Characterization of Endogenous Gibberellins and Molecular Cloning of a Putative Gibberellin 3-Oxidase Gene in Bunching Onion

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ADDITIONAL INDEX WORDS. Allium fistulosum, bolting, chromosome 7A, active gibberellin, GA, 13-non-hydroxylation pathway

ABSTRACT. To clarify the role of gibberellin (GA) in the growth of bunching onion (Allium fistulosum), identification of endogenous GAs and expression analysis of a putative gibberellin 3-oxidase (AfGA3ox1) were conducted. GA1, GA3, GA4, GA9, GA20, and GA34 were identified with levels of GA4 and GA9 being higher than those of GA1, GA3, and GA20. The young seedlings were clearly elongated by exogenous GA4 treatment but not by GA3. These results indicate that the 13-non-hydroxylation pathway of GA biosynthesis may be predominant in shoots with GA4 playing an important role in the growth of bunching onion. Expression of AfGA3ox1 was higher in leaf sheaths than leaf blades during vegetative growth. In reproductive organs, expression of AfGA3ox1 was higher at early and middle development stages in the stalks but was detected at a late development stage in the umbels. AfGA3ox1 was mapped on chromosome 7A from shallot, a bunching onion-related species.

Bunching onion is an important leafy vegetable crop in East Asian countries such as Japan, China, and Korea (Inden and Asahira, 1990). Bunching onions are classified into two types on the basis of their edible parts, leaf and leaf sheath types.

At present, there are many cultivars and strains adapted to different regions with year-round cultivation being common in Japan. Bunching onion is biennial, usually vernalizing in winter and bolting early the next spring. Plants that have bolted lose their marketability because their flower stalks become too hard to eat. Requirements for bolting have been extensively studied under a number of environmental conditions in various varieties. For example, Yamasaki et al. (2000a, 2000b) investigated the effects of temperature and photoperiod during vernalization on flower initiation and revealed that a long-day photoperiod was needed for flower initiation and revealed that a long-day photoperiod was induced for flower initiation.

Gibberellins are known to play important roles in a number of physiological processes during plant growth. For example, Yamasaki et al. (2000a, 2000b) investigated the effects of temperature and photoperiod during vernalization on flower initiation and revealed that a long-day photoperiod was induced for flower initiation.

Received for publication 13 July 2011. Accepted for publication 11 Oct. 2011.
This work is supported in part by Grants-in-Aid from the Japanese Society for the Promotion Science (No. 19580042).
We thank Ms. S. Sanagawa and T. Araki (National Institute of Vegetable and Tea Science) for their technical assistance. We also thank Kyowa Hakko Co., Ltd., for donating GA1.

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GAs are considered to be essential for seed germination, leaf growth, inflorescence stem elongation, and seed development in bunching onion. Therefore, it is important to investigate the identification of the bioactive GAs and the molecular regulation of GA biosynthetic genes as a first step toward understanding the physiological role of GA in bunching onion. However, endogenous GAs in bunching onion have yet to be studied. In this report, we investigated endogenous GAs and analyzed the expression of a putative GA 3-oxidase in vegetative and reproductive tissues of bunching onion.

Materials and Methods

Table 1. Classification of flower developmental stages [according to Eguchi et al. (1958) and Yamasaki et al. (2000b)] used in the present study of bunching onion.

| Code | Developmental stage               |
|------|----------------------------------|
| 0    | Vegetative                      |
| I    | Apical dome enlarging to spathe formation |
| II   | Floret formation                |
| III  | Perianth and stamen formation   |
| IV   | Anther and pistil formation     |
| V    | Pollen and ovule formation      |
| VI   | Pollen grain and early stage of stigma formation |
| VII  | Flower opening                  |

Fig. 1. Distribution of GA-like activity in extracts of bunching onion shoots fractionated by high-performance liquid chromatography. Each fraction was bioassayed using a modified rice microdrop assay with the dwarf rice ‘Tanginbouzu’. Responses to GA$_3$: 50 pg/plant = 143% of the control, 100 pg/plant = 200% of the control. GA = gibberellin.

Table 2. Gas chromatography–mass spectrometry identification of GAs in the shoots of bunching onion.

| Identified GAs | Retention time (min) | KRI$^a$ | Principal ions and relative abundance (% base peak)$^a$ |
|----------------|----------------------|--------|------------------------------------------------------|
| GA$_{34}$      | 15                   | 2692   | 504 (M$^+$, 100), 475 (7.6), 387 (6.0), 370 (10.7), 208 (19.4) |
| GA$_3$         | 16                   | 2670   | 506 (M$^+$, 100), 491 (16.6), 448 (11.2), 376 (14.2), 207 (5.2) |
| GA$_{20}$      | 22–23                | 2494   | 418 (M$^+$, 100), 404 (10.5), 375 (23.1), 359 (12.0), 301 (9.3) |
| GA$_{34}$      | 25–26                | 2667   | 506 (M$^+$, 100), 459 (4.6), 431 (4.7), 416 (3.8), 372 (7.3) |
| GA$_4$         | 27                   | 2515   | 418 (M$^+$, 40.6), 386 (33.1), 328 (26.3), 284 (100), 225 (58.6) |
| GA$_3$         | 30                   | 2327   | 330 (M$^+$, 6.0), 298(100), 286 (18.4), 270 (53.7), 243 (30.7) |

$^a$KRI = Kovats’ retention indices (Kovats, 1958). KRI of authentic GAs: GA$_{34}$ = 2692, GA$_3$ = 2668, GA$_{20}$ = 2491, GA$_{34}$ = 2665, GA$_4$ = 2510, GA$_3$ = 2326.

$^b$Identified as methyl ester trimethylsilyl ether derivatives by comparison with reference spectra and KRI data (Gaskin and MacMillan, 1991). GA = gibberellin.
Quantification of endogenous gibberellins. ‘Cho-etsu’ was sown three seeds per hole on a 288-hole plug tray (10 mL/plug; Landmark Plastics, Akron, OH) containing commercial soil mixture (Napra; Yamier, Tokyo, Japan) and grown in a phototron [20/15 °C, 12/12 h (light/dark), 158 μmol-m⁻²-s⁻¹]. Ten d after sowing, 5 μg GA₃ (Tokyo Kasei, Tokyo, Japan) and GA₄ (Kyowa Hakko Co. Ltd., Tokyo, Japan) in 0.5% (v/v) methanol were applied to each plug. Fourteen d after treatment, plant height was measured.

Molecular cloning of putative gibberellin 3-oxidase cDNA (AfGA3ox1). Total RNA was isolated from shoots of ‘Cho-etsu’ using the hot borate method described by Wan and Wilkins (1994). First-strand cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan). Partial cDNA fragments of GA 3-oxidase were amplified by reverse transcription polymerase chain reaction (PCR) using two degenerate primers; sense: 5'-ATGTGGYMNGARGGNT-TYAC-3' and antisense: 5'-GT-RTGNGCNGCNAGNCCCAT-3' (Y = C/T, M = A/C, N = A/C/G/T, R = A/G). Degenerate oligonucleotide primers were designed from conserved regions of A. thaliana GA4 (Chiang et al., 1995, 1997) and GA4H (Yamaguchi et al., 1998), pea LE (Lester et al., 1997; Martin et al., 1997), rice OsGA3ox2 (AB056519; Itoh et al., 2001), and barley HvGA3ox1 [AY551430 (Spielmeyer et al., 2004)]. GA = gibberellin.
The second RACE primer was 5'-CGTGCATGATCGAGAGGGGA-3'. The 5' RACE PCR products (500 bp) were gel-purified, subcloned into the pGEM-T easy vector, and sequenced.

Expression analysis of \textit{AfGA3ox1}. Gene expression of \textit{AfGA3ox1} was analyzed in vegetative tissues of bunching onion. ‘Cho-etsu’ was planted in 10-L plastic boxes on 15 Sept. 2005 and grown under natural conditions in a glasshouse until 26 Nov. Total RNA was then isolated from the roots, leaf sheaths, and leaf blades. For Northern blot analysis, 10 µg of total RNA was fractionated on 1.2% agarose gel containing 0.66 M formaldehyde and blotted onto Hybond N’ nylon membranes (GE Healthcare, Little Chalfont, U.K.). Hybridization of the DNA blots was performed using a \textsuperscript{32}P-labeled \textit{AfGA3ox1} full-length cDNA fragment. Post-hybridization washes were as follows: 2 × 15 min at 65 °C in 0.2 × SSC (0.15M NaCl and 0.015M sodium citrate, pH7) and 0.1% sodium dodecyl sulphate, after which the membranes were exposed to an imaging plate (Fuji Film, Tokyo, Japan). Signals were detected with an image analyzer (FLA5000; Fuji Film).

Gene expression analysis of \textit{AfGA3ox1} at different reproductive stages was then conducted. Developmental flower stages and flower stalk length were scored according to Table 1 and sampled at each stage from Dec. 2004 until May 2005. After removal of the roots, leaf sheaths, and leaf blades, flower stalks from approximately five plants were divided into umbels and stalks, placed in a freezer, and kept at −80 °C. Northern blot analysis was conducted as described previously.

Chromosomal location of \textit{AfGA3ox1} in shallot. A series of alien monosomic addition lines of bunching onion carrying single chromosomes of shallot (\textit{Allium cepa} var. \textit{aggregatum}; 2n = 17, FF + 1A to FF + 8A) was used (Shigyo et al., 1996). PCR amplifications of genomic DNA of shallot and bunching onion were achieved using \textit{AfGA3ox1}-specific primers; sense: 5’-ATGCCCTCATTCTCAATGGGAAA-3’ and antisense: 5’-CTTCCCATTCAATTGGGATGC-3’. Amplified fragments were digested with \textit{RsaI}, and the products were separated by electrophoresis in 2.0% agarose gel and then stained with ethidium bromide to identify the chromosomal location of the \textit{AfGA3ox1} gene.

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) according to treatment. All ANOVAs were performed with JMP statistical software (Version 6.0; SAS Institute, Cary, NC) using treatments as a statistical parameter at a significant level of \( P < 0.05 \). Means were separated using Tukey’s multiple range test.

Results

Qualitative analysis of endogenous gibberellins. Extracts from the shoots of bunching onion were shown to contain several fractions with GA-like activity (Fig. 1). Endogenous gibberellins, GA1, GA3, GA4, GA9, GA20, and GA34, were detected by comparison of their mass spectra and KRI with those from a spectral library (Gaskin and MacMillan, 1991) (Table 2).

Fig. 3. The precursor (GA\textsubscript{9} and GA\textsubscript{20}) and active GA (GA\textsubscript{1}, GA\textsubscript{3}, and GA\textsubscript{4}) contents of bunching onion leaf sheaths and leaf blades 30 d after transplanting. Vertical bars indicate SD (\( n = 3 \)). GA = gibberellin.

Fig. 4. Expression analysis of the \textit{AfGA3ox1} gene in vegetative tissues of bunching onion. RNA was extracted from roots (R), leaf sheaths (LS), and leaf blades (LB) at 72 d after transplanting, and then 10 µg of total RNA was loaded per lane and stained with ethidium bromide.

Fig. 5. Effects of GA\textsubscript{3} and GA\textsubscript{4} (5 µg in 0.5% methanol) applied 10 d after sowing on plant height 14 d after treatment of bunching onion seedlings. Vertical bars indicate SD (\( n = 15 \)). Values with different letters are significantly different by Tukey’s multiple range test (\( P < 0.05 \)). GA = gibberellin.
However, GAs were not identified in fractions 17 to 19 and 28 to 29 despite their higher GA-like activity.

**CLONING OF cDNA ENCODING GIBBERELLIN 3-OXIDASE.** The sequence of the PCR fragment (290 bp) had high homology with onion expressed sequence tag (EST) sequences (CF440541 and CF445692). We obtained a full-length cDNA using a combination of 3' and 5' RACE and PCR based on the EST information. The resulting fragment was 1208 bp and was named *AfGA3ox1*. *AfGA3ox1* contained an open reading frame consisting of 365 amino acids, and as shown in Figure 2, the deduced amino acid sequence shared high homology with other GA 3-oxidases as follows: 62% with *A. thaliana* GA4, 53% with pea LE, 45% with rice OsGA3ox1, and 43% with barley Hv3ox1.

**QUANTITATIVE ANALYSIS OF ENDOGENOUS GIBBERELLINS AND *AfGA3ox1* EXPRESSION ANALYSIS DURING VEGETATIVE GROWTH.** In leaf sheaths and leaf blades, the GA9 content was higher than GA20; that of GA4 was higher than GA1 and GA3 (Fig. 3). The content of GA9 and GA4 in the leaf sheaths was higher than in the leaf blades. Expression of *AfGA3ox1* was high in leaf sheaths but low in leaf blades of bunching onion (Fig. 4). This was in accordance with the levels of GA4, which were higher in leaf sheaths than leaf blades (Fig. 2). The roots showed no expression of *AfGA3ox1*. GA4 had a stronger promoting effect than GA3 on first leaf length in bunching onion (Fig. 5).

**EXPRESSION OF THE *AfGA3ox1* GENE DURING REPRODUCTIVE GROWTH.** Flower stalks grew rapidly from flower developmental Stage II (Table 1; Fig. 6A). Expression of *AfGA3ox1* was high at early to middle stages (length 3–15 cm) in the stalks (Fig. 6B–C) and observed at flower developmental Stage V (length 15–40 cm) in the umbels. These results indicate that expression of *AfGA3ox1* plays a role in stalk extension and the late stage of floret (umbel) development in bunching onion.

**CHROMOSOMAL LOCATION OF *AfGA3ox1* IN SHALLOT.** PCR gave same-sized bands of 1.23 kb (data not shown) in both shallot and bunching onion. In PCR–restriction fragment length polymorphism (RFLP) analysis, bands of 0.5, 0.25, 0.2, 0.15, and 0.13 kb were observed in shallot and 0.6, 0.4, and 0.23 kb in bunching onion. In addition, the FF + 7A line contained bands from both bunching onion (FF) and shallot (AA) (Fig. 7). This result indicates that the *AfGA3ox1* gene is located on chromosome 7 in shallot.

**Discussion**

Three C-13-hydroxylated GAs (GA1, GA3, and GA20) and three C-13-non-hydroxylated GAs (GA4, GA9, and GA34) were identified in bunching onion. These results indicate that both early 13-hydroxylation and non-hydroxylation pathways of GA biosynthesis (Yamaguchi, 2008) may operate in bunching onion. However, higher levels of non-13-hydroxylated GAs were detected in the leaf sheaths and leaf blades. A higher level of GA4 in leaf sheaths was in accordance with higher expression of *AfGA3ox1* in leaf sheaths. Moreover, exogenous GA4 had a stronger effect than GA3 on first leaf length in seedlings. This suggests that the 13-non-hydroxylation pathway of GA biosynthesis may be predominant with GA4 playing an important role in the growth of bunching onion. However, further studies on feedback regulation of GA biosynthetic and catabolism genes including GA 3-oxidase and GA 2-oxidase are needed to clarify this hypothesis.

In onion (*Allium cepa*), GA1, GA4, GA6, GA9, GA12, GA15, GA20, GA24, GA34, GA44, GA51, and 3-epi-GA4 have been identified in the leaf sheath of 7-month-old seedlings.
was relatively higher than that of GA1 + GA3 (Yamazaki et al., 1993). Furthermore, in 4-week-old onion plants, the levels of GA4 were shown to be approximately five to eight and two to six times higher than those of GA1 in the leaf sheaths and leaf blades, respectively. In Allium wakegi, GA1, GA3, GA4, GA12, GA15, GA19, and GA20 were identified as endogenous GAs in basal leaf sheaths, and the level of GA4 was relatively higher than that of GA1 + GA3 (Yamazaki et al., 2002). These findings further confirm that in Alliaceae, including bunching onion, there are both 13-hydroxy and non-hydroxylation pathways of GA biosynthesis with the non-hydroxylation pathway to GA4 being predominant. Identification of GA3 suggests that the biosynthetic pathway to GA3 also exists in bunching onion. The biosynthetic pathway from GA20 to GA3 through GA5 has been reported in maize (Zea mays) (Fujioka et al., 1990) and other plant species (MacMillan, 1997) and from GA3 to GA9 in Prunus cerasus (Nakayama et al., 1996). GA3 was also identified in A. wakegi (Yamazaki et al., 2002) but not in onion plants (Nojiri et al., 1993). Tashiro et al. (1995) reported that A. wakegi is a hybrid between bunching onion and shallot; thus, GA3 biosynthesis in this species could have originated from bunching onion.

GAs play an important role in bolting in other cold-requiring plants such as R. sativus (Nishijima et al., 1998), cabbage (Hamano et al., 2002), and tulip (Rebers et al., 1995). In the present study, expression of AfGA3ox1 was observed during flower stalk growth. In addition, the level of AfGA3ox1 transcript was higher during early-stage bolting in the stalk and was also detected during late-stage bolting (pollen and ovule formation stages) in the umbel. In Cucurbita maxima female flowers, higher levels of GA4 were identified along with increased expression of CmGA20ox3 and CmGA3ox3 transcripts (Pimenta and Lange, 2006). Itoh et al. (2001) and Kobayashi et al. (1990) also reported that mature anthers of rice metabolize GAs with increased GA 3-oxidase mRNA expression during pollen differentiation in rice. Similarly, the findings presented here suggest that GAs also play important roles in pollen formation in bunching onion.

The putative Allium GA 3-oxidase gene was found on chromosome 7A of shallot by PCR-RFLP analysis using alien addition lines. It has also been reported that very high genome synteny exists in bunching onion and onion (Tsukazaki et al., 2008). Accordingly, the AfGA3ox1 gene is also thought to be located on chromosome 7F in bunching onion. Shigyo et al. (1997) reported that the morphological characteristics of the FF + 7A line include “fast expansion of leaves” and “elongation of axillary buds from autumn to winter.” We are therefore interested in determining the morphological changes related to the additional effects of the AfGA3ox1 gene on chromosome 7A. Further studies are thus needed to clarify the relationship between the GA 3-oxidase gene and morphological changes using gene knockout in bunching onion and onion.

In conclusion, the regulation of the expression of GA 3-oxidase and endogenous GAs in bunching onion has been investigated. Bunching onion contains two biosynthetic sequences for GA synthesis, the early-13-hydroxylation and the non-hydroxylation pathways. Higher levels of non-13-hydroxylated GAs were observed in the leaf sheaths and leaf blades. When combined with higher responsiveness to GA4 in seedlings, the non-13-hydroxylation pathway is considered to be the predominant route of GA metabolism in bunching onion. A relative correlation between AfGA3ox1 mRNA levels and GA4 content in the leaf was observed. AfGA3ox1 was also involved in the regulation of stalk growth. Our results suggest that AfGA3ox1 is a candidate responsible for the growth regulation in bunching onion.

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