Increase in the Expression of an Alpha-Amylase Gene and Sugar Accumulation Induced during Cold Period Reflects Shoot Elongation in Hyacinth Bulbs

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Abstract. The aim of this study was to investigate physiological and biochemical mechanisms of shoot elongation after cold period in hyacinth (Hyacinthus orientalis L. cv. Delft Blue). Hyacinth shoot rapidly elongated during hydro-culture period in cooled bulbs, but not in non-cooled bulbs. Alpha-amylase (EC 3.2.1.1.) is a key enzyme involved in starch hydrolysis. Alpha-amylase activity increased during the cold storage period and was low during rapid shoot elongation in hyacinth. In the non-cooled bulbs, its activity remained at the similar level. Sucrose content increased during the cold storage period in the shoot, but not in the scales. We, for the first time, isolated cDNA for cold-responsive alpha-amylase gene (HoAmy1A, accession No. AB198975) from hyacinth, and presented that HoAmy1A expression increased in the scale during the cold storage period, but the level was very low during shoot elongation. We also found that promoter region of HoAmy1A contained CArG element, which is related to the response to low temperature. In tulip (Tulipa gesneriana L.), the most studied bulbous plant, dramatic increase in alpha-amylase activity and translocation of sugars from the scales to shoot occurred during the shoot growth, following cold treatment (Komiyama et al., 1997; Lambrechts et al., 1994). Our results suggest that there are two types (tulip and hyacinth types) of sprouting mechanisms in bulbous plants.

Many bulbous plants require a cold period to stimulate rapid shoot elongation under suitable growth conditions. When bulbs are planted outdoors in a temperate climate, their shoots grow quite slowly during the winter, then elongate rapidly in the spring. Since the manipulation of bulb temperature to control flowering is very important for the horticultural industry, cold treatment conditions have been studied extensively. Hyacinth is one of these flower bulbs, requiring a cold period (-0.5 to 10 °C for 10 to 18 weeks) for optimal shoot elongation in spring (De Hertogh and Le Nard, 1993). It has not been demonstrated, however, how the metabolism would proceed during the cold period for subsequent shoot elongation nor what connects the length of the cold period with shoot length after planting.

Many studies on the mechanisms of germination have been reported in cereal plants. During seed germination, alpha-amylase in the aleurone layer plays an important role in hydrolyzing the endosperm starch into metabolizable sugars, which provide the energy for the growth of shoots (Akazawa and Hara-Nishimura, 1985; Beck and Ziegler, 1989). There are also a few reports on alpha-amylase in bulbous plants. Tulip, one of the most studied bulbous plants, requires cold period as in hyacinth, alpha-amylase activity in the storage organ increases slightly during the cold period and increases dramatically during shoot elongation (Komiyama et al., 1997; Lambrechts et al., 1994). Lily (Lilium cultivars Casablanca and Mona) bulblets regenerated in vitro showed increase in alpha-amylase activity in response to low temperature (Shin et al., 2002). However, studies on alpha-amylase at the mRNA transcription level in bulbous plants have not been reported, in spite of some reports available on the enzyme activity.

Low temperature causes conversion of starch to sugars in many higher plants. For example, lily and tulip bulbs and potato (Solanum tuberosum L.) tubers cause increase in reducing sugars during the cold period involved with starch breakdown (Isherwood, 1973; Komiyama et al., 1997; Lambrechts et al., 1994; Matsuura-Endo et al., 2004; Miller and Langhans, 1990; Shin et al., 2002).

Gene transcription is commonly controlled by the interplay of promoter DNA of general and gene-specific components of the mRNAsynthetic machinery (Orphanides et al., 1996). Cis-regulatory DNA elements involved in GA induction of alpha-amylase genes have been studied extensively in barley (Hordeum vulgare L.) and rice (Oryza sativa L.) (Gubler et al., 1995, 1997, 1999, 2002; Gómez-Cadenas et al., 2001; Skriver et al., 1991; Sutliff et al., 1993), GARE was reported as the GA response element. But there have been no reports on the promoter region of cold-induced alpha-amylase.

To demonstrate the cold-required sprouting mechanism in hyacinth bulbs, we investigated the effect of low temperature on the activity and gene expression of alpha-amylase, and the contents of starch and sugars in hyacinth bulbs. And we analyzed the promoter regions of HoAmy1A, whose transcription was induced by low temperature.
Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. Hyacinth bulbs, the products of The Netherlands, were stored at 5°C (cooled) or 25°C (non-cooled) for 12 weeks from 1 Oct. and then grown on aluminum trays with distilled water (hydro-culture) at 25°C with 70% relative humidity under a natural light-dark photoperiod for 5 weeks in the phytotron of Kyushu Univ., Fukuoka, Japan. Six bulbs were harvested each time (0, 7, 12, 13, 15, and 17 weeks) and separated into scales, leaves and basal plate. They were frozen in liquid nitrogen and stored at –80°C until analysis.

ALPHA-AMYLASE ACTIVITY ASSAYS. Scales of four bulbs per assay were used and extraction was conducted per bulb. Frozen powder tissue (500 to 600 mg) taken from the scales was homogenized at 0 to 4°C in 2 mL buffer containing 50 mM Hepes-NaOH (pH 7.5), 10 mM β-mercaptoethanol. The enzyme extract (5 μL) was mixed with 25 μL of 200 mM citrate-phosphate buffer (pH 4.5) containing 4 mM CaCl₂, and the reaction was started by adding 30 μL of BP-NPG (Megazyme, Bray, Ireland) as a substrate. Then 30 μL of α-glucosidase, Amylase HR Reagent (Megazyme), was added and conducted according to manufacturer’s instructions. The activity was determined as liberated p-nitrophenol detected spectrophotometrically at 405 nm (Sirou et al., 1990) with a spectrophotometer (model V-530; JASCO, Tokyo). One unit of alpha-amylase activity was taken to be the amount of enzyme required to provide 1 μmol of p-nitrophenol per minute. Protein concentrations were determined using a Bio-Rad Protein assay kit (Bio-Rad, Richmond, Calif.) with bovine serum albumin as a standard.

CARBOHYDRATE ANALYSIS. Scales and shoots of four hyacinth bulbs per analysis were used and the analysis was conducted per bulb. Frozen powder tissue (500 to 600 mg) was extracted at 60°C in 2 mL of 80% (v/v) ethanol following the procedure described by Lambrechts et al. (1994). After each extraction the suspension was centrifuged at 2000 g, for 5 min. The supernatants were pipetted off and combined. The ethanol was evaporated at 50°C. The residue was twice submitted to a 5chloroform : 8 water mixture. The water phases were combined for analysis of sucrose, glucose, and fructose. The ethanol-insoluble pellet was used for analysis of starch. Sugars and starch were analyzed using an imager (Molecular Imager FX; Bio-Rad). Experiments were repeated three times.

ISOLOATION OF cDNA CLONES FOR ALPHA-AMYLASE GENES. Frozen scales from hyacinth bulbs cooled for 7 weeks were ground into fine powder in liquid nitrogen, and total RNA was extracted by phenol/SDS method (Ausubel et al., 1997), and then treated with DNasel (RNase free, Roche Diagnostics) to remove the contaminating sequences of genomic DNA. First-strand cDNA synthesis using template 900 ng total RNA from hyacinth scales was performed in a 20-μL volume with RNA PCR kit (AMV version 2.1; Takara). Reverse transcription was performed using mixed primer (oligo dT/random primer [24:1 (v/v)]). Each cDNA diluted to 1/50 concentration and used as a template for a PCR reaction with degenerated primers. The sequence of HoAmy1A forward primer was a 5’-TCA AAT GTG TCG CGG ACA TAG TCA TAA ACC-3’ and that of the reverse primer was a 5’-ACT GTG CAG GTC ACT CCA GAT TCA AAC-3’, and these were designed for 387-bp fragment. HoAmy1B forward primer was a 5’-TTA AGG GTT AAT GAT ATT GCT AG-3’, and its reverse primer was a 5’-TTA AGC CAG TTC AGC CAC T-3’, and these were designed for 437-bp fragment. 18S rRNA was used as an internal control (Sato et al., 2005). Number of cycles was set at 20, 25, 30, 31, and 32. Those PCR products were quantified using an imager (Molecular Imager FX; Bio-Rad). Experiments were repeated three times.

RESULTS

Shoot growth of hyacinth bulbs during temperature treatment and hydro-culture period. Shoot elongation in non-cooled bulbs was low during temperature treatment and hydro-culture periods (Fig. 1). Cooled bulbs showed a similar growth pattern to non-cooled bulbs during temperature treatment period, but they grew rapidly and the shoots reached about twice the length of those of the non-cooled bulbs at 5 weeks of hydro-culturing. Neither cooled nor non-cooled bulbs flowered until 5 weeks of hydro-culturing.

ALPHA-AMYLASE ACTIVITY IN THE SCALES OF HYACINTH BULBS. Alpha-amylase activity remained at the similar level throughout temperature treatment and hydro-culture period in the non-cooled

Hyacinth bulbs and its full-length cDNA was obtained using a non-cooled plants during hydro-culture (Fig. 3D). Starch content remained constant in the cooled and cooled plants at 12 weeks of treatment period (Fig. 3D). The total amount of soluble sugars (sucrose, glucose, and fructose) in cooled and non-cooled bulbs was approximately twice that of the non-cooled plants at 12 weeks of temperature treatment (Fig. 3D). After the onset of hydro-culture, glucose and fructose contents gradually increased in cooled bulbs, whereas they remained at low levels in non-cooled plants (Fig. 3A, 3C, 3E). Sucrose content increased during temperature treatment and decreased after temperature treatment (Table 1). The amounts of glucose and fructose were comparatively low. Glucose showed the largest content at 12 weeks in the cooled treatment bulbs. There was no difference in the fructose content (Table 1). Sucrose contents in cooled and non-cooled bulbs showed similar values at 12 weeks of temperature treatment, and decreased after temperature treatment (Table 1). The amounts of glucose and fructose were comparatively low. Glucose showed the largest content at 12 weeks in the cooled treatment bulbs. There was no difference in the fructose content (Table 1). Sucrose was the major carbohydrate in the leaves of shoots throughout temperature treatment and hydro-culture periods (Fig. 3A, 3B, 3C, 3E). Sucrose content increased during temperature treatment and decreased during hydro-culture in cooled plants, whereas it increased gradually in non-cooled plants (Fig. 3A). Sucrose content increased during temperature treatment in cooled plants, but not in non-cooled plants (Fig. 3B). Little fructose was detected during temperature treatment (Fig. 3C). After the onset of hydro-culture, glucose and fructose contents gradually increased in accordance with the decrease in sucrose content in cooled plants, but they remained at low levels in non-cooled plants (Fig. 3A, 3B, 3C). There was no difference in the total amount of soluble sugars (sucrose, glucose, and fructose) in cooled and non-cooled plants at 7 weeks of temperature treatment period, but the content in the cooled plants was approximately twice that of the non-cooled plants at 12 weeks of treatment period (Fig. 3D). The total amount of soluble sugars remained constant in the cooled and non-cooled plants during hydro-culture (Fig. 3D). Starch content was relatively low in the shoot leaves (Fig. 3E).

**Isolation of a cDNA for alpha-amylase genes in the scales.** We cloned a 714-bp fragment of from the scales of cooled hyacinth bulbs and its full-length cDNA was obtained using a 5’- and 3’-RACEs protocol. The gene corresponding its cDNA sequence contained an open reading frame coding for a putative protein of 419 amino acids. An amino acid alignment was performed with DNASIS software using HoAmy1A and other amino acid sequences identified as alpha-amylases (data not shown). HoAmy1A showed identity to barley2 (Swiss-Prot/PIR P04063) (66%), a mung bean (Vigna radiata L.) (Swiss-Prot/PIR 1803517A) (64%), potato Amy23 (GenBank/EMBL/DDBJ M79328) (47%), and apple (Malus xdomestica Borkh.) Amy8 (GenBank/EMBL/DDBJ AF153828) (46%).

**Expression of HoAmy1A in the scales.** The expression of HoAmy1A estimated by a semi-quantitative RT-PCR method in the scales in cooled bulbs increased markedly during temperature treatment at 5 °C, but decreased rapidly and remained low after the onset of hydro-culturing at 25 °C (Fig. 4A). Expression of HoAmy1A in non-cooled scales was always low (Fig. 4B). 18S rRNA was used as an internal control.

<table>
<thead>
<tr>
<th>Starch (g/100 g fresh wt)</th>
<th>Sucrose (g/100 g fresh wt)</th>
<th>Glucose (g/100 g fresh wt)</th>
<th>Fructose (g/100 g fresh wt)</th>
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</thead>
<tbody>
<tr>
<td>12 weeks of cooled treatment</td>
<td>11.1 b</td>
<td>1.04 b</td>
<td>0.0300 b</td>
</tr>
<tr>
<td>12 weeks of non-cooled treatment</td>
<td>13.3 c</td>
<td>1.02 b</td>
<td>0.0078 a</td>
</tr>
<tr>
<td>3 weeks of culturing after cooled treatment</td>
<td>9.4 a</td>
<td>0.68 a</td>
<td>0.0068 a</td>
</tr>
<tr>
<td>3 weeks of culturing after non-cooled treatment</td>
<td>11.2 b</td>
<td>0.61 a</td>
<td>0.0188 a</td>
</tr>
</tbody>
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<sup>**</sup>Statistical analysis was performed using the Tukey’s HSD test. Means followed by the same letter are not significantly different at <sup>**</sup>P < 0.05. <sup>**</sup>Nonsignificant.
ISOLATION AND CHARACTERIZATION OF THE PROMOTER REGIONS OF TWO ALPHA-AMYLASE GENES. A genomic DNA fragment around the 5′-flanking sequence was amplified by inverse PCR to obtain the 5′-upstream region of the HoAmy1A cDNA. Two fragments were obtained; one was the 5′-upstream region of the HoAmy1A (DDBJ accession No. AB222269, 1042-bp), the other (DDBJ accession no. AB222270, 697-bp) was that of another putative gene for alpha-amylase (designated as HoAmy1B) based on comparing each exon sequence with the HoAmy1A cDNA. Partial sequences of the 5′-upstream regions of HoAmy1A and HoAmy1B are shown in Fig. 5. The region of HoAmy1A from -1 through -299 showed 89.2% similarity to that of HoAmy1B from -1 through -297, while HoAmy1B had no region similar to that of HoAmy1A from -300 through to -623, or from -653 through -669.

Computational analysis of the 5′-upstream regions of HoAmy1A and HoAmy1B, using PLACE Web Signal Scan program, could not identify classical TATA boxes, common core promoter elements. GARE (TAACAG/AA), a GA response element, was located between -103 and -97 of HoAmy1B (TAACAGA), but not HoAmy1A (CAGCAGA) (Fig. 5). CARe (CCWWWWWWGG; W=A or T) is cold-responsive element; this box motif was located between -527 and -536 in the upstream region of the HoAmy1A. HoAmy1B did not contain CARe in the upstream region (Fig. 5) and its expression was not detected during the cold period at all (data not shown).

Discussion

Alpha-amylase is a key enzyme involved in the hydrolysis of starch into sugars that can be metabolized. It plays an important role in germination and sprouting in many higher plants (Akazawa and Hara-Nishimura, 1985; Beck and Ziegler, 1989; Hagenimana and Simard, 1994; Karrer et al., 1991). Sucrose is utilized as an energy source and is essential for sprouting and shoot elongation. Our findings that alpha-amylase activity and gene expression increased during a cold treatment period and low during rapid shoot elongation (Fig. 1, Fig. 2, Fig. 4) were unexpected from previous reports that alpha-amylase activity increases at the time of germination or sprouting and during subsequent growth in many plants (Akazawa and Hara-Nishimura, 1985; Beck and Ziegler, 1989; Hagenimana and Simard, 1994; Komyama et al., 1997). The changes in transcription level of HoAmy1A corresponded with that in alpha-amylase activity (Fig. 2, Fig. 4) suggesting that the enzymatic activity is mainly regulated by mRNA level.

No sucrose accumulation in the scales was caused by low temperature, whereas starch content decreased during cooling period (Table 1), suggesting that most sucrose is transported from the scales to the shoot during cold period in hyacinth. Starch breakdown and sucrose accumulation in the scales of tulip occurred during cold period, and total amount of sugars (sucrose, glucose and fructose) dramatically increased after planting (Lambrechts et al., 1994). It is therefore indicated that carbohydrate metabolism in hyacinth is different from that in tulip. The more the sucrose accumulation in shoot leaves was, the more the shoot leaves elongated in hyacinth (Fig. 1, Fig. 3A). And the longer the cold treatment period was, the more sucrose accumulation in shoot leaves was (Fig. 3A). From these results, we suppose that sucrose accumulation might recognize cold requirement, and thus the shoot length might reflect the length of cold period in hyacinth.

Carbohydrates in the endosperm of seeds are completely consumed for germination. Mother-bulbs of tulips are replaced...
is the organ and sugars in the shoot increase during cold period. This plant species). In the other, alpha-amylase activity in the storage the shoot increase after planting (representative of the majority of In one, alpha-amylase activity in the storage organ and sugars in sugar transportation to the shoots during cold storage period. We show that there are thus two types of sprouting mechanisms. of hyacinth might be due to early alpha-amylase induction and degradation of sugars via starch hydrolysis and transportation to the shoot occurs during or after the cold period may reflect the difference in the bulb replacement system. Its relationship to hyacinth being a more rapidly flowering species than tulip after completion of the cold requirement (Lambrechts et al., 1994; Morris and Arthur, 1984). Hyacinth bulbs have perennial scales, and a part of the carbohydrate in the scales is preserved for years. The difference in whether the increase in perennial scales, and a part of the carbohydrate in the scales is preserved for years. The difference in whether the increase in alphamy selected TATA boxes. However, putative initiator elements that overlaps the transcription start site compensates for the lack of a TATA box, and directs basal transcription initiation (Smale, 1997; Smale and Baltimore, 1989; Smale et al., 1998). Our results showed that the 5'-upstream regions of neither HoAmy1A nor HoAmy1B had classical TATA boxes. However, putative initiator motifs were located overlapping the transcription initiation sites between -2 and +5 in both HoAmy1A and HoAmy1B. The expression of HoAmy1B was much less than that of HoAmy1A during cold period, suggesting that some sequences of promoter regions of HoAmy1A different from HoAmy1B should contain gene-specific regulatory elements for cold-induced gene expression. There have been no reports on the promoter region of cold-induced alpha-amylase, neither in bulbous or other plants. We here report on two promoter regions of alpha-amylase genes, HoAmy1A and HoAmy1B, with parts of their 5'-untranslated regions. Putative transcription factor binding sites and transcription initiation sites are labeled. The DNA analysis was used to make the alignment. Identical nucleotides are shaded in gray. Dashed lines indicate gaps introduced to achieve maximum alignment. genes are classified into two categories: common core promoter elements that are needed for basal transcription initiation, and gene-specific regulatory elements located upstream (Roeder, 1996). The core promoter is located near or at the transcription initiation site. A TATA box is essential in most cases. However, many genes lack a TATA box at the expected positions (Azizkhan et al., 1993). In some TATA-less promoters, an initiator element that overlaps the transcription start site compensates for the lack of a TATA box, and directs basal transcription initiation (Smale, 1997; Smale and Baltimore, 1989; Smale et al., 1998). Our results showed that the 5'-upstream regions of neither HoAmy1A nor HoAmy1B had classical TATA boxes. However, putative initiator motifs were located overlapping the transcription initiation sites between -2 and +5 in both HoAmy1A and HoAmy1B. The expression of HoAmy1B was much less than that of HoAmy1A during cold period, suggesting that some sequences of promoter regions of HoAmy1A different from HoAmy1B should contain gene-specific regulatory elements for cold-induced gene expression. There have been no reports on the promoter region of cold-induced alpha-amylase, neither in bulbous or other plants. We here report on two promoter regions of alpha-amylase genes, HoAmy1A is cold-responsiveness, and HoAmy1B is not. Some studies have suggested that many cold-responsive genes have in their promoter regions one or several copies of the CRT/DRE cis-element, which has the core sequence CCGAC (Stockinger et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). A family of transcription factors, CBFs or DREBs, binds to this element and activates transcription of the downstream cold and dehydr-
tion-responsive genes (Liu et al., 1998; Stockinger et al., 1997). *HoAmy1A* had no CTR/DRE *cis*-element in the promoter region, suggesting that *HoAmy1A* expression is controlled independently in this pathway.

Another report about cold-induced genes is on the flowering-time gene *SOC1* (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). Extended exposure to low temperature is required for flowering in arabidopsis Hephworth et al., 2002, as well as for shoot elongation in hyacinth or tulip. FLC is a transcription factor (Michaels and Amasino, 1999) and the mRNA level is reduced in response to low temperature (2 to 4 °C) (Gendall et al., 2001; Michaels and Amasino, 1999). FLC binds to CARG (Hephworth et al., 2002) and acts as a negative regulator of *SOC1* in arabidopsis (Hephworth et al., 2002, Lee et al., 2000; Samach et al., 2000). The expression of *SOC1* increases under low temperature conditions due to the reduction of FLC. Our results showed that *HoAmy1A* has a CARG motif in the promoter region, suggesting that the expression of *HoAmy1A* might be regulated by a similar mechanism to that mentioned above.

We propose based on the results in this study that 1) starch in hyacinth scales is hydrolyzed by cold-induced alpha-amylase, and sugars are transported to the shoot during cold period, 2) sucrose accumulation might recognize cold requirement, and thus the shoot length might reflect the length of cold period in hyacinth, and 3) there are two types (tulip- and hyacinth-types) of sprouting mechanisms in bulbous plants.

**Literature Cited**


