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Characterization of the starch branching enzymes in Arabidopsis thaliana

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Characterization of the starch branching enzymes in Arabidopsis thaliana

by

Qinglei Gan

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:
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Ames, Iowa
2007

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# TABLE OF CONTENTS

**LIST OF FIGURES**

**LIST OF TABLES**

## CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW
- Starch Composition and Structure  2
- Starch Biosynthesis  5
- Thesis Organization  12
- References  13

## CHAPTER 2. ANALYSIS OF MUTATIONS OF STARCH BRANCHING ENZYME ISOFORMS IN ARABIDOPSIS THALIANA
- Abstract  23
- Introduction  24
- Materials and Methods  26
- Results  30
- Discussion  34
- References  42

## CHAPTER 3. EXPRESSION PATTERNS OF DIFFERENT STARCH BRANCHING ENZYME ISOFORMS IN ARABIDOPSIS
- Abstract  46
- Introduction  47
- Materials and Methods  49
- Results  54
- Discussion  60
- References  81

## CHAPTER 4. IN VITRO ACTIVITY ASSAYS OF ARABIDOPSIS STARCH BRANCHING ENZYME 2 AND STARCH BRANCHING ENZYME 3 ISOFORMS
- Abstract  83
- Introduction  84
- Materials and Methods  86
- Results  91
- Discussion  97
- References  108

## CHAPTER 5. SUMMARY AND GENERAL CONCLUSIONS


### LIST OF FIGURES

| Figure 1.1 | Representative partial structures of amylase and amylopectin | 3 |
| Figure 1.2 | Architecture structures of starch granules | 4 |
| Figure 1.3 | Two types of amylopectin double-helical chains | 5 |
| Figure 1.4 | General pathway of starch biosynthesis | 6 |
| Figure 1.5 | Diagrammatic representation of the chemical reactions catalyzed by enzymes involved in amylopectin biosynthesis | 7 |
| Figure 2.1 | Molecular structures of Atbe mutants | 37 |
| Figure 2.2 | Characterization of Arabidopsis T-DNA insertion sbe mutants | 38 |
| Figure 2.3 | Western blots of wild type, Atbe2 and Atbe3 mutants | 39 |
| Figure 2.4 | Zymogram analysis of wild type and Atbe mutant plants | 40 |
| Figure 3.1 | Promoter regions of promoter-GUS constructs for BE genes | 66 |
| Figure 3.2 | Plasmid map of each BE promoter insertion to the pBGWSF7 vector | 67 |
| Figure 3.3 | BE gene expression in different plant tissues at various developmental stages | 70 |
| Figure 3.4 | Expression levels of BEs genes from microarray analysis | 74 |
| Figure 3.5 | Diurnal cycle analysis of BE2 & BE3 expression in wild type plants | 75 |
| Figure 4.1 | Expression of Arabidopsis BE proteins in E. coli cells | 102 |
| Figure 4.2 | In-plate phosphorylase a assay of purified Arabidopsis BE2 and BE3 proteins. | 103 |
| Figure 4.3 | Activity of purified recombinant Arabidopsis BE2 and BE3 protein in phosphorylase a assay | 104 |
| Figure 4.4 | FACE analysis of the products from Arabidopsis BE2 or BE3 with corn amylose reactions | 105 |
| Figure 4.5 | FACE analysis of the products from Arabidopsis BE2 or BE3 with typeIII reactions | 106 |
Figure 4.6 Homology modeling and structure comparison of Arabidopsis BE2 and BE3 107


**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Summary of all the investigated Atbe mutants</td>
<td>41</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Relative BE genes expression levels in different plants tissues</td>
<td>76</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Starch metabolism related genes with similar expression patterns with each BE gene across all the experiments in database</td>
<td>77</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Promoter analysis of BE genes</td>
<td>80</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Starch is a complex insoluble carbohydrate reserve in plant tubers and seed endosperm as a way to store excess glucose. It constitutes more than half of the carbohydrates supplied by traditional staple foods. The four major resources for starch production and consumption in the USA are corn, potatoes, rice, and wheat. In industry, starch and its derivatives are used in the manufacturing of adhesives, paper and textiles. It is also used as a mold in the manufacture of sweets such as wine gums and jelly beans.

Starch can be hydrolyzed into simpler carbohydrates by acids or various enzymes. The extent of conversion is typically quantified by dextrose equivalent (DE), which is roughly the fraction of the glycoside bonds in starch that have been broken. Food products made in this way include: maltodextrin (DE 10–20), various corn syrups (DE 30–70), dextrose (DE 100) and high fructose syrups.

Starch is generally classified as transient starch or storage starch based on the length of time persisting to degradation. They are different in amylopectin structure and the ratio of amylopectin and amylose (Zeeman et al., 2002). Transient starch is first produced from sun light in chloroplasts of leaf tissue and then broken down to maintain the supply of carbon to other parts while the storage starch is accumulated in some specific organs as an energy reserve for the plants growth or seed germination.

We have known about thirty distinct enzymes are involved in starch biosynthesis in higher plants. The starch synthases and branching enzymes are involved directly in amylopectin biosynthesis. In addition, starch debranching enzymes and disproportionating enzyme show potential roles in amylopectin biosynthesis (Myers et al., 2000). Therefore, understanding the function and interaction of these enzymes will provide us with helpful information about the mechanism of starch biosynthesis and structure controls and therefore enable us to customize the starch structures and functions.
Starch Composition and Structure

Starch is composed of two categories of glucose homopolymers, amylose (normally 20-30%) and amylopectin (normally 70-80%). The relative proportion of these two molecules is dependent on the species and tissues. Amylose and amylopectin are inherently distinct molecules: amylose has lower molecular weight with a relatively extended shape while amylopectin has larger but compact conformation. Most of their structure consists of α-(1-4)-D-glucose units. Although the α-(1-4) links are capable of relatively free rotation around the (φ) phi and (ψ) psi torsions, hydrogen bonding between the O3' and O2 oxygen atoms of sequential residues tends to encourage a helical conformation. These helical structures are relatively stiff and may present contiguous hydrophobic surfaces. (Martin Chaplin et al., 2001)

Amylose molecules consist of single mostly-unbranched chains with 500-20,000 α-(1-4)-D-glucose units dependent on source. A very few α-(1-6) branches and linked phosphate groups may be found (Hoover, 2001), but these have little influence on the molecule's behavior (Buléon et al., 1998). Amylose can form an extended shape but generally tends to wind up into a rather stiff left-handed single helix or form even stiffer parallel left-handed double helical junction zones (Figure 1.1). Single helical amylose has hydrogen-bonding O2 and O6 atoms on the outside surface of the helix with only the ring oxygen pointing inwards. Hydrogen bonding between aligned chains causes retrogradation and releases some of the bound water. The aligned chains may then form double stranded crystallites that are resistant to amylases. These make extensive inter- and intra-strand hydrogen bonding, resulting in a fairly hydrophobic structure of low solubility. (Martin Chaplin et al., 2001)

Amylopectin is formed by non-random α-(1-6) branching of the amylose-type α-(1-4)-D-glucose structure. This branching is determined by branching enzymes that leave each chain with up to 30 glucose residues. Each amylopectin molecule contains up
to two million glucose residues in a compact structure with hydrodynamic radius 21-75 nm (Parker and Ring, 2001) (Figure 1.1). The molecules are oriented radially in the starch granule and as the radius increases, the number of branches increases to fill up the space, with the consequent formation of concentric regions of alternating amorphous and crystalline structure (Figure 1.2).

**Figure 1.1** Representative partial structure of amylose and amylopectin. Above is the structure of amylase and below is the structure of amylopectin (Adapted from Imberty et al., 1988.)
Figure 1.2  Architectural structure of starch granules. A. The essential features of amylopectin. There are usually slightly more 'outer' unbranched chains (called A-chains) than 'inner' branched chains (called B-chains). There is only one chain (called the C-chain) containing the single reducing group. B. The organization of the amorphous and crystalline regions of the structure generates the concentric layers that contribute to the “growth rings” visible by light microscopy. C. The orientation of the amylopectin molecules in a cross section of an idealized entire granule. D. The proposed double helix structure taken up by neighboring chains and giving rise to the extensive degree of crystallinity in granule. (Adapted from Tang et al., 2006)
Some amylopectin has phosphate groups attached to some hydroxyl groups, which increase its hydrophilicity and swelling power. Amylopectin double-helical chains can either form the more open hydrated Type-B hexagonal crystallites or the denser Type-A crystallites, with staggered monoclinic packing (Figure 1.3) (Parker and Ring, 2001). Type-A is found in most cereals. Type-B is found in banana, some tubers such as potato and high amylose cereal starches. There is also a type C structure, which is a combination of types A and B and found in peas and beans (Tang et al., 2006).

![Figure 1.3](image)

**Figure 1.3** Two types of amylopectin double-helical chains. Type-A has unbroken chain lengths of about 23-29 glucose units. Type-B has slightly longer unbroken chain lengths of about 30-44 glucose units. (Adapted from Parker and Ring, 2001)

**Starch Biosynthesis**

Starch biosynthesis must be examined within the framework of the overall hierarchical structure of the products. Thus, it is necessary to understand not only the basic mechanism of each enzyme, but also the relationship between the synthesis of the primary product and subsequent higher order packed structures. Genetic and biochemical analyses revealed that multiple enzymes are involved in the starch biosynthesis pathway, including ADP-Glc pyrophosphorylase (AGPase), starch synthases (SSs), starch branching enzymes (BEs), starch debranching enzymes (DBEs) and disproportionating enzyme (D-enzyme) are also considered to have critical roles in forming the highly organized starch granules in higher plants. (Martin and Smith, 1995; Myers et al., 2000; Ball and Morell, 2003; James et al., 2003; Tetlow et al., 2004) (Figure 1.4, 1.5)
Figure 1.4 General pathway of starch biosynthesis. Numbered activities are as follows: 1, sucrose synthase; 2, UDP-Glc pyrophosphorylase; 3, glycolytic enzymes including phosphoglucomutase; 4, ADPGlc pyrophosphorylase; 5, hexose phosphate transporters; 6, ADPGlc transporters. Not all plants possess both the indicated transporters and cytosolic ADPGlc pyrophosphorylase. Transport of Glc-1-P is depicted as a possible example and is not meant to imply that hexose phosphate transporters are necessarily specific for this molecule. Ap, amylopectin; Am, amyllose (adapted from Myers et al., 2000).
Figure 1.5 Diagrammatic representation of the chemical reactions catalyzed by enzymes involved in amylopectin biosynthesis. Starch synthase (SS), starch branching enzyme (BE), debranching enzyme (DBE), and disproportionating enzyme (D-enzyme). Donated glucosyl units are shown in red, and asterisks indicate reducing carbons through which these glucans are transferred. Structures are shown only to illustrate changes in linkage structure and are not intended to indicate substrate specificities (adapted from Myers et al., 2000).
**ADP-Glc pyrophosphorylase (AGPase)** catalyzes the first step in starch synthesis which produces the activated glucosyl donor ADP-glucose from ATP and Glc-1-phosphate (Figure 1.4). This reaction is generally considered to be the committed step in starch biosynthesis (James et al., 2003). In the Arabidopsis genome, six genes encode proteins with homology to AGPase in other species. Two of them code for small subunits, and four encode for large subunits (Crevillen et al., 2005). AGPase enzymes are allosterically regulated by 3-phosphoglycerate (3-PGA) as an activator while inorganic phosphate (Pi) is an inhibitor (Preiss, 1996). The subcellular location of AGPase is not consistent between all higher plants. In cereal endosperm the enzyme is mostly extra-plastidial while in all other plants it is primarily plastidial (Beckles et al., 2001).

**Starch synthases (SSs)** catalyze the transfer of the glucose moiety from ADP-Glc to the end of an existing α-(1-4) or α-(1-6) linked glucan (Figure 1.5). Among the published literature the most popular opinion is that the glucose residue is added to the non-reducing end of the chain rather than the reducing end. However, no direct evidence has ever been shown to prove any of these models. Five distinct classes of starch synthases have been identified in higher plants based on their primary sequences. They are GBSS, SSI, SSII, SSIII, and SSIV/V (Li et al., 2001). These isoforms are highly conserved in the plant kingdom indicating each of them have a specific function. Granule bound starch synthase (GBSS) is involved in the synthesis of amylose chains as a granule bound protein. There are two closely related GBSS forms, GBSSI and GBSSII. GBSSI is mostly confined to storage organs while GBSSII coded by a distinct gene is in leaves and other non-storage tissues that accumulate transient starch (Fujita and Taira, 1998; Nakamura et al., 1998; Vrinten and Nakamura, 2000). GBSS were found to be completely within the starch granule matrix while others are found in soluble phases or both the granule and the soluble phases. Starch synthase I (SSI) is primarily responsible
for the synthesis of the shortest glucan chains (DP5 to DP10) in the amylopectin molecule (Commuri and Keeling, 2001). Its activity varies among different species. Starch synthase II (SSII) is required for production of chains in the range of DP12 to DP25 (Umemoto et al., 2002). Although SSII is a minor contributor to total SS activities in cereal endosperms, the loss of its activity has a dramatic impact on both the amount and composition of starch. Starch synthase III (SSIII) plays different roles in different species. In maize, mutations eliminating SSIII lead to slight but reproducible changes in kernel appearance (Gao et al., 1998) and the starch granules are enriched in intermediate-size highly branched polyglucans (Inouchi et al., 1984; Wang et al., 1993). The reduction of SSIII activity in potato resulted in a major impact on the amylopectin with modified chain length distribution. For the group of SSIV/V, no mutant was reported from any of plant species.

**Starch branching enzymes (BEs)** catalyze the internal cleavage of a α-(1,4) linkages and transfer of the released reducing end to a C-6 hydroxyl group of glucose in a linear glucan, creating a new α-(1,6) branch linkage (Figure 1.5). There are two classes of BE (BEI and BEII) that differ in terms of the lengths of chains transferred in vitro, with BEII transferring shorter chains than BEI (Takeda et al., 1993; Guan H and Preiss J, 1993). We use the nomenclature based on the maize endosperm isoforms as was recommended earlier by a commission on plant gene nomenclature (Smith White B and Preiss J, 1994). Therefore, maize BEI and related enzymes such as potato BEI, rice BEI and pea BEII belong to class I. Maize BEII, and related enzymes such as potato BEII, rice BEIII, pea BEI and *Arabidopsis thaliana* BE2.1 and BE2.2 (Fisher DK et al., 1996), belong to class II. In cereals, there are two closely related forms of BEII (BEIIa and BEIIb) (Mizuno et al., 2001; Rahman S et al., 2001). They also differ in chain-length specificity in vitro, with BEIIb transferring shorter chains than BEIIa during extended incubation. The temporal and spatial patterns of expression vary between BE isoforms. BEI and BEIIa are expressed in the endosperm and several other cereal tissues, whereas
BEIIb is only expressed in the endosperm and reproductive tissues.

There is good evidence that the different BE isoforms have distinct roles in endosperm starch synthesis. First, in vitro assay of maize BEs showed that BEI preferentially transfers longer chains (DP16), and BEII generates shorter chains (DP11-12) (Takeda and Preiss, 1993; Guan and Preiss, 1993). Second, biochemical analyses of maize BEIIb-deficient mutants revealed that the reduction of BEIIb activity led to a specific decrease in short chains DP≤13, with the greatest decrease in chains of DP 8-11 (Shi et al., 1995; Klucinec et al., 2002; Yao et al., 2004) indicating that BEIIb has a critical role in the formation of short A chains and this role cannot be complemented by BEIIa and/or BEI (Nakamura, 2002). Third, genetic analysis of BEI in rice showed that when BEI is lacking, the amyllopectin contains fewer intermediate size (16≤DP≤23) and long chains, which suggests that BEI has an important role in the synthesis of B1 chains and B2-B3 chains (Nakamura, 2002). However, other studies showed that reduction or elimination of BEI activity in both monocots and dicots had minimal effects on starch synthesis and composition (Tetlow et al., 2004). In maize, a BEI mutant was shown to have an effect on amyllopectin structure only in an amylase extender background, which suggests that BEIIb is dominant to BEI in affecting amyllopectin structure (Yao et al., 2004). Recent study of a maize BEIIa mutant showed a clear phenotype in the leaf starch, but no apparent effects on the storage starch of endosperm tissue (Blauth et al., 2001), which is consistent with the preliminary result in rice with inhibited BEIIa activity (Nakamura, 2002). These results suggest a distinct role for BEIIa in the synthesis of amyllopectin in transitory starch (Tetlow et al., 2004). Furthermore, in addition to granule bound starch synthase I (GBSSI), a fraction of soluble starch synthases and also of SBEI and SBEII have been observed to be strongly associated to starch granules from maize (Mu C et al., 1994), pea (Denyer et al., 1995; Dry I et al., 1992; Edwards A et al., 1996), wheat (Rahman S et al., 2002) and potato
tuber (Flipse E et al., 1996; Larsson CT et al., 1996; Marshall J et al., 1996). Recently, it was reported that 45% of SBEII from maize seeds was granule bound. Soluble SBEII has been isolated from extracts of maize, pea and rice seeds (Mizuno K et al., 1992; Nakamura Y et al., 1992; Yamanouchi H et al., 1992), whereas soluble SBEII from potato has hitherto not been detected in tuber juice. This is in contrast to potato SBEI, which is soluble and present in the tuber juice (Khoshnoodi J et al., 1993; Khoshnoodi J et al., 1996; Larsson CT et al., 1996).

The structure of BEs has not been solved. However, crystal structure of glycogen branching enzyme from Escherichia coli which is highly homologous with starch branching enzyme has been reported. The enzyme consists of three major domains, an NH2-terminal seven-stranded β-sandwich domain, a COOH-terminal domain, and a central α/β-barrel domain containing the enzyme active site. While the central domain is similar to that of all the other amylase family enzymes, branching enzyme shares the structure of all three domains only with isoamylase. While most of the oligosaccharides modeled well in the branching enzyme structure, an approximate 50° rotation between two of the glucose units was required to avoid steric clashes with a Trp residue of branching enzyme. A similar rotation was observed in the mammalian α-amylase structure caused by an equivalent tryptophan residue in this structure (Marta C et al., 2002).

In vitro assay with isolated amyloplasts suggests that the activities of BEs might be regulated by protein phosphorylation. Immunoprecipitation experiments showed that BEIIb and starch phosphorylase each coimmunoprecipitated with BEI in a phosphorylation-dependent manner. Conversely, dephosphorylation of immunoprecipitated protein complex led to its disassembly, and reduced the activity of BEIIa and BEIIb in amyloplasts. This data suggests that these enzymes may form protein complexes to function within the amyloplast in vivo (Tetlow et al., 2004).
Starch debranching enzymes (DBEs) hydrolyze \( \alpha-(1,6) \) glycosidic linkages of \( \alpha \)-polyglucans (Figure 1.5). Based on their substrate specificities, DBEs are classified into two types: isoamylase-type \( \alpha-(1,6) \) glycohydrolase and pullulanase-type \( \alpha-(1,6) \) glycohydrolase. Isoamylase type DBEs can debranch glycogen, phytoglycogen and amylopectin (Manners, 1971) while pullulanase type DBEs can attack pullulan and amylopectin rather than glycogen and phytoglycogen (Manners et al., 1970; Manners, 1971). There are some different models to explain how DBEs are involved in starch synthesis. Among the most popular models the “glucan-trimming” model indicated that DBEs can selectively remove improperly positioned branches during amylopectin synthesis, thus they have direct functions in amylopectin formation (Myers et al., 2000; Nakamura, 2002); the “water-soluble polysaccharide (WSP) clearing” model proposed that DBEs eliminate the soluble glucan from the stroma, thus they remove the competition for the binding of SSs and BEs in the soluble phases (Zeeman et al., 1998).

Disproportionating enzyme (D-enzyme) catalyzes the transfer of \( \alpha-(1,4) \) linked oligosaccharides from the end of one linear glucan chain to the end of another chain (Figure 1.5). The function of D-enzyme on starch biosynthesis is still unclear. Study of *Chlamydomonas* that lacks D-enzyme activity indicated that D-enzyme was involved in starch synthesis (Colleoni et al., 1999; Colleoni et al., 1999; Wattiebled et al., 2003). However, the study of Arabidopsis D-enzyme showed that it only has function in starch degradation (Critchley et al., 2001; Chia et al., 2004).

**Thesis Organization**

This thesis consists of four chapters. Chapter 1 is an introductory chapter that reviews the relevant literature about the importance and industrial applications of starch, starch composition and structure, the known pathway of starch biosynthesis. Chapter 2 is a research study of the characterization of six T-DNA insertion mutants of BE genes in
Arabidopsis thaliana. Chapter 3 is the description of the study of the expression patterns of different BE isoforms in Arabidopsis. Chapter 4 presents research comparing the in vitro activities of purified recombinant forms of the Arabidopsis BE2 and BE3 enzymes. All chapters will be organized in a research paper format consisting of the following sections: Abstract, Introduction, Materials and Methods, Results, Discussion and References.

I performed all the experimental work in the thesis with one exception. In Chapter 2 two pairs of primers (JW041/042 and JW087/088) for genotyping were designed by Ms. Jennifer Walker-Daniels, a former postdoctoral research associate in the laboratory. All other results described in chapter 2 were obtained by my own efforts. I was responsible for all the experiments described in chapter 3 and chapter 4. All of the thesis was written by me with the guidance and assistance from my co-major professors, Drs. Alan Myers and Martha James.

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CHAPTER 2. ANALYSIS OF MUTATIONS OF STARCH BRANCHING ENZYME ISOFORMS IN *ARABIDOPSIS THALIANA*

Abstract

Starch consists of two polymers amylose and amylopectin. Multiple enzymes are involved in the starch synthesis pathway. Starch branching enzymes (BEs) catalyze the internal cleavage of α-(1,4) linkages and formation of α-(1,6) linkages in amylopectin. Three isoforms of starch branching enzyme, AtBE1, AtBE2 and AtBE3, have been found in *Arabidopsis thaliana*. BE2 and BE3 are two highly conserved proteins, whereas the amino acid sequence similarity between BE1 and the other two BEs is quite low. The roles of these three isoforms in *Arabidopsis* leaf starch metabolism were investigated by characterizing the effects of mutations at all three AtBE genomic loci. Six AtBE mutants were identified from T-DNA insertion collections and four homozygous (HM) mutants were identified and isolated for further analysis. In this study, one Atbe1 homozygous mutant, designated Atbe1-1, was identified and the mutant plants showed environmentally sensitive property and hardly survived. Two Atbe2 homozygous mutants, referred to as Atbe2-1 and Atbe2-2, also were identified. Neither was a null mutation; however, in gel activity assays revealed that both mutations had effects on the activities of starch modifying enzymes. One Atbe3 homozygous mutant, referred to as Atbe3-1, was identified, which was demonstrated to be a null mutation. BE enzyme activities in the homozygous Atbe mutant plants were examined by starch zymogram analysis, which revealed that the homozygous Atbe2 and Atbe3 mutations had no detectable BE activities compared with wild type. This indicates that the BE2 protein produced in the Atbe2-1 and Atbe2-2 mutants is not active or functional. Furthermore, the activities of other starch metabolism related enzymes in these three homozygous mutants had different changes, which indicates the possible interaction between BEs and other starch modifying enzymes.
enzymes in *Arabidopsis*.

**Introduction**

Starch is a polymer of glucose and is the major carbohydrate storage product found in higher plants such as corn, potatoes, wheat and rice. It accumulates in the plastids of plants as water insoluble semicrystalline granules in different sizes and shapes based on the botanical species (Gallant, 1992). The starch granules are composed of two structurally distinct homopolymers: amylose (Am) and amylopectin (Ap). Amylose is a minor component of starch and is essentially a linear polymer consisting of \(\alpha-(1,4)\) O-glycosidic bonds linked glucose units with rare \(\alpha-(1,6)\) branch points. The degree of polymerization (DP) of amylose is between 600 and 5000 glucose units. Amylopectin is the major component of starch and is a highly branched polymer consisting of 10,000-100,000 glucose units. The linear backbone chains of \(\alpha-(1,4)\) linked glucose units are linked together by \(\alpha-(1,6)\) bonds. These \(\alpha-(1,6)\) linkages are commonly referred to as the branching points of starch. The starch granule shape and size in mutants that lack amylose is normal, which indicates that the crystalline nature of starch granules is only related to amylopectin.

The starch biosynthesis pathway is very complex and involves multiple enzymes, which include starch synthases (SSs, chain elongation step), starch branching enzymes (BEs, introduction of branches), starch debranching enzymes (DBEs, isoamylase type and pullulanase type), ADP-Glc pyrophosphorylase (AGPase, first synthesis step) and disproportionating enzyme (D-enzyme) (Martin, 1995); (Myers et al., 2000; Ball and Morell, 2003; James et al., 2003; Tetlow et al., 2004).

This study focused on starch branching enzymes (BEs; EC 2.4.1.18), which catalyze the formation of \(\alpha-(1,6)\) linkages within the polymer by cleaving a pre-existing \(\alpha-(1,4)\) linkage and transferring it to the C6 position of glucose in a liner glucan and creating a
new α-(1,6) linked branch. Several genetically independent isoforms of BEs are observed in the developing storage tissues of plants: Arabidopsis (Fisher et al., 1996); maize (Stinard, 1993; Blauth et al., 2001; Blauth et al., 2002); rice and pea (Nakamura, 1992; Martin, 1995); potato (Larsson, 1996); wheat (Morell et al., 1997); barley (Sun, 1996, 1997). Based on the peptide sequences and structural and catalytic properties, BEs are categorized into two classes. One is referred as family B or BEI, the other is family A or BEII which can be further separated into two subfamilies named BEIIa and BEIIb in maize (Boyer and Preiss, 1978; Gao et al., 1997). Each different isoform has its own specificity and branching activity needed for various plant tissues or developmental stages which was demonstrated in pea (Burton, 1995); maize (Guan and Preiss, 1993; Guan et al., 1994; Guan et al., 1997); rice (Guarente, 1983; Takeda et al., 1993; Mizuno et al., 2001) and potato (Ryberg, 2001). These studies showed that BEI is preferentially active on longer glucan chains, whereas BEII more likely acts on shorter chains. In addition, the minimum chain length requirement for transfers of BEI is longer than BEII. BEI requires at least DP16 for transfer, while DP12 is the minimum chain length for BEII transfer (Guan et al., 1997).

Effects of loss of function of each BE isoform on starch metabolism were studied in maize (Stinard, 1993; Blauth et al., 2001; Blauth et al., 2002; Yao, 2004) and rice (Nakamura, 2002; Satoh et al., 2003). In both species the absence of BEI has little or no effect on starch metabolism in the endosperm of plants. BEIIb mutation, however, accumulates 20% less starch within the endosperm, which has a reduced amount of branching and longer branches (Garwood et al. 1976). BEIIa mutants display an early senescence phoenotype and accumulate leaf starch with a reduced amount of branching and longer branches (Blauth et al., 2001). BE mutant lines were also analyzed in Arabidopsis (Fisher et al., 1996; Khoshnoodi, 1998; Dumez, 2006). There are three genes putatively encoding BEs in Arabidopsis: \textit{AtBE1}, \textit{AtBE2} and \textit{AtBE3}. AtBE2 and AtBE3 proteins corresponding to the \textit{Atbe2} and \textit{Atbe3} genes have more than 80% amino acid
sequence identity with each other and both belong to the SBEII family, while AtBE1 protein is not related to any of the standard SBE families described before depending on the amino acid sequence alignment, which is less than 28% identity, but AtBE1 is more related to the bacterial glycogen branching enzymes. Mutation of AtBE1 showed no apparent function on starch metabolism in Arabidopsis leaves, so it is commonly considered as a non-functional BE. AtBE2 and AtBE3 play an important role in the starch synthesis (Dumez, 2006).

The function and genomic arrangement of the three BE isoforms in Arabidopsis are not well known. This work was designed to investigate the function of the Arabidopsis SBE isoforms on leaf starch metabolism by mutant analysis. A series of Atbe mutant alleles were characterized at genomic DNA, mRNA and protein levels. Starch modifying enzymes activities were analyzed in some of the mutants.

Materials and Methods

Plant Materials

Wild type Arabidopsis thaliana plants used in this study were ecotype Columbia and all mutant lines are from the same genetic background. Seeds of six BE T-DNA insertion mutant lines were ordered from the SALK Institute (http://www.salk.edu/). Seeds were surface sterilized in sterilization solution (50% bleach, 0.02% Triton X-100 in sterile distilled H2O) for seven minutes and rinsed in sterile dH2O three times. After the sterilization, seeds were sown in a sterile potting medium (LC1 Sunshine Mix, Sun Gro, Horticulture, Inc., Bellevue, WA). The soil trays were incubated at 4°C for 2 days and transferred to the growth room at 21°C, 60% relative humidity and 16-hr light/8-hr dark photoperiod.
DNA Isolation and PCR

DNA was isolated from individual plants. Leaf tissues were harvested and ground in 500ul extraction buffer (0.2M Tris-HCl, pH 9.0, 0.4M LiCl, 25mM EDTA, 1% SDS), then centrifuged for 10 min at 13,000 rpm. 350ul of the supernatant was transferred to a new tube containing 350ul isopropanol. The mixture was centrifuged again. The pellet was dried and resuspended in 400ul dH2O.

Mutant’s identification was done by PCR screening. For each BE mutant allele, one pair of gene specific primers flanking the T-DNA insertion region was designed: QL003 (GAT TTC TTT CTC AAG GGG TCC T) and QL004 (ACC TCT GTG AAG GCG TGA TT) for Atbe1-1; QL005 (GTA AAA GGG GGC ATC ACA AA) and QL006 (TGG TTG TGA TTT TCG GCA TA) for Atbe1-2; JW053 (TGC GTA AAT TTC TTA TGC TTG G) and JW054 (AGC CAG ACA CAC ACC GTG CT) for Atbe2-1; JW087 (TGA ATG TAG GAG GTT TAG CA) and JW088 (GAC AAG GAG CCA GAC ACA CA) for Atbe2-2; JW041 (TGC CTG ACT TGT TTG CTT GTT GA) and JW042 (GCA TGG GGC TAC ACT AAA GGC) for Atbe3-1; QL007 (CGA TTT CAG GCG TAC GTT TT) and QL008 (TAA GCC AGG AGA AGG ATG GA) for Atbe3-2. T-DNA left border primer (LBa1, TGG TTC ACG TAG TGG GCC ATC G) was used together with one of those gene specific primers to detect T-DNA insertions. All PCR reactions were one cycle at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, and a final extension cycle at 72°C for 10 min.

RNA Isolation and RT-PCR

Total RNA was isolated from about 100 mg leaf tissues using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer’s instructions. Briefly, leaves were ground thoroughly in liquid nitrogen and then lysed in 450ul RLT buffer (β-Mercaptoethanol (β-ME) added). The lysate was applied to a QIAshredder spin column and centrifuged for 2 min at maximum speed. The supernatant of the flow-through fraction was
transferred to a new tube and 0.5 volume ethanol was added and mixed by pipetting. All the mixture was applied to an RNeasy mini column and centrifuged for 15 seconds at 10,000 rpm. 700ul Buffer RW1 was added and centrifuged for 15 seconds at 10,000 rpm, followed by another centrifugation under the same conditions with 500ul Buffer RPE. The RNeasy silica-gel membrane was dried by centrifuging the column with 500ul Buffer RPE for 2 min at 10,000 rpm. RNA was eluted to a new collection tube by pipetting 30 ul RNase-free water directly to the membrane, waiting for 1 min, and centrifuging for 1 min at 10,000 rpm.

DNase treatment was done to remove any contaminating genomic DNA after RNA isolation. DNase buffer and DNase (Promega) were added to the isolated RNA and incubated at 37°C for 30 min. All the RNA isolation steps were repeated again. RNA was ready to use after the last elution.

First-strand cDNA was synthesized from total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA synthesis was performed with total RNA and oligo(dT). PCR was performed using the two pairs of gene specific primers (upstream and downstream primers) as follows: QL009 (TCA AGG AGA GAG GGG TGA AA) and QL010 (CAG GCG CCC ACT CTC TAT AA) as Atbe2-1 and Atbe2-2 upstream primers; QL011 (CAT GAG TAG CAC GGA ACC AA) and QL012 (CTG CAT CCC CAA GAT CAA AT) as Atbe2-1 and Atbe2-2 downstream primers; QL013 (CGA TTT CAG GCG TAC GTT TT) and QL014 (CGA TCA GTG ATG CTG CCT TA) as Atbe3-1 upstream primers; QL015 (TCT TCT TTC CAA CGC GAG AT) and QL016 (GCG GGA TAT GTA CTG GTG CT) as AtBE3-1 downstream primers.

**Protein Extraction**

Protein was extracted from *Arabidopsis* mature leaves by grinding about 300 mg fresh weight leaf tissue with a mortar and pestle under liquid nitrogen. The ground tissue was homogenized in 2 volumes of extraction buffer (50 mM Tris-acetate, pH7.5, 10 mM
DTT) (described before in Zhang et al., 2005). The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged again. The second supernatant was stored at –20°C. Protein concentration was determined by using the Bradford assay with a bovine serum albumin (BSA) standard curve (Bradford, 1976).

**SDS-PAGE and Western Blot**

SDS-PAGE was performed using the methods modified from Laemmli (Laemmli, 1970). Total protein was loaded onto 7.5% polyacrylamide resolving gels. SDS-PAGE was performed at 70-100 V at room temperature. After running, the gel was electrophotransferred onto nitrocellulose membrane for 50 minutes at 4°C. The membrane was blocked in blocking buffer (5% milk in TTBS (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 30 minutes with gentle shaking at room temperature. To detect BE2 and BE3 proteins, a maize BEIIa/b antibody (rabbit, polyclonal, produced in our laboratory by Rebekah S. Marsh) was used as the primary antibody at 1:5000 dilution. The membrane was incubated in the primary antibody solution overnight with gentle shaking at room temperature or 4°C, and then washed four times with TTBS. After washing, the membrane was incubated in the secondary antibody (goat anti-rabbit alkaline phosphatase conjugate, Bio-Rad Laboratories, Hercules, CA., catalog no. 170-6432) for 1 hour with gentle shaking at room temperature. Two wash steps with TTBS and two wash steps with TBS (10 mM Tris, pH 7.5, 150 mM NaCl) were performed with gentle shaking. Detection was performed following the provided protocol of the alkaline phosphatase conjugate kit (Bio-Rad Laboratories, Hercules, CA, catalog no. 170-6432).

**Starch Zymogram Analysis**

Protocols followed were described previously (Dinges et al., 2001). 50-100 ug total
protein from leaf extracts was loaded onto a 7% (w/v) native polyacrylamide gel containing 0.3% (w/v) potato starch (Sigma-Aldrich, catalog no. S-2630). After running in the native running buffer (25mM Tris, 192mM Glycine and 2mM DTT) at 100V for about 3 hours in the cold room (4°C), the gel was incubated in the incubation buffer (50 mM Tris-HCl, pH 7.0, 1 mM CaCl₂, 1 mM DTT, 1 mM MgCl₂) with gentle shaking overnight at room temperature. The next day the gel was stained with iodine solution (I₂/KI: 1g/10g for 1L) and photographed.

Results

Identification of Homozygous Atbe Mutants

Six Arabidopsis starch branching enzyme mutants were investigated in this study (Table 2.1). All mutations were obtained from the SALK T-DNA insertion lines collection (ref). The genomic locus At3g20440, 7,240 bp nucleotides comprising 20 exons and 19 introns, is referred to here as Atbe1. SALK_142429 (T-DNA insertion located in exon 18) and SALK_113250 (T-DNA insertion located in intron 10) are designated as Atbe1-1 and Atbe1-2, respectively (Figure 2.1 A). The genomic locus At5g03650, 5,704 bp nucleotides comprising 20 exons and 19 introns, is referred to here as Atbe2. SALK_034880 (T-DNA insertion located in intron 5) and SALK_107255 (T-DNA insertion located in intron 4) are designated as Atbe2-1 and Atbe2-2, respectively (Figure 2.1 B). The genomic locus At2g36390, 5,856 bp nucleotides comprising 18 exons and 17 introns, is referred to here as Atbe3. SALK_048089 (T-DNA insertion located in exon 10) and SALK_098395 (T-DNA insertion located in intron 1) are designated as Atbe3-1 and Atbe3-2, respectively (Figure 2.1 C).

To identify these mutations, a pair of gene specific primers was designed for each mutation to be used in genotyping PCR analysis together with a T-DNA border primer LBA1. Each pair of gene specific primers flanks the approximate region of T-DNA
insertion. PCR amplification of genomic DNA with two gene specific primers reveals the wild type allele, and one gene specific primer with LBa1 primer identifies the mutant allele. Plants from original SALK seeds and successive generations were genotyped and homozygous plants were isolated based on PCR results. From these experiments, Atbe1-1, Atbe2-1, Atbe2-2 and Atbe3-1 homozygotes were confirmed and the PCR results of Atbe2-1 homozygous were shown (Figure 2.2 A). Atbe2-2 and Atbe3-1 homozygotes had the same PCR result patterns but different band sizes (data now shown). Homozygous seeds were not obtained for two mutations, Atbe1-2 and Atbe3-2, s. Atbe1-1 homozygous plants were very weak and sensitive to the environment and died before F2 homozygous seeds could be obtained. Further analysis was mostly done on Atbe2-1, Atbe2-2 and Atbe3-1 homozygous plants.

Identification of Null Mutation for Atbe Mutants

The mRNA accumulation in the Atbe2-1, Atbe2-2 and Atbe3-1 mutants was investigated by RT-PCR. Three different pairs of primers were used for the two Atbe2 mutants, including primers amplifying downstream and upstream regions and theregions flanking the T-DNA insertion sites. The wild type allele had the normal transcript that was used as the positive control and sterile water was the negative control. The same primer pairs were used for RT-PCR amplifications of Atbe2-1 and Atbe2-2 because their T-DNA insertion locations were very close. The bands indicating the same transcripts as wild type were detected with the upstream primer pairs for both Atbe2-1 and Atbe2-2 mutants. The transcript signal from downstream and flanking primer pairs of Atbe2-1 was somewhat decreased but still observable, and Atbe2-2 showed normal transcript patterns with these primers (Figure 2.2 B). Thus, mRNA accumulations were not prevented in Atbe2-1 and Atbe2-2 mutants.

In the case of Atbe3-1, upstream and downstream primer pairs were used for RT-PCR. Normal amplification was detected with upstream primers. However, the
RT-PCR signal was completely absent with the downstream primers, indicating that no normal transcripts are present in *Atbe3-1* mutants (Figure 2.2 C). *Atbe3-1* is likely to be a null allele based on these results.

The *Atbe2-1* and *Atbe2-2* mutants were also examined at the level of protein accumulation. Immunoblot analysis of total leaf protein was used to investigate the BE protein accumulation in the wild type and mutant plants. Proteins were separated by SDS-PAGE and hybridized with a maize anti-BeIIa/b polyclonal antibody. Wild type protein extracts produced an approximately 100 kDa band, which is likely to be the predicted molecular mass of both the mature AtBE2 and AtBE3 protein. Both *Atbe2-1* and *Atbe2-2* homozygous mutant plant protein extracts revealed the same band as the wild type plant (Figure 2.3 A). It should be pointed out that the antibody used for the western blot detection was a maize BeIIa/b antibody, which might recognize both the maize BeIIa and BeIIb protein. Thus, it was not clear whether the band observed in the western blot was *Arabidopsis* BE2 or BE3. It could also be both although the wild type plant only gave one band, since BE2 and BE3 proteins might co-migrate. If this band was AtBE2, it supports the mRNA evidence that *Atbe2-1* and *Atbe2-2* are not null mutations. On the other hand, if this band identifies AtBE3, the Atbe2 mutants would show this band no matter if they were null mutations or not. To address this question, another western blot was performed on *Atbe3-1* mutants with the same antibody and it showed no band (Figure 2.3 B). Thus, this band is assumed to indicate BE3. Taken with the RT-PCR results, the data indicates that *Atbe3-1* is a null allele.

**Effects of *Atbe* Mutations on BE and Other Starch Metabolism Enzymes Activities**

Starch Zymogram analysis was employed to determine the effects of *Atbe2-1*, *Atbe2-2* or *Atbe3-1* mutations on starch metabolizing enzymes activities. Equal volumes of leaf protein extracts were loaded onto a native polyacrylamide gel containing 0.3% potato starch. After separation, the gel was incubated in the incubation buffer with gentle
shaking overnight at room temperature and stained with iodine solution the next day. The colorful staining pattern revealed the activities of different starch modifying enzymes. Two different protein amounts (50 ug and 100 ug) were used for the zymogram and the same patterns were revealed (data not shown). The zymogram analysis with 50 ug protein is shown (Figure 2.4). Based on the missing activity bands in the corresponding BE mutants, the bands referring to each BE activity were revealed.

In both Atbe2 homozygous mutants, BE activity bands were clearly missing in the upper part of the gel, which matches the result reported by (Dumez, 2006). The very top bluish activity band, corresponding to isoamylase activity, ISA1 and ISA2 gene products (Wattebled et al., 2005), was significantly reduced in both Atbe2 mutants. No significant changes were detected for the two unknown activity bands in the middle of the gel. The putative β-amylase activity band (Dumez, 2006) in the lower part of the gel completely disappeared in the Atbe2-1 mutant, and was significantly reduced in the Atbe2-2 mutant.

A predicted BE3 activity band displayed an obvious pink color below the putative BE2 activity band and it co-migrates with another unknown activity band. The fact that this pink band was missing in the zymogram analysis of the Atbe3-1 mutant suggests that it represents the activity of BE3 (Figure 2.4). Interestingly, the slowly migrating ISA activity band was increased in the Atbe3-1 mutant, resulting in a strong blue color covering the putative BE2 band region. None of the other activity bands were significantly affected by the Atbe3-1 mutation.

BE3 activity was affected by Atbe2 mutation. The BE3 activities in both Atbe2 mutants were decreased comparing to the wild type plants. On the other hand, the BE2 activity band was observed normally in the Atbe3-1 mutant. The band of pullulanase-type (limit dextrinase) (At5g04360) DBE activity reported by (Wattebled et al., 2005) was very weakly detected in this work because of the sensitivity of the assay.
Discussion

In this study, six Atbe T-DNA insertion mutations (Table 2.1) from SALK, two for each gene, were obtained. Figure 2.1 shows the T-DNA insertion location of each mutation and also the locations of primers used for genotyping. After genotypic screening, three homozygous mutations were identified (Figure 2.2 A). They were Atbe2-1, Atbe2-2 and Atbe3-1. These three homozygotes were further characterized by RT-PCR and western blot to address the question of whether or not they are null mutations. Atbe3-1 was identified as a null mutation from the RT-PCR result (Figure 2.2 C), which showed no detectable downstream transcription signal in the Atbe3-1 mutant plants. However, RT-PCR results for Atbe2-1 and Atbe2-2 mutants showed that it is likely that neither is a null mutation (Figure 2.2 B). The downstream and flanking transcription signal of Atbe2-1 was somewhat eliminated compared to wild type signal but still observable, whereas Atbe2-2 had the same as wild type signals.

To examine whether the two Atbe2 alleles had BE2 protein translations besides transcription accumulation, western blot was performed on their mutant plants protein extracts. From the immunodetection (Figure 2.3), Atbe2-1 and Atbe2-2 mutants both showed the same bands as wild type. As mentioned above in the results section, this band was confirmed to be the BE3 band because it was missing in the Atbe3-1 mutant. This result suggested that either the antibody could not recognize the AtBE2 band or the Atbe3-1 mutation could also cause the loss of AtBE2 protein in Arabidopsis leaves.

Native starch zymogram analysis was used to test if the corresponding BE activity was lost or reduced in the three Atbe homozygous mutants (Figure 2.4). Some of the zymogram bands were identified based on previously reported data (Wattebled et al., 2005; Dumez, 2006). It is interesting that Atbe2-1 and Atbe2-2 had no BE2 activities, although it was evident that there were BE2 mRNA and protein accumulations in both mutants. BE2 activity bands were clearly missing or reduced to undetectable signal in
both Atbe2 mutants, which is in agreement with the result reported by (Dumez, 2006), although the Atbe2 mutants they used were from a different genetic background and the mutants were null mutants from mRNA level. The zymogram analysis also revealed that the BE3 activity band was missing in the Atbe3-1 mutant. Taken together, our results on the analysis of mutant lines specifically defective of one of the three putative Arabidopsis BE genes indicate that Atbe3-1 is a truly null mutation, and Atbe2-1 and Atbe2-2 can be considered as null mutations too, because the BE2 proteins produced in these two mutations are not active or functional.

The effects of loss of function of each Arabidopsis BE isoform on other starch metabolism related enzymes activities could also be shown in the starch zymogram analysis result (Figure 2.4). First, the effects of each BE mutations on the activity of the other BE was examined. No significant effects were observed. The BE2 activity band was shown normally in the Atbe3-1 mutant. Moreover, the BE3 activity bands in both Atbe2 mutants were slightly weaker but not significantly different compared to wild type. These indicate that Atbe2 or Atbe3 mutation does not affect the activity of another protein, which suggests that, for Arabidopsis BE2 and BE3 proteins, the function of one does not require the other’s existence in plants.

Other starch modifying enzymes activities shown on the zymogram result were isoamylase activity, pullulanase-type DBE activity, β-amylase activity (from up to down) and three unknown activities. The isoamylase activity band, which is a blue band on the very top of the starch-containing zymogram gel, was partially eliminated in both Atbe2 mutants. This band corresponds to the isoamylase activity lacking in the dbe1 mutant, which is localized within the plastid (Zeeman et al., 1998). Both ISA1 and ISA2 genes are responsible for this isoamylase activity in Arabidopsis leaves. It is not known yet whether both proteins represent subunits of the same heteromultimeric complex or their activities are simply interdependent ( Wattebled et al., 2005). However, it was reported that the isoamylase activity has a possible direct function on starch synthesis because the
loss of this isoamylase activity leads to a significant decrease of starch content and the residual starch is composed of a structurally modified amylopectin (Wattebled et al., 2005). The β-amylase activity band, a blueish band on the bottom of gel corresponding to the β-amylase activity, completely disappears in the Atbe2-1 mutant, while remaining very little in the Atbe2-2 mutant. The pullulanase-type DBE activity was hardly detectable in our assay because of low sensitivity, although it was clearly shown on other similar experiments (Dumez, 2006). Other three unknown activities shown on the gel did not have significant differences from wild type. Taken together, these above results showed that the effects on other starch-metabolizing enzyme activities caused by the absence of BE2 activity are as follows: decrease of isoamylase activity, and decrease or loss of β-amylase activity.

Conversely, the isoamylase activity was increased in the Atbe3-1 mutant. This indicated that the absence of BE3 activity leads to higher isoamylase activity in Arabidopsis. This isoamylase activity is the product of ISA1 and ISA2 genes, which are both starch synthesis genes. It was reported that the Arabidopsis mutant Atisa1 and Atisa2 both lack the same isoamylase and influence the branch point distribution of amylopectin during starch synthesis (Delatte et al., 2006). Our result raises the possibility that BE3 might acts as an inhibitor of this isoamylase, or BE3 might have some physical interaction with isoamylase and the loss of BE3 gives the opportunity of the interaction of isoamylase with other proteins that could increase the activity of isoamylase. None of other activity bands was significantly affected by the Atbe3-1 mutation.

BE2 and BE3 mutations had opposite effects on the isoamylase activity in Arabidopsis leaves, which strongly suggests that different BE isoforms have distinct properties and play specific role in starch synthesis. However, the roles remain unknown. Further investigation needs to be done to find out the functions of branching enzymes by characterizing other aspects of the Atbe homozygous mutants such as starch content, starch granule structure and activity assays of more other starch-related enzymes.
Figure 2.1 Molecular structures of Atbe mutants. Exons are shown as black boxes and introns are shown as black lines. T-DNA insertions are indicated as triangles. The arrows show the locations of primer pairs used for the PCR reactions of genotyping of each Atbe mutant.

A: Gene map of Atbe1 allele and Atbe1 mutations. The Atbe1 gene has 7,240 bp nucleotides. It is composed with 20 exons and 19 introns.

B: Gene map of Atbe2 allele and Atbe2 mutations. The Atbe2 gene has 5,704 bp nucleotides. It is composed with 20 exons and 19 introns.

C: Gene map of Atbe3 allele and Atbe3 mutations. The Atbe3 gene has 5,856 bp nucleotides. It is composed with 18 exons and 17 introns.
Figure 2.2 Characterization of *Arabidopsis* T-DNA insertion *sbe* mutants. dH₂O was used as all negative controls.

A: Detection of homozygous mutants. T-DNA insertion mutants were screened by PCR amplification with a pair of gene-specific primers (left), and with one gene-specific primer with T-DNA insertion border primer LBa1 (right).

B: Detection of *Atbe2*-*1* and *Atbe2*-*2* homozygous mutants transcription by RT-PCR. First-strand cDNA was synthesized from total isolated from leaf tissues. cDNA synthesis was performed with total RNA and oligo(dT). PCR was performed using upstream primers (left), downstream primers (middle) and flanking primers (right).

C: Detection of *Atbe3*-*1* homozygous mutants transcription by RT-PCR using upstream primers (left) and downstream primers (right).
**Figure 2.3** Western blots of wild type, *Atbe2* and *Atbe3* mutants.
Total protein extracted from plant leaf tissues were separated by SDS-PAGE, and transferred onto nitrocellulose membrane for immunoreaction with a maize BEIIa/b antibody at 1:5000 dilution. The goat anti-rabbit alkaline phosphatase conjugate was used as the secondary antibody.
A: Western blot of wild type, *Atbe2*-1 and *Atbe2*-2 mutants.
B: Western blot of wild type, *Atbe2*-1, *Atbe2*-2 and *Atbe3*-1 mutants.
Figure 2.4  Zymogram analyses of wild type and Atbe mutant plants.
Total protein extracts (containing 50 ug protein) were extracted from mature wild type and mutant plants leaf tissues, separated by native PAGE in a starch-containing gel and electroblotted to another native gel. The second gel was stained with I2/KI solution to detect the activities of starch modifying enzymes. Different enzyme activities were shown as different colored bands. Iso1: isoamylase activity (ISA1 and ISA2 gene products) as reported by Wattebled et al. (2005); Unknown: unknown activity; LDA: Limit dextrinase (At5g04360) reported by Wattebled et al. (2005); BAM1: β-amylase (At4g15210) as described by Dumez et al. (2006).
### Table 2.1  Summary of all the investigated *Atbe* mutants

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<th>Gene</th>
<th>Mutants</th>
<th>Allele</th>
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<th>SALK ID</th>
<th>Location of T-DNA</th>
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CHAPTER 3. EXPRESSION PATTERNS OF DIFFERENT STARCH BRANCHING ENZYME ISOFORMS IN *ARABIDOPSIS*

Abstract

In higher plants, starch is synthesized in the plastids in the form of dense, semi-crystalline, water-insoluble granules. Within the granule, two classes of polymers, amylose and amylopectin, are organized to form the highly ordered and complex structure. Amylopectin is the primary determinant of the structure and physical properties of the starch granules. Starch branching enzymes are responsible for catalyzing one of the basic chemical steps for amylopectin synthesis, the cleavage of an $\alpha$-(1,4) linkage and formation of a new $\alpha$-(1,6) linkage which is the branch linkage. Three isoforms of BE exist in *Arabidopsis*, and their distinct properties suggest that each isoform may have a specific function in the starch biosynthesis, although these roles are not well understood yet. Different isoforms have different expression with tissue and developmental stage specificity. The main aim of this study was to determine the expression patterns of starch branching enzymes in *Arabidopsis*. First, we investigated the tissue specificity and levels of gene expression of $BE1$, $BE2$ and $BE3$ genes during various developmental stages. The promoter region of each BE gene was fused to the GUS reporter gene and the expression of each fusion protein was visualized by GUS staining in the plant tissues. We found that $BE2$ is highly expressed in the seedling, and $BE1$ and $BE3$ have opposite expression patterns in seedlings. $BE1$ is mainly expressed in newer tissues, while most of the expression of $BE3$ is in older tissues. After seedlings grow, $BE2$ and $BE3$ are co-expressed in almost all tissues and this pattern is maintained throughout development. In general, $BE2$ has a higher expression level than $BE3$. $BE1$ has a very different expression pattern compared with $BE2$ and $BE3$. Second, BE expression patterns were examined by analysis of over 1000 individual microarray
experiments in the public database. These analyses indicate that the expression patterns of the three BE isoforms are consistent with the GUS staining data. Third, the possible correlation of BE gene expression with the expression of other genes in the Arabidopsis genome was analyzed to identify putative genes that are functionally correlated with BEs. Fourth, motifs in the promoters of the Arabidopsis BE genes were identified to better understand their expression differences. Fifth, BE2 and BE3 expression levels during a diurnal cycle were evaluated at the protein level by western blot analysis with isoform specific antibodies. Both proteins showed highest accumulation in the middle of the day, which is consistent with the co-expression results from the GUS staining and suggests that they may function together under this growth condition.

**Introduction**

Starch is the most significant storage form of carbohydrate in plants. It accumulates in the leaf chloroplasts in the day and is degraded to soluble glucose monomers in the dark. Both starch biosynthesis and degradation are highly regulated processes, although the mechanisms are not completely clear yet. Starch is synthesized as semi-crystalline granules with different degrees of crystallinity and polymorphic types. Starch consists of two types of molecules: amylose (normally 18-33%) which is a linear polymer of \( \alpha-(1,4) \) O-glycosidic bonds linked glucose units with rare \( \alpha-(1,6) \) branch points, and amylopection (72-82%) which is a highly branched polymer formed though chains of \( \alpha-D \)-glucopyranosyl residues linked together mainly by \( \alpha-(1,4) \) linkages but with 5-6% of \( \alpha-(1,6) \) bonds at the branch points (Buleon et al., 1998). Amylopection is the major contributor to both mass and granule structure.

Starch formation begins from ADP-glucose synthesis from ATP and Glucose-1-phosphate through ADP-glucose pyrophosphorylase (AGPase). Then a glucose unit is transferred from ADP-glucose to the non-reducing end of a growing \( \alpha-(1,4) \) or
α-(1,4) linked glucan by the catalytic activity of starch synthases (SSs). Branching enzymes (BEs) create new α-(1,6) branch linkages by catalyzing the cleavage of α-(1,4) linkages and transfer of the released reducing ends to the C-6 hydroxyl group in a linear glucan. Debranching enzymes (DBEs) and disproportionating enzyme (D-enzyme) also have potential roles in amylopecton biosynthesis suggested by genetic data. Although amylopectin is chemically similar to glycogen, they have a major difference in that amylopecton is organized into large, insoluble, semicrystalline granules. So the understanding of the biochemical mechanisms of both the amylopecton synthesis and crystallization is important. The role of the BEs, along with SSs and potentially DBEs and D-enzymes, is important to this crystallization process, for example, the placement of branching points provides architectural organization that enables the crystallization to occur.

Simply mixing of these starch-synthesizing enzymes in vitro does not produce amylose and amylopectin, let alone an organized starch granule. The process of producing Am and Ap must require considerable additional complexity, and one obvious source of such complexity is the existence of multiple isoforms of the related enzymes. In all species that have been investigated, there are isoforms for each enzyme of the committed steps of starch biosynthesis. The existence of different isoforms could provide flexibility and specialization and control in starch biosynthesis (Martin, 1995). The isoforms may differ in their substrate preferences, their products, their kinetic properties, their timing of expression during starch biosynthesis and granule formation, the organs in which they are expressed and active, and the proteins they interact with.

Starch branching enzymes (BEs), as one of the primary enzymes of starch biosynthesis, have multiple isoforms existing in the plant kingdom. The isoforms were mostly identified based predominantly on the activities in biochemically fractionated extracts and also by molecular analysis. Three forms of BE were purified and characterized in developing wheat endosperm, and they are differentially expressed
during endosperm development and kinetic characteristics differences were observed (Morell et al., 1997). Two isoforms of purified BE from mature embryos of round peas were identified and one isoform from mature embryo of wrinkled peas was found, and the enzyme activities raise rapidly at different developmental stages (Smith, 1988). Pea BEI was relatively highly expressed in young embryos while maximum expression of BEII occurs in older embryos (Burton, 1995). The contribution of each BE isoform to starch biosynthesis changes during embryo development, and the nature of amylpectin changes during pea embryo development and this correlates with the differential expression of BE isoforms (Burton, 1995). Three BE isoforms have been identified in maize: BEI, BEIIa and BEIIb. Maize be2 expression peaked earlier than that of Sbe1 in developing endosperm and embryos (Gao et al., 1996). Taken together, in these species each BE isoform has different expression with tissue and developmental stages specificity.

Three BE isoforms have been identified in Arabidopsis (termed BE1, BE2 and BE3 respectively), and the functions of BE2 and BE3, which both belong to the SBEII class, are largely redundant (Dumez, 2006).

To further analyze the expression of Arabidopsis BE isoforms in vivo and characterize the difference and potential interaction between the three isoforms, the expression patterns of BE genes were investigated in this work from three aspects: expression patterns in plant tissues of various developmental stages, expression patterns and correlation analysis of BE gene expressions with other starch modifying enzymes from microarray data, and BE expression levels during a diurnal cycle in wild type plants.

**Materials and Methods**
Plant Materials

Wild type Arabidopsis thaliana plants used in this study were of the ecotype Columbia and all transgenic plants are from the same genetic background. Seeds were surface sterilized in sterilization solution (50% bleach, 0.02% Triton X-100 in sterile distilled H₂O) for seven minutes and rinsed in sterile dH₂O three times. After the sterilization, seeds were sown in a sterile potting medium with Sunshine Soil (LC1 Sunshine Mix, Sun Gro, Horticulture, Inc., Bellevue, WA). The soil trays were incubated at 4°C for 2 days and transferred to a growth room at 21°C, 60% relative humidity and 16-hr light/8-hr dark photoperiod.

Promoter-GUS Fusion Vectors Construction

Promoter-GUS fusion constructs were made for all three BE genes by cloning PCR amplified promoter region into a binary vector pBGWSF7 (Karimi et al., 2002). One construct was made for each gene. PCR primers were designed to cover the promoter region of each BE gene with attB sites (Gateway™ Cloning Technology, Invitrogen). The 5’-UTR and the first intron of each gene were also included in the amplified promoter region because of their possible regulatory and enhancer functions to gene expression. Primers used for Atbe1 were QL029 (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG ACT CCA GCG AGA AGA GTG G) and QL030 (GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CGA AAT GGC GTT TGA AAA). Primers used for Atbe2 were QL023 (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CGA AGC ATC CAC TTT CAA T) and QL024 (GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CCT CAA GAC CAC CCT CAT A). Primers used for Atbe3 were QL027 (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT TAA ATT TTC TTT TGT TCA) and QL028 (GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT GAT CAG AGG CAG ATG AAG AGC). High fidelity PCR and DNA gel extraction were performed to get pure PCR fragments, a 1282 bp, 1752 bp, 1369 bp
fragment for *Atbe1, Atbe2, Atbe3* respectively. All PCR reactions were one cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 68°C for 2 min, and a final extension cycle at 68°C for 10 minutes. Invitrogen’s Gateway™ Cloning Technology was used to make the promoter-GUS constructs. The constructs fused GUS (β-glucuronidase) under the control of each BE gene promoter region. After confirmation of the predicted PCR products using restriction digestion followed by agarose gel electrophoresis, the constructs were first cloned into the entry vector pDONR201, then into a binary destination vector pBGWSF7, and finally transformed into *Agrobacterium tumefaciens* stain AgroGV3101 cells using electroporation and were ready for plant transformation. There is a *bar* gene on the pBGWSF7 vector, which is used for the later herbicide selection after transformation.

**Plant Transformation and Selection**

The AgroGV3101 cell strains carrying the constructs were grown in 250ml LB with specific antibiotics gentamycin and spectinomycin. The cells were separated by centrifugation and the pellet was resuspended in 250ml 5% sucrose solution. Transformation was done by dipping method (Clough et al., 1998) to about one month old wild type Columbia *Arabidopsis* plants. Before dipping, 0.05% Silwet L-77 was added to the solution and mixed. The above-ground parts of plants were dipped into AgroGV3101 solution for 3-4 seconds with gentle agitation. Then the plant pots were put with one side down in the tray underneath a plastic dome for one day at room temperature. After that, they were taken back to the growth room and grown and watered as usual. Seven days later, a second transformation was repeated following the same procedures on the same plants. When seeds were mature, watering was stopped and the seeds were harvested.

Putative transformants were planted to the soil and selected by spraying with 0.04% Liberty herbicide based on *bar* resistance of the pBGWSF7 vector. Seeds of successfully
transformed plants were harvested and planted again for GUS staining.

**GUS Staining**

Six independent transgenic lines for each $BE$ gene in the T2 generation were screened for GUS staining. 3-5 plants from each line were used for this experiment. The staining procedure was based on the methods described by Li Ling from Eve Wurtele’s lab (Iowa State University). Different plant tissues at various developmental stages were harvested and put in appropriate sized tubes. X-Gluc (5-bromo-4-chloro-3-indolylglucuronide) stock solution was made by dissolving 100 mM X-Gluc in dimethylsulfoxide (DMSO). GUS stain solution (0.1% Triton X-100, 0.4% Ethanol, 0.5 M KPO$_4$ Buffer pH 7.0, 0.4 mM K$_3$Fe(CN)$_6$, 0.4 mM K$_4$Fe(CN)$_6$, 1 mM X-Gluc stock solution) was added to cover tissues. Air bubbles were removed from the tissue under vacuum. The samples were foil wrapped and incubated at 37°C for 2 days. GUS stain solution was replaced by 70% ethanol to remove chlorophyll. Ethanol washing was repeated 3-5 times for 2 days. The stained samples were stored in 50 mM KPO$_4$ pH 7.0 and the staining patterns were documented by an Olympus stereomicroscope in the Bessey Microscopy Facility (Ames, IA).

**Microarray Analysis**

Software MetaOmGraph (http://www.metnetdb.org/MetNet_MetaOmGraph.htm) was used to analyze data from 963 microarray chips representing 70 Arabidopsis Affymetrix ATH1 microarray experiments that are available in the Nottingham Arabidopsis Stock Center microarray (NASCArray) database (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) (Li et al., in press). These experiments include various mutants, development stages, organ types, stress conditions and others. Expression patterns of all three BE genes were analyzed. Among all the genes in the database, pairwise Pearson correlation coefficients were visualized by
software MetaOmGraph using the provided method in the software. The genes most correlated with each BE protein expression pattern were identified.

**Promoter Motif Analysis**

Putative motifs that are in the promoters of the *Arabidopsis* BE genes were identified by Plant Care (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/visualize_select.pl).

**Protein Extraction and Immunodetection**

Protein was extracted from leaves by grinding about 300 mg fresh weight leaf tissue with a mortar and pestle under liquid nitrogen. The ground tissue was homogenized in 2 volumes of extraction buffer (50 mM Tris-acetate, pH7.5, 10 mM DTT). The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged again. The second supernatant was stored at –20°C. Protein concentration was determined by the Bradford assay (Bradford, 1976).

SDS-PAGE was performed (modified from Laemmli, 1970). Total protein was loaded onto 7.5% polyacrylamide resolving gels. SDS-PAGE was performed at 70-100 V at room temperature. After running, the gel was electrotransferred to nitrocellulose membrane for 50 minutes at 4 °C. To detect BE2 and BE3 proteins, a specific Arabidopsis AtBE2 antibody (mouse monoclonal, produced in our lab by Rebekah S. Marsh) and a specific Arabidopsis AtBE3 antibody (rabbit polyclonal, produced in our lab) were used as the primary antibodies respectively. The goat anti-rabbit alkaline phosphatase conjugate kit (Bio-Rad Laboratories, Hercules, CA, catalog no. 170-6432) was used as the secondary antibody for the BE3 immunoblot analysis following the manufacturer’s instructions. The goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) was used as the secondary antibody for the BE2 immunoblot analysis and followed by
the detection using the ECL™ Western Blotting Detection Reagents kit (Amersham, UK) according to the provided protocols.

Results

Promoter-GUS Fusion Protein Constructs

Three promoter-GUS fusion constructs were made in this study. The predicted promoter regions for each of the three Arabidopsis BE genes (shown in Figure 3.1) were PCR amplified and then inserted into pDONR201 (Invitrogen) via BP recombination sites. The insertions were verified by DNA sequencing and then LR reactions were performed to insert each promoter region to the expression vector pBGWSF7 which contains GUS and GFP sequences located downstream of the insertion sites. Three pBGWSF7-BE promoter constructs were therefore made and verified by DNA sequencing (Figure 3.2). These are designated pBGWSF7-BE1pro, pBGWSF7-BE2pro and pBGWSF7-BE3pro, respectively.

BE Genes Expression Patterns Based on GUS Staining

The three BE promoter-GUS plasmid constructs were expressed in Agrobacterium tumefaciens stain AgroGV3101, which was used to transform wild type Arabidopsis plants. After selection by liberty herbicide spray, seeds of the successful transformations were collected and replanted. For each construct, six independent transgenic lines in the T2 generation were analyzed (18 lines total) and 3-5 plants in each line were used for the experiments. Because GUS expression was under the control of the BE gene promoters in the constructs, GUS staining was used for the analysis at various developmental stages of these transgenic lines with wild type plants as the background control (Figure 3.3).

Analysis of BE2-GUS transformed plants showed BE2 expression is extremely strong in all parts of young seedling, cotyledon, first leaf, hypocotyls, root and root tip
The expression of BE2 in root tip is stronger than in other root parts. In contrast, BE1 and BE3 showed opposite expression patterns in young seedling tissue: BE1 is primarily expressed in the first leaves with little expression in the cotyledon, hypocotyls, root or root tip, whereas BE3 is mostly expressed in the cotyledon, hypocotyls, root and root tip and only a little in the first leaves (Figure 3.3 A).

As the plants grew to three-weeks-old, BE expression patterns changed. BE2 and BE3 exhibited similar expression patterns in small plants, with BE1 also showing a similar pattern but a weaker expression level and little observable expression in the root or the meristem (Figure 3.3 B). The only difference detected between BE2 and BE3 gene expression at this developmental stage is the stronger expression of BE3 in meristem relative to BE2.

When plants were 4-weeks-old, the BE expression patterns are maintained. All three BEs have very similar expression patterns at this developmental stage (Figure 3.3 C). In leaves, the expression levels are clearly higher in the veins. Meristems have the strongest expression for all three BEs. The expression levels in hydathode are examined for this developmental stage. All three BEs have about average expression level in hydathode. Hydathode permits the release of water through pores in the epidermis or margin of leaves, and it is connected to the plant vascular system by a vascular bundle. However, little is known about the function and structure of *Arabidopsis* hydathode.

After the plants reached maturity at 6-weeks-old stage, the expression pattern in young flowers, floral buds, young siliques and stem branches were examined (Figure 3.3 D). BE1 was found to be expressed in floral buds and in the ovary, but the GUS signal was absent in petals, sepals, filaments, pedicels and secondary branches. BE2 and BE3 were expressed similarly but for BE2 GUS staining was at a little higher level. No expression in the stigma could be detected for any of the three BEs.

As plants grow old after 8 weeks, BE1 expression was still absent in petals, sepals, ovary, filaments, pedicels, and the expression of BE2 and BE3 in these tissues decreased
a lot, particularly for BE2, for which there was almost no expression observed at all (Figure 3.3 E).

In the old leaves from the 8-week-old plants, BE genes are expressed mostly in the vasculature tissues (Figure 3.3 F). BE2 has the strongest expression while BE1 is the lowest one. Leaf edges have higher expression than other parts. Three BEs show different expression patterns in hydothodes. BE1 has very low expression, and BE2 has a moderate expression level, and BE3 has very high expression in hydothodes comparing to other leaf tissues. No expression was detected for any of the BEs in the trichomes.

In the old siliques also from the 8-week-old plants, BE2 and BE3 show nearly identical expression patterns (Figure 3.3 G). They are expressed in silique walls and even higher in the seeds inside. No expression exists in the remaining parts of the stigma uncovered by the silique wall. Both BE2 and BE3 also express in the pedicles and stem branches. Overall, BE2 has the same expression pattern but a slightly higher level of expression than BE3 in all silique and branch tissues. In comparison, BE1 has a very different pattern (Figure 3.3 G). BE1 has weak expression in the silique wall, but no detectable expression at all in other parts, including pedicle, seeds, remains of stigma, and branches.

A general overview of the expression patterns of all the various tissues and developmental stages (Figure 3.3) indicates that BE2 is highly expressed throughout the seedling, and BE1 and BE3 have different expression in seedlings. BE1 is mainly expressed in younger tissues, while most of the expression of BE3 expression is in older tissues. As the seedlings grow, BE2 and BE3 become co-expressed in almost all tissues (with a few exceptions, noted above), and this pattern is maintained throughout plant development. In all tissues examined, however, BE2 has a higher expression level relative to BE3 and this difference in magnitude of expression is maintained at all developmental stages. BE1 is a relatively poorly expressed isoform, but it does exhibit expression in specific tissues such as the young meristem, floral buds, the ovary, and leaf
vasculature. Taken together, these experimental results indicate that BE1 has expression preference in newly developing tissues, in particular in the young seedling stages.

**BE Genes Expression Patterns Based on Microarray Analysis**

To further understand the BE gene expression patterns, data from 963 microarray chips representing 70 Arabidopsis Affymetrix ATH1 microarray experiments that are available in the Nottingham Arabidopsis Stock Center microarray (NASCArray) database (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) were analyzed by a open source software component of the MetNet platform, MetaOmGraph (http://www.metnetdb.org/MetNet_MetaOmGraph.htm). This database includes experiments on various mutants, development stages, organ types, stress conditions and others. Expression patterns of all three BE genes in different plant tissues were analyzed (Figure 3.4). A summary of BE gene expression patterns and levels is presented in Table 3.1. Among all the experiments in the database, the expression levels of BE1 are lowest, ranging from 9 to 125; BE2 expression levels range from 5 to 790; and BE3 range from 80 to 1420. The highest expression level for BE3 is much higher than that of either of the other two BE isoforms. However, the average expression level for BE3 is about the same as BE2, while both are much higher than the average level for BE1 which is consistent with the BE genes expression patterns from GUS staining analysis.

Relative to the average BE1 expression level in all of the microarray experiments analyzed, BE1 expression is highest in the seed and male gametophyte, lowest in fruit, and is close to the expression average in other tissues. Relative to the average BE2 expression level in all of the microarray experiments analyzed, BE2 is highly expressed in seedling and shoot. BE2 has low expression levels in root, and has moderate expression levels in other tissues. Relative to the average BE3 expression level in all of the microarray experiments analyzed, BE3 is extremely highly expressed in male
gametophyte, highly expressed in rosette leaf, whereas it is lowly expressed in root, hypotocyl and cotyledon. In other tissues, BE3 is expressed moderately. The high and low in table 3.1 are defined based on their difference from the average level of each gene (more than 1 standard deviation). Extremely high indicates over 2 standard deviation higher than the average level.

**Correlation of Expression of BE Genes with Other Genes in the Arabidopsis Genome**

Gene correlation analysis of expression pattern could provide evidence for identification of genes that are functionally correlated. Pearson correlations were generated from the microarray data by the software MetaOmGraph. First, the correlations of the expression of every two BE isoforms were analyzed. The Pearson correlation between BE1 and BE2 is 7%, between BE1 and BE3 is 19%, between BE2 and BE3 is 31%. Correlations higher than 38.2% across the 22,746 genes on these 22,000 chips are considered to be highly significant (p-value < 3.2e-41 after the adjustment for parallel testing of multiple genes on the chip). Using this standard, none of the BE isoforms exhibit significant correlation, although BE2 and BE3 expressions are the most highly correlated among the three BE genes.

All other genes with expression patterns similar to the BEs across all the experiments in the database were searched. Expression of nine genes was found to be highly correlated (Pearson correlation greater than 60%) with BE1 expression (Table 3.2 A). Among them, only one gene (At1g69200, 62%) participates in starch metabolism and it encodes a kinase involved in the sucrose biosynthesis, while others code for mostly ATP-binding or DNA binding proteins. Many genes were found to be highly correlated with BE2. The nine most highly correlated genes among them were chosen for further analysis (Table 3.2 B). These include two genes (At1g12800, 74% and At5g48300 (ADG1), 73%) related to starch metabolism. The former codes a S1 RNA-binding
domain-containing protein participating in glycolysis and sucrose degradation. The latter codes the small subunit of ADP-glucose pyrophosphorylase, which has glucose-1-phosphate adenylyltransferase activity and involves in starch biosynthesis. Eight genes were found to be highly correlated with expression of BE3 (Table 3.2 C), and six of these are starch metabolism genes. They are: At4g09020 (74%), ISA3 protein involved in starch breakdown; At1g10760 (SEX1, 68%), α-glucan functions in gluconeogenesis and starch degradation; At3g52180 (67%), plant-specific protein phosphatase and binds starch; At3g46970 (67%), cytosolic α-glucan phosphorylase related with starch synthesis pathway; At5g26570 (PWD, 63%), similar to SEX1, chloroplastidic phosphogulcan involved in degradation of leaf starch; At2g40840 (62%), cytosolic amylomaltase, essential component of the pathway from starch to sucrose in leaves at night. The functions and related pathways of these genes highly correlated with all three AtBE genes are also presented in Table 3.2.

**Promoter Motif Analysis of BE Genes**

Promoter motifs are the DNA sequences within promoters that serve to regulate gene expression, such as transcription factor binding sites. Pattern and positioning of promoter motifs are important factors for gene regulation. To better understand AtBE genes transcription and expressions, putative motifs that are present in the BE genes promoters were identified using two publicly available promoter analysis programs: Plant Care (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/visualize_select.pl) (Table 3.3). All three BE genes were found to have light responsive elements in their promoters: BE1 has one, and BE2 and BE3 both have two. All three BE genes also have stress responsive elements in their promoters. BE1 and BE2 have cold stress elements, and BE3 has water stress elements. Isoform specific motifs present in all BE genes are: ABA motifs in BE3; GA motifs in BE2 and BE3; and a MYB binding site motif in BE1.
BE2 and BE3 Expressions in Arabidopsis Plants during a Diurnal Cycle

Leaf protein extracts were harvested from wild type Arabidopsis every four hours over the course of a diurnal cycle. A specific Arabidopsis BE2 antibody and a specific Arabidopsis BE3 antibody were used for western blot detection of each protein at each time point. BE2 and BE3 both show highest signals at the 2 PM time point (Figure 3.5), which is in the middle of the light period. This result suggests that BE2 and BE3 may be co-expressed and have some related functions in wild type plants under the long day (16h day/8h night) growth condition. BE3 also shows a detectable signal at the 2 AM time point, which means that BE3 is expressed at relatively high level at night. No signal was detected for either protein at any of the other time points.

Discussion

Arabidopsis is an excellent model system for studying starch biosynthesis (Zeeman et al., 2002). To examine the three starch branching enzymes (BE1, BE2 and BE3) expression patterns in Arabidopsis, the gene promoter regions of each BE gene (Figure 3.1) was fused to a GUS reporter gene and the constructs (Figure 3.2) were expressed in the Agrobacterium tumefaciens stain AgroGV3101 cells and then transformed into wild type Arabidopsis plants. The pBGWSF7 vector contains both GUS reporter genes which are under the control of the BE promoter, and a BAR selection gene which provides a convenient way for the selection of successful transformants.

The T2 generation of each BE promoter transformed plants were analyzed for GUS expression. In young seedlings, BE2 expression is throughout the seedling, cotyledon, first leaf, hypocotyls, root and root tip and the expression level is quite high. Root tip has stronger BE2 expression than other root parts. Interestingly, BE1 and BE3 are expressed at totally opposite parts in the young seedlings. The former is mostly expressed in the newer tissues such as first leaves and only a little in cotyledon, hypocotyls, root and root
tip, whereas the latter is mostly expressed in older tissues, for example cotyledon, hypocotyls, root and root tip (Figure 3.3 A). These observations suggest that BE2 might play the primary branching enzyme role in young seedlings. On the other hand, BE1 specifically acts in new tissues and BE3 functions in older tissues, which suggests that BE1 and BE3 might be compensatory for the function of BE3 at this certain developmental stage. Although BE1 is reported to have no apparent function in Arabidopsis leaf starch biosynthesis based on the biochemical analysis of mutants (Dumez, 2006), it shows good expression level and tissue specificity here, which suggests that BE1 very possibly has some unknown functions in starch synthesis pathway.

At the later stages of development examined in this study, some common features of all three BE expression patterns were observed: 1) all are preferentially expressed in the leaf vascular tissues, with no expression in the stigma or the trichomes; 2) all are coincidentally expressed at certain developmental stages, such as in hydathodes in the leaves of young plants. These experimental results suggest that all three BE isoforms might be needed and take part in the starch synthesis at some tissues and developmental stages.

This research also reveals clear differences between the expression patterns of BE1 and the other two BEs. In general, the overall expression level of BE1 is much lower than that of either BE2 or BE3. What’s more, BE2 and BE3 are co-expressed in some tissues where BE1 is not detected, for example petals, sepals, ovary, filaments, pedicels, seeds, secondary branches and most siliques. Considering that starch synthesis is abolished in the mutants with the combined loss of both BE2 and BE3 (Dumez, 2006), this result supports the idea that the functions of BE2 and BE3 are largely but not fully redundant in Arabidopsis leaves.

No previous study showed that BE2 and BE3 form a protein complex involved in starch synthesis in Arabidopsis. From our observations of their expression patterns, BE2
and BE3 have very similar expression patterns throughout the development and they are co-expressed in most plant tissues. BE2 generally has a higher expression level than BE3, but there is not significant difference. However, BE2 is detected in some tissues where BE3 has no expression at all, especially in young seedlings, which indicates that BE2 might have a broader function. The \textit{in vivo} expression of BE2 and BE3 during a diurnal cycle was analyzed at four-hour time points, which showed that both proteins had the highest accumulation in the middle of the day (Figure 3.5). With all these considerations in mind, combined with those of another study (see chapter 4), which indicated that mixing of recombinant BE2 and BE3 proteins \textit{in vitro} leads to higher enzyme activity than either protein alone, the results raise the possibility that BE2 and BE3 might have some functional interaction or dependence with each other. Apparently, further work is needed.

From our diurnal cycle experiment result, BE3 has obvious protein accumulation at the 2 AM time point during the night period (Figure 3.5 B). Previous mRNA profiling data (http://starchmetnet.org/Datapages/AtBE3/AtBE3Frameset.htm) showed that BE3 had very high expression at the beginning of the dark period under short day (8 h day/16 h night) growth condition. These data suggests that BE3 not only has a role in starch biosynthesis in the light period but also might participate in the starch degradation process during the dark period.

Our growth condition for GUS staining is under a long day regimen (16 h day/8 hr night). Nevertheless, BE expression levels are known to exhibit changes according to differences in growth conditions (Khoshnoodi, 1998). To get an idea of BE expression patterns across a variety of conditions, the public data from the Nottingham Arabidopsis Stock Center microarray (NASCArray) database were analyzed using the software MetaOmGraph (http://www.metnetdb.org/MetNet_MetaOmGraph.htm). The average expression levels of BE2 and BE3 are very similar, while both are much higher than the average level for BE1 (Figure 3.4). This finding is in general agreement with the results
from GUS staining experiments. Each BE has its specific relevantly high or low expressed specific tissues and different timings of expression (Table 3.1), which demonstrates that the existence of several isoforms in plants could provide flexibility for specialization and control in starch biosynthesis (Martin, 1995). BE3 has extremely high expression in male gametophyte and high expression in rosette leaf, which suggests that BE3 might play unique role in these certain tissues. The highly expressed tissues for BE1 and BE2 are seed and male gametophyte, seedling and shoot, respectively. These differences of tissue or conditional specificity of the expression of each BE isoform, indicate that the three BEs have overlapping and unique roles in *Arabidopsis* starch metabolism.

Using the same approach and database above, we also investigated all genes in *Arabidopsis* genome that had highly correlated expression patterns with each BE protein (Table 3.2). Firstly, none of the BE isoform is significantly correlated with others. However, the correlation between BE2 and BE3 expressions are higher than either of them with BE1 expression, which is consistent with our experimental evidences about expression patterns in this study. Other genes that have similar expression patterns across all the experiments in the database were searched and the most highly correlated genes were chosen for analysis (Table 3.2). Among those genes, the expression of only one starch metabolism gene was correlated with =BE1 (Table 3.2 A). This gene (At1g69200) encodes a kinase involved in the sucrose biosynthesis, which suggests that BE1 might be regulated by post-translational phosphorylation and this kinase might play a role in phosphorylation in starch metabolism.

Expression profiles for many genes were found highly correlated with BE2 (Table 3.2 B). Among the nine most highly correlated genes, two genes, At1g12800 and At5g48300 (ADG1), are involved in starch metabolism. The former codes an S1 RNA-binding domain-containing protein participating in glycolysis and sucrose degradation. The ADG1 gene codes the small subunit of ADP-glucose
pyrophosphorylase (AGPase), which is responsible for the catalytic function, and the small subunit is required for the large subunit stability (Kavakli et al., 2002). AGPase is a key enzyme in starch biosynthesis of higher plants, which catalyzes the first committed and rate-limiting step in starch biosynthesis. Since the reaction catalyzed by AGPase is followed by the reaction catalyzed by BEs in *Arabidopsis* starch biosynthesis pathway, the high correlation between BE2 and AGPase expression might avoid the accumulation of the intermediate product, ADP-glucose, in order to get high efficiency of starch biosynthesis.

Eight genes were found to be highly correlated with expression of BE3 (Table 3.2 C), and six of these are starch metabolism genes. They are: At4g09020, ISA3 protein involved in starch breakdown; At1g10760 (SEX1), α-glucan functions in gluconeogenesis and starch degradation; At3g52180, plant-sepcific protein phosphatase and binds starch; At3g46970, cytosolic α-glucan phosphorylase related with starch synthesis pathway; At5g26570 (PWD), similar to SEX1, chloroplastidic phosphogulcan involved in degradation of leaf starch; At2g40840, cytosolic amylomaltase, an essential component of the pathway from starch to sucrose in leaves at night. Five of these eight genes are involved in the starch degradation, which strongly suggests that BE3 may play a role in the starch degradation. On the other hand, three genes among these eight highly correlated genes are regulatory genes including kinase, phosphatase and phosphorylase. This gives possibility that BE3 expression may be regulated by post-translational phosphorylation or dephosphorylation.

Putative promoter motifs in all three BE genes were identified by Plant Care and Athena programs (Table 3.3), which revealed that light responsive elements exist in all BE gene promoters. This is as expected, because plant starch biosynthesis is presumed to be a highly regulated diurnal process and BEs are all starch biosynthesis involved genes. In addition, all three *AtBe* genes have stress responsive elements in their promoters: cold stress motifs in *BE1* and *BE2*, and water stress motifs in *BE3*. On the other hand,
some specific promoter motifs were identified for each BE gene. BE3 specifically contains five ABA (abscisic acid) signaling elements; BE2 and BE3 have GA (gibberellic acid) elements; and BE1 has specific MYB binding sites element. The plant hormone ABA regulates many essential processes, including inhibition of germination, control of stomatal closure, maintenance of seed dormancy and adaptive responses to various of environmental stresses (Finkelstein et al., 2002). In the promoters of known ABA-activated genes, a conserved ABA responsive element (ABRE; PyACGTGGC), which controls ABA-regulated gene expression, has been identified (Bray, 1994; Giraudat et al., 1994; Busk and Pages, 1998). In the promoter of AtBE3 gene, two ABA or ABA-like binding site elements and three other ABA signaling elements were identified, which suggests that AtBE3 might be an ABA-inducible gene. ABA is produced under dehydration conditions and plays pivotal roles in response to drought stress (Finkelstein et al., 2002; Xiong et al., 2002), which is in agreement with our result that a water stress element exists in AtBE3 gene promoter. AtBE2 and AtBE3 promoters both have GA elements (GAREAT). GA is a hormone found in plant promoting growth and elongation of cells. The occurrence of this GA-responsive element GAREAT in GA-inducible and GA-responsive genes found in Arabidopsis seed germination are 20% and 18%, respectively (Yoshiharu et al., 2007). Based on this result, AtBE2 and AtBE3 genes both could be putative GA-inducible and GA-responsive genes. One of the plant MYB proteins, ATMYB2, is involved in regulation of genes that are responsive to water stress in Arabidopsis (Yoshiharu et al., 2007). Therefore, the specific MYB binding site element in AtBE1 gene promoter suggests that AtBE1 also might be a water stress responsive gene. The related functions of these promoter motifs might provide information for understanding the regulation of the transcription and expression of the different BE genes and their possible roles in Arabidopsis starch metabolism.
Figure 3.1  Promoter regions of promoter-GUS constructs for *AtBE* genes. Arrows indicate the starting sites of transcription.
A: Promoter-GUS Construct for *AtBE1* gene.
B: Promoter-GUS Construct for *AtBE2* gene.
C: Promoter-GUS Construct for *AtBE3* gene.
Figure 3.2  Plasmid map of BE1 promoter insertion to the pBGWSF7 vector.
Figure 3.2 (continued)  Plasmid map of BE2 promoter insertion to the pBGWSF7 vector.
Figure 3.2 (continued)  Plasmid map of BE3 promoter insertion to the pBGWSF7 vector.
Figure 3.3 BE gene expression in different plant tissues at various developmental stages.

A: BE expression in young seedlings, cotyledons, first leaves, hypocotyls, roots and root tips.

B: BE expression in 3-week-old plants, meristems and roots.
**Figure 3.3 (continued)** BE gene expression in different plant tissues at various developmental stages.

C: BE expression in 4-week-old plants, young leaves, hydothodes, meristems and roots.

D: BE expression in 6-week-old plants, young flowers, floral buds, young siliques and branches.
Figure 3.3 (continued)  BE gene expression in different plant tissues at various developmental stages.

E: BE expression in 8-week-old plants, floral buds, old flowers and pedicels.
F: BE expression in old leaves, hydothode and trichomes from 8-week-old plants.
Figure 3.3 (continued) BE gene expression in different plant tissues at various developmental stages.
G: BE expression in old siliques and branches from 8-week-old plants.
Figure 3.4 Expression levels of *AtBE* genes from microarray analysis.  
A: Expression level of *AtBE1* genes from microarray analysis.  
B: Expression level of *AtBE2* genes from microarray analysis.  
C: Expression level of *AtBE1* genes from microarray analysis.
Figure 3.5  Diurnal cycle analysis of BE2 & BE3 expression in wild type plants under long day growth condition (16h day/8h night). The day phase is from 6 AM to 10 PM and the night phase is from 10 PM to 6 AM.
A: Western blot of total leaf protein extracted at different time points with specific AtBE2 antibody.
B: Western blot of total leaf protein extracted at different time points with specific AtBE3 antibody.
### Table 3.1  Relative BE genes expression levels\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>BE1(^b)</th>
<th>BE2(^c)</th>
<th>BE3(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rossete Leaf</td>
<td>Normal</td>
<td>Normal</td>
<td>High</td>
</tr>
<tr>
<td>Shoot apex</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Seedling</td>
<td>Normal</td>
<td>High</td>
<td>Normal</td>
</tr>
<tr>
<td>Root</td>
<td>Normal</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Shoot</td>
<td>Normal</td>
<td>High</td>
<td>Normal</td>
</tr>
<tr>
<td>Seed</td>
<td>High</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Leaf</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Flower</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Hypocotyl &amp; Cotyledon</td>
<td>Normal</td>
<td>Normal</td>
<td>Low</td>
</tr>
<tr>
<td>Leaf apex</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Male gametophyte</td>
<td>High</td>
<td>Normal</td>
<td>Extremely high</td>
</tr>
<tr>
<td>Fruit</td>
<td>Low</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

\(^a\) The relative levels shown in this table are all compared to the average expression level of each gene in all of the microarray experiments analyzed. The definitions of low, normal, high here are evaluated by the statistics deviation of all experiments.

\(^b\) For BE1, the average expression level is 41.8.

\(^c\) For BE2, the average expression level is 241.1.

\(^d\) For BE3, the average expression level is 265.
Table 3.2  Starch metabolism related genes with similar expression patterns with each BE gene across all the experiments in database. (The genes involved in the carbohydrate metabolism are in bold.)

A: Starch metabolism related genes with similar expression patterns with BE1 (At3g20440) across all the experiments in database.

<table>
<thead>
<tr>
<th>Pearson correlation</th>
<th>Gene</th>
<th>Function and related pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.19</td>
<td>BE3</td>
<td></td>
</tr>
<tr>
<td>0.07</td>
<td>BE2</td>
<td></td>
</tr>
<tr>
<td>0.69</td>
<td>At5g08610</td>
<td>ATP binding; ATP-dependent helicase; nucleic acid binding</td>
</tr>
<tr>
<td>0.65</td>
<td>At3g56330</td>
<td>S-adenosylmethionine-dependent methyltransferase activity; tRNA (guanine-N2-) - methyltransferase activity</td>
</tr>
<tr>
<td>0.64</td>
<td>At2g24120</td>
<td>DNA binding; DNA-directed RNA polymerase</td>
</tr>
<tr>
<td><strong>0.62</strong></td>
<td><strong>At1g69200</strong></td>
<td><strong>Kinase; sucrose biosynthesis</strong></td>
</tr>
<tr>
<td>0.62</td>
<td>At1g48570</td>
<td>Zinc ion binding</td>
</tr>
<tr>
<td>0.61</td>
<td>At1g10522</td>
<td>Expressed protein (PPR repeat-containing)</td>
</tr>
<tr>
<td>0.60</td>
<td>At3g13150</td>
<td>Unknown</td>
</tr>
<tr>
<td>0.60</td>
<td>At1g66520</td>
<td>hydroxymethyl-, formyl - and related transferase activity</td>
</tr>
<tr>
<td>0.60</td>
<td>At1g09900</td>
<td>ATP binding (PPR repeat-containing)</td>
</tr>
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</table>
Table 3.2 (continued)  

B: Starch metabolism related genes with similar expression patterns with BE2 (At5g03650) across all the experiments in database.

<table>
<thead>
<tr>
<th>Pearson correlation</th>
<th>Gene</th>
<th>Function and related pathways</th>
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<tr>
<td>0.31</td>
<td>BE3</td>
<td></td>
</tr>
<tr>
<td>0.07</td>
<td>BE1</td>
<td></td>
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<tr>
<td>0.76</td>
<td>At1g08450 (CRT3)</td>
<td>Calcium ion binding</td>
</tr>
<tr>
<td>0.75</td>
<td>At1g51110</td>
<td>Plastid-lipid associated protein; structural molecule activity</td>
</tr>
<tr>
<td>0.75</td>
<td>At5g22830</td>
<td>Magnesium ion transporter activity</td>
</tr>
<tr>
<td>0.75</td>
<td>At5g62790 (DXR)</td>
<td>1-deoxy-D-cylulose-5-phosphate reductoisomerase activity; isoprenoid biosynthesis</td>
</tr>
<tr>
<td>0.74</td>
<td>At1g60990</td>
<td>Aminomethyltransferase activity; glycine catabolism</td>
</tr>
<tr>
<td><strong>0.74</strong></td>
<td>At1g12800</td>
<td><strong>S1 RNA-binding domain-containing protein; calvin cycle; glycolysis; sucrose degradation</strong></td>
</tr>
<tr>
<td>0.74</td>
<td>At1g80030</td>
<td>Heat shock protein binding; protein folding</td>
</tr>
<tr>
<td>0.73</td>
<td>At1g52510</td>
<td>Hydrolase activity; aromatic compound metabolism</td>
</tr>
<tr>
<td><strong>0.73</strong></td>
<td>At5g48300 (ADG1)</td>
<td><strong>Small subunit of ADP-glucose pyrophosphorylase; glucose-1-phosphate adenylyltransferase activity; starch biosynthesis</strong></td>
</tr>
</tbody>
</table>
**Table 3.2 (continued)**  C: Starch metabolism related genes with similar expression patterns with BE3 (At2g36390) across all the experiments in database.

<table>
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<th>Pearson correlation</th>
<th>Gene</th>
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<tr>
<td>0.31</td>
<td>BE2</td>
<td></td>
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<tr>
<td>0.19</td>
<td>BE1</td>
<td></td>
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<tr>
<td>0.74</td>
<td>At4g09020</td>
<td>ISA3 protein; strongly involved in starch breakdown</td>
</tr>
<tr>
<td>0.68</td>
<td>At1g10760 (SEX1)</td>
<td>Alpha-glucan; gluconeogenesis; starch degradation</td>
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<tr>
<td>0.67</td>
<td>At3g52180</td>
<td>Plant-specific protein phosphatase; binds starch</td>
</tr>
<tr>
<td>0.67</td>
<td>At3g46970</td>
<td>Cytosolic alpha-glucan phosphorylase; starch synthesis</td>
</tr>
<tr>
<td>0.63</td>
<td>At5g26570 (PWD)</td>
<td>Chloroplastidic phosphoglucon; normal degradation of leaf starch’ similar to SEX1</td>
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<tr>
<td>0.62</td>
<td>At2g40840</td>
<td>Cytosolic amylomaltase; essential component of the pathway from starch to sucrose in leaves at night</td>
</tr>
<tr>
<td>0.61</td>
<td>At1g27630</td>
<td>Cyclin-dependent protein kinase activity</td>
</tr>
<tr>
<td>0.61</td>
<td>At4g19120</td>
<td>Early-responsive to dehydration stress protein (ERD3)</td>
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### Table 3.3  Promoter analysis of *AtBE* genes

<table>
<thead>
<tr>
<th>Motif</th>
<th>Function</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>BE2</th>
<th>BE3</th>
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<td>-</td>
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<td>MYB1AT</td>
<td>stress response</td>
<td>WAACCA</td>
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<td>+</td>
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<tr>
<td>MYB binding site promoter</td>
<td>cold stress response</td>
<td>MACCWAMC</td>
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<td>MYB4 binding site motif</td>
<td>promoter element</td>
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<td>+</td>
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<td>TATA-box Motif</td>
<td>TATAAA</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>ABFs binding site motif</td>
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<td>BACGTGKM</td>
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<td>GBOXLERBCS</td>
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<td>W-box promoter motif</td>
<td>TTGACY</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

<sup>a</sup> W=A/T, M=A/C
References


CHAPTER 4. IN VITRO ACTIVITY ASSAYS OF ARABIDOPSIS BRANCHING ENZYME 2 AND BRANCHING ENZYME 3 ISOFORMS

Abstract

Three starch branching enzyme isoforms, encoded by three genes AtBE1, AtBE2, and AtBE3, exist in Arabidopsis thaliana. BE2 and BE3 are two isoforms with high amino acid sequence similarity and both belong to the SBEII class, while the conservation of BE1 sequence is very low and BE1 cannot be classified to either plant SBE class. BE1 is reported to have little or no apparent function on starch biosynthesis in maize or Arabidopsis, although tissue-specific gene expression for AtBe1 has been demonstrated. In this study, the in vitro biochemical activities of purified recombinant BE2 and BE3 proteins were investigated by three different assays to better understand their specific functions in starch biosynthesis. An in-plate phosphorylase a assay provided a direct conclusion that these two proteins both have branching enzyme activity with maize amylose as the substrate, and that BE2 activity is higher than BE3 activity. This result was confirmed by the phosphorylase a stimulation assay, which uses C\textsuperscript{14}-labeled glucose-1-phosphate. According to this assay, the activity of BE2 is about 30% higher than that of BE3 at the point of maximal activity for both proteins. Analyses of the structures of the final products from the in vitro BE-amylose reactions by fluorescence-assisted capillary electrophoresis (FACE) analysis with two different forms of amylose as the reaction substrates revealed that the two BE proteins had different reaction termination points for the substrates. Moreover, BE2 and BE3 exhibited different catalytic activities toward the different substrates. The possible reasons of the products difference were investigated by analysis of 3-D structure predictions for both BE proteins, accomplished using online program servers for homology modeling and structure comparisons.
Introduction

Starch is the primary form for carbon storage in plants. Starch branching enzymes (SBE, EC 2.4.1.18), which cleave the $\alpha$-1,4-glucosidic linkages and create new $\alpha$-1,6-glucosidic linkages, play a fundamental role in starch biosynthesis. Multiple forms of starch branching enzyme have been identified in many species, including spinach leaf (Hawker, 1974), maize endosperm and leaf (Boyer and Preiss, 1978; Preiss, 1991); pea seed and embryo (Smith, 1988; Burton, 1995); potato tuber (Preiss, 1988, 1991); rice endosperm, seed and other vegetative tissues (Mizuno, 1992; Nakamura, 1992; Yamanouchi, 1992; Mizuno et al., 2001) and germinating castor bean endosperm (Goldner, 1989). These SBEs from various higher plants can be divided into two classes based on their chromatographic properties and genes and encoded proteins sequence comparisons, named class B (SBEI; e.g. potato SBEI, maize SBEI, pea SBE II), and class A (SBEII; e.g. potato SBEII, maize SBEII, pea SBE I) (Smith, 1988; Nakamura, 1992; Burton, 1995; Rydberg, 2001). The two classes have highly similar amino-acid sequences with major differences on the N-terminal extension of the class B and the C-terminal extension of class A (Burton, 1995; Gao et al., 1996; Larsson, 1998).

The first SBE cDNA isolated was the pea SBEI at the rugosus ($r$) locus (Bhattacharyya et al., 1990). After that, the genes coding for SBEs have been cloned from different plant sources, such as rice seed (Mizuno, 1992; Nakamura, 1992; Kawasaki, 1993; Mizuno, 1993), maize endosperm (Baba, 1991; Fisher et al., 1993; Guan et al., 1994, 1994), rice endosperm (Nakamura, 1992; Mizuno, 1993), potato tuber (Kossmann, 1991) and cassava (Salehuzzaman, 1992). In addition, the expression patterns of several cloned SBE genes were well characterized at various developmental stages in maize (Gao et al., 1996), rice (Mizuno, 1992; Kawasaki, 1993; Mizuno, 1993), pea embryo (Burton, 1995), and potato (Kossmann, 1991; Salehuzzaman, 1992; Visser, 1994).
Biochemical analysis of purified SBE isofroms from maize endosperm, BEI and BEII, revealed that they differed in their actions on amylopectin (Guan and Preiss, 1993; Takeda et al., 1993; Guan et al., 1997). BEI had the highest activity in branching amylose, but its rate of branching amylopectin was less than 5% of that of branching amylose. In contrast, BEII had lower rates in branching amylose (about 9-12% of that of BEI) and had higher rates of branching amylopectin (about 6-fold) than BEI. That indicates that SBEI preferentially branches amylose, whereas SBE II preferentially branches amylopectin (Guan and Preiss, 1993). SBEI also was shown to be responsible for longer chain transfer than SBEII in vitro, and SBEI takes part in the long and intermediate chains synthesis during amylopectin biosynthesis (Takeda et al., 1993). The analysis of amylopectin in the maize amylose-extender mutants indicated an increased average chain length (Hizukuri, 1985), which supported the above results. The in vitro activities of purified potato SBEI and SBEII were compared and it was revealed that SBEI was more active than SBEII on a long linear substrate and SBEII was more active than SBEI on an amylopectin substrate (Rydberg, 2001). Debranching of the products showed that both potato SBEI and SBEII produced a small fraction of dp 6-7 and a larger fraction of chains of dp 11-14, and SBEI produced a population of chains of dp 29-30 (Rydberg, 2001). Biochemical and genetic analysis of the effects of amylose-extender mutations in rice endosperm indicated that the function of BEIIb can not be complemented by BEIIa and BEI, and strongly suggested that BEIIb plays a specific role in the transfer of short chains (Aiko, 2001). These studies have greatly facilitated the investigation of the role of SBE isoforms in starch biosynthesis in plant species.

In Arabidopsis, there are three SBE isoforms: BE1, BE2 and BE3. BE1 cannot be easily classified according to any plant SBE class and this isoform was reported to have no apparent function for starch synthesis in leaves (Dumez, 2006). BE2 and BE3 both belong to the SBEII class and their functions are largely redundant in Arabidopsis (Dumez, 2006). However, the particular role in starch biosynthesis of each SBE isoform
remains unclear.

The aim of this study was to compare the in vitro activities of Arabidopsis BE2 and BE3, and investigate their substrate preferences and product differences. The two isoforms were expressed in E. coli cells and purified. Several distinct BE activity assays were performed to characterize and differentiate the catalytic properties of BE2 and BE3. The structures of the products obtained from in vitro BE-amylase reactions were analyzed, and fluorescence-assisted capillary electrophoresis (FACE) was used to measure and compare the chain length distributions of the products.

Materials and Methods

Plasmid Construction

The coding region of each BE cDNA without the predicted transit peptide sequence was PCR amplified using gene-specific primers containing Gateway’s specific attB sites. The PCR product was purified using Qiagen’s gel extraction kit following the manufacturer’s protocol. The cDNA sequence was cloned into a pDONR vector via a BP (based on attB and attP recombination sites) reaction using Invitrogen Gateway cloning technology. The insert was transferred into the destination vector pDEST15 via an LR reaction, resulting a fusion of each BE with a N-terminal glutathione-S-transferase (GST) tag. All plasmids were sequenced by the Iowa State University DNA Sequencing Facility to confirm the identity of the insertion.

Protein Expression

Each construct was transformed into the competent E. coli BL21 AI cell strains by electroporation. Single colonies were selected to inoculate 3 ml liquid LB cultures containing 50 µg/ml carbenicillin and incubated overnight at 30°C with shaking (200 rev/min). 1 ml of the 3 ml cultures were used to subculture into 20 ml of LB containing
50 µg/ml carbenicillin and incubated overnight at 30°C with shaking (200 rev/min). All the 20 ml cultures were used to subculture into 1 L of LB containing 50 µg/ml carbenicillin and grown at 30 °C with shaking (200 rev/min) until the OD at 595 reached 0.4-0.5. Protein expression was induced by the addition of 0.1% Arabinose and the LB cultures were incubated at 16 °C overnight with shaking (200 rev/min). Cells were spun down at 5,000 rpm, 4°C for 15 minutes and the pellets were suspended in 15 ml cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) containing 5 mM DTT and 0.5% Triton X-100. Cell lysates were prepared by sonication using an Artek sonic dismembrator model 150 with 45 seconds X4 with 30m seconds rest. Another centrifugation (12,000 rpm, 4 °C, 25 minutes) was done to separate the supernatant and pellet and the supernatant was filtered by a 0.45 µm filter to a clean container and kept on ice.

**GST Column Preparation**

Glutathione Sepharose 4B matrix (GE Healthcare) was gently shaken and 800 ul of the slurry was transferred to a new tube. After washing with cold PBS twice, the beads/PBS mixture was pipetted to a new 10 ml disposable column and allowed to settle. The column was washed with at least 5 bed volumes of cold 20% EtOH and stored at 4°C.

**GST Column Purification**

The GST column was washed with 10 bed volumes of cold PBS before any purification procedure. Afterwards, the supernatant was applied into the column and flow-through was collected which was applied to the column again later. The matrix containing the protein of interest was washed three times with 10 bed volumes of cold PBS. Protein was eluted by the addition of 500 µl Glutathione Elution Buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) to the closed column for 10 minutes and
this elution step was repeated twice. All three eluates were pooled and the buffer was changed to 10 mM sodium acetate using Amicon microcon concentrators (YM 50,000). All steps were performed at 4 °C except the elution steps. Protein concentration was determined by using the Bradford assay with a BSA standard curve (Bradford, 1976).

**Immunodetection**

Purified protein was loaded onto 7.5% polyacrylamide SDS-PAGE gels and run at 70-100 V at room temperature until the dye ran off. For Commassie Blue staining, the gel was washed with dH2O for several minutes and then stained for 1 hour with gentle shaking using the Bio-Safe™ Coomassie (Bio-Rad Laboratories, Hercules, CA., catalog no. 161-0786). Destaining was performed by changing the staining buffer to dH2O for overnight incubation with gentle shaking. For western blot analysis, the proteins in the gel were electrotransferred onto nitrocellulose membrane for 50 minutes at 4 °C. Membrane was blocked in blocking buffer (5% milk in TTBS (10 mM Tris, pH 7.5, 150 mM NaCl2, 0.1% Tween 20) for 30 minutes with gentle shaking at room temperature. To detect recombinant BE proteins, a GST specific antibody (rabbit polyclonal), which can recognize GST protein, was used as the primary antibody. A goat anti-rabbit alkaline phosphatase conjugate was used as the secondary antibody.

**Assay of BE Activity**

Two different BE activity assays were used. The phosphorylase a stimulation assay is based on the increased rate of synthesis of α-D-glucan from α-D-glucose-1-phosphate catalyzed by rabbit phosphorylase a when BE is present in the reaction mixture (Guan and Preiss, 1993). BE can increase the number of non-reducing ends of the substrate by adding branch points. This assay is modified from the previously published protocol (Guan and Preiss, 1993). 10 mM AMP, 6 units of rabbit phosphorylase a and branching enzyme were mixed in 0.1 M sodium citrate buffer (pH 7.0). The reaction was started by
addition of 50 mM \(^{14}\)C-G-1-P and incubating at 30°C in a water bath. After certain time points, a 50 µl aliquot was taken out and a solution of 1 ml of 75% methanol, 1% KCl and 100 µl 10 mg/ml glycogen was added. After vortexing and centrifugation, the glucan pellet was dissolved in 200 µl dH\(_2\)O and centrifugation was repeated with the addition of 1 ml of 75% methanol and 1% KCl. The final pellet was dissolved in 500 µl dH\(_2\)O and transferred to a scintillation vial containing 10 ml of scintillation fluid to count the \(^{14}\)C present. The amount of radioactivity incorporated into precipitated glucan was quantified by liquid scintillation counter (1600 TR, Tri-Carb; Packard/PerkinElmer, Downers Grove, IL). An in-plate phosphorylase \(a\) assay was also used. This assay combined the following in a 100 µl volume reaction mixture: 10% (v/v) glycerol, 50 mM G-1-P, 10 mM AMP, 6 units of rabbit phosphorylase \(a\), recombinant BE2 or BE3 protein (singly or together), and Hepes buffer. The mixture was incubated at 30 °C overnight, after which 100 µl iodine solution (I\(_2\)/KI: 1g/10g for 1L) was added.

The amylose assay is based on the determination of the number of branching linkages produced in the amylose after incubation with starch branching enzyme. Two types of amylose were used: maize amylose (Sigma, A-7043) and type III amylose from potato(Sigma, A0512), which is completely free of amylopectin. Purified recombinant BE and the substrate solution (50 mg amylose in 9 ml MOPS buffer) were mixed and incubated at 30 °C. Aliquots were taken out at specific time points from 1 hour to 48 hours and the reaction terminating time point of each type of amylose was chosen for further analysis. The aliquots were boiled for 2 minutes to stop the reaction. The reducing ends in the debranched product were quantified according to the assay of Fox & Robyt (Fox and Robyt, 1991).

**BE-Amylose Reaction Products Structural Analysis**

Fluorophore-assisted capillary electrophoresis (FACE) was used for the chain length distribution analysis of in vitro BE-amylose reaction products according to a previously
published procedure (O’Shea et al., 1998; Dinges et al., 2003) with some modifications. Reaction products were debranched with *Pseudomonas amylofera* isoamylase (1 µl, 0.3 units) (Megazyme International, Bray, Ireland, catalog no. E-ISAMY), which was added to the BE-amylose reaction aliquot with 500 mM sodium acetate buffer (pH 4.4) to final volume 50 µl. The reaction was incubated at 42°C overnight, after which 15 µl was removed and dried in a Speed-Vac (Savant Instruments, Holbrook, NY) for 30 minutes. The reducing ends of the linear chains in the dried sample were fluorescently labeled by suspension in 2 µl sodium cyanoborohydride (1 M in tetrahydrofuran) and 2 µl 8-amino-1, 3, 6-pyrenetrisulfonic acid (APTS) (10 mg in 96 µl of 15% acetic acid) (Sigma-Aldrich, St. Louis, MO, catalog no. 09341). The reaction was wrapped in foil and incubated overnight at 42°C. The labeled reaction was diluted with 46 µl milliQ water, vortexed and centrifuged for 2 minutes at 10,000 rpm. 5 µl of solution from the top of the supernatant of the centrifuged sample was combined with 1 µl labeled arabinose and 194 µl milliQ water then applied to a Beckman P/ACE capillary electrophoresis instrument. Software 32 Karat 5.0 was used for the results integration.

**Homology Modeling and Structure Comparison**

Three-dimensional structures for AtBE2 and AtBE3 were predicted by the homology modeling method (http://molbiol-tools.ca/Protein_tertiary_structure.htm). This method includes 3 steps: template selection, target-template alignment and model construction. A protein-protein blast search was performed on the NCBI website (http://www.ncbi.nlm.nih.gov) to identify proteins having known 3-D structure and high amino acid sequence alignment similarity with the target protein. Four different online program servers were used to make the structure prediction:

EsyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/);
3D-JIGSAW (http://www.bmm.icnet.uk/servers/3djigsaw/);
Geno3D2 (http://geno3d-pbil.ibcp.fr/cgi-bin/geno3d_automat.pl?page=/GENO3D/geno3d_home.html);
GenomeCBS (http://www.cbs.dtu.dk/services/CPHmodels/).

Except Geno3D2, other three servers gave very similar results. The predicted 3-D structures of BE2 and BE3 from EsyPred3D were chosen to do the structural comparison. FATCAT (Flexible structure alignment by chaining aligned fragment pairs allowing twists) (http://fatcat.ljcrf.edu/fatcat/) was the approach used for protein structure comparison.

Results

Expression of *Arabidopsis* BE Proteins in *E. coli* Cells

Three plasmid constructs coding for full length AtBE1, AtBE2, or AtBE3 sequence (without the predicted transit peptide sequence) were expressed in *E. coli* BL21 AI cells. Each BE sequence was linked to a GST tag coding sequence at the 3’ end to generate a BE-GST tagged fusion protein for the purpose of purification. Large scale protein expression was performed for all three Arabidopsis BE isoforms, BE1, BE2 and BE3, to get sufficient quantities of protein for analysis. Protein expression was induced by the addition of 0.1% Arabinose and the cell cultures were incubated at 16°C overnight with shaking (200 rev/min) to get good soluble expression. After expression and cell lysis by sonication, the soluble fusion proteins were identified by SDS-PAGE with Coomassie blue staining (Figure 4.1 A) and western blot analysis with a GST specific antibody (Figure 4.1 B). A no plasmid cell culture was used as a negative control throughout the expression process. The predicted sizes of the GST fusions for BE1, BE2 and BE3 are 120 kDa, 108 kDa, and 120 kDa, respectively. All three proteins showed good total expression (Figure 4.1 A and B) During the expression, protein degradation was observed from the western blot result for all three BE expression. This was reported to be...
a common feature of SBEs purification from biochemically fractionated extracts (Blennow, 1991; Martin, 1995). BE2 and BE3 had good expression as soluble proteins. However, BE1 expression was not stable and the protein was not soluble but contained in inclusion bodies. Because BE1 amino acid sequence homology is considered low compared with both BE2 and BE3 (27-28%) BE1 is not related to either class A or class B plant SBEs, and BE1 has no reported function in starch metabolism in *Arabidopsis* leaves, no further experiments were using BE1 in this study.

Soluble GST-tagged BE2 and BE3 were purified using affinity chromatography with glutathione sepharose™ 4B beads. After a series purification steps, the purified protein was identified again by SDS-PAGE and Coomassie blue staining (Figure 4.1 C) followed by immunodetection with GST specific antibody (Figure 4.1 D). Once the purified recombinant BE2 and BE3 proteins were confirmed, they could then be used for the *in vitro* activity assays. The Coomassie blue staining and immunodetection with GST specific antibody were done after every single time of protein expression to verify the production and purity of BE2 and BE3 recombinant proteins.

**In-plate Phosphorylase a Assay of Purified *Arabidopsis* BE2 and BE3**

The in-plate phosphorylase *a* assay is not a quantitative assay of BE activity, but it does provide a direct and simple way to visualize BE activity, which is shown by the coloration changes of the glucan-iodine complex. For this assay, a reaction mixture of glycerol, G-1-P, AMP, phosphorylase *a*, recombinant BE2 and/or BE3 protein, and Hepes buffer was combined in the well of 96-well plate and incubated at 30°C overnight. Iodine solution was added to the wells the next day and the plate was photographed immediately. Different combinations of BE2 and BE3 protein concentrations were used and compared to a no enzyme control (Figure 4.2).

Keeping the purified recombinant BE3 protein amount constant at 10 µg, the BE2 protein amount was increased from 0 to 20 µg in 5 µg increments. The iodine stain
results, shown in the first four wells in Figure 4.2 A, show that the color turned very dark blue to clear yellow from with increasing amounts of BE2. The combination of 20 ug BE2 with 10 ug BE3 showed very close color as the no enzyme control in the first lane of Figure 4.2 B.

The reverse experiment was also performed, with the amount of BE2 held at a constant level. The results shown in the wells two through five of Figure 4.2B reveal a similar pattern of color change but with a different level of intensity, indicating that BE2 clearly has higher activity relative to BE3.

Assays of BE2 or BE3 alone indicated that 20 ug BE2 protein has much higher enzyme activity than 20 ug BE3 (Figure 4.2 A well 5, Figure 4.2 B well 6). The activity of BE3 activity still appeared to be lower than that of 20 ug BE2 even when the BE3 amount was increased to 50 ug (Figure 4.2 A well 6 compared with Figure 4.2 B well 6).

Comparison of well 3 and well 5 in Figure 4.2 A or well 3 in Figure 4.2 B and the well 6 in Figure 4.2 B, both revealed the same result that the combination of BE2 and BE3 gave higher activity than only one of them was present in the reaction with same total amount of enzymes.

It needs to be pointed out that there might be some experimental differences in this assay. For example, the third well in Figure 4.2 A and the fourth well in Figure 4.2 B, which are both the combination of 10 ug BE2 and 10 ug BE3, showed slightly different colors. However, the significant color changes mentioned above were confirmed by triplicate experiments.

**Activity Measurement of Purified Arabidopsis BE2 and BE3 by Phosphorylase a Assay**

The phosphorylase a stimulation assay is a very sensitive assay of BE activity. This assay is based on measurement of the increased rate of $\alpha$-D-glucan synthesis from $\alpha$-D-glucose-1-phosphate catalyzed by rabbit phosphorylase a. When BE is present in
the reaction mixture, it can increase the number of non-reducing ends of the substrate by adding branch points. So BE activity can be calculated by measuring the product formation of this reaction. AMP, rabbit phosphorylase a, recombinant BE2 or BE3, and C-G-1-P were mixed in sodium citrate buffer (pH 7.0). Several BE protein concentrations were used in the experiment: 0, 0.05, 0.1, 0.15, and 0.2 mg/ml. Unlike the above in-plate phosphorylase a assays, no BE2 and BE3 combinations was examined here.

Both proteins had highest activity at a concentration of 0.05 mg/ml (Figure 4.3 A). The enzyme activities increased from 0 to 0.05 mg/ml, and started to decrease at concentrations higher than 0.05 mg/ml. Two different time points were chosen to do the same assay, 1 hour and 1.5 hours, and they showed the same result. At a concentration of 0.2 mg/ml, the activities for both BE2 and BE3 were at the same level as the background or even lower. Comparing the highest activity of each, BE2 activity is higher than BE3, which is in agreement with the in-plate phosphorylase a assay results.

Four lower concentrations in the 0 to 0.05 mg/ml concentration range then were analyzed for each protein: 0, 0.01, 0.02, 0.05 mg/ml. These experiments showed a linear increase in activity for both BE2 and BE3 (Figure 4.3 B). The rates of increase of BE2 and BE3 do not differ significantly.

**FACE Analysis on BE-amylose Reaction Products**

Two kinds of amylose were used for analysis of the products of the BE-amylose reactions: one is corn amylose which is not very pure and contains some amylopectin, and the other is type III amylose, which is from potato and free of amylopectin. The purified recombinant enzyme and the amylose solution were mixed and incubated at 30 °C, along with a no enzyme control experiment. For these experiments, BE2 itself, BE3 itself, and BE2+BE3 combinations were analyzed. Aliquots were removed for analysis after 2 hours and 4 hours incubation (for corn amylose) or after overnight or 2 day
incubation (for type III amylose). After removal of each aliquot, additional enzyme was added to the remaining reaction in the same amount as had been added at the beginning of the experiment. Prior to analysis, all samples were linearized by debranching with a commercial isoamylase and the resulting linear chains were labeled with a fluorophore. FACE analysis of the distribution of the linear chains in each sample was performed at the ISU Metabolomics Facility with a Beckman P/ACE capillary electrophoresis instrument. The data analysis contains four steps: Integration of all peaks; normalization of the distribution of the linear chains to arabinose peak area; calculation of molar percentage of each DP relative to the total; and subtraction of the no enzyme control background.

The chain distribution profiles of the products of the 2-hour and 4-hour BE2-corn amylose reactions showed no significant differences (Figure 4.4 A left), which suggests that the reaction was finished at 2 hours and the products could not be used as substrates. Therefore, the 2-hour products were used for comparisons of the chain profiles. The comparison of the molar of products from BE2 or no enzyme control reactions showed the in vitro branching enzyme activity of BE2 (Figure 4.4 A right). Similar results were observed for the BE3-corn amylose reactions and the BE2+BE3-corn amylose reactions (data not shown). Comparison of the BE2 and BE3 reaction products was made by subtracting the BE3 profile from that of BE2 (Figure 4.4 B). This showed that BE2 produces more short chains (DP 7-14) and fewer medium length chains (DP 15-36) than BE3. Subtracting either the BE2 or the BE3 chain profile from that of the BE2+BE3 combination reaction product confirmed this result (Figure 4.4 C). These results suggest that BE3 preferentially transfers longer chains whereas BE2 transfers more shorter chains when the corn amylose is the substrate.

In reactions that utilized type III amylose as the substrate, the products of overnight and 2-day reactions with BE2 also showed no significant differences (Figure 4.5 A left). Again, this suggests that the reaction was finished at the end of the overnight incubation
and the products could not be used as substrates. Similarly, the in vitro BE2 activity on this substrate was shown (Figure 4.5 A right). These were also true for reactions involving BE3 or a combination of BE2+BE3 (data not shown). Therefore, the overnight reaction products were used for all analyses. Comparison of the FACE chain distribution profiles of the reaction products (Figure 4.5 B) showed that BE3 specifically produced large amounts of chains of DP 7 whereas BE2 produced more chains in the ranges of DP 5-6 and DP 8-14. Subtracting the BE2 or BE3 chain profile from that of the BE2+BE3 combination profile (Figure 4.5 C) provided confirmation for the result that large amount of chains of DP 7 were produced by BE3. Another significant change observed from Figure 4.5 C was that the addition of either BE2 or BE3 to the other in the reaction cause fewer chains of DP 13-15. In addition, by combining BE2 and BE3, more long chains of DP 31-33 were produced, relative to BE3 singly in the reaction.

**Homology Modeling and Structure Comparison**

Arabidopsis BE2 and BE3 are two highly conserved proteins with 83% amino acid sequence identity. However, they do have some functional differences and specific roles for each are postulated. No known 3-dimensional structures have been reported for these two proteins. To get better understanding of their in vitro products differences might be achieved, 3-dimensional structures for BE2 and BE3 were predicted by homology based modeling methods (http://molbiol-tools.ca/Protein_tertiary_structure.htm). This included 3 steps: template selection, target-template alignment and model construction. Protein-protein blast searches were done on the NCBI website (http://www.ncbi.nlm.nih.gov) to find the protein with high sequence similarity and known 3-D structure, and the *E. coli* BE was chosen to be the target protein for both BE2 and BE3. Four different online program servers were used to do the structure predictions and three of them gave similar results as mentioned before. The result obtained from one of these, EsyPred3D, is shown (Figure 4.6 A for BE2 and Figure 4.6 B for BE3).
These predicted structure results were then compared in order to identify specific differences that might account for the observed changes in their enzymatic activities. FATCAT (Flexible structure alignment by chaining aligned fragment pairs allowing twists) (http://fatcat.ljcrf.edu/fatcat/) was the approach used for this structure comparison (Figure 4.6 C). Two major structural differences were identified based on the comparison results. One is, K625-P639 of BE3 form a loop in the putative BE3 structure, while at the same position BE2 only contains 4 amino acids D500-Y504 to form a short turn (indicated by the red rectangle in Figure 4.6 C). The other major difference is another loop structure with identical amino acid sequences in both BE2 (H317-S329) and BE3 (H441-S453) but at different spatial positions (indicated by the green rectangle in Figure 4.6 C).

**Discussion**

In this study, two isoforms of starch branching enzyme, BE2 and BE3, from Arabidopsis leaves were expressed in *E. coli* cells and purified to compare their enzymatic activities. This was done using several different activity assays and also by analysis of the chain length distribution profiles of the products generated from *in vitro* enzyme-substrate reaction.

At first, we expressed all three BE isoforms and gained good soluble expressions for BE2 and BE3 and some soluble expression for BE1 (Figure 4.1 A and B). Degradation appeared in all BE protein expressions before purification. Since BE1 is believed to have little obvious function in the Arabidopsis leaf starch biosynthesis pathway (Dumez, 2006), our biochemical experiments were focused on BE2 and BE3, using purified BE2 and BE3 protein with GST tags (Figure 4.1 C and D).

A combinational assay of phosphorylase *a* assay and iodine staining assay, termed the in-plate phosphorylase *a* assay, is a easy and convenient method to visualize BE
activity. Both purified BE2 and BE3 showed branching enzyme activities from this assay (Figure 4.2). The assay, although non-quantitative, indicated that BE2 is more active than BE3 and that the mixing of these two enzymes leads to higher enzymatic activity than occurs when either enzyme acts alone. This result suggests that BE2 and BE3 functions might have synergistic effect on each other.

The activities of BE2 and BE3 were also examined by a standard phosphorylase $a$ assay that measures the incorporation of radiolabeled $^{14}$C-glucose-1-phosphate (ref). In these reactions, a series of protein concentrations were used to measure the BE activities. Our investigations showed a maximum activity at 0.05 mg/ml concentration for both BE2 and BE3 (Figure 4.3 A). Enzymes at higher concentrations than 0.05 mg/ml showed reduced BE activity relative to the peak. This result may due to the maximum limitation of the scintillation counter for $^{14}$C present or product inhibition. Four enzyme concentrations in the range from 0 to 0.05 mg/ml were chosen to do this assay. It gave a linear result of activity over concentration (Figure 4.3 B). The maximum activity of BE2 is about 30% higher than BE3, which is in agreement of the general result of the in-gel phosphorylase $a$ assay.

The differentiation of properties of maize BE isoforms, maize BEI, BEIIa and BEIIb, were investigated in earlier analyses that showed they differ in their action on amylopectin (Guan and Preiss, 1993; Takeda et al., 1993). Ranking the activity from highest to lowest, BEIIb > BEIIa >BEI, in the branching of amylopectin. The maize enzymes also differed in the chain length transferred, with BEI preferentially transferring longer chains than BEIIa and BEIIb (Takeda et al., 1993). Arabidopsis BE2 and BE3 are two isoforms that very high amino acid sequence similarity (83%). However, they do show activity level differences based on the above two activity assays. To further investigate their catalytic differences and final product properties, the in vitro reaction products of BE2 or BE3 or BE2+BE3 with amylose were analyzed by FACE. Two types of amylose were used here. One is corn amylose, the other is type III amylose from
potato. The former is amylose mixed with some amylopectin, and the latter is free of amylopectin. The results indicate that the reaction with the corn amylose substrate is faster and ends in 2 hours, whereas the reaction with type III amylose requires an overnight incubation until saturation is reached (Figure 4.4 A and Figure 4.5 A). Comparison of the chain distribution profiles of the reaction products in which corn amylose was the substrate indicates that BE2 produces a polymer with more short chains (DP 7-14) and fewer medium length chains (DP 15-36) relative to BE3 (Figure 4.4 B and C). When potato type III amylose was the substrate, comparison of the chain profiles indicates that BE3 produces a large percentage of chains of DP 7 whereas BE2 produces more short chains than BE3 that are of lengths DP 5-6 and 8-14. BE2 shows a preference of transferring shorter chains with both types of substrate in our investigation (with the exception of DP 7 with type III amylose). The reason why BE3 specifically produces very large amounts of chains of DP 7 is not clear now. In addition, the reaction products of both BEs on the two different substrates have very different chain length profiles (Figure 4.4 B and Figure 4.5 B), which is probably due to the substrate difference leading to different functional patterns of the two BEs.

The in vitro BE-amylose reactions do not really resemble the physiological action of BE in starch biosynthesis in Arabidopsis leaves because that is a complex process that requires the activities of multiple enzymes. However, these results could help us to better understand the substrate preference and specificity of the individual BE isoforms and the possible mechanism of their roles in starch biosynthesis. Based on our FACE results, the products of the same BE on different substrates could be very different. Considering starch biosynthesis in plants, the substrates of BE are changing all the time with the effects of other starch metabolism enzymes. Therefore, the catalytic properties of BEs may change during the starch biosynthesis process. The finding from the in vitro reactions on amylopectin-free amylose that BE3 specifically produces chains of DP 7 suggests that, under certain substrate condition in plants, each BE might have very
unique role or catalytic property. In addition, the FACE results show that the combination of BE2 and BE3 act differently from either BE singly. This suggests that the plant tissues having the expression of both BE isoforms might differ from other tissues in which only one BE is expressed in the starch metabolism.

One possible explanation for the different products of BE2 and BE3 is that their 3-dimensional structures are different and this could determine the selection of substrate and alter the catalytic properties. Neither of the two AtBEs has yet had a 3-D structure revealed. We used homology modeling as the method to obtain a prediction of the AtBE2 and AtBE3 3-D structures. The *E. coli* branching enzyme was used as the template and the prediction was based on its known X-ray crystallographic structure (Abad, 2002). Online program servers of homology modeling were utilized to achieve the putative structures of BE2 and BE3. Three out of four servers gave very similar results and these structures (Figure 4.6 A and B) were chosen to make the structure comparisons also using online tools. One major structural difference (Figure 4.6 C) is a loop structure shown in the predicted BE3 3-D structure (K625-P639) instead of a turn in BE2 (D500-Y504). The other difference is another loop existing in both BE structures, BE2 (H317-S329) and BE3 (H441-S453), with the same amino acid sequences but at different 3-D positions (Figure 4.6 C). It has been suggested that BEs belong to the α-amylase family (Baba et al., 1991), and they contain the \((\beta/\alpha)_8\)-barrel structure with their central domains (Jespersen et al., 1993). The loops between \(\beta\)-strand 7 and \(\alpha\)-helix 7 and between \(\beta\)-strand 8 and \(\alpha\)-helix 8 have been implicated in determing the length of branches (Burton et al., 1995). Our result supports this idea, because the big loop in the putative AtBE3 structure while not in AtBE2 is the loop connecting the \(\beta\)-strand 7 and \(\alpha\)-helix 7. The smaller loop showing the structure difference between BE2 and BE3 is the connection of \(\beta\)-strand 3 and \(\alpha\)-helix 3. The central \((\beta/\alpha)_8\)-barrel structure is predicted to be involved in the hydrolysis, especially the loops connecting each barrel (Jespersen et
al., 1993). Therefore, the difference of this loop in the predicted structures of BE2 and BE3 might also cause different catalytic properties between them.

The results of the homology modeling and structural comparison experiment suggest that these two structural differences might be part of the reason of the difference of the catalytic properties and substrate preference of BE2 and BE3. To further test whether this is true, site-directed mutagenesis could be performed on these two structural regions on BE2 and/or BE3. Therefore, the FACE analysis on the products of mutated BE with amylose reaction would provide useful information about the testing of this hypothesis.
Figure 4.1 Expression of *Arabidopsis* BE proteins in *E. coli* cells. For all panels, N: no plasmid control; M: protein standard marker; 1: expressed recombinant AtBE1 protein; 2: expressed recombinant AtBE2 protein; 3: expressed recombinant AtBE3 protein. Red arrows indicate the bands of proteins of interest.

A: Coomassie blue staining gel of the total crude extracts of the expression of three recombinant AtBE proteins. B: Western blot of the soluble portion of extracts of three recombinant AtBE proteins with GST specific antibody.

C: Coomassie blue staining gel of the purified recombinant AtBE2 and AtBE3 proteins. D: Western blot of the purified recombinant AtBE2 and AtBE3 proteins with GST specific antibody.
Figure 4.2 In-plate phosphorylase a assay of purified *Arabidopsis* BE2 and BE3 proteins. A and B show the reactions with different amount combinations of AtBE2 and AtBE3 proteins in the assay.
**Figure 4.3** Activity of purified recombinant *Arabidopsis* BE2 and BE3 protein in phosphorylase a assay. Each point is the average of three replicates.
A: Phosphorylase a assay of purified BE2 and BE3 recombinant protein in 0-0.2 mg/ml concentration range.
B: Phosphorylase a assay of purified BE2 and BE3 recombinant protein in 0-0.05 mg/ml concentration range. The 0-hr controls are all lower than 726 and have been subtracted at each point.
Figure 4.4  FACE analysis of the products from Arabidopsis BE2 or BE3 with corn amylose reactions. The products were debranched with commercial isoamylase. The reducing ends of the linear chains were fluorescently labeled and separated by FACE. The frequency of individual chain lengths was normalized to total peak area. No enzyme control reaction background values were subtracted from all data.

A: Comparison of the FACE results of BE2 at two incubation times (left) and comparison of the normalized molar of no enzyme control and BE2 after 2-hours incubation (right).

B: Comparison of the FACE results of BE2 with BE3.

C: Comparison of the FACE results of the combination of BE2 and BE3 with either BE2 or BE3.
Figure 4.5  FACE analysis of the products from *Arabidopsis* BE2 or BE3 with typeIII reactions. The products were debranched with commercial isoamylase. The reducing ends of the linear chains were fluorescently labeled and separated by FACE. The frequency of individual chain lengths was normalized to total peak area. No enzyme control reaction background values were subtracted from all data.

A: Comparison of the FACE results of BE2 at two incubation times (left) and comparison of the normalized molar of no enzyme control and BE2 after 2-hours incubation (right).

B: Comparison of the FACE results of BE2 with BE3.

C: Comparison of the FACE results of the combination of BE2 and BE3 with either BE2 or BE3.
Figure 4.6  Homology modeling and structure comparison of *Arabidopsis* BE2 and BE3. Only backbone is shown in the structures.

A: The predicted 3-D structure of AtBE2.
B: The predicted 3-D structure of AtBE3.
C: Structure comparison of the predicted AtBE2 and AtBE3 3-D structures. Yellow indicates BE2 and white indicates BE3. The red and green rectangles indicate the two major structural differences between BE2 and BE3.
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CHAPTER 5. SUMMARY AND GENERAL CONCLUSIONS

The overall goal of this thesis was to increase the understanding of the molecular mechanism of starch biosynthesis in higher plants, specially focusing on the characterization of the expression and functions of starch branching enzyme (BE) isoforms that have been identified in Arabidopsis. To achieve this goal, three different approaches were used (described in Chapters 2-4 in this thesis). Based on our results, a clear conclusion is that the three BE isoforms have some overlapping and unique functions in starch biosynthesis in Arabidopsis.

1) T-DNA insertion mutant lines of BE isoforms were identified and analyzed. One mutant allele (Atbe3-1) in the BE3 gene was shown to be null mutation because it has abnormal transcript and no BE3 protein accumulation. Two mutant alleles in the BE2 gene were not shown to be null mutations based on transcripts and BE2 protein detection. However, no BE2 activities were shown in these two mutants. The effects of loss of function of BE2 or BE3 on activities of other starch metabolism enzymes were determined. Two BE isoforms showed opposite effects on the activities of isoamylase which suggests that they might have different functional relations with isoamylase. Also, BE2 cause the decrease of the activities of putative β-amylase which suggests that BE2 might have functional relation with β-amylase (Chapter 2).

2) The expression patterns of each BE isoform in Arabidopsis under long day growth condition were examined. BE2 is the most strongly expressed isoform in the seedling, and BE1 and BE3 are expressed in different parts of seedling, which indicates that they might have complementary functions at this developmental stage. BE2 and BE3 are co-expressed in most plant tissues throughout development after seedling grows. BE1 expression pattern has clear differences from the other two isoforms. Some common features of all three BE expression pattern were observed, which suggest that they all might have some overlapping functions in the starch synthesis at certain tissues and
developmental stages, whereas the specific expression pattern of each BE suggests that each BE might have unique roles in starch biosynthesis in Arabidopsis (Chapter 3).

3) The microarray experiments from the NASCArray database were analyzed to get further understanding of BEs expression patterns. Each BE isoform has its specific relevantly high or low expressed tissues, which confirmed that they have distinct expression patterns and might play unique roles in starch biosynthesis (Chapter 3).

4) The expression of BE2 and BE3 during a diurnal cycle was detected with isoform specific antibodies. They both showed the highest expression levels in the middle of the light phase. This suggests that BE2 and BE3 may have some related functions in wild type plants. BE3 specifically showed detectable expression at night (Chapter 3).

5) Other genes mostly correlated with each BE expression pattern in the Arabidopsis genome were identified and among them the starch metabolism genes were further analyzed. BE1 and BE3 both have some regulatory genes highly correlated with them. A kinase is highly correlated with BE1 which suggests that BE1 might be regulated by phosphorylation. Some phosphatase and phosphorylase expression patterns are highly correlated with BE3, which suggests that BE3 might play a role in starch degradation. This gives possibility that they may be regulated by post-translational phosphorylation or dephosphorylation. Three of the genes highly correlated with BE3 expression are involved in the starch degradation, which suggests that BE3 might also play a role in the starch degradation (Chapter 3).

6) Putative promoter motifs in the three BE genes were identified. They all have common light responsive elements. On the other hand, there are specific promoter motifs in each BE gene, which indicates they might be inducible or responsive by different plant hormone or stress conditions (Chapter 3).

7) The in vitro enzyme activities of purified recombinant BE2 and BE3 proteins were examined. The two proteins both showed branching enzyme activities and BE2 activity is higher. In addition, they show synergistic effects in the reaction assays instead of
working independently (Chapter 4).

8) The chain length profiles of the products of in vitro reaction of BE2 and BE3 (together or singly) with two types of amylose as substrate were analyzed. Two BE proteins have different reaction termination points and exhibited different catalytic properties toward different substrates. With a substrate of amylose mixed with some amylopectin, BE2 produces more short chains (DP 7-14) and fewer medium length chains (DP 15-36) relative to BE3. With a substrate of pure amylose without amylopectin, BE3 specially produces a large amount of DP 7 while BE2 producing more short chains of DP 5-14 except DP 7. The chain length preferences of each BE protein suggest their unique functions and catalytic properties (Chapter 4).

9) The possible reason of the products differences was investigated by the 3-D structure predictions of BE2 and BE3. From the comparison of the predicted structures, two major differences were identified. They both are the loops connecting the β-strand and α-helix structures in the central domains, which were previously predicted to be involved in determining the length of branches or other catalytic properties (Chapter 4).