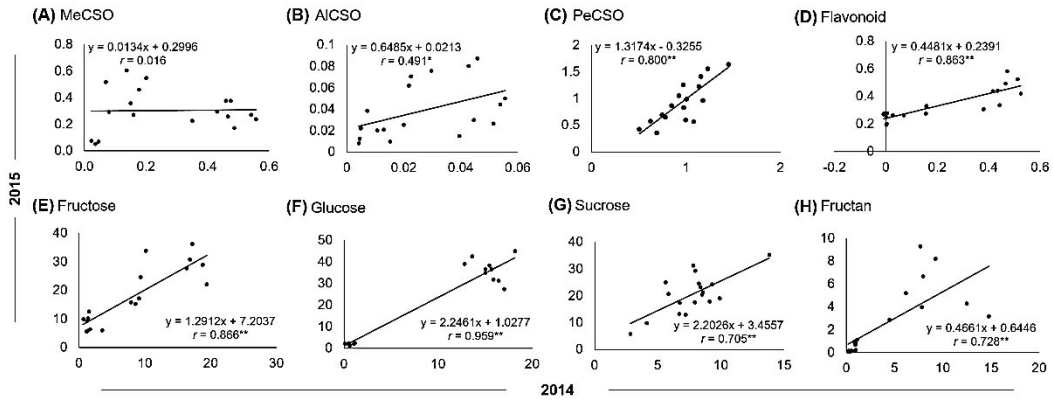


Fig. 3. Relationships between data from 2014 and 2015 in MeCSO (A), AlCSO (B), PeCSO (C), flavonoid (D), fructose (E), glucose (F), sucrose (G) and fructan (H) contents of bulb onions and shallots in 2014 and 2015 * Significant at P < 0.05 and ** Significant at P < 0.01.

Table 3. Contents of soluble sugars (mg.g^{-1} FW) in bulb onions and shallots for 2014 and 2015

Variety or landrace	2014				2015			
	Fructose	Glucose	Sucrose	Fructan	Fructose	Glucose	Sucrose	Fructan
Short-day bulb onion								
'Shippo wase 7 go'	16.35 ± 1.73[y]a[z]	15.03 ± 0.48 abc	6.71 ± 0.75 bcde	0.78 ± 0.13 h	27.63 ± 2.00 abc	34.97 ± 0.86 cde	13.19 ± 3.26 de	0.12 ± 0.03 f
'Advance'	19.42 ± 1.11 a	16.43 ± 0.66 ab	2.85 ± 1.22 e	0.18 ± 0.04 h	22.14 ± 0.72 cde	31.28 ± 1.83 ef	5.75 ± 3.57 e	0.06 ± 0.02 f
'Answer'	18.83 ± 0.70 a	17.03 ± 0.98 ab	5.64 ± 1.78 cde	0.29 ± 0.03 h	28.98 ± 3.07 abc	27.30 ± 1.99 f	24.98 ± 0.76 abcd	0.15 ± 0.05 f
'Shippo Ama 70'	16.85 ± 0.28 a	15.88 ± 0.78 abc	4.13 ± 0.68 de	0.39 ± 0.03 h	30.81 ± 2.46 abc	31.79 ± 0.87 def	9.78 ± 2.96 de	0.05 ± 0.02 f
'Tarzan'	17.22 ± 0.46 a	18.18 ± 0.87 a	5.86 ± 1.22 cde	0.53 ± 0.07 h	36.23 ± 1.32 a	44.90 ± 0.89 a	20.72 ± 1.23 abcde	0.16 ± 0.02 f
Momijinokagayaki'	9.33 ± 1.15 b	15.44 ± 1.32 abc	8.38 ± 0.81 bcd	0.91 ± 0.09 h	24.50 ± 0.74 bcd	38.28 ± 2.65 abcde	23.14 ± 1.23 abcd	0.69 ± 0.11 ef
'Momiji 3 go'	10.10 ± 1.98 b	15.69 ± 1.16 abc	7.21 ± 0.82 bcde	0.98 ± 0.11 h	33.77 ± 4.78 ab	36.60 ± 2.64 bcde	12.95 ± 5.60 de	0.22 ± 0.14 f
Long-day bulb onion								
'Okhotsk 222'	8.55 ± 0.38 b	12.82 ± 0.21 c	9.11 ± 1.20 bc	0.81 ± 0.27 h	15.21 ± 1.73 defgh	39.09 ± 093 abcd	17.84 ± 4.91 bcde	0.94 ± 0.05 ef
'Kitamomiji 2000'	7.84 ± 0.14 bc	15.06 ± 1.07 abc	8.59 ± 0.78 bc	1.01 ± 0.22 h	15.81 ± 3.10 defg	36.53 ± 3.02 bcde	21.22 ± 2.39 abcde	0.73 ± 0.08 ef
'Super Kitamomiji'	9.10 ± 0.92 b	13.64 ± 0.61 bc	8.53 ± 1.02 bcd	1.10 ± 0.25 h	17.14 ± 1.75 def	42.56 ± 1.36 ab	20.34 ± 2.56 abcde	1.10 ± 0.13 ef
Vietnamese shallot								
'Lyson'	8.57 ± 0.62 b	8.61 ± 0.33 d	6.32 ± 0.18 bcde	4.23 ± 1.01 efg	na	na	na	na
'Quangai'	9.21 ± 0.54 b	8.71 ± 0.25 d	6.84 ± 0.27 bcde	3.49 ± 0.65 fg	na	na	na	na
'Soc trang'	10.04 ± 0.45 b	8.25 ± 0.32 d	9.70 ± 0.32 abc	2.05 ± 0.47 gh	na	na	na	na
Indonesian shallot								
'Biru'	0.59 ± 0.12 d	0.11 ± 0.04 e	7.82 ± 0.45 bcd	14.80 ± 0.54 a	9.92 ± 0.87 fgh	2.18 ± 0.48 g	31.24 ± 3.23 ab	3.16 ± 0.40 de
'Philip'	0.74 ± 0.05 d	0.72 ± 0.25 e	5.91 ± 0.22 cde	13.14 ± 0.93 ab	na	na	na	na
'Tiron'	1.64 ± 0.40 d	0.62 ± 0.04 e	6.73 ± 0.27 bcde	12.49 ± 0.29 b	6.37 ± 0.50 gh	1.66 ± 0.20 g	17.36 ± 3.19 bcde	4.29 ± 1.39 cd
'Thailand'	3.46 ± 0.08 d	1.16 ± 0.07 e	13.84 ± 0.67 a	4.47 ± 0.29 ef	5.96 ± 0.40 gh	2.25 ± 0.07 g	35.16 ± 2.52 a	2.84 ± 0.42 de
'Bima Juna'	1.16 ± 0.14 d	1.07 ± 0.09 e	7.95 ± 0.53 bcd	6.19 ± 0.20 de	9.17 ± 1.31 fgh	2.01 ± 0.24 g	17.50 ± 1.23 bcde	5.20 ± 0.84 cd
'Bauji Bunder'	1.07 ± 0.30 d	0.52 ± 0.16 e	9.92 ± 0.29 abc	7.68 ± 0.83 cd	5.76 ± 0.65 h	1.16 ± 0.36 g	24.46 ± 1.92 abcd	8.20 ± 0.75 ab
'Bauji Plompong'	1.25 ± 0.09 d	0.61 ± 0.20 e	8.28 ± 0.40 bcd	9.28 ± 0.25 c	5.59 ± 0.43 h	2.11 ± 0.28 g	19.02 ± 2.71 bcde	9.30 ± 0.72 a
'Crok Kuning'	1.33 ± 0.49 d	0.53 ± 0.06 e	7.98 ± 0.14 bcd	7.83 ± 0.29 cd	10.16 ± 0.82 fgh	2.21 ± 0.19 g	29.21 ± 1.91 abc	3.98 ± 0.33 d
'Probolinggo'	1.47 ± 0.17 d	0.58 ± 0.23 e	9.29 ± 0.27 bc	7.98 ± 1.13 cd	12.50 ± 1.18 efgh	1.95 ± 0.19 g	24.20 ± 0.93 abcd	6.65 ± 0.56 bc

[z] Means with the same letter in a column are not significantly different according to Tukey's test ($P < 0.05$).

[y] Each value is the mean ± SE (n = 3).

na : not analyzed.

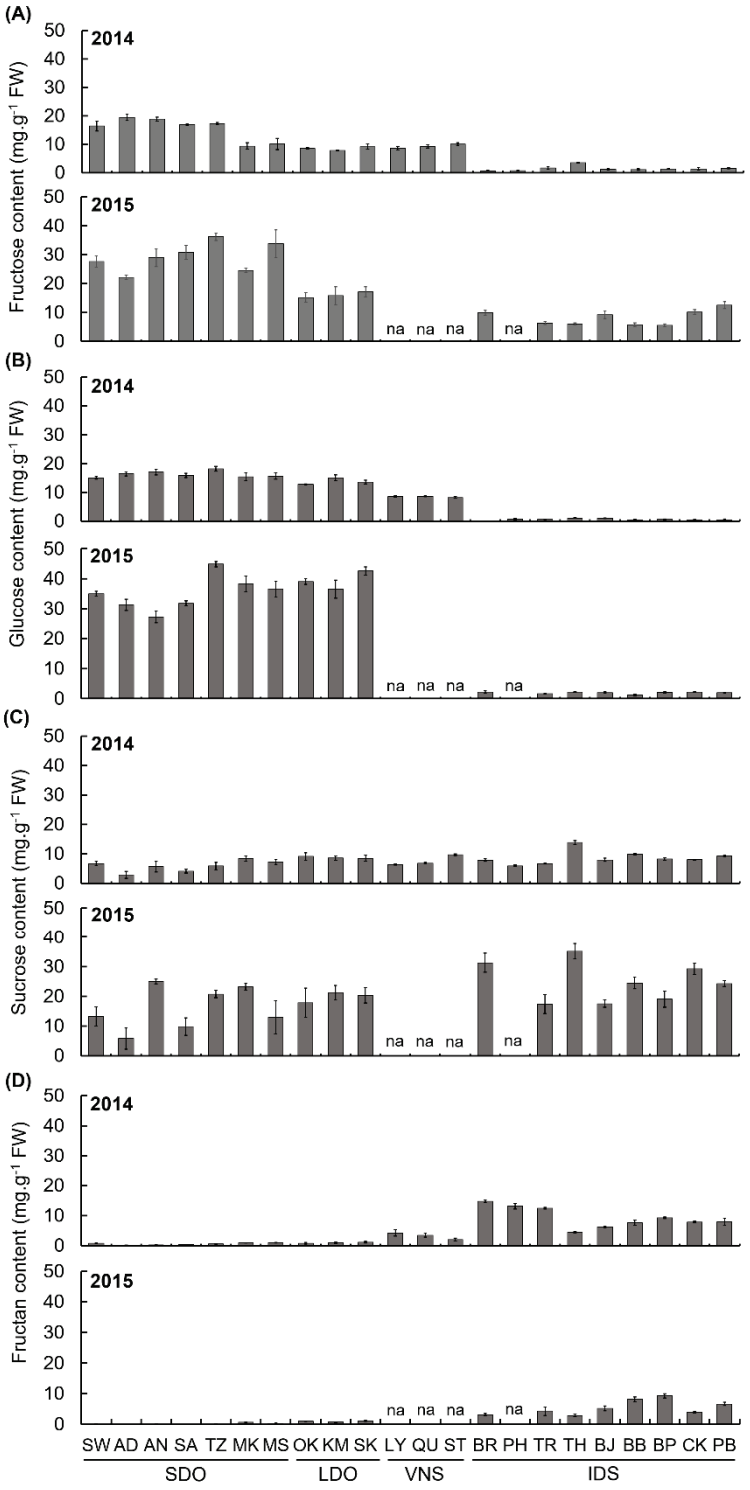


Fig. 4. Quantification of sugar contents including fructose (A), glucose (B), sucrose (C), and fructan (D) in seven short-day onions (SDO), three long-day onions (LDO), three Vietnamese shallots (VNS) and nine Indonesian shallots (IDS) in 2014 and 2015. Each value is the mean ± SE of three replications. na = not analyzed

More than 50% of the sugar contents of bulb onions were monosaccharides (fructose and glucose), and the major sugar content in shallot landraces was sucrose. This result was derived from the qualification of soluble sugar content using the TLC method for the 2014 samples. Figure 5 shows that bulb onion varieties had higher amounts of monosaccharides, and that shallot landraces were rich in disaccharides (sucrose) and poly- saccharides (1-kestose and nystose). A significantly higher amount of fructans, known as fructose polymers, could be detected in shallot landraces from Indonesia. The coefficient correlation of fructans contents between 2014 and 2015 was significantly high (r = 0.728**) (Fig. 3H).

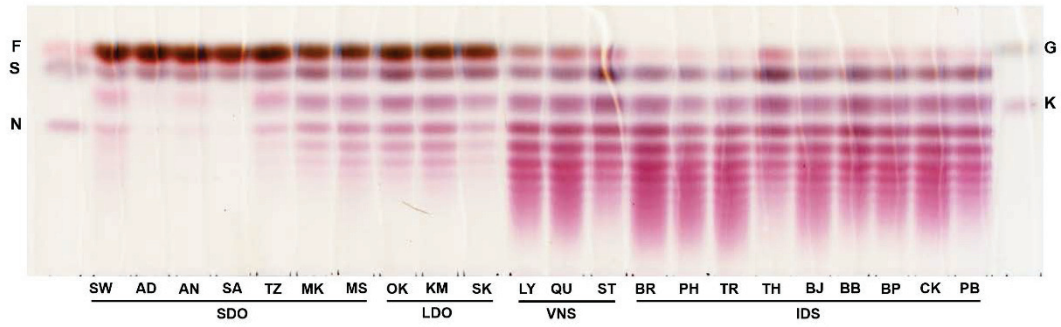


Fig. 5. TLC profile of soluble sugars in seven short-day bulb onions (SDO), three long-day bulb onions (LDO), three Vietnamese shallots (VNS), and nine Indonesian shallots (IDS). Fructose (F), glucose (G), sucrose (S), 1-kestose (K), and nistose (N) were used as standard compounds.

Principal component analysis

The phytochemical properties—including ACSOs, flavonoids, and sugars—of 10 long-day bulb onion varieties, 3 short-day bulb onion varieties, 3 Vietnamese shallot landraces, and 9 Indonesian shallot landraces were analyzed in 2014 and 2015. The PCA of 2014 yielded 2 principal components, accounting for 83.9% of the total variations, divided into 68.3% of the PC1 and 15.6% of the PC2 (Fig. 6A). The loading

values of fructose and glucose in the PC1 were found to be negative (−0.812 and −0.920, respectively), as also observed in the PC1 of the 2015 PCA (−0.814 and −0.971, respectively). The PC2 in 2014 consisted of sucrose (0.918), PeCSO (0.750), and AlCSO (0.577). However, in the PC2 of 2015, which was only 10.3% of the total variation, this component consisted of MeCSO (−0.925) and PeCSO (0.802). In the PCA analysis of 2015, the PC1 explained 65.5% of the total variations (Fig. 6B). Both the PCA analyses of 2014 and 2015 could clearly discriminate short-day bulb onions from long-day bulb onions and shallots from a phytochemical point of view.

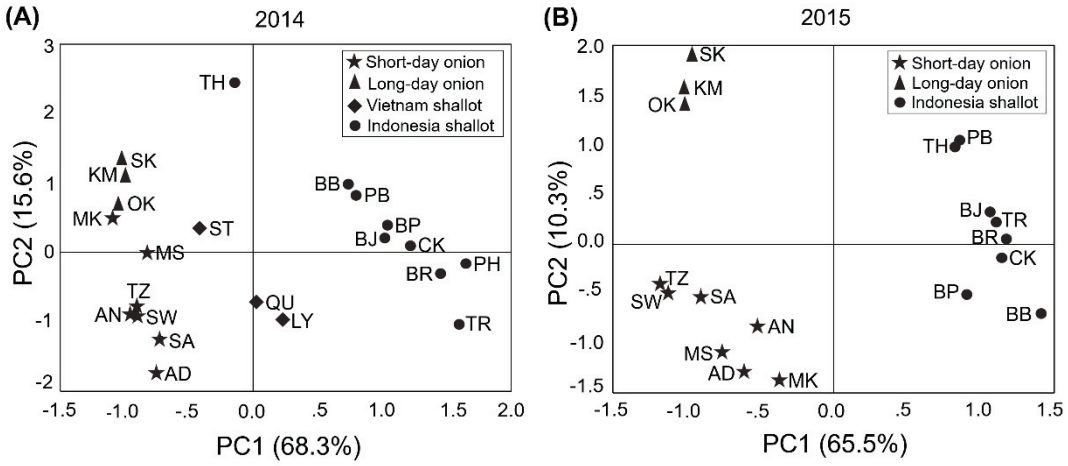


Fig. 6. Plot of the first and second principal components obtained from a data set of chemical composition analyses in bulb onions and shallots for 2014 (A) and 2015 (B).

Discussion

The two groups of bulb onion varieties examined in this study could be differentiated by their photoperiod requirements for bulb formation. Short-day onions initiate bulbs with days 11–12 hours long, while long-day onion need days longer than 16 hours for bulb formation (Brewster, 2008). By employing two bulb onion types, Marotti and Piccaglia (2002) reported that the different photoperiods of the tested cultivars did not influence the bulb yield, but did affect the dry matter content. The same tendency was also found in this study where the dry matter content of long-day onions was higher (±11%) than that of short-day onions (±9%) (data not shown).

Organosulfur compounds, known as flavor precursors (Brewster, 2008), are related to the pungent taste of bulb onions and shallots (Block, 1992). In previous studies, MeCSO and PeCSO were found to be the main compounds correlated to pungency in bulb onions and shallots (Lee et al., 2009). However, AlCSO content did not lead to a stronger pungent taste as it was found at low concentrations or even undetected (Yoo and Pike, 1998). Lee et al. (2009) reported that the high pungency level achieved in their research occurred with 0.15 $mg \cdot g^{-1}$ FW of MeCSO and 0.60 $mg \cdot g^{-1}$ FW of PeCSO in bulb onions. Therefore, our shallot landraces, which possessed high levels of MeCSO (0.49 $mg \cdot g^{-1}$ FW) and PeCSO (1.45 $mg \cdot g^{-1}$ FW) contents were very pungent. Furthermore, the amounts of organosulfur compounds are affected by the variety, maturity, soil fertility, and other growing conditions (Saghir et al., 1965). In this study, long-day bulb onions, cultivated from March to September and grown during the summer, showed a higher PeCSO content than short-day bulb onions cultivated from September to March. It is reasonable to say that the level of pungency in bulb onions and shallots increases with temperature (Brewster, 2008). Shallots from Indonesia, cultivated in the dry season (June to September), also

possessed high amounts of PeCSO and MeCSO. Regarding their edibility, Kimura et al. (2014) mentioned the importance of pungency for salad use of bulb onions and shallots. Actually, less pungent bulb onions are suitable for raw consumption, while strong pungency is needed for shallots, which are mainly used as a condiment.

Flavonoids are responsible for the bitterness of bulb onions and shallots (Astraya et al., 2007). Leighton et al. (1992) reported that, in addition to genetic back- ground, environmental conditions also affect flavonoid production. Furthermore, the bulb onions and shallots used in this study were cultivated at different latitudes, possibly leading to the different flavonoid contents produced (Jaakola and Hohtola, 2010). Differences in latitude may account for the differences in day length, light quality, UV radiation, and temperature. However, in agreement with the results of this research, it has been reported that shallots produce higher levels of flavonoids than bulb onions (Fattorusso et al., 2002; Leighton et al., 1992). In fact, shallots from Indonesia, which were cultivated near the equator and exposed to high UV radiation, produced the highest levels of flavonoids. Long-day bulb onions cultivated in Hokkaido also produced a higher amount of flavonoids than short-day types. Flavonoids play an important role in plant defense mechanisms against abiotic stresses such as high UV radiation (Harborne and Williams, 2000). The high flavonoid content found in Indonesian shallots makes them more bitter than other shallots and bulb onions. Moreover, long-day bulb onions, which are more suitable for processed food, also possessed a more bitter taste than short-day bulb onions. Further organoleptic analysis will be needed to explore the palatability of bulb onions as compared with shallots.

The quality of bulb onions and shallots as horticultural products depends on the dry matter contents of their bulbs. Darbyshire and Steer (1990) explained that most of the dry matter in bulb onions (65–80%) consists of non-structural carbohydrates,

including fructose, glucose, sucrose, and fructans. As shown in Table 3, the F- test of this study detected fructose and glucose as major contents, followed by sucrose, in short-day bulb onions. This result was similar to that of Lee et al. (2009). Fructose and glucose contents in bulb onions were positively correlated with sweetness (Terry et al., 2005). However, the combination of high PeCSO and flavonoid contents with the low amount of monosaccharides in shallots may create a specific taste different from bulb onions. Such differences lead to different consumer preferences for bulb onions and shallots in the human diet. Therefore, it is recommended that the amino acid content be analyzed to better understand the difference in taste and flavor precursors in bulb onions and shallots. Furthermore, a high level of fructan content was also observed in shallots. This result is very interesting because Brewster (2008) reported that most species with high fructan accumulations are from temperate regions and not from tropical regions. However, Raines et al. (2009) reported a positive correlation between dry weight and fructan content in bulb onions. The shallots used in this study also had dry weights higher than those of bulb onions (data not shown). Moreover, the accumulation of fructans could be highly correlated with drought or cold stress tolerance (Livingston III et al., 2009). As a practical matter, transgenic tobaccos with a drought-resistant gene produce high levels of fructans due to an upregulatory effect that protects the membrane and other cellular components by inducing cell wall hardening and limiting cell growth to reduce the water demand (Pilon-Smits et al., 1995). Therefore, we assumed that the higher production of fructans in the Indonesian shallots was closely related to cultivation conditions. It was suggested that the high fructan content of these shallots is a kind of plant-defense mechanism against fungal and pathogenic attacks (Kawakami and Yoshida, 2012; Van den Ende et al., 2004). Based on Brewster (2008), the degree of polymerization (DP) of fructans in bulb onions and shallots is up to 20. However,

Yaguchi et al. (2009) reported that the DP of shallots was less than 12, as we observed in the lower part of sugar profiling using the TLC method. Further research will be needed to measure the effect of different climatic conditions on the production of fructans in shallots.

The results of PCA analyses clearly discriminate between bulb onions and shallots based on their area of cultivation. Indonesian shallots tended to accumulate soluble sugars in a polysaccharide form and produce high levels of flavonoids, as also reported by Leighton et al. (1992). Furthermore, in our previous report, we proved that 75–80% of shallot dry matter consisted of carbohydrates with a high level of sugars (Rabinowitch and Kamenetsky, 2002). Moreover, the fructan content represented 33–83% of the total sugars (Vu et al., 2013). In general, bulb onions accumulated high levels of monosaccharaides, along with low levels of sulfur compounds and flavonoids, resulting in a mild and sweet taste. On the other hand, shallots produced a higher amount of polysaccharides, flavonoids, and ACSOs, leading to strong pungency and more bitter tastes. Between short-day and long-day bulb onion types, the PeCSO content became the main differentiating factor. Higher amounts of PeCSO content in long-day bulb onions clearly distinguished them from short-day bulb onions.

In conclusion, bulb onions and shallots possessed different taste characteristics based on their chemical com- positions. The results of this research will be very important for producers and consumers in determining the quality of Japanese bulb onion varieties and shallot landraces in production fields, as well as at markets. Moreover, these results also underscore the importance of developing an F_1 hybrid between shallots and bulb onions to improve the taste quality of bulb onions. The similarities in the morphological characteristics of inflorescence and flowers, karyotype, and chromosome behaviors in the meiosis of the shallot and the bulb onion

suggest indirectly that they are closely related (Tashiro et al., 1982). There is no serious internal barrier between shallots and bulb onions, as F1 and F2 plants exhibited hybrid vigor and full fertility. Further study of an F1 hybrid between a cytoplasmic male sterile shallot with *A. galanthum* cytoplasm and the common onion reported three morphological characteristics of haploid plants—shallots, hybrid, and common onion types— and double haploid plants displayed integrated characteristics between shallots and bulb onions (Sulistyaningsih et al., 2002). The future challenge in onion breeding is to produce an F1 bulb onion hybrid with a common shape and size, but with higher eating quality derived from the shallot. All in all, we have elucidated here the partial metabolomic profile of all leading bulb onion varieties in Japan, together with some representative shallot landraces for Southeast Asia, which will allow us to provide a novel indicator for characterizing some genotypes of *A. cepa*. Further research related to amino acid content and intermediate forms of flavor precursors in bulb onions and shallots will be beneficial in better understanding the regulatory system for taste and flavor.

Chapter 3. PHYTOCHEMICAL ANALYSES OF AMINO ACIDS AND PRECURSOR COMPOUNDS FOR *S*-ALK(EN)YL-L-CYSTEINE SULFOXIDES IN BULB ONIONS AND SHALLOTS

Introduction

From more than 800 species of the *Allium* genus, bulb onions (*Allium cepa* Common onion group) and shallots (*Allium cepa* Aggregatum group) are two of the most important *Allium* species in the world, as they are used mainly in our food to enhance the taste and palatability. Their organic sulfur compound and amino acids are known to be the source of their unique taste and flavor characteristics (Corzo-Martinez et al., 2007; Lanzotti, 2006). In a previous study, Ariyanti et al. (2017) exploited 10 F_1 bulb onion varieties (7 short-day types and 3 long-day types) and some shallot landraces from Indonesia and Vietnam to understand the variation in their chemical properties responsible for the taste. The results indicated that bulb onions and shallots produce different amounts of flavor precursors—*S*-alk(en)yl-L-cysteine sulfoxides (ACSOs)—flavonoids, and sugars. The differences lead to the different tastes of bulb onions and shallots. Those differences are possibly the main reason for consumers' preferences. However, eating quality is considered based on odor, taste, and flavor which are generated from many kinds of phytochemicals including amino acids.

Solms et al. (1965) first reported the taste of L- and D-amino acids. They differentiated the amino acids into three big groups: amino acids without taste, amino acids with varying tastes, and amino acids with a bitter or sweet taste. Later on, Schiffman et al. (1981) elaborated the existing amino acid taste description by comparing the taste of amino acids as well as their thresholds. The results indicate that the large differences in the amino acid thresholds were not followed by quality

differences. Moreover, between L- and D-amino acids, there were parallel threshold values. The amino acid cysteine is one of the primary source of ACSO biosynthesis, together with antioxidant glutathione (GSH) (Hesse et al., 2004). In cysteine biosynthesis, serine also contributes as the source of the *O*-acetylserine together with sulfide in the catalyzed process. During GSH biosynthesis, γ-glutamylcysteine (γ-GC) was formed. Later on, the intermediate product of GSH is *S*-methylglutathione and/or *S*-2-carboxypropylgluthathione. Moreover, the intermediate product of γ-GC is γ-glutamyl-*S*-2-carboxypropylcysteine (Rose et al., 2005).

As the ACSO contents of bulb onions and shallots were found different, a comprehensive study of the differences of ACSO biosynthesis pathways is needed. In order to achieve a better understanding of the taste and flavor regulatory system of bulb onions and shallots, we analyzed amino acids using an HPLC method and analyzed some intermediate compounds constructed during the ACSO biosynthesis using LC-MS/MS method.

Materials and Methods

Plant materials

Ten cultivars of the Japanese bulb onion (*A. cepa* Common onion group) including 7 cultivars of short-day bulb onions and 3 cultivars of long-day bulb onions, were studied. The short-day bulb onions were cultivated in Kagawa prefecture (N 34º, E 134º), and the long-day bulb onions were grown in Hokkaido (N 44º, E 142º). Fourteen cultivars of shallots (*A. cepa* Aggregatum group) divided into 9 cultivars from Indonesia (S 6º, E 106º), 3 cultivars from Vietnam (N 21º, E 105º), and 2 cultivars of European shallots (N 42º, W 86º) were collected from farmers and local markets. The

name and origin of each sample as well as the cultivation periods are indicated in Table 1.

Extraction and determination of amino acid content

In order to obtain the free amino acid content in the bulb onion and shallot samples, 70% hot ETOH extraction was used. The method of extraction used was described by Vu et al. (2012). A 100µL sample and 100µL of amino acid standard were vacuum dried using Spin Dryer Lite VC-36R (Taitec Co., Ltd., Saitama, Japan). For derivatization, 20µL of freshly prepared methanol : H_2O : TEA : PITC (7 : 1 : 1 : 1) was added to the dried sample and vortexed. The mixture was incubated for 20 minutes at temperature for the reaction process before drying again in vacuum conditions. The supernatant was dissolved in 100µL of 5mM sodium phosphate, pH7.6, containing 5% acetonitrile. The quantitative analyses using an HPLC machine were obtained as described by Masamura et al. (2011), with some minor modifications. The injection volume was 50µL, and the column temperature was 43ºC.

The HPLC analysis was run using the following solvent system. Solvent A: 19 g sodium acetate trihydrate and 2mL TEA were dissolved in 1L highpurity water. The solution was adjusted to pH6.08 by the addition of glacial acetic acid. To make up a 10% acetonitrile solution, 950 ml of solution was added to 50ml of acetonitrile. Solvent B: 60% acetonitrile and 40% high purity water (v/v) were mixed. The transmission of the gradient elution and the flow rate were obtained as described by Masamura et al. (2011).

A total of 21 standard amino acid were used to identify the retention time of each amino acid compound. Most of the amino acid standards were synthesized by

Sigma-Aldrich, Inc. (Japan), while the cysteine standard was purchased from Ajinomoto Co., Inc. (Japan), and the histidine, threonine, tyrosine, methionine, and phenylalanine standards were purchased from Wako Pure Chemical Industries Ltd. (Japan). Quantifications of various amino acids per-1g FW were measures by the absolute calibration method.

Determination of ACSOs and their intermediate compounds with LC-MS-MS

The ACSOs, together with their intermediate compounds, were analyzed using water extracts. Lyophilized extract was dissolved with 500 μL of Milli-Q water. The dissolved extract was then diluted 20 or 200 times with Milli-Q water before being filtrated through a 0.45 μm aqueous phase filter to remove the solids. The LC conditions were the same as those described by Vu et al. (2013). Reference standards—γ-glutamylcysteine (Sigma-Aldrich, USA), glutathione (Sigma-Aldrich, USA), *S*-methylglutathione (Sigma-Aldrich, USA), *S*-2-carboxypropylglutathione (WuXiAppTec, China), and γ-glutamyl-S-2 carboxypropylcysteine (wuXi AppTec, China)—were prepared for ESI (electrospary ionization; positive mode) MS (mass spectrometry) analyses. Typical ESI-MS voltage settings for detection and analysis were as follows: curtain gas (CUR), 40 psig; collision gas (CAD), medium; ionspray voltage (IS), 5000 V; temperature (TEM), 600 ºC; ion source gas1 (GS1), 60 psig; ion source gas2 (GS2), 60 psig.

Statistical analysis

The data obtained were analyzed by *t*-test, Tukey's test and correlation test using SPSS 19.0 (SPSS, Japan).

Results

Free amino acid contents

Twenty-one amino acids were detected from the bulb onion and shallot sample using the HPLC method. Figure 7 shows a chromatogram of the amino acid analysis from the long-day bulb onion 'Superkitamomiji' and the Indonesian shallot 'Bima Juna.' From those 21 amino acids, four amino acids—β-alanine (β-Ala), glutamine (Gln), tryptophan (Trp), and lysine (Lys) —were detected in very low amounts, and even zero in some cultivars, as shown in Fig. 8 in both years. Seven amino acids—aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), glycine (Gly), arginine (Arg), and alanine (Ala)—were found in high quantities, ranging from 0.014 to 1.573 $mg.g^{-1}$ FW. Another 7 amino acids—histidine (His), threonine (Thr), proline (Pro), cysteine (Cys), valine (Val), leucine (Leu) and phenylalanine (Phe)—were detected in moderate amounts, ranging from 0.002 to 0.30 $mg.g^{-1}$ FW. The research results show that shallots produce higher amounts of the first 7 amino acids than bulb onions do. In both 2014 and 2015, shallots produced higher Asp, Glu, Asn and Ala, as shown in Fig. 8, ranging from 0.142 to 1.717 $mg.g^{-1}$ FW. A similar trend was also found for other amino acids where shallots produce higher amounts than blub onions do.

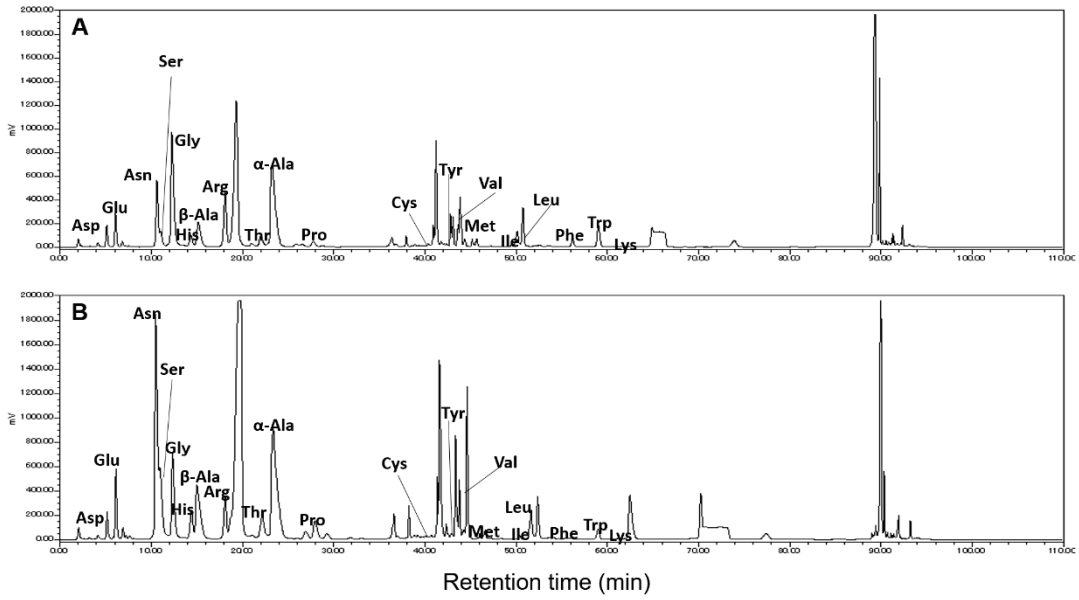


Fig. 7. A chromatogram of amino acids in the long-day bulb onion ‘Superkitamomiji’ (A), and the Indonesian shallot landrace ‘Bima Juna’ (B). The names of amino acids— listed based on their retention times— are aspartic acid (Asp); glutamic acid (Glu), asparagine (Asn), serine (Ser), glycine (Gly), β-alanine (β-Ala), glutamine (Gln), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), cysteine (Cys), tyrosine (Tyr), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), tryptophan (Trp), and lysine (Lys).

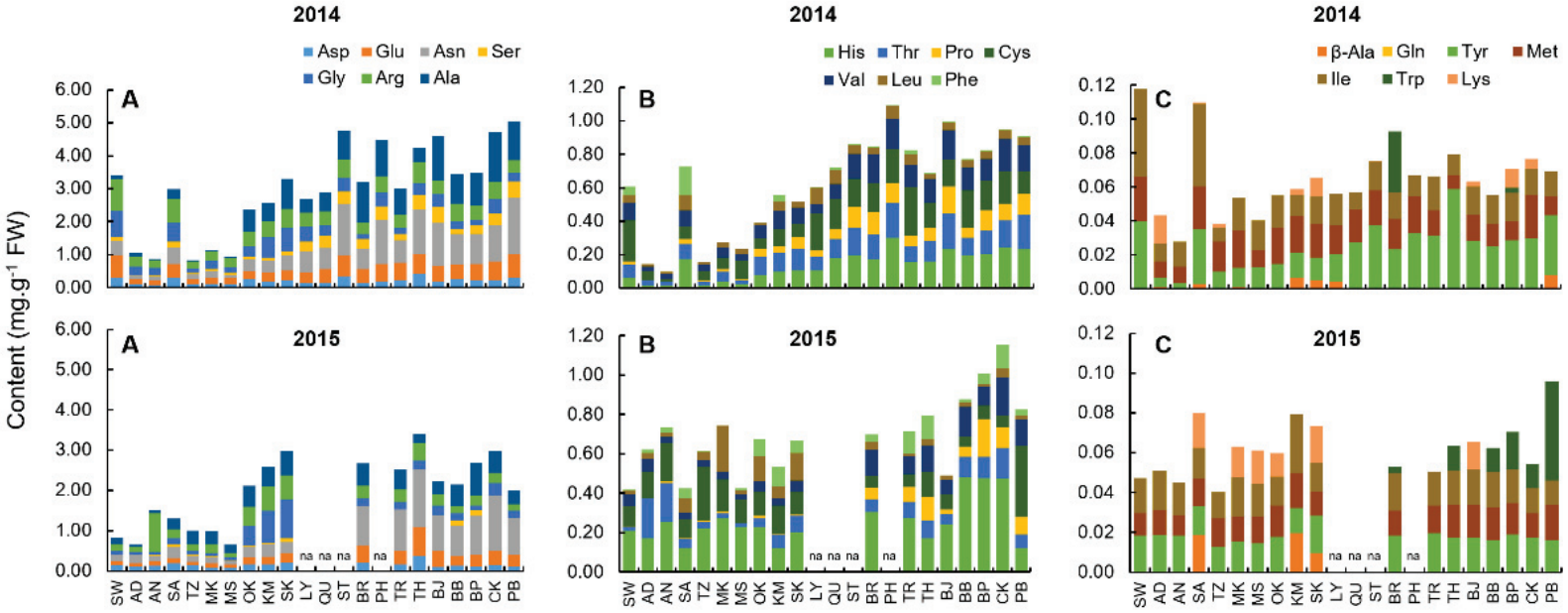


Fig. 8. Quantification of amino acid contents of bulb onions and shallots landraces in 2014 and 2015. Based on their quantity, there were high (A), average (B), and low (C) amounts of amino acids. na = not analyzed

S-alk(en)yl-L-cysteine sulfoxides (ACSOs) and their intermediate compounds

A different method, LC-MS/MS, was used to determine ACSOs—MeCSO, PCSO, AlCSO, and PeCSO—and some intermediate compounds related to their biosynthesis pathway. The intermediate compounds are *S*-methylglutathione (*S*-MG), *S*-2-carboxypropylglutathione (*S*-2-GPC), γ-glutamyl-*S*-2-carboxypropylcysteine (γ-G-S-*2*-CPC), glutathione (GSH), and γ-glutamylcysteine (γ-GC). On average, Vietnamese shallots produced significantly lower amounts of MeCSO than Indonesian shallots and both short-day and long-day bulb onions in 2014 (Table 4). In both years, all bulb onions and shallots produced low amount of PCSO and AlCSO. In 2014, the AlCSO was detected in almost all samples except two short-day bulb onions, 'Advance' and 'Answer,' while in 2015, AlCSO could not be detected in any bulb onions, short-day or long-day types (Table 5). In 2014, the PeCSO content detected in Indonesian shallots was significantly higher than in Vietnamese shallots, but no significant differences were found in the PeCSO content among bulb onion varieties. However, in 2015, the PeCSO content of long-day bulb onion cultivars was significantly higher than in short-day bulb onions and Indonesian shallots.

Table 4. Contents of ACSOs and their intermediate compounds (mg/g FW) in bulb onions and shallots in 2014

Variety or landraces	MCSO	PCSO	PeCSO	ALCSO	S-MG	S-2-CPG	γ-G-S-2-CPC
Onion short-day type							
'Shippo wase 7 go'	0.085	0.042	0.588	0.010	n.d	0.047	n.d
'Advance'	0.097	0.042	0.723	n.d	n.d	0.540	n.d
'Answer'	0.219	0.050	0.916	n.d	n.d	0.872	n.d
'Shippo Ama 70'	0.163	0.040	0.784	0.011	n.d	0.565	n.d
'Tarzan'	0.159	0.042	0.735	0.012	n.d	0.870	n.d
'Momijinokagayaki'	0.149	0.052	1.076	0.021	n.d	0.928	n.d
'Momiji 3 go'	0.208	0.052	1.008	0.022	n.d	0.973	n.d
Average	**0.154 a**[y]	**0.046 a**	**0.833 ab**	**0.011 a**		**0.717 b**	
Onion long-day type							
'Okhotsk 222'	0.182	0.043	0.779	0.030	0.004	1.469	n.d
'Kitamomiji 2000'	0.164	0.047	0.912	0.026	0.004	1.105	n.d
'Super Kitamomiji'	0.237	0.063	1.080	0.034	n.d	1.322	n.d
Average	**0.194 a**	**0.051 a**	**0.923 ab**	**0.030 a**	**0.0012 a**	**1.299 a**	
Vietnamese shallot							
'Lyson'	0.005	0.040	0.585	0.016	n.d	0.597	n.d
'Quangai'	0.030	0.044	0.684	0.017	n.d	0.864	n.d
'Soc trang'	0.164	0.079	1.085	n.d	0.001	1.323	0.037
Average	**0.067 b**	**0.055 a**	**0.785 b**	**0.011 a**	**0.0005 b**	**0.928 b**	**0.037 a**
Indonesian shallot							
'Biru'	0.110	0.045	0.877	1.319	0.002	1.191	0.040
'Philip'	0.199	0.072	1.373	n.d	n.d	1.615	0.041
'Tiron'	0.047	0.043	0.680	n.d	0.001	0.970	0.029
'Thailand'	0.253	0.105	1.484	0.022	n.d	1.190	0.041
'Bima Juna'	0.135	0.064	1.093	1.652	0.002	1.354	0.030
'Bauji Bunder'	0.186	0.052	1.026	n.d	n.d	1.215	0.025
'Bauji Plompong'	0.240	0.056	1.166	n.d	n.d	1.323	0.026
'Crok Kuning'	0.063	0.042	0.825	n.d	n.d	1.340	0.032
'Probolinggo'	0.350	0.098	1.395	0.025	n.d	1.711	0.047
Average	**0.176 a**	**0.064 a**	**1.102 a**	**0.348 a**	**0.0005 b**	**1.323 a**	**0.035 a**

[y] Means with the same letters in a column are not significantly different according to Tukey's test ($P < 0.05$)

n.d: not detected

In 2014, the *S*-MG was only detected in some samples—'Okhotsk 222,' 'Kitamomiji 2000,' 'Soc trang,' 'Biru,' 'Tiron,' and 'Bima juna'—but not detected in other samples (Table 4). Moreover, γ-G-*S*-2-CPC was only detected in Indonesian shallot landraces and one Vietnamese shallot, 'Soc trang.' The *S*-2-CPG in Indonesian shallot landraces and bulb onion long-day cultivars was significantly higher than in bulb onion short-day cultivars and Vietnamese shallot landraces. Slight differences were

found in the 2015 research results. *S*-MG and GSH were not detected in all shallot samplse but were traced in bulb onion samples. A significantly high amount of γ-GC was found in Indonesian shallot landraces, while only trace amounts were detected in bulb onion samples. Significantly higher amounts of *S*-2-CPG and γ-G-*S*-2-CPC were detected in Indonesian shallot landraces than in both bulb onion cultivars.

Regarding the intermediate compound of ACSO biosynthesis detected in the 2015 experimental year, there was a high correlation between the *S*-2-CPG and γ-G-*S*-2-CPC of bulb onions (r = 0.727**), whereas the correlation between them was low and insignificant in shallots (r = 0.317) (Table 6). Moreover, a highly significant correlation between γ-GC and γ-G-*S*-2-CPC was also detected in shallot samples (r = 0.569**), but no correlation was found in bulb onions, as γ-GC was only detected in trace amount.

Table 5. Contents of ACSOs and their intermediate compounds (mg/g FW) in bulb onions and shallots from 2015

Variety or landraces	MCSO	PCSO	PeCSO	ALCSO	S-MG	S-2-CPG	γ-G-S-2-CPC	GSH	γ-GC
Onion short-day type									
'Shippo wase 7 go'	trace	0.002	0.623	n.d.	trace	0.656	0.003	trace	trace
'Advance'	0.118	0.031	0.863	n.d.	trace	0.662	0.007	trace	trace
'Answer'	0.050	0.013	0.924	n.d.	trace	0.922	0.005	trace	trace
'Shippo Ama 70'	0.145	0.015	0.783	n.d.	trace	0.438	0.001	trace	trace
'Tarzan'	0.155	0.034	0.978	n.d.	trace	0.782	0.006	trace	trace
'Momojinokagayaki'	0.222	0.024	0.928	n.d.	trace	1.182	0.024	trace	trace
'Momiji 3 go'	0.244	0.051	1.345	n.d.	trace	1.064	0.011	trace	trace
Average	**0.131 a**[y]	**0.024 b**	**0.920 b**	—	—	**0.815 b**	**0.008 b**	—	—
Onion long-day type									
'Okhotsk 222'	0.172	0.038	1.249	n.d.	trace	0.640	0.009	trace	trace
'Kitamomiji 2000'	0.214	0.038	1.297	n.d.	trace	0.570	0.006	trace	trace
'Super Kitamomiji'	0.238	0.049	1.555	n.d.	trace	0.736	0.013	trace	trace
Average	**0.208 a**	**0.042 b**	**1.367 a**	—	—	**0.649 b**	**0.009 b**	—	—
Indonesian shallot									
'Biru'	0.302	0.064	0.447	0.009	n.d.	1.486	0.015	n.d.	0.008
'Tiron'	0.347	0.087	0.488	0.012	n.d.	1.695	0.021	n.d.	0.010
'Thailand'	0.327	0.075	0.525	0.008	n.d.	0.482	0.012	n.d.	0.004
'Bima Juna'	0.077	0.070	0.548	0.009	n.d.	1.339	0.025	n.d.	0.011
'Bauji Bunder'	0.130	0.046	0.462	0.010	n.d.	2.549	0.012	n.d.	0.008
'Bauji Plompong'	0.069	0.026	0.215	0.010	n.d.	1.546	0.006	n.d.	0.004
'Crok Kuning'	0.147	0.042	0.372	0.009	n.d.	2.024	0.013	n.d.	0.004
'Probolinggo'	0.191	0.072	0.716	0.007	n.d.	1.850	0.023	n.d.	0.005
Average	**0.199 a**	**0.060 a**	**0.472 c**	**0.009 a**	—	**1.621 a**	**0.016 a**	—	**0.007**

[y] Means with the same letters in a column are not significantly different according to Tukey's test ($P < 0.05$)
n.d: not detected

Table 6. The Pearson correlation among ACSO content and their intermediate compounds of bulb onions and shallots from 2015 data

Atrribute		PCSO	PeCSO	AlCSO	S-2-CPG	γ-G-S-2-CPC	γ-GC
MeCSO	Onion	0.783**	0.704**	—	0.166	0.491**	—
	Shallot	0.733**	0.418	0.359	-0.024	0.316	0.200
PCSO	Onion		0.889**	—	0.128	0.333	—
	Shallot		0.825	0.374	0.137	0.759**	0.532**
PeCSO	Onion			—	0.113	0.306	—
	Shallot			0.125	0.369	0.812**	0.368
AlCSO	Onion				—	—	—
	Shallot				0.416*	0.186	0.464*
S-2-CPG	Onion					0.727**	—
	Shallot					0.317	0.225
γ-G-S-2-CPC	Onion						—
	Shallot						0.569**

* significant at $P < 0.05$ and ** significant at $P < 0.01$

Discussion

Bulb onions and shallots were elucidated to have different amounts of phytochemicals, which leads to their unique flavor and specific taste. In our previous report, we could clearly discriminate between bulb onions and shallots based on their phytochemical contents (Ariyanti et al., 2017). Bulb onions generate a lower amount of PeCSO and flavonoid than shallots. Moreover, bulb onions produce more monosaccharides, while shallots produce large quantities of polysaccharides. Basically, those differences are controlled by many factors, including their genetic differences, environmental conditions, and stresses leading to their different metabolic pathways and products (Bernaert et al., 2012; Speiser et al., 2015).

In general, amino acids were translocated from senescing foliage to the bulb (Nilsson, 1980). In addition to ACSOs, amino acid content is also known to be responsible for most of the food's taste, including sweetness, sourness, bitterness, saltiness, and umami (Nishimura and Kato, 1988). In this study, there were seven high content amino acids, included aspartic acid, glutamic acid, asparagine, serine, glycine, arginine, and alanine, as also reported by Kuon et al. (1963) and Matikkala et al. (1967). However, their contents were found to be higher in shallots than in bulb onions. Schiffman et al. (1981) compared and described the taste quality and thresholds of all kinds of amino acids. Aspartic acid, glutamic acid, and asparagine were described as having a unique, meaty, and salty taste, known to be *umami* or "delicious." Serine, glycine, alanine, and proline are amino acids that contribute to a sweet taste; whereas histidine, arginine, threonine, cysteine, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, tryptophan, and lysine are free amino acids responsible for a

bitter taste. In total, shallots produce a higher amount of amino acids than do bulb onions. Genetic factors and environmental conditions could be the two main factors affecting amino acid production in bulb onions and shallots (Lancaster and Boland, 1990). Although the significance of amino acids' effect on how bulb onions and shallots taste is still unknown, the higher amino acid content of shallots, together with ACSOs and other intermediate compounds, seems to make shallots more valuable as a new source of food-enhancing compounds.

Moreover, the non-volatile sulfur storage compounds, called *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs), will generate volatile chemicals through a process of enzymatic hydrolysis during cell destruction (Rose et al., 2005). Those volatile compounds are very important for generating the specific and unique taste of *Allium* species, including bulb onions and shallots. Ariyanti et al. (2017) reported that MeCSO and PeCSO became the main compound in bulb onions and shallots, while AlCSO was detected in a very small amount. Furthermore, the PeCSO produced by shallots was not significantly higher than that of bulb onions. A different tendency was found for MeCSO, which was detected at higher levels in shallots than in bulb onions. However, the PeCSO content in long-day bulb onions was significantly higher than in short-day bulb onions. In this current research, we analyzed ACSOs—MeCSO, PCSO, AlCSO, and PeCSO— using the LC-MS/MS method. The results indicate the same tendency, where AlCSO was hardly found in bulb onions and a small amount was detected in shallots, for both years. An interesting result was found for PCSO, where it could be detected in all bulb onions and shallot samples, and shallots produced significantly higher amounts than did bulb onions in 2015. The same tendency was also reported by Randle (1995) where PCSO was detected in a lower amount in three different S fertility

stages. PCSO was barely detected when using HPLC analysis, as also reported by Vu et al. (2012).

The different amounts of ACSOs in bulb onions and shallots are strongly related to their ACSO biosynthesis pathways. γ-glutamylcysteine (γ-GC) and glutathione (GSH), the starting compounds (Rose et al., 2005) in the biosynthetic precursor, were barely detected in bulb onions and shallot samples. This could have happened because most of the contents were used to produce ACSOs in both bulb onions and shallots. The remaining GSH and γ-GC detected in bulb onions was evidence that not all ACSO starting compounds could be synthesized, possibly as a result of less effective biosynthesis in bulb onions than in shallots. Furthermore, the methylation of GSH produces *S*-methylglutathione (*S*-MG), called line 1, and a decarboxylation process will turn it into γ-glutamyl-*S*-1-methyllcysteine. At the same time *S*-2-carboxypropylglutathione (*S*-2-CPG) could be generated from a reaction of GSH and methylacrylate. Later on, an intermediate reaction changes it into γ-glutamyl-*S*-2-carboxypropylcysteine (γ-G-*S*-2-CPC), and we call it line 2. Line 3 was a reaction between γ-GC and methylacrylate to produce γ-G-*S*-2-CPC. ACSOs in *Allium* species form this compound via decarboxylation, oxidation, and a reaction with glutamyl transpeptides (Blok, 1992; Rose et al., 2005).

In this study, we first reported differences in the metabolic pathways of the ACSOs of bulb onions and shallots, especially for the γ-G-*S*-2-CPC biosynthesis. Bulb onion go through line 2, as the correlation between *S*-2-CPG and γ-G-*S*-2-CPC detected was significantly high, whereas in shallots, γ-G-*S*-2-CPC was synthesized via line 3, with the role of methylacrylate from γ-GC. In these cases, the biosynthesis of ACSOs in onions may mostly happen via glutathione biosynthesis, while in shallots, it is

biosynthesized directly from γ-GC. Previous reports on shallots double haploid (DHA) and onion double haploid (DHC) growth in abiotic stress conditions have revealed that metabolic traits and their related genes were found to be more highly interconnected in DHA than in DHC (Abdelrahman et al., 2015). This means that, in this case, shallots were more tolerant of abiotic stress conditions than were bulb onions. The fact that the shallot landraces exploited in this study were cultivated in the dry season (June to September) is a legitimate explanation for that. During that sort of stress, plants try to save energy by using a shorter biosynthesis pathway. ACSOs biosynthesis increases linearly in response to temperature, indicating that ACSOs are important for defense compounds (Randle et al., 1993; Coolong and Randle, 2003). In this case, Indonesian shallots that were cultivated in a dry season chose to take a shorter ACSO biosynthetic pathway rather than the long one as chosen by bulb onions. This action gave more advantages for balancing its growth rate and survival capacity by saving more energy.

The purpose of our experiments was to analyze differences in amino acids between bulb onions and shallots, as they possess different characteristics in some phytochemical compounds (Ariyanti et al., 2017), and to discover any differences in ACSO biosynthesis. In line with other phytochemical compounds, such as sugars and flavonoids, shallots also produce higher amounts of free amino acids than do bulb onions. Both environmental differences during the cultivation process and their genetic backgrounds might play the same role in the process of metabolizing phytochemicals. The results of this experiment prove that there are differences between the ACSO biosynthesis of bulb onions and shallots, especially γ-glutamyl-*S*-2-carboxyprpylcysteine synthesis, where bulb onions took a long way by glutathione biosynthesis rather than the more direct γ-glutamylcysteine route used by shallots. The

reason shallots take a shorter pathway is highly related to their abiotic stress resistance ability as reported before by Abdelrahman et al. (2015). However, further investigation using the radiolabeled method would be needed to build a clear understanding of the differences between the ACSO biosynthesis of bulb onions and shallots.

Chapter 4. MORPHOLOGYCAL AND PHYTOCHEMICALS CHARACTERIZATION OF HEAT-TOLERANT CULTIVARS IN THE JAPANESE BUNCHING ONION (*ALLIUM FISTULOSUM* L.)

Introduction

Climate change, as a result of global warming, influences a number of agricultural activities in direct and indirect ways. The elevated CO_2 level, precipitation, air humidity, potential evaporation, temperature, and solar radiation are all important climatic conditions required for plant growth (Rötter and van de Geijn, 1999; Ryan, 1991). An extreme condition in one or some of them could induce an environmental stress called abiotic stress. Abiotic stress decreased the photosynthesis rate in tomatoes (Camejo et al., 2005), St. John's wort (Zobayed et al., 2005), and wheat (Almeselmani et al., 2006). Atkinson and Urwin (2012) reported a 50% plant-yield reduction induced by abiotic stress for most major crop plants, including the *Allium* species.

The Japanese bunching onion (JBO; *Allium fistulosum* L.) is one of the most famous *Allium* species; it is used in many countries, especially in East Asia (Inden and Asahira, 1990), to enhance the taste of food and is consumed for its health benefit (Chen et al., 1999; Štajner et al., 2006). In Japan, annual production of the JBO in 2014 (482.900 MTN) was almost half of that of bulb onions (Ministry of Agriculture, Forestry and Fisheries). Japanese plant breeders and producers could recognize two types of JBOs, leeks and chives, called nebuka-negi and ao-negi, respectively. There are three main production areas for ao-negi in Japan: Fukuoka, Shizuoka, and Chiba prefectures. Moreover, the optimum temperature for growth and development of the JBO is around 15–20°C (Brewster, 2008). A problem will arise during summer

cultivation in Japan, when the temperatures increase drastically, over 30°C in the daytime. The heat and UV radiation will cause crop damage, especially in morphological appearance, which causes a reduction in yield and quality (Wahid et al., 2007). The physiological process of plants is also affected by environmental stress.

Heat stress is known to cause the accumulation of secondary metabolites in plants (Wahid et al., 2007), such as the phenolic compound including flavonoids and phenylpropanoids (Rivero et al., 2001). The elevation of antioxidant enzyme activities was found, together with the accumulation of secondary metabolites such as α-tocopherol, carotenoid, glutathione, and ascorbic acid, to minimize cell damage from various reactive oxygen species (ROS) (Nakabayashi and Saito, 2015; Ramakrishna and Ravishankar, 2011). Recently, a Japanese breeding company has been trying to develop new cultivars suitable for summer cultivation. However, the response of those cultivars to heat stress seems unclear based on their morphological and phytochemical characteristics. Our research output will be beneficial for breeders and farmers when determining suitable cultivars for their own purposes, especially for summer cultivation. In order to study the effect of summer cultivation on the morphological and phytochemical characteristics of several JBO summer cultivars, we conducted an examination in 2013 and 2014.

Materials and Methods

Plant materials

The plant materials were grown under a plastic house at Kurume Research Station, Kyushu Okinawa Agricultural Research Center (NARO/KARC) Japan (33°19′N, 130°31′E), in the summers of 2013 and 2014. The average daily temperature and UV radiations during cultivation are shown in Fig. 9. The dates of sowing and harvesting for cultivars used in this study are shown in Table 7. In each year, there were three different cultivation times: early, middle, and late summer. From those different cultivation times, we collected 10 plants randomly for morphological observations and phytochemical analyses. The names of the cultivars were not all similar in each year due to the selection process.

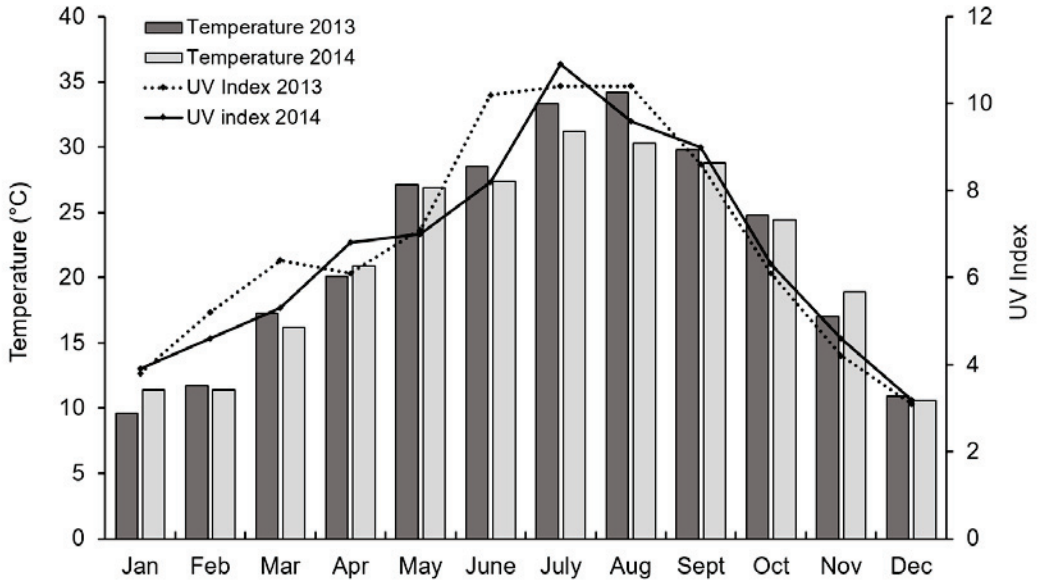


Fig. 9. Average monthly temperature and UV index of the Kurume area in 2013 and 2014, collected by the Japan Meteorological Agency (jma.go.jp).

Table 7. Cultivars name, date of sowing and harvesting of Japanese bunching onion cultivated in summer 2013 and 2014

Year	Cultivar	Date					
		Early		Middle		Late	
		Sowing	Harvesting	Sowing	Harvesting	Sowing	Harvesting
2013	'Fuyuhiko' 'Kuronegi' 'Sanpeinegi' 'Kaminari'	27-May	5-Aug	10-Jun	19-Aug	24-Jun	4-Sep
2014	'Fuyuhiko'					−	−
	'Kuronegi'						
	'Kaminari'						
	'Kaminari II'					−	−
	'NE 40'	11-Jun	25-Aug	26-Jun	17-Sep	28-Jul	9-Oct
	'NE 15'	−	−	−	−		
	'NE 71'	−	−	−	−		
	'YSG'	−	−	−	−		
	'Fukuichi'	−	−	−	−		

− : not carried out

Morphological observation

All morphological parameters were evaluated from 10 plants in each replication. The total fresh weight (TFW) was measured using a digital balance directly after harvesting. The upper leaf-tip burns (UPTB) and under leaf-tip burns (UDTB), leaf color (LC), thrips damage (TD), total length (TL), white-part length (WPL), and white-part diameter (WPD) were measured after simple cleaning. The upper leaf-tip burns were measured at the second leaf from the top and the under leaf-tip burns at the oldest leaf using a damage rating score: 0 = no damage, 0.5−0.9 = light damage, 1−2 = in damage, >2 = serious damage. The SPAD-502Plus (Konica Minolta Inc., Tokyo, Japan) was used to measure the green leaf color. The thrips damage was quantified by adjusting the symptoms of insect damage via the usage of silver-gray blotches on the leaf sheet, and same scoring system was used as with the leaf-tip burns. After morphological

assessment, all the samples were transported to Yamaguchi University for phytochemical analyses.

Extraction and determination of ascorbic acid content

The ascorbic acid content was quantified as described by Yaguchi et al. (2008) with a slight modification. Five grams of JBO leaf blades was mashed with 10 mL of 10% metaphosphoric acid. The pulp was then filtrated through filter paper (ADVANTEC, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) by adding 35 mL of distilled water. One milliliter of the filtrate was used for analysis using the 2,4-dinitrophenylhydrazine method (Roe et al., 1948). The experiment was separated into two blocks, an oxidized ascorbic acid block and a total ascorbic acid block. For the oxidized ascorbic acid, two drops of 0.2% 2,6-dichloroindophenol sodium solution were mixed with a 1 mL sample. Another 1 mL of 2% metaphosphoric acid and 1 mL of thiourea were added to the mixture before incubating for 70 min at 50°C. After the incubation process, samples were added by 2.5 mL of 85% H_2SO_4. Then the blank tubes were added by 0.5 mL of 2,4-dinitrophenylhydrazine as the sample tubes was added before incubation. The ascorbic acid contents were quantified according to their absorbance at a 530 nm wavelength. To obtain the main values, all chemical extractions were prepared for three biological replicates. Each extraction was applied to a chemical determination three times.

Extraction and determination of phenolic content

The phenolic analysis was conducted by using the Folin and Denis (1915) method with a slight modification. One milliliter of the hot 70% ethanol extract was diluted five times with distilled water. Next, 1 mL of the diluted sample solution was added to 1 mL of a 1 N phenol reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan) before incubation for 3 min. After 3 min, 1 mL of 10% sodium carbonate was added and kept for one hour at room temperature. Spectrophotometric analysis at a wavelength of 530 nm was performed using a HITACHI U-2001 spectrophotometer (HITACHI High-Technologies Corporation, Tokyo, Japan). For each sample, there were three biological replications, and each was analyzed three times.

Extraction and determination of flavonoid content

The total flavonoid content was determined via a colorimetric method using a hot 70% ethanol extract. The extraction methods were applied as described by Vu et al. (2013). Fifty microliters of the extract sample was taken to analyze with 50 µL of 70% ethanol and 200 µL of 2% aluminum chloride. The mixture was thoroughly homogenized by pipetting in the micro plate and incubated in the dark for one hour before analysis. Different concentrations of quercetin, 5, 10, 20, and 40 $\mu L.mL^{-1}$, were used as standards. Moreover, 100 µL of 70% ethanol and 200 µL of 2% aluminum chloride solution were used as blanks. The total flavonoid content was quantified using an iMark Microplate Reader (serial number 16548; BIO-RAD Laboratories, Tokyo, Japan) at 420 nm, and the data was read by Microplate Manager 6. To obtain the mean values, all chemical extractions were prepared for three replications. Each extraction was applied to triplicate chemical determinations.

Measurement of antioxidant capacity

The total antioxidant capacity of JBO heat-tolerant varieties was assessed based on the scavenging activity of the chemically stable 2,2-dyphenyl-1-picrylhidrazyl (DPPH) free radical antioxidant (Blois, 1958). The assessment used 8-, 24-, and 40-times dilutions of the sample using 70% ethanol. A mixture of 30% ethanol, 2-morpholinoenaesulfonic acid (MES), and DPPH was used as the blank. The mixture of the sample with MES and sample with a mixing solvent was used as the control. The determination was performed by an iMark Microplate Reader (serial number 16548; BIO-RAD Laboratories, Tokyo, Japan) at 520 nm, and the data was read by Microplate Manager 6. The DPPH radical scavenging activity was expressed as IC_{50}^{-1}.

Extraction and determination of ACSO content

The green part of each JBO heat-tolerant variety (2–5 g) was used for water extraction as described by Vu et al. (2013). The 10-times dilution of the sample was used for HPLC analysis to determine the *S*-alk(en)yl-L-cystein sulfoxide (ACSO) content. One milliliter of the diluted sample was filtered using a disposable membrane filter (Dismic®-13 HP ADVANTEC, Toyo Roshi Kaisha, Ltd.). The HPLC system included a pump, a degasser, a column oven, a diode array detector set (220 nm, HITACHI L-2450; HITACHI High-Technologies Corporation, Tokyo, Japan), a data collection system (EZchrom Elit™; HITACHI High-Technologies Corporation, Tokyo, Japan), and an AQUASIL column (4.6 mm Ø x 25 cm long). The solvent was 0.005% trifluoroacetic acid (TFA), flowed for 15 min at a rate of 0.6 mL/min. Twenty

$mg.mL^{-1}$ of 1-propil CSO and a mix of allyl (Al) and propenyl (Pe) CSO, produced by House Foods Corporation, Japan, was used as the standard.

Statistical analysis

An *F*-test followed by Tukey's honest significant difference (HSD) test was used to analyze the collected data. Correlations among morphological and phytochemical variables were done to study their interdependence. A principal component analysis (PCA) was used to discriminate between the JBO cultivars tested in this study. All of the statistical analyses were performed using IBM SPSS Statistics 19 (IBM, New York, NY, USA).

Results

Morphological condition

All of the morphological characteristics mentioned in the materials and methods were evaluated by a triplicate examination in early, mid, and late summer cultivation in each year, 2013 (Table 8) and 2014 (Table 9). In 2013, ‘Kuronegi’ had significantly more leaf damage than other cultivars, as shown in its UPTB scores (0.63 in early and 0.67 in mid-summer cultivations). These scores showed that ‘Kuronegi’ had a moderate leaf-tip burn area as compared with other cultivars, which had less leaf-tip burn. The UPTB and UDTB scores increased in mid-summer and then decreased in late summer. The same tendency was found in the experimental year of 2014.

In the summer cultivation of JBO cultivars, the leaf color (LC) values varied from 50.47 to 64.90 in 2013 and from 36.68 to 48.59 in 2014. In 2013, ‘Kuronegi’ and ‘Kaminari’ showed lower LC values than ‘Fuyuhiko’ and ‘Sanpeinegi’ at three different cultivation times, as shown in Table 8. No significant difference in LC values was detected between any cultivars for two terms (early and mid-summer) in 2014. Moreover, there was no significant difference in thrips damage (TD) values between any of the JBO cultivars throughout two years, except in the late summer of 2013. On average, the TD score was less than 0.5, indicating relatively light damage by thrips attack.

For the yield components, there were no significant differences in the total length (TL) of leaves among all cultivars in three different cultivation times throughout two years. The same tendency was found on the white-part length (WPL) of the JBO leaf or leaf sheath length. In 2013, the white-part diameter (WPD) of the leaf varied from 5.92 to 8.47 mm. However, the WPD of ‘Kaminari’ was always significantly higher

than of ‘Fuyuhiko.’ The same tendency was observed in the total fresh weight (TFW) of the above-ground part over two years. In 2013, ‘Kaminari’ produced a significantly higher TFW (21.91 g) than ‘Fuyuhiko’ did (15.42 g) in the early summer cultivation. In 2014, ‘Kaminari’ and ‘Kaminari II’ produced a higher TFW (10.99 g and 10.66 g, respectively) than other cultivars, and the fresh weight was reduced in mid-summer. New cultivars tasted in 2014 (‘NE 40,’ ‘NE 15,’ ‘NE 71,’ ‘YSG,’ and ‘Fukuichi’) produced a moderate range of TFW from 5.13 g to 7.63 g.

Table 8. Morphological traits of Japanese bunching onion cultivars cultivated in summer 2013

Cultivars' name	Upper leaf-tip burns	Under leaf-tip burns	Leaf color	Thrips' damage	Total length (cm)	White-part length (cm)	White-part diameter (mm)	Total fresh weight (g)
Early summer								
'Fuyuhiko'	0.23±0.08[y]bc[z]	0.73±0.09 ab	62.19±1.28 a	0.38±0.05 a	61.02±1.34 a	1.63±0.07 ab	7.57±0.15 b	15.42±0.77 b
'Kuronegi'	0.63±0.08 a	0.87±0.05 a	57.24±1.10 b	0.43±0.08 a	57.63±1.10 a	1.51±0.13 b	8.23±0.21 a	19.52±1.25 ab
'Sanpeinegi'	0.08±0.04 c	0.50±0.07 b	62.51±0.59 a	0.50±0.05 a	57.27±0.85 a	1.69±0.12 ab	7.68±0.21 b	15.13±1.06 b
'Kaminari'	0.48±0.08 b	0.79±0.10 a	57.08±0.92 b	0.61±0.06 a	57.84±1.13 a	2.02±0.18 a	8.47±0.25 a	21.91±1.35 a
Mid summer								
'Fuyuhiko'	0.18±0.07 b	0.53±0.08 b	64.90±0.70 a	0.30±0.06 a	47.86±0.89 a	0.91±0.07 a	5.92±0.14 c	7.21±0.31 c
'Kuronegi'	0.67±0.06 a	0.93±0.03 a	56.00±1.23 b	0.45±0.06 a	51.21±0.96 a	1.00±0.08 a	6.90±0.11 b	10.93±0.38 b
'Sanpeinegi'	0.58±0.08 a	0.95±0.10 a	59.23±0.97 b	0.40±0.06 a	42.68±0.78 b	1.04±0.10 a	6.30±0.15 b	8.04±0.38 c
'Kaminari'	0.53±0.07 a	0.92±0.03 a	58.68±0.89 b	0.48±0.04 a	47.45±1.00 a	0.87±0.06 a	7.81±0.16 a	12.75±0.47 a
Late summer								
'Fuyuhiko'	0 a	0.32±0.06 c	56.67±0.97 a	0.23±0.06 ab	41.79±0.76 a	1.17±0.09 a	5.95±0.13 b	8.19±0.40 b
'Kuronegi'	0.05±0.03 a	0.60±0.07 ab	51.19±0.71 ab	0.42±0.05 a	45.04±0.88 a	1.27±0.05 a	6.52±0.19 ab	12.39±0.82 a
'Sanpeinegi'	0 a	0.50±0.05 b	50.47±0.67 ab	0.18±0.04 b	44.13±1.40 a	1.16±0.09 a	6.72±0.17 a	12.32±1.05 a
'Kaminari'	0 a	0.65±0.06 a	53.08±0.61 b	0.28±0.06 ab	42.86±0.80 a	1.27±0.10 a	6.82±0.25 a	14.68±0.70 a

[z] Means with the same letter in a column are not significantly different according to Tukey's test ($P<0.05$).

[y] Each value is the mean ± SE (n = 3).

Table 9. Morphological traits of Japanese bunching onion cultivars cultivated in summer 2014

Cultivars' name	Upper leaf-tip burns	Under leaf-tip burns	Leaf color	Thrips' damage	Total length (cm)	White-part length (cm)	White-part diameter (mm)	Total fresh weight (g)
Early summer								
'Fuyuhiko'	0.92±0.24[y]a[z]	0.53±0.10 a	46.48±0.53 a	0.28±0.04 a	41.38±1.02 a	1.42±0.14 a	5.13±0.14 a	6.27±0.35 a
'Kuronegi'	1.32±0.04 a	0.63±0.08 a	43.26±0.24 a	0.23±0.01 a	44.61±2.59 a	1.49±0.07 a	5.49±0.40 a	7.66±1.05 a
'Kaminari'	1.00±0.02 a	0.38±0.01 a	46.65±0.83 a	0.22±0.04 a	50.71±4.48 a	1.56±0.12 a	5.93±0.23 a	10.99±2.27 a
'Kaminari II'	0.83±0.11 a	0.28±0.07 a	48.59±0.54 a	0.37±0.07 a	47.89±8.49 a	1.21±0.27 a	6.24±0.55 a	10.66±3.29 a
'NE 40'	0.72±0.09 a	0.55±0.18 a	42.09±0.99 a	0.28±0.04 a	39.38±3.02 a	1.18±0.26 a	6.17±0.30 a	7.79±1.17 a
Mid summer								
'Fuyuhiko'	0.37±0.02 a	0.98±0.13 a	45.61±1.99 a	0.32±0.16 a	37.99±0.91 a	1.37±0.18 a	4.04±0.15 b	3.25±0.32 b
'Kuronegi'	0.53±0.07 a	0.97±0.08 a	46.02±0.23 a	0.18±0.03 a	40.39±3.15 a	1.17±0.10 a	4.66±0.20 ab	5.25±0.69 ab
'Kaminari'	0.60±0.07 a	0.94±0.03 a	41.47±0.45 a	0.15±0.02 a	41.35±3.59 a	1.36±0.08 a	4.53±0.18 ab	5.16±0.62 ab
'Kaminari II'	0.15±0.02 a	0.92±0.04 a	46.82±0.28 a	0.22±0.10 a	43.31±2.02 a	1.33±0.14 a	5.21±0.36 a	6.75±1.09 a
'NE 40'	0.33±0.09 a	0.92±0.14 a	44.37±1.32 a	0.17±0.08 a	38.84±1.71 a	1.32±0.11 a	5.35±0.11 a	5.70±0.30 ab
Late summer								
'Kuronegi'	0.18±0.09 a	0.99±0.06 a	39.41±0.30 b	0.08±0.01 a	37.78±0.66 a	1.37±0.27 a	4.94±0.44 a	4.84±0.70 a
'Kaminari'	0.28±0.01 a	0.73±0.06 a	41.16±0.81 ab	0.08±0.06 a	40.55±1.73 a	1.24±0.21 a	5.07±0.34 a	6.23±1.21 a
'NE 40'	0.67±0.06 a	0.88±0.12 a	40.08±0.44 b	0.00±0.00 a	34.60±1.77 a	1.32±0.10 a	4.85±0.15 a	5.13±0.10 a
'NE 15'	0.05±0.02 a	0.53±0.16 a	42.82±2.68 ab	0.22±0.16 a	42.31±4.70 a	0.98±0.25 a	6.17±0.76 a	7.80±1.36 a
'NE 71'	0.00±0.00 a	0.98±0.01 a	37.81±2.19 b	0.03±0.01 a	36.56±1.33 a	1.12±0.01 a	5.68±0.24 a	6.69±0.19 a
'YSG'	0.00±0.00 a	0.68±0.22 a	52.72±3.03 a	0.13±0.01 a	34.34±0.97 a	0.69±0.23 a	5.30±0.46a	5.35±0.53 a
'Fukuichi'	0.02±0.01 a	0.42±0.16 a	36.68±0.02 b	0.03±0.01 a	42.86±1.47 a	1.31±0.02 a	5.34±0.66 a	7.63±1.31 a

[z] Means with the same letter in a column are not significantly different according to Tukey's test ($P<0.05$).

[y] Each value is the mean ± SE (n = 3).

Phytochemical contents

The enhancement of some antioxidant metabolites such as ascorbic acid, flavonoids, and total phenol was observed in mid-summer (Fig. 10). The antioxidative activity indicated by the IC_{50}^{-1} value of DPPH radical scavenging capacity also increased during mid-summer. In the mid-summer of 2013, a significantly lower amount of ascorbic acid (0.38 $mg.g^{-1}$ FW) was found in 'Sanpeinegi.' Furthermore, 'Kuronegi' produced a significantly lower amount of flavonoids (0.24 $mg.g^{-1}$ FW) than other cultivars. In both the early and mid-summer of 2014, relatively high levels of ascorbic acid (0.37 $mg.g^{-1}$ FW), flavonoid (0.23 $mg.g^{-1}$ FW), and phenolic (0.78 $mg.g^{-1}$ FW) contents were observed in 'Kaminari.' In the late cultivation of 2014, such a high level of ascorbic acid (0.41 $mg.g^{-1}$ FW), flavonoid (0.72 $mg.g^{-1}$ FW), and phenolic (0.72 $mg.g^{-1}$ FW) contents was observed in 'YSG.'

Three different ACSOs—*S*-methyl-L-cysteine sulfoxide (MeCSO), *S*-2-propenyl(allyl)-L-cysteine sulfoxide (AlCSO), and *S*-1-propenyl-L-cysteine sulfoxide (PeCSO)—were detected on the JBO cultivars in 2013 and 2014 (Table 10). The contents of AlCSO were extremely low in comparison with other two compounds. In 2013, 'Kuronegi' produced a significantly higher PeCSO content than other cultivars in early and late summer. Further, very low amounts of MeCSO and PeCSO were detected in 'Fuyuhiko.' The increase in these two ACSOs could not be detected in mid-summer. Interestingly, in mid-summer, the ACSO accumulation decreased in 'Kuronegi.'

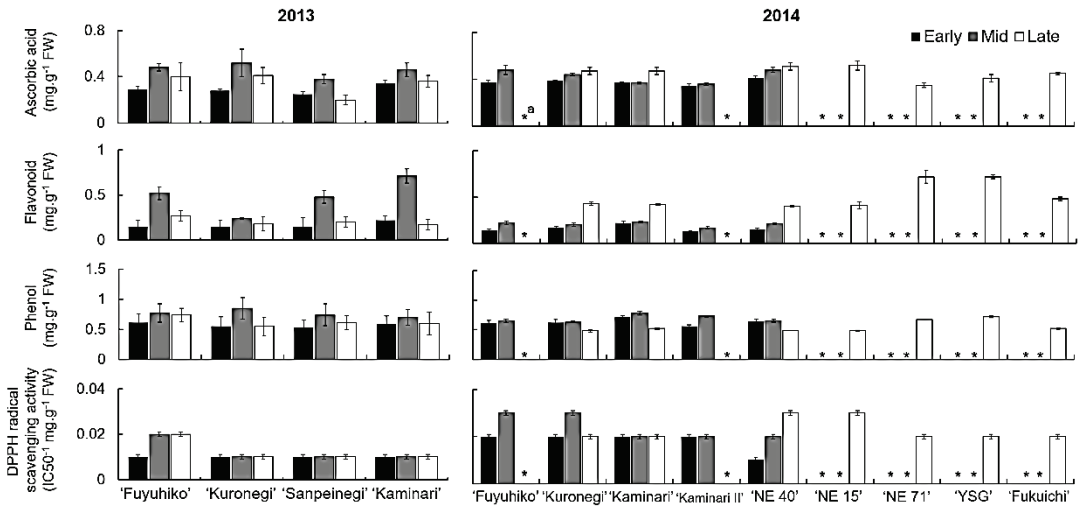


Fig. 10. Ascorbic acid, flavonoid, and phenolic contents of heat-tolerant JBO cultivars as well as their DPPH radical scavenging activity in 2013 and 2014. Each value is the mean ± SE of three replications. a = Not carried out

Correlations between morphological and phytochemical traits

A simple Pearson correlation test was used to determine the interrelationship between morphological and phytochemical traits of heat-tolerant JBO cultivars (Table 11). In 2013, the radical scavenging activity of mid-summer JBO cultivation was discovered to have a significant negative correlation with leaf-tip burn (r = -0.603*) and white-part diameter (0.618*). Furthermore, a significant correlation between MeCSO and the UPTB (0.628*) and UDTB (0.622*) was also observed. The total fresh weight was found to have a negative correlation with ascorbic acid (-0.497), flavonoid (-0.491), and phenolic (-0.698*) contents and DPPH radical scavenging activity (-0.574). Figure 11 shows the correlation between each antioxidant metabolite—ascorbic acid (0.365), flavonoids (0.277), and phenol (0.461)—and the antioxidant activities in 2013. All of the antioxidant metabolites showed a positive correlation with the antioxidant activity. The same tendency was observed in the following year (data

not shown), where the DPPH radical scavenging activity showed a positive correlation with ascorbic acid (0.310), flavonoid (0.077), and phenolic (0.306) contents.

In 2014, the flavonoid content in JBO showed a significant negative correlation with leaf-tip burn (-0.693*). Moreover, the flavonoid content indicated has a significant negative correlation with thrips damage (-0.605*) and white-part length (-0.543*). In opposition to the 2013 data, the MeCSO content shows a significant negative correlation with the UPTB (-0.538*) and UDTB (-0.484*). The total fresh weight of the observed JBO had a significant correlation with radical scavenging activity (0.503*).

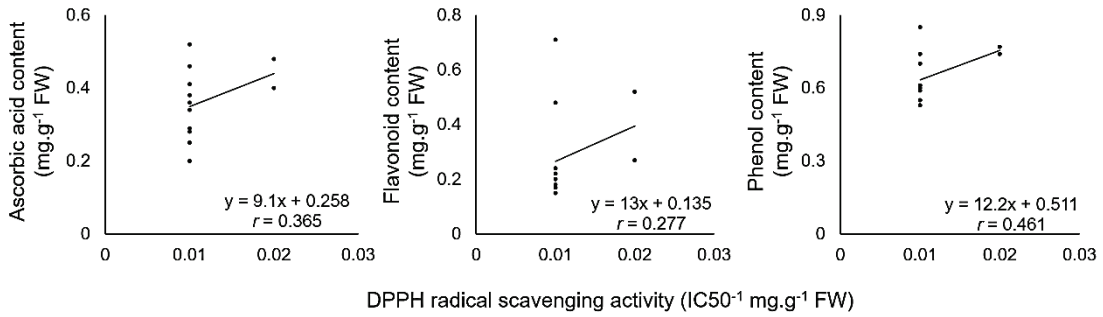


Fig. 11. Relationship between DPPH radical scavenging activity and phenol content of heat-tolerant JBO cultivars in 2013.

Table 10. Three kind of ACSOs (MeCSO, AlCSO and PeCSO) content (mg.g^{-1} FW) of JBO cultivated early, mid and late of summer 2013 and 2014

Cultivar	2013			Cultivar	2014		
	MeCSO	AlCSO	PeCSO		MeCSO	AlCSO	PeCSO
Early summer				*Early summer*			
'Fuyuhiko'	0.23±0.05[y]a[z]	0.02±0.01 b	0.65±0.01 b	'Fuyuhiko'	0.12±0.04 a	0.05±0.02 a	1.36±0.27 ab
'Kuronegi'	0.26±0.04 a	0.08±0.01 a	1.05±0.01 a	'Kuronegi'	0.09±0.02 a	0.09±0.02 a	1.27±0.07 ab
'Sanpeinegi'	0.16±0.02 a	0.01±0.01 b	0.64±0.01 b	'Kaminari'	0.10±0.03 a	0.05±0.01 a	1.04±0.19 b
'Kaminari'	0.21±0.06 a	0.01±0.01 b	0.83±0.01 b	'Kaminari II'	0.08±0.02 a	0.06±0.00 a	0.98±0.07 b
				'NE 40'	0.11±0.01 a	0.06±0.00 a	1.90±0.11 a
Mid summer				*Mid summer*			
'Fuyuhiko'	0.28±0.02 a	0.10±0.01 a	0.73±0.13 a	'Fuyuhiko'	0.09±0.01 c	0.04±0.01 a	0.62±0.14 a
'Kuronegi'	0.19±0.03 b	0.04±0.01 b	0.68±0.07 a	'Kuronegi'	0.17±0.03 ab	0.06±0.02 a	0.66±0.10 a
'Sanpeinegi'	0.23±0.02 ab	0.05±0.01 b	0.73±0.06 a	'Kaminari'	0.17±0.01 a	0.01±0.01 a	0.80±0.04 a
'Kaminari'	0.25±0.04 ab	0.09±0.01 a	0.86±0.07 a	'Kaminari II'	0.16±0.01 bc	0.03±0.00 a	0.47±0.12 a
				'NE 40'	0.09±0.01 bc	0.02±0.01 a	0.44±0.11 a
Late summer				*Late summer*			
'Fuyuhiko'	0.04±0.01 c	0.03±0.01 b	0.49±0.15 b	'Kuronegi'	0.26±0.03 a	0.14±0.04 a	0.67±0.06 a
'Kuronegi'	0.20±0.01 a	0.12±0.01 a	1.11±0.12 a	'Kaminari'	0.13±0.04 ab	0.03±0.01 b	0.44±0.11 ab
'Sanpeinegi'	0.13±0.02 b	0.06±0.02 ab	0.86±0.11 ab	'NE 40'	0.06±0.01 b	0.02±0.01 b	0.33±0.10 ab
'Kaminari'	0.09±0.01 bc	0.07±0.01 ab	0.71±0.14 ab	'NE 15'	0.14±0.04 ab	0.02±0.01 b	0.45±0.09 ab
				'NE 71'	0.12±0.02 ab	0.03±0.01 b	0.23±0.04 b
				'YSG'	0.21±0.01 ab	0.01±0.00 b	0.32±0.02 ab
				'Fukuichi'	0.17±0.06 ab	0.04±0.01 b	0.70±0.12 a

[z] Means with the same letter in a column are not significantly different according to Tukey's test (P<0.05).

[y] Each value is the mean ± SE (n = 3).

Table 11. Correlation between morphological traits and phytochemical compounds of JBO cultivars cultivated in summer 2013 and 2014

Phytochemical contents	Morphological traits							
	UPTB	UDTB	LC	TD	TL	WPL	WPD	TFW
					2013			
Ascorbic Acid	0.334	0.247	0.153	0.136	-0.325	-0.598*	-0.376	-0.497
DPPH	-0.338	-0.603*	0.351	-0.471	-0.328	-0.339	-0.618*	-0.574
Flavonoid	0.334	0.303	0.356	0.053	-0.364	-0.657*	-0.210	-0.491
Phenolic	0.350	0.198	0.223	-0.194	-0.378	-0.697*	-0.530	-0.698*
MeCSO	0.628*	0.622*	0.493	0.505	0.455	-0.036	0.336	0.147
AlCSO	-0.058	0.060	-0.269	-0.247	-0.463	-0.591*	-0.325	-0.298
PeCSO	0.216	0.327	-0.432	0.255	0.068	0.062	0.324	0.387
					2014			
Ascorbic Acid	-0.254	0.114	0.111	-0.075	0.055	-0.053	-0.324	-0.147
DPPH	-0.151	0.464	-0.057	-0.325	-0.338	0.125	-0.682**	-0.503*
Flavonoid	-0.693**	0.208	-0.411	-0.605*	-0.449	-0.543*	0.134	-0.159
Phenolic	0.117	0.022	0.060	0.377	0.452	0.175	0.130	0.232
MeCSO	-0.538*	-0.484*	-0.243	-0.410	-0.299	-0.242	-0.188	-0.316
AlCSO	0.459	-0.283	0.165	0.372	0.318	0.216	0.157	0.213
PeCSO	0.704**	-0.532*	0.230	0.571*	0.363	0.244	0.317	0.376

Abbreviations are upper leaf-tip burns (UPTB), under leaf-tip burns (UDTB), leaf color (LC), thrips damage (TD), total length (TL), white-part length (WPL), white-part diameter (WPD) and total fresh weight (TFW). * significant at $P < 0.05$ and ** significant at $P < 0.01$

Principal component analysis

The PCA was done for each year based on the morphological and phytochemical data. In both 2013 and 2014, the analyses yielded two principal components, PC1 and PC2 (Fig. 12). In 2013, those PCs accounted for 87.61% of the total variations (PC1: 58.65% and PC2: 28.96%). PC1 consisted of all morphological traits except for total length, DPPH scavenging activity, and phenol and PeCSO contents of the JBO. Both scavenging activity and phenol had a negative loading value. PC2 included MeCSO, AlCSO, ascorbic acid, and flavonoids. The flavonoids and AlCSO were found to have a negative loading value. The PCA could clearly discriminate 'Fuyuhiko' from the other three cultivars.

In 2014, the total variation was 56.82% from PC1 (34.01%) and PC2 (22.81%). Flavonoids were also found to have a negative loading score in PC1, together with ACSOs, upper leaf-tip burns, white-part length, and thrips damage. PC2 consisted of phenolic content, under leaf-tip burns, total fresh weight, total length, ascorbic acid, scavenging activity, and leaf color. Phenol, tip burns, and leaf color were found to have a negative loading score. In this PCA, three groups of heat-tolerant JBO cultivars were differentiated.

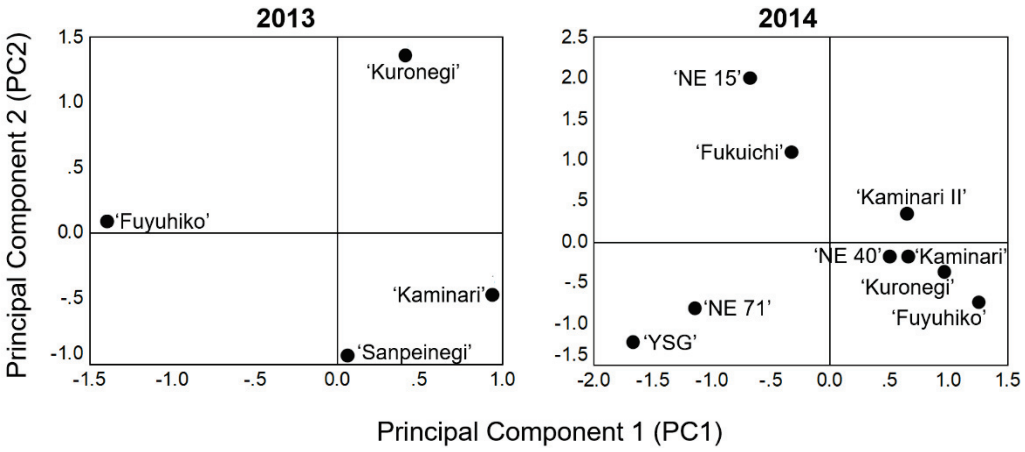


Fig. 12. Scatter plot of the principal components resulting from the morphological and phytochemical data of 2013 and 2014.

Discussion

Abiotic stress in plants triggers a range of responses, from modifications in gene expression and cellular metabolism to changes in growth rate and crop yield (Wahid et al., 2007). High temperatures during summer cultivation lead to abiotic stress conditions for JBOs, as their optimum temperature is 15–20°C (Brewster, 2008). Under this condition, plants will adapt by using a certain physiological process, such as decreasing their photosynthetic rate and increasing their transpiration rate (Zhang et al., 2010). As a result, the yield traits, including quality and quantity, will be affected.

The temperature and UV radiation were increasing during cultivation in summer, as shown in Fig. 9. The temperature and UV radiation (shown as the UV index) in 2013 and 2014 reached their maximum in July (31.2°C and UV index 10.4 in 2014) and/or August (34.2°C and UV index 10.9 in 2013). In order to adapt to such stress conditions, plants produce more antioxidant metabolites, such as ascorbic acid and phenolic compounds, including flavonoids (Almeselmani et al., 2006; Bernaert et al., 2012; Sairam et al., 2000; Wahid et al., 2007). The results show that both ascorbic acid and flavonoid contents increased during mid-summer cultivation. The increase of those secondary metabolites was beneficial to protect the cell and subcellular system from the cytotoxic effect of the active oxygen radicals (Alselmani et al., 2006). Furthermore, tolerance to heat stress in plants was reported to be associated with the increase in antioxidant metabolites (Sairam et al., 2000).

Negative correlations were found between antioxidant metabolites with morphological and yield traits. Inden and Asahira (1990) reported the effect of high temperature on the epicuticular wax layers on JBO leaves. Modification of the epicuticular wax from a dendrite type to a cocoon type affected the possibility of leaf

damage. In this research, the leaf damage happened especially on the leaf tip with a burning symptom. Guilioni et al. (2003) explained that high temperatures can cause sunburn on leaves, shoot and growth inhibition, and reduced yield. The heat stress also leads to anatomical changes such as reduced cell size, closure of stomata, and greater xylem vessels of both root and shoot (Wahid et al., 2007). All of these conditions lead to yield losses, including quality and quantity. In the present study, the increase in antioxidant metabolites such as ascorbic acid, flavonoids, and phenol was an indicator of the heat stress–resistant mechanism in JBO.

Moreover, DPPH analysis successfully determined the total antioxidant capacity of JBO cultivars cultivated in summer and showed negative correlations with leaf-tip burn and total fresh weight. It is known that heat stress could damage the photosynthesis apparatus, including chlorophyll *a* and *b*. The degradation occurred in relation to the production of reactive oxygen species (ROS). Therefore, plants produced more antioxidants to increase their antioxidant activity to protect their cells from those ROS (Wahid et al., 2007). In this research, a positive correlation between total antioxidant capacity of the JBO and antioxidant metabolites (ascorbic acid, flavonoids, and phenols) was observed in both years. The correlation between total antioxidant capacity and phenolic compounds was positive and higher than that for other metabolites. This research result confirmed our previous data on the eight different *Allium* addition lines (Yaguchi et al., 2013), where the DPPH scavenging activity showed a positive and high correlation with the polyphenol content. Thus, in *A. fistulosum*, polyphenol seems to make a high contribution to the antioxidant activity. Furthermore, the influence of the temperature on the increase in total S-accumulations, pungency, and ACSO production of *A. cepa* was reported by Randle et al. (1993). Coolong and Randle (2003) also

reported a high accumulation per °C of MeCSO in comparison with AlCSO and PeCSO in response to temperature. However, we could not find the same tendency in this research, as the ACSO contents varied depending on the cultivars.

Based on the PCA results, the characteristics of all JBO cultivars examined in 2013 and 2014 were clearly differentiated. 'Kuronegi,' 'Sanpeinegi,' and 'Kaminari' were three cultivars that could produce a high total fresh weight in comparison with 'Fuyuhiko.' However, 'Sanpeinegi' was more susceptible to thrips attack and leaf-tip damage, as shown in the 2013 PCA result. The result from the following year confirmed that 'Fuyuhiko,' 'Kuronegi,' 'Kaminari,' 'Kaminari II,' and 'NE 40' were cultivars with a high accumulation of green leaf color and phenolic compounds. The other group, consisting of 'NE 15' and 'Fukuichi,' was observed to have a high accumulation of total fresh weight with a longer and bigger size. Both of those cultivars also accumulated high ascorbic acid and high antioxidant activity. However, they were more vulnerable to thrips attack and leaf-tip burn. The last group, including 'NE 71' and 'YSG,' tended to have a smaller size with a low white-part diameter. However, 'YSG' and 'NE 71' accumulated high MeCSO, flavonoids, and phenols, as well as high green leaf color, which is important for the market.

During summer cultivation, all JBO cultivars showed tolerance by producing a higher amount of antioxidant metabolites such as ascorbic acid and flavonoids. As a response to the stress, plants increased their antioxidant activity to protect their cellular systems. Due to that physiological response, the morphological traits were also affected. All morphological traits assessed in this study exhibited some correlations with the phytochemical traits. Although the environmental conditions were susceptible to thrips attack, in this study, thrips damage was in the tolerable range. Moreover,

genetic factors play an important role in the plant resistance shown by the different morphological and phytochemical profiles of each cultivar. From the PCA analysis results, we could determine the specific cultivar for specific purposes. ‘Kuronegi,’ ‘Kaminari,’ ‘NE 15,’ and ‘Fukuichi’ are suitable for high-yield production, as they could produce a higher TFW. ‘Fuyuhiko,’ ‘NE 71,’ and ‘YSG’ show high resistance to leaf-tip burn and thrips attack in comparison with other tested cultivars. All cultivars examined in this research showed heat tolerance by producing a high amount of antioxidant metabolites.

Chapter 5. PRODUCTION AND CHARACTERIZATION OF ALIEN CHROMOSOME ADDITION LINES IN *ALLIUM FISTULOSUM* CARRYING EXTRA CHROMOSOMES OF *ALLIUM ROYLEI* USING MOLECULAR AND CYTOGENETIC ANALYSES

Introduction

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

In the breeding of cultivated *Allium* species, wild relatives are important sources for introducing new desirable traits via interspecific hybridization (Kik, 2002). *Allium roylei*, a wild species originating in India, has attracted considerable attention in onion breeding for downy mildew resistance (Scholten et al., 2007) and alloplasmic male sterility (Vu et al., 2011). This wild species also possesses other useful characteristics such as partial resistance to leaf blight (De Vries et al., 1992) and moderate resistance to *Fusarium* basal rot (Galvan et al., 2008). Therefore, exploitation of *A. roylei* for the

breeding of *A. fistulosum* would be valuable. Recently Khrustaleva and Kik (1998, 2000) reported the successful uses of *A. roylei* as the bridging species in order to transfer some important genes from *A. fistulosum* to *A. cepa*. Long before, McCollum (1982) reported successful crosses of *A. roylei* with *A. fistulosum*. However, no further backcrossing generation has been reported since then. Meiotic irregularities, which were moderately frequent in the *A. roylei–A. fistulosum* hybrid (McCollum, 1982), may hamper the introgression process of genes from *A. roylei* to *A. fistulosum* via backcrossing. Doubling of the sterile F_1 hybrid is one way to overcome these barriers (Singh, 2003). In a previous study, a high number of alien addition lines of *A. cepa* carrying extra chromosomes from *A. roylei* were produced by backcrossing the doubled F_1 hybrid (Vu et al., 2012). Alien addition lines, which carry the extra chromosomes of wild species and the normal chromosome complement of recipient species, would speed up the introgression process of the wild species by producing chromosome substitution and translocation lines (Singh, 2003). In this study, we first report the use of *A. roylei* for the production of alien addition lines in *A. fistulosum*. A preliminary study on the variation of the biochemical content and antifungal activities against four isolates of *Fusarium oxysporum* f. sp. *cepae* was also conducted on the alien addition lines.

Materials and Methods

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

Figure 13 describes the crossing procedure for the production of *A. fistulosum* addition lines with extra chromosomes from *A. roylei*. *Allium fistulosum* 'Kujyo-kodaikei' (genomes FF, $2n = 2x = 16$, seed parent) was crossed with *A. roylei* '97175' (genomes RR, $2n = 2x = 16$, pollen parent) to produce F_1 hybrids (genomes FR, $2n = 2x = 16$). The chromosomes of an F_1 hybrid were doubled using colchicine to produce amphidiploids (genomes FFRR, $2n = 4x = 32$). The colchicine was applied by culturing a primordial stem in the Linsmaier and Skoog (LS) media containing 0.1 % colchicine in a dark condition for 4 days before being transferred to LS free hormone media and cultured for 2 months. After that, the amphidiploids were backcrossed with three different *A. fistulosum* cultivars ('Kujyo-Hoso,' 'Banchusei-Hanegi-Keitou,' and 'Nebuka-Negi-Keitou') to produce BC_1 progenies. The BC_1 plants were then backcrossed with the three *A. fistulosum* cultivars to produce BC_2 progenies. Crosses were carried out by hand pollination in a screen-covered isolation greenhouse in Yamaguchi, Japan (N34º11′, E131º280′). One month after pollination, the ovules of the BC_2 were cultured and generated on an MS solid medium (Murashige and Skoog, 1962) containing 3.0 % (w/v) sucrose and 2.0 % (w/v) agar at 25 ºC in dark conditions until germinated, between May and August. After germination, the cultures were treated with 8 h day length and 50 % humidity. Healthy seedlings were then planted in sand in plastic trays and transplanted to pots from November to December.

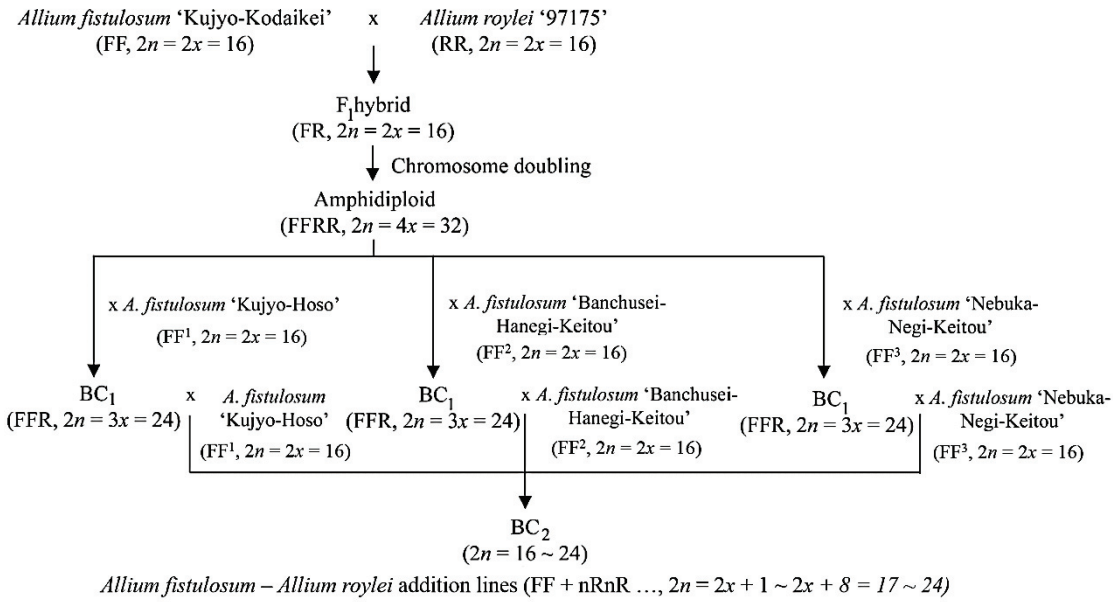


Fig. 13. Method for producing alien addition lines of *Allium fistulosum* with extra chromosome of *A. roylei*.

The BC_2 plants were grown in a greenhouse and fertilized each week with a nutrient solution containing 15: 8: 17 (N: P_2O_5:K_2O, w/w/w) (OK-F-1; Otsuka Chemical Co., Osaka, Japan) or 6.5: 6: 19 (w/w/w) (Hyponex; Hyponex Co., Marysville, OH, USA). The chromosome numbers of the BC_2 plants were counted using Feulgen nuclear staining followed by the squash method. The karyotype analyses were undertaken according to the standard nomenclature system for the chromosomes of *Allium* (Kalkman, 1984), which was agreed upon at the Eucarpia 4th Allium Symposium (De Vries, 1990).

Characterization of alien chromosomes using isozyme and DNA markers

The BC_2 plants with $2n$ = 17–23 were further characterized using five isozymes and five DNA markers. The chromosomal locations of the five DNA markers were reported in *A. cepa* or *A. fistulosum* as shown in Table 12. Chromosomal locations of

the two isozymes and five DNA markers in *A. roylei* were determined from those that had been assigned in *A. cepa* and *A. fistulosum* because of the close genetic relationship between the species. Extraction of enzymes, electrophoresis, and staining were carried out following the method of Shigyo et al. (1995) and Van Heusden et al. (2000b). For DNA marker analyses of *A. fistulosum–A. roylei* addition lines, the total genomic DNA of the parental and BC_2 plants was isolated from fresh leaf tissue using a miniprep DNA-isolation method (Van Heusden et al., 2000a). The polymerase chain reaction (PCR) amplifications of the markers F3H, CHS-B, and AMS12 were evaluated as described previously (Masuzaki et al., 2006a, b). For amplification of the marker ACM024, the reaction mixture (20 μL) contained 100 ng of DNA, 2 mM 10 x PCR buffer, 0.2 mM dNTP mixture, 0.8 μM each of forward and reverse primers, 1.5 mM $MgCl_2$, and 0.5 units of r *Taq* polymerase. Touchdown PCR was performed to amplify the marker ACM024 as follows: initial denaturation at 94 ºC for 2 min, followed by 10 cycles at 94 ºC for 0.5 min, 65 ºC for 0.5 min, and 72 ºC for 0.5 min, where the annealing temperature is reduced by 1 ºC per cycle; then 35 cycles at 94 ºC for 0.5 min, 55 ºC for 0.5 min, and 72 ºC for 0.5 min, and a final extension at 72 ºC for 4 min on a program thermal cycler iCycler™ (Bio-Rad, Hercules, CA, USA). To amplify the marker SiR-1, the reaction mixture (25 μL) contained 50 ng of DNA, 2 mM 10 x ExPCR buffer, 0.2 mM dNTPs, 0.5 μM each of forward and reverse primers, and 0.625 units of Ex *Taq* polymerase. The PCR condition for SiR-1 was as follows: initial denaturation for 3 min at 94 ºC and 40 cycles of PCR amplification (1 min denaturation at 94 ºC, 1 min annealing at 70 ºC, and 1 min primer extension at 72 ºC). The PCR products were separated on 2 % agarose or 5 % polyacrylamide gel electrophoresis according to the method of Yaguchi et al. (2009).

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

Plant materials used for the preliminary analysis included *A. fistulosum*, *A. roylei*, the F_1 hybrid, the amphidiploid, and different *A. fistulosum–A. roylei* multiple addition lines. The preliminary analysis was done to analyze the sugar content, including fructose, sucrose, and glucose. The multiple addition lines was cultivated for a year so the number of new plants multiplied from vegetative propagation were very limited. Only one sample for each line was collected in December of the next year. The leaf blades were cut into small pieces and mixed thoroughly. Two grams of the leaf-blade tissues were extracted using hot 70 % ethanol as described by Hang et al. (2004). Every extract was stored at -20 ºC until analysis. The 70 % hot-ethanol extract was filtered through a Sep-Pak C18 cartridge column followed by a 0.5 μm filter (Katayama Chemical, Osaka, Japan) to remove pigments prior to HPLC analysis. Sugars in each filtrate were analyzed three times using an HPLC system (Hitachi LaChrom Elite) equipped with a refractive index detector (Hitachi L-7490). An aliquot of the filtrate (20 μL) was injected into the HPLC apparatus fitted with a LiChrospher 100 NH_2 (Merck) column of 4 x 250 mm with a column temperature of 35 ºC. The mobile phase was acetonitrile: water (80:20, v/v) at a flow rate of 0.8 mL/min with a retention time of 30 min. The internal standards were prepared by dissolving glucose, fructose, and sucrose at a concentration of 0.5 % in 70 % aqueous ethanol.

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

The plant materials for analyses of flavonoids and ACSOs were the same as those for the sugar analysis. Five grams of leaf-sheath tissues from each plant were extracted with hot 70 % ethanol as described by Hang et al. (2004). The 70 % hot-ethanol extractions were then used for flavonoid analysis. To analyze the ACSOs, two grams of the leaf-blade tissues were microwaved for 2 min to denature the alliinase and extracted with distilled water. The flavonoid and ACSO contents were determined using HPLC according to the method described by Vu et al. (2013).

Table 12. DNA markers for identification of extra chromosomes from *A. roylei* in BC_2 progenies

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

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

Roots of *A. fistulosum*, *A. roylei*, an amphidiploid (FFRR), and an allotriploid (FFR) were collected a year after the chemical content analysis and used for saponin extraction. Freeze-dried root tissues (0.2–0.4 g) were ground thoroughly using a blender and then extracted three times with 100 mL of *n*-hexane. The remaining root materials were extracted three times with 100 mL of 70 % methanol and filtered. The filtrate was vacuum dried and dissolved in 100 mL of water. After that, *n*-butanol with the same volume of water (100 mL) was added. The *n*-butanol fraction was separated three times using a separation funnel. The *n*-butanol fractions were vacuum dried to give crude saponins. The saponins were visualized by spotting the butanol fraction on a thin layer chromatography (TLC) and then developed using a system of chloroform: methanol: water (6: 3: 1). The TLC plates were sprayed with *p*-anisaldehyde reagents and heated at 100 ºC for 10 min. The saponin contents were determined using a spectrophotometer in accordance with Ebrahimzadeh and Niknam (1998). Diosgenin (purity: approx. 95 %, Sigma, USA) was used as a standard for establishing a calibration curve. The ANOVA for saponin data was conducted with the General Linear Model of SPSS statistical software version 18.0 with advanced models (SPSS Japan Inc., Tokyo, Japan). Differences between means were located using Tukey's multiple range test.

The antifungal activities of the crude saponins were tested on four F. *oxysporum* f. sp. *cepae* pathogens (Takii and AC214 isolated from bulb onions; AF60 and AF22 isolated from A. *fistulosum*). Pathogens were obtained from the Laboratory of Molecular Plant Pathology, Faculty of Agriculture, Yamaguchi University, Japan. The antifungal activity was evaluated by an agar-plate diffusion method, using 3.2 cm diameter Perspex plates of potato dextrose agar (PDA). Crude saponin was added to obtain a final concentration of 1000 ppm. The plates were inoculated with a 5 mm plug

containing the fungi grown on a PDA for 5 days. Plates were incubated at 25 ºC, and the fungal radical growth wasmeasuredafter1 week by measuring the diameter of the fungal hypha that was grown on the plate. Each experiment was performed in triplicate with the water treatment as a control. Dunnett's multiple test was used for comparison of antifungal activities between *A. fistulosum* and the amphidiploid and allotriploid.

GISH analysis

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

Selfing and backcrossing of the addition lines

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

Results

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

A. fistulosum 'Kujyo-kodaikei' set germinable F_1 hybrid seeds when crossed with *A. roylei* '97175' as the pollen parent. After doubling the chromosomes of the F_1 hybrid, amphidiploid plants were obtained. In the backcrossing between the amphidiploids and three different cultivars of *A. fistulosum*, 31 BC_1 plants were produced (Table 13). The chromosome numbers ($2n$) of the BC_1 plants were 24 (29 plants) and 32 (two plants) (Table 14). Subsequently, 29 BC_2 plants were produced from backcrossing between allotriploid BC_1 plants and *A. fistulosum* (Table 13). The chromosome numbers ($2n$) of the BC_2 plants ranged from 16 to 23 (Table 14). The plants with $2n = 17$ appeared with the highest frequency (eight plants). Lower frequencies (one to six plants) were observed in plants with $2n = 16$, 18, 19, 20, 21, 22, and 23 (Table 14).

Table 13. Seed set, seed germination, and number of seedlings survival in the backcrossings of amphidiploids ($2n = 32$, genomes FFRR) and triploids ($2n = 24$, genomes FFR) to three cultivars of *A. fistulosum*: 'Kujyo-Hoso' (F^1F^1), 'Banchusei-Hananegi-Kei-Tou' (F^2F^2), and 'Nebuka-Negi-Keitou' (F^3F^3)

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

Table 14. Variation of chromosome numbers in BC_1 and BC_2 progenies

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

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

Van Heusden et al. (2000b) reported that isozyme loci *Lap-1*, *6-Pgdh*, and *Pgi-1* are located on chromosomes 1, 2, and 5, respectively, in *A. roylei*. Furthermore, the two isozyme loci, *Got-2* and *Gdh-1*, were allocated on chromosomes 6 and 8 of *A. cepa* (Shigyo et al., 1994, 1995). *Allium fistulosum* and *A. cepa* had different band patterns of the five isozymes *Lap-1*, *6-Pgdh*, *Pgi- 1*, *Got-2*, and *Gdh-1*. The introgression of gene encoding for *Lap-1* from *A. roylei* in the BC_2 plants was determined by the presence of bands from both *A. fistulosum* and *A. roylei* (Fig. 14). Meanwhile, the BC_2 plants that possessed encoding genes of *6-Pgdh*, *Pgi-1*, and *Got-2* in *A. roylei* showed bands from the parental bands with additional bands of intermediate mobility between the two parents. The presence of gene encoding for *Gdh-1* from *A. roylei* in the BC_2 plants was confirmed by bands at intermediate positions between the parental bands. There are two pattern types of intermediate mobility (Fig. 15). The results of isozyme analysis in the BC_2 progenies are included in Table 15. With the five isozyme markers, the three AMALs (FF + 1R, FF + 5R, and FF + 8R) were characterized, and the presence of extra chromosomes 1R, 2R, 5R, 6R, and 8R was detected in a double-monosomic addition line ($2n = 18$) and other MALs ($2n = 20$, 21, 22, and 23).

All of the DNA markers used in this study were able to show polymorphism between *A. fistulosum* and *A. roylei*. The DNA fragments derived from *A. roylei* were

used to confirm the presence of *A. roylei* respective chromosomes. Two AMALs, FF + 3R and FF + 4R, were identified by one EST and one SCAR marker (Si-R and CHS-B, respectively) (Table 15). Furthermore, extra chromosomes of *A. roylei* (2R, 3R, 4R, and 7R) were also detected in the double-monosomic addition line and the other MALs via DNA markers.

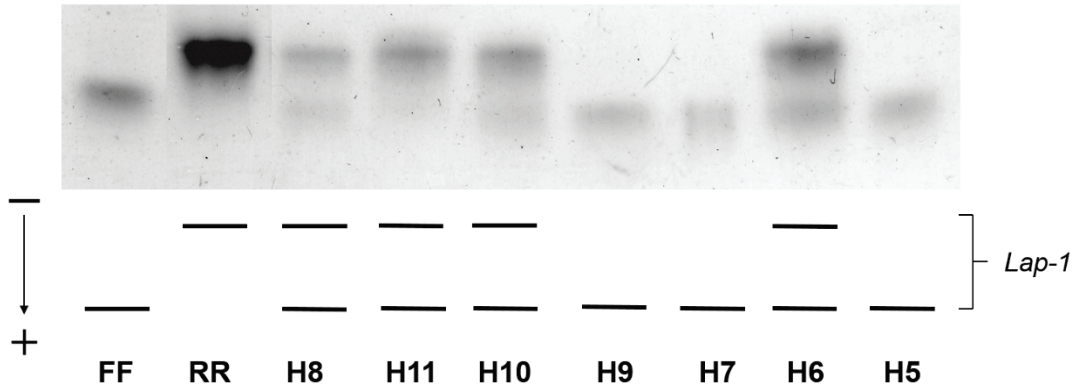


Fig. 14. *Lap-1* zymograms and the schematic illustration of *A. fistulosum* 'Kujyo-Hoso' (FF), *A. roylei* '97175' (RR), MALs (H8, H11, H10, H6, and H5), double-monosomic addition line (H9) and BC_2 FF (H7)

In summary, with the use of five isozyme and five DNA markers, five AMALs ($2n = 17$), one double-monosomic addition line ($2n = 18$), and nine MALs ($2n = 20$, 21, 22, and 23) were characterized. GISH analyses were carried out with one AMAL ($2n = 17$, FF + 3R) and a double-monosomic addition line ($2n = 18$, FF + 3R + 8R) for further confirmation of the chromosome constitutions of these lines (Fig. 16). FF + 3R showed an intact chromosome 3 of *A. roylei*, one recombinant *A. roylei–A. fistulosum* chromosome, and other intact chromosomes of *A. fistulosum*. The double-monosomic line FF + 3R + 8R had two intact chromosomes of *A. roylei*, in addition to a complete set of 16 chromosomes from *A. fistulosum*, without any translocation.

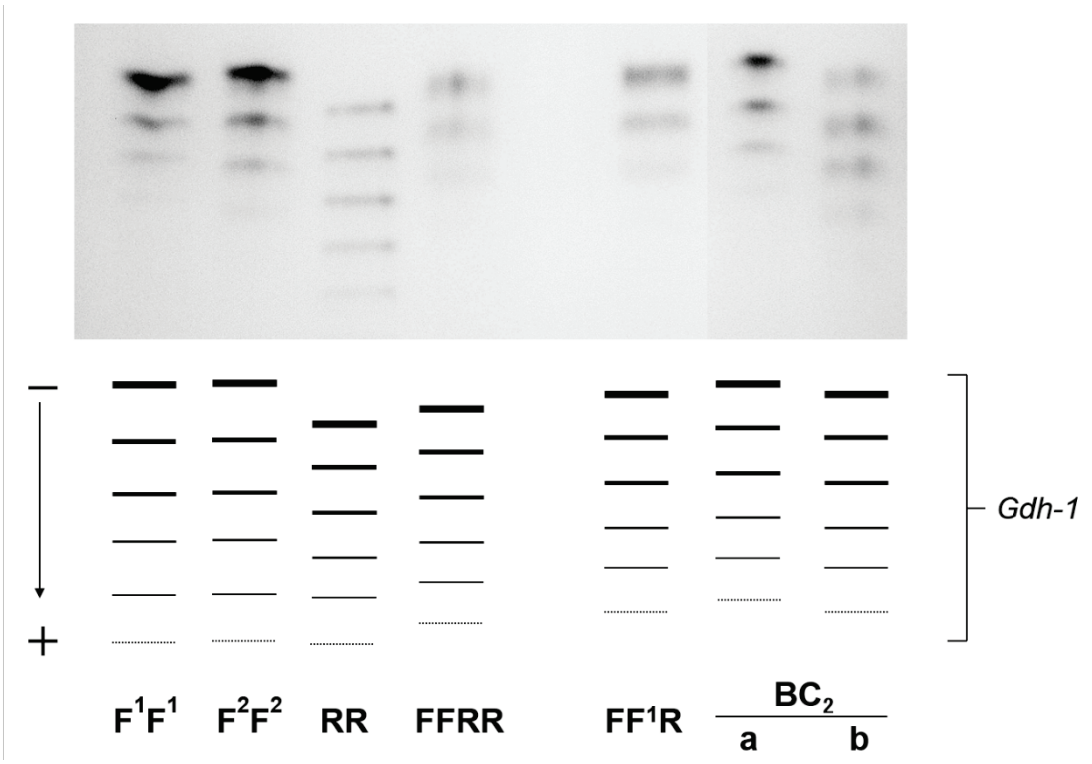


Fig. 15. *Gdh-1* zymograms and the schematic illustration of *A. fistulosum* 'Banchusei-Hanegi-Keitou' (F^1F^1), *A. fistulosum* 'Nebuka-Negi-Keitou' (F^2F^2), *A. roylei* '97175' (RR), amphidiploid (FFRR), and allotriploid (FF1R). BC_2 plants showed two patterns (a and b)

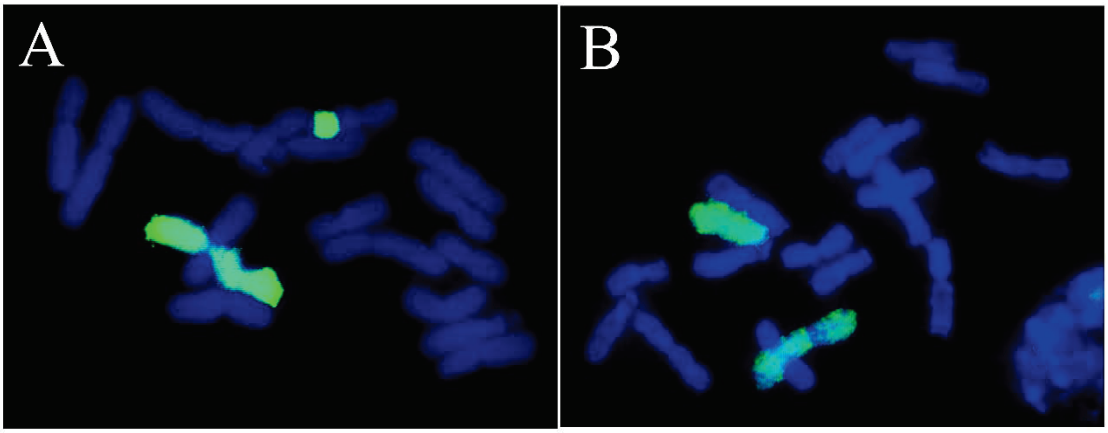


Fig. 16. Somatic metaphase cells of a monosomic addition line ($2n$ = 17, genomes FF + 3R) (a) and a double-monosomic addition line ($2n$ = 18, genomes FF 3R + 8R) (b) in the BC_2 generation after genomic in situ hybridization.

Selfing and backcrossing of the addition lines

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

Biochemical characteristics of the alien addition lines

The contents of some chemical compounds (sugars, ACSOs, flavonoids, and saponins) were preliminarily investigated in multiple addition lines together with the parental, allotriploid, and amphidiploid lines. Preliminary investigation was done because only one plant survived. Consequently, only one replication could be done for the analysis. However, variations of the chemical contents were observed among the investigated lines (Fig. 17).

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

Table 15. Identification of extra chromosomes in *A. cepa-A. roylei* addition lines via chromosome-specific isozyme and DNA markers

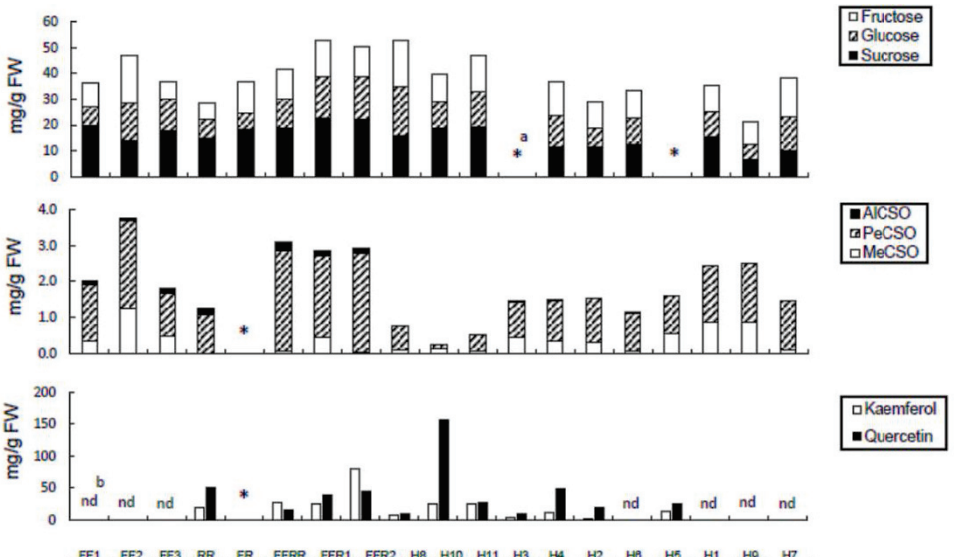
Chromosome number	Group	Frequency of plants	Chromosome specific markers											Extra chromosome
			1R	2R		3R		4R	5R	6R		7R	8R	
			Lap	*6-Pgdh*	ACM024	F3H	Si-R	CHS-B	*Pgi-1*	*Got-2*	Karyotype	AMS-12	*Gdh-1*	
17	1	1	+[a]	o[c]	-	-	-	-	-	-	-	-	-	1R
	2	3	-[b]	o	-	o	+	-	-	-	-	-	-	3R
	3	1	-	o	-	o	-	+	-	-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

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

Table 16. Seed set, seedlings survival, and chromosome number of seedlings in backcrossing of a double-monosomic addition line (2*n* = 18, genomes FF + 3R + 8R) to *A. fistulosum* 'Senbon-Negi' (F^4F^4) and selfing of a monosomic addition line (2*n* = 17, genomes FF+3R)

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

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



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

Fig. 17. Sugar, cysteine sulfoxide, and flavonoid contents of the three cultivars of *A. fistulosum*: 'Kujyo-Hoso' (FF1), 'Banchusei-Hanegi-Keitou' (FF2), 'Nebuka-Negi-Keitou' (FF3); *A.roylei* '97175' (RR); F1 hybrid (FR); amphidiploid (FFRR); allotriploids (FFR1, FFR2); and *A. fistulosum–A. roylei* chromosome addition lines (H8–H7). [a] Not carried out. [b] Not detected.

Differences were observed between *A. fistulosum*, *A. roylei*, the allotriploid (FFR), and the amphidiploid (FFRR) in the total amount of saponins extracted from the roots (Fig. 18). Significantly higher saponin content was observed in the allotriploid plant in comparison with the *A. fistulosum* and the amphidiploid plant.

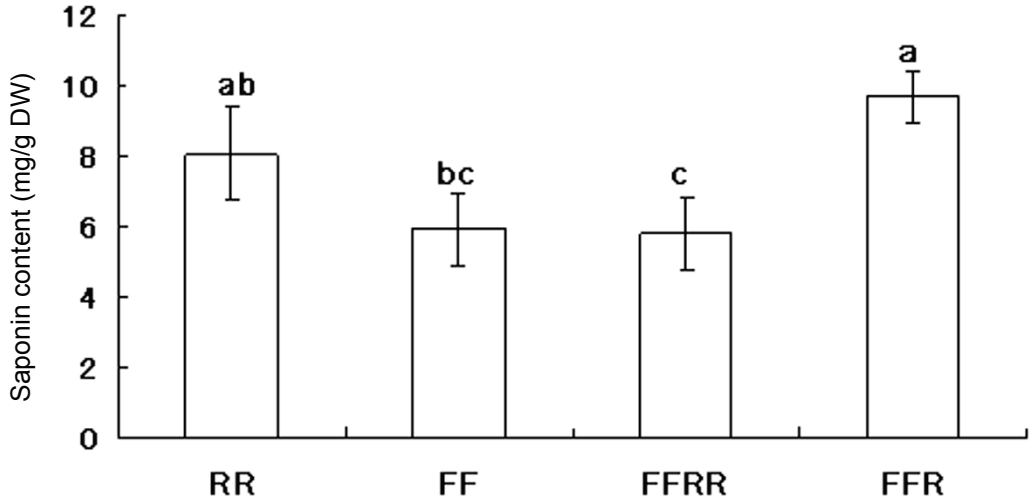


Fig. 18. Saponin contents in the roots of *A. roylei* '97175' (RR), *A. fistulosum* 'Kujyo-Hoso' (FF), amphidiploid (FFRR) and allotriploid (FFR). Vertical bars indicate + and – standard error. Different *letters* indicate a significant difference among the lines according to Tukey's multiple range test.

Saponins of *A. fistulosum* showed higher antifungal activities than those of *A. roylei* against all four fungal isolates (Fig. 19). Meanwhile, saponins of *A. fistulosum* and the amphidiploid had the same levels of fungal inhibition against the four isolates. Saponins of the allotriploid had significantly higher antifungal activities against the two isolates AC Takii and AF22 in comparison with those of *A. fistulosum*.

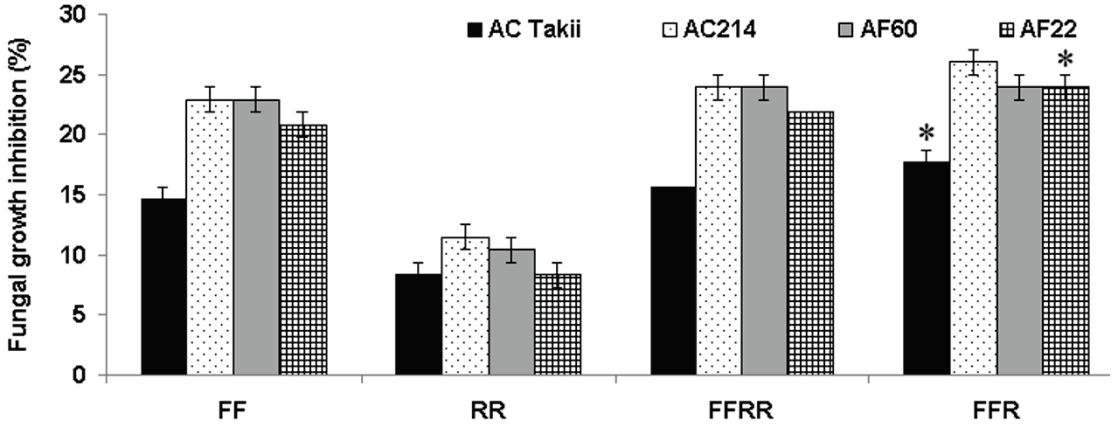


Fig. 19. Antifungal activities of saponins in the roots of *A. fistulosum* 'Kujyo-Hoso', *A. roylei* '97175', amphidiploid (FFRR), and allotriploid (FFR) against the four isolates of *F. oxysporum* f. sp. *cepae*. Dunnett's multiple test was used for comparison of antifungal activities between *A. fistulosum* and each of the amphidiploid and the allotriploid. *Asterisk* indicates significant higher antifungal activity than *A. fistulosum* at $p < 0.05$.

Discussion

This study reports, for the first time, the successful production of *A. fistulosum–A. roylei* chromosome addition lines. In crossings between the amphidiploids (FFRR) and the diploids *A. fistulosum* (FF), the seed set was high, ranging from 25 to 40 %. In the case of crossings between the allotriploids (FFR) and the diploids *A. fistulosum* (FF), the seed set was extremely low (0.48–2.6 %). This phenomenon might be due to the high proportion of non-functional female gametes produced by the allotriploids. A similar result was also described in the backcrossings of *A. cepa–A. fistulosum* allotriploids (Hang et al. 2004). In contrast, backcrossings of *A. cepa–A. roylei* allotriploids had relatively high rates of germinated seeds (Vu et al. 2012). We did not succeed in completing the eight possible types of AMALs, but we found that AMALs with $2n = 17$ appeared with the highest frequency among the BC_2 plants. The MALs of *A. fistulosum* with extra chromosomes from *A. roylei* also appeared with lower frequencies. Therefore, we think the addition of chromosomes from *A. roylei* does not decrease the survival ability of the female gametes produced from the *A. fistulosum– A. roylei* allotriploids. These results differed from those of Vu et al. (2012), who found a high number of plants with $2n = 16$, followed by $2n = 17$ in the BC_2 generation.

Employing isozyme and DNA markers in this study enabled us to successfully identify the presence of *A. roylei* in the BC_2 plants in most cases. However, seven BC_2 plants showed differences between the cytogenetic and molecular data. These plants might be derived from chromosome substitution or recombination during meiosis of the *A. fistulosum–A. roylei* allotriploids. This result is in agreement with a previous study, which reported frequent chromosome parings and moderately frequent meiotic irregularities (e.g., univalents) in pollen mother cells of the *A. fistulosum–A. roylei*

hybrid (McCollum, 1982). As an example, the GISH result of our study showed a recombination in an AMAL (FF + 3R) and a true double-monosomic addition line without recombination (FF + 3R + 8R). The recombination probably resulted from the chiasma formation during the meiosis of the allotriploid as also reported by Vu et al. (2012). Further GISH analyses are required to reveal the genomic constitutions and recombination frequencies of all of the BC2 plants.

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

in *A. fistulosum*. The overall flavor of *Allium*-derived plants is determined by the ratios and amounts of ACSOs (Block, 2010). Therefore, additional chromosomes from *A. roylei* would actually alter the flavor of *A. fistulosum*. Some addition lines with very lowACSO content could be mildly pungent. These lines would be very good breeding material for developing low-pungency cultivars of *A. fistulosum*. Shigyo et al. (1997a) reported that only one *A. fistulosum*–shallot monosomic addition line FF + 5A showed a reddish-yellow leaf sheath and suggested that chromosome 5 of the shallot possesses important genes for controlling pigment production. Furthermore, the authors found a large number of peaks attributable to flavonoids in the FF + 5A (Shigyo et al., 1997b). This study reported a similar result, that only the *A. fistulosum–A. roylei* addition lines that carry the extra chromosome 5R of *A. roylei* show a red leaf sheath. Chromosome 5R of *A. roylei* would also carry important genes related to flavonoid synthesis in *A. roylei*. Further determination of chromosomal locations of structural enzyme-coding genes and regulatory genes in the pigment biosynthetic pathways of *A. roylei* is needed to confirm this result. The saponin content in leaves of *A. roylei* was higher than in those of *A. fistulosum* (Vu et al., 2013). Thin-layer chromatography also showed qualitative differences in saponins between these two species. The present study found that the FFR triploids had significantly higher saponin content and saponin antifungal activities than did *A. fistulosum*. The additional saponin content of the FFR allotriploids would be derived from *A. roylei* saponin biosynthesis controlled by the introgressed genes located on the extra chromosomes of *A. roylei*. The observations of chemical modifications in the *A. fistulosum–A. roylei* addition lines would bring helpful information regarding chromosome manipulation to improve the consumer quality as well as the disease resistance of *A. fistulosum*. The introgression of desirable traits, such as *Fusarium* or downy mildew resistance, from *A. roylei* to *A. fistulosum* is promising

for the future, as a BC_3 generation could be produced via initial trials of selfing and backcrossing of the *A. fistulosum–A. roylei* addition lines.

Chapter 6. GENERAL DISCUSSION

As opposed to animals, humans, or other living organisms, plants cannot avoid an unfavorable environment by moving or running. Therefore, they must use their own ability to keep alive by developing other mechanisms, such as programmed cell death, or increase their self-tolerance. A failure to protect themselves either from biotic or abiotic stress will decrease their ability to continue their life cycle, including growing and developing, which affect production quantity and quality (Shinozaki et al., 2015). For that reason, proper research related to plant defense and tolerance mechanisms against biotic and abiotic stress, especially for *Allium* species, is important.

In this study, we used *Allium* germplasm, including *A. fistulosum* L. (Japanese bunching onion), *A. roylei*, *Allium cepa* L. Common onion (onion), and *A. cepa* L. Aggregatum group (shallot) to reveal the effect of genetic background and abiotic stress to biochemical compounds as an indicator of quality assessment. The extra chromosome(s) of *A. roylei* in *A. fistulosum* seems to affect some chemical compound and the ability to inhibit *Fusarium oxysporum* f. sp. *cepae* infection. Moreover, *A. fistulosum* heat-tolerant varieties could protect from the bad effects of high temperatures and UV radiation in summer cultivation by producing secondary metabolites that play a role as biochemical defense mechanisms. Furthermore, with the exploitation of Japanese bulb onion F_1 varieties and shallot landraces from East-Asian countries, we gain more understanding on the similarities and differences between bulb onion and shallot phytochemical compound characteristics that will be important and beneficial for further research, breeding improvement, and their potential as an abiotic stress-tolerant strain.

Phytochemical differences in bulb onions and shallots

Bulb onions and shallots have different shapes, skin colors, and phytochemical contents (Benkeblia, 2004), as each is used for many different purposes. Bulb onions are of two types, short-day and long-day types, as their photoperiod requirements are different (Brewster, 2008). Despite their genetic background differences, environmental factors also have a big effect on their phenotypic characteristics, including their phytochemical compounds. In this research, we have obtained two years of data sets with high correlation between them. The fact that MeCSO and PeCSO were the major sulfur compounds found in bulb onions and shallots, respectively, was in line with a previous report (Lee et al., 2009). As sulfur metabolism is very sensitive to temperature (Brewster, 2008), long-day bulb onions were observed with higher PeCSO than short-day bulb onions. Moreover, shallot landraces from Indonesia produce higher PeCSO content, as they were cultivated in a dry season. Higher PeCSO and MeCSO and their proportions with other compounds determine the pungency level and nutraceutical value of bulb onions and shallots (Kimura et al., 2014).

Furthermore, between bulb onions and shallots, there were some noticeable differences where most of the sugars in bulb onions were in a monosaccharide form, while there were higher polysaccharide contents in shallots. An interesting result was found in the fructan content of shallots, where fructan is hardly found in species cultivated in tropical regions (Brewster, 2008). However, abiotic stress—in this case, a dry season with high temperatures—triggered shallot landraces from Indonesia to produce more fructan as a defense mechanism. High UV radiation in the tropical region also generated high flavonoid production in shallots. This was a natural response to

environment conditions such as different latitudes, light intensities, and day lengths (Harborne and Williams, 2000; Jaakola and Hohtola, 2010). It seems that shallots have higher tolerance to abiotic stress conditions, as they produce higher amounts of ACSOs, flavonoids, and fructan than do bulb onions.

Characteristics of amino acids and ACSO metabolism pathways between bulb onions and shallots

Further research on the differences between bulb onions and shallots, especially their amino acid contents and ACSO biosynthesis pathways, is necessary to better understand their phytochemical differences as related to taste. Apart from sugars, free amino acids also correlate positively with the taste of many foods, including *Allium* species (Maga, 1990). In line with other phytochemicals, the production of amino acids was also affected by genetic background, soil fertility, maturity, and other growing conditions (Saghir et al., 1965). The higher amino acid contents observed in shallot landraces were also related to their lower water content than that in bulb onions. Furthermore, some important amino acids possessing a meaty taste, known as umami taste, were also observed to be higher in content in shallots than in bulb onions. This could explain why the taste of shallots is stronger than that of bulb onions. Moreover, increased amino acid biosynthesis is highly related to abiotic stress tolerance (Abdelrahman et al., 2015).

Another possible explanation for shallots' tolerance to extreme environmental conditions, such as high temperatures and draught, is their ACSO content. In this research, the biosynthesis pathway of shallots was discovered to be shorter than the pathway in bulb onions. ACSOs have been known as one defense compound, especially

against heat stress (Coolong and Randle, 2003), as ACSO production increases in line with increasing temperature. Based upon stress conditions, plants will build their defense mechanisms in two categories: avoidance and tolerance. Shallots build a tolerance mechanism by producing higher amounts of fructan (Ariyanti et al., 2017) and other metabolites, as their gene expression was found to be upregulated (Abdelrahman et al., 2015).

Morphological and phytochemical response of some JBO heat-tolerant varieties under summer cultivation

Global climate change is the biggest challenge for future agriculture production. Christensen and Christensen (2007) predicted that, in some important agriculture regions, the annual temperature will be increased by 2.5°C to 4.3°C between 2080 and 2099. Many important plants, including barley, wheat, and maize, have responded negatively to climate change in terms of observations from 1981 to 2002 (Lobell and Field, 2007). The Japanese bunching onion (*Allium fistulosum* L.) is one important *Allium* species used in many Asian countries, including China, Korea, Japan, and Indonesia, as an ingredient in soup or ramen. As a vegetable and a spice, production continuity throughout the year is very important. However, as their optimum condition for growing is in moderate to low temperatures (Brewster, 2008), cultivation in summer is quite difficult, especially in Japan. For that reason, breeders have tried to produce new cultivars that have high tolerance in conditions of heat stress.

During a two-year experiment, F_1 heat-tolerant varieties of JBO exploited in this study exhibited a biochemical defense mechanism by enhancing the synthesis of ascorbic acid, flavonoids, phenolic compounds, and antioxidant activity. This was a

natural response for plants to produce more antioxidant metabolites (Almeselmani et al., 2006; Bernaert et al., 2012; Sairam et al., 2000; Wahid et al., 2007) to protect cells from active oxygen radicals (Almeselmani et al., 2006). Those antioxidant metabolites protect plants from heat damage that negatively affects *Allium* production. Moreover, highly resistant varieties will produce higher antioxidant compounds and cysteine sulfoxides, which has an important role in the plant defense mechanism against abiotic stress (Randle et al., 1993). The antioxidant compound itself could prevent thrip attacks, as they could not be digested and would become toxic (Miles, 1999; Leiss et al., 2009). Each cultivar observed in this study showed different adaptability to high-temperature conditions. Principal component analyses helped us to identify which cultivars are suitable for high yields and which cultivars are suitable for thrip resistance. Further research will be needed to understand the gene responsible for this defense mechanism.

Allium roylei: promising wild relative for improving *Allium fistulosum* tolerance against biotic stress

The exploitation of a wild relative's gene to improve the performance of some cultivated plants via interspecific hybridization has been reported for some species. In *Solanum tuberosum*, the purpose was to improve resistance to some pathogens (Mattheij et al., 1992). Rice wild relatives were exploited to intensify genetic variability for tolerance to some stresses (Brar and Khush, 1997); in wheat, the aim was to upgrade salt tolerance (Colmer et al., 2006). In *Allium* species, *A. roylei* was used as the chromosome donor for *A. cepa* (Vu et al., 2012); it was also used as the mediator for *A. fistulosum–A. cepa* introgression (Khrustaleva and Kik, 2000). This study reported the first successful production of *A. fistulosum–A. roylei* chromosome addition lines. Some

constraints, such as the low number of seeds set from the crossing between FFR and FF, happened because of the high proportion of non-functional female gametes produced by the FFR, which also occurred in the backcrossing of *A. cepa* and *A. roylei* (Hang et al., 2004).

Moreover, the fact that *A. roylei* extra chromosome clearly altered the biochemical characteristics of MALs was discovered. Variations in sugar, cysteine sulfoxide, and flavonoid contents were observed among MALs in various amounts. Regarding sugar production, chromosome 8 of *A. roylei* seems not to have a significant role, like that of chromosome 8 of *A. cepa* and *A. fistulosum* (Yaguchi et al., 2008, 2009). The additional chromosome from *A. roylei* in *A. fistulosum* also affected ACSO production by reducing the amount of ACSOs. Moreover, only one addition line carrying chromosome 5 of *A. roylei* showed a red leaf sheath. This result was in line with that of Shigyo et al. (1997b) that chromosome 5 of *A. cepa* in *A. fistulosum* is also responsible for flavonoid synthesis. Furthermore, the allotriploids (2*n*=24, FFR) of *A. fistulosum–A. roylei* showed significantly higher saponin content and antifungal activities against isolates of *Fusarium oxysporum* f. sp. *cepae* than did *A. fistulosum*. Nevertheless, producing a complete set of alien chromosome addition lines of *A. fistulosum* carrying extra chromosomes of *A. roylei* is highly recommended for further study.

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SUMMARY

Among the edible *Allium* species, bulb onions (*A. cepa* L. Common onion group), shallots (*A. cepa* L. Aggregatum group) and Japanese bunching onion (JBO) (*A. fistulosum* L.) are top three species with high production and consumption worldwide. To accomplish the consumer preference together with the unfavourable conditions that may affect *Allium* crops production, breeders need to create new strains which are not only high productivity, but also have better taste and flavour, high beneficial chemical compound and tolerance for many biotic and abiotic stresses. Moreover, global climate changes have been given a big impact for the agriculture system. Not only because they influence climatic conditioned but also the pathogen behaviour. One of many possibilities is by exploiting wild relatives as the chromosome donor which can contribute to enhancing the quality. It has been known *Allium roylei* is one of *Allium* wild relative that has many diseases tolerance. The exploitation of *A. roylei* in *Allium cepa* could increase the disease tolerance and also the production of the beneficial chemical compound, such as flavonoid, phenolic compound, and antioxidant activity.

1. Phytochemicals variations in bulb onions and shallots

To meet the demand of high nutraceutical of bulb onions and shallots, a study on their phytochemical contents along with better understanding of their biosynthesis are important. For that reasons, 10 F_1 commercial bulb onion varieties (seven short-day and three long-day varieties) from Japan, 12 shallots landraces from Indonesia (nine landraces) and Vietnam (three landraces) were exploited in two years on 2014 and 2015. Due to their photoperiod's differences, short-day and long-day bulb onions

possess different phytochemical characteristics. In general, the sweetness of bulb onions come from their high content of monosaccharaides than polysaccharides sugar. Otherwise, shallot produces more polysaccharides including fructans which is very important for the abiotic stress tolerance. Moreover, shallots also produce higher flavonoid and ACSOs, especially PeCSO which lead to the strong pungent and bitter taste on them in compare with bulb onions.

2. Amino acids and ACSOs biosynthesis differences between bulb onion and shallot

Highly accurate phytochemical composition analysis might be responsible for positioning *A. cepa* varieties favorably. Furthermore, the taste and quality of the foods are also influenced by their amino acids content. It has been known that free amino acids are essential for human health as essential as their role to construct taste. Broadly, shallots produce higher in total 21 free amino acids than bulb onions. Most free amino acids possess meaty taste were also detected higher in shallots than in bulb onions. The genetic background together with environmental conditions, while they were grown, gave a huge impact on this characteristics. However, the more interesting results were found in this study. We revealed that shallots have different ACSOs biosynthesis pathway than bulb onions. By detecting their intermediate compounds—glutathione, γ-glutamylcysteine (γ-GC), *S*-methylglutathione (*S*-MG), *S*-2-carboxypropylglutathione (S-2-CPG), and γ-glutamyl-S-2-carboxypropylcysteine (γ-G-S-2-CPC)—it was confirmed that shallots use a shorter pathway by directly catabolized γ-GC to γ-G-S-2-CPC without passing through the glutathione biosynthesis. We assumed that these differences are strongly related to the capability of shallots to grown under abiotic stress

condition since they were cultivated in Indonesia during the dry season. In that stressful environment, shallots try to reduce their energy usage by choosing a more efficient biosynthesis pathway. Due to that cellular mechanism, shallots could be more resistant to drought and high temperatures.

3. Evaluation on promising Japanese bunching onion heat tolerance cultivars

To meet the challenges of global warming issue, there were several F_1 varieties of *A. fistulosum* with high heat tolerance has produced by the breeder because high summer temperature limit JBO production. Heat stress, followed by biotic stress such as attacks by thrips, can seriously damage JBO production in Japan. In order to evaluate the morphological and phytochemicals characteristics those JBO heat tolerance varieties, two years experiment by using four varieties in 2013 and nine varieties in 2014 of JBO was conducted. The results indicated that all of varieties could survive under heat summer cultivation by producing higher antioxidant compounds such as flavonoid, ascorbic acid and other phenolic compound. Those phytochemicals were detected higher in the second harvesting time, which was the peak of the summer time, as the result of overeactivation of antioxidant activity. Positive correlations were found between the antioxidant activities, shown by the 2,2-dyphenyl-1-picrylhidrazyl (DPPH) radical scavenging capacity, and the phenolic content of the JBO throughout the year. The principal component analysis (PCA) could segregate JBO cultivars based on their morphology and phytochemical characteristics. ‘Kuronegi,’ ‘Kaminari,’ ‘NE 15,’ and ‘Fukuichi’ are suitable for high-yield production purposes, as they could produce a higher total fresh weight (TFW) during summer cultivation. ‘Fuyuhiko,’ ‘NE 71,’ and ‘YSG’ show lower leaf-tip burn and thrips damage than other tested cultivars, a

promising development for thrips-resistant cultivars, since thrips damage usually worsens in summer.

4. Production and characterization of *A. fistulosum*−*A. roylei* alien chromosome addition lines

The last part of this study reported that the employment of *A. roylei* in the *A. fistulosum* to create alien chromosome addition line via interspecific hybridization. By using this method, we successfully produced five alien monosomic addition lines (AMAL, 2*n* = 17) and ten monosomic addition lines (MAL, 2*n* = 18-23). The presence of the extrachromosome of *A. roylei* in *A. fistulosum* was characterized by using isozyme and DNA markers. The extrachromosome of *A. roylei* clearly altered the biochemical characteristics in the MALs. Variation in sugar, cystein sulfoxide, and flavonoid contents were observed among the MALs. *Allium fistulosum – A. roylei* allotriploids (2*n* = 24, FFR) showed significant higher saponin contents and antifungal activities of saponin extracts against isolates of *Fusarium oxysporum* f. sp. *cepae*, in comparison with *A. fistulosum*. This report of *A. fistulosum – A. roylei* addition lines opens possibility for development of novel *A. fistulosum* cultivars with enhanced nutritional value and disease resistance.

All in all, the results obtained thus far could be exploratory data upon which to build further research to improve the breeding of bulb onions, shallots, and JBOs and to enhance their biotic and abiotic stress tolerances and nutraceutical value. Moreover, the results achieved would be beneficial for breeders, producers and consumers in the industrial society of *Allium*.

生物的・非生物的ストレス適応性の改良に向けた主要ネギ属野菜の植物化学的特性評価

熱帯気候での栽培に適応しているシャロットの食味は濃厚で，刺激臭も強く，タマネギとは異なる化学内容成分組成を示すと思われるが，実験的に実証された報告はない．そこで本研究では，広範なタマネギとシャロットの品種および系統を供試し，耐暑性や食味に影響を及ぼす化合物の変化の把握を試みた．植物材料として，10 種類のタマネギ F_1 栽培品種と 12 種類の海外産のシャロット在来系統の鱗茎サンプルを 2014 年と 2015 年にそれぞれ一度ずつ収集したものを用いた．硫黄化合物（ACSO），総フラボノイドおよび可溶性糖の含量が，タマネギとシャロットの間や品種・系統間の変化を検出するために利用された．一方で，両年の試験結果を用いた主成分分析により，タマネギとシャロットを植物化学的視点から明確に区別することができ，特にタマネギは単糖類を蓄積する傾向が強いことが確認された．対照的に，シャロットは多くの多糖類を蓄積する傾向にあった．また，シャロットにおけるフラボノイドとイソアリインの含量はタマネギより高くなっており，この熱帯野菜の強い辛味や苦味は，これらの化合物の過剰生産に起因することが示唆された．

高精度植物化学成分分析はネギ属栽培種の品種・系統を機能性の観点でより好ましい位置付けに押し上げる重要な知見を与える．そこで，シャロットとタマネギの鱗茎サンプル中のアミノ酸および ACSO 合成経路における幾つかの中間生成物の組成をアミノ酸分析装置および LC-MS/MS によりそれぞれ明らかにしたところ，ネギ属野菜に特徴的な食味に関与する幾つかの主要アミノ酸が比較的高いレベルで検出された．また，シャロットはタマネギよりアミノ酸を多く含有するという興味深い知見が得られた．さらに，シャロットでは幾つかの特異な中間産物が得られたので，同植物の推定 ACSO 合成経路はタマネギの既知経路から分化していることが考えられた．すなわち，タマネギではグルタチオン生合成を介して γ-グルタミル-S-2-カルボキシプロピルシステインが合成される経路が利用されているが，シャロットでは直接 γ-グルタミルシステインから同化合物が合成されていることが推察された．この様に，シャロットは効率的な生合成経路を選択してエネルギー利用効率を高めることで，高温・乾燥下での栽培に適応している可能性が示唆された．

ネギは，ヒトの健康に有益な化合物を豊富に含有し，広く栽培されているネギ属の栽培種である．高温ストレスによる障害に引き続いて起こるアザミウマ被害のような生物的ストレスは，日本のネギ生産に深刻な被害を及ぼしている．本研究では，ネギ耐暑性品種の形態的および植物化学的特性を評価するために，2013 年に 4 品種ならびに 2014 年に 9 品種を用いて 2 年間に及ぶ試験が実施された．その結果，DPPH ラジカル捕捉活性とほとんど全ての形態形質との間で高い負の相関がみられたが，ACSO 含量と形態形質の間では高い正の相関がみいだされた。本研究で用いたネギ品種は 8 種類の農業形質と 7 種類の植物化学物質データを用いた主成分分析によって特徴づけられた．‘くろねぎ’，‘かみなり’，‘Ne-15’ と ‘ふくいち’ は夏季栽培時に総新鮮重が重くなる傾向があり，高収量性を備えていた．‘ふゆひこ’ および ‘さんぺいねぎ’ を他の品種と比較すると，アザミウマ被害の

程度が低く, これらの品種は一般的に夏季の植物体の状態を悪化させる害虫への抵抗性を有していると思われた.

北西インドに自生する *Allium roylei*（RR, 2*n*=2*x*=16）は 2 種類の栽培種，タマネギとネギ（FF, 2*n*=2*x*=16）の間の橋渡し植物として知られている．本研究では，ネギと *A. roylei* の種間交雑で F_1 雑種を作出し，その後，複二倍体を得るために F_1 雑種の染色体倍加を行った．2 度にわたって複二倍体にネギを戻し交雑した結果，BC_2 集団が得られ，それらの染色体数（2*n*）は 16 から 23 の間で変化した．この集団を遺伝マーカーにより分析したところ，5 種類の単一異種染色体添加系統（FF+nR, 2*n*=17）と 10 種類の複数添加系統（2*n*=18〜23）の添加染色体がそれぞれ同定された．また，異種染色体の添加はネギの ACSO，フラボノイドと糖の含量の変化を促し，生化学的な特性を改変することが明らかになった。さらに，異質三倍体（2*n*=3*x*=24）では，ネギと比較して明確に高いサポニン含量やタマネギ乾腐病菌に対する抗菌活性がみられた.

以上より，ネギ属植物がもつ代謝物とストレス耐性の関係が明らかにされ，一連の成果は染色体工学的手法を絡めた代謝育種の計画推進に役立つことが示された.

LIST OF PAPERS RELATED TO THE THESIS

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(In relation to chapter 5)

Ariyanti, N. A., K. Torikai, R. P. Kirana, S. Hirata, E. Sulistyaningsih, S. I. Ito, N. Yamauchi, N. Kobayashi and Shigyo, M., 2017. Comparative Study on Phytochemical Variations in Japanese F_1 Varieties of Bulb Onions and South-East Asian Shallot Landraces. Hort. J. DOI: 10.2503/hortj.OKD-066.

(In relation to chapter 2)

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