Changes in the expression of carbohydrate metabolism genes during three phases of bud dormancy in leafy spurge

Wun S. Chao • Marcelo D. Serpe

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Abstract Underground adventitious buds of leafy spurge (Euphorbia esula) undergo three well-defined phases of dormancy, para-, endo-, and ecodormancy. In this study, relationships among genes involved in carbohydrate metabolism and bud dormancy were examined after paradormancy release (growth induction) by decapitation and in response to seasonal signals. Real-time PCR was used to determine the expression levels of carbohydrate metabolism genes at different phases of bud dormancy. Among differentially-regulated genes, expression of a specific Euphorbia esula β-amylase gene (Ee-BAM1) increased 100-fold after growth induction and 16,000-fold from July (paradormancy) to December (ecodormancy). Sequence data analysis indicated that two genes, Ee-BAM1 and Ee-BAM2, could encode this β-amylase. However, real-time PCR using gene-specific primer pairs only amplified Ee-BAM1, indicating that Ee-BAM2 is either specific to other organs or not abundant. The deduced amino acid sequences of these two genes are very similar at the N-terminal but differ at the C-terminal. Both contain a nearly identical, predicted 48-amino acid plastid transit peptide. Immunoblot analyses identified a 29 kD (mature Ee-BAM1 after cleavage of the transit peptide) and a 35 kD (unprocessed EeBAM1) protein. Both 35 and 29 kD proteins were constitutively expressed in growth-induced and seasonal samples. Immunolocalization indicated that Ee-BAM1 is in the cytosol of cells at the shoot tip of the bud. Ee-BAM1 also surrounds the amyloplasts in mature cells toward the base of the bud. These observations suggest that Ee-BAM1 may have dual functions; serving as reserve protein in the cytosol and as a degrading enzyme at the surface of amyloplasts.

Keywords β-amylase • Carbohydrate metabolism • Dormancy • Leafy spurge

Introduction

Leafy spurge (Euphorbia esula L.) is a deep-rooted perennial weed that infests range and recreational lands in the northern Great Plains of North America. Underground adventitious buds on the root and crown (i.e. root and crown buds) undergo well-defined phases of dormancy throughout the year (Anderson et al. 2005; Chao et al. 2006). Dormancy has been described as temporary suspension of growth of any plant structure containing a meristem (Lang et al. 1987). Dormancy is subdivided into three categories: (1) ecodormancy in which growth cessation is controlled by external environmental factors, (2) paradormancy or correlative inhibition in which growth cessation is controlled by physiological factors external to the affected structure, and (3) endodormancy or innate dormancy in which growth cessation is controlled by internal physiological factors. In leafy spurge, paradormancy inhibits buds from developing into new shoots through signals generated from the actively growing aerial portion of the plant, namely auxin and sugars (Horvath 1998, 1999; Chao et al. 2006). In comparison,
endodormancy may be triggered by cold temperature (Foley et al. 2009).

A major shift in starch and free soluble sugars were observed during paradormancy release (Chao et al. 2006). Root buds of intact (control) plants contained the highest level of sucrose. Sucrose levels decreased significantly (40%) 1 day after paradormancy release by decapitation and stayed at similar levels until day 5. Amyloplasts were also abundant in control buds, and they were drastically reduced after decapitation (Chao et al. 2006). During seasonal cycles, starch levels were greater during the period of paradormancy (Jul to late Sep), but during the transition from para- to endodormancy (Sep to early Oct), an inverse shift in starch and free soluble sugars began to occur. As starch levels decreased, total soluble sugars increased until reaching maximum levels in Nov to Dec and remained elevated through the ecodormant period (Dec to Mar). As crown buds began to show increased growth after overwintering (Apr), a corresponding shift back to increased levels of starch and decreased levels of soluble sugars (mainly sucrose) occurred (Anderson et al. 2005).

Plants convert fixed carbon to starch and sucrose, which can be metabolized to provide energy and organic carbon to synthesize other molecules. Starch is a highly branched, with straight chains of polysaccharides made of glucose. Amylose consists of (mainly sucrose) occurred (Anderson et al. 2005). Plants convert fixed carbon to starch and sucrose, which can be metabolized to provide energy and organic carbon to synthesize other molecules. Starch is composed of amylopectin and amylose, which are both long chain polysaccharides made of glucose. Amylose consists of straight chains of α-1,4-linked glucan, while amylopectin is highly branched, with α-1,4-linked glucan chains connected by α-1,6-bonds. The branches are packed into semi-crystalline macromolecular granules, which have to be broken down to component sugars for plant use.

There are many enzymes responsible for the breakdown of starch granules (Fig. 1) (for reviews, see Lloyd et al. 2005; Smith et al. 2005; Zeeman et al. 2007a, b). Glucan-water dikinase (GWD) phosphorylates a small portion of glucose residues in amylopectin, whereas the activity of phosphorylase (PWD) is tightly dependent on starch being previously phosphorylated by GWD. Glucan phosphorylation may alter the granule surface, making the granule matrix more accessible to starch degrading enzymes (Lloyd et al. 2005). Recently, Edner et al. (2007) provided evidence for an interdependence between the activities of GWD and starch degrading enzymes such as β-amylases. Amylases include α- and β-amylases. α-Amylases can cleave the α-1,4 linkage in amylopectin molecules. α-Amylases have been shown to play an important role in starch degradation in cereal endosperm and rice leaves (Oryza sativa) (Asatsuma et al. 2005); however, it does not appear to play an essential role in starch degradation in Arabidopsis leaves (Yu et al. 2005). β-Amylases are exoamylases that hydrolyze alternate α-1,4 linkages sequentially from the non-reducing end of amylopectin molecules, producing maltose. RNAi and mutant studies indicate that α-amylases are important enzymes for starch degradation in leaves of potato and Arabidopsis (Scheidig et al. 2002; Kaplan and Guy 2005). Debranching enzymes, including isoamylases and pullulanases (limit dextrinases) cleave the α-1,6 linkage in amylopectin molecules. Isoamylase3 may work on granular starch directly at night to degrade starch and is probably the major debranching enzyme in Arabidopsis leaves (Delatte et al. 2006). Disproportionating enzyme (DPE1) (also called amylolmaltsase, α-enzyme, and 4-α-glucanotransferase) catalyzes the transfer (disproportionation) of maltose units from one 1,4-α-1,6-glucan (the donor) to another (the acceptor). The preferred substrate for DPE1 is maltotriose, which is a malto-oligosaccharide (MOS) containing three glucose units, and a glucose is produced when DPE1 acts on maltotriose. The reaction changes the size distribution of MOS molecules, potentially generating suitable substrates for other enzymes involved in starch metabolism such as starch phosphorylase and β-amylase (Takahata and Smith 1999; Chia et al. 2004; Lu and Sharkey 2004).

Maltose (produced by the action of β-amylase) and glucose (produced by the action of DPE1) are the major degradation products of starch in plastids and the major metabolites exported to the cytosol. Maltose and glucose are exported into the cytosol through the maltose transporter MEX1 and the glucose transporter, respectively (Niittyla¨ et al. 2004; Weber et al. 2000). In the cytosol, maltose is further metabolized by transglucosidase DPE2 in Arabidopsis. DPE2 uses maltose as a donor molecule to transfer one glucose moiety to a polysaccharide and release the other glucose moiety for phosphorylation and conversion to sucrose (Chia et al. 2004; Lu and Sharkey 2004).

The shift in carbohydrate levels during paradormancy release and seasonal cycles and the inhibitory effect of some carbohydrates (i.e. sucrose and glucose) on the growth of underground adventitious buds of leafy spurge suggest that sugar molecules may play important roles in the control of bud dormancy. Sugars serve as a primary source of carbon and energy, but also may act as signaling molecules that affect bud growth. This notion is supported by observations in other systems where sugars play regulatory roles in processes such as embryo growth during seed development and in the control of seed germination (Wobus and Weber 1999; Dekkers et al. 2004; Koch 2004). Furthermore, plant responses to environmental factors are often mediated by changes in sugar levels (Moore et al. 2003; Rolland et al. 2006). These changes can activate various pathways that regulate transcription, translation, and protein stability (Rolland et al. 2006; Hummel et al. 2009). Characterization of the expression of enzymes involved in the metabolism of carbohydrates can thus provide an insight into mechanisms that regulate the levels of molecules with critical functions as both carbon and...
energy sources, and signaling molecules. In this study, we used real-time PCR to examine the expression of genes involved in carbohydrate metabolism after growth induction by decapitation and in response to seasonal signals. In addition, the protein levels of $\beta$-amylase, Ee-BAM1, and the activities of $\alpha$- and $\beta$-amylases were determined to examine if the abundance and activities of these enzymes correlated with changes in bud dormancy.

**Materials and methods**

**Real-time PCR**

Primer pairs were designed from 21 clones annotated to carbohydrate metabolism genes (Table 1) based on sequences obtained from a leafy spurge EST-database (Anderson et al. 2007). Primers (20-24 nucleotides) were designed using Lasergene (DNASTAR, Inc., Madison, WI) sequence analysis software. Expression of these genes was examined using total RNA prepared from underground adventitious buds of growth-induced and seasonal samples. Growth-induced bud samples were harvested from greenhouse-grown plants 0, 2, 4, 8, 16 h, 1, 2, 3, and 4 days after shoot removal, whereas seasonal bud samples were harvested monthly (July to December) from field-grown leafy spurge (Anderson et al. 2005). Two sets of growth-induced (2003 and 2004) and two sets of seasonal (2003 and 2004) bud samples were collected. All samples were harvested between 10 a.m.–12 p.m. to avoid circadian effects on gene expression.

Total RNA was extracted from underground adventitious buds of growth-induced and seasonal samples, quantified, and used to prepare cDNA template through reverse transcription (RT) reaction. The details of cDNA preparation and real-time PCR parameters were described previously by Chao (2008). Briefly, the comparative $C_T$ method was used to determine changes in target gene expression in test samples relative to a control sample. Fold difference in gene expression of test versus control sample is $2^{-\Delta C_T}$, where $\Delta C_T = C_{T,\text{test}} - C_{T,\text{control}}$. SYBR green chemistry was used to produce a fluorescent signal, and three technical replicates were used per sample for the real-time PCR experiments. Heat-maps of the RT-PCR results were created based on log2 values using Eisen Lab software, Cluster and TreeView (Stanford University, Stanford, CA, USA) as described by Eisen et al. (1998).

**Antibody preparations, immunoprecipitation, and immunoblot analysis**

For preparation of antibodies, two peptides were designed based on the DNA sequence of an amplified $\beta$-amylase. The amino acid sequence of peptide 1 (P1) was PLDTITLGGKLNRPRA, and the amino acid sequence of peptide 2 (P2) was KASAEAIKKD. Peptide synthesis, antibody production, and affinity purification were performed by Affinity Bioreagents (Golden, CO) according to the company’s general protocols. Briefly, P1 and P2 were injected jointly into two rabbits. For antibody purification, crude antiserum from two rabbits was combined and passed through a column containing immobilized P1 then a column...
containing immobilized P2 to obtain P1 and P2 antibodies. Using these antibodies, immunoprecipitation was carried out according to Chao et al. (2007). For immunoblot analysis, protein samples were phenol extracted as described by Wang et al. (1992). SDS–PAGE was carried out according to the method of Laemmli (1970) using a 10% polyacrylamide resolving gel and a 4% stacking gel. The immunoblot procedures were described by Wang et al. (1992).

Immunolocalization

For immunolocalization studies, underground adventitious buds were fixed in 4% (w/v) paraformaldehyde in 50 mM Pipes (pH 6.9) containing 5 mM MgSO4 and 5 mM EGTA and embedded in 4:1 (v/v) butyl methacrylate to methyl methacrylate (Polysciences, Inc., Warrington, PA, USA) as described by Baskin et al. (1992). Samples were sectioned at a thickness of 3 μm and affixed to slides coated with 3-aminopropyltriethoxysilane. After removal of the methacrylate, sections were treated with a blocking buffer (pH 7.4) consisting of 100 mM K-phosphate, 138 mM NaCl, 2.7 mM KCl, 0.5% Tween 20 (Pierce, Rockford, IL, USA) and 10% horse serum (Sigma). The sections were then incubated for 2 h with a 1:500 dilution of the P2 antibody in blocking buffer. After rinsing with blocking buffer, bound primary antibody was detected using goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Molecular Probe, Eugene, OR). To ascertain the specificity of the antibodies to the sections, we conducted two controls. Some sections were incubated with secondary antibody only, while others were incubated with primary antibody that was preabsorbed overnight with the P2 peptide at a molar ratio of blocking peptide to antibody of 50 to 1. After immunolabeling, sections were counter stained with 4',6-diamidino-2-phenylindole (DAPI) to facilitate localization of P2 antibody binding at the subcellular level.

x- and β-amylase activity assays

Two sets of seasonal (2005 and 2006) and time point (Mar-07 and Apr-07) crown bud samples were used to examine the activities of x- and β-amylase activities. x- and β-amylase were extracted according to the Megazyme protocol (Wicklow, Ireland) with a minor modification. Prior to extraction, 0.25% NP-40 (Fluka, St. Louis, MO), 2% PVPP (Sigma) and 1% Protease Inhibitor Cocktail (Sigma) was added to the extraction buffers.

Table 1 Primers used to determine expression of carbohydrate metabolism related genes using real-time PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer 5′–3′</th>
<th>Reverse primer 5′–3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H3</td>
<td>GAGGTGTGAAGAAGCCCCATCGTT</td>
<td>TAGGCCGAAAGGGTAGATCCTCC</td>
</tr>
<tr>
<td>1. α-amylase (CV03118A1E01)</td>
<td>AATCAACCATAGAACCTGCCGAGAA</td>
<td>CATTTTGCGCCTGCTGAGTGGAGAT</td>
</tr>
<tr>
<td>2. α-amylase (CV03059A1C08)</td>
<td>CTGGCTACCGCCATCGCCTGAGACCT</td>
<td>CTCCCTCGGAAAGATACCCCATCA</td>
</tr>
<tr>
<td>3. α-amylase (CV03094A1H11)</td>
<td>TCAATCCAACGGTTCCAAAACGAG</td>
<td>TAAACTTCACCACGCCCCCATTCA</td>
</tr>
<tr>
<td>4. α-amylase (CV03106B1G03)</td>
<td>CTTAAGGAAGACGCAGAGTTATGG</td>
<td>AGAGATCGGTTTTTCTGTTTAT</td>
</tr>
<tr>
<td>5. β-amylase (CV03118B2G07)</td>
<td>GGAGACGACAGAGAAGACCTACAGAT</td>
<td>CATTTTGCGCCTGCTGAGTGGAGAT</td>
</tr>
<tr>
<td>6. β-amylase (CV03058A2C08)</td>
<td>ATGCCGGAATACCAATGGGTGCTCT</td>
<td>ATGGTGTCGGTATGAGTGTGCT</td>
</tr>
<tr>
<td>7. β-amylase (CV03052A1F10)</td>
<td>AGCAGGTTGTTAGGGAGGAGAAGGG</td>
<td>GTTGGGTTCTCTAGTAGTGTGCT</td>
</tr>
<tr>
<td>8. β-amylase (CV031388B1G11)</td>
<td>TTGGATACCATAGAACGCTGAA</td>
<td>CTCTACATGCGCTGCTGCT</td>
</tr>
<tr>
<td>9. β-amylase (CV03126A2E05)</td>
<td>TGGTTAAGGAGGTTTGTAGGAAG</td>
<td>GTTGGGTTCTCTAGTAGTGTGCT</td>
</tr>
<tr>
<td>10. Glucanotransferase (CV03069A1D07)</td>
<td>GGAGATCCTGCTATTTGCGTAGT</td>
<td>ATCCGCGGGTCGGAAGATTAG</td>
</tr>
<tr>
<td>11. Glucanotransferase (CV03120A2H06)</td>
<td>GGGGCTGCTTTGCTTCATCTCTAC</td>
<td>TGGTGTCGGTATGAGTGTGCT</td>
</tr>
<tr>
<td>12. Glucanotransferase (CV03047B2C01)</td>
<td>AATTTTCGGGGAATCACAATTCGCT</td>
<td>CTCTACATGCGCTGCTGCT</td>
</tr>
<tr>
<td>13. Glucose transporter (CV03093A2G04)</td>
<td>CCGGCTTGGTGTCTGGGAGACTGCT</td>
<td>ATGGTGTCGGTATGAGTGTGCT</td>
</tr>
<tr>
<td>14. Glucose transporter (CV03131A1E10)</td>
<td>TCTACTTCCGCGGCCCTTGTGTTT</td>
<td>TCTATTTTCGCGGAGACCCACTT</td>
</tr>
<tr>
<td>15. Glycosyl hydroxylase (CV03036B2D01)</td>
<td>ATTCGCGGCGCTTTCACCTGCT</td>
<td>GATATTTCGCGGACTGCGGACT</td>
</tr>
<tr>
<td>16. Glycosyl hydroxylase (CV03041A1D06)</td>
<td>GCAAAACTATGATGAGGAGAGCTCA</td>
<td>CATTTTCGCTGCTGCTGCT</td>
</tr>
<tr>
<td>17. Glycoside hydroxylase (CV03018B2C08)</td>
<td>AAGCCTCTCATACTCTTCATCCT</td>
<td>GATATTTCGCGGACTGCGGACT</td>
</tr>
<tr>
<td>18. Hexokinase (CV03077B2C08)</td>
<td>GCGGCTTGGTGTGATGAGATACGAT</td>
<td>CTGTGATAGGCTGGTCTGTCG</td>
</tr>
<tr>
<td>19. Isoamylase (CV03098B2H11)</td>
<td>TTCAGACGCTTGGGAGTGTGGA</td>
<td>CGACGGCCTGCTTCTCCTCAC</td>
</tr>
<tr>
<td>20. Isoamylase (CV03134B1D02)</td>
<td>CTTCCGTCTTTTCTAATTCCTCAC</td>
<td>TGGTGTCGGTATGAGTGTGCT</td>
</tr>
<tr>
<td>21. Pullulanase (CV03111A2G12)</td>
<td>ACCTGCGGAATATGAGTGTGACT</td>
<td>ATGGTGTCGGTATGAGTGTGCT</td>
</tr>
<tr>
<td>22. Ee-BAM1 gene specific primer pair</td>
<td>CTAGTACAAGCAAATTTTCTCAGGA</td>
<td>AAATATCCATGCGGACTGCTG</td>
</tr>
<tr>
<td>23. Ee-BAM2 gene specific primer pair</td>
<td>GCTGCTTCTGCTACTACCTCTG</td>
<td>ATGATTTTCGCGGACTGCGGACT</td>
</tr>
</tbody>
</table>
z-Amylase activity was determined according to the Ceralpha method (Megazyme). The procedure uses the "non-reducing end blocked p-nitrophenyl maltoheptaoside" (BPNPG7) as substrate in the presence of excess levels of α-glucosidase. The assay was performed by incubating 100 μl of extract with 100 μl of substrate for 20 min at 40°C. Released p-nitrophenol was determined by measuring the absorbance at 400 nm after adding 1.5 ml 1% trisodium phosphate solution, pH 11. β-amylose activity was assayed according to the Betamyl method (Megazyme). The procedure uses the "p-nitrophenyl maltoheptaoside" (PNPG5) as a substrate with excess levels of α-glucosidase and in the presence of stabilizers to reduce the rate of cleavage of PNPG5 by α-glucosidase. The assay was performed by incubating 100 μl of extract with 100 μl of 1,250-fold diluted extract with 100 μl of substrate for 10 min at 40°C. Released p-nitrophenol was determined by measuring the absorbance at 400 nm after adding 1.5 ml 1% Trizma base solution, pH 8.5.

Results

Differential expression of carbohydrate metabolism genes

Growth of underground adventitious buds of leafy spurge into new shoots is inhibited by sucrose and glucose at 30 mM (Chao et al. 2006). In addition, carbohydrate contents shift drastically after paradormancy release. We thus examined the expression of a number of genes involved in carbohydrate metabolism using real-time PCR to determine whether changes in gene expression are linked to shifts in carbohydrate levels.

The transcript profiles of 21 individual clones including 4 α-amylases, 5 β-amylases, 3 glucanotransferases, 2 glucose transporters, 1 glycosidase hydrolase, 2 glycosyl hydrolases, 1 hexokinase, 2 isoamylases, and 1 pullulanase were examined. Histone H3 (HisH3) was used as a control since its expression pattern was determined previously (Anderson et al. 2005; Jia et al. 2006). After growth induction (Fig. 2), HisH3 transcript started to increase 1 day after decapitation and reached a plateau by day 2, showing the expected expression pattern in both 2003 and 2004. During seasonal progression (Fig. 3), the HisH3 transcript levels were up-regulated in Aug, down-regulated in Sep or Oct, and lowest in Dec, exhibiting a similar pattern in two seasonal cycles (2003 and 2004). In 2003, the log2 value for the Dec sample was −5.71, which was 53-fold less than that of the Jul sample. Since HisH3 exhibited the expected expression pattern after growth induction (2003 and 2004) and in two seasonal cycles (2003 and 2004), total RNA and cDNA samples were thus considered adequate for examining the expression of other genes.

mRNA expression after growth induction

In Fig. 2 (also in Supplementary Table 1), among four α-amylase genes, two (3: CV03094A1H11 and 4: CV03106B1G03) were slightly up-regulated after 2–4 h and stayed at similar levels until day 4. One α-amylase (2: CV03059A1C08) was up-regulated 2 or 4 h after induction and then down-regulated drastically at day 2. Another α-amylase (1: CV03118A1E01) was down-regulated after growth induction, up-regulated after 4 h or 2 day, and declined to control level at day 4. Among the five β-amylases, one β-amylase (5: CV03118B2G07) was quickly and drastically up-regulated after growth induction and maintained high expression levels until day 4. Two β-amylases (6: CV03058A2C08 and 7: CV03052A1F10) were up-regulated 2 h after growth induction and down-regulated to levels slightly above (2003) or equal (2004) to the control. The two remaining β-amylases (8: CV03118B2G07 and 9: CV03126A2E05) were down-regulated immediately after growth induction and then up-regulated slightly after 4 h or 1 day to a level slightly above (2003) or equal to (2004) the control.

For glucanotransferases, the expression patterns for CV03069A1D07 (10) and CV03047B2C01 (12) were not consistent between 2003 and 2004. In the 2003 samples these two genes were up-regulated after growth induction, whereas in the 2004 samples, they were either down-regulated (10: CV03069A1D07) or stayed at the control levels (12:
A difference is designated as log2 value. Seasonal bud samples were used to examine the expression of these genes. The fold was the July buds. Two sets of seasonal (2003 and 2004) crown bud samples relative to a control sample. The control for seasonal samples was harvested from July through December. The comparative Cβ method was used to determine changes in transcript profiles in test samples relative to a control sample. The control for seasonal samples was the July buds. Two sets of seasonal (2003 and 2004) crown bud samples were used to examine the expression of these genes. The fold difference is designated as log2 value. Red color indicates up-regulated genes and green color indicates down-regulated genes.

CV03047B2C01. In contrast to all other genes, glucanotransferase CV03120A2H06 (11) was constitutively expressed. One glucose transporter (13: CV03093A2G04) was up-regulated after growth induction, whereas the expression pattern of the other (14: CV03131A1E10) was inconsistent between the 2003 and 2004 samples. Both glycosyl hydrolases (15: CV03036B2D01 and 16: CV03041A1D06) were slightly up-regulated 2–4 h after growth induction and stayed at a similar level until day 4. Glucoside hydrolase (17: CV03108B2C08) was down-regulated immediately after growth induction and up-regulated after 4 h or 1 day. Hexokinase (18: CV03077B2C08) was transiently up-regulated after growth induction and returned to control levels. Both isoamylase (19: CV03098B2H11 and 20: CV03134B1D02) transcripts were transiently down-regulated and stayed slightly up-regulated afterwards. The expression of pullulanase (21: CV03111A2G12) was not the same between 2003 and 2004, but can be considered as transiently and slightly up-regulated after growth induction.

mRNA expression in response to seasonal signals

In Fig. 3 (also in Supplementary Table 2), among the four α-amylase genes examined, one (3: CV03094A1H11) was up-regulated and three were down-regulated as the season progressed from Jul to Dec; however, the down-regulation of two (1: CV03118A1E01 and 4: CV03106B1G03) of the 2003 samples was insignificant. Among the five β-amylases, two (5: CV03118B2G07 and 6: CV03058A2C08) were up-regulated, two (8: CV03138B1G11 and 9: CV03126A2E05) were down-regulated, and one (7: CV03052A1F10) exhibited inconsistent expression patterns between 2003 and 2004. For glucanotransferases, two (10: CV03069A1D07 and 12: CV03047B2C01) showed minor up-regulation and one (11: CV03120A2H06) was constitutively expressed. Both glucose transporters were slightly up-regulated. One glycosyl hydrolase (15: CV03036B2D01) was slightly down-regulated and another one (16: CV03041A1D06) was slightly up-regulated during ecodormancy. Pullulanase (21: CV03111A2G12) was slightly up-regulated between July and December. The rest of the clones (2 glycosyl hydrolases, 1 hexokinase, and 2 isoamylases) were differentially regulated to some extent; however, the expression patterns were inconsistent between 2003 and 2004.

Among these 21 clones, β-amylase (5: CV03118B2G07) exhibited the highest increase in gene expression after both growth induction and in response to seasonal signals. These large changes in gene expression suggested that CV03118B2G07 has an important role in the control of bud dormancy. Consequently, this gene was selected for further studies.

Sequence comparison of β-amylases

Sequencing data revealed that the differentially expressed β-amylase (5: CV03118B2G07) can be encoded by two genes, Ee-BAM1 (GU062394) and Ee-BAM2 (GU062395) (Fig. 4). The deduced amino acid sequences of these two genes are very similar at the N-terminal end but differ at the C-terminal. Based on the predictions of ChloroP software, Ee-BAM1 and Ee-BAM2 also contain a nearly identical, 48-amino acid plastid transit peptide. The deduced amino acid sequences of Ee-BAM1 and Ee-BAM2 are highly similar to a plastid-targeted potato β-amylase PCT-BMY1 (AF393847) and a plastid-targeted Arabidopsis β-amylase AtCt-BAM3 (also named At4g17090, CT-BMY, and BMY8). PCT-BMY1 and AtCt-BAM3 are major enzymes involved in leaf starch degradation in these two species. Their functions were confirmed using transgenic plants; i.e. down-regulation of these two transcripts resulted in high levels of starch accumulation in leaves (Scheidig et al. 2002; Kaplan and Guy 2005). Interestingly, the deduced amino acid sequences of the two leafy spurge β-amylases are significantly shorter than the potato and Arabidopsis homologs (Fig. 4).
Real-time PCR, using gene-specific primer pairs for each gene (Table 1, 22 and 23), only amplified Ee-BAM1, indicating that Ee-BAM2 is not abundant or is expressed in other parts of the plant. In contrast, the expression patterns of Ee-BAM1 (Fig. 5) are almost identical to the expression patterns of the β-amylase gene (5: CV03118B2G07, see Figs. 2, 3), indicating that primer pairs for Ee-BAM1 and for CV03118B2G07 amplified the same cDNA template. As shown in Fig. 5, Ee-BAM1 responded to growth induction very quickly. For example, 2 h after decapitation, the Ee-BAM1 transcript increased 8- to 16-fold with expression levels of some time points as high as 100-fold. During the seasonal cycle, the transcripts of Ee-BAM1 increased in Sep or Oct with the highest expression levels in Dec. In 2003, the log$_2$ value for Dec sample was 14, which was 16,000 fold higher than that of the July sample.

Ee-BAM1 protein expression

Two peptides (P1 and P2, see Fig. 4) were designed from the Ee-BAM1 sequence, and subsequently synthesized, and used to generate antibodies. Immunoblot analyses of crude protein samples from seedlings and crown buds showed that both antibodies identified two major proteins with molecular weights of 35 and a 29 kD (data not shown). The 29 kD band may represent the mature form of Ee-BAM1 protein samples from seedlings and crown buds showed that both antibodies identified two major proteins with molecular weights of 35 and a 29 kD (data not shown). The 29 kD band may represent the mature form of Ee-BAM1 protein (lane 4). In contrast, this 35 kD protein was not present in the supernatant after immunoprecipitation (lane 3). Although the P2 antibody immunoprecipitated the 35 kD protein samples from seedlings, the immunoprecipitate contained a 35 kD protein (lane 4). In contrast, this 35 kD protein was not present in the supernatant after immunoprecipitation (lane 3). Although the P2 antibody immunoprecipitated the 35 kD protein, it did not precipitate the 29 kD protein. One possible reason for this result is that the P2 epitope is buried within the mature native Ee-BAM1. Neither the 35 nor the 29 kD proteins were precipitated by the P1 antibody (data not shown), indicating that this antibody was weak (as we

![Fig. 4 Deduced amino acid sequence comparison among β-amylase genes. The deduced amino acid sequences of two leafy spurge β-amylases (Ee-BAM1 and Ee-BAM2), one potato β-amylase (PCT-BMY1), and one Arabidopsis β-amylase (AtCT-BAM3) were compared. Plastid transit peptides are shown in red. Peptide 1 (P1) and peptide 2 (P2) were used to generate antibodies](image-url)
observed in the immunoblot analysis) or that the P1 epitope is buried in the native Ee-BAM1. Since the P2 antibody was a better antibody than P1, P2 was used for protein expression studies.

Ee-BAM1 abundance was investigated in various tissues as well as during paradormancy release and seasonal dormancy progression. The 35 and 29 kD proteins were identified in all tissues examined (Fig. 6b). The levels of the 29 kD protein were similar, while the levels of the 35 kD protein were slightly lower in root, stem, hypocotyl and cotyledon (6B). Unlike the transcripts, which showed drastic differences in expression following growth-induction and through the seasons, the levels of the 35 and 29 kD proteins appeared similar (Fig. 6c, d).

To determine Ee-BAM1 protein expression at the cellular and subcellular levels, sections of adventitious buds were incubated with the P2 antibody (Fig. 7). The green fluorescence indicates binding of the P2 antibody to the sections and the red fluorescence reveals staining of the nuclei with DAPI (4′,6-Diamidino-2-phenylindole). In paradormant (non-growing) buds, binding of the P2 antibody occurred at the tip of the bud including leaf primordia and the procambium (B). Higher magnification in meristematic cells (close to the tip of the bud) shows that P2 antibody labeling was in the cytoplasm (C). There was also labeling of cells toward the base of the bud (D). Cells in this region are mature, larger, and contain many amyloplasts. In these cells, the P2 antibody predominantly labeled the periphery of the amyloplasts. Preincubation of the P2 antibody with the P2 peptide completely inhibited binding of the antibody to the sections (A); the faint green color was due to autofluorescence in some of the scales.

Three days after decapitation, buds grew bigger. However, the labeling was still mainly restricted to meristematic cells and again the labeling was in the cytosol (Fig. 7e, f). In the base of the bud, most of the labeling was around the amyloplasts and some around the periphery of the cytoplasm (G). In general, the pattern of labeling was the same as prior to decapitation, but there was less labeling in mature cells, which correlates with a decrease in the number of amyloplasts (Chao et al. 2006). Seasonal crown bud samples were also examined (data not shown). The pattern of labeling in para, endo, and eco dormant buds was similar to those shown in Fig. 7 for non-growing and growing buds.

**α- and β-amylase activity**

During paradormancy release or seasonal endodormancy progression, a rapid breakdown of starch was observed (Anderson et al. 2005; Chao et al. 2006). Since α- and β-amylases play important roles in starch breakdown, the activities of these two enzymes were measured after growth induction (Fig. 8a, b) and during seasonal progression (Fig. 8c, d). Two sets of time point (Mar-07 and Apr-07) and seasonal (2005 and 2006) crown bud samples were used to examine the activities of these enzymes. After growth induction, α-amylase activity decreased progressively to the lowest level in day 4. In contrast, β-amylase activity was slightly up and then down after growth induction, but increased on day 4. During seasonal cycles, α-amylase activity increased during seasonal progression but decreased in Dec, whereas β-amylase activity increased in Dec.

**Fig. 5** Transcript levels for Ee-BAM1. The expression of Ee-BAM1 transcript was examined using real-time PCR after growth induction and in response to seasonal signals. Two sets of growth-induced (2003 and 2004) and two sets of seasonal (2003 and 2004) crown bud samples were used. Time points after growth induction are indicated in hours (h) and days (d). Seasonal bud samples were harvested from July (Jul) through December (Dec). The comparative C(T) method was used to determine changes in transcript expression levels in test samples relative to a control sample. The control for growth-induced samples was 0 h buds (0 h) and for seasonal samples was the July buds. The fold difference is designated as log2 value.
Discussion

We have examined the expression of several genes associated with carbohydrate metabolism to determine whether their expression correlates with changes in dormancy (Figs. 2, 3). We found that most of these genes were differentially expressed and exhibited a consistent expression pattern after growth induction and during seasonal progression. Among these genes, only glucanotransferase (10: CV03069A1D07) and isoamylase (19: CV03098B2H11) showed inconsistent expression patterns between different years after growth induction and during seasonal progression. The expression of these two genes is therefore unlikely to be controlled by growth induction or seasonal signals. Similarly, glucanotransferase (11: CV03120A2H06) appeared not responsive to environmental and developmental signals since it was constitutively expressed. This gene can thus be a useful tool for normalizing the expression of other genes in Northern blot or real-time PCR analyses.

Changes in transcript abundance within a gene family are not always coordinated; in fact, inverse expression patterns can be frequently observed. For example, two β-amylases (5: CV03118B2G07 and 6: CV03058A2C08) were up-regulated immediately after growth induction and during seasonal progression, whereas the other two β-amylases (8: CV03138B1G11 and 9: CV03126A2E05) were down- and up-regulated after growth induction and
**Fig. 7** Immunolocalization of the Ec-BAM1 protein in root buds of leafy spurge. Sections were labeled with the anti-Ec-BAM1 (P2) antibody (green) and the nuclei were stained with DAPI (false color, red). In a, the P2 antibody was preincubated with the P2 peptide. Micrograph b–d are paradormant buds and e–g are growth-induced buds. Amyloplasts appear as green circles with a dark center. Bars in a, b, e, 200 μm; in c, d, f, g, 20 μm.

**Fig. 8** α- and β-amylase activities. The activities of α- and β-amylase were measured after growth induction (a, b) and during seasonal cycles (c, d). Two sets of time points (Mar-07 and Apr-07) and seasonal (2005 and 2006) crown bud samples were used. Time points after growth induction are indicated in hours (h) and days (d). Seasonal bud samples were harvested from July (Jul) through December (Dec). α- and β-amylase activities are presented as Ceralpha units (CU, a and c) and Betamyl units (BU, b and d), respectively. One Ceralpha unit is defined as the amount of enzyme required to release 1 μmol of p-nitrophenol from BPNPG7 per min under the defined assay conditions. One Betamyl activity is defined as the amount of enzyme required to release 1 μmol of p-nitrophenol from PNPG5 per minute under the defined assay conditions. The activities presented were the average values of two technical replicates.
down-regulated during seasonal progression. Within a gene family, genes that differ in expression may have specific developmental roles, which can be similar or not at the biochemical level. For the two $\beta$-amylases that were up-regulated, especially Ee-BAM1 (5: CV0311BB2G07), there was also a good correlation with the decrease in starch that is known to occur after growth induction and during seasonal progression (Anderson et al. 2005; Chao et al. 2006). These two $\beta$-amylases thus could play critical roles in the process of starch degradation.

Surprisingly, although an apparent link was observed between starch levels and the shifts in the Ee-BAM1 transcript, changes in these transcripts did not correlate with alterations in the expression of the Ee-BAM1 protein. No major changes were found for Ee-BAM1 after either growth induction or during seasonal progression (Fig. 6c, d). This result suggests that Ee-BAM1 abundance is regulated at the post-transcriptional level. A possible explanation for this phenomenon is that the rates of Ee-BAM1 turnover and synthesis increase during periods of rapid starch hydrolysis such as following growth induction. Perhaps, Ee-BAM1 undergoes oxidative damage and carbonylation leading to rapid degradation upon the resumption of metabolic activity (Vanita et al. 2008). This increase in protein degradation would maintain similar levels of Ee-BAM1 despite an increase in protein synthesis due to the rapid increase in transcript levels. Lack of correspondence between transcription and translation has been observed in other starch degrading enzymes (Smith et al. 2004; Lu et al. 2005). These authors found that levels of transcripts encoding many starch degrading enzymes, i.e., GWD, AMY3, DPE2, and R1 protein (SEX1) showed strong diurnal and circadian rhythms, but enzyme activities and protein levels changed very little over the course of the day. In the winter, the increase in Ee-BAM1 transcript is particularly high (16,000-fold increase in Dec vs. July). Such an increase may be needed to produce sufficient amount of Ee-BAM1 when the overall activity for protein synthesis is low under cold winter conditions.

Immunolocalization showed that Ee-BAM1 is present in the cytosol of meristematic cells at the tip of the bud, in leaf primordia, and the procambium. The function of Ee-BAM1 in these cells is unclear. Ee-BAM1 was rather abundant in the cytosol of young cells, thus it may serve as a nitrogen and sulfur source during growth. In addition, Ee-BAM1 is observed in the mature and bigger cells, where it appears to be surrounding the amyloplasts (Fig. 7). In these areas, Ee-BAM1 may participate in starch hydrolysis although this activity appears to be regulated by factors other than the presence of the enzyme. For example, paradormant buds show high levels of Ee-BAM1, but appear to have little metabolic activity since many amyloplasts are present and their degradation is only apparent after growth induction. This suggests that the activity of Ee-BAM1 is controlled by mechanisms that regulate accessibility to the substrate or post-translation modifications. The notion that Ee-BAM1 has a dual function as a reserve protein in the cytosol and as a starch-degrading enzyme in the amyloplasts requires further investigation. Nevertheless, some support from this notion comes from findings in tubers and corms, organs similar to underground adventitious buds that are involved in asexual reproduction. Some tubers and corms have storage proteins that also have enzymatic activity; these proteins usually play roles in plant defense via modifications of lipids and carbohydrates (Shewry 2003). It is also known that barley $\beta$-amylases accumulate during seed maturation accounting for 1–2% of the total nitrogen present in the seed (Fincher and Stone 1993). Furthermore, Schmitt and Marinac (2008) showed that a specific class of proteinases, serine endoproteinases, mediates the hydrolysis of these $\beta$-amylases during seed germination.

The activities of both $\alpha$- and $\beta$-amylases were somewhat altered during the course of short-term growth induction or seasonally imposed bud development (Fig. 8). Since the values represented total activity, minor disparity in activity could be due to significant changes in the activity of one or more isoform(s) within a gene family. After growth induction, $\alpha$-amylase activity decreased progressively to the lowest level in day 4, which parallels the decrease in cellular starch levels. $\beta$-amylase activity, on the other hand, increased slightly and then decreased after growth induction, but increased again on day 4. Since amyloplasts were virtually absent 3 days after decapitation (Chao et al. 2006) and the levels of Ee-BAM1 also declined significantly in these cells (compare Fig. 7d, g), the increase in $\beta$-amylase activity may be derived from the cytosol-localized Ee-BAM1 or other $\beta$-amylases within the gene family. During seasonal cycles, $\alpha$-amylase activities decreased in Dec whereas $\beta$-amylase activities increased. These results suggest that $\alpha$-amylase expression may be inhibited by severe cold temperature. In contrast, the expression of $\beta$-amylases (Ee-BAM1 in particular) may be cold induced.

Most studies of starch metabolism have been conducted in leaves, where alterations in starch biosynthesis and degradation are reflected by diurnal variations in leaf starch and sucrose contents. Starch levels in root buds of leafy spurge do not change diurnally (data not shown), but these levels decreased markedly after growth induction and at the transition between para- and endormancy. The expression of Ee-BAM1 transcript changed markedly in a manner suggesting that this enzyme is involved in starch degradation during these periods. However, the expression of the Ee-BAM1 protein did not parallel the changes in transcript level. Nevertheless, the general pattern of carbohydrate in leafy spurge root buds is that higher sucrose levels appear
to be associated with growth arrest regardless of high or low starch levels for the three types of dormancy. This phenomenon raises an interesting question “Is sucrose directly involved in dormancy development?” Study of the regulation of Ee-BAM transcript expression and rate of Ee-BAM activities during various environmental and developmental conditions may provide valuable insights for sucrose involvement in the development of bud dormancy.

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References


