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Single Alien Chromosome Additions from Shallot (*Allium cepa* L. Aggregatum group) Increase Endogenous Polyphenol Contents in Japanese Bunching Onion

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Eight members of the *Allium fistulosum* L. – shallot (*Allium cepa* L. Aggregatum group) monosomic addition line ($2n=17$, FF+1A–FF+8A) proved to be very effective in revealing the effects of single alien chromosomes from *A. cepa* on the production of polyphenol in the green leaf tissues of *A. fistulosum*. The determination of polyphenol content in the green leaf tissue of these monosomic additions was carried out monthly from January 2002 to December 2003. Throughout the 2-year period, every monosomic addition accumulated polyphenols in green leaf tissues. Two-way ANOVA revealed significant differences, at the 1% level, in the polyphenol contents of the various monosomic addition types as well as in various months. Dunnett's test showed that four monosomic additions (FF+2A, FF+5A, FF+6A, and FF+8A) caused a greater increase in the amount of polyphenol content than did *A. fistulosum*. The levels of polyphenol accumulation in the remaining four monosomic additions and *A. fistulosum* were almost the same. A phenylalanine ammonialyase gene of shallot was allocated to chromosome 2A using two sets of the monosomic additions. These results indicate that the genes related to polyphenol production may be located on the 2A, 5A, 6A, and 8A chromosomes of shallot.

Key Words: alien monosomic addition line, *Allium fistulosum* L., leaf vegetable, phenylalanine ammonialyase, polyphenol.

Introduction

Polyphenols are secondary plant metabolites and bioactive molecules ubiquitously distributed in all plants. Polyphenols influence the morphology, growth, and reproduction of plants, as well as their resistance to parasites and environmental stresses (Bravo, 1998). The most common and important low-molecular-weight phenolic compounds are simple phenolic derivatives and flavonoids. The presence of polyphenols in plants is influenced by genetic factors and environmental conditions (Tomas-Barberan and Espin, 2001).

Recent interest in food polyphenols has increased owing to their roles as antioxidants and scavengers of free radicals. Onions (*Allium cepa* L.) contain a large number of flavonoids, which are present in the colored scales of the edible part (Bahorun et al., 2004; Chu et al., 2000). Among 11 onion varieties, shallot (*A. cepa* Aggregatum group) was found to have the highest polyphenol content (Yang et al., 2004). On the other hand, the Japanese bunching onion (*A. fistulosum* L.) has a white leaf sheath and low polyphenol content. *A. fistulosum* is frequently used raw in salads or as an herb to flavor soups and plays an important role in the Japanese diet; therefore, it is desirable to increase the polyphenol contents of *A. fistulosum* in order to increase the intake of food polyphenols by Japanese people.

A complete set of *A. fistulosum* – shallot (*A. cepa* Aggregatum group) monosomic addition lines ($2n=17$, FF + 1A–FF + 8A) was established in a previous study (Shigyo et al., 1996). Previous studies also revealed that several lines with alien chromosomes which possessed the key regulatory and structural genes for flavonoid metabolism showed different flavonoid compositions in the leaf sheath tissues (Masuzaki et al., 2006a, 2006b; Shigyo et al., 1997b).

An investigation of the polyphenol substance in the leaf blade (green leaf) tissues of the *Allium* monosomic additions should reveal changes in the polyphenol contents of *A. fistulosum* and show the chromosomal locations of the shallot genes, which are important for polyphenol metabolism. In the present study, the polyphenol contents in a complete set of *A. fistulosum* – shallot monosomic additions were evaluated to determine the effect of a single alien chromosome from

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the shallot on polyphenol production in *A. fistulosum*.

Materials and Methods

Plant materials

The plant materials were a complete set of *A. fistulosum* – shallot monosomic additions ($2n = 2x + 1 = 17$, FF + 1A–FF + 8A) and a control plant, Japanese bunching onion (*A. fistulosum* 'Kujyo-hoso', $2n = 2x = 16$, FF). The monosomic additions were grown in an experimental field at Yamaguchi University (34°N, 131°E) over a 2-year period (January 2002–December 2003). Cultivation and fertilizer applications were carried out according to the procedures of Shigyo et al. (1997a).

Polyphenol extraction and quantitative analyses

Extraction was performed monthly from January 2002 to December 2003. Two grams of green leaf tissue was extracted by 70% ethanol according to the method of Hang et al. (2004). After the extract was adequately diluted with water, polyphenol contents were determined as described by Folin and Denis (1915), with minor modifications. One milliliter of 1 N phenol reagent (Wako Pure Chemical Industries Ltd., Osaka, Japan) was mixed with 1 mL diluted extract. After 3 min, 1 mL of 10% sodium carbonate aqueous solution was added to the extract solution, and the mixture was incubated for 60 min at room temperature. The polyphenol contents were quantified according to absorbance at 530 nm on a U-2001 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan). Quantification was achieved by comparison in a catechol calibration curve.

DNA isolation

Total genomic DNA of two complete sets of *A. fistulosum* – shallot monosomic additions and control plants was isolated from fresh green leaf tissue using a mini-prep DNA-isolation method (van Heusden et al., 2000).

PCR primer design

Primer sets to amplify a segment of the gene-encoding enzyme of phenylalanine ammonialyase (PAL) in a possible sulfur assimilation pathway were designed with the GENETYX 6.1.3 software (Genetyx, Tokyo, Japan) based on the GenBank™ accession number AY541031 (complete cDNA sequence of *Allium cepa* PAL). Primer sets were as follows: forward; 5'-CTC CTC CAA GGC TAT TCG GGT ATC-3', reverse; 5'-GGG TGG TGC TTT AGC TTG TGG-3'.

PCR amplification and digestion of PCR products

PCR amplifications were conducted with 50 ng genomic DNA, each of the primers at 1 μM, 0.25 mM dNTPs, a 1 × Ex *Taq* buffer (Takara Bio Inc., Japan), and 0.5 units Ex *Taq* polymerase in a volume of 25 μL. PCR was performed as follows: initial denaturation of 3 min at 94°C, 35 cycles of PCR amplification (1 min denaturation at 94°C, 1 min annealing at 68°C, and 1 min primer extension at 72°C) on a Program Thermal Cycler iCycler™ (Bio-Rad Laboratories Inc., USA). The ramp times were measured under default conditions that adjusted temperature at the maximum ramp rate with the minimum ramp time. Nine microliters of the PCR products was incubated for 2 h at 37°C in a volume of 15 μL using 2 units of a restriction enzyme and was subsequently resolved by electrophoresis on 2% agarose gels. Restriction digestion with *Alu* I was used in an attempt to generate polymorphisms.

Statistical analyses

Monthly polyphenol content data for the eight addition lines and *A. fistulosum* were used for two-way analysis of variance (ANOVA). Dunnett's test was employed to compare the polyphenol contents of *A. fistulosum* and each monosomic addition. Statistical analyses were performed using SPSS 11.5 software with advanced models (SPSS Japan Inc., Tokyo, Japan).

Results

Determination of polyphenol contents in monosomic additions

As shown in Figure 1, the polyphenol contents in *A. fistulosum* (control) varied from 41.5 to 74.6 mg/100 g fresh weight (FW) (average, 56.7 mg/100 g FW) as catechol equivalents. Seasonal changes in the polyphenol contents of *A. fistulosum* did not vary widely. Polyphenol accumulation in all monosomic addition lines showed a small decrease from May to June with the exception of FF + 6A in 2003. The polyphenol contents in FF + 6A (average, 83.1 mg/100 g FW) were higher than in *A. fistulosum* throughout the 2-year study. FF + 2A, FF + 5A, and FF + 8A also accumulated high volumes of polyphenol (averages, 73.3, 67.2, and 76.2 mg/100 g FW, respectively). These monosomic addition lines showed that alien chromosomes, such as 2A, 5A, 6A, and 8A, caused the polyphenol contents to increase. The polyphenol contents of FF + 7A were slightly higher than those of *A. fistulosum*. Polyphenol accumulation in the remaining three monosomic additions (FF + 1A, FF + 3A, and FF + 4A) showed the same tendency as *A. fistulosum*. Two-way ANOVA revealed a significant difference at the 1% level in the monthly contents of the polyphenol substance as well as among plant materials (Table 1). Dunnett's test showed significant differences between the control and the four monosomic additions (FF + 2A, FF + 5A, FF + 6A, and FF + 8A).

Chromosomal assignment of a candidate gene involved in polyphenol production

The primer set for the PAL gene of *A. cepa* amplified a single band of approximately 1,000 bp in *A. fistulosum* and shallot. After both PCR products were digested with the enzyme *Alu* I, a polymorphism between *A. fistulosum*

and shallot was detected via 2% agarose gel electrophoresis. In the two complete sets of monosomic additions, the same restriction fragment length polymorphism (RFLP) patterns as in shallot were present only in FF + 2A (Fig. 2). This result revealed that the PAL gene of the shallot was located on chromosome 2A.

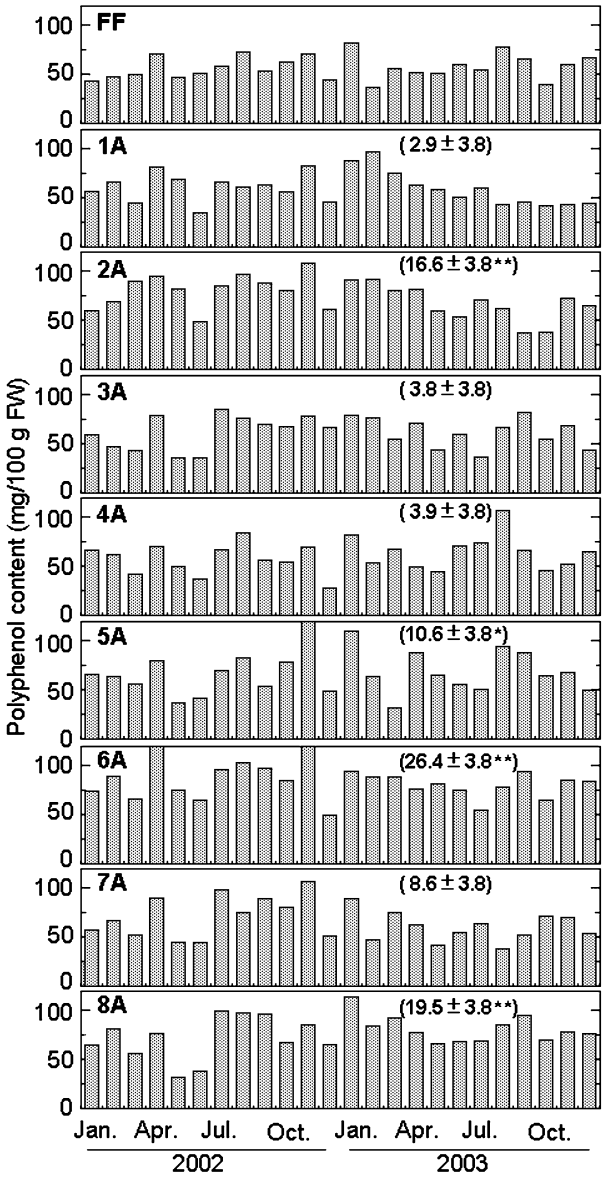

Fig. 1. The year-round variations of polyphenol contents in a complete set of monosomic additions (1A–8A) compared with *Allium fistulosum* (FF). Values in parentheses show the mean difference ± SE of polyphenol content between *A. fistulosum* and each monosomic addition. Dunnett's multiple comparison test was used for mean separation. *, ** significant at $P \leq 0.05$, 0.01, respectively.

Discussion

Different effects of a single alien chromosome addition were observed in several chemical composition productions in previous studies. The production of L-ascorbic acid (vitamin C) in three monosomic additions (FF + 1A, FF + 2A, and FF + 8A) showed a significant increase than in *A. fistulosum* and the remaining five monosomic additions showed the same tendency as *A. fistulosum* (Yaguchi et al., 2008a). In the production of non-reducing sugar, including sucrose and fructan, FF + 8A showed significantly higher sucrose accumulation but FF + 2A hardly accumulated non-reducing sugar (Hang et al., 2004; Yaguchi et al., 2008b). Almost all monosomic addition lines showed a decrease of the content of *S*-alk(en)yl-L-cystein sulfoxides, which is known as a flavor precursor of *Allium* crops, compared with *A. fistulosum* (Yaguchi et al., unpublished data). Four monosomic additions (FF + 2A, FF + 5A, FF + 6A, and FF + 8A) showed an increase of polyphenol contents compared with the control in this study; therefore, it could be expected that the increase of polyphenol contents in *A. fistulosum* would be accomplished by alien chromosome addition from shallot to *A. fistulosum*.

In the present study, several monosomic addition lines showed a small decrease of polyphenol content from May to June. Previous studies of the seasonal change of sugar accumulation in the monosomic addition lines revealed that the sugar content in the green leaf tissues of monosomic addition lines decreased from spring to summer and then increased from autumn to winter (Hang et al., 2004; Yaguchi et al., 2008b). Bolting and flowering were observed in all plant materials between March and May during the term of this study. These changes of the growth period in spring would affect the decrease of polyphenol and sugar content in green leaf tissues from several monosomic addition lines and *A. fistulosum*.

Sakakibara et al. (2003) reported that *A. fistulosum* polyphenols consisted of kaempferol glycosides (79.1–95.4 μmol/100 g FW) and caffeic acids (8.8–10.0 μmol/100 g FW). Based on these data, the total polyphenol content was 44.3–52.8 mg/100 g FW. Yang et al. (2004) reported that shallot had the highest polyphenol content (114.7 ± 10.0 mg of gallic acid equivalent/100 g FW) among 11 onion varieties. In the present study, FF + 6A

Table 1. Analysis of variance for total polyphenol contents in monosomic additions and *Allium fistulosum*.

Source	Degree of freedom	Sum of squares	Mean square	F value
Total	215			
Months	23	33670.60	1463.94	8.66[z]
Plant materials	8	15161.09	1895.14	11.22[z]
Error	184	31091.58	168.98	

[z] for significance at the 1% level

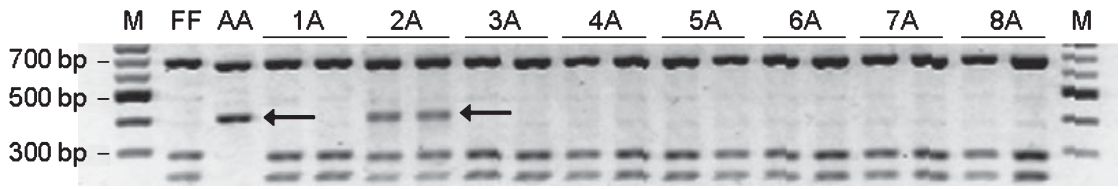

Fig. 2. Electrophoretogram showing the chromosomal location of phenylalanine ammonialyase gene in shallot. FF, *Allium fistulosum*; AA, shallot; 1A–8A, two complete sets of *A. fistulosum* – shallot monosomic addition lines. M, molecular size marker (100 bp DNA ladder). Arrows point to the shallot-specific band.

showed a higher accumulation of polyphenol (average, 83.1 mg/100 g FW) than *A. fistulosum* (average, 56.7 mg/100 g FW). The respective polyphenol contents of broccoli (*Brassica oleracea* L.), spinach (*Spinacia oleracea* L.), and yellow onion, which are considered to have high polyphenol levels, were 80.8, 79.6, and 68.9 mg of gallic acid equivalent/100 g FW (Chu et al., 2002). FF + 6A contained a substantial amount of polyphenol compounds compared with those vegetables; therefore, FF + 6A could be utilized as a desirable dietary source of polyphenols in fresh vegetables, as in the case of the new *A. fistulosum* cultivars.

Sakakibara et al. (2003), who developed a simultaneous determination method of all polyphenols in vegetables, reported that the major polyphenols were caffeic acid (a simple polyphenol) and kaempferol glycoside in *A. fistulosum* and quercetin glycoside in *A. cepa*. Ren et al. (2001) found caffeic acid in the green leaf tissues of *A. fistulosum*. Caffeic acid and glycosides of kaempferol and quercetin are a phenylpropanoid and a flavonoid, respectively. The ubiquitous plant enzymes PAL (Langer et al., 1997) and chalcone synthase (CHS) (Ferrer et al., 1999) are key biosynthetic catalysts in phenylpropanoid and flavonoid assembly, respectively. In the present study, the PAL gene was allocated to chromosome 2A of shallot. Masuzaki et al. (2006a) revealed that the CHS-A gene was assigned to chromosome 2A of shallot. The higher polyphenol accumulation observed in the green leaf tissue of FF + 2A could have been due to the higher expression of these enzyme genes. Thus, anonymous genes by the gene dosage effect related to the upregulation of polyphenol production, other than PAL, could be located on chromosome 5A, 6A, and 8A.

Rice-Evans et al. (1995) noted that polyphenolic compounds were active mainly due to their redox properties, which allowed them to act as reducing agents, hydrogen donators, and reactive oxygen quenchers. Wang et al. (2005) reported that the polyphenol compounds in *A. fistulosum* were the main active compounds contributing to antioxidant activity. Further biochemical analyses and examinations of the chromosomal assignment of genes associated with polyphenol production are necessary to reveal the polyphenol production related to antioxidant activity in *A. fistulosum* and shallot.

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