ABSTRACT

The Role of Pullulanase and SSIII in the Formation of the Vitreous Endosperm Phenotype in Quality Protein Maize

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Despite being a major food source, maize does not provide a balanced source of protein, because maize lacks some essential amino acids, such as lysine and tryptophan. *opaque-2* (*o2*) mutants highly increased the lysine content, but they were not suitable for commercial development, due to their soft and chalky endosperm. Modifier genes were introgressed into *o2* maize that restored hard and vitreous endosperms, while maintaining the high lysine content and these lines were called Quality Protein Maize (QPM). In this study two candidate factors associated with the formation of the vitreous endosperm phenotype in QPM were characterized; *Zpu1*, encoding pullulanase-type starch debranching enzyme and SSIII, encoding Starch Synthase III. The data showed that the QPM inbred line, K0326Y, had higher pullulanase activity and SSIII abundance than W64A*o2*. Recombinant inbred lines (RILs) generated from a W64A*o2* X K0326Y cross demonstrated that kernel vitreousness was positively correlated with pullulanase activity, indicating pullulanase may be one of the key factors affecting vitreousness. Pullulanase activity was highly dependent on *Zpu1* alleles and *SSIII* alleles, indicating that *SSIII* might

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The Role of Pullulanase and Starch Synthase III in the Formation of the Vitreous Endosperm Phenotype in Quality Protein Maize

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LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl coenzyme A
ADP-Glc/ADPG	Adenosine diphoaphate glucose
ae1	amylose-extender 1
AGPase/ ADPG-PPase	ADP-glucose pyrophosphorylase
ANOVA	Analysis of variance
Ap	Amylopectin
APTS	8-amino-1,3,6-pyrenetrisulfonic acid
bt2	brittle-2
CID	Collision-induced dissociation
CTAB	Cetyltrimethylammonium bromide
cyPPDK1	Cytoplasmic pyruvate orthophosphate dikinase 1 gene
DAP	Days after pollination
DBE	Starch debranching enzymes
DMSO	Dimethyl sulfoxide
DP	Degree of Polymerization
DSC	Differential Scanning Calorimetry
du1	dull1
EIL	Ethylene insensitive 3-like protein
ER	Endoplasmic reticulum
EREBP	Ethylene responsive element binding protein

ERF1	Ethylene response-related factor 1
FACE	Fluorescence-assisted capillary electrophoresis
G1P	Glucose-1-phosphate
GBSSI	Granule bound starch synthase I
HSPs	Heat shock proteins
IMAC	Immobilized-metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISA	Isoamylase-type starch debranching enzyme
kDa	Kilodalton
m/z	Mass-to-charge ratio
mo2	Opaque-2 modifier
MS	Mass spectrometry
o2	opaque-2
PDI	Polydispersity index
PFP	Pyrophosphate-Dependent Fructose-6-Phosphate1-Phosphotransferase
PPDK	Pyruvate orthophosphate dikinase
QPM	Quality Protein Maize
qRT-PCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative Trait Loci
RIL	Recombinant inbred line
RIP	Ribosome-inactivating Protein
RNAi	RNA interference

RNA-seq	RNA Sequencing
SBE(I, IIa, IIb)	Starch branching enzymes (I, IIa, IIb)
SEM	Scanning electron microscope
sh1	shrunken-1
sh2	shrunken-2
SNP	Single nucleotide polymorphism
SP	Starch phosphorylase
SS (I-IV)	Starch synthases (I-IV)
su1	sugary-1
su2	sugary-2
UDP-Glc/UDPG	Uridine diphoaphate glucose
WSP	water-soluble polysaccharide
wx1	waxy1
Zpu1	Zea mays pullulanase type debranching enzyme

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DEDICATION

To My Lord, Jesus Christ

CHAPTER ONE

Introduction

Maize

Maize (*Zea mays* L. ssp. mays) is a cereal plant of the Poaceae family that was originated from teosinte growing in tropical Mexico (Beadle, 1939; Wang et al., 2005). Maize is one of the main staple food crops for humans and the demand for maize will continue to increase as a result of expanding human population around the world (Pingali, 2001).

Recent reports showed that the United States was responsible for about a third (31.7%) of the world's maize production (US Grains Council, 2013). And in the year of 2014/15, the production of maize was 14,215.532 million bushels, accounting for 95.4% of total feed grain production (USDA-ERS, 2015). About 12% of that was used for human diet that was either consumed directly (e.g. corn chips) or indirectly (e.g. high fructose corn syrup) in US (National Corn Grower's Association, 2013). In some developing countries, maize makes up about 50% of the caloric intake in local diets (McCann, 2001). A study performed by International Service for the Acquisition of Agri-biotech Applications (ISAAA) showed that the global demand for maize will increase by 45% by 2020. Especially, this demand in developing countries will increase by 72% (James, 2003). To meet the challenge, it is essential to maintain nutritional quality and high yields of maize.

Maize and Nutrition

Carbohydrates and proteins are two major nutrients in maize kernels, which provide 66%-76% and 5%-13% of the kernel weight, respectively (Cortez and Wild-Altamirano, 1972). However, maize does not a provide a nutritionally balanced protein source, because the lysine content in normal maize lines is so limiting that it cannot meet the requirement of optimal human nutrition and animal feed (Bhan et al.,2003; Young et al., 1998).

Lysine is one of the essential amino acids for monogastric animals and humans. They are not able to synthesize it, so they have to obtain it from their diet (Galili, 1995). Studies on chickens and rats showed that lysine deficiency could disrupt protein synthetic pathways and result in poor growth, retarded bone formation, anemia, and hypoproteinemia (Tesseraud et al., 1996; Gillespie et al., 1945; Borum and Broquist, 1977). Therefore, in order to correct the lysine deficiency of cereals, traditi onally, farmers add soybeans or other common beans as supplements, because those food legumes contain relatively rich source of lysine (Bressani and Elías, 1974; Bressani, 1975), but maize-bean mixed diet increases the feed cost, and it may not be applicable for some developing countries where maize is their major source of diets. Therefore, it is important to understand the mechanism of lysine metabolism, and develop stable maize lines with high lysine content.

Lysine in developing kernels is mainly acquired by synthesis through the aspartate pathway and translocation from other tissues (Azevedo et al., 2003; Arruda and Silva, 1979). On the one hand, a very limited amount of lysine is involved in biosynthesis of endosperm proteins. The major protein fraction in endosperm is zeins, which compose up to 70% of the total endosperm proteins (Gibbon and Larkins, 2005). Zeins are highly hydrophobic prolamin proteins and are deposited in the form of protein bodies within rough

endoplasmic reticulum (Argos et al., 1982; Larkins and Hurkman, 1978). They consist of four structurally distinct types α-zeins (19 and 22-kDa), β-zeins (15-kDa), γ-zeins (50, 27 and 16-kDa) and δ-zeins (18 and 10 kDa), among which α-zeins are the most abundant, and 16-kDa γ-zein and 15-kDa β-zein play an essential role in binding and assembly of α-zeins (Wu et al., 2010, Kim et al., 2002). However, zeins are devoid of lysine (Burr and Burr, 1976). The second largest fraction is glutelins, which compose 15% up to 45% of total endosperm proteins (Prat et al., 1985; Ludevid et al., 1984). They are hydrophobic proteins that have relatively high lysine content (Osborne and Mendel, 1914; Yau et al., 1999). On the other hand, the rest of free soluble lysines are degraded through the saccharopine pathway into acetyl-CoA and produce glutamate (Arruda et al., 2000), which could explain the reason why normal maize lines have relatively low lysine content in their endosperms.

Maize opaque-2 Mutant

In the early 1960s, the *opaque-2* (*o2*) mutant lines were discovered to have increased lysine content in endosperm when compared with the endosperm of normal maize lines (Mertz et al., 1964). Several studies on chicks, swine, rats and humans suggested that the *o2* lines are nutritionally superior to normal lines (Cromwell et al., 1967-1, Cromwell et al., 1967-2; Mertz et al., 1965; Young et al., 1971; Kies and Fox, 1972). The nutritional value of *o2* maize is nearly equivalent to that of the traditional maize-bean mixed diet (Cromwell et al., 1967-1). If the *o2* maize were widely applied in commercial farming, it would largely reduce the feed cost and help solve the protein malnutrition problem in developing countries. Therefore, intensive studies of *O2* gene were done in past

decades in order to reveal the mechanism responsible for the elevated high lysine content in *o2* maize endosperm.

Early studies showed that the increase of lysine content in *o2* endosperm was associated with a reduction in the ratio of zein to glutelin (Mertz et al., 1964). In *o2* mutant lines, zein protein content decreased by approximately 50% compared with normal maize lines, especially, a significant reduction of 22-kDa α-zein was observed (Kodrzycki et al., 1989). *O2* gene encodes a transcription factor with a "leucine-zipper" motif that binds a specific target site in 22-kDa α-zein gene promoters (Hartings et al., 1989, Schmidt et al., 1990; Schmidt et al., 1992). *o2* is a recessive mutation of *O2* allele, and it substantially reduced the expression of 22-kDa α-zein, causing the decreased size of zein protein bodies, which could be responsible for the opaque kernel phenotype (Geetha et al., 1991, Segal et al., 2003).

The *o2* mutant also influences the expression of many other target genes (Hunter et al., 2002). Prior studies reveal that the mutant reduced the transcription of *b-32* gene, which encodes a ribosome-inactivating protein (RIP) as an immunotoxin and antifungal agent (Lohmer et al., 1991; Bass et al., 1992). Also, the expression of the cyPPDK1 gene was reduced in *o2* mutant endosperm. This gene encodes a cytoplasmic pyruvate orthophosphate dikinase (PPDK) isoform involved in C4 photosynthesis and amino acid interconversion (Maddaloni et al., 1996). In recent years, RNA-seq, gene microarray techniques and proteomic analysis facilitated us to better characterize a broader spectrum of target genes affected by *o2* mutant (Hartings et al., 1989; Hunter et al., 2002; Jia et al., 2013). Those genes were associated with starch biosynthesis, stress response and defense,

ER maintenance, and etc (Jia et al., 2013), which need to be further analyzed to obtain deeper insight into the *o2* maize lines.

Quality Protein Maize

Despite the improvement in the lysine composition and nutrition value, most *o2* lines were not commercially developed, due to the following physical drawbacks compared with normal counterparts: 1) lower kernel density, weight and yields, 2) soft, chalky and dull endosperm texture, 3) more susceptible to ear rots and grain pests, 4) slower grain drying after mature (Villegas, 1994; Habben and Larkins, 1995). To solve those problems, from the early 1970s, plant biologists, agronomists and breeders in US Illinois, South Africa, and Mexico developed several modified *o2* lines by introgressing genes that alter the soft and opaque endosperm, producing a hard and vitreous endosperm while maintaining high lysine content of *o2* lines (Vasal et al., 1980). Also, genes were accumulated in those lines that restored the grain yield and developed resistance against major disease and pest attack (Borlaug, 1994). Those genes were designated *o2* modifiers (*mo2*), and the *o2* lines with those genes were called Quality Protein Maize (QPM) (Prasanna et al., 2001).

Both in human and animal diets, QPM is nutritionally superior to normal maize lines, because it keeps the high lysine content of *o2* lines, and compared with o2 counterparts, QPM has better dry-milling properties, because its hard kernel texture makes it easier to separate pericarp from endosperm (Serna-Saldivar and Rooney, 1994). By the mid-1990s, QPM was prevalent in Mexico, Sub-Saharan Africa, US, China and many other countries around world, and became an effective way to alleviate global hunger and malnutrition (Villegas, 1994).

However, the genetic complexity of QPM and technical difficulties of introducing multiple *mo2* loci, while monitoring the presence of *o2* gene to maintain the high lysine level in endosperm were major obstacles to efficiently develop stable QPM lines (Vasal et al., 1980). This process could be accelerated if the key mechanisms by which the *mo2* genes produce a hard and vitreous endosperm were characterized, but so far, the location of most modifier genes and their specific downstream effects are not known.

Studies revealed that a locus near the centromere region of the long arm of chromosome 7 was linked with a gene encoding 27-kDa γ-zein, which may be associated with the hard and vitreous endosperm phenotype in QPM lines (Lopes et al., 1995). Compared with *o2* lines, QPM maintained low level of 22-kDa α-zein, and high level of lysine-rich non-zein proteins, but QPM accumulated twice up to three times as much 27-kDa γ-zein as its *o2* counterparts (Wallace et al., 1990; Geetha et al., 1991). In recent studies, a γ-RNAi transgene was introduced into QPM lines to knock down the expression of 27-kDa γ-zein, resulted in an opaque phenotype in RNAi treated QPM endosperm (Wu et al., 2010). Also, Dr. Holding's group performed a genome-wide deletion of 27-kDa and 50-kDa γ-zein, causing dramatic decrease of protein body number, and generating a QPM variant with an opaque endosperm phenotype (Yuan et al., 2014). Those findings further confirmed the vital role of γ-zein proteins in the vitreous and hard endosperm formation.

However, the mechanisms by which the γ-zein contributes to the formation of the vitreous and hard endosperm phenotype have been difficult to characterize. One hypothesis was: as the maturation of kernels, the desiccation process enhances the interaction between protein bodies. Cysteine‐rich γ‐zeins are located at the surface of protein bodies and form disulfide linkages between protein bodies. The highly packed protein bodies around

starch granules may form a dense and hard proteinaceous matrix, which could be a continuous medium for light transmission. Since *o2* lines have lower amount of γ - zein than QPM, less interconnection are formed between protein bodies and may leave air spaces between starch granules, causing a soft endosperm texture and generating a discontinuous matrix that disrupts the transmission of light (Figure 1-1) (Larkins et al., 1994).

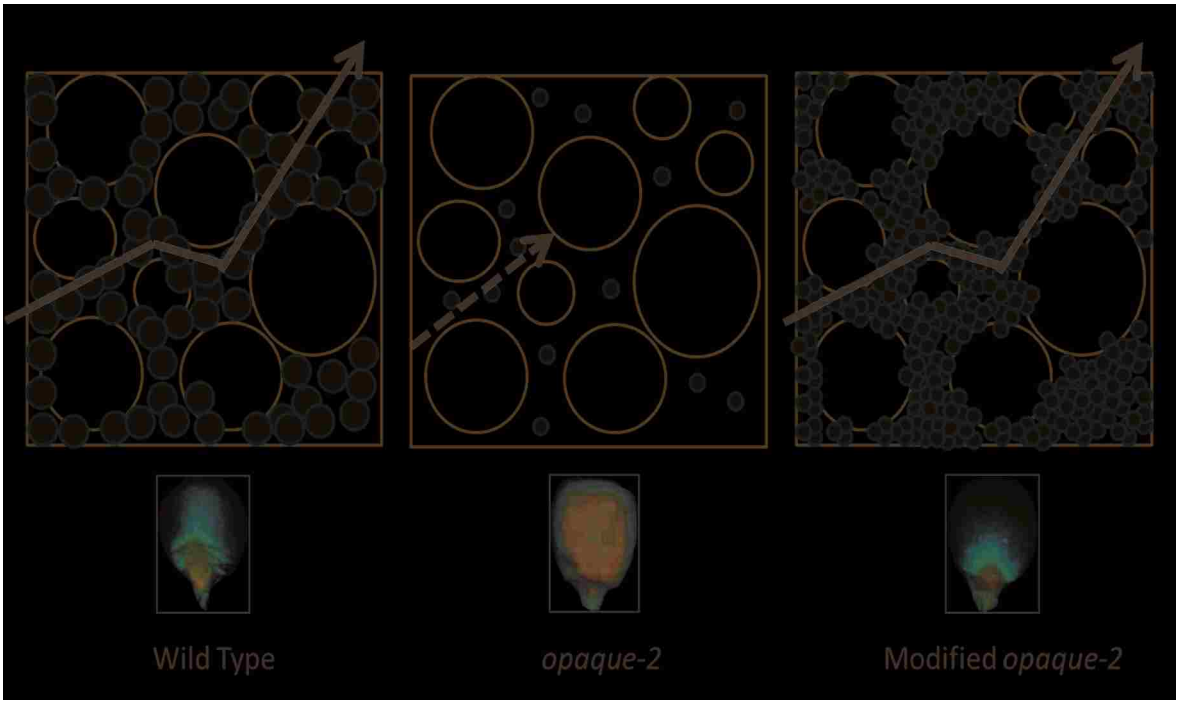

Figure 1-1 Model of maize endosperm structure (Larkins et al., 1994 and Gibbon and Larkins, 2005) The white spheres represents starch granules and the orange spheres represents protein bodies. The black solid line with an arrow represents the light that transmits through the endosperm and the dash line with an arrow represents the light that cannot transmits through the endosperm. Wild type and modified opaque-2 endosperms form a dense proteinaceous matrix, which could be a continuous medium for light transmission, resulting in vitreous phenotype; whereas opaque-2 endosperms have lower content of protein bodies and interconnections between starch granules, which produce a discontinuous matrix that disrupts the transmission of light, resulting in opaque phenotype.

Other Factors Associated with Kernel Vitreousness

Pyrophosphate-Dependent Fructose-6-Phosphate1-Phosphotransferase (PFP)

Genome-wide QTL mapping and expression analysis identified several loci and genes associated with the vitreous phenotype of QPM, including the genes encoding glucose transporter, α-subunit of pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (PFPα) Type-2c protein phosphatase, ethylene responsive element binding protein (EREBP), ethylene response-related factor 1 (ERF1) and ethylene insensitive 3-like (EIL) protein (Holding et al., 2011). Those genes were significantly up-regulated in QPM lines compared with their *o2* counterparts, which indicated that multiple biochemical pathways may be involved to influence the kernel vitreousness.

The PFPα was the only one among them that was characterized (Guo et al., 2012). The gene was mapped closed to the quantitative trait loci (QTL) on Chromosome 9 (Holding et al., 2011) and the product functioned as an ATP-independent enzyme that catalyzes a reversible reaction between fructose-6-phosphate and fructose-1,6-bisphosphate in glycolysis. PFPα was coinduced with heat shock proteins (Hsps) and may increase glycolytic flux, which regulates the redox energy balance in QPM lines. However, the underlying mechanism how PFPα affects the formation of vitreousness endosperm phenotype was not clear.

Starch

Apart from zeins and other protein factors, several studies revealed that starch biosynthesis and structure may also influence endosperm vitreousness. A homozygous recessive mutation in the *waxy1* gene (*wx1*), encoding granule bound starch synthase I

(GBSS I), disrupted the biosynthesis of amylose (Nelson and Rines, 1962). This mutant reduced the vitreousness of endosperm, while in the regions that were normally vitreous in wildtype counterparts, the endosperm remained hard. Also, proteomic analysis of *o2* and QPM lines showed that the extractability of GBSS I from QPM starch granules was elevated compared with *o2*, suggesting that the interior of QPM starch granules was more accessible to solvent (Gibbon et al., 2003). In addition, recent research on QTL mapping for *o2* modifiers showed that the *Wx1* gene was mapped on Chromosome 9 near the marker phi022. This gene is positively correlated with the kernel vitreousness and may influence the lysine and tryptophan composition in maize endosperm (Babu et al., 2015).

Mutation of *sugary-1* (*su1*), encoding an isoamylase‐type starch debranching enzyme, decreased the concentration of amylopectin and increased the concentration of highly branched glucopolysaccharide phytoglycogen (Pan and Nelson, 1984; James et al., 1995). In the ears from self-pollinated heterozygous lines (+/*su1*), around 25% of kernels had shrunken and vitreous phenotype (Dinges et al., 2001), which may result from pleiotropic effect of *sugary-1* mutation, but the underlying pathway were so far poorly understood.

ADP-glucose pyrophosphorylase (AGPase) is an enzyme involved in the formation of ADP-glucose, which is the precursor for the starch biosynthesis (Dickinson and Preiss, 1969; Stark et al., 1992). The enzyme is composed of two subunits encoded by *Shrunken-2* (*Sh2*) and *Brittle-2* (*Bt2*), respectively.

And a directed interaction between those two subunits was observed by yeast two-hybrid expression (Greene and Hannah, 1998). Double mutation of *sh2* and *bt2*

substantially decreased the activity of AGP, which resulted in a vitreous endosperm phenotype (Hannah and Nelson, 1976; Giroux and Hannah, 1994).

Scanning electron microscopy of mature maize endosperm showed that QPM starch granules formed several contacts and interconnections between one another, whereas only smooth starch granules with protein bodies embedded were observed in wildtype and *o2* endosperms (Gibbon et al., 2003).

Although both wildtype and QPM have a vitreous endosperm phenotype, the mechanism that affects their kernel vitreousness could be different. The physical connections between starch granules might be one of the essential factors that contribute the vitreous endosperm in QPM lines.

Interactions between Gene*s Encoding Starch Biosynthetic Enzymes and Zeins*

Studies showed that in bt2, o2 double mutants, RNase activity was about 2 times higher than o2 mutant alone and 10 times greater than the normal lines, suggesting that the bt2 mutant might have pleiotropic effects that increase the RNase level, which in turn may partially contribute the decrease of zein protein level (Lee and Tsai, 1984).

Further studies in maize mutants with genes encoding starch biosynthetic enzymes or zeins, such as *sh1* (*shrunken-1*), *sh2*, *bt2*, *wx1*, *ae1* (*amylose-extender-1*) and *o2*, revealed that the expression of one set of those genes may affect the expression of the other. The data indicated that those genes acted in a concerted manner and formed a metabolic network to influence the endosperm phenotype (Giroux et al., 1994). However, proteomic studies needed to be done in order to further understand the physical and functional interactions between the starch biosynthetic enzymes and storage proteins.

Amylose and Amylopectin

Starch is the major component of the maize kernel, and it provides up to 72~73% of the kernel weight (FAO, 1992). Endosperm starch in normal maize lines consists of two types: amylose and amylopectin. Amylose molecules are linear polymers of α-D-glucopyranosyl units linked by α-1,4-glycosidic bonds, but researchers also discovered some amylose slightly branched by α-1,6-linkages, but the presence of branches did not significantly affect the iodine affinities and solubility (Takeda et al., 1988). Amylopectin is a highly branched polymer with short chains of α-D-glucopyranosyl units interlinked by α-1,6-glycosidic bonds. There are three types of glucan chains: A chains are outer glucan chains without any branch, and have only one α-1,6-linkage with B chains, which are inner chain bearing at least one α-1,6-linkages; C chains are a special type of B chain with free reducing end (Mouille et al., 1996). These chains are not distributed randomly, but form cluster structures (Figure 1-2) (Myers et al., 2000).

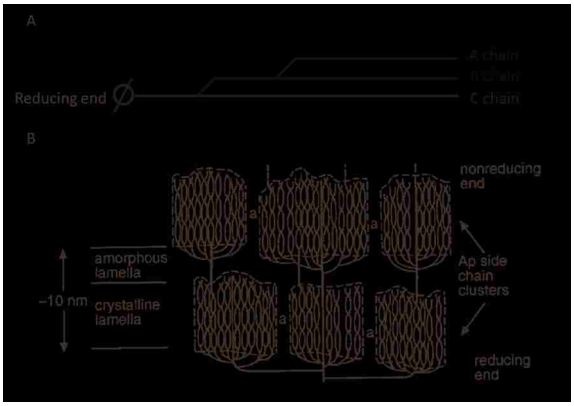

Figure 1-2 Branch types and clusters of amylopectin. A. Three types of amylopectin branch. A chain is the outer chain without any branch; B chain bear at least one branches and C chain is a special type of B chain with free reducing end. B. Branches are densely packed and form clusters structures (Myers et al., 2000).

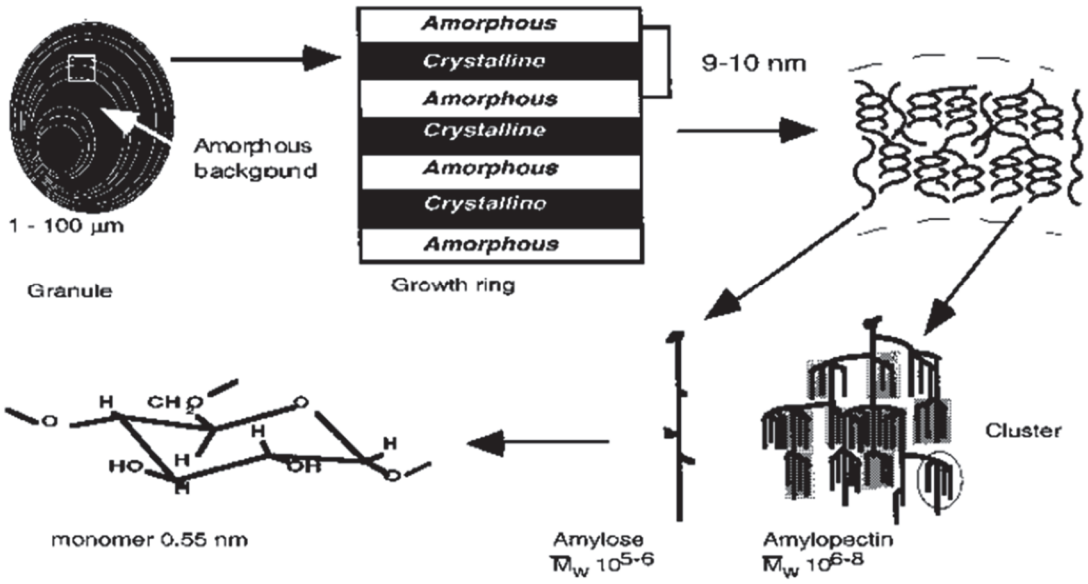

Figure 1-3 Structural Organization of the starch granule (Buleon et al., 1998) Multiple growth rings observed in starch granules are formed by alternating amorphous and crystalline lamellae. Crystalline lamellae are formed by clusters of amylopectin and single helix linear amylose, while amorphous lamellae are formed by less densely packed branches. Both amylose and amylopectin are polymers of glucopyranosyl units.

Starch Granule

Starch granules are a water-insoluble complex with semicrystalline structure formed by starch polymers (Smith, 2001). Figure 1-3 shows the structural organization of starch granules (Buleon et al., 1998). Electron microscopy studies revealed rippled fibrous structures like growth rings with alternating crystalline and amorphous lamellae (Yamaguchi et al., 1979). Crystalline lamellae are formed by tightly packed double helix side chains of amylopectin and single helix linear amylose, while amorphous lamellae are formed by less densely packed branches (Myers et al., 2000), thus amorphous lamellae are more susceptible to acid hydrolysis than crystalline lamellae (Utrilla-Coello et al., 2014). Previous data showed that CM105*o2* lines were significantly more resistant to acid

hydrolysis than CM105*mo2* QPM lines, indicating that CM105*o2* had greater proportion of crystalline lamellae in the starch granules (Figure 1-4) (Gibbon et al., unpublished data).

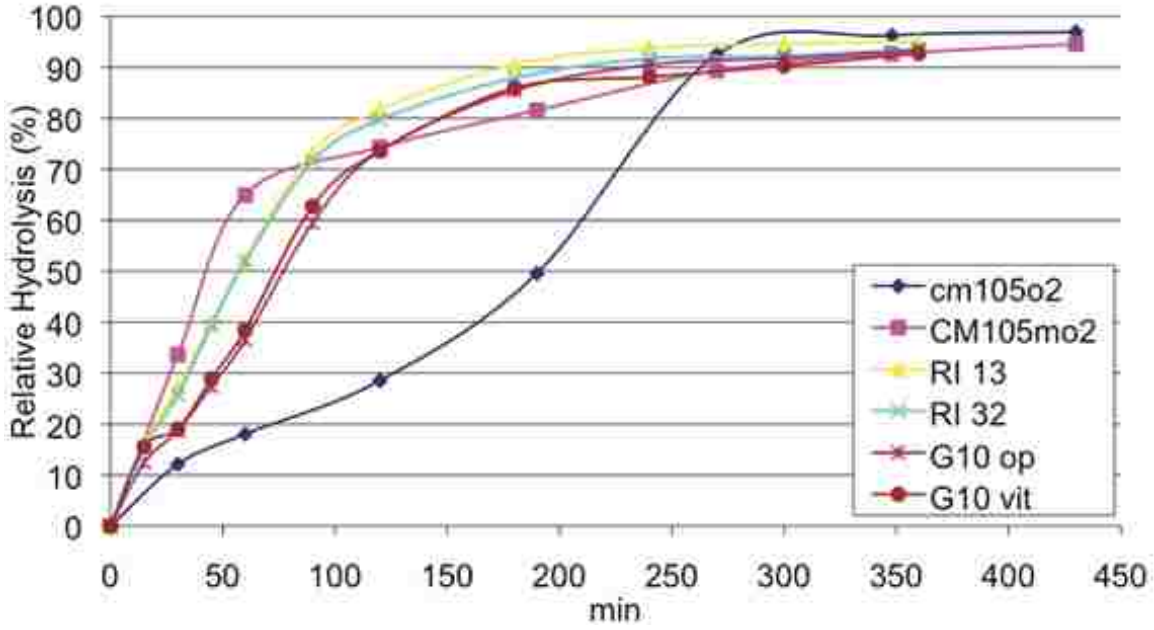

Figure 1-4 Acid Hydrolysis of Starches. (Gibbon unpublished data) Relative hydrolysis is calculated by (starting mass-insoluble mass)cX100%/(starting mass). CM105o2, RI13 and G10 op are lines with opaque kernels, and CM105mo2, RI 32 and G10 vit are lines with vitreous kernels. RI13 and RI32 are recombinant inbred lines derived from W22o2 and Pool33 QPM cross, and G10 op and G10 vit are F2 ear from W64Ao2 and K0326Y cross.

Differential Scanning Calorimetry (DSC)

Under the treatment of heat and water, the semicrystalline nature of starch granules is reduced and starch suspension becomes a viscous solution, and this process called gelatinization (Ratnayake and Jackson, 2008). Gelatinization is associated with starch thermal properties that can be analyzed by DSC, in which the process can be illustrated as a curve of endotherm (Figure 1-5) (Stevens and Elton, 1971; Liu et al., 1990). The temperature at which semicrystalline structure of starch granules begin to break is called the onset temperature, and the temperature at which starch reaches to an order-disorder

transition point with the peak endotherm is called the maximum temperature (green curve). The area between green curve and red base line is proportional to the enthalpy and is positively related to the degree of crystallinity. Also, the phase transition point can be visualized by the change of derivative of heat flow (blue curve). Since starch thermal properties are highly related with the starch structure, DSC provides an efficient way to detect the structural difference between starch granules from different lines.

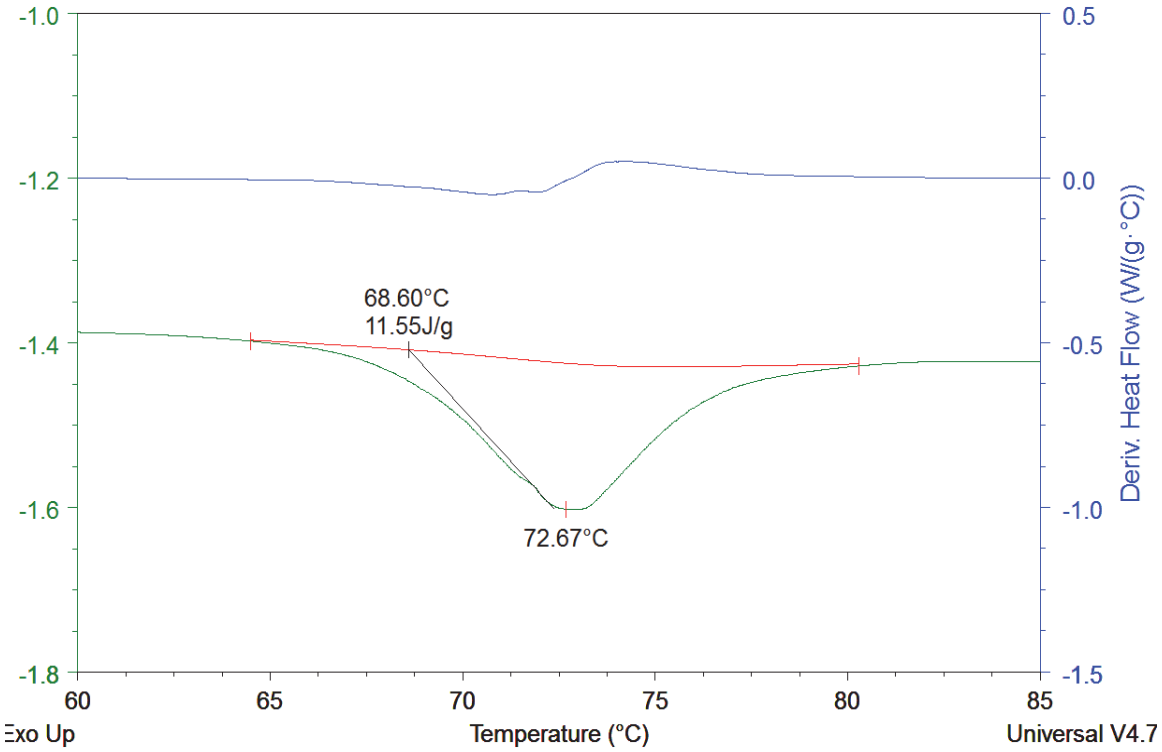

Figure 1-5 Curve of endotherm by differential scanning calorimetry. The green curve illustrate endotherm values as the temperature increase. The onset temperature is the temperature when gelatinization process begins (e.g. 68.60°C in the figure). Maximum temperature is the temperature when endotherm curve reach to a peak (e.g. 72.67°C in the figure). Enthalpy is reflected by the area between the curve of endotherm (green) and the base line (red) (e.g. 11.55J/g in the figure). Derivative of heat flow (blue curve) is used to monitor the phase transition during gelatinization process.

Starch Biosynthetic Pathway

Studies discovered two types of precursors for starch biosynthesis, uridine diphoaphate glucose (UDP-Glc) and adenosine diphoaphate glucose (ADP-Glc), and the latter is the preferred substrate for the starch biosynthetic enzymes (Recondo and Leloir, 1961). ADP-Glc in amyloplasts (organelles for starch synthesis and storage) is either translocated from cytoplasm or derived from glucose-1-phosphate (G1P) catalyzed by AGP (ADPG-PPase) (Hannah and Nelson, 1975; Wang et al., 2007).

As previously mentioned, the elongation of the α-1,4-linked glucans to produce amylose is mainly catalyzed by granule bound starch synthase I (GBSS-I) (Nelson and Rines, 1962). The biosynthesis of amylopectin involves several starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (DBE).

SSI and SSIII account for the majority of soluble starch synthase activity in developing maize kernels, and they were also found to be bound in starch granules (Cao et al., 1999; Commuri and Keeling, 2001; Grimaud et al., 2008). Studies revealed that SSI was responsible for the biosynthesis of relatively short A or B chains, but the glucan chains could not be elongated further by SSI when reach a certain threshold (DP~20) (Commuri and Keeling, 2001). SSIII was associated with the formation of longer chain and regulation of clusters (Zhu et al., 2013-1; Zhu et al., 2013-2). The *dull1* (*du1*) mutants deficient in *SSIII* generated dull kernels with reduced vitreousness, and their amylopectin had larger clusters with more singly branched building blocks, compared with their wildtype background (Gao et al., 1998; Zhu et al., 2013-2). Additionally, Coimmuno-precipitation and yeast two-hybrid analysis showed that SSIII functions as a scaffolding protein that

binds other starch biosynthetic enzymes, like SSI, SSII, BEIIa, and BEIIb, to form a protein complex (Hennen-Bierwagen et al., 2008).

SSII may also play an important role in the kernel phenotype. So far, there are two isoforms of SSII identified in maize. SSIIa is mainly expressed in endosperms, whereas SSIIb in leaves but at relatively low level (Harn et al., 1998). The *sugary-2* (*su2*) mutation resulted in SSIIa deficiency, which caused an increase in abundance of short glucan chains (DP 6-11) and a corresponding decrease in intermediate length chains (DP 12-30) (Zhang et al., 2004), indicating that SSIIa might function to produce intermediate length glucan chains. SSIIb was also discovered in maize endosperm but so far its specific role has not been characterized (James et al., 2003).

SBE catalyzes the branch formation by cleaving the α-1,4-glycosidic bond in a glucans chain and reattaching the chain with another chain by an α-1,6- glycosidic bond. In maize endosperm, there are two types of SBE, SBEI and SBEII, and SBEII has two isoforms, SBEIIa and SBEIIb (Joen et al., 2010). In vitro studies revealed that SBEI preferentially transfers longer chains (DP>10), whereas SBEII transfers smaller chains (DP 3-9) (Guan et al., 1997). However, the SBEI mutant (*sbe1*) did not show a significant difference in glucan chain length distribution compared with the wildtype background (Blauth et al., 2002), suggesting that there might be other factors in endosperm influencing the glucan chain length. SBEIIa and SBEIIb are highly similar in maize, and exhibit similar immunological properties (Liu et al., 2012; Fisher and Boyer, 1983). However, they mainly function at different locations. The mutation *sbe2* with an SBEIIa deficiency, the starch in leaves has dramatically decreased branching and the leaves showed a senescence-like phenotype, indicating that SBEIIa is mainly responsible for branching the starch in leaves

(Yandeau-Nelson et al., 2011). SBEIIb has been relatively well-characterized by recent studies. Compared with SBEIIa, SBEIIb is predominantly expressed in maize endosperm during kernel development (Makhmoudova et al., 2014). The mutation of the gene *amylose-extender* (*ae*), encoding SBEIIb, highly reduces the branching of amylopectin (Kim et al., 1998; Yao et al., 2004), suggesting that SBEIIb played an important role in amylopectin A chain formation in endosperms (Jeon et al., 2010).

Two types of DBEs have been identified in maize developing endosperm, isoamylase-type and pullulanase-type. Isoamylase hydrolyzes α-1,6-glycosidic bonds in amylopectin. The mutants of the *sugary-1* (*su1*) gene, encoding isoamylase, produced higher amount of short chains (DP 3-12) compared with their wildtype background, suggesting the isoamylase is associated with the branching chain editing of starch (Dinges et al., 2001). Pullulanase (R-enzyme or limit-dextrinase), encoded by the *Zpu1* gene, hydrolyzes the α-1,6 glycosidic bonds of pullulan, which is a linear polymer of maltotriose units produced during starch metabolism (Beatty et al., 1999). In maize, the pullulanase activity can be mainly observed in developing kernels. A null mutation of pullulanase *Zpu1-204* accumulated branched maltooligosaccharides and altered the normal starch catabolism (Dinges et al., 2003).

Figure 1-6 summarizes the starch biosynthetic pathway in maize developing endosperms (Regina et al., 2004). ADP-Glc is the preferred precursor for starch biosynthesis and it is derived by G1P via ADPG-PPase or translocated from the cytoplasm. GBSS-I is mainly involved in the production of linear amylose and several types of SSs (SSI, SSIIa, SSIIb and SSIII), SBEs (SBEI, SBEIIa and SBEIIb) and DBEs (Isoamylase

and pullulanase) participate in the synthesis of highly branched amylopectin. They function in a concerted fashion and contribute the normal formation of starch granules.

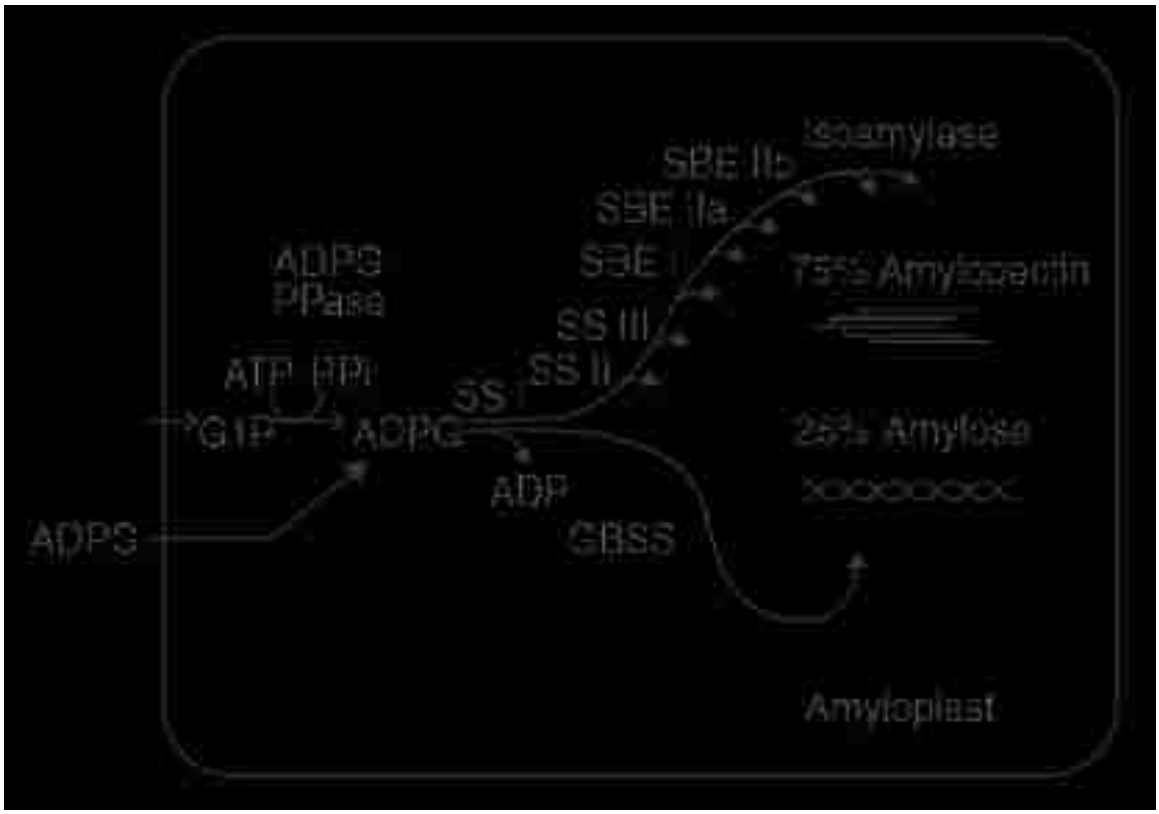

Figure 1-6 Starch biosynthetic pathway (Regina et al., 2004). ADPG is the precursor of starch biosynthesis. It's either translocated from cytoplasm or produced from G1P by ADPG-PPase. GBSS is involved in the biosynthesis of amylose and SSI, SSII, SSIII, SBEI, SBEIIa, SBEIIb, Isoamylase and Pullulanase are involved in the biosynthesis of amylopectin.

Significance and Hypothesis

The modifier genes in QPM alter the soft and starchy endosperm in *o2*, giving rise to a hard and vitreous phenotype. The starch structure, as well as the starch-protein interaction, is different between wildtype and QPM, although kernels of both wildtype and QPM are hard and vitreous. The study of modifier genes and their specific protein products is key to understand the mechanism responsible for the modification of the *o2* endosperms.

The hardness and vitreousness of maize kernels is believed to relate to many valuable traits, including resistance to mechanical damage and insects. Knowing the modifier genes and their specific functions will make the introgression of *mo2* genes much more efficient and accurate by marker-assisted selection, while maintaining the high lysine content. Also, once we get sufficient information on the *mo2* genes, it may be possible to genetically engineer plants to obtain the desired characteristics. Then the lysine content can be controlled more finely, which will improve the nutrition value of maize and benefit subsistence farmers from developing countries.

Sequence analysis of starch biosynthetic enzymes reveals that reveals that distinct alleles of four enzymes, Pullulanase, SSIII, SSIIa and SSIIb, were present in *mo2* (Gibbon et al, unpublished data). Previous data showed that pullulanase activity had positive correlation with kernel vitreousness, and the activity of pullulanase might also influenced by SSIII and other factors (Wu et al., unpublished data). Therefore, in this project, it is reasonable to hypothesize that pullulanase is one of the key factors associated with the formation of vitreous endosperm phenotype in QPM.

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CHAPTER TWO

Pullulanase and Starch Synthase III are Associated with Formation of Vitreous Endosperm in Quality Protein Maize

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Abstract

The *opaque-2* (*o2*) mutation of maize increases lysine content, but the low seed density and soft texture of this type of mutant are undesirable. Lines with modifiers of the soft kernel phenotype (*mo2*) called "Quality Protein Maize" (QPM) have high lysine and kernel phenotypes similar to normal maize. Prior research indicated that the formation of vitreous endosperm in QPM might involve changes in starch granule structure. In this study, we focused on analysis of two starch biosynthetic enzymes that may influence kernel vitreousness. Analysis of recombinant inbred lines derived from a cross of W64A*o2* and K0326Y revealed that pullulanase activity had significant positive correlation with kernel vitreousness. We also found that decreased Starch Synthase III abundance may decrease the pullulanase activity and average glucan chain length given the same *Zpu1* genotype. Therefore, Starch Synthase III could indirectly influence the kernel vitreousness by affecting pullulanase activity and coordinating with pullulanase to alter the glucan chain length distribution of amylopectin, resulting in different starch structural properties. The glucan chain length distribution had strong positive correlation with the polydispersity index of glucan chains, which was positively associated with the kernel vitreousness based

on nonlinear regression analysis. Therefore, we propose that pullulanase and Starch Synthase III are two important factors responsible for the formation of the vitreous phenotype of QPM endosperms.

Introduction

Normal maize lines cannot provide a nutritionally balanced source of protein because of the deficiency of lysine (Bhan et al., 2003). Most cereal grains contain 1.5%-2% lysine, which is less than a half of the amount required for human nutrition (Young et al., 1998). The opaque2 (o2) mutation of maize markedly changes the amino acid balance, and results in a substantial increase in the lysine content (Cromwell et al., 1967). Studies on rats and pigs showed that the o2 lines are nutritionally superior to normal lines (Young et al., 1971). However, the o2 mutation gives rise to a starchy, soft endosperm that is opaque when viewed on a light box. Previous studies discovered that the starchy (or opaque) phenotype is associated with reduced protein body size and lower amounts of 22-kD α-zein, a prolamin storage protein in the endosperm (Geetha et al., 1991; Segal et al., 2003). The o2 gene encodes a transcription factor that influences the expression of 22 kDa α-zein and many other genes (Schmidt et al., 1990; Schmidt et al., 1992). However, the cellular and biochemical mechanisms that cause the opaque phenotype are not well understood but recent evidence indicates that an important factor is the ratio and arrangement of zein protein isoforms within the substructure of protein bodies (Holding et al., 2007; Guo et al., 2013).

Despite the improvement in lysine composition and nutritional value, most o2 lines were not commercially developed, due to their low grain yields, soft, chalky endosperm, and insect susceptibility (Habben and Larkins 1995; Hasjim et al., 2009). To solve those

problems, breeders developed modified o2 lines by introgressing genes that alter the soft and opaque endosperm, producing a hard and vitreous endosperm while maintaining high lysine content of o2 lines (Vasal et al., 1980). Those genes were designated o2 modifiers (mo2), and the o2 lines with those genes are called Quality Protein Maize (QPM; Prasanna et al., 2001). However, it is time-consuming to develop QPM lines, because of the difficulty of introducing multiple mo2 loci, while simultaneously maintaining the amino acid level in kernels (Vasal et al., 1980; Prasanna et al., 2001)

Improvement of QPM would be easier if the key mechanisms by which the mo2 genes produce a hard and vitreous endosperm were characterized, but so far, the location of each modifier gene and their specific downstream effects are not well understood. Genetic mapping of mo2 revealed the linkage between a locus close to the centromere of chromosome 7 and the gene encoding 27-kD γ-zein (Lopes et al., 1995). QPMs maintain the reduced level of 22-kD α-zein as o2 mutants, but accumulate twice to three times as much 27-kD γ-zein as wild type and unmodified o2 lines (Geetha et al., 1991; Holding et al., 2008). A dominant RNAi transgene was introduced into QPM lines to eliminate the expression of the gene encoding 27-kD γ-zein, resulted in an opaque phenotype in RNAi treated QPM endosperm (Wu et al., 2010). Therefore, 27-kD γ-zein was believed to be an essential component of QPM endosperm modification associated with the vitreous phenotype. Additionally, genome-wide QTL mapping and expression analysis identified several loci and genes associated with the vitreous phenotype of QPM, including glucose transporter, α-subunit of pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (PFPα) and ethylene insensitive 3-like (EIL) protein (Holding et al.,

2011). And recent study showed that PFPα was coinduced with heat shock proteins (Hsps), which regulates the redox energy balance in QPM lines (Guo et al., 2012).

Apart from zeins, starch granule synthesis and structure may also influence the endosperm texture. Starch granules are composed of two types of glucan polymers: amylose, a linear α-1,4 linked polymer and amylopectin, a branched glucan polymer with clusters of α-1,6 linkages. The organization of amylose and amylopectin forms starch granules with alternating crystalline and amorphous lamellae (Buleon et al., 1998). A mutation of gene encoding Granule-Bound Starch Synthase I (GBSS I), encoded by waxy1 (wx1) prevents the production of amylose, resulting in partially opaque phenotype (Nelson et al., 1962). Also a mutation of the isoamylase-type starch debranching enzyme, encoded sugary 1 (su1), results in reduced activity of both isoamylase-type and pullulanase-type debranching enzymes, and produces a vitreous and shrunken endosperm (Dinges et al., 2001; Wu et al., 2002). Double mutants of shrunken2 and brittle2 decrease the activity of ADP-glucose pyrophosphorylase, causing a vitreous kernel phenotype (Hannah and Nelson, 1976). Those mutations change the pattern of starch synthesis and starch structure, which in turn alter the kernel vitreousness.

Although both wild type and QPM have vitreous kernels, the starch structure of QPM is substantially different from its wild type counterparts (Gibbon et al., 2003). Scanning electron microscopy shows that QPM starch granules form contacts between one another, which are not observed in wild type starch granules. Also, proteomic analysis of QPM lines shows an increased extractability of GBSS I from starch granules, suggesting that the interior of QPM starch granules was more accessible to solvent (Gibbon et al., 2003). Those data indicate that protein-starch interactions may be different between QPM

and wild type. Our goal in this study was to identify and analyze genes and their corresponding protein products associated with the vitreous endosperm phenotype of QPM lines. Previous sequence analysis of starch synthases (SS), starch branching enzymes (BE) and starch debranching enzymes (DBE) revealed that distinct alleles of four enzymes: pullulanase-type starch debranching enzyme (*ZPU1*); starch synthase IIa (SSIIa); starch synthase IIb (SSIIb); and starch synthase III (SSIII), are present in mo2. A population of recombinant inbred lines (RILs) was developed from K0326Y (QPM inbred line) crossed to W64Ao2, followed by seven generations of self-pollination (Holding et al., 2011). These RILs showed a broad range of phenotypes for vitreousness and allow characterization of the relationship between specific gene expression, enzyme activities, starch structure and kernel vitreousness. In this study, these RILs were used to analyze the role of pullulanase and SSIII on kernel modification. We found that the activity of pullulanase-type starch debranching enzyme was positively correlated with the kernel vitreousness and SSIII may influence pullulanase to affect starch structure and properties. Therefore, we propose that pullulanase and SSIII could play an important role in promoting formation of vitreous endosperm by altering the fine structure of starch.

Materials and Methods

Gene*tic Materials*

All maize lines in the study were grown and harvested in summer, 2011 in Elm Mott, TX. The parental lines used in the pullulanase activity assay, SSIII abundance assay, DSC and the measurement of glucan chain length distribution were W64A*o2* (an *opaque 2* mutant line in the W64A inbred, an early maturing dent developed by the Wisconsin

Agricultural Experiment Station) and K0326Y (a tropical QPM inbred line developed in South Africa by Hans Geevers) and harvested at 18 days after pollination (DAP). 14 recombinant inbred lines (RILs) were originally obtained from University of Arizona. RILs were derived from F2 kernels of a K0326Y x W64A*o2* cross, followed by seven generations of self-pollination, which manifest a broad range of soft, opaque to vitreous, hard phenotypes (Holding et al., 2011). RILs involved in the assay mentioned above were also harvested at 18 DAP. Additionally, W64A+ and the SSIII null mutant W64A*du1-M4* were harvested at 18 DAP and used as references for assays on parental lines. All developing kernels were frozen in liquid nitrogen and stored at -80^{o} C.

Full Length Zpu1 cDNA Sequencing and Sequence Alignment

For each reverse transcription reaction, isolated *Zpu1* mRNA (1µg) was mixed with 0.5 µg of Oligo(dT)15 primer and RNase-free H_2O to make final volume of 5µL. The mRNA/primer mixture was preheated at 70^{o}C for 5 min and chilled on ice for 5 min, and then mixed with reverse transcription mixture containing 4 µL of ImProm-II™ (Promega Inc. Madison, WI) 5X Reaction Buffer, 1 µL of dNTP, 1.5 µL of 50 mM MgCl2, 0.5 µL of Recombinant RNasin® Ribonuclease Inhibitor, 1 µL of ImProm-II™ Reverse Transcriptase (Promega Inc.) and 7 µL RNase-Free H_2O (to a final volume of 15 µL). Reverse transcription was performed in an S1000TM Thermal Cycler (Bio-Rad Laboratories, Inc.) with priming at 25 ˚C for 5 min, reverse transcription at 42 ˚C for 60 min and inactivation at 70 ˚C for 15 min.

Eight pairs of primers were designed via primer designing tool using Geneious (v. 5.6.4, Biomatters, Aukland, NZ) and synthesized by the Midland Certified Reagent Company (Midland, TX). The sequences are listed in Table S2-1. The PCR reaction

mixture contained 10 µL of 5X Phusion HF Buffer (New England BioLabs, Inc. Ipswich, MA), 1 µL of dNTP, 1 µL of forward and reverse primers, 0.5 µL of Phusion High-Fidelity DNA Polymerase (New England BioLabs, Inc.), 2 µL of cDNA template, 1.5 µL of DMSO and diH_2O to make final volume of 50 µL. PCR was also performed in an S1000™ Thermal Cycler for 40 cycles with pre-denaturation at 98 ˚C for 1 min, denaturation at 98 ˚C for 20s, annealing at 60 ˚C for 25s, extension at 72 ˚C for 45s, and final extension at 72 ˚C for 10 min. The PCR products were tested by 1% agarose gel electrophoresis at 120V for 30 min, and purified via Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE healthcare) following the manufacturer's manual. The purified fragments were sequenced by Macrogen Inc. (Rockville, MD). Geneious was used to assemble sequence of fragments, to align them with B73 reference sequence from MaizeGDB (http://www.maizegdb.org/) and to perform the multiple alignment between W64A+, W64A*o2* and K0326Y with the default parameter setup.

Restriction Analysis of Zpu1 Allele*s*

For each restriction reaction, 1 µg of *Zpu1* gene 3' end fragment, the PCR product by the primer *Zpu1*_3'F and *Zpu1*_3'R (S1 Table), was mixed with 0.5 µL BslI restriction enzyme, 5 µL CutSmart™ Buffer (New England BioLabs, Inc.) and diH2O to make the final volume of 50 µL. The reaction mixture was incubated at 55 ˚C for 1 hour.

Full-length SSIII ORF Sequencing and multiple alignments

Total RNA was extracted and isolated from frozen developing endosperm (18 DAP) of W64Ao2 and K0326Y following the conventional procedure according to Jia et al. (2013). The cDNA was synthesized using ImProm-II™ Reverse Transcription System

(Promega Corp. Madison, WI) according to the manufacturer's instructions. SSIII full-length ORF was synthesized in 25 μL reaction system with 5 μL of Phusion® HF Buffer, 0.5 μL of dNTP, 0.5 μL of each primer (Du1_Forward with XhoI restriction site: ACTAACCTCGAGACCCTTCTTTTCTTCCCCTTC and Du1_Reverse with EcoRI restriction site: GCACGTGAATTCTTACAATTTGGACG-CTGAAC), 0.25 μL of Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, MA), 0.75 μL of 100% DMSO, 2 μL of cDNA and up to 25 μL diH2O. The reaction condition was set as pre-denaturation 98ºC 30s, denaturation 98 ºC 10s, annealing 60 ºC 25s, extension 72 ºC 6min, final extension 72ºC 10min, and the cycle number was set as 35. The PCR product was gel purified using Illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, United Kingdom), and digested by XhoI and EcoRI (NEB, Ipswich, MA), then ligated with vector pRSET-C (Life Technologies, Grand Island, NY) digested by same set of restriction enzyme. The recombinant plasmid was transformed into 10-beta Competent *E. coli* (NEB, Ipswich, MA). Then recombinant plasmids purified from overnight culture of positive clones using EZNA® Plasmid DNA Mini Kit (Omega Bio-Tek, Inc. Norcross, GA) were sent to Macrogen Inc. (Rockville, MD) for sequencing. The primers sequences for sequencing were from Lin et al.(2012) The multiple alignments of nucleotide sequences and conceptual translations were performed using Geneious (v. 5.6.4, Biomatters, Aukland, NZ) and compared with the W64A+ SSIII sequence available at GenBank (acc. JF273457) (Lin et al.,2012). The gene sequences of the W64Ao2 and K0326Y SSIII coding regions were submitted to Genbank with the accession numbers KR350619 and KR350620, respectively.

Protein Extraction and Pullulanase Activity Assay

Pericarps and embryos of developing kernels (18 DAP) from parental lines (three independent ears) and RILs (three independent ears) were removed. Their endosperms were ground into fine powder in liquid nitrogen. The powders were weighed and suspended with kernel protein extraction buffer (50 mM Tris-Acetate solution, pH 8.0, 10 mM EDTA, and 5 mM DTT, 2 μL/mg ground powder). The crude homogenates were centrifuged at 16,000 g for 10 min at 4˚C, and the supernatants with water-soluble proteins were collected. The concentration of total water-soluble proteins was determined by NanoDrop Spectrophotmeter ND-100 (Thermo Fisher Scientific, Inc.) at 280 nm and assuming an OD of 1.0 equaled 1 mg/ml total protein in the extracts.

The kernel protein extracts were diluted with sodium acetate buffer (200 mM sodium acetate, pH 5.0 adjusted by glacial acetic acid) to make the final concentration of total water-soluble proteins 10 mg/mL and the final volume of the extract solution 50 μL. Red-pullulan (Megazyme International. Wicklow, Ireland) was used to measure the activity of pullulanase. A 2% [w/v] red-pullulan, 50 mM KCl solution and diluted kernel protein extracts were pre-equilibrated at 40 ˚C for 5 min. Then 50 μL extract was mixed with 50 μL of red pullulan solution. The mixture was incubated at 40 ˚C for 10 min for pullulanase digestion. The reaction was terminated with 125 μL of 100% ethanol to precipitate undigested red-pullulan molecules for 10 min. The precipitate was spun down at 16,000 g for 10 min at room temperature. The supernatant, containing the ethanol-soluble small dyed oligosaccharides, was collected and 80 μL of the supernatant was transferred into a 96-well plate, and absorption measured at a wavelength of 490 nm.

Western Immunoblotting and Zymogram Analysis of SSIII

Kernel protein extracts were diluted with distilled water to total protein concentration of 15 mg/mL before being loaded onto a 7.5% denaturing gel with 0.4% [w/v] SDS. SDS-PAGE was performed at room temperature for 2.5 hours at 120V. Then the gel was transferred onto nitrocellulose sheets using standard methods (Towbin et al., 1979). The antisera used in western immunoblotting were rabbit anti-SSIII at 1:2,000 dilution and mouse anti-actin (Sigma-Aldrich Co. St. Louis, MO) with 1:4,000 dilution as control. The secondary anti-sera were HRP-Goat anti-Rabbit (Life Technologies, Grand Island, NY) with 1:30,000 dilution and HRP-Goat anti-Mouse with 1:40,000 dilution (Life Technologies, Grand Island, NY). Bands were visualized using a chemiluminescence substrate on an ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ) For zymogram analysis, kernel protein extracts were diluted with distilled water to a total protein concentration of 12mg/mL before being loaded onto a 7.5% native polyacrylamide gel with 0.5% oyster glycogen. Native PAGE was performed at 4° C for 4 hours at 120V. The gel then was incubated in starch synthase reaction buffer (100 mM Bicine, pH 8.0, 0.5 M citrate, 25 mM potassium acetate, 0.5 mg/mL BSA, 5 mM ADPGlc, 5 mM 2-mercaptoethanol, and 20 mg/mL glycogen) for 36 hours at 30° C. The gel was stained with iodine solution (0.2% iodine and 2% potassium iodide in 10 mM HCl) and photographed on a light box.

Starch Granule Isolation

Starch was isolated using a small-scale wet milling procedure (Gutierrez et al., 2002). Maize kernels (5g), were covered with 0.45% [w/v] $Na_2S_2O_5$ solution to about 2.5 cm above the kernels and were incubated in a 50 ˚C water bath for 24 hours. The pericarp

and embryo were removed and the endosperms were homogenized in 10 mL of 50 mM NaCl solution for 2 min with an IKA® T-18 Basic Ultra Turrax® Homogenizer (Cole-Parmer, Vernon Hills, IL). The homogenate was filtered through 2 layers of cheesecloth to remove large debris and washed with an additional 10 mL of 50 mM NaCl. The starch granules were allowed to settle at room temperature for 3 hours. Then the supernatant was removed and the starch was washed 5 times by suspending the starch in a 5:1 ratio of 50 mM NaCl: Toluene (10 mL NaCl and 2 mL Toluene), followed by centrifugation at maximum speed in a clinical centrifuge (VWR, Radnor, PA) and the supernatant was discarded. The starch was washed once with 10 mL H_2O followed by 10 mL acetone. The purified starch was dried in a fume hood for 72 hours. The purified, dried starch was stored at room temperature before use.

Differential Scanning Calorimetry

Purified starch from 3 independent ears of each line was suspended in a 1:3 slurry with H_2O and transferred to a sample pan and weighed. The mass of suspended starch was recorded and the sample pan was sealed in a crimping device. The sample pan and a reference pan were placed on the sample pedestals of a differential scanning calorimeter (Q200, TA Instruments, New Castle, DE).

Scans were performed using a heating rate of 5°C/min from 35-95°C. The onset, peak endotherm and total enthalpy of melting were determined using the DSC built-in analysis software (TA Instruments, New Castle, DE). Statistical analysis of the data was performed using JMP (Version Pro 9.0, SAS Institute Inc., Cary, NC).

Measurements of Kernel Vitreousness

Kernel vitreousness was measured by digital image analysis as described previously (Holding et al. 2008). Kernels were mounted over holes in a black card stock mask and placed over a light box. Digital images were acquired using a digital camera with fixed shutter, aperture and ISO exposure settings, which were empirically determined to be below saturation for very vitreous kernels. The images were imported into Photoshop (Adobe Inc., San Jose, CA) or NIH ImageJ (NIH, Bethesda, MD) and the freehand selection tool was used to outline the endosperm. Average pixel intensity was recorded in arbitrary units.

Glucan Chain Length Distribution

The distribution of glucan chain length was determined by fluorescence-assisted capillary electrophoresis (FACE) as described previously (O'Shea et al., 1998). Starch was solubilized by boiling 4 mg in 200 µl 100% dimethyl sulfoxide. Then 2 µl of the solubilized starch was mixed with 38 µl 500 mM sodium acetate pH 4.4, and debranched by incubating with 2 µL of Pseudomonas isoamylase (Megazyme International. Wicklow, Ireland) at 42 ˚C overnight. Then 10 µl of the debranched starch was dried in Eppendorf Vacufuge® vacuum concentrator (Eppendorf, Hauppauge, NY) at 30 ˚C for 1 hour. Then 2 µL of 1 M sodium cyanoborohydride (Sigma-Aldrich Co., St. Louis, MO) and 2 µL of 10 % [w/v] 8-amino-1,3,6-pyrenetrisulfonic acid (APTS; Biotium, Inc., Scarborough, ON, Canada) solution made by resuspending 10 mg powdered APTS in 96 µL of 15% [v/v] acetic acid. The samples were separated on a Beckman P/ACE capillary electrophoresis instrument equipped with a laser activated fluorescence detector. The analysis software was used to extract percent peak area as a measure of the relative abundance of glucan chains.

Statistical Tests and Calculation of Polydispersity Index

The starch thermal properties, and average chain length were tested by each pair t test with the significance level $p<0.05$. Pullulanase activity and polydispersity index (PDI) for W64A+, W64Ao2 and K0326Y were tested by all pairs Tukey HSD analysis with a significance level of $p<0.05$. All correlation data was tested by ANOVA of the slope with the significance level of $p<0.05$. All above tests were performed using JMP (Version 9.0, SAS Institute Inc., Cary, NC) or SPSS (Version 16.0.0, SPSS Inc., Armonk, New York). Nonlinear regression analysis of PDI and kernel vitreousness was performed using SAS 9.2 (SAS Institute Inc. Cary, NC) with 95% confidence interval of Growth Rate.

PDI was more precisely defined as degree-of-polymerization dispersity, $Đ_X$ (Gilbert et al., 2009), which is a quantitative measurement to reflect the characteristic of degree-of-polymerization distribution. $Đ_X$ was described as the ratio of the mass-average degree of polymerization, $\bar{X}_W$, to the number-average degree of polymerization, $\bar{X}_n$. The equation is as follows:

$$Đ_X = \frac{\bar{X}_W}{\bar{X}_n} = \frac{\Sigma\left(\frac{A \times DP}{\Sigma(A \times DP)} \times DP\right)}{\frac{\Sigma(A \times DP)}{\Sigma A}}$$

Where DP represents the degree of polymerization of each linear glucan chain, and A represents the percent area, which demonstrates the frequency, corresponding to each linear glucan chain. $\bar{X}_W$ is expressed by the sum of product of weight fraction of each linear glucan chain, $\frac{\mathrm{A} \times \mathrm{DP}}{\Sigma(\mathrm{A} \times \mathrm{DP})}$, and its corresponding degree of polymerization, DP;

and $\bar{X}_n$ is expressed by the ratio of total degree of polymerization $\sum(A \times DP)$, to total percent area, $\sum A$.

Scanning Electron Microscopy

Maize endosperms were cut medial-longitudinally with a razor blade and mounted on SEM pedestals with double-sided carbon tapes. After sputter-coating with gold, the samples were observed with a JEOL JSM5410 scanning electron microscope at 25mm working distance and 10kV.

Results

Multiple Alignment and Restriction Analysis of Zpu1 Alleles

The full length mRNA of the *Zpu1* gene has 3,261 nt (acc. AF080567, Beatty et al., 1999). Full length cDNA contigs for *Zpu1* from W64A+, W64Ao2 (KP872821) and K0326Y (KP872822) were assembled from 8 overlapping RT-PCR fragments. Multiple sequence alignment of full-length *Zpu1* cDNA sequences from W64A+, W64Ao2 and K0326Y was performed using Geneious 5.6.4. The results showed that W64A+ and W64Ao2 had identical *Zpu1* alleles (W64A-derived *Zpu1* allele), while the *Zpu1* allele in K0326Y (QPM-derived *Zpu1* allele) had 4 single nucleotide polymorphisms (SNPs; Fig. 2-1A). Three of the SNPs were silent, whereas the A→C SNP at position 2864 introduced a change in amino acid sequence from alanine to proline (Fig. 2-1B). This SNP also created a new BslI restriction endonuclease site (Hsieh et al., 2000). A DNA

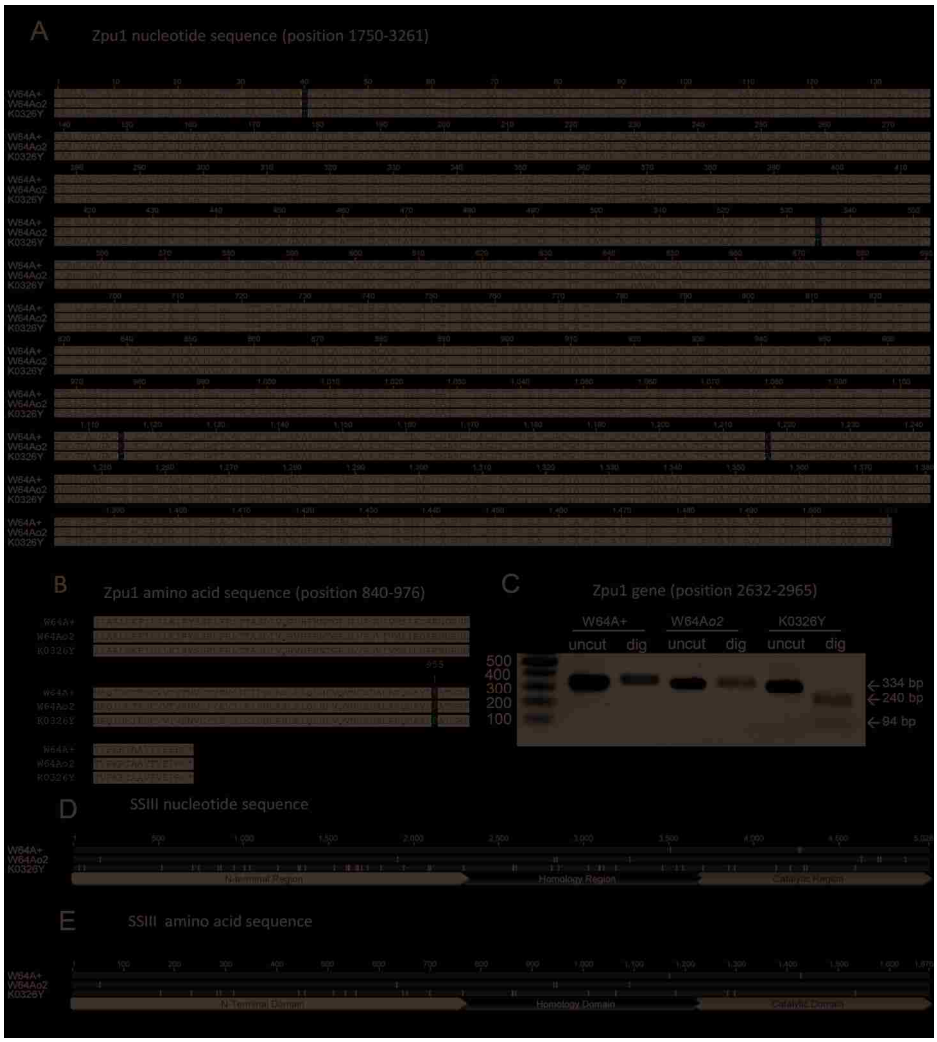

Figure 2-1. Sequence analysis of maize *Zpu1* and *SSIII* genes. (A) Multiple alignments of *Zpu1* gene sequences between W64A+, W64Ao2 and K0326Y. The highlighted positions showed four single nucleotide polymorphisms in K0326Y. (B) Multiple alignments of translated amino acid sequences of pullulanase. One amino acid difference (highlighted) was found in K0326Y due to the substitution of nucleotide from A to C at position 2864. (C) Restriction analysis of a 3' end fragment (position 2632-2965) of the *Zpu1* gene. The gel bands represent the size of fragments before (uncut) and after digestion (dig) with *Bsl*I. (D) Multiple alignments of *SSIII* gene sequences between W64A+, W64Ao2 and K0326Y. Hash marks on each sequence showed nucleotide polymorphisms. Prior studies identified three regions of the nucleotide sequences: N-terminal region (base 1-2304), homology region (base 2305-3679), and catalytic region (base 3680-5025). Full nucleotide sequence alignments are provided in Supplemental information. (E) Multiple alignments of translated amino acid sequences of SSIII, and hash marks on each sequence showed amino acid changes. Sequence annotation showed three domains of the amino acid sequences: N-terminal domain (amino acid 1-768) and homology domain (amino acid 769-1226), and catalytic domain (amino acid 1227-1674). Full protein sequence alignments are provided in Supplemental information.

fragment located at the 3' end of *Zpu1* gene (nucleotides 2632-2965) was amplified, purified and digested by BslI to confirm the presence of the SNP. The size did not change for W64A+ and W64Ao2, while K0326Y generated two fragments 240 bp and 94 bp, respectively, indicating the presence of the QPM SNP (Fig. 2-1C).

Multiple Alignment of SSIII allele*s*

The full length coding region of the *SSIII* gene from W64A*o2* (5028 bp) and K0326Y (5025 bp) were cloned into the pRSET-C vector and sequenced. Their sequences were aligned with the full length W64A+ *SSIII* cDNA sequence (acc. JF273457). W64A+ and W64A*o2* (acc. KR350619; W64A*o2*-derived *SSIII* allele) had similar *SSIII* gene sequences, except for the presence of 12 SNPs 5 of which were silent. In contrast, the K0326Y *SSIII* gene (acc KR350620; QPM-derived *SSIII* allele) showed much more variation throughout the sequence (Fig. 2-1D) with 51 SNPs and a single 3 bp deletion. The alteration of some nucleotides resulted in change in the resulting amino acid sequence. Compared with the conceptual translation of the W64A-derived *SSIII* sequence, the QPM-derived SSIII sequence had 25 amino acid substitutions and one amino acid deletion at position 654 in the N-terminal domain (Fig. 2-1E).

Pullulanase Activity and Starch Synthase III Abundance

In parental Lines, the activity of pullulanase and the abundance of SSIII were tested by quantitative enzyme activity assays and Western blots, respectively. In the parental lines of the RIL population, pullulanase enzyme activity of K0326Y was significantly higher than W64A*o2*, and W64A+ (Fig. 2-2A). The abundance of SSIII in K0326Y was significantly higher than W64A*o2* and W64A+, but W64A*o2* and W64A+ did not show

any significant difference in SSIII protein abundance; whereas *du1-M4*, an *SSIII*-null mutant, had no detectable SSIII (Fig.2-2C). SSIII enzyme activity in the parental lines was tested by a zymogram based on native PAGE, and showed that K0326Y had higher SSIII activity than W64A*o2*. W64A+ and W64A *du1-M4* did not have any detectable SSIII activity (Fig. 2-2C).

In recombinant Inbred Lines, pullulanase activity and SSIII abundance were also tested in RIL populations homozygous for either the W64A allele or the QPM allele of the *Zpu1* gene and *SSIII* gene, respectively. The pullulanase activity was influenced by both *Zpu1* gene allele itself and also by the *SSIII* gene allele. RILs homozygous for QPM-derived alleles of *Zpu1* gene and W64A-derived alleles of *SSIII* gene (Q-W) showed significantly lower pullulanase activity than others. Also, among the RILs with the same *Zpu1* allele, those homozygous for QPM-derived alleles of *SSIII* gene tended to have higher pullulanase activity than those homozygous for W64A-derived alleles of the *SSIII* gene (Fig. 2-2A). Pullulanase activity showed an approximately threefold variation, and the kernel vitreousness of the RIL population had an approximately 50-fold variation (Holding et al., 2011). The activity of pullulanase was positively correlated with kernel vitreousness (Fig. 2-2B), based on ANOVA analysis and correlation tests (R^2=0.3762, $p<0.05$), indicating that pullulanase activity could be a candidate factor that influences the vitreous phenotype. Previous studies showed that kernel vitreousness, density and hardness were correlated with one another (Holding et al., 2011). However, our data showed that the pullulanase activity was not significantly correlated with kernel density or hardness (Fig. S2-1), suggesting that other factors affect hardness and density. SSIII abundance was highly dependent on which *SSIII* allele was present in the genetic

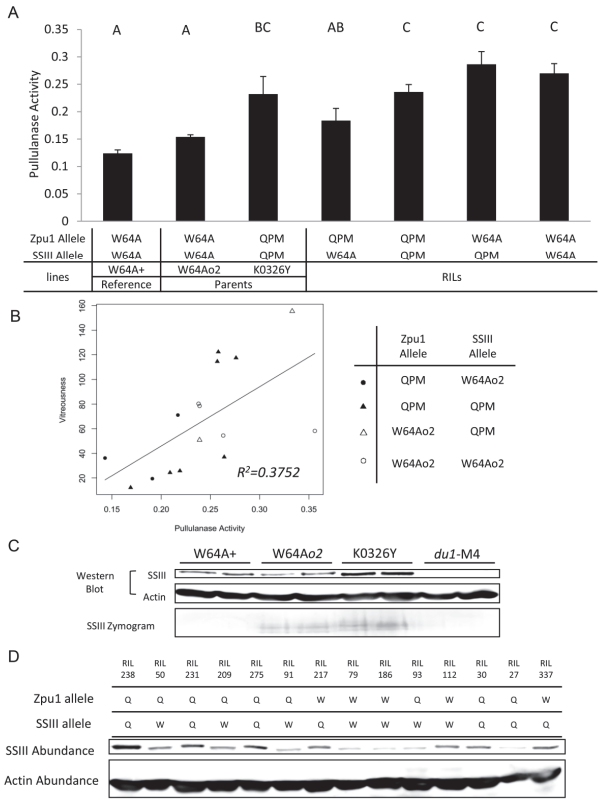

Figure 2-2. Pullulanase activity and SSIII abundance in parental lines and RILs. (A) Pullulanase activity of W64A+, W64Ao2, K0326Y and RILs with four *Zpu1*-SSIII genotypes. Pullulanase activity was measured by the spectrophotometer at 490 nm absorbance (y-axis). For the first three columns, each represents mean pullulanase activity of three independent ears of the corresponding lines; and for the remaining four columns, each represents mean pullulanase activity of all RILs with the corresponding genotype from three independent ears of each individual RIL. The letters above each column represent statistically significant differences among the lines with $p<0.05$ by pairwise two-tailed t-test. Columns with the same letter are not significantly different from one another. The error bars represent standard error. (B) Positive correlation between pullulanase activity and kernel vitreousness. The significance level of the correlation was tested by ANOVA of the slope at $p<0.05$. Each data point represents mean pullulanase activity of three independent ears of each individual RIL. (C) SSIII abundance and SSIII activity of W64A+, W64Ao2, K0326Y and *du1-M4*. SSIII abundance was tested by SDS-PAGE followed by Western blot (Images of the whole blots and SDS-PAGE gels are provided in Figure S2-2A). SSIII activity was tested by native PAGE followed by a zymogram for the parental lines. (Image of whole zymogram gel is provided in Figure S2-2B) (D) Western blot of SSIII among RILs homologous for W64Ao2 (W) or QPM (Q) – derived *Zpu1* or *SSIII* alleles. The order of samples corresponded to their kernel vitreousness, from most opaque to most vitreous (left to right; images of whole blots and SDS-PAGE gels are provided in Figure S2-3 and S2-4).

background. The abundance of SSIII was higher in RILs homozygous for the QPM allele compared to those homozygous for W64A alleles (Fig. 2-2D). However, the *Zpu1* allele in the background did not appear to have a significant effect on SSIII abundance. The order of RILs in the Figure 2-2D corresponded to their kernel vitreousness, from most opaque to most vitreous. However, the SSIII abundance did not show significant correlation with kernel vitreousness, suggesting that SSIII was not a factor directly affecting the kernel vitreousness.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to evaluate the starch gelatinization and measure the starch thermal properties (Krueger et al., 1987; Jenkins and Donald, 1995). When heated in water, the starch gelatinization can be reflected as a curve of endotherm. The temperature at which starch begins melting is called the onset temperature, and the temperature at which starch reaches to an order-disorder transition point with the peak endotherm is called the maximum temperature. The area of the curve is proportional to the enthalpy and is positively related to the degree of crystallinity (Krueger et al., 1987; Stevens and Elton, 1971).

DSC analysis of the parental lines W64Ao2 and K0326Y showed that there was no significant difference between o2 and QPM parental lines for onset and maximum endotherm, but K0326Y had significantly lower enthalpy (Fig. 2-3 A-C), indicating that QPM starch granules have lower overall crystallinity. In addition, DSC analysis of RILs with varying vitreousness showed that the thermal properties of the starch were influenced by both pullulanase and SSIII (Fig. 2-3 D-F). The onset temperature and maximum temperature of the RILs homozygous for QPM-derived *Zpu1* alleles and W64A-derived

SSIII alleles (Q-W) were significantly higher than others (Fig. 2-3 D and E). Also, among the RILs with homozygous QPM-derived *Zpu1* alleles, those homozygous for QPM-derived *SSIII* alleles (Q-Q) showed significantly lower onset and maximum temperature than those homozygous for W64A-derived *SSIII* alleles (Q-W). Among the RILs homozygous for the W64A-derived *Zpu1* alleles, those with homozygous QPM-derived *SSIII* alleles (W-Q) showed significantly higher enthalpy than those homozygous for the W64A-derived *SSIII* alleles (W-W). Likewise, among the RILs homozygous for the QPM-derived *SSIII* alleles, those homozygous for the QPM-derived *Zpu1* alleles (Q-Q) had significantly lower enthalpy than those with W64A-derived *Zpu1* alleles (W-Q). These data suggest that pullulanase and SSIII could have an interaction between one another resulting in different glucan chain length and crystallinity of starch granules, which in turn influence the starch thermal properties and starch granule structure.

Glucan Chain Length Distribution

In order to understand what factors more directly influence the kernel vitreousness, the glucan chain length distribution of debranched amylopectin from the RIL populations was measured via fluorescence-assisted capillary electrophoresis (FACE). The results showed that the average degree of polymerization (DP; the number of α-D-glucopyranosyl units in glucan chains) of glucan chains was affected by both pullulanase and SSIII. The RILs homozygous for both W64A-derived *Zpu1* alleles and QPM-derived *SSIII* alleles (W-Q) showed the highest average glucan chain length among all selected RIL samples. Also, among the RILs homozygous for W64A-derived *Zpu1* alleles, those homozygous for W64A-derived *SSIII* alleles (W-W) showed significantly

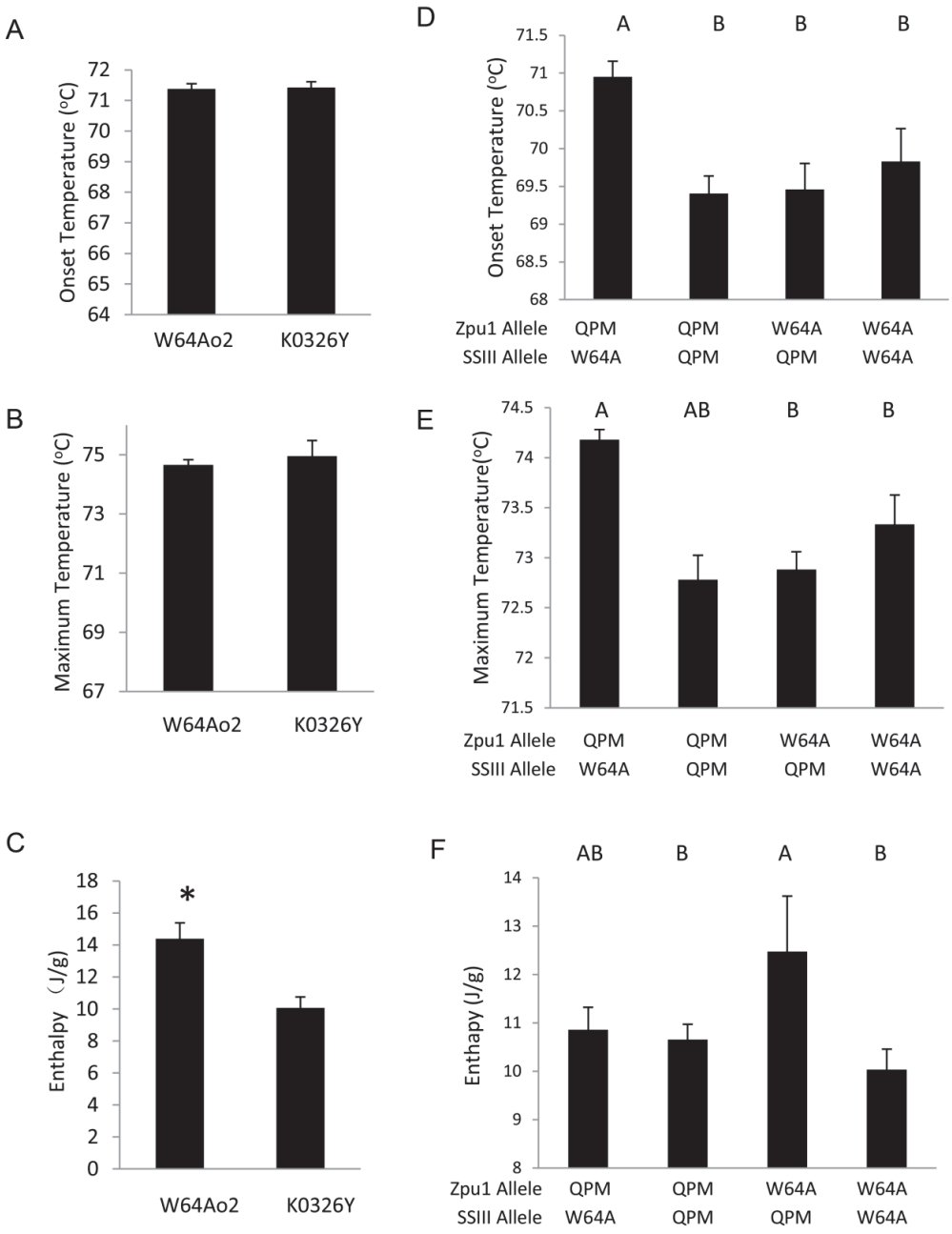

Figure 2-3 Thermal properties of starch. (A-C) Comparison of starch thermal properties, including onset temperature, maximum temperature and enthalpy, between W64Ao2 and K0326Y. Significant level indicated by asterisks at $p<0.05$ by two-tailed t test (D-F) Comparison of the starch thermal properties among RILs. For parental lines, each column represents mean thermal property values of three independent ears of corresponding lines; and for RILs, each column represents mean thermal property values of all RILs of the corresponding genotype with three independent ears for each individual RIL. The letters above each column represent statistically significant differences among the lines for $p<0.05$ by pairwise two-tailed t-test. Columns sharing the same letter are not significantly different from one another. The error bars represent standard error.

lower average glucan chain length than those homozygous for QPM-derived *SSIII* alleles (W-Q). Among the RILs homozygous for QPM-derived *SSIII* alleles, those homozygous for W64A-derived *Zpu1* alleles (W-Q) showed significantly higher average glucan chain length than those homozygous for QPM-derived *Zpu1* alleles (Q-Q or Q-W) (Fig. 2-4A). The average glucan chain length among those four types of RILs had similar trend to their enthalpy values (Fig. 2-3F), indicating the glucan chain length could influence the crystallinity of starch granule, which in turn affects the enthalpy change during gelatinization.

DP and corresponding percent peak area were used to calculate the polydispersity index (PDI). The PDI measures the width of molecular weight distributions (Rogosic et al., 1996), and the PDI of glucan chain length was highly correlated with average glucan chain length (Fig. 2-4B), making PDI a good parameter to describe the distribution of glucan chain length. The PDI analysis of parental lines showed that the PDI of K0326Y was significantly higher than that of W64Ao2 (Fig. 2-4C). The trend correlated well with the comparison of kernel vitreousness between W64A+, W64Ao2 and K0326Y (Gibbon et al., 2003). Also, the PDI of RILs showed a significant positive non-linear exponential relationship with kernel vitreousness (Fig. 2-4D), based on non-linear regression modeling using SAS 9.2 (SAS Institute Inc., Cary, NC). According to the non-linear analysis, the approximate 95% confidence interval of Growth Rate (lower 3.0167, upper 5.1769) did not include 0, indicating that the Growth Rate was greater than 0 at p-value = 0.05 level. Fig. 2-4 E-G showed the comparison of glucan chain length distribution between opaque (low vitreousness) lines and vitreous (high vitreousness) lines among parental lines and some RILs. From the figures, the curves of vitreous lines (K0326Y,

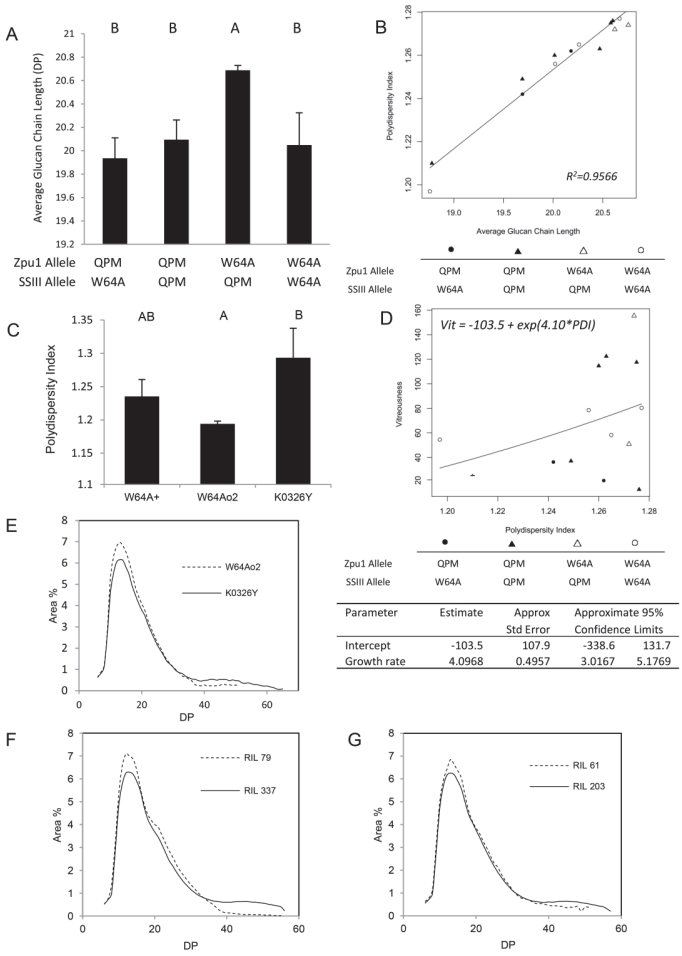

Figure 2-4 Average glucan chain length and polydispersity index (PDI). (A) Comparison of the average glucan chain length among RILs. Each column contains multiple lines homozygous for the parental alleles (W64Ao2 or QPM) of *Zpu1* or SSIII. Each column represents mean glucan chain length of all RILs of the corresponding genotype with three independent ears of each individual RIL. (B) Positive correlation between average glucan chain length and PDI. The correlations are significant, tested by ANOVA of the slope at $p<0.05$. Each data point represents mean glucan chain length of three independent ears of each individual RIL. (C) Comparison of PDI between parental lines (W64A+, W64Ao2 and K0326Y). Each column represents the mean PDI value of three independent ears of corresponding lines. The letters above each column of (A) and (C) represent statistically significant differences among the lines for $p<0.05$ by each pair t test. Columns sharing the same letter are not significantly different from one another. The error bars represent corresponding standard error. (D) Nonlinear regression analysis between PDI and kernel vitreousness among RILs, test statistics are listed below. The relationship was significantly positive, because 0 was excluded from the 95% confidence interval of Growth Rate, indicating that the Growth Rate was greater than 0 at p-value = 0.05 level. Each data point represents mean PDI of three independent ears of each individual RIL. (E-G) Glucan chain length distribution between opaque lines (dash curves) and vitreous lines (solid curves).

RIL 337 and RIL203) were flatter, with longer tail and lower peak, than corresponding opaque lines (W64Ao2, RIL 79 and RIL 61), indicating vitreous lines had higher glucan chain heterogeneity (PDI) than opaque lines, which was consistent with the relationship between vitreousness and PDI on Fig. 2-4D.

Scanning Electron Microscopy

Scanning electron microscopy was performed to analyze the gross structure and organization of starch granules in W64Ao2, K0326Y, RIL 217, RIL 27, RIL 209 and RIL 112 endosperms. The starch granules of W64Ao2 were smooth and separate, except for a small amount of matrix material surrounding them (Fig. 2-5A), whereas there were many contacts (arrows) and interconnections (asterisks) forming between adjacent starch granules in K0326Y endosperm (Fig. 2-5B). RIL 217 homozygous for the W64A-derived *Zpu1* alleles and homozygous for the QPM-derived *SSIII* alleles (W-Q) had opaque kernels, and showed smooth and separate starch granules (Fig. 2-5C), similar to W64Ao2. In contrast, RIL 27 homozygous for the QPM-derived *Zpu1* alleles and homozygous for the QPM-derived *SSIII* alleles (Q-Q) had vitreous kernels, and showed marked contacts and interconnections between starch granules (Fig. 2-5D), similar to K0326Y. In addition, RIL 209 homozygous for the QPM-derived *Zpu1* alleles and homozygous for the W64A-derived *SSIII* alleles (Q-W) had opaque kernels, and showed smooth and separate starch granules (Fig. 2-5E); Whereas RIL 112 homozygous for the W64A-derived *Zpu1* alleles and homozygous for the W64A-derived *SSIII* alleles (W-W) had semi-vitreous kernels, but showed smooth and separate starch granules (Fig. 2-5F) similar to opaque kernels.

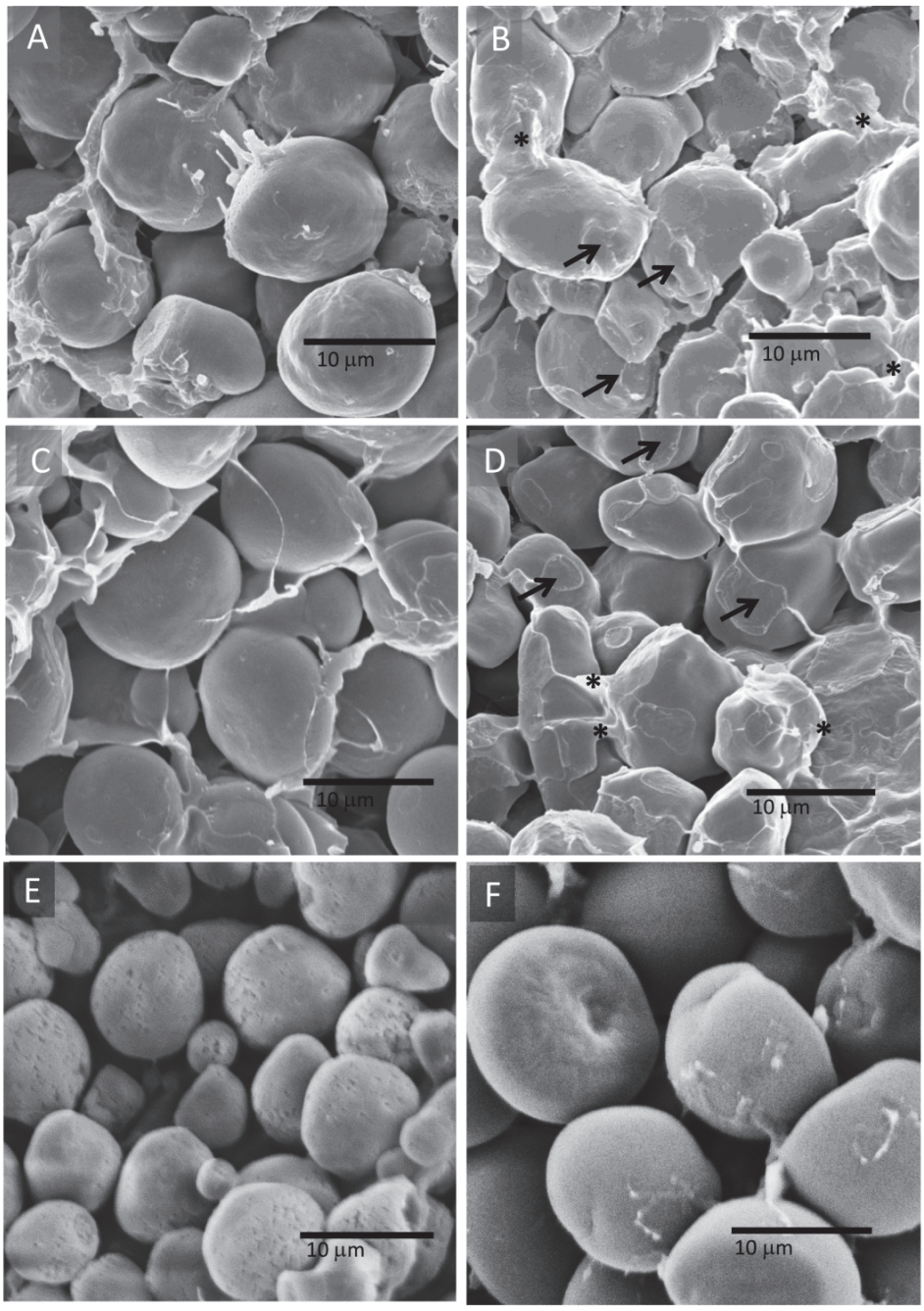

Figure 2-5 Scanning electron microscopy of starch granules from parental lines and RILs. (A) Smooth starch granules in W64Ao2 endosperm with a little matrix surrounding them. (B) Contacts (arrows) and interconnections (asterisks) formed between adjacent starch granules in K0326Y endosperm. (C) Starch granules in the endosperm of the opaque RIL 217 showed similar smooth surface as in W64Ao2. (D) Starch granules in the endosperm of RIL 27 showed similar contact and interconnection structure as in K0326Y. (E-F) Starch granules in the endosperm of RIL 209 (E) and RIL 112 (F) showed similar smooth surface as in W64Ao2.

Discussion

The modifier genes in QPM alter the soft and starchy endosperm in o2, giving rise to a hard and vitreous phenotype (Vasal et al., 1980). The study of modifier genes and their specific protein products is key to understand the mechanism responsible for the modification of the o2 endosperms. Previous sequence analysis of starch biosynthesis genes showed that SSIIa, SSIIb, SSIII and *Zpu1* were hypothesized to be the candidate genes that influence the kernel phenotypes of QPM lines. Some evidence showed that the QPM amylopectin branching pattern was most similar to the SSII mutant in maize and rice (Gibbon et al., 2003), and the SSIIa mutation (sugary2, or su2) increased the proportion of short amylopectin branches (Gibbon et al., 2003; Zhang et al., 2004). Although those starch synthases might have potential effect on kernel phenotypes, this study focused on the influence of pullulanase and SSIII on kernel vitreousness, because they were the only two enzymes that appeared to be associated with changes in vitreousness.

Pullulanase-type debranching enzymes hydrolyze the α-1,6 glycosidic bonds of pullulan, a linear polymer of maltotriose units produced during starch metabolism (Beatty et al., 1999). In maize, pullulanase activity is mainly observed in developing kernels. A null mutation of pullulanase, *Zpu1*-204, alters the normal starch catabolism and accumulates branched maltooligosaccharides, although compared with the wild type, it does not exhibit any obvious morphological differences in kernels (Dinges et al., 2003). A su1/*Zpu1*-204 double mutation, however, produces kernels with wrinkling, vitreous crown, even extending further into the central region (Dinges et al., 2003), suggesting that both pullulanase and isoamylase are involved in starch branching pattern editing and determination, and can affect the endosperm texture and kernel phenotype.

SSIII is an important starch synthase involved in glucan chain elongation and also functions as a scaffolding protein that brings together multiple components of the starch biosynthetic pathway, and evidence showed that SSIII mutants altered the branching pattern and resulted in larger amylopectin clusters (Jeon et al., 2010; Hennen-Bierwagen et al., 2008; Zhu et al., 2013). The protein binding activity was found to me mediated at the highly diverse N-terminal domain and the relatively conserved homology domain, and glucan chain length elongation was mediated by the highly conserved catalytic domain at the C-terminal region (Lin et al., 2012; Gao et al., 1998; Hennen-Bierwagen et al., 2008). The sequence analysis revealed some amino acid changes in the N-terminal domain and in the homology domain of W64Ao2. It is not clear if the polymorphisms between W64a+ and W64Ao2 are the result of new mutations arising in the many generations that the lines have been used by independent laboratories or if the W64Ao2 allele is a remnant from a parental line used for introgression of the o2 mutation into the W64A background. In contrast, the QPM-derived SSIII had amino acids altered in the catalytic domain compared with the W64A-derived SSIII (Fig. 2-1E), which could be one of other factors causing the increase of SSIII catalytic activity in K0326Y, apart from higher enzyme abundance (Fig. 2-2C). The amino acid changes in the N-terminal domain and homology domain of QPM-SSIII may influence the formation of starch biosynthetic complex by changing the binding activity to other starch biosynthetic enzymes (Fig. 2-1E). The underlying mechanisms need to be further characterized, but SSIII could be one factor that influences the endosperm texture and kernel phenotype.

This study showed that there was significant positive correlation between pullulanase activity and kernel vitreousness according the quantitative pullulanase activity

assay, as well as kernel vitreousness assays of RILs generated by crossing K0326Y QPM lines to W64Ao2 opaque lines (Fig. 2-2B). The parental lines of RILs, K0326Y and W64Ao2, showed significantly different pullulanase activity. With higher kernel vitreousness, K0326Y had higher pullulanase activity than W64Ao2 with lower kernel vitreousness (Fig. 2-2A). Multiple sequence alignment revealed that the QPM-derived *Zpu1* allele was different from the W64Ao2-derived allele in some SNPs, one of which altered the threonine to proline (Fig. 2-1A and B). Compared with threonine, proline has a unique closed ring structure making it difficult to form many of the main-chain conformations and adopt normal helical conformations (Betts and Russell, 2003), thus it is possible that the substitution might alter the pullulanase 3D structure, which in turn affects pullulanase activity.

Pullulanase activities of parental lines and RILs were compared in Fig 2-2A. The pullulanase activity of K0326Y was consistent with RILs homozygous for QPM-derived *Zpu1* allele and *SSIII* allele (Q-Q), but W64Ao2 was not consistent with RIL homozygous for W64Ao2-derived *Zpu1* allele and *SSIII* allele (W-W). Also from Fig 2B, pullulanase activity showed a quantitative trait pattern, but its allele was not mapped in one of the QTLs reported by Holding et al (2011), suggesting that there might be one or more upstream QTL factors affecting pullulanase activity. This factor(s) might be segregated during the formation of RILs, which causes the discrepancy of the pullulanase activities of W64Ao2 and W-W RILs.

In addition, SSIII also could affect the pullulanase activity. The RILs homozygous for QPM-derived *SSIII* alleles had higher SSIII abundance than those homozygous for W64Ao2-derived *SSIII* alleles (Figs. 2-2C and 2-2D), and among those RILs with same

Zpu1 genotype, those homozygous for QPM-derived *SSIII* alleles tended to have higher pullulanase activity than those homozygous for W64Ao2-derived *SSIII* alleles (Fig. 2-2A). Specifically, RILs showed in both 3rd and 4th column had QPM derived pullulanase allele (Q), but those with QPM derived *SSIII* allele (Q) have significant higher pullulanase activity than those with W64Ao2 derived *SSIII* allele (W); whereas RILs showed in last two columns had W64Ao2 derived pullulanase allele (W), although they didn't have significant difference, those with QPM derived *SSIII* allele (Q) still had a relative trend of higher pullulanase activity than those with W64Ao2 derived *SSIII* alleles (W) suggesting that high abundance of SSIII might promote the activity of pullulanase. However, there was no significant correlation between kernel vitreousness and SSIII abundance (Fig. 2-3D), indicating that SSIII might not influence the kernel vitreousness directly. The structure of amylopectin in these likes were analyzed by DSC, glucan chain length measured by FACE and the PDI was calculated based on the glucan chain length distribution. The DSC analysis of RILs showed that pullulanase and SSIII were two of the factors affecting the starch thermal properties. Data showed that RILs homozygous for the same type of *Zpu1* and *SSIII* alleles (both derived from either W64Ao2 or QPM, Q-Q or W-W) did not have significantly different onset and maximum temperature (Fig. 2-3 D and E), which was consistent with the corresponding parental data (Fig. 2-3 A and B), whereas those homozygous for different types of *Zpu1* and *SSIII* allele (Q-W or W-Q) showed significant differences (Fig. 2-3 D and E), which could be explained by a counterbalance effect between pullulanase and SSIII. Previous studies revealed that the *Zpu1* mutant reduced the amount of water-soluble polysaccharides (WSP) (Dinges et al., 2003), suggesting that higher pullulanase activity could increase the amount of WSP. Pullulanase

functions to hydrolyze α-1, 6 linkages in small, branched polysaccharides, producing short water-soluble glucans consist mainly of maltose, maltotriose, and maltotetraose (Dinges et al., 2003). These small glucans could be recycled and used as building blocks for starch chain elongation and branch formation by collaborating with SSs and SBEs. Therefore, higher pullulanase activity tends to have higher capacity to produce small glucans used to form short branches. SSIII, however, functions to elongate glucan chains, so higher the amount of SSIII tends to have higher capacity to produce long branches, which therefore might counterbalance the effect of pullulanase to some extent. In addition, the onset and maximum temperature did not show a strong correlation with average glucan chain length (Fig. 2-4A), indicating that these parameters are not only affected by glucan chain length, as many studies have shown (Fujita et al., 2006; Liu et al., 2012), but also affected by other factors, such as branching pattern, polydispersity and starch composition (Vasanthan et al., 2012).

The enthalpy of starch gelatinization reflects the starch crystallinity. This study showed that average glucan chain length had similar trend comparing with enthalpy among four types of RILs (Figs. 2-3F and 2-4A), suggesting that glucan chain length could be one of the important factors associated with the starch crystallinity, which was consistent with prior studies (Jane et al., 1999). Average glucan chain length was also depend on both pullulanase and *SSIII* alleles (Fig. 2-4A), and had strong correlation with PDI (Fig. 2-4B), which reflects the heterogeneity of glucan chains. Prior studies revealed that kernels of W64Ao2 had significantly lower vitreousness than W64A+ and K0326Y (Gibbon et al., 2003), and this trend correlated well with the PDI among parental lines (Fig. 2-4C). Similar results were observed in analysis of the RILs (Fig. 2-4D), which suggests that the

heterogeneity of glucan chains could be an important factor contributing to the kernel vitreousness.

Scanning electron microscopy analysis revealed dramatic differences in starch granules between opaque and vitreous kernels. In the study, compared with W64Ao2, RIL217 (W-Q), RIL 209 (Q-W) and RIL 112 (W-W) with smooth and separate starch granule (Fig. 2-5 A, C, E and F), both K0326Y and RIL 27 (Q-Q) had contacts and interconnections between adjacent starch granules (Fig. 2-5 B and D). These observations are consistent with previous findings using independently developed modified lines CM105o2, CM105mo2, RIL 5 (opaque kernels) and RIL 32 (vitreous kernels) (Gibbon et al., 2003). Together these data suggest that the contacts and interconnections between starch granules were associated with the formation of vitreous endosperms in QPM lines and RILs with QPM-derived *Zpu1* and *SSIII* alleles, although the formation and composition of those contacts and interconnections have not yet been fully characterized.

In conclusion, the study revealed that pullulanase activity was positively correlated with the kernel vitreousness, and SSIII could influence kernel vitreousness indirectly by affecting the pullulanase activity and by altering the starch fine structure. Data showed that pullulanase and SSIII influenced glucan chain length distribution, resulting in diverse PDI, thermal properties and starch granule surface profiles. Therefore it is possible that pullulanase and SSIII represented two factors associated with the formation of vitreous kernel phenotype. Nevertheless, more studies are needed to elucidate the mechanism of any pullulanase-SSIII interaction and the detailed mechanism for pullulanase and SSIII to promote kernel vitreousness.

Authors Contribution

Kasi Clay performed starch isolation and DSC. Stephanie S. Thompson performed RNA isolation of RILs. Dr. Tracie Hennen-Bierwagen performed FACE to analysis glucan chain length distribution. Bethany Andrews and Dr. Bernd Zechmann performed SEM. Bryan C. Gibbon is the advisor of the project and revised the article.

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Supplemental Data

1. Nucleotide sequence Alignments of *Zpu1* gene. Letters with gray background represent silent SNPs

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          1         10         20         30         40         50         60
          |         |          |          |          |          |          |
 W64A+    CCCGCCTTCTCTCTCCCTCCGAATCCAAACGCGGACGCAAATGTTGCTCCACGCCGGTCC
W64Ao2    CCCGCCTTCTCTCTCCCTCCGAATCCAAACGCGGACGCAAATGTTGCTCCACGCCGGTCC
K0326Y    CCCGCCTTCTCTCTCCCTCCGAATCCAAACGCGGACGCAAATGTTGCTCCACGCCGGTCC

 W64A+    CTCGTTCCTGCTCGCACCACCTCCGCGCTTTGCCGCCGCTCCGTCGTCAGCTTCGCCGAG
W64Ao2    CTCGTTCCTGCTCGCACCACCTCCGCGCTTTGCCGCCGCTCCGTCGTCAGCTTCGCCGAG
K0326Y    CTCGTTCCTGCTCGCACCACCTCCGCGCTTTGCCGCCGCTCCGTCGTCAGCTTCGCCGAG

 W64A+    GCGATCCAGGACACCGCAATCCTCGCCGCCGACGTCGCATTTCGCGCGCCCCGCTGATCC
W64Ao2    GCGATCCAGGACACCGCAATCCTCGCCGCCGACGTCGCATTTCGCGCGCCCCGCTGATCC
K0326Y    GCGATCCAGGACACCGCAATCCTCGCCGCCGACGTCGCATTTCGCGCGCCCCGCTGATCC

 W64A+    CGTGGCCCAAAGGGTGCGTCCCGTCGCGCCGAGGCCCCCCATGGCGACGGCGGAGGAGGG
W64Ao2    CGTGGCCCAAAGGGTGCGTCCCGTCGCGCCGAGGCCCCCCATGGCGACGGCGGAGGAGGG
K0326Y    CGTGGCCCAAAGGGTGCGTCCCGTCGCGCCGAGGCCCCCCATGGCGACGGCGGAGGAGGG

 W64A+    CGCCAGCTCTGACGTCGGCGTCGCCGTCGCCGAGTCCGCACAGGGGTTCTTGTTGGATGC
W64Ao2    CGCCAGCTCTGACGTCGGCGTCGCCGTCGCCGAGTCCGCACAGGGGTTCTTGTTGGATGC
K0326Y    CGCCAGCTCTGACGTCGGCGTCGCCGTCGCCGAGTCCGCACAGGGGTTCTTGTTGGATGC

 W64A+    GAGGGCTTACTGGGTGACAAAATCCTTGATTGCATGGAATATCAGTGATCAGAAAACTTC
W64Ao2    GAGGGCTTACTGGGTGACAAAATCCTTGATTGCATGGAATATCAGTGATCAGAAAACTTC
K0326Y    GAGGGCTTACTGGGTGACAAAATCCTTGATTGCATGGAATATCAGTGATCAGAAAACTTC

 W64A+    TCTCTTCTTATATGCAAGCAGAAATGCTACAATGTGCATGTCGAGTCAGGATATGAAAGG
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W64Ao2   TCTCTTCTTATATGCAAGCAGAAATGCTACAATGTGCATGTCGAGTCAGGATATGAAAGG
K0326Y   TCTCTTCTTATATGCAAGCAGAAATGCTACAATGTGCATGTCGAGTCAGGATATGAAAGG

 W64A+   TTATGATTCCAAAGTTGAGCTGCAACCAGAAAATGATGGACTTCCATCCAGTGTGACCCA
W64Ao2   TTATGATTCCAAAGTTGAGCTGCAACCAGAAAATGATGGACTTCCATCCAGTGTGACCCA
K0326Y   TTATGATTCCAAAGTTGAGCTGCAACCAGAAAATGATGGACTTCCATCCAGTGTGACCCA

 W64A+   GAAATTCCCTTTTATCAGCTCTTATAGAGCCTTCAGAATTCCGAGCTCCGTTGATGTTGC
W64Ao2   GAAATTCCCTTTTATCAGCTCTTATAGAGCCTTCAGAATTCCGAGCTCCGTTGATGTTGC
K0326Y   GAAATTCCCTTTTATCAGCTCTTATAGAGCCTTCAGAATTCCGAGCTCCGTTGATGTTGC

 W64A+   CACCTTGGTGAAATGTCAACTTGCTGTTGCTTCATTTGATGCTCATGGGAACAGGCAAGA
W64Ao2   CACCTTGGTGAAATGTCAACTTGCTGTTGCTTCATTTGATGCTCATGGGAACAGGCAAGA
K0326Y   CACCTTGGTGAAATGTCAACTTGCTGTTGCTTCATTTGATGCTCATGGGAACAGGCAAGA

 W64A+   TGTTACTGGGTTGCAACTACCTGGAGTATTGGATGACATGTTCGCCTACACTGGACCGCT
W64Ao2   TGTTACTGGGTTGCAACTACCTGGAGTATTGGATGACATGTTCGCCTACACTGGACCGCT
K0326Y   TGTTACTGGGTTGCAACTACCTGGAGTATTGGATGACATGTTCGCCTACACTGGACCGCT

 W64A+   TGGTACTATTTTTAGTGAAGAAGCTGTGAGTATGTACCTATGGGCTCCTACAGCACAGGA
W64Ao2   TGGTACTATTTTTAGTGAAGAAGCTGTGAGTATGTACCTATGGGCTCCTACAGCACAGGA
K0326Y   TGGTACTATTTTTAGTGAAGAAGCTGTGAGTATGTACCTATGGGCTCCTACAGCACAGGA

 W64A+   TGTAAGTGTGAGCTTCTATGATGGTCCAGCTGGCCCTTTACTGGAAACAGTTCAACTCAA
W64Ao2   TGTAAGTGTGAGCTTCTATGATGGTCCAGCTGGCCCTTTACTGGAAACAGTTCAACTCAA
K0326Y   TGTAAGTGTGAGCTTCTATGATGGTCCAGCTGGCCCTTTACTGGAAACAGTTCAACTCAA

 W64A+   CGAGTTAAATGGTGTTTGGAGTGTTACTGGTCCAAGGAACTGGGAGAACCGGTATTATCT
W64Ao2   CGAGTTAAATGGTGTTTGGAGTGTTACTGGTCCAAGGAACTGGGAGAACCGGTATTATCT
K0326Y   CGAGTTAAATGGTGTTTGGAGTGTTACTGGTCCAAGGAACTGGGAGAACCGGTATTATCT

 W64A+   ATATGAAGTCACAGTATATCATCAAACTACAGGAAACATTGAGAAATGTTTAGCCGCTGA
W64Ao2   ATATGAAGTCACAGTATATCATCAAACTACAGGAAACATTGAGAAATGTTTAGCCGCTGA
K0326Y   ATATGAAGTCACAGTATATCATCAAACTACAGGAAACATTGAGAAATGTTTAGCCGCTGA

 W64A+   TCCTTATGCTAGAGGGCTTTCTGCAAATAGCACACGAACTTGGTTGGTTGATATTAATAA
W64Ao2   TCCTTATGCTAGAGGGCTTTCTGCAAATAGCACACGAACTTGGTTGGTTGATATTAATAA
K0326Y   TCCTTATGCTAGAGGGCTTTCTGCAAATAGCACACGAACTTGGTTGGTTGATATTAATAA

 W64A+   TGAAACATTAAAGCCACTTGCCTGGGATGGATTGGCGGCTGAAAAGCCAAGGCTTGATTC
W64Ao2   TGAAACATTAAAGCCACTTGCCTGGGATGGATTGGCGGCTGAAAAGCCAAGGCTTGATTC
K0326Y   TGAAACATTAAAGCCACTTGCCTGGGATGGATTGGCGGCTGAAAAGCCAAGGCTTGATTC

 W64A+   CTTCTCTGACATAAGCATATATGAATTGCACATTCGTGATTTCAGTGCCCATGATAGCAC
W64Ao2   CTTCTCTGACATAAGCATATATGAATTGCACATTCGTGATTTCAGTGCCCATGATAGCAC
K0326Y   CTTCTCTGACATAAGCATATATGAATTGCACATTCGTGATTTCAGTGCCCATGATAGCAC

 W64A+   AGTGGACTGTCCTTTCCGAGGAGGTTTCTGTGCATTTACATTTCAGGATTCTGTAGGCAT
W64Ao2   AGTGGACTGTCCTTTCCGAGGAGGTTTCTGTGCATTTACATTTCAGGATTCTGTAGGCAT
K0326Y   AGTGGACTGTCCTTTCCGAGGAGGTTTCTGTGCATTTACATTTCAGGATTCTGTAGGCAT

 W64A+   AGAACACCTAAAGAAACTATCTGATGCCGGTTTGACTCATGTCCATTTGTTGCCAAGCTT
W64Ao2   AGAACACCTAAAGAAACTATCTGATGCCGGTTTGACTCATGTCCATTTGTTGCCAAGCTT
K0326Y   AGAACACCTAAAGAAACTATCTGATGCCGGTTTGACTCATGTCCATTTGTTGCCAAGCTT

 W64A+   TCAATTTGGTGGTGTTGATGACATAAAGAGCAATTGGAAATGTGTTGATGAGATTGAACT
W64Ao2   TCAATTTGGTGGTGTTGATGACATAAAGAGCAATTGGAAATGTGTTGATGAGATTGAACT
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K0326Y   TCAATTTGGTGGTGTTGATGACATAAAGAGCAATTGGAAATGTGTTGATGAGATTGAACT

 W64A+   GTCAAAACTCCCTCCAGGGTCAGATTTGCAACAAGCTGCAATTGTGGCTATTCAGGAAGA
W64Ao2   GTCAAAACTCCCTCCAGGGTCAGATTTGCAACAAGCTGCAATTGTGGCTATTCAGGAAGA
K0326Y   GTCAAAACTCCCTCCAGGGTCAGATTTGCAACAAGCTGCAATTGTGGCTATTCAGGAAGA

 W64A+   GGACCCTTATAATTGGGGGTATAACCCTGTGGTTTGGGGCGTTCCAAAAGGAAGCTATGC
W64Ao2   GGACCCTTATAATTGGGGGTATAACCCTGTGGTTTGGGGCGTTCCAAAAGGAAGCTATGC
K0326Y   GGACCCTTATAATTGGGGGTATAACCCTGTGGTTTGGGGCGTTCCAAAAGGAAGCTATGC

 W64A+   AAGTAACCCAGATGGTCCAAGTCGTATCATTGAGTACCGGCTGATGGTGCAGGCCTTGAA
W64Ao2   AAGTAACCCAGATGGTCCAAGTCGTATCATTGAGTACCGGCTGATGGTGCAGGCCTTGAA
K0326Y   AAGTAACCCAGATGGTCCAAGTCGTATCATTGAGTACCGGCTGATGGTGCAGGCCTTGAA

 W64A+   TCGCTTAGGTCTTCGAGTTGTCATGGATGTTGTATACAATCATCTATACTCAAGTGGCCC
W64Ao2   TCGCTTAGGTCTTCGAGTTGTCATGGATGTTGTATACAATCATCTATACTCAAGTGGCCC
K0326Y   TCGCTTAGGTCTTCGAGTTGTCATGGATGTTGTATACAATCATCTATACTCAAGTGGCCC

 W64A+   TTTTGCCATCACTTCCGTGCTTGACAAGATTGTACCTGGATACTACCTCAGAAGGGACTC
W64Ao2   TTTTGCCATCACTTCCGTGCTTGACAAGATTGTACCTGGATACTACCTCAGAAGGGACTC
K0326Y   TTTTGCCATCACTTCCGTGCTTGACAAGATTGTACCTGGATACTACCTCAGAAGGGACTC

 W64A+   TAATGGTCAGACTGAGAACAGCGCGGCTGTGAACAATACAGCAAGTGAGCATTTCATGGT
W64Ao2   TAATGGTCAGACTGAGAACAGCGCGGCTGTGAACAATACAGCAAGTGAGCATTTCATGGT
K0326Y   TAATGGTCAGACTGAGAACAGCGCGGCTGTGAACAATACAGCAAGTGAGCATTTCATGGT

 W64A+   TGATAGATTAATCGTGGATGACCTTCTGAATTGGGCAGTAAATTACAAAGTTGACGGGTT
W64Ao2   TGATAGATTAATCGTGGATGACCTTCTGAATTGGGCAGTAAATTACAAAGTTGACGGGTT
K0326Y   TGATAGATTAATCGTGGATGACCTTCTGAATTGGGCAGTAAATTACAAAGTTGACGGGTT

 W64A+   CAGATTTGATCTAATGGGACATATCATGAAAAAGACAATGATTAGAGCAAAATCGGCTCT
W64Ao2   CAGATTTGATCTAATGGGACATATCATGAAAAAGACAATGATTAGAGCAAAATCGGCTCT
K0326Y   CAGATTTGATCTAATGGGACATATCATGAAAAAGACAATGATTAGAGCAAAATCGGCTCT
                                                      1789
                                                       |
 W64A+   TCAAAGCCTTACAATTGATGAACATGGAGTAGATGGTTCAAAGATATACTTGTATGGTGA
W64Ao2   TCAAAGCCTTACAATTGATGAACATGGAGTAGATGGTTCAAAGATATACTTGTATGGTGA
K0326Y   TCAAAGCCTTACAATTGATGAACATGGAGTAGATGGTTCAAAGATATATTTGTATGGTGA

 W64A+   AGGATGGAACTTCGGTGAAGTTGCGGAAAATCAACGTGGGATAAATGGATCCCAGCTAAA
W64Ao2   AGGATGGAACTTCGGTGAAGTTGCGGAAAATCAACGTGGGATAAATGGATCCCAGCTAAA
K0326Y   AGGATGGAACTTCGGTGAAGTTGCGGAAAATCAACGTGGGATAAATGGATCCCAGCTAAA

 W64A+   TATGAGTGGCACTGGGATTGGTAGTTTCAACGATAGAATCCGTGATGCTATAAATGGTGG
W64Ao2   TATGAGTGGCACTGGGATTGGTAGTTTCAACGATAGAATCCGTGATGCTATAAATGGTGG
K0326Y   TATGAGTGGCACTGGGATTGGTAGTTTCAACGATAGAATCCGTGATGCTATAAATGGTGG

 W64A+   CAGTCCGTTTGGGAATCCACTGCAACAAGGTTTCTCTACTGGATTGTTCTTAGAGCCAAA
W64Ao2   CAGTCCGTTTGGGAATCCACTGCAACAAGGTTTCTCTACTGGATTGTTCTTAGAGCCAAA
K0326Y   CAGTCCGTTTGGGAATCCACTGCAACAAGGTTTCTCTACTGGATTGTTCTTAGAGCCAAA

 W64A+   TGGATTTTATCAGGGCAATGAAACAGAGACAAGGCTCACGCTTGCTACATACGCTGACCA
W64Ao2   TGGATTTTATCAGGGCAATGAAACAGAGACAAGGCTCACGCTTGCTACATACGCTGACCA
K0326Y   TGGATTTTATCAGGGCAATGAAACAGAGACAAGGCTCACGCTTGCTACATACGCTGACCA

 W64A+   TATACAGATTGGATTAGCTGGCAATTTGAAGGACTATGTAGTTATATCTCATACTGGAGA
W64Ao2   TATACAGATTGGATTAGCTGGCAATTTGAAGGACTATGTAGTTATATCTCATACTGGAGA
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K0326Y   TATACAGATTGGATTAGCTGGCAATTTGAAGGACTATGTAGTTATATCTCATACTGGAGA

 W64A+   AGCTAGAAAAGGATCTGAAATTCGCACCTTCGATGGCTCACCAGTTGGCTATGCTTCATC
W64Ao2   AGCTAGAAAAGGATCTGAAATTCGCACCTTCGATGGCTCACCAGTTGGCTATGCTTCATC
K0326Y   AGCTAGAAAAGGATCTGAAATTCGCACCTTCGATGGCTCACCAGTTGGCTATGCTTCATC

 W64A+   CCCTATAGAAACAATAAACTACGCCTCTGCTCATGACAATGAAACACTATTTGATATTAT
W64Ao2   CCCTATAGAAACAATAAACTACGCCTCTGCTCATGACAATGAAACACTATTTGATATTAT
K0326Y   CCCTATAGAAACAATAAACTACGCCTCTGCTCATGACAATGAAACACTATTTGATATTAT

 W64A+   TAGTCTAAAGACTCCGATGGACCTCTCAATTGACGAGCGATGCAGGATAAATCATTTGTC
W64Ao2   TAGTCTAAAGACTCCGATGGACCTCTCAATTGACGAGCGATGCAGGATAAATCATTTGTC
K0326Y   TAGTCTAAAGACTCCGATGGACCTCTCAATTGACGAGCGATGCAGGATAAATCATTTGTC
          2284
            |
 W64A+   CACAAGCATGATTGCATTATCCCAGGGAATACCATTTTTTCATGCTGGTGATGAGATACT
W64Ao2   CACAAGCATGATTGCATTATCCCAGGGAATACCATTTTTTCATGCTGGTGATGAGATACT
K0326Y   CACTAGCATGATTGCATTATCCCAGGGAATACCATTTTTTCATGCTGGTGATGAGATACT

 W64A+   ACGATCTAAGTCGCTTGATCGAGATTCATATGACTCTGGTGATTGGTTTAACAAGATTGA
W64Ao2   ACGATCTAAGTCGCTTGATCGAGATTCATATGACTCTGGTGATTGGTTTAACAAGATTGA
K0326Y   ACGATCTAAGTCGCTTGATCGAGATTCATATGACTCTGGTGATTGGTTTAACAAGATTGA

 W64A+   TTTTACCTATGAAACAAACAATTGGGGTGTTGGGCTTCCACCAAGAGAAAAGAACGAAGG
W64Ao2   TTTTACCTATGAAACAAACAATTGGGGTGTTGGGCTTCCACCAAGAGAAAAGAACGAAGG
K0326Y   TTTTACCTATGAAACAAACAATTGGGGTGTTGGGCTTCCACCAAGAGAAAAGAACGAAGG

 W64A+   GAGCTGGCCTTTGATGAAGCCAAGATTGGAGAACCCGTCGTTCAAACCTGCAAAACATGA
W64Ao2   GAGCTGGCCTTTGATGAAGCCAAGATTGGAGAACCCGTCGTTCAAACCTGCAAAACATGA
K0326Y   GAGCTGGCCTTTGATGAAGCCAAGATTGGAGAACCCGTCGTTCAAACCTGCAAAACATGA

 W64A+   CATTATTGCTGCCTTAGACAAATTTATTGATATCCTCAAGATCAGATACTCATCACCTCT
W64Ao2   CATTATTGCTGCCTTAGACAAATTTATTGATATCCTCAAGATCAGATACTCATCACCTCT
K0326Y   CATTATTGCTGCCTTAGACAAATTTATTGATATCCTCAAGATCAGATACTCATCACCTCT

 W64A+   CTTTCGCCTAACTACAGCAAGTGATATTGTGCAAAGGGTTCACTTTCACAACACAGGGCC
W64Ao2   CTTTCGCCTAACTACAGCAAGTGATATTGTGCAAAGGGTTCACTTTCACAACACAGGGCC
K0326Y   CTTTCGCCTAACTACAGCAAGTGATATTGTGCAAAGGGTTCACTTTCACAACACAGGGCC

 W64A+   CTCCTTGGTTCCAGGAGTTATTGTCATGAGCATCGAAGATGCACGAAATGATAGGCATGA
W64Ao2   CTCCTTGGTTCCAGGAGTTATTGTCATGAGCATCGAAGATGCACGAAATGATAGGCATGA
K0326Y   CTCCTTGGTTCCAGGAGTTATTGTCATGAGCATCGAAGATGCACGAAATGATAGGCATGA

 W64A+   TATGGCCCAGATAGATGAAACATTCTCTTGTGTCGTTACAGTCTTCAATGTATGTCCGTA
W64Ao2   TATGGCCCAGATAGATGAAACATTCTCTTGTGTCGTTACAGTCTTCAATGTATGTCCGTA
K0326Y   TATGGCCCAGATAGATGAAACATTCTCTTGTGTCGTTACAGTCTTCAATGTATGTCCGTA

 W64A+   CGAAGTGTCTATAGAAATCCCTGATCTTGCATCACTGCGGCTTCAGTTGCATCCAGTGCA
W64Ao2   CGAAGTGTCTATAGAAATCCCTGATCTTGCATCACTGCGGCTTCAGTTGCATCCAGTGCA
K0326Y   CGAAGTGTCTATAGAAATCCCTGATCTTGCATCACTGCGGCTTCAGTTGCATCCAGTGCA
                                                  2864
                                                    |
 W64A+   GGTGAATTCATCGGATGCGTTAGCCAGGCAGTCTGCGTACGACACCGCCACAGGTCGATT
W64Ao2   GGTGAATTCATCGGATGCGTTAGCCAGGCAGTCTGCGTACGACACCGCCACAGGTCGATT
K0326Y   GGTGAATTCATCGGATGCGTTAGCCAGGCAGTCTGCGTACGACCCCGCCACAGGTCGATT

 W64A+   CACCGTGCCGAAAAGGACAGCAGCAGTGTTCGTGGAACCCAGGTGCTGATGGATGCCTTT
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W64Ao2   CACCGTGCCGAAAAGGACAGCAGCAGTGTTCGTGGAACCCAGGTGCTGATGGATGCCTTT
K0326Y   CACCGTGCCGAAAAGGACAGCAGCAGTGTTCGTGGAACCCAGGTGCTGATGGATGCCTTT
                              2966
                               |
 W64A+   CGCTAGCGAGCAAGTGCATTCGGCATCCAAGTCGAAGCAAACGAATGAAATAAGAGAAGG
W64Ao2   CGCTAGCGAGCAAGTGCATTCGGCATCCAAGTCGAAGCAAACGAATGAAATAAGAGAAGG
K0326Y   CGCTAGCGAGCAAGTGCATTCGGCAACCAAGTCGAAGCAAACGAATGAAATAAGAGAAGG

 W64A+   CCATCGAATAAAACGAAGTATATAAATAGATTGAATAAGACGTTGCCCAAGTTGCCAAGG
W64Ao2   CCATCGAATAAAACGAAGTATATAAATAGATTGAATAAGACGTTGCCCAAGTTGCCAAGG
K0326Y   CCATCGAATAAAACGAAGTATATAAATAGATTGAATAAGACGTTGCCCAAGTTGCCAAGG

 W64A+   CACGCTTTGCCATATGTATGCGTTGAAAAATAAATAAATAAATAAATAAATAAATAAATA
W64Ao2   CACGCTTTGCCATATGTATGCGTTGAAAAATAAATAAATAAATAAATAAATAAATAAATA
K0326Y   CACGCTTTGCCATATGTATGCGTTGAAAAATAAATAAATAAATAAATAAATAAATAAATA

 W64A+   AATAAATGATGTTATAGAGGTACAAAAGCATTGGAACATTTCTTTATAGAGGTGAACCAC
W64Ao2   AATAAATGATGTTATAGAGGTACAAAAGCATTGGAACATTTCTTTATAGAGGTGAACCAC
K0326Y   AATAAATGATGTTATAGAGGTACAAAAGCATTGGAACATTTCTTTATAGAGGTGAACCAC

 W64A+   CCTATTTTCCAGTTTCCATGTGTGAATTGTGATTAGCATATGTATGGAATAATAATATAA
W64Ao2   CCTATTTTCCAGTTTCCATGTGTGAATTGTGATTAGCATATGTATGGAATAATAATATAA
K0326Y   CCTATTTTCCAGTTTCCATGTGTGAATTGTGATTAGCATATGTATGGAATAATAATATAA

 W64A+   ATTAATTTTATGCAAAAAAAA
W64Ao2   ATTAATTTTATGCAAAAAAAA
K0326Y   ATTAATTTTATGCAAAAAAAA
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2. Amino acid sequence alignment of *Zpu1* gene product

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         1        10        20        30        40        50        60
         |        |         |         |         |         |         |
 W64A+   PPSLSLRIQTRTQMLLHAGPSFLLAPPPRFAAAPSSASPRRSRTPQSSPPTSHFARPADP
W64Ao2   PPSLSLRIQTRTQMLLHAGPSFLLAPPPRFAAAPSSASPRRSRTPQSSPPTSHFARPADP
K0326Y   PPSLSLRIQTRTQMLLHAGPSFLLAPPPRFAAAPSSASPRRSRTPQSSPPTSHFARPADP

 W64A+   VAQRVRPVAPRPPMATAEEGASSDVGVAVAESAQGFLLDARAYWVTKSLIAWNISDQKTS
W64Ao2   VAQRVRPVAPRPPMATAEEGASSDVGVAVAESAQGFLLDARAYWVTKSLIAWNISDQKTS
K0326Y   VAQRVRPVAPRPPMATAEEGASSDVGVAVAESAQGFLLDARAYWVTKSLIAWNISDQKTS

 W64A+   LFLYASRNATMCMSSQDMKGYDSKVELQPENDGLPSSVTQKFPFISSYRAFRIPSSVDVA
W64Ao2   LFLYASRNATMCMSSQDMKGYDSKVELQPENDGLPSSVTQKFPFISSYRAFRIPSSVDVA
K0326Y   LFLYASRNATMCMSSQDMKGYDSKVELQPENDGLPSSVTQKFPFISSYRAFRIPSSVDVA

 W64A+   TLVKCQLAVASFDAHGNRQDVTGLQLPGVLDDMFAYTGPLGTIFSEEAVSMYLWAPTAQD
W64Ao2   TLVKCQLAVASFDAHGNRQDVTGLQLPGVLDDMFAYTGPLGTIFSEEAVSMYLWAPTAQD
K0326Y   TLVKCQLAVASFDAHGNRQDVTGLQLPGVLDDMFAYTGPLGTIFSEEAVSMYLWAPTAQD

 W64A+   VSVSFYDGPAGPLLETVQLNELNGVWSVTGPRNWENRYYLYEVTVYHQTTGNIEKCLAAD
W64Ao2   VSVSFYDGPAGPLLETVQLNELNGVWSVTGPRNWENRYYLYEVTVYHQTTGNIEKCLAAD
K0326Y   VSVSFYDGPAGPLLETVQLNELNGVWSVTGPRNWENRYYLYEVTVYHQTTGNIEKCLAAD

 W64A+   PYARGLSANSTRTWLVDINNETLKPLAWDGLAAEKPRLDSFSDISIYELHIRDFSAHDST
W64Ao2   PYARGLSANSTRTWLVDINNETLKPLAWDGLAAEKPRLDSFSDISIYELHIRDFSAHDST
K0326Y   PYARGLSANSTRTWLVDINNETLKPLAWDGLAAEKPRLDSFSDISIYELHIRDFSAHDST
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 W64A+   VDCPFRGGFCAFTFQDSVGIEHLKKLSDAGLTHVHLLPSFQFGGVDDIKSNWKCVDEIEL
W64Ao2   VDCPFRGGFCAFTFQDSVGIEHLKKLSDAGLTHVHLLPSFQFGGVDDIKSNWKCVDEIEL
K0326Y   VDCPFRGGFCAFTFQDSVGIEHLKKLSDAGLTHVHLLPSFQFGGVDDIKSNWKCVDEIEL

 W64A+   SKLPPGSDLQQAAIVAIQEEDPYNWGYNPVVWGVPKGSYASNPDGPSRIIEYRLMVQALN
W64Ao2   SKLPPGSDLQQAAIVAIQEEDPYNWGYNPVVWGVPKGSYASNPDGPSRIIEYRLMVQALN
K0326Y   SKLPPGSDLQQAAIVAIQEEDPYNWGYNPVVWGVPKGSYASNPDGPSRIIEYRLMVQALN

 W64A+   RLGLRVVMDVVYNHLYSSGPFAITSVLDKIVPGYYLRRDSNGQTENSAAVNNTASEHFMV
W64Ao2   RLGLRVVMDVVYNHLYSSGPFAITSVLDKIVPGYYLRRDSNGQTENSAAVNNTASEHFMV
K0326Y   RLGLRVVMDVVYNHLYSSGPFAITSVLDKIVPGYYLRRDSNGQTENSAAVNNTASEHFMV

 W64A+   DRLIVDDLLNWAVNYKVDGFRFDLMGHIMKKTMIRAKSALQSLTIDEHGVDGSKIYLYGE
W64Ao2   DRLIVDDLLNWAVNYKVDGFRFDLMGHIMKKTMIRAKSALQSLTIDEHGVDGSKIYLYGE
K0326Y   DRLIVDDLLNWAVNYKVDGFRFDLMGHIMKKTMIRAKSALQSLTIDEHGVDGSKIYLYGE

 W64A+   GWNFGEVAENQRGINGSQLNMSGTGIGSFNDRIRDAINGGSPFGNPLQQGFSTGLFLEPN
W64Ao2   GWNFGEVAENQRGINGSQLNMSGTGIGSFNDRIRDAINGGSPFGNPLQQGFSTGLFLEPN
K0326Y   GWNFGEVAENQRGINGSQLNMSGTGIGSFNDRIRDAINGGSPFGNPLQQGFSTGLFLEPN

 W64A+   GFYQGNETETRLTLATYADHIQIGLAGNLKDYVVISHTGEARKGSEIRTFDGSPVGYASS
W64Ao2   GFYQGNETETRLTLATYADHIQIGLAGNLKDYVVISHTGEARKGSEIRTFDGSPVGYASS
K0326Y   GFYQGNETETRLTLATYADHIQIGLAGNLKDYVVISHTGEARKGSEIRTFDGSPVGYASS

 W64A+   PIETINYASAHDNETLFDIISLKTPMDLSIDERCRINHLSTSMIALSQGIPFFHAGDEIL
W64Ao2   PIETINYASAHDNETLFDIISLKTPMDLSIDERCRINHLSTSMIALSQGIPFFHAGDEIL
K0326Y   PIETINYASAHDNETLFDIISLKTPMDLSIDERCRINHLSTSMIALSQGIPFFHAGDEIL

 W64A+   RSKSLDRDSYDSGDWFNKIDFTYETNNWGVGLPPREKNEGSWPLMKPRLENPSFKPAKHD
W64Ao2   RSKSLDRDSYDSGDWFNKIDFTYETNNWGVGLPPREKNEGSWPLMKPRLENPSFKPAKHD
K0326Y   RSKSLDRDSYDSGDWFNKIDFTYETNNWGVGLPPREKNEGSWPLMKPRLENPSFKPAKHD

W64A+   RSKSLDRDSYDSGDWFNKIDFTYETNNWGVGLPPREKNEGSWPLMKPRLENPSFKPAKHD
W64Ao2   RSKSLDRDSYDSGDWFNKIDFTYETNNWGVGLPPREKNEGSWPLMKPRLENPSFKPAKHD
K0326Y   RSKSLDRDSYDSGDWFNKIDFTYETNNWGVGLPPREKNEGSWPLMKPRLENPSFKPAKHD

 W64A+   IIAALDKFIDILKIRYSSPLFRLTTASDIVQRVHFHNTGPSLVPGVIVMSIEDARNDRHD
W64Ao2   IIAALDKFIDILKIRYSSPLFRLTTASDIVQRVHFHNTGPSLVPGVIVMSIEDARNDRHD
K0326Y   IIAALDKFIDILKIRYSSPLFRLTTASDIVQRVHFHNTGPSLVPGVIVMSIEDARNDRHD
                                                              955
                                                               |
 W64A+   MAQIDETFSCVVTVFNVCPYEVSIEIPDLASLRLQLHPVQVNSSDALARQSAYDTATGRF
W64Ao2   MAQIDETFSCVVTVFNVCPYEVSIEIPDLASLRLQLHPVQVNSSDALARQSAYDTATGRF
K0326Y   MAQIDETFSCVVTVFNVCPYEVSIEIPDLASLRLQLHPVQVNSSDALARQSAYDPATGRF

 W64A+   TVPKRTAAVFVEPRC*
W64Ao2   TVPKRTAAVFVEPRC*
K0326Y   TVPKRTAAVFVEPRC*
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3. Nucleotide sequence Alignments of *SSIII* gene

N-terminal region (base 1-2304), homology region (base 2305-3679), and catalytic region (base 3680-5025). Letters with gray background represent silent SNPs

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          1         10         20         30         40         50         60
          |         |          |          |          |          |          |
 W64A+    ATGGAGATGGTCCTACGGTCGCAGAGCCCTCTATGCCTTCGGAGTGGGCCGGTGCTCATT
W64Ao2    ATGGAGATGGTCCTACGGTCGCAGAGCCCTCTATGCCTTCGGAGTGGGCCGGTGCTCATT
K0326Y    ATGGAGATGGTCCTACGGTCGCAGAGCCCTCTCTGCCTTCGGAGTGGGCCGGTGCTCATT

 W64A+    TTTCGGCCAACCGTCGCGGGCGGAGGAGGGGGCACTCAGTCTTTGTTGAGGACTACCAGA
W64Ao2    TTTCGGCCAACCGTCGCGGGCGGAGGAGGGGGCACTCAGTCTTTGTTGAGGACTACCAGA
K0326Y    TTTCGACCAACCGTCGCGGGCGGAGGAGGGGGCACTCAGTCTTTGTTGAGGACTACCAGA

 W64A+    TTTGCGAGAAGAAGGGTCATTCGATGCGTTGTAGCAAGTCCAGGTTGTCCTAATAGGAAA
W64Ao2    TTTGCGAGAAGAAGGGTCATTCGATGCGTTGTAGCAAGTCTAGGTTGTCCTAATAGGAAA
K0326Y    TTTGCGAGAAGAAGGGTCATTCGATGCGTTGTAGCAAGTCCAGGTTGTCCTAATAGGAAA

 W64A+    TCTAGGACAGCGTCTCCCAACGTAAAAGTAGCTGCTTATAGCAACTATGCGCCAAGACTC
W64Ao2    TCTAGGACAGCGTCTCCCAACGTAAAAGTAGCTGCTTATAGCAACTATGCGCCAAGACTC
K0326Y    TCTAGGACAGCGTCTCCCAACGTAAAAGTAGCTGCTTATAGCAACTATGCGCCAAGACTC

 W64A+    CTCGTTGAGTCAAGCTCCAAGAAGAGCGAACACCATGATAGCAGCAGACACCGTGAAGAA
W64Ao2    CTCGTTGAGTCAAGCTCCAAGAAGAGCGAACACCATGATAGCAGCAGACACCGTGAAGAA
K0326Y    CTCGTTGAGTCAAGCTCCAAGAAGAGCGAACACCATGATAGCAGCAGACACCGTGAAGAA

 W64A+    ACTATTGATACATACAATGGGCTGTCAGGTTCTGATGCAGCAGAATTGACAAGTAATAGA
W64Ao2    ACTATTGATACATACAATGGGCTGTCAGGTTCTGATGCAGCAGAATTGACAAGTAATAGA
K0326Y    ACTATTGATACATACAATGGGCTGTCAGGTTCTGATGCAGCAGAATTGACAAGTAATAGA

 W64A+    GATGTAGAAATTGAAGTGGATTTGCAGCACATTTCTGAGGAGGAATTGCCAGGAAAAGTA
W64Ao2    GATGTAGAAATTGAAGTGGATTTGCAGCACATTTCTGAGGAGGAATTGCCAGGAAAAGTA
K0326Y    GATGTAGAAATTGAAGTGGATTTGCAGCACATTTCTGAGGAGGAATTGCCAGGAAAAGTA

 W64A+    TCGATTAATGCATCATTAGGAGAAATGGAAACAGTGGATGAAGCTGAGGTCGAGGAGGAT
W64Ao2    TCGATTAATGCATCATTAGGAGAAATGGAAACAGTGGATGAAGCTGAGGTCGAGGAGGAT
K0326Y    TCGATTAATGCATCATTAGGAGAAATGGAAACAGTGGATGAAGCTGAGGTCGAGGAGGAT

 W64A+    AAGTTTGAGGTAGATACCTCAGGAATTGTATTGCGCAATGCTGCAGTTCGGGAAGTGGAT
W64Ao2    AAGTTTGAGGTAGATACCTCAGGAATTGTATTGCGCAATGCTGCAGTTCGGGAAGTGGAT
K0326Y    AAGTTTGAGGTAGATACCTCAGGAATTGTATTGCGCAATGTTGCAGTTCGGGAAGTGGAT

 W64A+    CCAAAGGATGAACATAATGCTAAAGATGTATTTGTGGTAGATTCGTCAGGAACTGCACCA
W64Ao2    CCAAAGGATGAACATAATGCTAAAGATGTATTTGTGGTAGATTCGTCAGGAACTGCACCA
K0326Y    CCAAAGGATGAACATAATGCTAAAGATGTATTTGTGGTAGATTCGTCAGGAACTGCACCA

 W64A+    GATAATGCTGCAGTGGAGGAAGTGGTAGATGAAGCTGAGGTTGAAGAGGATATGGTTGAT
W64Ao2    GATAATGCTGCAGTGGAGGAAGTGGTAGATGAAGCTGAGGTTGAAGAGGATATGGTTGAT
K0326Y    GATAATGCTGCAGTGGAGGAAGTGGTAGATGAAGCTGAGGTTGAAGAGGATATGGTTGAT

 W64A+    GTGGATATCTTGGGACTTGACTTGAATAATGCAACGATGGAGGAAATTGATTTGATGGAA
W64Ao2    GTGGATATCTTGGGACTTGACTTGAATAATGCAACGATGGAGGAAATTGATTTGATGGAA
K0326Y    GTGGATATCTTGGGACTTGACTTGAATAATGCAACGATCGAGGAAATTGATTTGATGGAA

 W64A+    GAGGCTTTACTGGAGAATTTCGACGTGGATTCACCAGGCAATGCTTCTAGTGGTCGAACC
W64Ao2    GAGGCTTTACTGGAGAATTTCGACGTGGATTCACCAGGCAATGCTTCTAGTGGTCGAACC
K0326Y    GAGGCTTTACTGGAGAACTTCGACGTGGATTCACCAGGCAATGCTTCTAGTGGTCGAACC

 W64A+    TATGGGGGTGTGGATGAGTTGGGTGAGCTGCCTTCAACATCCGTGGATTGCATCGCCATT
W64Ao2    TATGGGGGTGTGGATGAGTTGGGTGAGCTGCCTTCAACATCCGTGGATTGCATCGCCATT
K0326Y    TATGGGGGTGTGGATGAGTTGGGTGAGCTGCCTTCAACATCCGTGGATTGCATCGCCATT
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 W64A+   AACGGAAAACATAGAAGTTTGAAGCCTGAGCCCTTGCCAATTGTCAGGTTCCAGGAACAA
W64Ao2   AACGGAAAACATAGAAGTTTGAAGCCTGAGCCCTTGCCAATTGTCAGGTTCCAGGAACAA
K0326Y   AACGGAAAACGTAGAAGTTTGAAGCCTAAGCCCTTGCCAATTGTCAGGTTCCAGGAACAA

 W64A+   GAACAGATAGTTTTAAGCATTGTTGACGAAGAAGGGTTGATTGCTGGTTCATGTGAAGAA
W64Ao2   GAACAGATAGTTTTAAGCATTGTTGACGAAGAAGGGTTGATTGCTGGTTCATGTGAAGAA
K0326Y   GAACAGATAGTTTTAAGCATTGTTGACGAAGAAGGGTTGATTGCTAGTTCATGTGAAGAA

 W64A+   GGCCAACCGGTGGTAGATTACGATAAGCAAGAGGAAAACTCTACAGCTTTCGATGAACAG
W64Ao2   GGCCAACCGGTGGTAGATTACGATAAGCAAGAGGAAAACTCTACAGCTTTCGATGAACAG
K0326Y   GGCCAACCGGTGGTAGATTACGATAAGCAAGAGGAAAACTCTACCGCTTTCGATGAACAG

 W64A+   AAGCAATTAACTGACGATTTCCCTGAAGAAGGCATATCTATAGTTCACTTCCCTGAGCCA
W64Ao2   AAGCAATTAACTGACGATTTCCCTGAAGAAGGCATATCTATAGTTCACTTCCCTGAGCCA
K0326Y   AAGCAATTAACTGATTGATTTCCCTGAAGAAGGCATATCTATAGTTCACTTCCCTGAGCCA

 W64A+   AACAATGATATTGTTGGATCCTCAAAATTCTTGGAGCAAAAACAAGAATTGGATGGTTCT
W64Ao2   AACAATGATATTGTTGGATCCTCAAAATTCTTGGAGCAAAAACAAGAATTGGATGGTTCT
K0326Y   AACAATGATATTGTTGGATCCTCAAAATTCTTGGAGCAAAAACAAGAATTGGATGGTTCT

 W64A+   TATAAACAAGATCGATCAACCACTGGATTGCATGAACAAGATCAGTCTGTTGTTAGTTCA
W64Ao2   TATAAACAAGATCGATCAACCACTGGATTGCATGAACAAGATCAGTCTGTTGTTAGTTCA
K0326Y   TATAAACAAGATCGATCAACCACTGGATTGCATGAACAAGATCAGTCTGTTGTTAGTTCA

 W64A+   CATGGACAAGATAAATCAATTGTTGGTGTGCCTCAGCAAATCCAGTACAATGATCAATCT
W64Ao2   CATGGACAAGATAAATCAATTGTTGGTGTGCCTCAGCAAATCCAGTACAATGATCAATCT
K0326Y   CACGGACAAGATAAATCAATTGTTGGTGTGCCTCAGCAAATCCAGTACAATGATCAATCT

 W64A+   ATTGCTGGTTCTCATAGACAAGATCAATCAATTGCCGGTGCACCTGAGCAAATCCAATCT
W64Ao2   ATTGCTGGTTCTCATAGACAAGATCAATCAATTGCCGGTGCACCTGAGCAAATCCAATCT
K0326Y   ATTGCTGGTTCTCATAGACAAGATCAATCAATTGCCGGTGCACCTGAGCAAATCCAATCC

 W64A+   GTTGCTGGCTTTATAAAACCAAATCAATCTATTGTTGGTTCTTATAAACAACATGAATTG
W64Ao2   GTTGCTGGCTTTATAAAACCAAATCAATCTATTGTTGGTTCTTATAAACAACATGAATTG
K0326Y   GTTGCTGGCTATATAAAACCAAATCAATCTATTGTTGGTTCTTGTAAACAACATGAATTG

 W64A+   ATTATTCCTGAGCCTAAGAAAATCGAATCCATCATCAGTTACAATGAAATAGATCAATCT
W64Ao2   ATTATTCCTGAGCCTAAGAAAATCGAATCCATCATCAGTTACAATGAAATAGATCAATCT
K0326Y   ATTATTCCTGAGCCTAAGAAAATCGAATCCATCATCAGTTACAATGAAATAGATCAATCT

 W64A+   ATTGTTGGTTCTCACAAACAAGACAAATCTGTTGTTAGTGTGCCTGAGCAAATCCAATCC
W64Ao2   ATTGTTGGTTCTCACAAACAAGACAAATCTGTTGTTAGTGTGCCTGAGCAAATCCAATCC
K0326Y   ATTGTTGGTTCTCACAAACAAGACAAATCTGTTGTTAGTGTGCCTGAGCAAATCCAATCC

 W64A+   ATTGTTAGTCACAGCAAACCAAATCAATCTACTATTGATTCTTATAGACAAGCTGAATCA
W64Ao2   ATTGTTAGTCACAGCAAACCAAATCAATCTACTATTGATTCTTATAGACAAGCTGAATCA
K0326Y   ATTGTTAGTCACAGCAAACCAAATCAATCTACTGTTGATTCTTATAGACAAGCTGAATCA

 W64A+   ATTATTGGTGTGCCTGAGAAAGTCCAATCCATCACCAGTTACAATAAACTAGACCAATCC
W64Ao2   ATTATTGGTGTGCCTGAGAAAGTCCAATCCATCACCAGTTACAATAAACTAGACCAATCC
K0326Y   ATTATTGGTGTGCCTGAGAAAGTCCAATCCATCACCAGTTACGATAAACTAGACCAGTCC

 W64A+   ATCGTTGGTTCTCTTAAACAAGATGAGCCTATTATTAGCGTGCATGAGAAAATTCAATCC
W64Ao2   ATCGTTGGTTCTCTTAAACAAGATGAGCCTATTATTAGCGTGCATGAGAAAATTCAATCC
K0326Y   ATTGTTGGTTCTCTTAAACAAGATGAGCCTATTATTAGCGTGCCTGAGAAAATCCAATCC
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 W64A+   ATTGTCCATTACACAAAACCAAATCAGTCTATTGTTGGCTTGCCCAAACAGCAACAATCA
W64Ao2   ATTGTCCATTACACAAAACCAAATCAGTCTATTGTTGGCTTGCCCAAACAGCAACAATCA
K0326Y   ATTGTCCATTACACTAAACCAAATCAGTCTATTGTTGGCTTGCCCAAACAACAACAATCA

 W64A+   ATTGTTCATATCGTTGAACCAAAACAGTCCATAGATGGTTTCCCTAAACAAGATCTATCA
W64Ao2   ATTGTTCATATCGTTGAACCAAAACAGTCCATAGATGGTTTCCCTAAACAAGATCTATCA
K0326Y   ATTGTTCATATCGTTGAACCAAAACAGTCCATAGATGGTTTCCCTAAACAAGATCTATCA

 W64A+   ATCGTTGGAATCTCCAATGAGTTTCAAACAAAGCAACTGGCTACTGTTGGGACTCATGAT
W64Ao2   ATCGTTGGAATCTCCAATGAGTTTCAAACAAAGCAACTGGCTACTGTTGGGACTCATGAT
K0326Y   ATCGTTGGTATCTCCAATGAGTTTCAAACAAAGCAACTGGCTACTGTTGGGACTCATGAT

 W64A+   GGATTGCTTATGAAGGGTGTGGAAGCTAAGGAGACATCTCAAAAGACTGAAGGGGATACA
W64Ao2   GGATTGCTTATGAAGGGTGTGGAAGCTAAGGAGACATCTCAAAAGATTGAAGGGGATACA
K0326Y   GGATTGCTTATGAAGGGTGTGGAAGCTAAGGAGACATCTCAAAAGACTGAAGGGGATACA

 W64A+   CTTCAGGCAACGTTCAATGCCGACAACTTGTCACAGAAACATGAGGAAGGCTTAACTAAA
W64Ao2   CTTCAGGCAACGTTCAATGCCGACAACTTGTCACAGAAACATGAGGAAGGCTTAACTAAA
K0326Y   CTTCAGGCAACGTTCAATGTCGACAACTTGTCACAGAAAC---AGGAAGGCTTAACTAAA

 W64A+   GAAGCAGACGAGATAACAATTATTGAGAAAATCAATGATGAAGACCTTGTGATGATTGAA
W64Ao2   GAAGCAGACGAGATAACAATTATTGAGAAAATCAATGATGAAGACCTTGTGATGATTGAA
K0326Y   GAAGCAGACGAGATAACAATTATTGAGAAAATCAATGATGAAGACCTTGTGATGATTGAA

 W64A+   GAACAGAAAAGCATAGCCATGAATGAAGAACAGACGATTGTTACTGAAGAAGACATTCTA
W64Ao2   GAACAGAAAAGCATAGCCATGAATGAAGAACAGACGATTGTTACTGAAGAAGACATTCTA
K0326Y   GAACAGAAAAGCATAGCCATGAATGAAGAACAGACGATTGTTACCGAAGAAGACATTCCA

 W64A+   ATGGCTAAGGTTGAGATAGGAATTGACAAGGCCAAATTTTTACATCTGCTTTCTGAAGAA
W64Ao2   ATGGCTAAGGTTGAGATAGGAATTGACAAGGCCAAATTTTTACATCTGCTTTCTGAAGAA
K0326Y   ATGGCTAAGGTTGAGATAGGAATTGACAAGGCCAAATTTTTACATCTGCTTTCTGAAGAA

 W64A+   GAGAGTTCATGGGATGAAAATGAAGTGGGAATAATTGAGGCTGATGAACAGTATGAAGTC
W64Ao2   GAGAGTTCATGGGATGAAAATGAAGTGGGAATAATTGAGGCTGATGAACAGTATGAAGTC
K0326Y   GAGAGTTCATGGGATGAAAATGAAGTGGGAATAATTGAGGCTGATGAACAGTATGAAGTC

 W64A+   GATGAGACATCTATGTCCACTGAACAAGATATCCAGGAATCACCTAATGATGATTTGGAT
W64Ao2   GATGAGACATCTATGTCCACTGAACAAGATATCCAGGAATCACCTAATGATGATTTGGAT
K0326Y   GATGAGACATCTATGTCCACTGAACAAGATATCCAGGAATCACCTAATGATGATTTGGAT

 W64A+   CCACAAGCACTACAGAGTATGCTTCAAGAGCTTGCTGAAAAAAATTATTCGCTGGGAAAC
W64Ao2   CCACAAGCACTACAGAGTATGCTTCAAGAGCTTGCTGAAAAAAATTATTCGCTGGGAAAC
K0326Y   CCACAAGCACTATGGAGTATGCTTCAAGAGCTTGCTGAAAAAAATTATTCGCTGGGAAAC

 W64A+   AAGTTGTTTACTTATCCAGATGTATTGAAAGCTGATTCAACAATTGATCTCTATTTCAAT
W64Ao2   AAGTTGTTTACTTATCCAGATGTATTGAAAGCTGATTCAACAATTGATCTCTATTTCAAT
K0326Y   AAGTTGTTTACTTATCCAGATGTATTGAAAGCTGATTCAACAATTGATCTCTATTTCAAT

 W64A+   CGTGATCTATCAGCTGTGGCCAATGAGCCTGATGTACTTATCAAAGGAGCATTCAATGGG
W64Ao2   CGTGATCTATCAGCTGTGGCCAATGAGCCTGATGTACTTATCAAAGGAGCATTCAATGGG
K0326Y   CGTGATCTATCAGCTGTGGCCAATGAGCCTGATGTACTTATCAAAGGAGCATTCAATGGG

 W64A+   TGGAAGTGGAGATTTTTCACTGAAAAATTGCACAAGAGCGAGCTGGCAGGGGACTGGTGG
W64Ao2   TGGAAGTGGAGATTTTTCACTGAAAAATTGCACAAGAGCGAGCTGGCAGGGGACTGGTGG
K0326Y   TGGAAGTGGAGATTTTTCACTGAAAAATTGCACAAGAGCGAGCTGGCAGGGGACTGGTGG

 W64A+   TGCTGCAAACTATACATTCCTAAGCAGGCATACAGAATGGACTTTGTGTTTTTTAACGGA
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W64Ao2   TGCTGCAAACTATACATTCCTAAGCAGGCATACAGAATGGACTTTGTGTTTTTTAACGGA
K0326Y   TGCTGCAAACTATACATTCCTAAGCAGGCATACAGAATGGACTTTGTGTTTTTTAACGGA

 W64A+   CGCACGATATATGAAAATAATGACAATAATGATTTCGTGATACAAATAGAAAGCACCATG
W64Ao2   CGCACGATATATGAAAATAATGACAATAATGATTTCGTGATACAAATAGAAAGCACCATG
K0326Y   CACACGGTATATGAAAATAATAACAATAATGATTTCGTGATACAAATAGAAAGCACCATG

 W64A+   GATGAAAATTTATTTGAGGATTTCTTGGCTGAAGAAAAGCAACGAGAACTTGAGAACCTT
W64Ao2   GATGAAAATTTATTTGAGGATTTCTTGGCTGAAGAAAAGCAACGAGAACTTGAGAACCTT
K0326Y   GATGAAAATTTATTTGAGGATTTCTTGGCTGAAGAAAAGCAACGAGAACTTGAGAACCTT

 W64A+   GCAAATGAGGAAGCTGAAAGGAGGAGACAAACTGATGAGCAGCGGCGAATGGAGGAAGAA
W64Ao2   GCAAATGAGGAAGCTGAAAGGAGGAGACAAACTGATGAGCAGCGGCGAATGGAGGAAGAA
K0326Y   GCAAATGAGGAAGCTGAAAGGAGGAGACAAACTGATGAGCAGCGGCGAATGGAGGAAGAA

 W64A+   AGGGCCGCAGATAAAGCTGACAGGGTACAAGCCAAGGTTGAGGTAGAAACAAAGAAGAAT
W64Ao2   AGGGCCGCAGATAAAGCTGACAGGGTACAAGCCAAGGTTGAGGTAGAAACAAAGAAGAAT
K0326Y   AGGGCCGCAGATAAAGCTGACAGGGTACAAGCCAAGGTTGAGGTAGAGACGAAGAAGAAT

 W64A+   AAATTGTGCAATGTATTGGGTTTAGCCAGAGCCCCTGTTGATAATTTATGGTACATTGAG
W64Ao2   AAATTGCGCAATGTATTGGCTTTAGCCAGAGCCCCTGTTGATAATTTATGGTACATTGAG
K0326Y   AAATTGTGCAATGTATTGGGTTTAGCCAGAGCTCCTGTTGATAATTTATGGTACATTGAG

 W64A+   CCCATCACGACTGGACAAGAGGCTACTGTCAGATTGTATTATAACATAAACTCAAGACCT
W64Ao2   CCCATCACGACTGGACAAGAGGCTACTGTCAGATTGTATTATAACATAAACTCAAGACCT
K0326Y   CCCATCACGACTGGACAAGAGGCTACTGTCAGATTGTATTATAACATAAACTCAAGACCT

 W64A+   CTAGTTCACAGTACTGAGATATGGATGCATGGTGGCTATAACAATTGGATTGATGGACTC
W64Ao2   CTAGTTCACAGTACTGAGATATGGATGCATGGTGGCTATAACAATTGGATTGATGGACTC
K0326Y   CTAGTTCACAGTACTGAGATATGGATGCATGGTGGCTATAACAATTGGATTGATGGACTC

 W64A+   TCTTTTGCTGAAAGGCTTGTTCATCATAATGACAAAGATTGTGATTGGTGGTTTGCAGAT
W64Ao2   TCTTTTGCTGAAAGGCTTGTTCATCATAATGACAAAGATTGTGATTGGTGGTTTGCAGAT
K0326Y   TCTTTTGCTGAAAGGCTTGTTCATCATCATGACAAAGATTGTGATTGGTGGTTTGCAGAT

 W64A+   GTTGTCGTGCCTGAAAGAACATATGTGTTGGATTGGGTTTTTGCTGACGGCCCGCCAGGG
W64Ao2   GTTGTCGTGCCTGAAAGAACATATGTGTTGGATTGGGTTTTTGCTGACGGCCCGCCAGGG
K0326Y   GTTGTCGTGCCTGAAAGAACATATGTATTGGACTGGGTTTTTGCTGACGGCCCACCAGGG

 W64A+   AGTGCAAGGAATTATGACAACAATGGAGGACATGATTTTCATGCTACCCTTCCAAATAAC
W64Ao2   AGTGCAAGGAATTATGACAACAATGGAGGACATGATTTTCATGCTACCCTTCCAAATAAC
K0326Y   AGTGCAAGGAATTATGACAACAATGGAGGACATGATTTTCATGCTACCCTTCCAAATAAC

 W64A+   ATGACTGATGAAGAGTATTGGATGGAAGAAGAACAAAGGATCTATACAAGGCTTCAACAA
W64Ao2   ATGACTGATGAAGAGTATTGGATGGAAGAAGAACAAAGGATCTATACAAGGCTTCAACAA
K0326Y   ATGACTGAGGAAGAGTATTGGATGGAAGAAGAACAAAGGATCTATACAAGGCTTCAACAA

 W64A+   GAGAGGAGGGAAAGGGAGGAGGCTATTAAAAGGAAGGCTGAGAGAAATGCAAAAATGAAA
W64Ao2   GAGAGGAGGGAAAGGGAGGAGGCTATTAAAAAGAAGGCTGAGAGAAATGCAAAAATGAAA
K0326Y   GAGAGGAGGGAAAGGGAGGAGGCTATTAAAAGGAAGGCTGAGAGAAATGCAAAAATGAAA

 W64A+   GCTGAGATGAAGGAAAAGACTATGAGAATGTTCCTGGTTTCTCAGAAACACATTGTTTAC
W64Ao2   GCTGAGATGAAGGAAAAGACTATGAGAATGTTCCTGGTTTCTCAGAAACACATTGTTTAC
K0326Y   GCTGAGATGAAGGAAAAGACTATGAGAATGTTCCTGGTTTCTCAGAAACACATTGTTTAC

 W64A+   ACCGAACCACTTGAAATACATGCTGGAACTACTATTGATGTGCTTTATAATCCTTCTAAT
W64Ao2   ACCGAACCACTTGAAATACATGCTGGAACTACTATTGATGTGCTTTATAATCCTTCTAAT
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K0326Y   ACCGAACCACTTGAAATACATGCTGGAACTACTATTGATGTGCTTTATAATCCTTCTAAT

 W64A+   ACAGTTCTAACTGGAAAGCCAGAGGTTTGGTTTCGATGTTCCTTTAATCGTTGGATGTAT
W64Ao2   ACAGTTCTAACTGGAAAGCCAGAGGTTTGGTTTCGATGTTCCTTTAATCGTTGGATGTAT
K0326Y   ACAGTTCTAACTGGAAAGCCAGAGGTTTGGTTTCGATGTTCATTTAATCGTTGGATGTAT

 W64A+   CCAGGTGGGGTGTTGCCACCTCAGAGGATGGTACAAGCAGAAAATGGTTCACACCTAAAA
W64Ao2   CCAGGTGGGGTGTTGCCACCTCAGAAGATGGTACAAGCAGAAAATGGTTCACACCTAAAA
K0326Y   CCAGGTGGGGTGTTGCCACCTCAGAAGATGGTACAAGCAGAAAATGGTTCACACCTAAAA

 W64A+   GCAACAGTTTACGTTCCACGAGATGCCTATATGATGGACTTCGTTTTCTCGGAGTCAGAA
W64Ao2   GCAACAGTTTACGTTCCACGAGATGCCTATATGATGGACTTCGTTTTCTCGGAGTCAGAA
K0326Y   GTAACAGTTTACGTTCCACGAGATGCCTATATGATGGACTTTGTTTTCTCGGAGTCAGAA

 W64A+   GAAGGTGGAATTTATGATAACAGAAATGGGTTAGACTATCATATTCCTGTTTTTGGGTCA
W64Ao2   GAAGGTGGAATTTATGATAACAGAAATGGGTTAGACTATCATATTCCTGTTTTTGGGTCA
K0326Y   GAAGGTGGAATTTATGATAACAGAAATGGGTTAGACTATCATATTCCTGTTTTTGGGTCA

 W64A+   ATTGCAAAGGAACCACCTATGCACATTGTCCACATTGCTGTTGAGATGGCACCAATCGCA
W64Ao2   ATTGCAAAGGAACCACCTATGCACATTGTCCACATTGCTGTTGAGATGGCACCAATCGCA
K0326Y   ATTGCAAAGGAACCACCTATGCACATTGTCCACATCGCTGTTGAGATGGCACCAATCGCA

 W64A+   AAGGTTGGAGGTCTTGGTGATGTTGTCACTAGTCTTTCACGTGCTGTGCAAGATTTAGGA
W64Ao2   AAGGTTGGAGGTCTTGGTGATGTTGTCACTAGTCTTTCACGTGCTGTGCAAGATTTAGGA
K0326Y   AAGGTTGGAGGTCTTGGTGATGTTGTCACTAGTCTTTCACGTGCTGTGCAAGATTTAGGA

 W64A+   CACAATGTGGAGGTTATTCTTCCAAAGTACGGTTGCTTGAATCTAAGCAATGTCAAGAAT
W64Ao2   CACAATGTGGAGGTTATTCTTCCAAAGTACGGTTGCTTGAATCTAAGCAATGTCAAGAAT
K0326Y   CACAATGTGGAGGTTATTCTTCCAAAGTACGGTTGCTTGAATCTAAGCAATGTCAAGAAT

 W64A+   CTACAAATCCATCAGAGTTTTTCTTGGGGTGGTTCTGAAATAAATGTGTGGCGTGGACTA
W64Ao2   CTACAAATCCATCAGAGTTTTTCTTGGGGTGGTTCTGAAATAAATGTGTGGCGTGGACTA
K0326Y   CTACACATCCATCAGAGTTTTTCTTGGGGTGGTTCTGAAATAAAAGTGTGGCGTGGACTA

 W64A+   GTCGAAGGCCTTTGTGTTTACTTCCTGGAACCTCAAAATGGGATGTTTGGAGTCGGATAT
W64Ao2   GTCGAAGGCCTTTGTGTTTACTTCCTGGAACCTCAAAATGGGATGTTTGGAGTCGGATAT
K0326Y   GTCGAAGGCCTTTGTGTTTACTTCCTGGAACCTCAAAATGGGATGTTTGGAGTCGGATAT

 W64A+   GTATATGGCAGGGACGATGACCGCCGATTTGGCTTCTTCTGTCGTTCTGCTCTAGAGTTT
W64Ao2   GTATATGGCAGGGACGATGACCGCCGATTTGGCTTCTTCTGTCGTTCTGCTCTAGAGTTT
K0326Y   GTATATGGCAGGGACGATGACCGCCGATTTGGCTTCTTCTGTCGTTCTGCTCTAGAGTTT

 W64A+   CTCCTCCAAAGTGGATCTTCTCCTAACATAATACATTGCCATGATTGGTCAAGTGCTCCT
W64Ao2   CTCCTCCAAAGTGGATCTTCTCCTAACATAATACATTGCCATGATTGGTCAAGTGCTCCT
K0326Y   CTCCTCCAAAGTGGATCTTCTCCTAACATAATACATTGCCATGATTGGTCAAGTGCTCCT

 W64A+   GTTGCCTGGCTACACAAGGAAAACTACGCGAAGTCTAGCTTGGCAAACGCACGGGTGGTA
W64Ao2   GTTGCCTGGCTACACAAGGAAAACTACGCGAAGTCTAGCTTGGCAAACGCACGGGTGGTA
K0326Y   GTTGCCTGGCTACACAAGGAAAACTACGCGAAGTCTAGCTTGGCAAATGCACGGGTGGTA

 W64A+   TTCACCATCCACAATCTTGAATTTGGAGCGCATCATATTGGCAAAGCAATGAGATATTGT
W64Ao2   TTCACCATCCACAATCTTGAATTTGGAGCGCATCATATTGGCAAAGCAATGAGATATTGT
K0326Y   TTCACCATCCACAATCTTGAATTTGGAGCGCATCATATTGGCAAAGCAATGAGATATTGT

 W64A+   GATAAAGCAACAACTGTCTCTAATACATATTCAAAGGAAGTGTCAGGTCATGGTGCCATA
W64Ao2   GATAAAGCAACAACTGTCTCTAATACATATTCAAAGGAAGTGTCAGGTCATGGTGCCATC
K0326Y   GATAAAGCCACAACTGTCTCTAATACATATTCAAAGGAAGTGTCAGGTCATGGTGCCATC
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 W64A+   GTTCCTCATCCTGGGAAATTCTATGGCATTCTCAATGGAATTGATCCGGATATATGGGAT
W64Ao2   GTTCCTCATCTTGGGAAATTCTATGGCATTCTCAATGGAATTGATCCGGATATATGGGAT
K0326Y   GTTCCTCATCTTGGGAAATTCTATGGCATTCTGAATGGAATTGATCCAGATATATGGGAT

 W64A+   CCGTACAATGACAACTTTATCCCGGTCCACTACACTTGTGAGAATGTGGTTGAAGGCAAG
W64Ao2   CCGTACAATGACAACTTTATCCCGGTCCACTACACTTGTGAGAATGTGGTTGAAGGCAAG
K0326Y   CCGTACAATGACAACTTTATCCCGGTCCACTACACTTGTGAGAATGTGGTTGAAGGCAAG

 W64A+   AGGGCTGCTAAGAGGGCACTGCAGCAGAAGTTTGGGTTACAGCAAATCGATGTCCCCGTC
W64Ao2   AGGGCTGCTAAGAGGGCACTGCAGCAGAAGTTTGGGTTACAGCAAATCGATGTCCCCGTC
K0326Y   AGGGCTGCTAAGAGGGCACTGCAGCAGAAGTTTGGGTTACAGCAAATCGATGTCCCCGTC

 W64A+   GTAGGAATCGTCACTCGCCTGACAGCCCAAAAGGGGATCCACCTGATCAAGCATGCGATT
W64Ao2   GTAGGAATCGTCACTCGCCTGACAGCCCAAAAGGGGATCCACCTGATCAAGCATGCGATT
K0326Y   GTAGGAATCGTCACTCGCCTGACAGCCCAAAAGGGGATCCACCTGATCAAGCATGCGATT

 W64A+   CACCGTACACTCGAACGGAACGGACAGGTGGTTTTGCTTGGTTCAGCGCCGGACTCTCGA
W64Ao2   CACCGTACACTCGAACGGAACGGACAGGTGGTTTTGCTTGGTTCAGCGCCGGACTCTCGA
K0326Y   CACCGTACACTCGAACGGAACGGACAGGTGGTTTTGCTTGGTTCAGCGCCGGACTCTCGA

 W64A+   ATCCAAGCTGATTTTGTCAACCTGGCGAATACGCTCCACGGCGTAAACCATGGGCAAGTG
W64Ao2   ATCCAAGCTGATTTTGTCAACCTGGCGAATACGCTCCACGGCGTAAACCATGGGCAAGTG
K0326Y   ATCCAAGCTGATTTTGTCAACCTGGCGAATAAGCTCCACGGCGTAAACCATGGGCAAGTG

 W64A+   AGGCTTTCCTTGACCTACGACGAGCCTCTCTCGCATCTGATATACGCTGGCTCTGACTTC
W64Ao2   AGGCTGTCCTTGACCTACGACGAGCCTCTCTCGCATCTGATATACGCTGGCTCTGACTTC
K0326Y   AGGCTTTCCTTGACCTACGACGAGCCTCTCTCGCATCTGATATACGCTGGCTCTGACTTC

 W64A+   ATTCTGGTCCCATCTATATTTGAGCCTTGCGGCCTAACTCAGCTCGTCGCCATGCGGTAT
W64Ao2   ATTCTGGTCCCATCTATATTTGAGCCTTGCGGCCTAACTCAGCTGGTCGCCATGCGGTAT
K0326Y   ATTCTGGTCCCATCTATATTTGAGCCTTGCGGCCTAACTCAGCTCGTCGCCATGCGGTAT

 W64A+   GGGACCATCCCGATTGTCCGCAAGACTGGAGGGCTCTTCGACACTGTCTTCGATGTGGAC
W64Ao2   GGAACCATCCCGATTGTCCGCAAGACTGGAGGGCTCTTCGACACTGTCTTCGATGTGGAC
K0326Y   GGGACCATCCCGATTGTCCGCAAGACTGGAGGGCTCTTCGACACTGTCTTCGATGTGGAC

 W64A+   AATGACAAGGAACGAGCCCGAGATCGAGGCCTTGAGCCCAACGGGTTTAGCTTTGACGGA
W64Ao2   AATGACAAGGAACGAGCCCGAGATCGAGGCCTTGAGCCCAACGGGTTTAGCTTTGACGGA
K0326Y   AATGACAAGGAACGAGCCCGAGATCGAGGCCTTGAGCCCAACGGGTTTAGCTTTGACGGA

 W64A+   GCTGATAGCAACGGTGTTGACTACGCGCTGAACAGGGCGATCTCAGCTTGGTTCGATGCC
W64Ao2   GCTGATAGCAACGGTGTTGACTACGCCCTGAACAGGGCGATCTCAGCTTGGTTCGATGCC
K0326Y   GCTGATAGCAACGGTGTTGACTACGCGCTGAACAGGGCGATCTCAGCTTGGTTCGATGCC

 W64A+   CGGAGCTGGTTCCACTCCCTTTGCAAGAGAGTCATGGAGCAGGACTGGTCGTGGAACCGA
W64Ao2   CGGAGCTGGTTCCACTCCCTTTGCAAGAGAGTCATGGAGCAGGACTGGTCGTGGAACCGA
K0326Y   CGGAGCTGGTTCCACTCCCTTTGCAAGAGAGTCATGGAGCAGGACTGGTCGTGGAACCGA

 W64A+   CCTGCCCTCGACTACATCGAGCTCTACCGTTCAGCGTCCAAATTGTAA
W64Ao2   CCTGCCCTCGACTACATCGAGCTCTACCGTTCAGCGTCCAAATTGTAA
K0326Y   CCTGCCCTCGACTACATCGAGCTCTACCGTTCAGCGTCCAAATTGTAA
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4. Amino acid sequence alignment of *SSIII* gene product

N-terminal domain (amino acid 1-768), homology domain (amino acid 769-1226) and catalytic domain (amino acid 1227-1674)

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          1         10        20        30        40        50        60
          |         |         |         |         |         |         |
 W64A+    MEMVLRSQSPLCLRSGPVLIFRPTVAGGGGGTQSLLRTTRFARRRVIRCVVASPGCPNRK
W64Ao2    MEMVLRSQSPLCLRSGPVLIFRPTVAGGGGGTQSLLRTTRFARRRVIRCVVASLGCPNRK
K0326Y    MEMVLRSQSPLCLRSGPVLIFRPTVAGGGGGTQSLLRTTRFARRRVIRCVVASPGCPNRK

 W64A+    SRTASPNVKVAAYSNYAPRLLVESSSKKSEHHDSSRHREETIDTYNGLSGSDAAELTSNR
W64Ao2    SRTASPNVKVAAYSNYAPRLLVESSSKKSEHHDSSRHREETIDTYNGLSGSDAAELTSNR
K0326Y    SRTASPNVKVAAYSNYAPRLLVESSSKKSEHHDSSRHREETIDTYNGLSGSDAAELTSNR

 W64A+    DVEIEVDLQHISEEELPGKVSINASLGEMETVDEAEVEEDKFEVDTSGIVLRNAAVREVD
W64Ao2    DVEIEVDLQHISEEELPGKVSINASLGEMETVDEAEVEEDKFEVDTSGIVLRNAAVREVD
K0326Y    DVEIEVDLQHISEEELPGKVSINASLGEMETVDEAEVEEDKFEVDTSGIVLRNVAVREVD

 W64A+    PKDEHNAKDVFVVDSSGTAPDNAAVEEVVDEAEVEEDMVDVDILGLDLNNATMEEIDLME
W64Ao2    PKDEHNAKDVFVVDSSGTAPDNAAVEEVVDEAEVEEDMVDVDILGLDLNNATMEEIDLME
K0326Y    PKDEHNAKDVFVVDSSGTAPDNAAVEEVVDEAEVEEDMVDVDILGLDLNNATIEEIDLME

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W64Ao2    EALLENFDVDSPGNASSGRTYGGVDELGELPSTSVDCIAINGKHRSLKPEPLPIVRFQEQ
K0326Y    EALLENFDVDSPGNASSGRTYGGVDELGELPSTSVDCIAINGKRRSLKPKPLPIVRFQEQ

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W64Ao2    EQIVLSIVDEEGLIAGSCEEGQPVVDYDKQEENSTAFDEQKQLTDDFPEEGISIVHFPEP
K0326Y    EQIVLSIVDEEGLIASSCEEGQPVVDYDKQEENSTAFDEQKQLTDDFPEEGISIVHFPEP

 W64A+    NNDIVGSSKFLEQKQELDGSYKQDRSTTGLHEQDQSVVSSHGQDKSIVGVPQQIQYNDQS
W64Ao2    NNDIVGSSKFLEQKQELDGSYKQDRSTTGLHEQDQSVVSSHGQDKSIVGVPQQIQYNDQS
K0326Y    NNDIVGSSKFLEQKQELDGSYKQDRSTTGLHEQDQSVVSSHGQDKSIVGVPQQIQYNDQS

 W64A+    IAGSHRQDQSIAGAPEQIQSVAGFIKPNQSIVGSYKQHELIIPEPKKIESIISYNEIDQS
W64Ao2    IAGSHRQDQSIAGAPEQIQSVAGFIKPNQSIVGSYKQHELIIPEPKKIESIISYNEIDQS
K0326Y    IAGSHRQDQSIAGAPEQIQSVAGYIKPNQSIVGSCKQHELIIPEPKKIESIISYNEIDQS

 W64A+    IVGSHKQDKSVVSVPEQIQSIVSHSKPNQSTIDSYRQAESIIGVPEKVQSITSYNKLDQS
W64Ao2    IVGSHKQDKSVVSVPEQIQSIVSHSKPNQSTIDSYRQAESIIGVPEKVQSITSYNKLDQS
K0326Y    IVGSHKQDKSVVSVPEQIQSIVSHSKPNQSTVDSYRQAESIIGVPEKVQSITSYDKLDQS

 W64A+    IVGSLKQDEPIISVHEKIQSIVHYTKPNQSIVGLPKQQQSIVHIVEPKQSIDGFPKQDLS
W64Ao2    IVGSLKQDEPIISVHEKIQSIVHYTKPNQSIVGLPKQQQSIVHIVEPKQSIDGFPKQDLS
K0326Y    IVGSLKQDEPIISVPEKIQSIVHYTKPNQSIVGLPKQQQSIVHIVEPKQSIDGFPKQDLS

 W64A+    IVGISNEFQTKQLATVGTHDGLLMKGVEAKETSQKTEGDTLQATFNADNLSQKHEEGLTK
W64Ao2    IVGISNEFQTKQLATVGTHDGLLMKGVEAKETSQKIEGDTLQATFNADNLSQKHEEGLTK
K0326Y    IVGISNEFQTKQLATVGTHDGLLMKGVEAKETSQKTEGDTLQATFNVDNLSQK-QEGLTK

 W64A+    EADEITIIEKINDEDLVMIEEQKSIAMNEEQTIVTEEDILMAKVEIGIDKAKFLHLLSEE
W64Ao2    EADEITIIEKINDEDLVMIEEQKSIAMNEEQTIVTEEDILMAKVEIGIDKAKFLHLLSEE
K0326Y    EADEITIIEKINDEDLVMIEEQKSIAMNEEQTIVTEEDIPMAKVEIGIDKAKFLHLLSEE

 W64A+    ESSWDENEVGIIEADEQYEVDETSMSTEQDIQESPNDDLDPQALQSMLQELAEKNYSLGN
W64Ao2    ESSWDENEVGIIEADEQYEVDETSMSTEQDIQESPNDDLDPQALQSMLQELAEKNYSLGN
K0326Y    ESSWDENEVGIIEADEQYEVDETSMSTEQDIQESPNDDLDPQALWSMLQELAEKNYSLGN

 W64A+    KLFTYPDVLKADSTIDLYFNRDLSAVANEPDVLIKGAFNGWKWRFFTEKLHKSELAGDWW
W64Ao2    KLFTYPDVLKADSTIDLYFNRDLSAVANEPDVLIKGAFNGWKWRFFTEKLHKSELAGDWW
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K0326Y   KLFTYPDVLKADSTIDLYFNRDLSAVANEPDVLIKGAFNGWKWRFFTEKLHKSELAGDWW

 W64A+   CCKLYIPKQAYRMDFVFFNGRTIYENNDNNDFVIQIESTMDENLFEDFLAEEKQRELENL
W64Ao2   CCKLYIPKQAYRMDFVFFNGRTIYENNDNNDFVIQIESTMDENLFEDFLAEEKQRELENL
K0326Y   CCKLYIPKQAYRMDFVFFNGHTVYENNNNNDFVIQIESTMDENLFEDFLAEEKQRELENL

 W64A+   ANEEAERRRQTDEQRRMEEERAADKADRVQAKVEVETKKNKLCNVLGLARAPVDNLWYIE
W64Ao2   ANEEAERRRQTDEQRRMEEERAADKADRVQAKVEVETKKNKLRNVLALARAPVDNLWYIE
K0326Y   ANEEAERRRQTDEQRRMEEERAADKADRVQAKVEVETKKNKLCNVLGLARAPVDNLWYIE

 W64A+   PITTGQEATVRLYYNINSRPLVHSTEIWMHGGYNNWIDGLSFAERLVHHNDKDCDWWFAD
W64Ao2   PITTGQEATVRLYYNINSRPLVHSTEIWMHGGYNNWIDGLSFAERLVHHNDKDCDWWFAD
K0326Y   PITTGQEATVRLYYNINSRPLVHSTEIWMHGGYNNWIDGLSFAERLVHHHDKDCDWWFAD

 W64A+   VVVPERTYVLDWVFADGPPGSARNYDNNGGHDFHATLPNNMTDEEYWMEEEQRIYTRLQQ
W64Ao2   VVVPERTYVLDWVFADGPPGSARNYDNNGGHDFHATLPNNMTDEEYWMEEEQRIYTRLQQ
K0326Y   VVVPERTYVLDWVFADGPPGSARNYDNNGGHDFHATLPNNMTEEEYWMEEEQRIYTRLQQ

 W64A+   ERREREEAIKRKAERNAKMKAEMKEKTMRMFLVSQKHIVYTEPLEIHAGTTIDVLYNPSN
W64Ao2   ERREREEAIKKKAERNAKMKAEMKEKTMRMFLVSQKHIVYTEPLEIHAGTTIDVLYNPSN
K0326Y   ERREREEAIKRKAERNAKMKAEMKEKTMRMFLVSQKHIVYTEPLEIHAGTTIDVLYNPSN

 W64A+   TVLTGKPEVWFRCSFNRWMYPGGVLPPQRMVQAENGSHLKATVYVPRDAYMMDFVFSESE
W64Ao2   TVLTGKPEVWFRCSFNRWMYPGGVLPPQKMVQAENGSHLKATVYVPRDAYMMDFVFSESE
K0326Y   TVLTGKPEVWFRCSFNRWMYPGGVLPPQKMVQAENGSHLKVTVYVPRDAYMMDFVFSESE

 W64A+   EGGIYDNRNGLDYHIPVFGSIAKEPPMHIVHIAVEMAPIAKVGGLGDVVTSLSRAVQDLG
W64Ao2   EGGIYDNRNGLDYHIPVFGSIAKEPPMHIVHIAVEMAPIAKVGGLGDVVTSLSRAVQDLG
K0326Y   EGGIYDNRNGLDYHIPVFGSIAKEPPMHIVHIAVEMAPIAKVGGLGDVVTSLSRAVQDLG

 W64A+   HNVEVILPKYGCLNLSNVKNLQIHQSFSWGGSEINVWRGLVEGLCVYFLEPQNGMFGVGY
W64Ao2   HNVEVILPKYGCLNLSNVKNLQIHQSFSWGGSEINVWRGLVEGLCVYFLEPQNGMFGVGY
K0326Y   HNVEVILPKYGCLNLSNVKNLHIHQSFSWGGSEIKVWRGLVEGLCVYFLEPQNGMFGVGY

 W64A+   VYGRDDDRRFGFFCRSALEFLLQSGSSPNIIHCHDWSSAPVAWLHKENYAKSSLANARVV
W64Ao2   VYGRDDDRRFGFFCRSALEFLLQSGSSPNIIHCHDWSSAPVAWLHKENYAKSSLANARVV
K0326Y   VYGRDDDRRFGFFCRSALEFLLQSGSSPNIIHCHDWSSAPVAWLHKENYAKSSLANARVV

 W64A+   FTIHNLEFGAHHIGKAMRYCDKATTVSNTYSKEVSGHGAIVPHPGKFYGILNGIDPDIWD
W64Ao2   FTIHNLEFGAHHIGKAMRYCDKATTVSNTYSKEVSGHGAIVPHLGKFYGILNGIDPDIWD
K0326Y   FTIHNLEFGAHHIGKAMRYCDKATTVSNTYSKEVSGHGAIVPHLGKFYGILNGIDPDIWD

 W64A+   PYNDNFIPVHYTCENVVEGKRAAKRALQQKFGLQQIDVPVVGIVTRLTAQKGIHLIKHAI
W64Ao2   PYNDNFIPVHYTCENVVEGKRAAKRALQQKFGLQQIDVPVVGIVTRLTAQKGIHLIKHAI
K0326Y   PYNDNFIPVHYTCENVVEGKRAAKRALQQKFGLQQIDVPVVGIVTRLTAQKGIHLIKHAI

 W64A+   HRTLERNGQVVLLGSAPDSRIQADFVNLANTLHGVNHGQVRLSLTYDEPLSHLIYAGSDF
W64Ao2   HRTLERNGQVVLLGSAPDSRIQADFVNLANTLHGVNHGQVRLSLTYDEPLSHLIYAGSDF
K0326Y   HRTLERNGQVVLLGSAPDSRIQADFVNLANKLHGVNHGQVRLSLTYDEPLSHLIYAGSDF

 W64A+   ILVPSIFEPCGLTQLVAMRYGTIPIVRKTGGLFDTVFDVDNDKERARDRGLEPNGFSFDG
W64Ao2   ILVPSIFEPCGLTQLVAMRYGTIPIVRKTGGLFDTVFDVDNDKERARDRGLEPNGFSFDG
K0326Y   ILVPSIFEPCGLTQLVAMRYGTIPIVRKTGGLFDTVFDVDNDKERARDRGLEPNGFSFDG

 W64A+   ADSNGVDYALNRAISAWFDARSWFHSLCKRVMEQDWSWNRPALDYIELYRSASKL*
W64Ao2   ADSNGVDYALNRAISAWFDARSWFHSLCKRVMEQDWSWNRPALDYIELYRSASKL*
K0326Y   ADSNGVDYALNRAISAWFDARSWFHSLCKRVMEQDWSWNRPALDYIELYRSASKL*
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5. Comparison of *SSIII* gene sequence between standard line and K0326Y

Standard line sequence was from Gao et al., 1998, and its GenBank accession number is AF023159. Letters with gray background represent silent SNPs

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AF023159      1 ATGGAGATGGTCCTACGGTCGCAGAGCCCTCTCTGCCTTCGGAGTGGGCCGGTGCTCATT   60
K0326Y        1 ATGGAGATGGTCCTACGGTCGCAGAGCCCTCTCTGCCTTCGGAGTGGGCCGGTGCTCATT   60

AF023159     61 TTTCGACCAACCGTCGCGGGCGGAGGAGGGGGCACTCAGTCTTTGTTGAGGACTACCAGA  120
K0326Y       61 TTTCGACCAACCGTCGCGGGCGGAGGAGGGGGCACTCAGTCTTTGTTGAGGACTACCAGA  120

AF023159    121 TTTGCGAGAAGAAGGGTCATTCGATGCGTTGTAGCAAGTCCAGGTTGTCCTAATAGGAAA  180
K0326Y      121 TTTGCGAGAAGAAGGGTCATTCGATGCGTTGTAGCAAGTCCAGGTTGTCCTAATAGGAAA  180

AF023159    181 TCTAGGACAGCGTCTCCCAACGTAAAAGTAGCTGCTTATAGCAACTATGCGCCAAGACTC  240
K0326Y      181 TCTAGGACAGCGTCTCCCAACGTAAAAGTAGCTGCTTATAGCAACTATGCGCCAAGACTC  240

AF023159    241 CTCGTTGAGTCAAGCTCCAAGAAGAGCGAACACCATGATAGCAGCAGACACCGTGAAGAA  300
K0326Y      241 CTCGTTGAGTCAAGCTCCAAGAAGAGCGAACACCATGATAGCAGCAGACACCGTGAAGAA  300

AF023159    301 ACTATTGATACATACAATGGGCTGTCAGGTTCTGATGCAGCAGAATTGACAAGTAATAGA  360
K0326Y      301 ACTATTGATACATACAATGGGCTGTCAGGTTCTGATGCAGCAGAATTGACAAGTAATAGA  360

AF023159    361 GATGTAGAAATTGAAGTGGATTTGCAGCACATTTCTGAGGAGGAATTGCCAGGAAAAGTA  420
K0326Y      361 GATGTAGAAATTGAAGTGGATTTGCAGCACATTTCTGAGGAGGAATTGCCAGGAAAAGTA  420

AF023159    421 TCGATTAATGCATCATTAGGAGAAATGGAAACAGTGGATGAAGCTGAGGTCGAGGAGGAT  480
K0326Y      421 TCGATTAATGCATCATTAGGAGAAATGGAAACAGTGGATGAAGCTGAGGTCGAGGAGGAT  480

AF023159    481 AAGTTTGAGGTAGATACCTCAGGAATTGTATTGCGCAATGTTGCAGTTCGGGAAGTGGAT  540
K0326Y      481 AAGTTTGAGGTAGATACCTCAGGAATTGTATTGCGCAATGTTGCAGTTCGGGAAGTGGAT  540

AF023159    541 CCAAAGGATGAACATAATGCTAAAGATGTATTTGTGGTAGATTCGTCAGGAACTGCACCA  600
K0326Y      541 CCAAAGGATGAACATAATGCTAAAGATGTATTTGTGGTAGATTCGTCAGGAACTGCACCA  600

AF023159    601 GATAATGCTGCAGTGGAGGAAGTGGTAGATGAAGCTGAGGTTGAAGAGGATATGGTTGAT  660
K0326Y      601 GATAATGCTGCAGTGGAGGAAGTGGTAGATGAAGCTGAGGTTGAAGAGGATATGGTTGAT  660

AF023159    661 GTGGATATCTTGGGACTTGACTTGAATAATGCAACGATCGAGGAAATTGATTTGATGGAA  720
K0326Y      661 GTGGATATCTTGGGACTTGACTTGAATAATGCAACGATCGAGGAAATTGATTTGATGGAA  720

AF023159    721 GAGGCTTTACTGGAGAACTTCGACGTGGATTCACCAGGCAATGCTTCTAGTGGTCGAACC  780
K0326Y      721 GAGGCTTTACTGGAGAACTTCGACGTGGATTCACCAGGCAATGCTTCTAGTGGTCGAACC  780

AF023159    781 TATGGGGGTGTGGATGAGTTGGGTGAGCTGCCTTCAACATCCGTGGATTGCATCGCCATT  840
K0326Y      781 TATGGGGGTGTGGATGAGTTGGGTGAGCTGCCTTCAACATCCGTGGATTGCATCGCCATT  840

AF023159    841 AACGGAAAACGTAGAAGTTTGAAGCCTAAGCCCTTGCCAATTGTCAGGTTCCAGGAACAA  900
K0326Y      841 AACGGAAAACGTAGAAGTTTGAAGCCTAAGCCCTTGCCAATTGTCAGGTTCCAGGAACAA  900

AF023159    901 GAACAGATAGTTTTAAGCATTGTTGACGAAGAAGGGTTGATTGCTAGTTCATGTGAAGAA  960
K0326Y      901 GAACAGATAGTTTTAAGCATTGTTGACGAAGAAGGGTTGATTGCTAGTTCATGTGAAGAA  960

AF023159    961 GGCCAACCGGTGGTAGATTACGATAAGCAAGAGGAAAACTCTACCGCTTTCGATGAACAG 1020
K0326Y      961 GGCCAACCGGTGGTAGATTACGATAAGCAAGAGGAAAACTCTACCGCTTTCGATGAACAG 1020

AF023159   1021 AAGCAATTAACTGATGATTTCCCTGAAGAAGGCATATCTATAGTTCACTTCCCTGAGCCA 1080
K0326Y     1021 AAGCAATTAACTGATGATTTCCCTGAAGAAGGCATATCTATAGTTCACTTCCCTGAGCCA 1080

AF023159   1081 AACAATGATATTGTTGGATCCTCAAAATTCTTGGAGCAAAAACAAGAATTGGATGGTTCT 1140
K0326Y     1081 AACAATGATATTGTTGGATCCTCAAAATTCTTGGAGCAAAAACAAGAATTGGATGGTTCT 1140

AF023159   1141 TATAAACAAGATCGATCAACCACTGGATTGCATGAACAAGATCAGTCTGTTGTTAGTTCA 1200
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K0326Y     1141 TATAAACAAGATCGATCAACCACTGGATTGCATGAACAAGATCAGTCTGTTGTTAGTTCA 1200

AF023159   1201 CACGGACAAGATAAATCAATTGTTGGTGTGCCTCAGCAAATCCAGTACAATGATCAATCT 1260
K0326Y     1201 CACGGACAAGATAAATCAATTGTTGGTGTGCCTCAGCAAATCCAGTACAATGATCAATCT 1260

AF023159   1261 ATTGCTGGTTCTCATAGACAAGATCAATCAATTGCCGGTGCACCTGAGCAAATCCAATCC 1320
K0326Y     1261 ATTGCTGGTTCTCATAGACAAGATCAATCAATTGCCGGTGCACCTGAGCAAATCCAATCC 1320

AF023159   1321 GTTGCTGGCTATATAAAACCAAATCAATCTATTGTTGGTTCTTGTAAACAACATGAATTG 1380
K0326Y     1321 GTTGCTGGCTATATAAAACCAAATCAATCTATTGTTGGTTCTTGTAAACAACATGAATTG 1380

AF023159   1381 ATTATTCCTGAGCCTAAGAAAATCGAATCCATCATCAGTTACAATGAAATAGATCAATCT 1440
K0326Y     1381 ATTATTCCTGAGCCTAAGAAAATCGAATCCATCATCAGTTACAATGAAATAGATCAATCT 1440

AF023159   1441 ATTGTTGGTTCTCACAAACAAGACAAATCTGTTGTTAGTGTGCCTGAGCAAATCCAATCC 1500
K0326Y     1441 ATTGTTGGTTCTCACAAACAAGACAAATCTGTTGTTAGTGTGCCTGAGCAAATCCAATCC 1500

AF023159   1501 ATTGTTAGTCACAGCAAACCAAATCAATCTACTGTTGATTCTTATAGACAAGCTGAATCA 1560
K0326Y     1501 ATTGTTAGTCACAGCAAACCAAATCAATCTACTGTTGATTCTTATAGACAAGCTGAATCA 1560

AF023159   1561 ATTATTGGTGTGCCTGAGAAAGTCCAATCCATCACCAGTTACGATAAACTAGACCAGTCC 1620
K0326Y     1561 ATTATTGGTGTGCCTGAGAAAGTCCAATCCATCACCAGTTACGATAAACTAGACCAGTCC 1620

AF023159   1621 ATTGTTGGTTCTCTTAAACAAGATGAGCCTATTATTAGCGTGCCTGAGAAAATCCAATCC 1680
K0326Y     1621 ATTGTTGGTTCTCTTAAACAAGATGAGCCTATTATTAGCGTGCCTGAGAAAATCCAATCC 1680

AF023159   1681 ATTGTCCATTACACTAAACCAAATCAGTCTATTGTTGGCTTGCCCAAACAACAACAATCA 1740
K0326Y     1681 ATTGTCCATTACACTAAACCAAATCAGTCTATTGTTGGCTTGCCCAAACAACAACAATCA 1740

AF023159   1741 ATTGTTCATATCGTTGAACCAAAACAGTCCATAGATGGTTTCCCTAAACAAGATCTATCA 1800
K0326Y     1741 ATTGTTCATATCGTTGAACCAAAACAGTCCATAGATGGTTTCCCTAAACAAGATCTATCA 1800

AF023159   1801 ATCGTTGGTATCTCCAATGAGTTTCAAACAAAGCAACTGGCTACTGTTGGGACTCATGAT 1860
K0326Y     1801 ATCGTTGGTATCTCCAATGAGTTTCAAACAAAGCAACTGGCTACTGTTGGGACTCATGAT 1860

AF023159   1861 GGATTGCTTATGAAGGGTGTGGAAGCTAAGGAGACATCTCAAAAGACTGAAGGGGATACA 1920
K0326Y     1861 GGATTGCTTATGAAGGGTGTGGAAGCTAAGGAGACATCTCAAAAGACTGAAGGGGATACA 1920

AF023159   1921 CTTCAGGCAACGTTCAATGTCGACAACTTGTCACAGAAACAGGAAGGCTTAACTAAAGAA 1980
K0326Y     1921 CTTCAGGCAACGTTCAATGTCGACAACTTGTCACAGAAACAGGAAGGCTTAACTAAAGAA 1980

AF023159   1981 GCAGACGAGATAACAATTATTGAGAAAATCAATGATGAAGACCTTGTGATGATTGAAGAA 2040
K0326Y     1981 GCAGACGAGATAACAATTATTGAGAAAATCAATGATGAAGACCTTGTGATGATTGAAGAA 2040

AF023159   2041 CAGAAAAGCATAGCCATGAATGAAGAACAGACGATTGTTACCGAAGAAGACATTCCAATG 2100
K0326Y     2041 CAGAAAAGCATAGCCATGAATGAAGAACAGACGATTGTTACCGAAGAAGACATTCCAATG 2100

AF023159   2101 GCTAAGGTTGAGATAGGAATTGACAAGGCCAAATTTTTACATCTGCTTTCTGAAGAAGAG 2160
K0326Y     2101 GCTAAGGTTGAGATAGGAATTGACAAGGCCAAATTTTTACATCTGCTTTCTGAAGAAGAG 2160

AF023159   2161 AGTTCATGGGATGAAAATGAAGTGGGAATAATTGAGGCTGATGAACAGTATGAAGTCGAT 2220
K0326Y     2161 AGTTCATGGGATGAAAATGAAGTGGGAATAATTGAGGCTGATGAACAGTATGAAGTCGAT 2220

AF023159   2221 GAGACATCTATGTCCACTGAACAAGATATCCAGGAATCACCTAATGATGATTTGGATCCA 2280
K0326Y     2221 GAGACATCTATGTCCACTGAACAAGATATCCAGGAATCACCTAATGATGATTTGGATCCA 2280

AF023159   2281 CAAGCACTATGGAGTATGCTTCAAGAGCTTGCTGAAAAAAATTATTCGCTGGGAAACAAG 2340
K0326Y     2281 CAAGCACTATGGAGTATGCTTCAAGAGCTTGCTGAAAAAAATTATTCGCTGGGAAACAAG 2340

AF023159   2341 TTGTTTACTTATCCAGATGTATTGAAAGCTGATTCAACAATTGATCTCTATTTCAATCGT 2400
K0326Y     2341 TTGTTTACTTATCCAGATGTATTGAAAGCTGATTCAACAATTGATCTCTATTTCAATCGT 2400

AF023159   2401 GATCTATCAGCTGTGGCCAATGAGCCTGATGTACTTATCAAAGGAGCATTCAATGGGTGG 2460
K0326Y     2401 GATCTATCAGCTGTGGCCAATGAGCCTGATGTACTTATCAAAGGAGCATTCAATGGGTGG 2460

AF023159   2461 AAGTGGAGATTTTTCACTGAAAAATTGCACAAGAGCGAGCTGGCAGGGGACTGGTGGTGC 2520
K0326Y     2461 AAGTGGAGATTTTTCACTGAAAAATTGCACAAGAGCGAGCTGGCAGGGGACTGGTGGTGC 2520
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AF023159   2521 TGCAAACTATACATTCCTAAGCAGGCATACAGAATGGACTTTGTGTTTTTTAACGGACAC 2580
K0326Y     2521 TGCAAACTATACATTCCTAAGCAGGCATACAGAATGGACTTTGTGTTTTTTAACGGACAC 2580

AF023159   2581 ACGGTATATGAAAATAATAACAATAATGATTTCGTGATACAAATAGAAAGCACCATGGAT 2640
K0326Y     2581 ACGGTATATGAAAATAATAACAATAATGATTTCGTGATACAAATAGAAAGCACCATGGAT 2640

AF023159   2641 GAAAATTTATTTGAGGATTTCTTGGCTGAAGAAAAGCAACGAGAACTTGAGAACCTTGCA 2700
K0326Y     2641 GAAAATTTATTTGAGGATTTCTTGGCTGAAGAAAAGCAACGAGAACTTGAGAACCTTGCA 2700

AF023159   2701 AATGAGGAAGCTGAAAGGAGGAGACAAACTGATGAGCAGCGGCGAATGGAGGAAGAAAGG 2760
K0326Y     2701 AATGAGGAAGCTGAAAGGAGGAGACAAACTGATGAGCAGCGGCGAATGGAGGAAGAAAGG 2760

AF023159   2761 GCCGCAGATAAAGCTGACAGGGTACAAGCCAAGGTTGAGGTAGAGACGAAGAAGAATAAA 2820
K0326Y     2761 GCCGCAGATAAAGCTGACAGGGTACAAGCCAAGGTTGAGGTAGAGACGAAGAAGAATAAA 2820

AF023159   2821 TTGTGCAATGTATTGGGTTTAGCCAGAGCTCCTGTTGATAATTTATGGTACATTGAGCCC 2880
K0326Y     2821 TTGTGCAATGTATTGGGTTTAGCCAGAGCTCCTGTTGATAATTTATGGTACATTGAGCCC 2880

AF023159   2881 ATCACGACTGGACAAGAGGCTACTGTCAGATTGTATTATAACATAAACTCAAGACCTCTA 2940
K0326Y     2881 ATCACGACTGGACAAGAGGCTACTGTCAGATTGTATTATAACATAAACTCAAGACCTCTA 2940

AF023159   2941 GTTCACAGTACTGAGATATGGATGCATGGTGGCTATAACAATTGGATTGATGGACTCTCT 3000
K0326Y     2941 GTTCACAGTACTGAGATATGGATGCATGGTGGCTATAACAATTGGATTGATGGACTCTCT 3000

AF023159   3001 TTTGCTGAAAGGCTTGTTCATCATCATGACAAAGATTGTGATTGGTGGTTTGCAGATGTT 3060
K0326Y     3001 TTTGCTGAAAGGCTTGTTCATCATCATGACAAAGATTGTGATTGGTGGTTTGCAGATGTT 3060

AF023159   3061 GTCGTGCCTGAAAGAACATATGTATTGGACTGGGTTTTTGCTGACGGCCCACCAGGGAGT 3120
K0326Y     3061 GTCGTGCCTGAAAGAACATATGTATTGGACTGGGTTTTTGCTGACGGCCCACCAGGGAGT 3120

AF023159   3121 GCAAGGAATTATGACAACAATGGAGGACATGATTTTCATGCTACCCTTCCAAATAACATG 3180
K0326Y     3121 GCAAGGAATTATGACAACAATGGAGGACATGATTTTCATGCTACCCTTCCAAATAACATG 3180

AF023159   3181 ACTGAGGAAGAGTATTGGATGGAAGAAGAACAAAGGATCTATACAAGGCTTCAACAAGAG 3240
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AF023159   3241 AGGAGGGAAAGGGAGGAGGCTATTAAAAGGAAGGCTGAGAGAAATGCAAAAATGAAAGCT 3300
K0326Y     3241 AGGAGGGAAAGGGAGGAGGCTATTAAAAGGAAGGCTGAGAGAAATGCAAAAATGAAAGCT 3300

AF023159   3301 GAGATGAAGGAAAAGACTATGAGAATGTTCCTGGTTTCTCAGAAACACATTGTTTACACC 3360
K0326Y     3301 GAGATGAAGGAAAAGACTATGAGAATGTTCCTGGTTTCTCAGAAACACATTGTTTACACC 3360


AF023159   3361 GAACCACTTGAAATACATGCTGGAACTACTATTGATGTGCTTTATAATCCTTCTAATACA 3420
K0326Y     3361 GAACCACTTGAAATACATGCTGGAACTACTATTGATGTGCTTTATAATCCTTCTAATACA 3420

AF023159   3421 GTTCTAACTGGAAAGCCAGAGGTTTGGTTTCGATGTTCCTTTAATCGTTGGATGTATCCA 3480
K0326Y     3421 GTTCTAACTGGAAAGCCAGAGGTTTGGTTTCGATGTTCATTTAATCGTTGGATGTATCCA 3480

AF023159   3481 GGTGGGGTGTTGCCACCTCAGAAGATGGTACAAGCAGAAAATGGTTCACACCTAAAAGCA 3540
K0326Y     3481 GGTGGGGTGTTGCCACCTCAGAAGATGGTACAAGCAGAAAATGGTTCACACCTAAAAGTA 3540

AF023159   3541 ACAGTTTACGTTCCACGAGATGCCTATATGATGGACTTCGTTTTCTCGGAGTCAGAAGAA 3600
K0326Y     3541 ACAGTTTACGTTCCACGAGATGCCTATATGATGGACTTTGTTTTCTCGGAGTCAGAAGAA 3600

AF023159   3601 GGTGGAATTTATGATAACAGAAATGGGTTAGACTATCATATTCCTGTTTTTGGGTCAATT 3660
K0326Y     3601 GGTGGAATTTATGATAACAGAAATGGGTTAGACTATCATATTCCTGTTTTTGGGTCAATT 3660

AF023159   3661 GCAAAGGAACCACCTATGCACATTGTCCACATTGCTGTTGAGATGGCACCAATCGCAAAG 3720
                 GCAAAGGAACCACCTATGCACATTGTCCACAT GCTGTTGAGATGGCACCAATCGCAAAG
K0326Y     3661 GCAAAGGAACCACCTATGCACATTGTCCACATCGCTGTTGAGATGGCACCAATCGCAAAG 3720

AF023159   3721 GTTGGAGGTCTTGGTGATGTTGTCACTAGTCTTTCACGTGCTGTGCAAGATTTAGGACAC 3780
                 GTTGGAGGTCTTGGTGATGTTGTCACTAGTCTTTCACGTGCTGTGCAAGATTTAGGACAC
K0326Y     3721 GTTGGAGGTCTTGGTGATGTTGTCACTAGTCTTTCACGTGCTGTGCAAGATTTAGGACAC 3780
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AF023159   3781 AATGTGGAGGTTATTCTTCCAAAGTACGGTTGCTTGAATCTAAGCAATGTCAAGAATCTA 3840
                AATGTGGAGGTTATTCTTCCAAAGTACGGTTGCTTGAATCTAAGCAATGTCAAGAATCTA
K0326Y     3781 AATGTGGAGGTTATTCTTCCAAAGTACGGTTGCTTGAATCTAAGCAATGTCAAGAATCTA 3840

AF023159   3841 CAAATCCATCAGAGTTTTTCTTGGGGTGGTTCTGAAATAAATGTGTGGCGTGGACTAGTC 3900
K0326Y     3841 CACCATCCATCAGAGTTTTTCTTGGGGTGGTTCTGAAATAAAAGTGTGGCGTGGACTAGTC 3900

AF023159   3901 GAAGGCCTTTGTGTTTACTTCCTGGAACCTCAAAATGGGATGTTTGGAGTCGGATATGTA 3960
K0326Y     3901 GAAGGCCTTTGTGTTTACTTCCTGGAACCTCAAAATGGGATGTTTGGAGTCGGATATGTA 3960

AF023159   3961 TATGGCAGGGACGATGACCGCCGATTTGGCTTCTTCTGTCGTTCTGCTCTAGAGTTTCTC 4020
K0326Y     3961 TATGGCAGGGACGATGACCGCCGATTTGGCTTCTTCTGTCGTTCTGCTCTAGAGTTTCTC 4020

AF023159   4021 CTCCAAAGTGGATCTTCTCCGAACATAATACATTGCCATGATTGGTCAAGTGCTCCTGTT 4080
K0326Y     4021 CTCCAAAGTGGATCTTCTCCTAACATAATACATTGCCATGATTGGTCAAGTGCTCCTGTT 4080

AF023159   4081 GCCTGGCTACACAAGGAAAACTACGCGAAGTCTAGCTTGGCAAACGCACGGGTGGTATTC 4140
K0326Y     4081 GCCTGGCTACACAAGGAAAACTACGCGAAGTCTAGCTTGGCAAATGCACGGGTGGTATTC 4140

AF023159   4141 ACCATCCACAATCTTGAATTTGGAGCGCATCATATTGGCAAAGCAATGAGATATTGTGAT 4200
K0326Y     4141 ACCATCCACAATCTTGAATTTGGAGCGCATCATATTGGCAAAGCAATGAGATATTGTGAT 4200

AF023159   4201 AAAGCAACAACTGTCTCTAATACATATTCAAAGGAAGTGTCAGGTCATGGTGCCATAGTT 4260
K0326Y     4201 AAAGCCACAACTGTCTCTAATACATATTCAAAGGAAGTGTCAGGTCATGGTGCCATCGTT 4260

AF023159   4261 CCTCATCTTGGGAAATTCTATGGCATTCTCAATGGAATTGATCCGGATATATGGGATCCG 4320
K0326Y     4261 CCTCATCTTGGGAAATTCTATGGCATTCTGAATGGAATTGATCCAGATATATGGGATCCG 4320

AF023159   4321 TACAATGACAACTTTATCCCGGTCCACTACACTTGTGAGAATGTGGTTGAAGGCAAGAGG 4380
K0326Y     4321 TACAATGACAACTTTATCCCGGTCCACTACACTTGTGAGAATGTGGTTGAAGGCAAGAGG 4380

AF023159   4381 GCTGCTAAGAGGGCACTGCAGCAGAAGTTTGGGTTACAGCAAATCGATGTCCCCGTCGTA 4440
K0326Y     4381 GCTGCTAAGAGGGCACTGCAGCAGAAGTTTGGGTTACAGCAAATCGATGTCCCCGTCGTA 4440

AF023159   4441 GGAATCGTCACTCGCCTGACAGCCCAAAAGGGGATCCACCTGATCAAGCATGCGATTCAC 4500
K0326Y     4441 GGAATCGTCACTCGCCTGACAGCCCAAAAGGGGATCCACCTGATCAAGCATGCGATTCAC 4500

AF023159   4501 CGTACACTCGAACGGAACGGACAGGTGGTTTTGCTTGGTTCAGCGCCGGACTCTCGAATC 4560
K0326Y     4501 CGTACACTCGAACGGAACGGACAGGTGGTTTTGCTTGGTTCAGCGCCGGACTCTCGAATC 4560

AF023159   4561 CAAGCTGATTTTGTCAACCTGGCGAATACGCTCCACGGCGTAAACCATGGGCAAGTGAGG 4620
K0326Y     4561 CAAGCTGATTTTGTCAACCTGGCGAATAAGCTCCACGGCGTAAACCATGGGCAAGTGAGG 4620

AF023159   4621 CTTTCCTTGACCTACGACGAGCCTCTCTCGCATCTGATATACGCTGGCTCTGACTTCATT 4680
K0326Y     4621 CTTTCCTTGACCTACGACGAGCCTCTCTCGCATCTGATATACGCTGGCTCTGACTTCATT 4680

AF023159   4681 CTGGTCCCATCTATATTTGAGCCTTGCGGCCTAACTCAGCTCGTCGCCATGCGGTATGGA 4740
K0326Y     4681 CTGGTCCCATCTATATTTGAGCCTTGCGGCCTAACTCAGCTCGTCGCCATGCGGTATGGG 4740

AF023159   4741 ACCATCCCGATTGTCCGCAAGACTGGAGGGCTCTTCGACACTGTCTTCGATGTGGACAAT 4800
K0326Y     4741 ACCATCCCGATTGTCCGCAAGACTGGAGGGCTCTTCGACACTGTCTTCGATGTGGACAAT 4800

AF023159   4801 GACAAGGAACGAGCCCGAGATCGAGGCCTTGAGCCCAACGGGTTTAGCTTTGACGGAGCT 4860
K0326Y     4801 GACAAGGAACGAGCCCGAGATCGAGGCCTTGAGCCCAACGGGTTTAGCTTTGACGGAGCT 4860

AF023159   4861 GATAGCAACGGTGTTGACTACGCGCTGAACAGGGCGATCTCAGCTTGGTTCGATGCCCGG 4920
K0326Y     4861 GATAGCAACGGTGTTGACTACGCGCTGAACAGGGCGATCTCAGCTTGGTTCGATGCCCGG 4920

AF023159   4921 AGCTGGTTCCACTCCCTTTGCAAGAGAGTCATGGAGCAGGACTGGTCGTGGAACCGACCT 4980
K0326Y     4921 AGCTGGTTCCACTCCCTTTGCAAGAGAGTCATGGAGCAGGACTGGTCGTGGAACCGACCT 4980

AF023159   4981 GCCCTCGACTACATCGAGCTCTACCGTTCAGCGTCCAAATTGTAA 5025
K0326Y     4981 GCCCTCGACTACATCGAGCTCTACCGTTCAGCGTCCAAATTGTAA 5025
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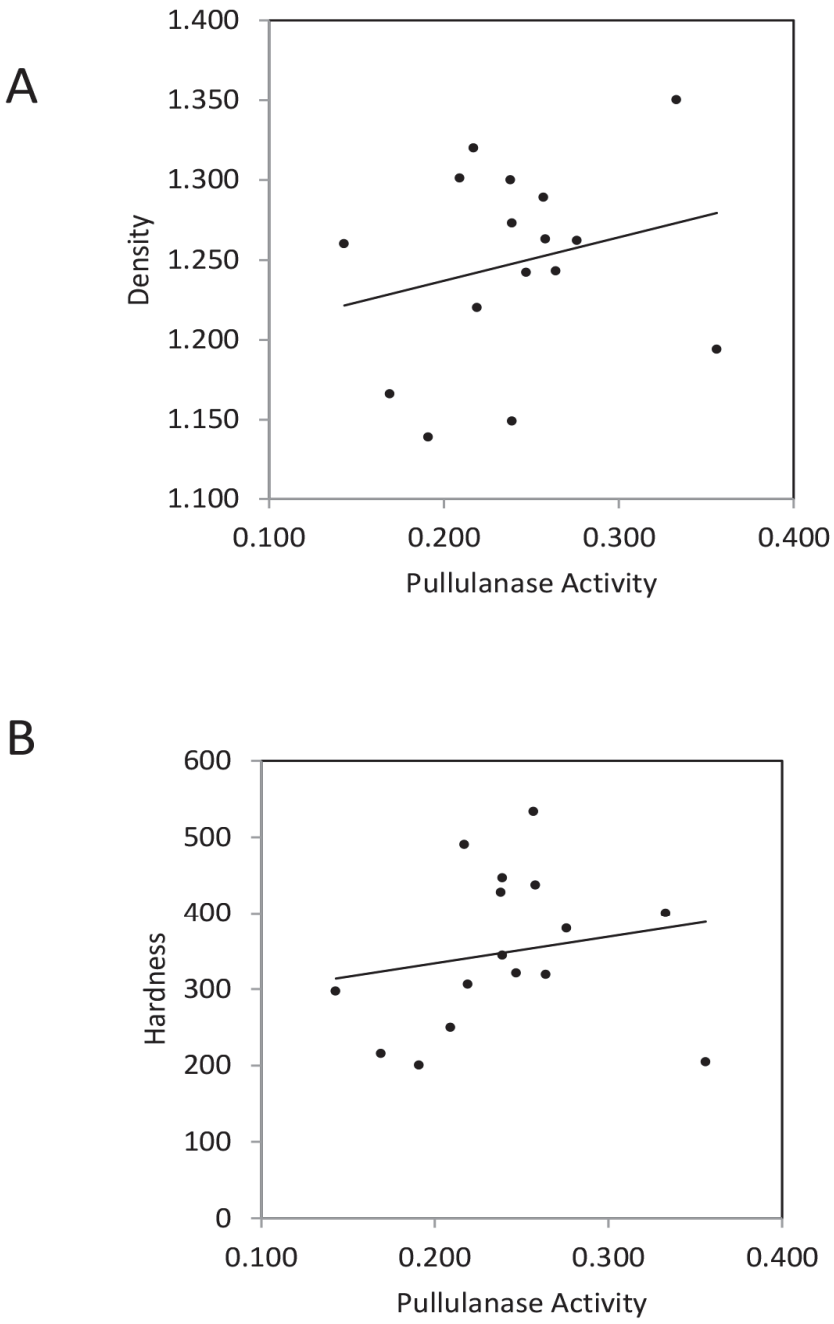

Figure S2-1 Correlation between kernel density and hardness and pullulanase activity. (A) No significant correlation was found between kernel density and pullulanase activity, tested by ANOVA of slope ($p>0.05$). (B) No significant correlation was found between kernel hardness and pullulanase activity tested by ANOVA of slope ($p>0.05$).

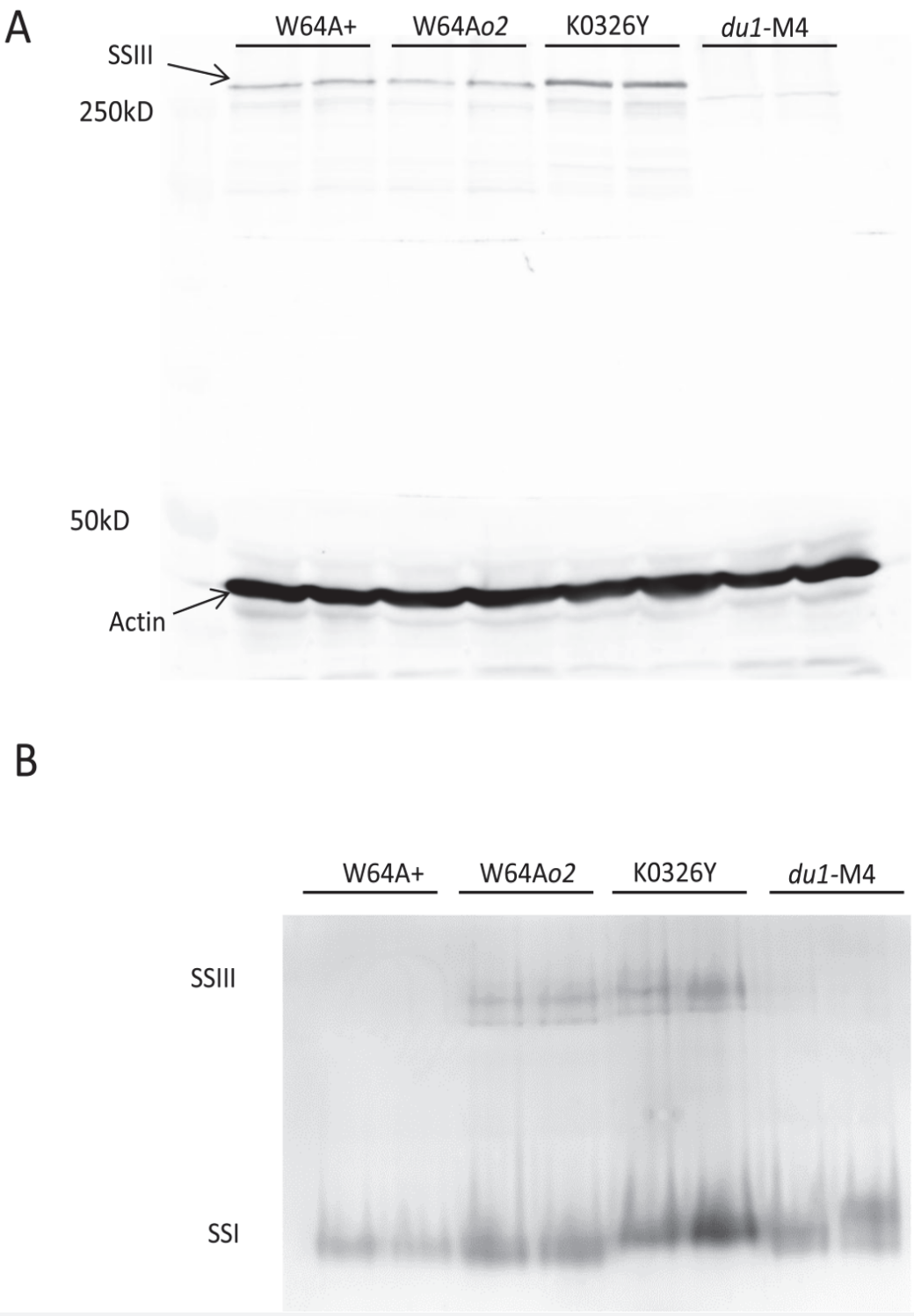

Figure S2-2 Full gel images of SSIII abundance and SSIII activity of W64A+, W64Ao2, K0326Y and *du1-M4*.

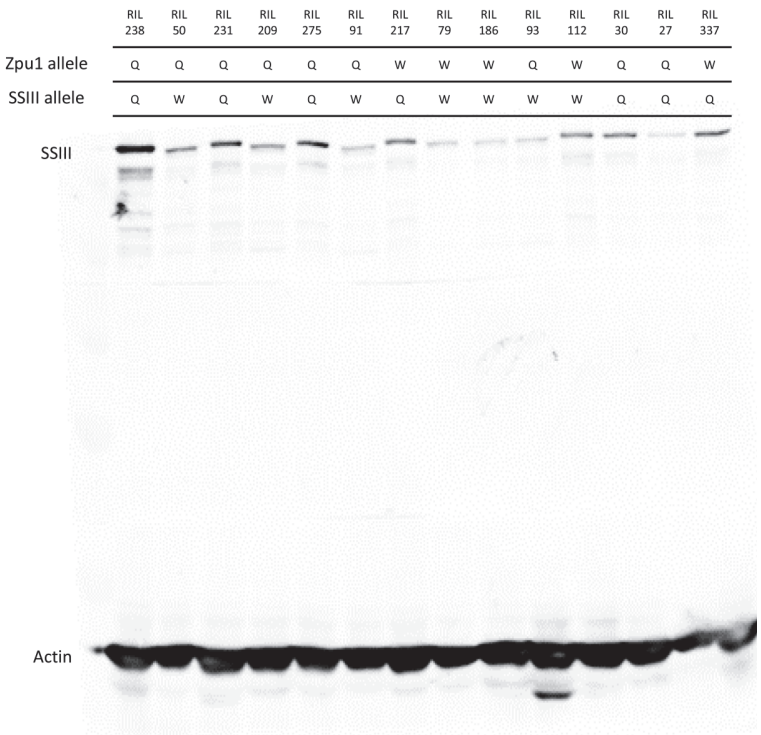

Figure S2-3 Full gel images for western blot of SSIII among RILs homologous for W64Ao2 (W) or QPM (Q) – derived *Zpu1* or *SSIII* alleles.

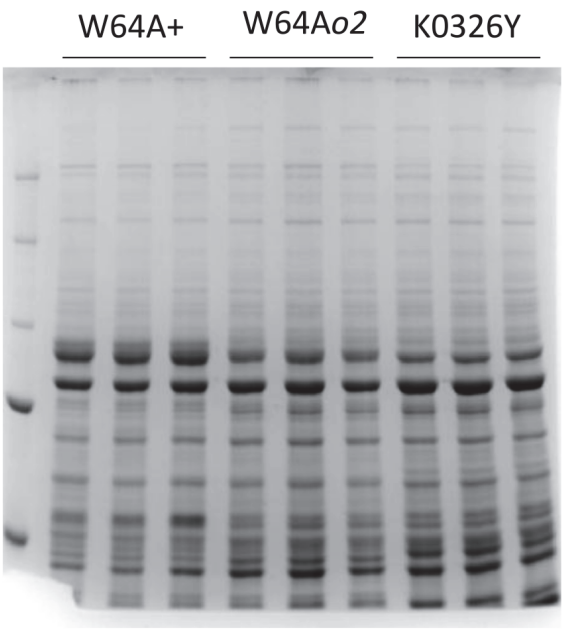

Figure S2-4 Full SDS-PAGE gel image of crude protein extracts from endosperms of parent lines, W64A+, W64Ao2 and K0326Y.

Table S2-1 *Zpu1* sequencing primers

Name	Sequence	Start	End	Product Size
*Zpu1*_1F	ACCGCCTTCTCTCTCCCTCCGA	-40*	276	317
*Zpu1*_1R	CACCCAGTAAGCCCTCGCATCCA			
*Zpu1*_5'F	CCCAAAGGGTGCGTCCCGTC	146	501	356
*Zpu1*_5'R	GGCAACATCAACGGAGCTCGGAA			
*Zpu1*_2F	GCCCAAAGGGTGCGTCCCGT	185	1000	816
*Zpu1*_2R	GCCGCCAATCCATCCCAGGCA			
*Zpu1*_3F	TCGCCTACACTGGACCGCTTG	642	1365	724
*Zpu1*_3R	GGAACGCCCCAAACCACAGGG			
*Zpu1*_4F	GCCTGGGATGGATTGGCGGCT	980	2040	1061
*Zpu1*_4R	TGGTCAGCGTATGTAGCAAGCGT			
*Zpu1*_5F	ACCCTGTGGTTTGGGGCGTT	1344	2464	1121
*Zpu1*_5R	GCTCCCTTCGTTCTTTTCTCTTGGTGG			
*Zpu1*_6F	CAGTTGGCTATGCTTCATCCCCT	2142	2904	763
*Zpu1*_6R	GCTGCTGTCCTTTTCGGCACG			
*Zpu1*_3'F	CACAGGGCCCTCCTTGGTTCC	2632	2965	334
*Zpu1*_3'R	TGCCGAATGCACTTGCTCGCT			

* The primer *Zpu1*_1F starts from 5’ promoter region

CHAPTER THREE

Bacterial Cloning and Expression of *Zpu1* Alleles Derived from W64A and QPM Alleles

Abstract

Five types of pullulanase were identified in Bacteria and Archaea, and they are starch debranching enzymes that hydrolyze pullulan. Systematic studies of 46 species, including bacteria and plants, revealed that the amino acid sequences of plant pullulanase were closer to most Gram negative bacteria than Gram positive bacteria. Sequence alignments of conserved regions of plant pullulanase showed that maize pullulanase had most similar sequence to the putative $(\beta/\alpha)_8$-barrel structure identified previously in barley, indicating that maize pullulanase was most likely to form an $(\beta/\alpha)_8$-barrel structure to catalyze the reaction with pullulan. *Zpu1* genes derived from W64A allele (from W64Ao2) and QPM allele (from K0326Y) were cloned and expressed in competent *Escherichia coli*. The target SDS-PAGE bands in elute samples after partial purification were processed by in-gel digestion and identified by Mass Spectrometry, which confirmed that the target bands were expected pullulanase. The time-course zymogram assay of partially purified pullulanase showed that the pullulanase encoded by W64A-derived *Zpu1* allele had greater reaction rate than the pullulanase encoded by QPM-derived *Zpu1* allele, which could be due to the one amino acid change in the C-terminal region of the pullulanase. However, this amino acid change was not located in $(\beta/\alpha)_8$-barrel domain, suggesting that C-terminal region might also regulate the reaction rate of pullulanase.

Introduction

Pullulanase was discovered in *Klebsiella pneumonia* isolated from media with pullulan as the only carbon source (Bender and Wallenfels, 1961; Michaelis et al., 1985). As an important type of starch debranching enzyme that hydrolyzes pullulan, amylopectin and other oligosaccharides, it was widely used in food industry (Contesini et al., 2013; Norman, 1982). Combined with β-amylase or glucoamylase, pullulanase played an essential role in saccharification of starch, in order to produce high-glucose or high-maltose syrups (Hii et al., 2012). Pullulanase also helped the preparation of starch to a form resistant to α-amylase hydrolysis. This form of starch was called resistant starch, which was nutritionally superior to normal starch because it was associated with reducing plasma glucose and increasing short-chain fatty acid production in large intestine (Zhang and Jin, 2011).

So far, five types of pullulanase have been identified. Type I pullulanase (EC. 3.2.1.41) was relatively well-characterized, and it mainly hydrolyzes α-1,6-glycosidic bond in pullulan producing maltotriose (Kuriki et al., 1988) Apart from *Klebsiella*, type I pullulanase isoforms were also discovered in several thermophilic bacteria, such as *Thermotoga neapolitana*, *Bacillus thermoleovorans*, *Fervidobacterium pennavorans* and etc. (Hope and Dean, 1974; Kang et al., 2011; Messaoud et al., 2002; Bertoldo et al., 1999). Type II pullulanase (also called amylopullulanase) (EC. 3.2.1.41) has two domains that can specifically hydrolyze α-1,6 and α-1,4-glycosidic bond in pullulan and some other polysaccharides, and produce not only maltotriose, but glucose and maltose as well (Motherway et al., 2008). This type of pullulanase was discovered from some thermophilic Archaea., such as *Thermococcus litoralis, Thermococcus hydrothermalis, Pyrococcus*

furiosus ,etc. (Brown and Kelly, 1993; Erra-Pujada et al., 1999; Dong et al., 1997) The pullulanase in those organisms was heat-stable and the optimum temperature can reach up to 90^{o}C, which played a vital role to improve the industrial starch bioconversion process, including gelatinization and saccharification (Bertoldo and Antranikian, 2002; Niehaus et al., 1999). Pullulan hydrolase type I (EC. 3.2.1.135) was first discovered as a novel α-amylase that specifically hydrolyzes α-1,4 glycosidic bonds of pullulan to produce panose from *Thermoactinomyces vulgari,* a thermophilic bacteria (Shimizu et al., 1978). Pullulan hydrolase type II (EC. 3.2.1.57) discovered from fungus *Aspergillus niger* also specifically hydrolyzes α-1,4 glycosidic bonds of pullulan, and forms isopanose (Sakano et al., 1971). And recently, a unique pullulan hydrolase (pullulan hydrolase type III) (EC. not available) was discovered from thermophilic archaea *Thermococcus aggregans*, could hydrolyze both α-1,4 and α-1,6 glycosidic bonds in pullulan, as well as amylopectin, producing a mixture of maltotriose, panose, maltose and glucose (Niehaus et al., 2000). Figure 3-1 summarized the action of those types of pullulanase on their substrate pullulan (Bertoldo and Antranikian, 2002).

In plants, pullulanase (EC 3.2.1.41) was also named R-enzyme (Manners, 1997). In 1950, Peat's group isolated R-enzymes that can hydrolyze the branch linkages in amylopectin and β-limit dextrin, when doing the preparation of Q-enzyme (or starch branching enzyme) in beans and potatoes (Hobson et al., 1950, Nakamura, 1996). In recent years, pullulanase in many other plants was isolated and characterized, and its function was often associated with isoamylase, another type of starch debranching enzyme. In 1996, a pullulanase was isolated from rice (*Oryza sativa*), and its gene was mapped on chromosome 4 (Nakamura et al., 1996). The pullulanase-deficient mutant did

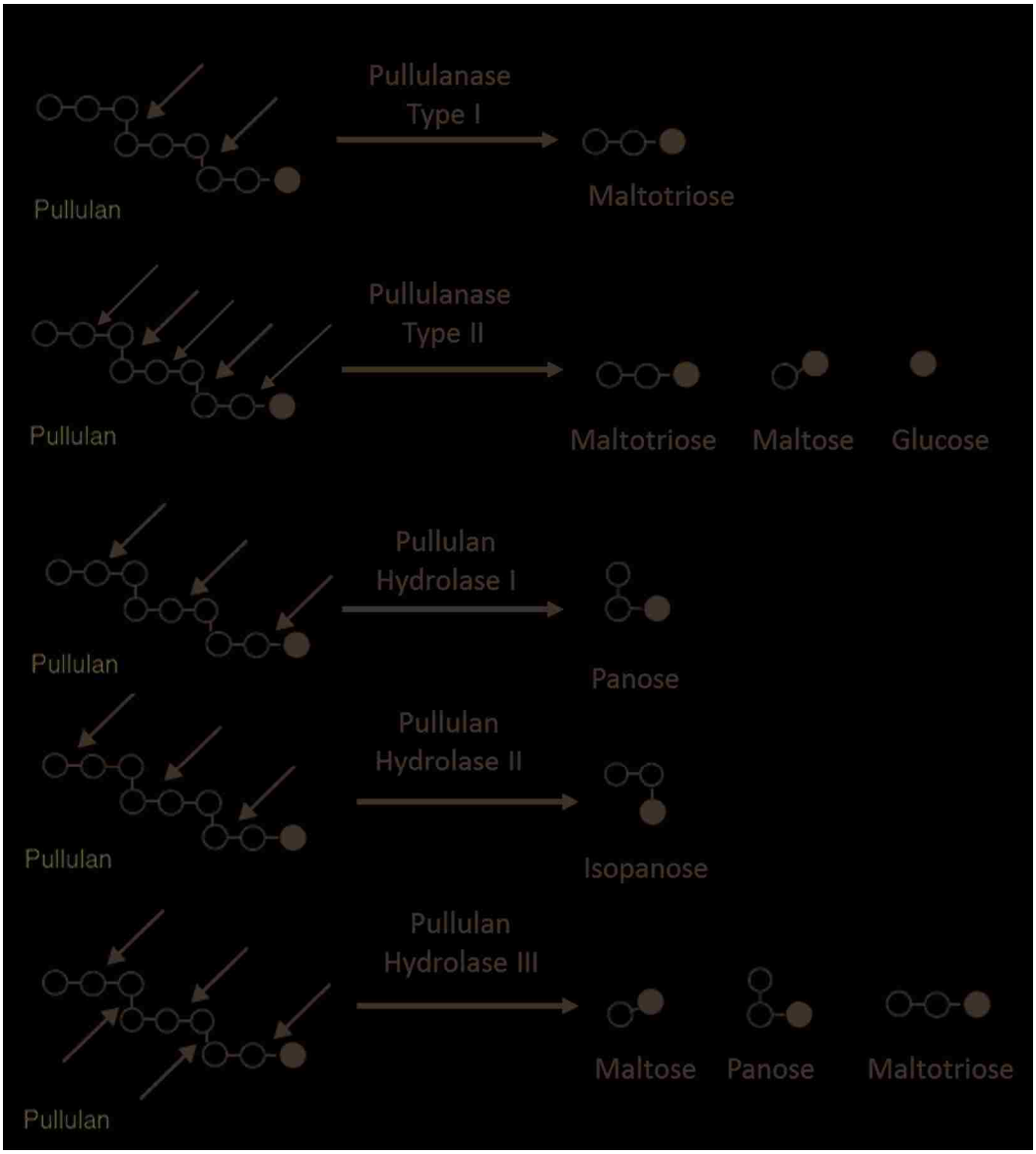

Figure 3-1. Five types of pullulanase and their action on the substrate pullulan (Bertoldo and Antranikian, 2002). The arrows point at the linkages on pullulan that the pullulanase may attack. The black (solid) ovals represent reducing glucopyranosyl units, and white (hollow) ovals represent non-reducing glucopyranosyl units.

not show significant effect on the activities of other starch biosynthetic enzymes, but compared with the wild type, the proportion of short chains (DP<13) of amylopectin in the mutants was increased, whereas the abundance of water-soluble polysaccharide (WSP) remained the same (Fujita et al., 2009). However, another study in a rice *sugary-1* mutant

background revealed that pullulanase could compensate for the role of isoamylase in amylopectin branching constructions (Kubo et al., 1999). Mutational studies of starch debranching enzymes in Arabidopsis leaves showed that three types of isoamylase and one type of pullulanase were functionally overlapped. In particular, Arabidopsis pullulanase has both starch synthetic activity and debranching activity partially redundant to isoamylase type I (ISA1) and type III (ISA3), respectively (Wattebled et al., 2008). Maize pullulanase was purified and characterized, and amino acid sequence alignments of pullulanase and isoamylase showed six motifs conserved between the two types of starch debranching enzymes, suggesting that they might function coordinately (Beatty et al., 1999). The study of pullulanase/isoamylase double mutants (*su1-st/Zpu1-204*) also revealed the functional association between the two types of starch debranching enzymes, which pullulanase may partially compensate for the defect in isoamylase. (Dinges et al., 2003)

In barley (*Hordeum vulgare*), pullulanase was usually named limit dextrinase, because its natural substrate was limit dextrin, not pullulan (Burton et al., 1999). Sequence alignments of the barley pullulanase sequence with amylolytic enzymes from other species revealed a conserved catalytic $(\beta/\alpha)_8$-barrel domain, with the active site at the C-terminal end of the β-barrel (Kristensen et al., 1999) The maize pullulanase amino acid sequence showed 11 conserved motifs according to the multiple alignment with the sequences from other species (Beatty et al., 1999). However, more structural studies need to be done in order to draw the relationship between those motifs. Previous data (see Chapter 2) showed that pullulanase from Quality Protein Maize (QPM) had one amino acid difference at C-terminal end, compared with the one from *opaque-2* (*o2*) lines. In this study, we cloned

and expressed the *Zpu1* gene (encoding pullulanase) derived from W64A*o2* (an *o2* line with W64A background) and K0326Y (a QPM inbred line) in *E. coli*, in order to explore if the alternation of one amino acid could influence the activity and properties of the pullulanase.

Materials and Methods

Gene*tic Materials*

All maize lines in the study were grown and harvested in summer, 2013 in Elm Mott, TX. The lines used in the study were W64A*o2* (an opaque 2 line with W64A background) and K0326Y (a tropical QPM inbred line). The *Escherichia coli* strain that hosted the recombinant plasmid was NEB 10-beta Electrocompetent *E. coli,* and its genotype is *(Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC))* (NEB, Ipswich, MA). The *E. coli* strain that expressed the recombinant plasmid is E. cloni® EXPRESS BL21(DE3) Competent Cells and its genotype is *(F– ompT hsdSB (rB- mB-) gal dcm lon λ(DE3 [lacI lacUV5-T7* gene *1 ind1 sam7 nin5]))* (Lucigen, Middleton, WI)

Sequence Alignments and Systematic Analysis of Pullulanase among Species

Amino acid sequences from 46 species, including 18 plants and 28 bacteria, were downloaded from GenBank, and their accession numbers and related information were listed at Table S3-1. Sequence alignments were performed and distance trees were established via Geneious (Biomatters, Auckland, New Zealand). Nine plant species (*Arabidopsis thaliana, Hordeum vulgare, Oryza sativa, Populus trichocarpa, Spinacia oleracea, Theobroma cacao, Triticum urartu, Zea Mays* line K0326Y*, and Zea Mays* line

W64A*o2*), whose pullulanase was well-characterized were selected, and sequence alignment was also performed via Geneious in order to reveal putative homologous domain conserved throughout those plants. The amino acid sequences of $(\beta/\alpha)_8$-barrel domains were extracted from the alignment data and combined, in order to predict if maize pullulanase had similar domains as barleys did.

Genomic DNA Extraction and Purification from W64Ao2 and K0326Y Endosperm

Developing endosperms (18 DAP) were grinded and homogenized in 300 μL of cetyltrimethylammonium bromide (CTAB) extraction buffer (100mM Tris-HCl, pH 7.5, 700mM NaCl, 50mM EDTA, pH 8.0, 1% CTAB (w/v) and 140mM β-mercaptoethanol). The homogenized samples were then incubated in 65ºC water bath for 1 hour. 400 μL of chloroform: isoamyl alcohol (24:1) was added in the samples, followed by centrifugation at 16,000Xg for 20 min at 4ºC. The supernatant was transferred into 300 μL of isopropanol, followed by centrifugation at 16,000Xg for 20 min at 4ºC. The pellet was washed with 70% ethanol for three times by centrifugation at 16,000Xg for 10 min at 4ºC each time. The pellet with purified DNA was dissolved in 100 μL diH_2O after air dry for 20 min.

PCR Amplification of Three Zpu1 Gene *Fragments*

Since first 205 bp at 5'- end of *Zpu1* gene doesn't contain any intron, but it was difficult to obtain through reverse transcription of RNA, this fragment could be amplified by PCR using Phusion® High-Fidelity (NEB, Ipswich, MA) system with corresponding genomic DNA as template (2 μL), Phusion® HF buffer (10 μL), dNTP (1 μL), each primer (1 μL) (*Zpu1*_F_XhoI and *Zpu1*_1R, see their sequences at Table S3-2), Phusion® High-Fidelity DNA Polymerase (0.5 μL), 100% DMSO (1.5 μL), and diH_2O (to 50 μL). The

reaction condition was set as pre-denaturation 98ºC 30s, denaturation 98 ºC 10s, annealing 69 ºC 25s, extension 72 ºC 1 min30sec, final extension 72ºC 10min, and the cycle number was set as 35. The product (Fragment #1) was 929 bp with XhoI site at 5'- end (position 16 on the 929 bp fragment) and NcoI site at position 200 (on the 929 bp fragment).

Next two fragments (#2 and #3) were synthesized based on *Zpu1* cDNA template reversed transcribed from RNA, which were extracted and isolated from frozen developing endosperm of W64Ao2 and K0326Y (18 DAP) following the conventional procedure according to Jia et al. (2013). The cDNA was synthesized using ImProm-II™ Reverse Transcription System (Promega Corp. Madison, WI) based on the manufacturer's instructions. Two *Zpu1* gene fragments were synthesized in 25 μL reaction system with 5 μL of Phusion® HF Buffer, 0.5 μL of dNTP, 0.5 μL of each primer (*Zpu1*_2F and *Zpu1*_2R_BstBI for 815 bp fragments; *Zpu1*_3F and *Zpu1*_3'R_BstBI for 2324 bp fragments, see their sequences at Table S3-2), 0.25 μL of Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, MA), 0.75 μL of 100% DMSO, 2 μL of cDNA and up to 25 μL diH_2O. The reaction condition was set as pre-denaturation 98ºC 30s, denaturation 98 ºC 10s, annealing 69 ºC 25s, extension 72 ºC 1 min/1kb, final extension 72ºC 10min, and the cycle number was set as 35. The fragment #2 was 815bp with restriction sites NcoI and BstBI at 5' and 3' end, respectively, and a PvuII site at the middle. And the fragment #3 was 2324bp with PvuII site and BstBI site at 5' and 3' end, respectively.

Plasmid Constructions for Cloning and Expression of Zpu1 Gene*s*

pRSET-A plasmid (Life Technologies, Grand Island, NY) was used to clone and express *Zpu1* gene. Three steps were involved to construct a recombinant plasmid inserted with full-length *Zpu1* gene. 1) Both pRSET-A and Fragment #2 were digested with NcoI

and BstBI and ligated with T4 DNA ligase. 2) Both the recombinant plasmid and Fragment #1 were digested with XhoI and NcoI followed by ligation with T4 DNA ligase. 3) Then both the new recombinant plasmid and Fragment #3 were digested with PvuII and BstBI followed by ligation with T4 DNA ligase. All restriction enzymes and T4 DNA ligase were from NEB (Ipswich, MA). Recombinant plasmid generated from each step was subject to transformation into NEB 10-beta Electrocompetent *E. coli* (Ipswich, MA), which was then incubated on LB plates with 100 μg/mL of ampicillin at 37ºC overnight. The positive colonies was selected and inoculated in 10 mL of LB media with 100 μg/mL of ampicillin, and incubated with shaking at 37ºC overnight. The plasmid was extracted and purified from LB culture using EZNA® Plasmid DNA Mini Kit (Omega Bio-Tek, Inc. Norcross, GA) following the manufacturer's instruction. The recombinant plasmids with full-length W64Ao2 or K0326Y *Zpu1* gene were sent to Macrogen Inc. (Rockville, MD) for sequencing, in order to confirm if the full-length ORF was in frame with upstream 6X his-tag and if there was any mismatch, deletion mutants, insertion mutants or frame-shifts in the sequences. The primers for sequencing were listed in Table S3-2.

Bacterial Expression and Partial Purification of Pullulanase

The recombinant pRSET-A with full-length *Zpu1* gene was transformed into E. cloni® EXPRESS BL21(DE3) Competent Cells (Lucigen, Middleton, WI) following the manufacturers instruction, and incubated on LB plate with 100 μg/mL of ampicillin at 37ºC overnight. The positive colonies was selected and inoculated in 10 mL of LB media with 100 μg/mL of ampicillin, and incubated with shaking at 37ºC overnight. The overnight culture was then subcultured at a 1:10 dilution into LB media with 100 μg/mL of ampicillin, as well as 1M of sorbitol to reduce the chance of target proteins entering the inclusion

bodies. The LB-sorbitol culture was incubated with shaking at 37°C until the OD600 (absorbance at 600nm) reached to 0.8-0.9 (approximately 7-8 hours). The expression of *Zpu1* gene was induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the final concentration of 1 mM, and the culture was incubated with shaking at 25°C for 18 hours. The bacterial cells were harvested by centrifugation at 5,000 X g at 4°C for 15 min. The pellet was resuspended with 10 mL of sonication buffer (Tris-HCl, pH7.5, 150 mM NaCl, 0.1% Triton X-100) (Wu et al. 2002) and lysed by sonication. Cell lysates were centrifuged at 30,000Xg for 20 min at 4°C. The pullulanase in the supernatant was purified by affinity chromatography through Thermo Scientific HisPur™ Ni-NTA Resin (Life Technologies, Grand Island, NY) following the manufacturer's protocol.

SDS-PAGE, Western Immunoblotting, and Zymogram

Purified pullulanase was diluted with diH_2O to total protein concentration of 10 mg/mL before being loaded onto a 7.5% denaturing gel with 0.4% [w/v] SDS. SDS-PAGE was performed at room temperature for 1.5 hours at 200V. Then the gel was transferred onto nitrocellulose sheets using conventional methods (Towbin et al., 1979). The antiserum used in western immunoblotting was 6x-His Epitope Tag Antibody (Thermo Fisher Scientific Inc., Rockford, IL) at 1:2,500 TBST dilution. The secondary anti-serum was HRP-Goat anti-Mouse with 1:30,000 TBST dilution (Life Technologies, Grand Island, NY). Bands were visualized on ImageQuant LAS 4000 imager (GE Healthcare, Piscataway, NJ). For zymogram analysis, purified pullulanase were diluted with diH_2O to a total protein concentration of 10 mg/mL before being loaded onto a 7.5% native polyacrylamide gel with 0.6% red pullulan (Megazyme, Wicklow, Ireland). Native PAGE was performed at 4° C for 4 hours at 120V. The gel then was incubated in pullan gel

incubation buffer (Tris 25 mM, pH 8.2, Glycine 192 mM, DTT 1 mM) for 0 up to 7 hours at 25° C and photographed every 30 min on Gel Doc™ EZ Imager (Bio-Rad Laboratories, Inc., Hercules, CA) except for last three data points (1 hour interval). The gel images were processed by Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA) to equilibrate the background exposure, and the band clearness was then measured by gray values that reflected the pullulanase activities. The t test for the gray values on each time point was conducted by JMP (Version Pro 9.0, SAS Institute Inc., Cary, NC).

Quantitative Pullulanase Activity Assay

Purified pullulanase was diluted with diH_2O to make the final concentration 10 mg/mL and the final volume 50 μL. Red-pullulan solution with 2% [w/v] pullulan (Megazyme International. Wicklow, Ireland) and 50 mM KCl, and diluted pullulanase solution were pre-equilibrated at 37 ˚C for 5 min. Then 50 μL of purified pullulanase solution was mixed with 50 μL of red pullulan solution. The mixture was incubated at 37 ˚C for 3 hours for pullulanase digestion. 25 μL of the reaction mixture was taken out every hour and was mixed with 125 μL of 100% ethanol to precipitate undigested red-pullulan molecules for 10 min. The precipitate was spun down at 16,000 X g for 10 min at room temperature. The supernatant, containing the ethanol-soluble small dyed oligosaccharides, was collected and 80 μL of the supernatant was transferred into a 96-well plate, and absorption measured at a wavelength of 490 nm.

Mass Spectrometry

To prepare the samples for mass spectrometry, the bands of pullulanase (W64Ao2 and K0326Y) were excised, trypsin digested and desalted using In-Gel Tryptic Digestion

Kit (Life Technologies, Grand Island, NY) following the manufacturer's instruction. The processed samples were sent to Mass Spectrometry Center of Baylor University to run the liquid chromatography--tandem mass spectrometry (LC-MS/MS). The samples were separated by liquid chromatography followed by ionization, then they were processed by MS in low energy mode followed by high energy mode.

The data was analyzed by MassLynx Mass Spectrometry Software (Waters Corp., Milford, MA) and the peak values of the mass spectra were compared with the predicted m/z values generated by ProteinProspector V5.14.1 (University of California San Francisco, San Francisco, CA). Since ProteinProspector only compared the m/z value of single charged substances, the m/z values of double charged substances (peak values on the mass spectrum) in the samples needed to be converted to single charged m/z values by the formula: Single charged m/z = (Double charged m/z X 2) -1. In low energy mode, the m/z value of each putative peptide was matched with the predicted value generated by MS-Digest Tool in ProteinProspector, whereas in high energy mode, the m/z value of each charged small fraction dissociated from the putative peptide was matched with the predicted value in Theoretical Peak Table generated by MS-Product Tool in ProteinProspector.

Results

Systematic Studies of Gene*s Encoding Pullulanase among Species*

Multiple alignment of pullulanase (or limit dextrinase) amino acid sequences was performed for 28 bacteria species and 18 plant species (Figure S3-1). The distance tree was built according to the alignment. There were three clusters shown in the tree (Figure 3-2A).

Most species in the first cluster was Gram positive bacteria, except *Thermotoga maritime* (Tm) and *Francisella philomiragia*, (Fp) which were Gram negative bacteria. All species in the second cluster were Gram negative bacteria and all species in the third cluster were plants. The tree revealed that the amino acid sequences of pullulanase were relatively conserved within each taxon, and the pullulanase gene of most Gram negative bacteria was closer to plants than Gram positive bacteria. In the full alignment figure (Figure S3-1), the outliers were all from the first cluster. The alignment between plants and most Gram negative bacteria revealed that the pullulanase of Gram negative bacteria were generally longer at N-terminal or/and C-terminal region than the pullulanase of plants (Figure S3-1).

The pullulanase of 9 out of 18 plant species have been previously characterized (others were putative sequences) (Table S3-1). The alignment of their amino acid sequences (Figure S3-2) showed that the pullulanase was highly conserved among plants. A conserved catalytic $(\beta/\alpha)_8$-barrel domain was identified in barley (*Hordeum vulgare*) (Kristensen et al., 1999), and was also annotated in Figure S3-2, where E1-E8 represented putative β-strands, and H1-H8 represented putative α-helices. The extraction and realignment of those 16 fragments among the 9 selected plants (Figure 3-2B) reveal that the $(\beta/\alpha)_8$-barrel structure may highly conserved among plants, and especially, maize has most similar amino acid sequence with barley (Table 3-1), which was consistent with the distance tree established according to the $(\beta/\alpha)_8$-barrel sequence alignment (Figure 3-2C), indicating that maize pullulanase was most likely to form an $(\beta/\alpha)_8$-barrel structure. Note that the $(\beta/\alpha)_8$-barrel domain between maize line W64Ao2 and K0326Y was identical, indicate that the amino acid difference may not influence the structure of this conserved domain.

Recombinant Plasmid Construction, Expression and Partial Purification of Pullulanase

The cloning and expression vector used in this study is pRSET-A (2897 bp) (Invitrogen, Life Technologies, Grand Island, NY) with T7 promoter, ribosomal binding site (RBS), and 6x His-Tag upstream of multiple cloning sites (MCS). Two fragments from *Zpu1* cDNA and one fragment from *Zpu1* genomic DNA were ligated with the vector, and the full length coding region was in frame with 6x His-Tag (Figure 3-3). Firstly, a 773 bp fragment digested with NcoI and BstBI from an 815 bp cDNA fragment (position 185-999) amplified by primer *Zpu1*_2F and *Zpu1*_2R_BstBI. Secondly, a 185 bp fragment (no intron included) digested with XhoI and NcoI from a 929 bp genomic DNA fragment (position 2247-3175 on genomic DNA) amplified by primer *Zpu1*_1F_XhoI and *Zpu1*_1R. Thirdly, a 2,220 bp fragment digested with PvuII and BstBI from a 2,324 bp fragment (position 642-2965) amplified by primer *Zpu1*_3F and *Zpu1*_3'R_BstBI. The size of full length inserted *Zpu1* gene was 2952 bp.

The full length open reading frame of *Zpu1* gene was in frame with the sequence of 6x His-Tag. The recombinant plasmids were then sequenced and the sequences of full length *Zpu1* genes (from W64Ao2 or K0326Y) were consistent with previous sequencing data (Chapter 2, Supplemental data 1), indicating that the Zpu1 genes were successfully inserted in the vector.

Expression of recombinant *Zpu1* gene was induced by IPTG during the log phase of bacteria growth, and the culture was incubated at 25^{o}C with constant shaking for 18 hours. W64Ao2 and K0326Y pullulanase activities from same volume of crude extracts were detected by quantitative enzyme activity assay, comparing with the negative control with empty pRSET-A plasmid. After incubated for 3 hours period of time, pRSET-A

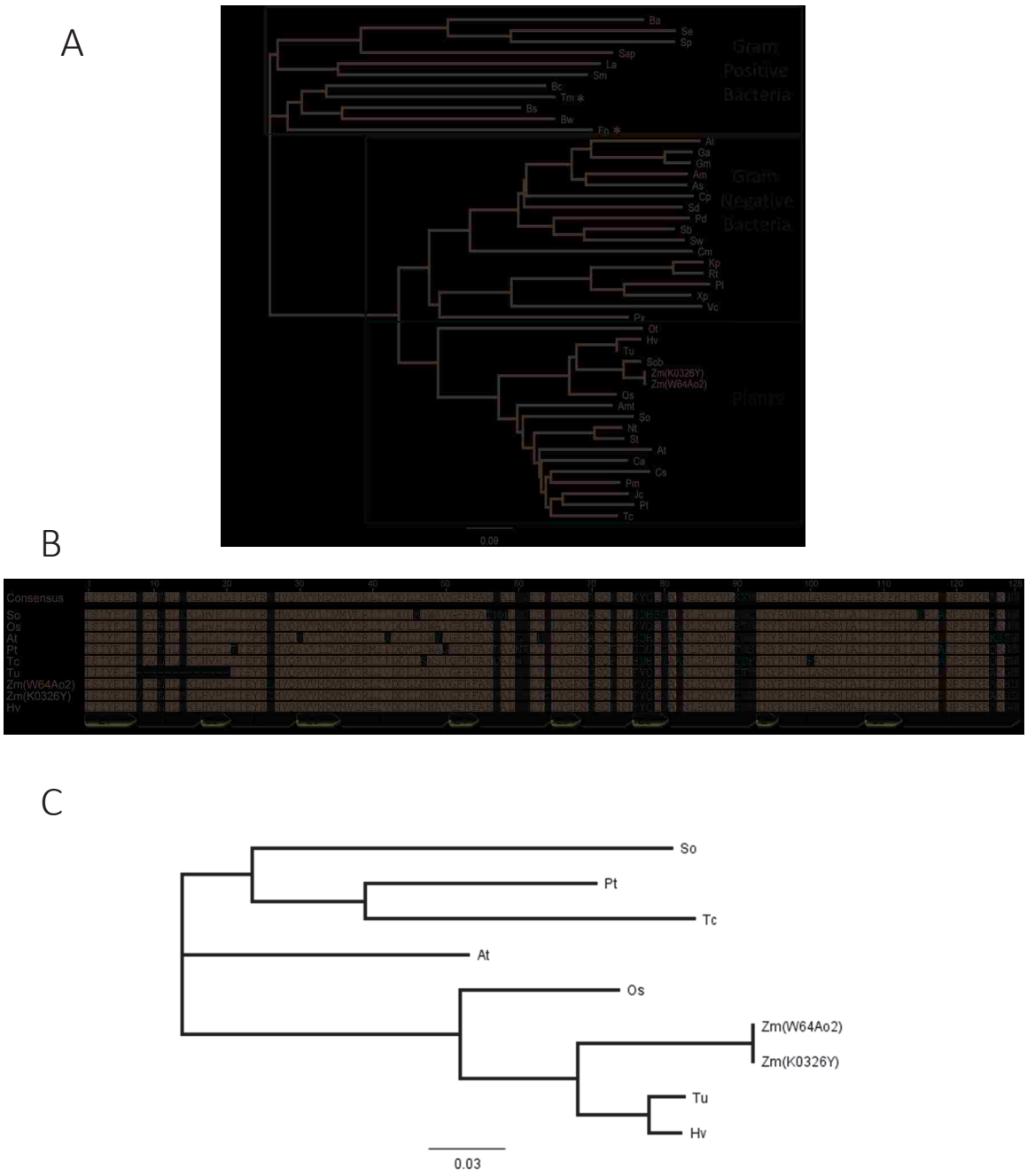

Figure 3-2 Systematic studies of pullulanase among bacteria and plants. (A) Distance tree based on the amino acid sequence alignment of pullulanase among bacteria and plants. Three clusters were highlighted with colors, red-Gram Positive bacteria, orange-Gram Negative bateria and green-plants. Asterisks (*) in positive bacteria represent the exceptions. Tm (*Thermotoga maritima*) and Fp (*Francisella philomiragia*) are gram negative bacteria. (B) Amino acid sequence alignments of 9 putative pullulanase (β/α) 8-barrel domains. Black highlights represent highly conserved regions; green and white highlights represent less conserved regions. The label E(1-8) and H(1-8) at the annotations represent putative β-strands and α-helices, respectively. (C) Unrooted distance tree based on B.

Table 3-1 The Similarities (%) of putative $(\beta/\alpha)_8$-barrel Domain among 9 Plants

Species	So	Os	At	Pt	Tc	Tu	Zm (W64Ao2)	Zm (K0326Y)	Hv
So	100	68	72.7	75	71.1	61.7	67.2	67.2	69.5
Os	68	100	76.6	72.7	68.8	78.1	83.6	83.6	85.9
At	72.7	76.6	100	76.6	75.8	65.6	71.9	71.9	73.4
Pt	75	72.7	76.6	100	80.5	62.5	68	68	70.3
Tc	71.1	68.8	75.8	80.5	100	59.4	66.4	66.4	67.2
Tu	61.7	78.1	65.6	62.5	59.4	100	80.5	80.5	87.5
Zm(W64Ao2)	67.2	83.6	71.9	68	66.4	80.5	100	100	89.8
Zm(K0326Y)	67.2	83.6	71.9	68	66.4	80.5	100	100	89.8
Hv	69.5	85.9	73.4	70.3	67.2	87.5	89.8	89.8	100

plasmid inserted with W64Ao2 *Zpu1* (pW64Ao2) had up to 5-fold increase of pullulanase activity, whereas pRSET-A plasmid inserted with K0326Y *Zpu1* (pK0326Y) had 2-fold increase, and no significant change detected in the control (Figure 3-4). The expression level of recombinant *Zpu1* varied among positive clones, and it might be affected by multiple factors (Duan et al., 2013). The cell extract of positive clones were purified via Ni-NTA resin with high affinity with 6xHis-tag. After purified by affinity chromatography, there were still some background proteins in elutes (Figure 3-5). Western immunoblotting with 6xHis-tag antibody showed that the molecular weight of the recombinant protein was a little greater than 100 kDa (Figure 3-5), which was consistent with the previous data (Beatty et al., 1999; Wu et al., 2002).

Mass Spectrometry Analysis of Pullulanase

In order to further confirm the identity of the target proteins with the molecular weight a little bit greater than 100 kDa, mass spectrometry analysis was performed following the in-gel digestion of the SDS-PAGE gel bands containing the target protein.

Compared with two blanks, each sample produced 10 peaks on chromatogram, and each peak represented a separated substance in the samples (Figure 3-6 A-B). Eight peaks

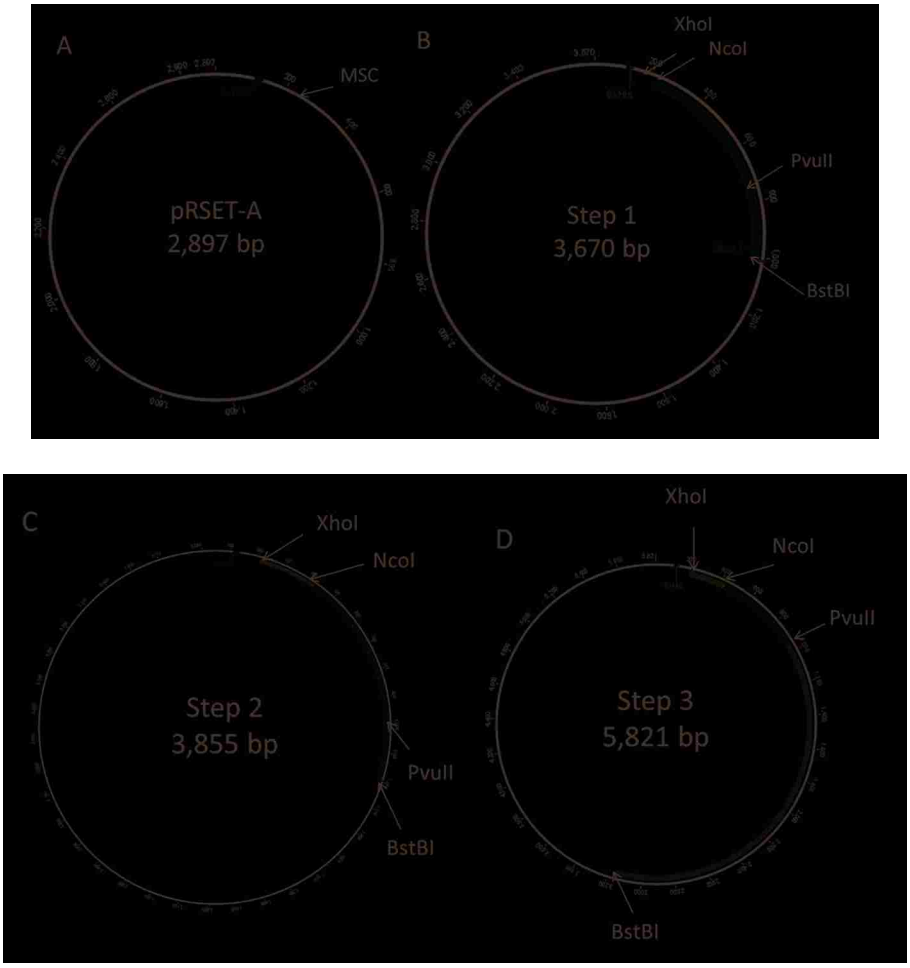

Figure 3-3 Plasmid construct for *Zpu1* cloning and expression. (A) pRSET-A (with 6xHis-Tag) is the vector used for *Zpu1* gene cloning and expression. Three steps were involved to obtain a full-length *Zpu1* clone. (B) A 773 bp fragment (green) was ligated at NcoI/BstBI site. (C) A 185 bp fragment (red) was ligated at XhoI/NcoI site. (D) A 2,220 bp fragment (yellow) was ligated at PvuII/BstBI site.

eluted between 9.5 min and 14.5 min, and the last two peaks were eluted between 33.7 min and 34.6 min. The relative signal intensity (x-axis) varied among peaks, and it was associated with the amount of each substance. Mass spectrum of each peak at low energy

mode was shown at Figure S3-3, where only major peak (with greatest intensity) represented the separated substance in the sample, whereas other small peaks were background noises. For double charged substances (PEAK#1-#8) the m/z value should be converted to single charged the m/z value before matching with the predicted values. For each sample (pW64Ao2 or pK0326Y), the single charged m/z value of 7 out of 10 peaks matched well with the corresponding predicted m/z value of a specific pullulanase peptide generated by built-in MS-Digest simulation tool of ProteinProspector so each positive peaks could represent a specific pullulanase peptide (Table 3-2 and Table 3-3). In high energy mode, the peptide was dissociated into smaller charged fragments, and generated peaks with smaller m/z on mass spectra (Figure S3-4). For each positive peptide, m/z values of the fragments were compared with the corresponding predicted m/z values generated by MS-Product Tool of ProteinProspector. The result showed that the fragment m/z values of each peptide matched well with the predicted values despite some background noises (Table 3-4 and Table 3-5), which further confirmed that the samples obtained from the target bands was the pullulanase. Note that in Table 3-4 and 3-5, the fragments generated via collision-induced dissociation (CID) under high energy mode could be single amino acids (such as F, Y, Q, etc.) or charged shorter oligopeptides. The oligopeptides were designated according to Roepstorff–Fohlmann–Biemann nomenclature (Roepstorff and Fohlmann, 1984; Steen and Mann, 2004). The letters a, b, c, x, y and z followed by a number denote where the peptide fragmented at and how many amino acids are kept. For instance “y7” represents the peptide bond between the amino acids at position 7 and 8 from C-terminus was broken, leaving 7 amino acids at the C-terminus, and “b2” represents the peptide bond between the amino acids at position

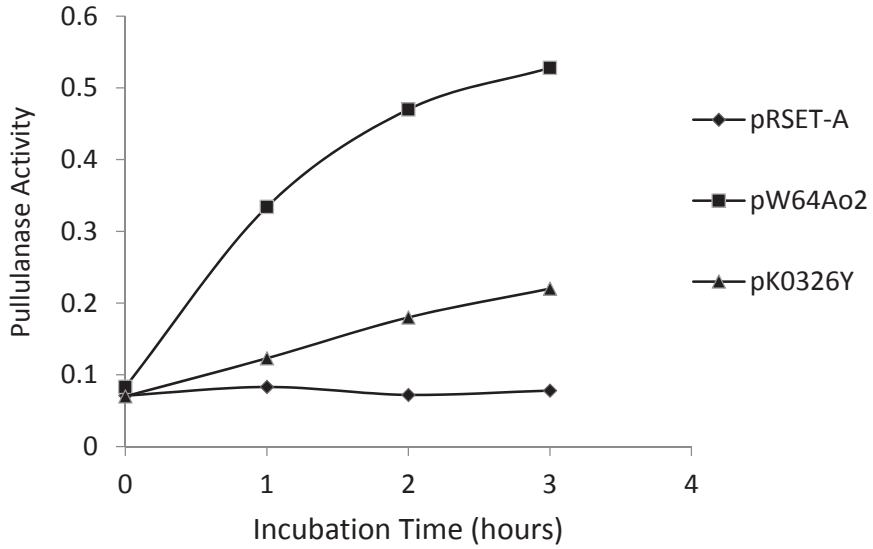

Figure 3-4 Time-course pullulanase activity assay. The assay used crude extracts of E. coli transformants with empty pRSET-A, pW64Ao2 (pRSET-A::W64Ao2 *Zpu1*) and pK0326Y (pRSET-A::K0326Y *Zpu1*). Pullulanase activity was measured by the spectrophotometer at 490 nm absorbance (y-axis) every hour.

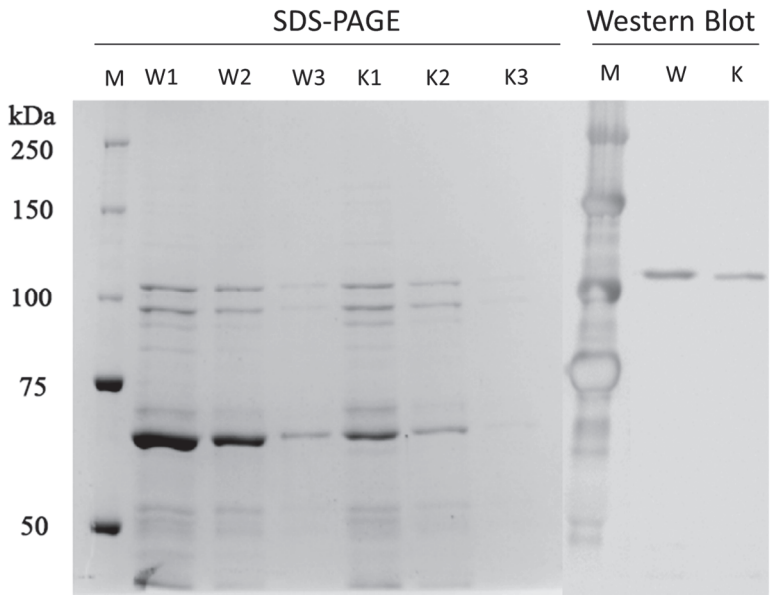

Figure 3-5 SDS-PAGE and Western Blot of partially purified elutes. Ni-NTA affinity chromatography was used in the purification and W1-3 and K1-3 represent the first, second and third elute of the products expressed from pW64Ao2 (W) and pK0326Y (K). The first elute (with highest protein concentration) was used in Western Blot. The primary antibody was 6x-His Epitope Tag Antibody and the second antibody was HRP-Goat anti-Mouse antibody.

2 and 3 from N-terminus was broken, leaving 2 amino acids at the N-terminus. Also, "-" (minus) followed by 28, H2O or NH3 represents the loss of C=O, H2O or NH3 respectively from an oligopeptides during CID.

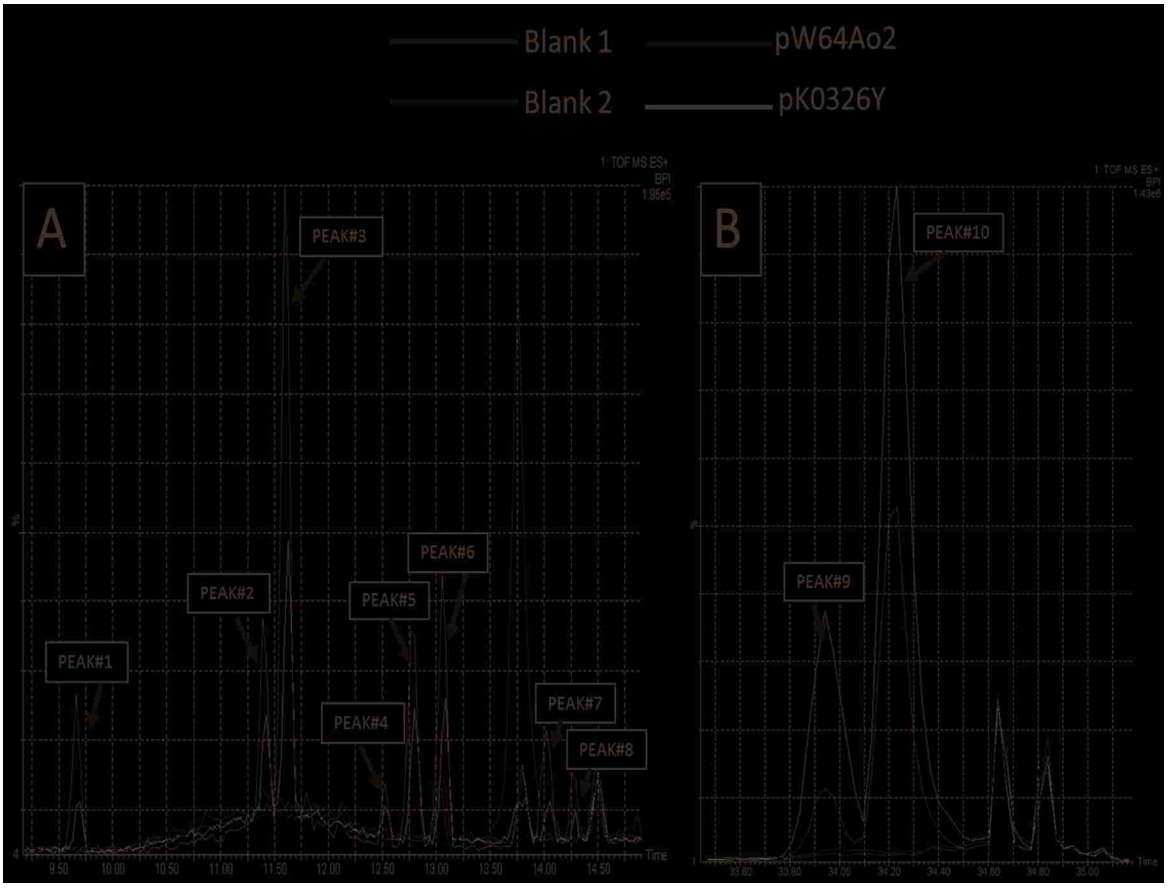

Figure 3-6 Chromatogram of the Mass Spectrometry. The target SDS-PAGE gel bands were excised and processed with in-gel digestion followed by mass spectrometry. Ten peaks were detected from the samples (green-pW64Ao2 and black-pK0326Y) compared with two blanks (red and blue). First eight peaks were detected at retention time 9.5 -14.5 min (A), and last two 33.7 - 34.6 min (B).

Table 3-2 Peak m/z values and putative peptide information of each chromatogram peak (for pW64Ao2)

Peak#	Predicted m/z	Measured m/z	Putative Peptide	From	To
PEAK#1	1061.5374	1061.6018	FAAAPSSASPR	17	27
PEAK#2	N/A	1050.5604	N/A	N/A	N/A
PEAK#3	1103.6055	1103.6746	LTTASDIVQR	850	859
PEAK#4	1107.5325	1106.6112	FDLMGHIMK	549	557
PEAK#5	989.5415	989.6000	TAAVFVEPR	953	961
PEAK#6	944.5346	944.5898	LMVQALNR	461	468
PEAK#7	869.4516	869.5104	YSSPLFR	843	849
PEAK#8	1176.5565	1176.6278	TPMDLSIDER	731	740
PEAK#9	N/A	387.2163	N/A	N/A	N/A
PEAK#10	N/A	415.2328	N/A	N/A	N/A

Note: N/A indicates no match with the predicted m/z values. From/To represent the position of putative peptide on pullulanase amino acid sequence.

Table 3-3 Peak m/z values and putative peptide information of each chromatogram peak (for pK0326Y)

Peak#	Predicted m/z	Measured m/z	Putative Peptide	From	To
PEAK#1	1061.5374	1061.5816	FAAAPSSASPR	17	27
PEAK#2	N/A	1050.5404	N/A	N/A	N/A
PEAK#3	1103.6055	1103.6540	LTTASDIVQR	850	859
PEAK#4	1107.5325	1106.5702	FDLMGHIMK	549	557
PEAK#5	989.5415	989.5806	TAAVFVEPR	953	961
PEAK#6	944.5346	944.5708	LMVQALNR	461	468
PEAK#7	869.4516	869.4922	YSSPLFR	843	849
PEAK#8	1176.5565	1176.6066	TPMDLSIDER	731	740
PEAK#9	N/A	387.2077	N/A	N/A	N/A
PEAK#10	N/A	415.2238	N/A	N/A	N/A

Note: N/A indicates no match with the predicted m/z values. From/To represent the position of putative peptide on pullulanase amino acid sequence.

Table 3-4 Fragment peak m/z values and matched fragment sequences (for pW64Ao2)

Peak#	Sequence	Predicted m/z	Measured m/z	Peptide Fragments
PEAK#1	FAAAPSSASPR	120.0808	120.0881	F
		147.0764	147.0764	SS-28
		191.1179	191.1261	a2
		255.1452	255.1704	y2-NH3
		341.1932	341.0336	y3-H2O
		413.2143	413.2866	y4-NH3
		701.3577	701.3973	y7
PEAK#3	LTTASDIVQR	129.0659	129.0581	Q
		211.1077	211.1452	VQ-NH3
		515.3300	515.3604	y4
		630.3570	630.3861	y5
		717.3890	717.4219	y6
		788.4261	788.4708	y7
		889.4738	889.5224	y8
		990.5214	990.5682	y9
PEAK#4	FDLMGHIMK	129.1022	129.0581	K
		147.1128	147.0711	y1
		505.2115	506.3016	b4-H2O
PEAK#5	TAAVFVEPR	120.0808	120.0881	F
		173.0921	173.1019	b2
		647.3511	647.3810	y5
		746.4196	746.4550	y6
		817.4567	817.4949	y7
PEAK#6	LMVQALNR	413.1853	413.2866	MVQA-NH3
		601.3416	601.3712	y5
		700.4100	700.4496	y6
PEAK#7	YSSPLFR	120.0808	120.0881	F
		136.0757	136.0855	Y
		619.3562	619.3846	y5
		706.3883	706.4247	y6
PEAK#8	TPMDLSIDER	153.1022	153.0288	a2-H2O
		304.1615	304.1754	y2
		847.4156	847.4630	y7
		978.4561	978.4988	y8

Table 3-5 Fragment peak m/z values and matched fragment sequences (for pK0326Y)

Peak#	Sequence	Predicted m/z	Measured m/z	Peptide Fragments
PEAK#1	FAAAPSSASPR	120.0808	120.0833	F
		147.0764	147.0711	SS-28
		157.0972	157.0900	SP-28/PS-28
		255.1452	255.1634	y2-NH3
		341.1932	341.0255	y3-H2O
		413.2143	413.2777	y4-NH3
		701.3577	701.3742	y7
PEAK#3	LTTASDIVQR	129.0659	129.0581	Q
		211.1077	211.1389	VQ-NH3
		515.3300	515.3505	y4
		630.3570	630.3861	y5
		717.3890	717.4102	y6
		788.4261	788.4464	y7
		889.4738	889.4963	y8
		990.5214	990.5408	y9
PEAK#4	FDLMGHIMK	129.1022	129.0581	K
		147.1128	147.0711	y1
PEAK#5	TAAVFVEPR	120.0808	120.0833	F
		173.0921	173.0962	b2
		647.3511	647.3699	y5
		746.4196	746.4431	y6
		817.4567	817.4824	y7
PEAK#6	LMVQALNR	129.0659	129.0581	Q
		413.1853	413.2777	MVQA-NH3
		601.3416	601.3605	y5
		700.4100	700.4264	y6
PEAK#7	YSSPLFR	120.0808	120.0833	F
		136.0757	136.0804	Y
		147.0764	147.0711	SS-28
		157.0608	157.0900	SS-H2O
		619.3562	619.3738	y5
		706.3883	706.4016	y6
PEAK#8	TPMDLSIDER	175.1190	175.1241	y1
		201.1056	201.1101	PM-28
		619.3046	619.3412	y5
		655.2933	655.2811	DLSIDE-H_2O

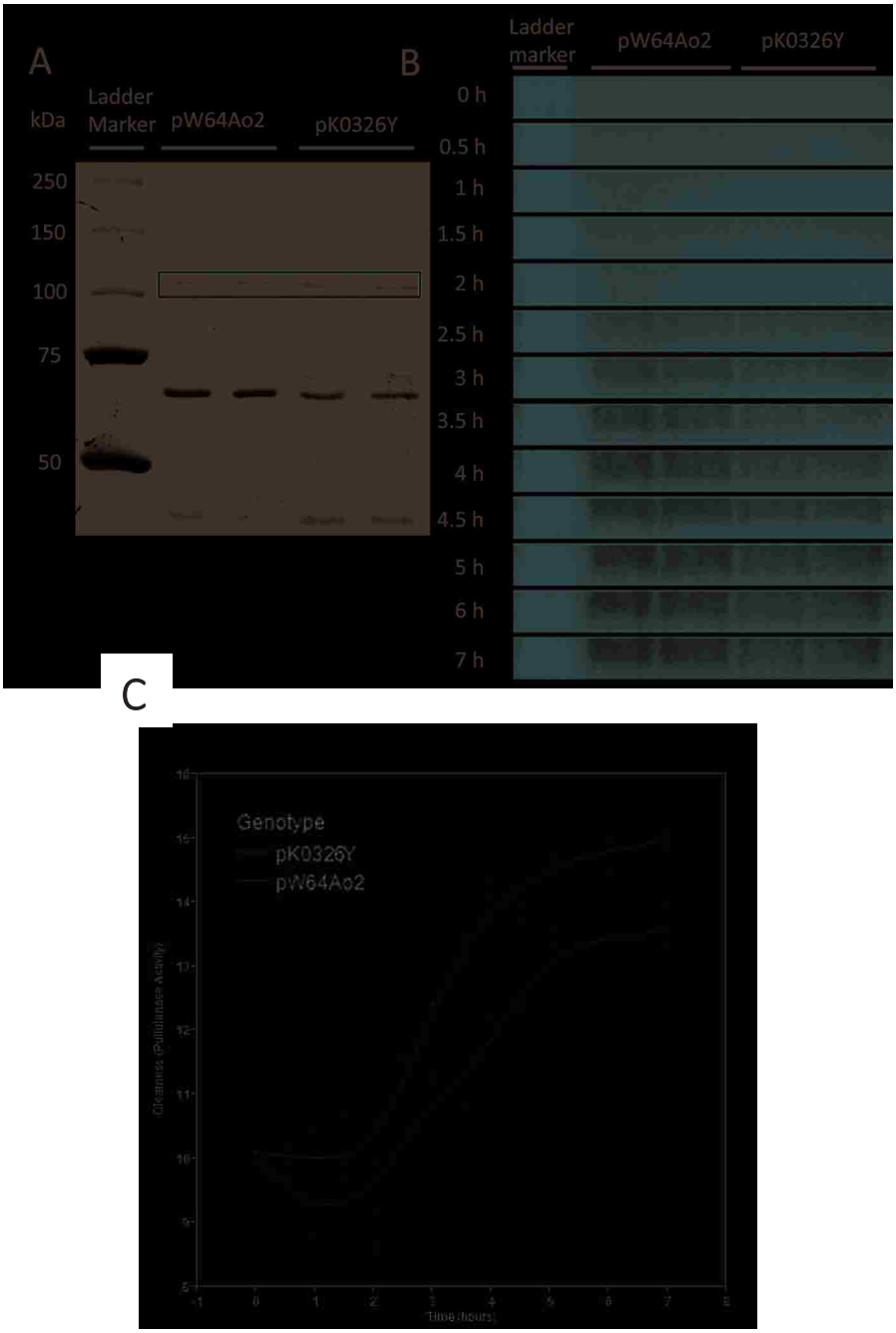

Figure 3-7 Time-course zymogram assay on partially purified elutes. (A) The amount of pullulanase expressed from pW64Ao2 and pK0326Y were equalized (same intensity on the gel band) (B) Equalized samples were loaded onto native-PAGE gel with red pullulan, and band clearness was recorded every 30 min (except last three data points, every one hour). The band clearness is proportional to the pullulanase activity. (C) The graph showed band clearness (y-axis) at each time point. The trend curves were generated by JMP 10.0 and the significant level of clearness values at each data point was analyzed by t test at $p<0.05$.

Time Course Study of Pullulanase Activities between pW64Ao2 and pK0326Y

The elutes were diluted to equalize the amount of pullulanase loaded on SDS-PAGE gel to rule out the effect of pullulanase abundance on the activity (Figure 3-7A). Native PAGE was performed with same amount of pullulanase loaded on 7.5% polyacrylamide gel with 0.6% red pullulan. The gel was then incubated in incubation buffer at room temperature (25^{o}C) and the gel image was taken every 30 min with the exception on last three data points, every hour. As the time went by, the red pullulan was gradually digested by pullulanase and formed clear bands where the pullulanase located (Figure 3-7B). The degree of clearness, whose values were measured by gray values (the brightness of a certain area on an image, the brighter, the greater the gray value), could reflect the activity of pullulanase. The pullulanase activity increased exponentially from hour 2 to 4.5, and reached to nearly equilibrium after then (Figure 3-7C). Given the same pullulanase abundance, activity of pW64Ao2 pullulanase was statistically higher than that of pK0326Y (Figure 3-7C), which could be caused by the amino acid difference between the two (Chapter 2).

Discussion

In bacteria, five types of pullulan digestive enzyme were discovered, while so far, only one type of pullulanase was discovered in higher plants (vascular plants). The amino acid sequence of plant pullulanase was homologous to the Pullulanase Type I in most Gram negative bacteria. Pullulanase in most Gram negative bacteria is a lipoprotein that can be secreted to the outer membrane and release to the surrounding environment, to digest large polysaccharides into relatively small carbohydrate molecules, which can be easily absorbed by the bacteria (Pugsley et al., 1986; Pugsley et al., 1997). The pullulanase in

genus *Klebsiella* has a 19-amino-acid transit signal peptide at N-terminal region that facilitates the secretion (Katsuragi et al., 1987). In developing maize kernels, pullulanase, together with other starch biosynthetic enzymes, is mainly located in amyloplast (an organelle for starch biosynthesis and storage) (Beatty et al., 1999; Yu et al., 1998). Studies showed that maize Granule-Bound Starch Synthase (GBSS) and Starch Synthase III (SSIII) may have N-terminal transit signal peptide targeting the enzymes to the amyloplast (Klösgen et al., 1986; Gao et al., 1998), but so far no N-terminal amyloplast-targeting peptide has been identified in pullulanase, which was consistent with the alignment data shown in Figure S3-1, where some bacterial pullulanase had N-terminal extension compared with plants. Since amyloplasts lack the protein biosynthetic apparatus, pullulanase should be synthesized in cytoplasm and then translocated into amyloplast during the endosperm development, but the mechanism needs to be further characterized. The sequence of pullulanase of Gram positive bacteria is largely different than that of Gram negative bacteria and plants, but there were fewer studies in pullulanase of Gram positive bacteria. Instead secreted out to the environment or located in the amyloplast matrix, Janulczyk's group identified a type of pullulanase attached to the cell wall of *Streptococcus*, and it had a cell wall target region, a membrane-spanning region, and a stop-transfer tail (Janulczyk and Rasmussen, 2001).

The sequence alignment of plant sequences revealed that the $(\beta/\alpha)_8$-barrel domains identified in Barley limit dextrinase (pullulanase) was conserved among the pullulanase of all other 8 selected species, and mostly closed to maize (Figure 3-2B, Table 3-1). Furthermore, the $(\beta/\alpha)_8$-barrel domain was also identified in isoamylase, dextran glucosidase, branching enzyme, etc, all associated with the interaction with the α-1,6-

glycosidic bonds (either hydrolysis or synthesis) in amylopectin (Jespersen et al., 1993). Those studies suggested that $(\beta/\alpha)_8$-barrel domain might be responsible for the catalytic activity on amylopectin branches, however, the one amino acid difference at C-terminal region of pullulanase between W64Ao2 and K0326Y was not within this domain, indicating that the difference may not directly influence the catalytic activity. In K0326Y, the position where threonine located in W64A allele was replaced with proline that tends to form tight turn in 3D structure, which may decrease the binding or activating ability of the enzyme (Bett and Russell, 2003). Several studies showed that the threonine-proline substitution could alter the protein 3D structure which in turn affects the activity and function of the protein (Cool et al., 1990; Osier et al., 2002; Prestona et al., 2006).

Mass spectrometry data showed that 7 out 10 chromatogram peaks matched with the predicted m/z value of pullulanase peptides digested by trypsin (Figure 3-7A-B), and the molecular weight of the target band in SDS-PAGE gel was consistent with the predicted molecular weight of maize pullulanase, which confirmed that the identity of the target band was the maize pullulanase. Peak #9 and Peak #10 had low m/z values and they did not seem to be peptides, because there was no small peptide predicted and the in low energy mode, it was difficult for peptide molecules to dissociate. PEAK #2 could be a peptide, but it may not be digested from pullulanase unless confirmed by further evidence such as post-transcriptional modification of the protein that caused possible alternation of the m/z value (Sun and Anderson, 2005). In high energy mode, collision-induced dissociation (CID) fragment ions were generated and matched with some of the predicted values, which further confirmed the identity of the pullulanase. However, due to the low sample concentrations after in-gel digestion, the signal of the peptides was not very high, and the

C-terminal peptide was missing, making it difficult to pinpoint and confirm the amino acid difference between pW64Ao2 and pK0326Y.

Affinity chromatography of 6xHis-tag protein, or metal-ion affinity chromatography (IMAC), was used to purified the recombinant pullulanase, but one of the limitations of the method is that the specificity is relatively lower than most other affinity method (Waugh, 2005), because some protein containing two or three consecutive histidine residues could be coeluted with the target protein (Magdeldin and Moser, 2012). Extract of negative control with intact pREST-A plasmid did not show detectable pullulanase activity, indicating that the pullulanase activity of pW64Ao2 and pK0326Y came solely from the expression of the recombinant *Zpu1* gene. Time course zymogram study showed that pullulanase of pW64Ao2 had higher reaction rate than the pK0326Y counterpart given the identical initial amount of pullulanase (Figure 3-7), which was consistent with the previous study on maize recombinant inbred lines (RILs) generated from W64Ao2 and K0326Y crossing. RILs with W64A-derived *Zpu1* allele (from W64Ao2) had significantly higher average pullulanase activity than those with QPM-derived *Zpu1* allele (from K0326Y) (Figure S3-5). However, this was contradictory with the parent data, where W64Ao2 had lower pullulanase activity than K0326Y (Figure 2-1A). The discrepancy between parents and RILs might be due to the background effect or/and post-transcriptional regulation. *Zpu1* gene was not in one of the quantitative trait loci (QTL) reported in prior studies (Holding et al., 2011), but showed a quantitative trait pattern (Figure 2-1B), indicating that there might be a factor (or several factors) in QTL that regulates the activity of pullulanase. And pullulanase could be involved in multiunit starch biosynthetic enzyme

complex in amyloplasts, so its activity may also influenced by other starch biosynthetic enzymes (Hennen-Bierwagen et al., 2008).

In summary, pullulanase is conserved among Gram negative bacteria and vascular plants. In maize, pullulanase may contain a $(\beta/\alpha)_8$-barrel domain similar to the one identified in Barley limit dextrinase (pullulanase), and this domain may be involved in the catalytic reaction with α-1,6-glycosidic bonds in amylopectin. The domain was not affected by the amino acid difference in C-terminal region between W64Ao2 and K0326Y, but the difference could influence the activity of *E. coli* expressed recombinant pullulanase. The function of C-terminal region needs to be further characterized, in order to understand the mechanism of the interaction between pullulanase and its substrates.

Authors' Contributions

Hao Wu, writing, experimental design and data (All figures and tables included).

Bryan C. Gibbon, experimental design and writing.

Christopher M. Kearney, experimental design and funding support

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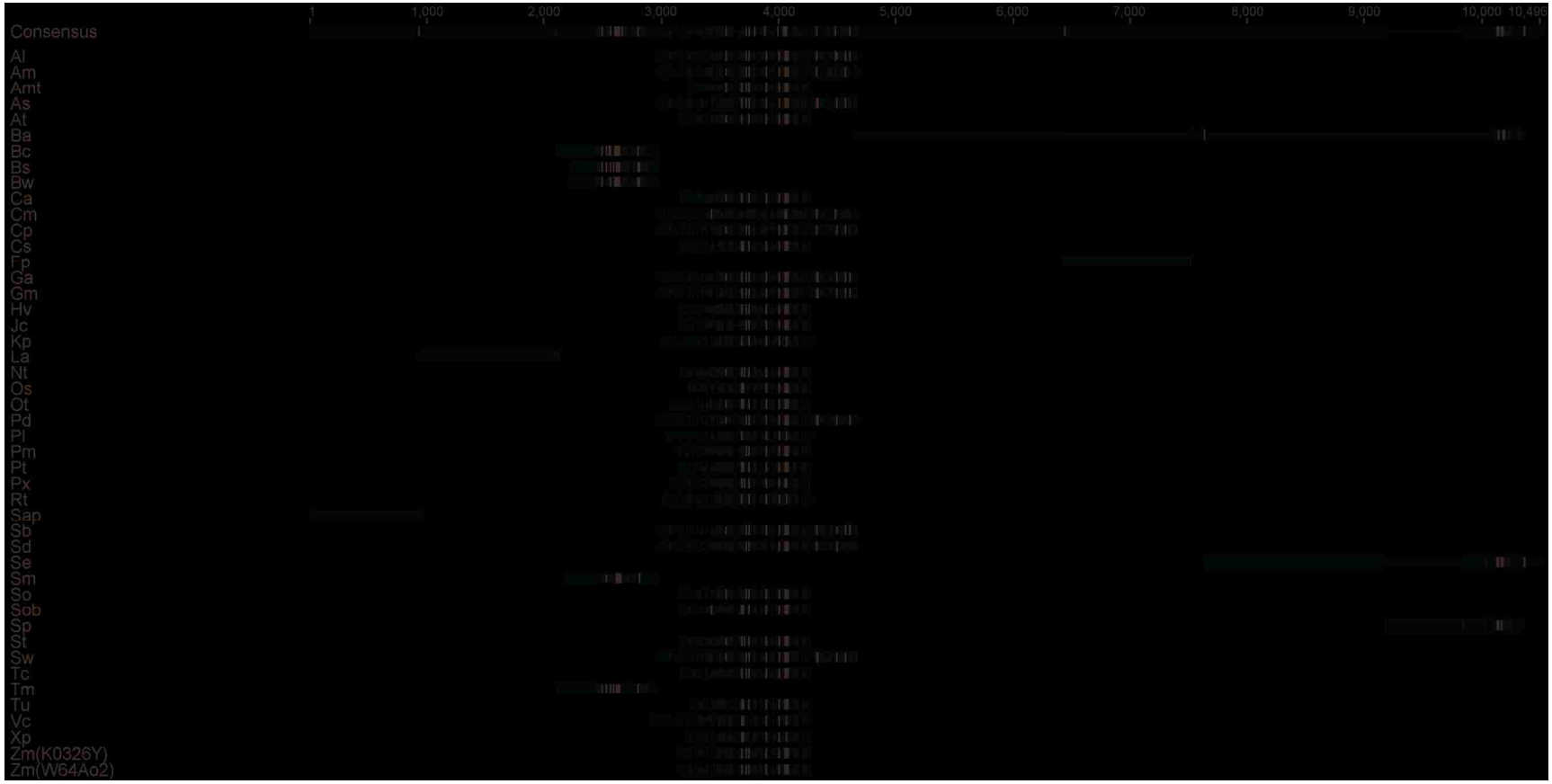


Figure S3-1 Pullulanase amino acid sequence alignments of 46 species. The black regions represent highly conserved regions, whereas other colors represent less conserved regions. Most of the outliers are Gram Positive bacteria (except Tm and Fp, which are Gram Negative). The full names of the species are at Table S3-1.

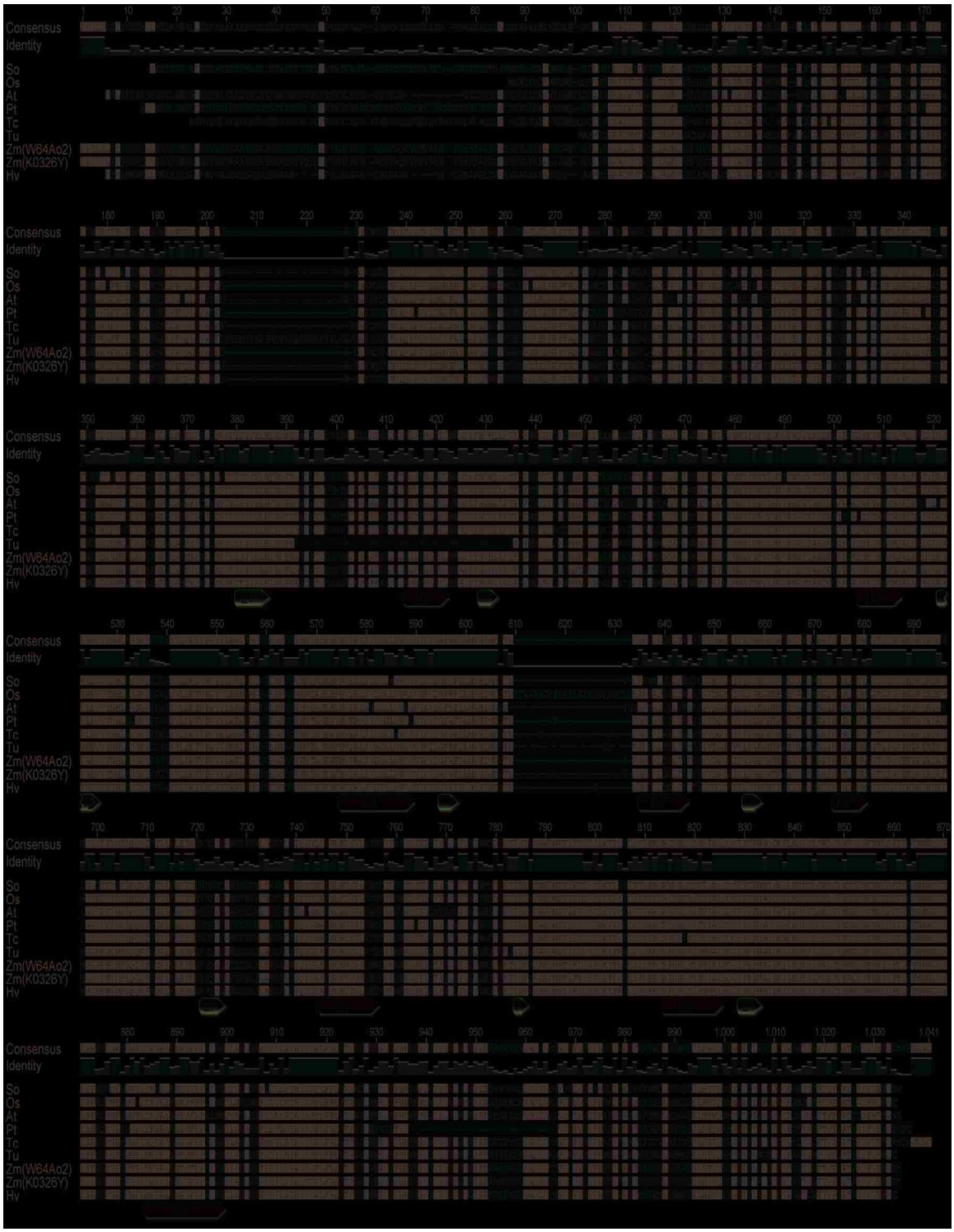
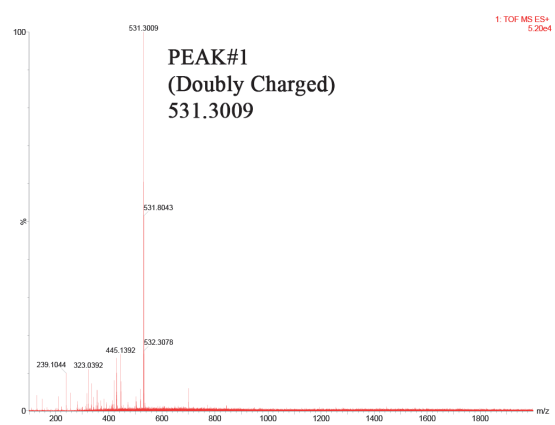


Figure S3-2 Pullulanase amino acid sequence alignments of 9 selected plants. Black highlights represent highly conserved regions, and other colors represent less conserved regions. The label E(1-8) and H(1-8) at the annotations represent putative β-strands and α-helices, respectively.

A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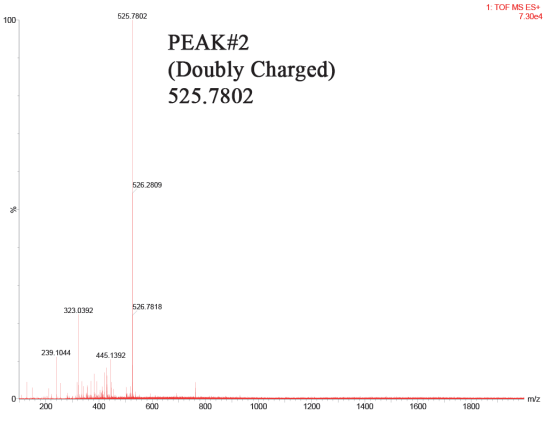
600
800
1000
1200
1400
1600
1800
m/z

B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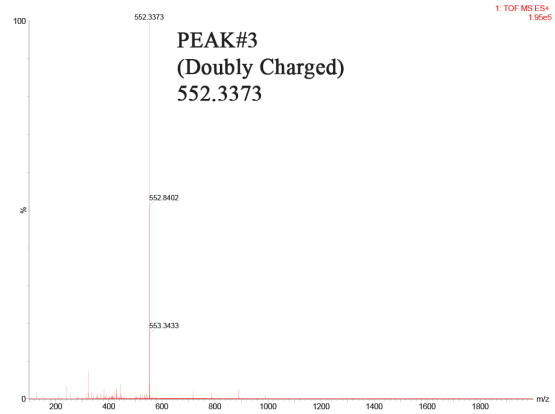
1800
m/z

C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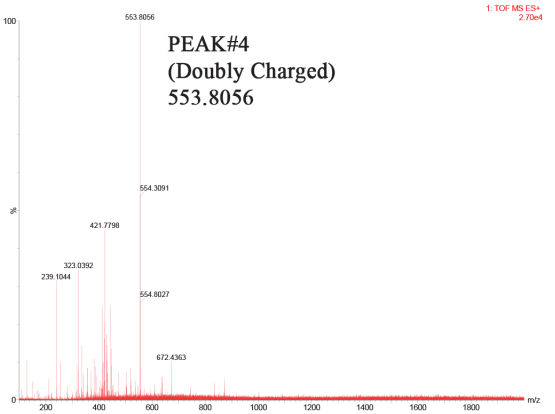
m/z

D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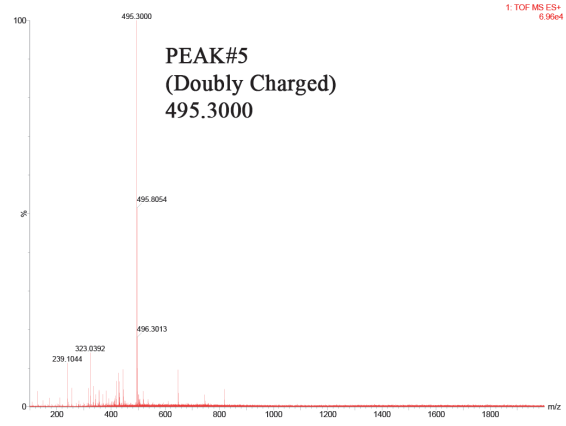
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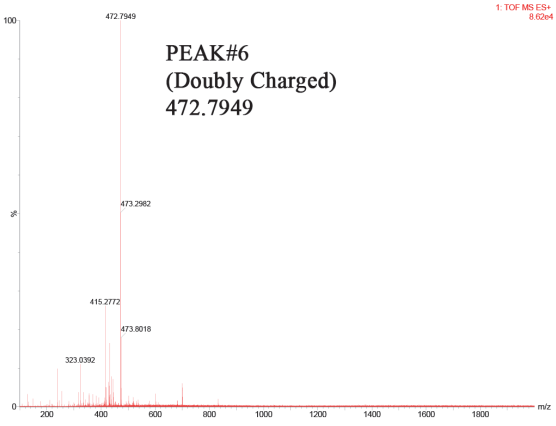
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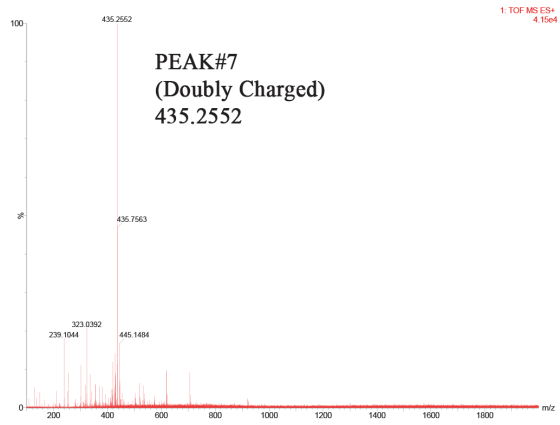
G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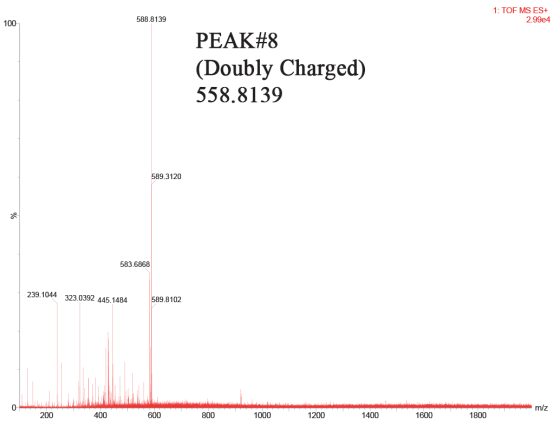
H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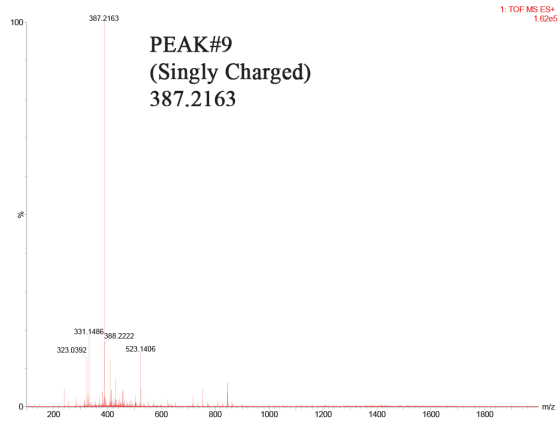
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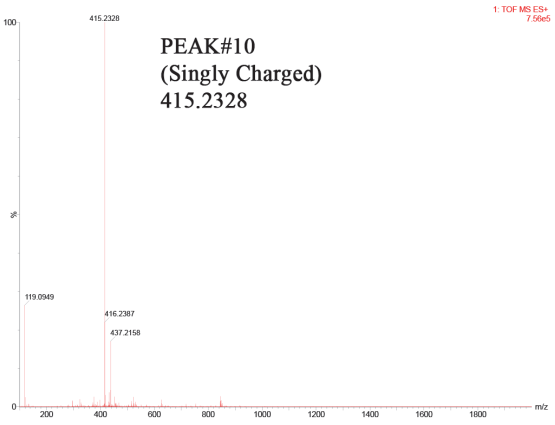
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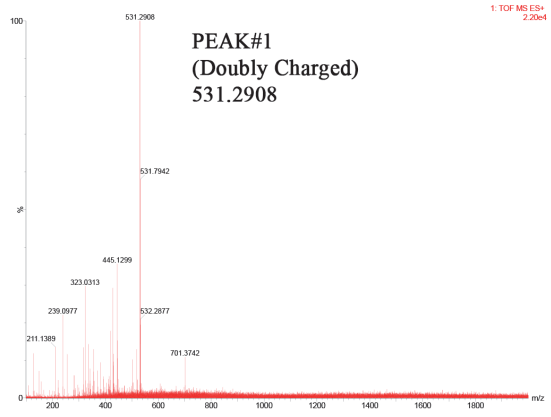
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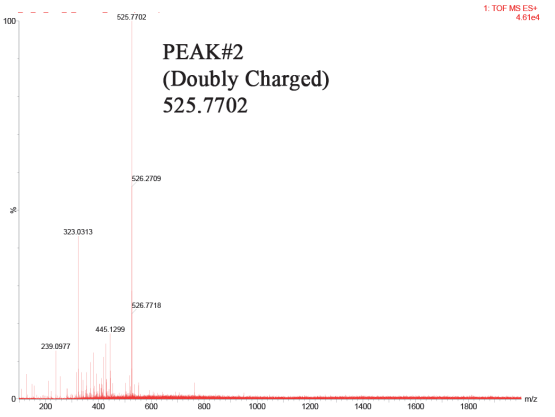
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m/z

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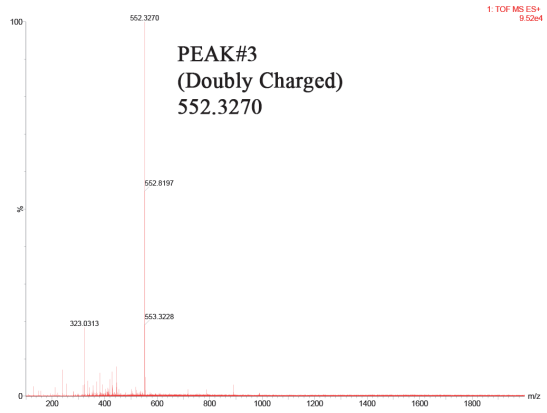
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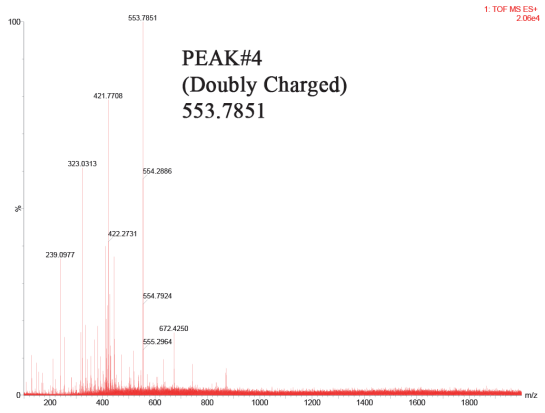
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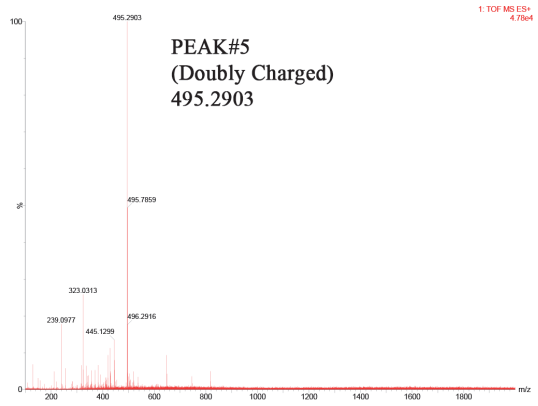
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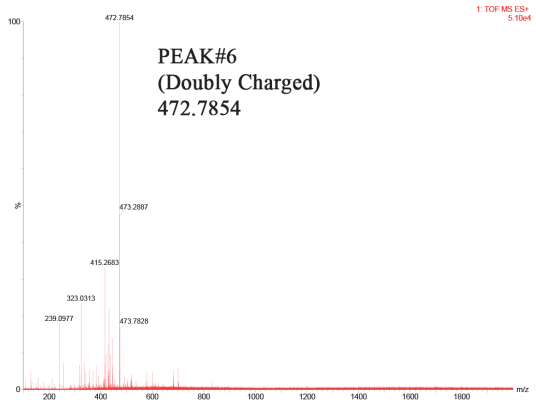
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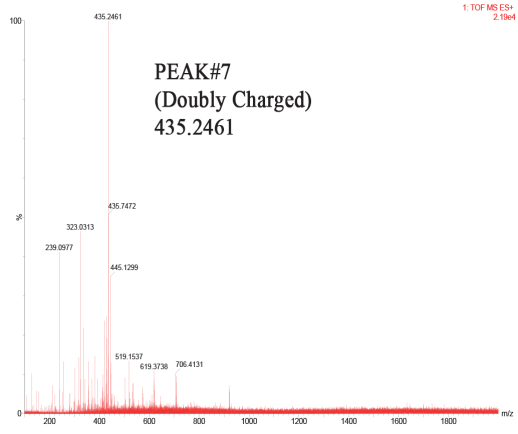
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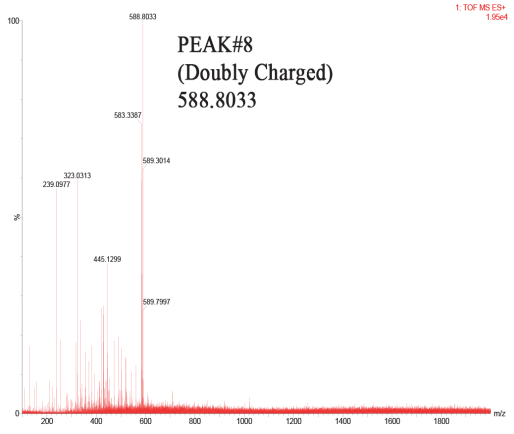
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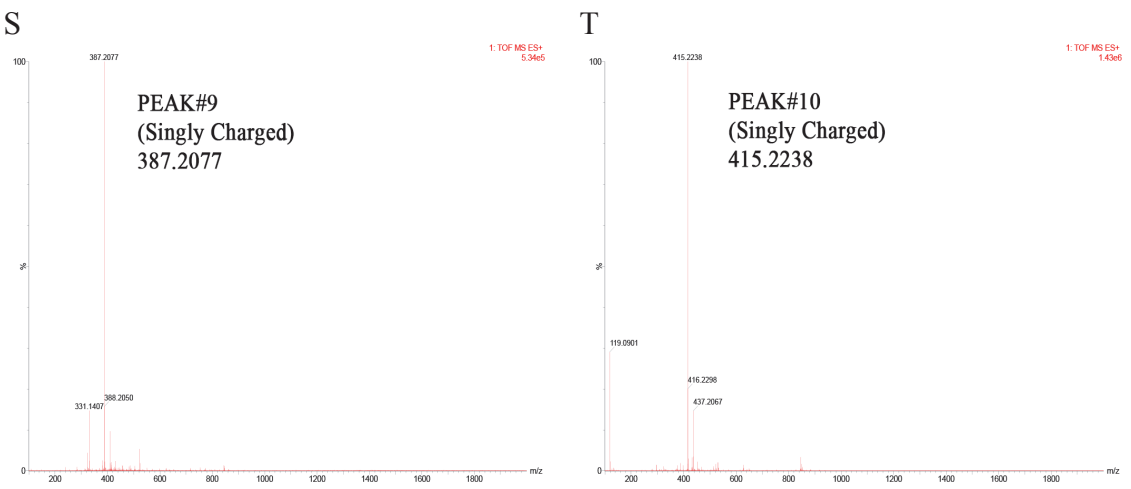
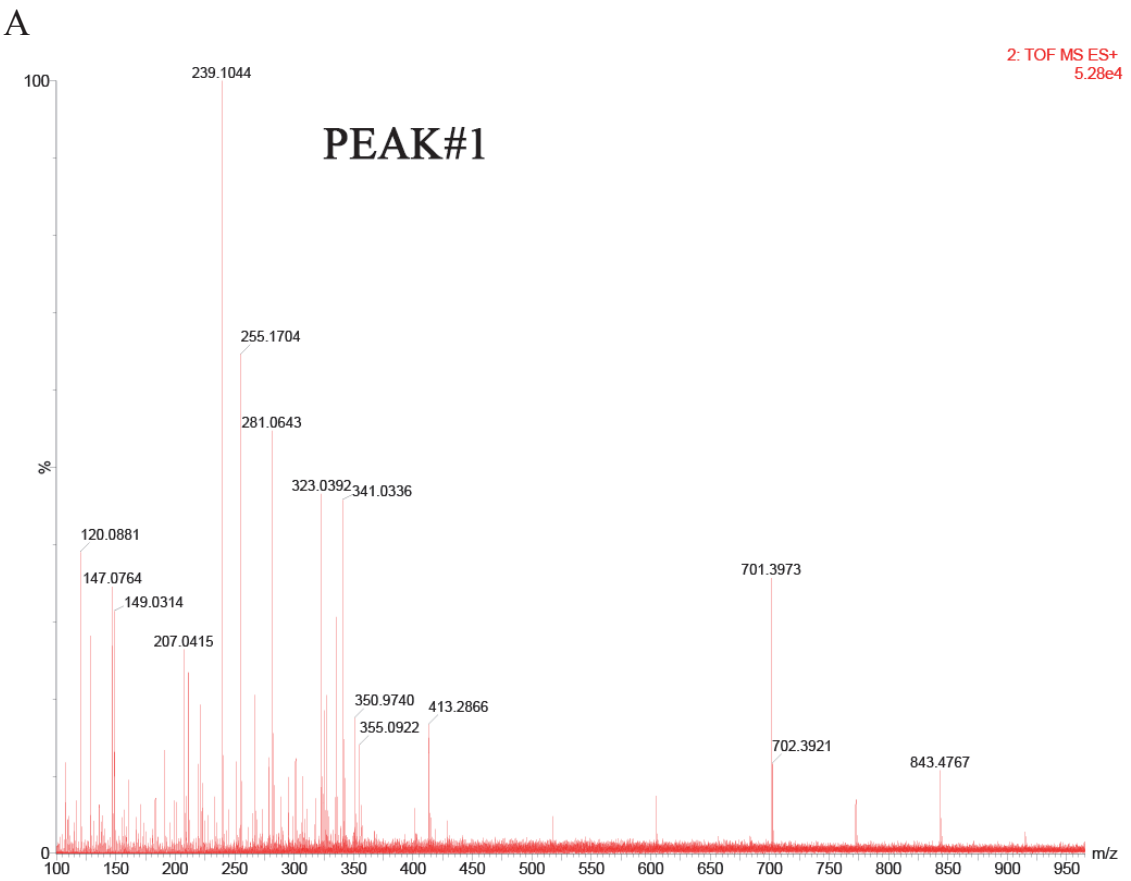


Figure S3-3 Mass spectrum of each chromatogram peak at low energy mode. (A-J) In-gel digested sample expressed from W64Ao2; (K-T) in-gel digested sample expressed from pK0326Y. Only major peak (with greatest intensity) represented the separated substance in the samples, whereas other small peaks were background noises.



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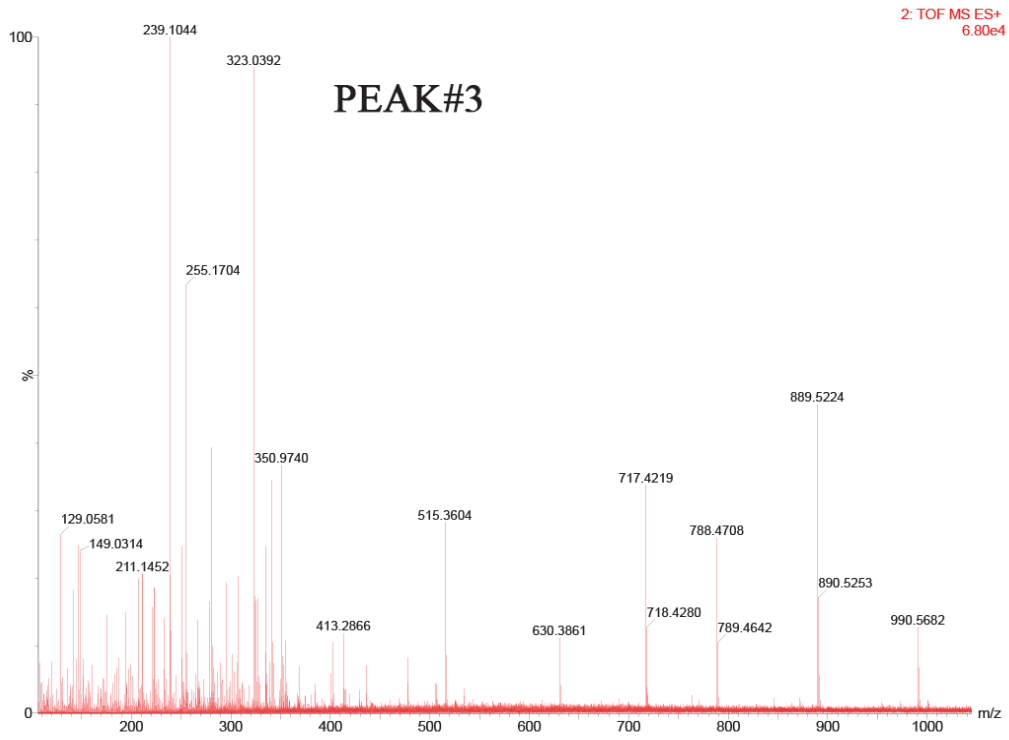
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129.0581
515.3604
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211.1452
890.5253
718.4280
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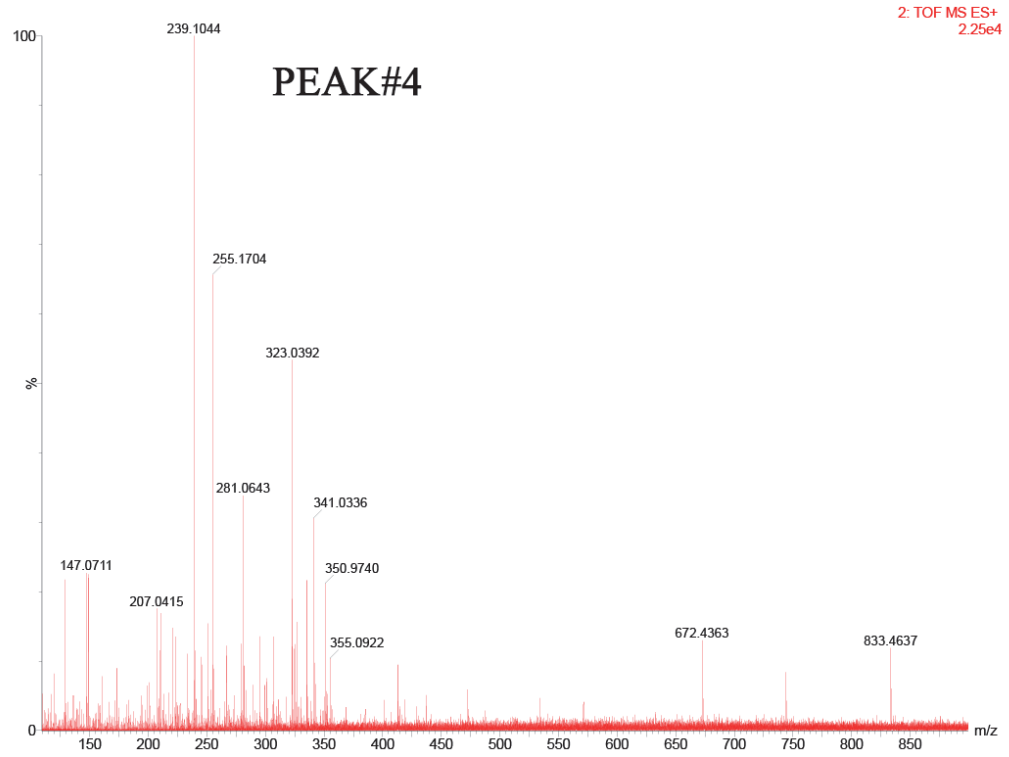
400
500
600
700
800
900
1000
m/z

C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550
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650
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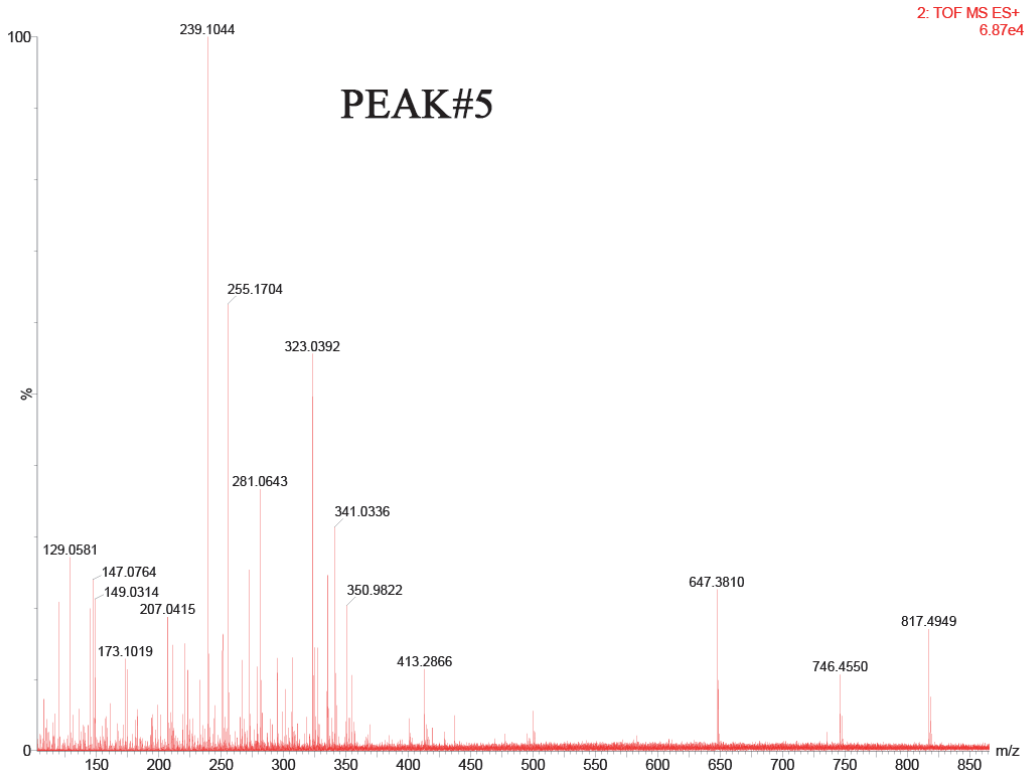
850
m/z

D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m/z

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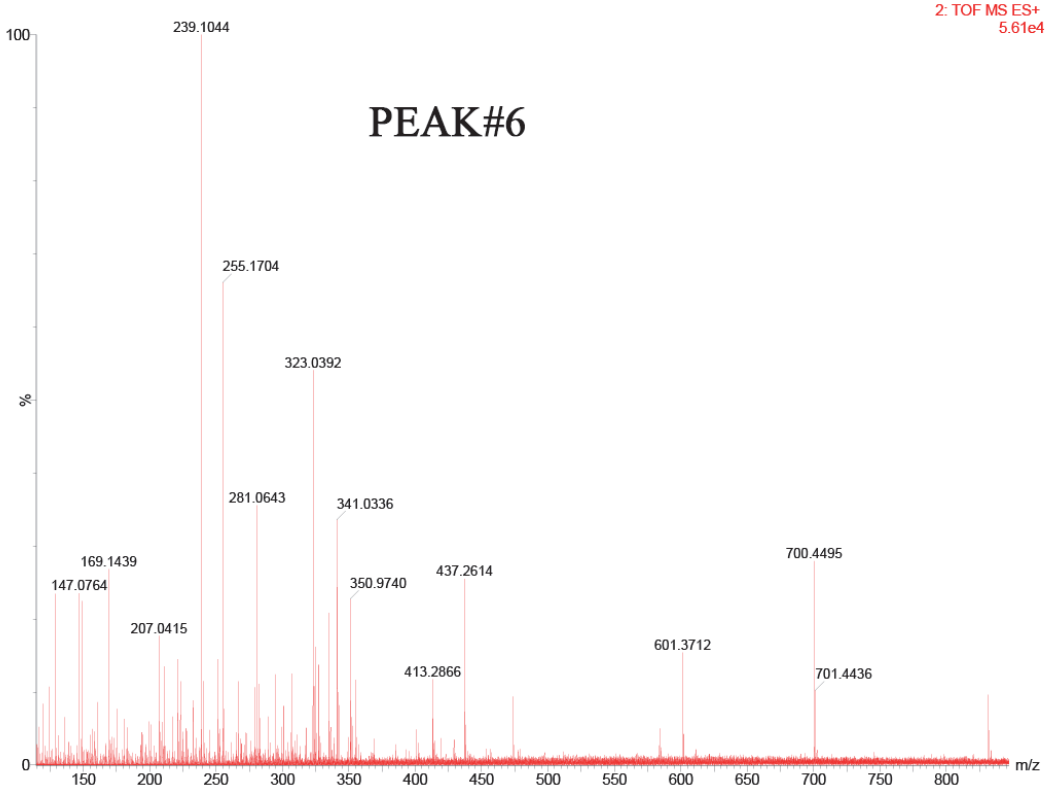
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m/z

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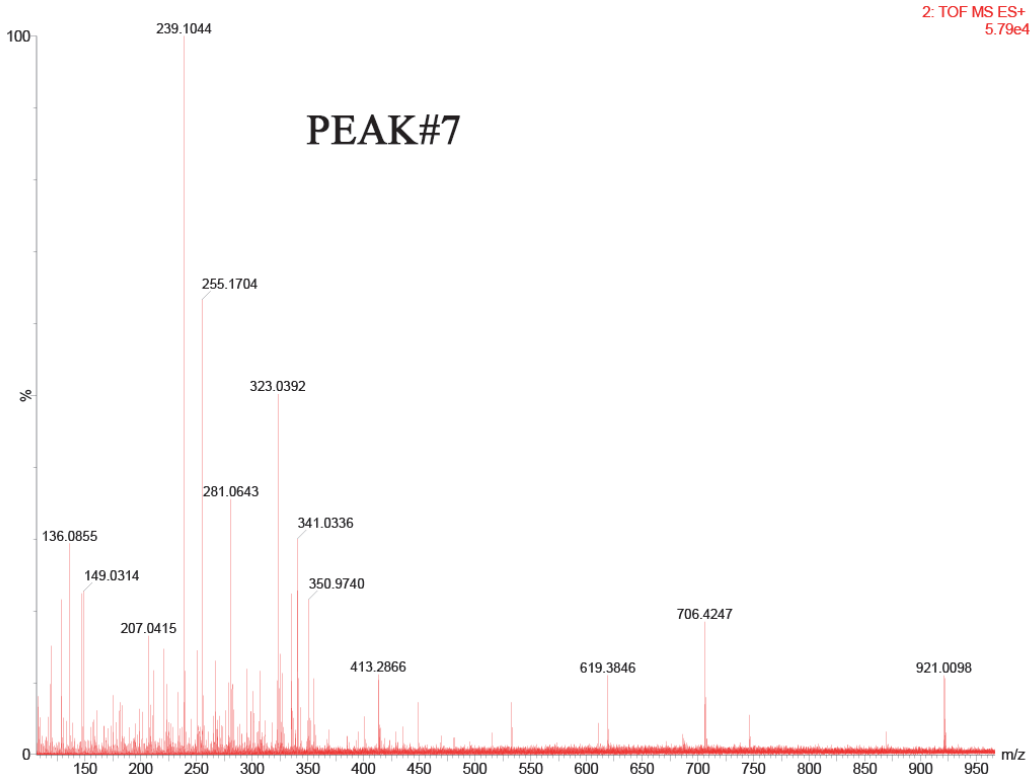
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m/z

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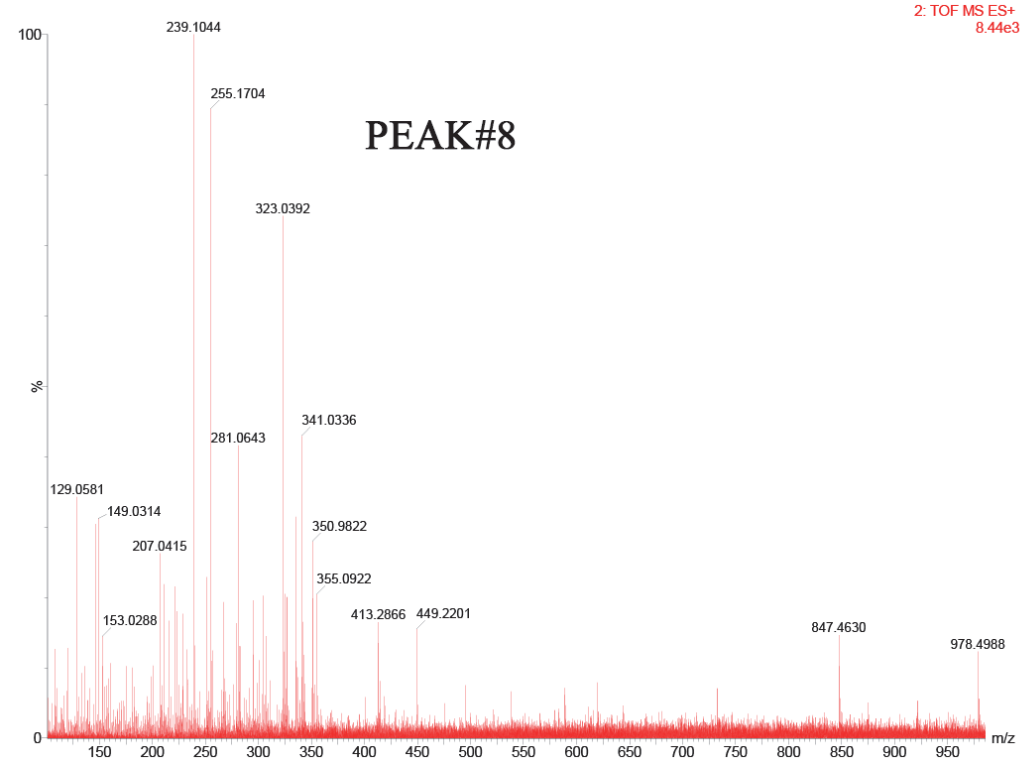
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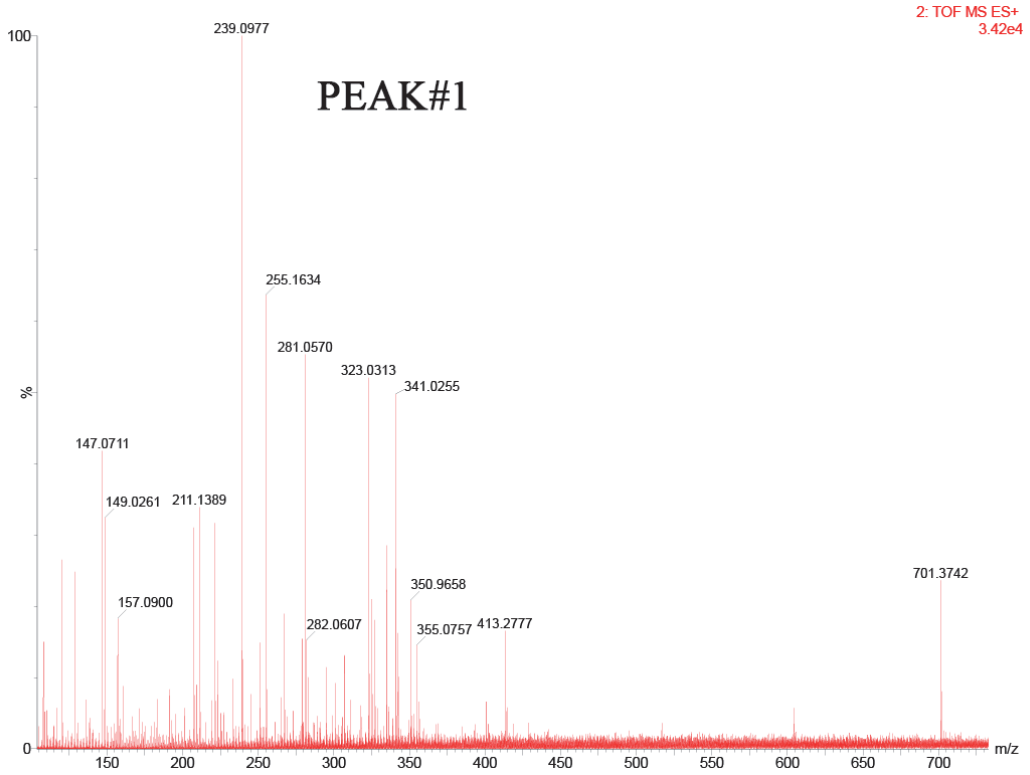
H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m/z

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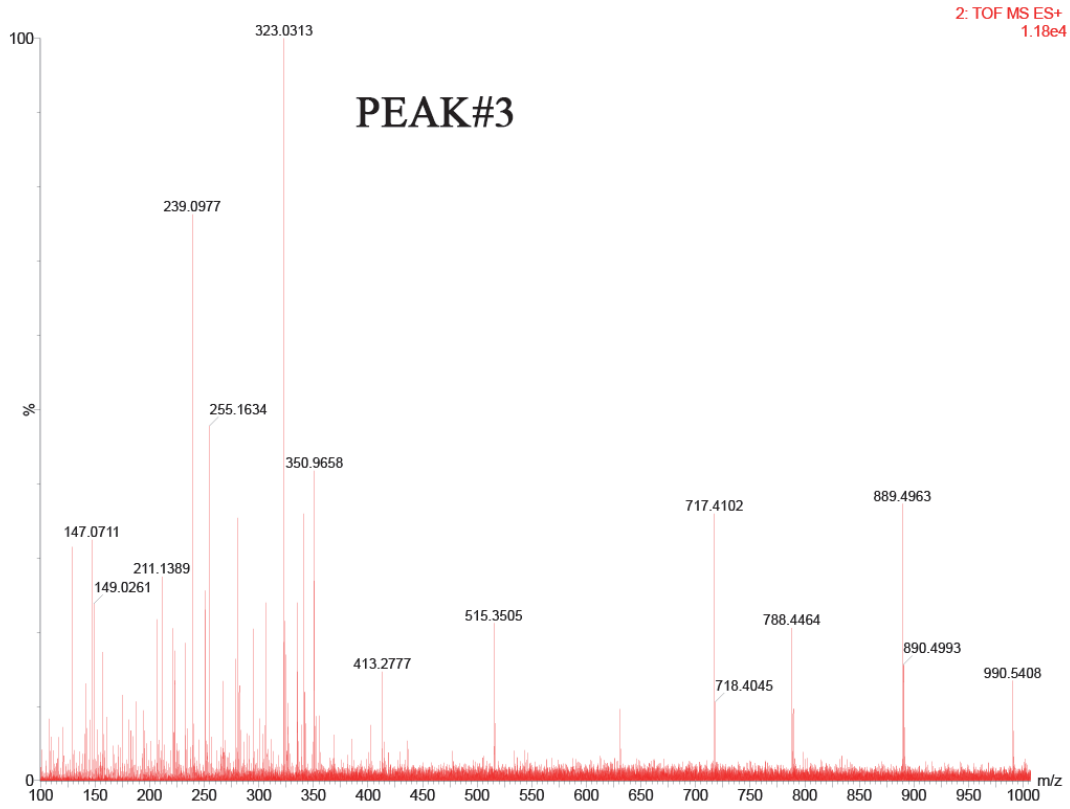
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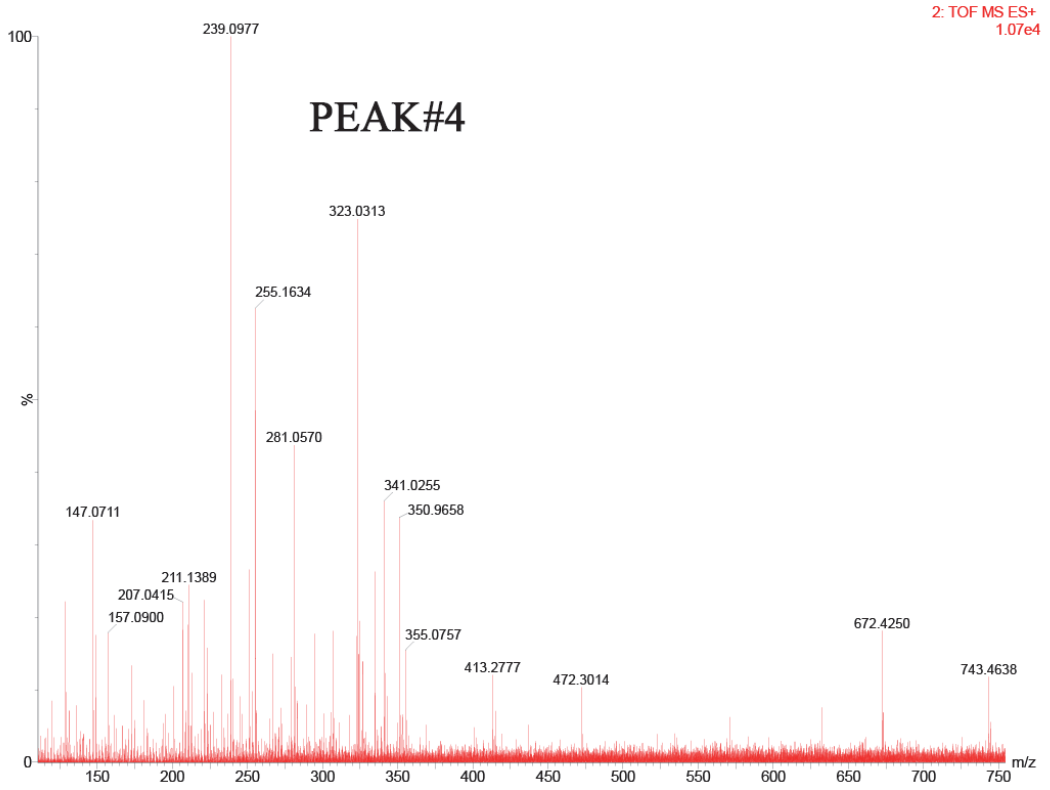
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m/z

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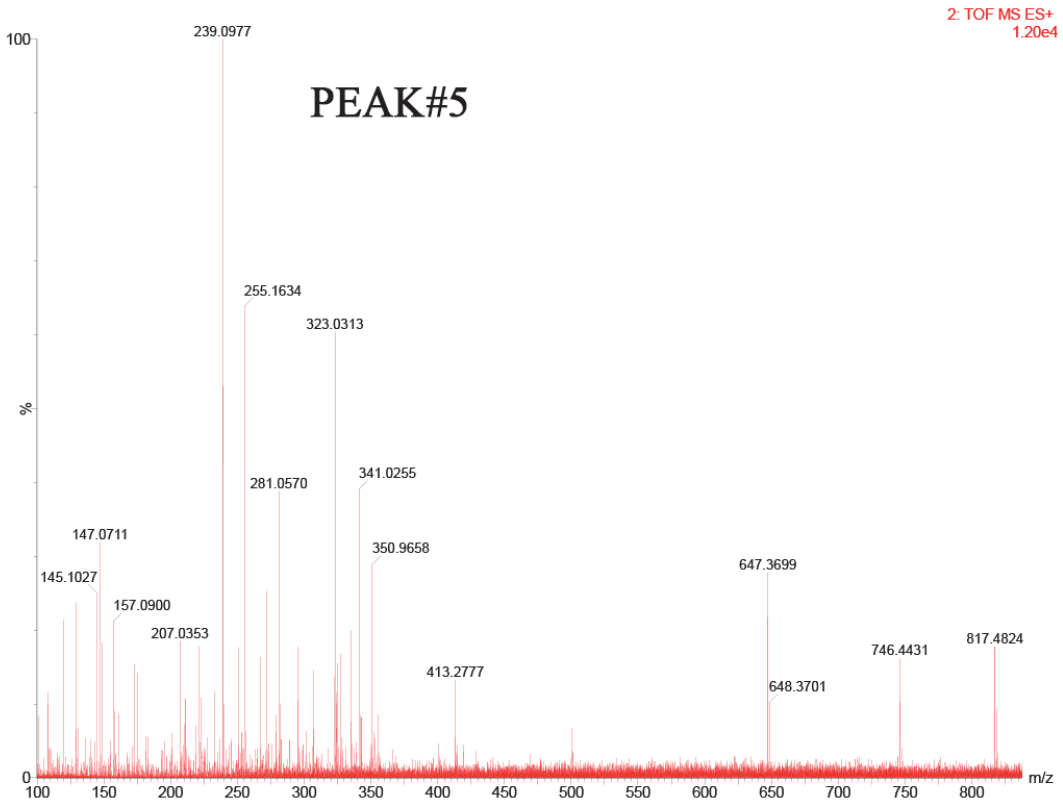
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746.4431
817.4824
413.2777
648.3701
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%
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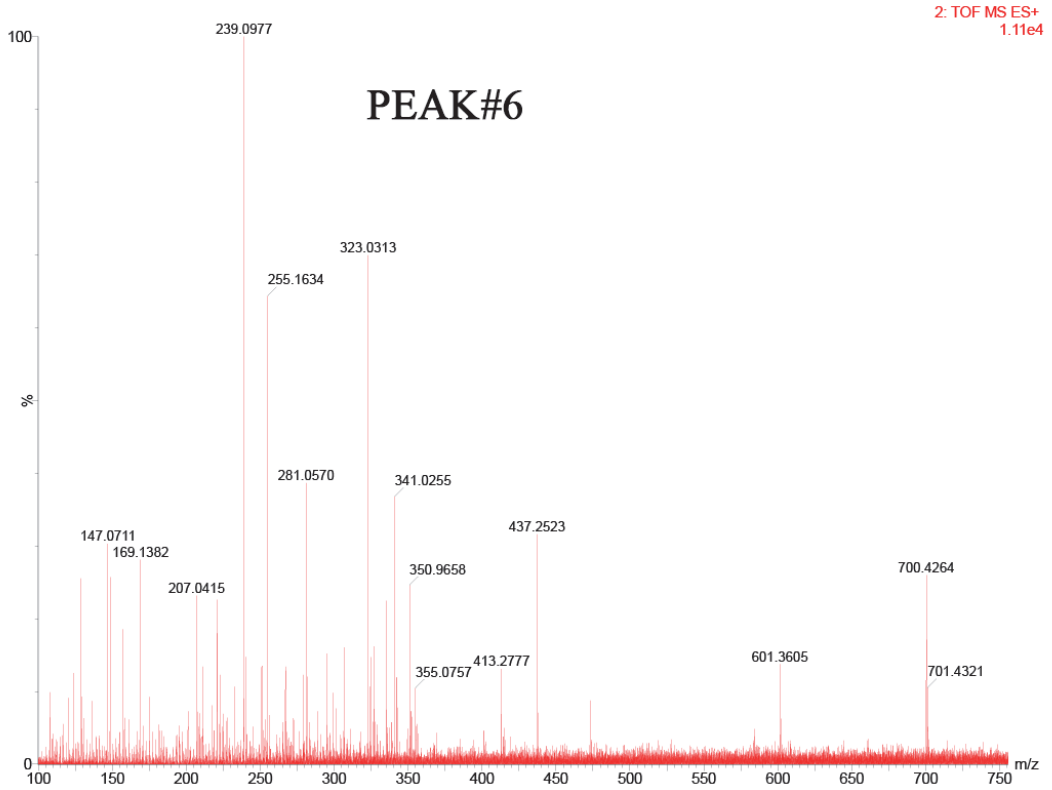
550
600
650
700
750
800
m/z

L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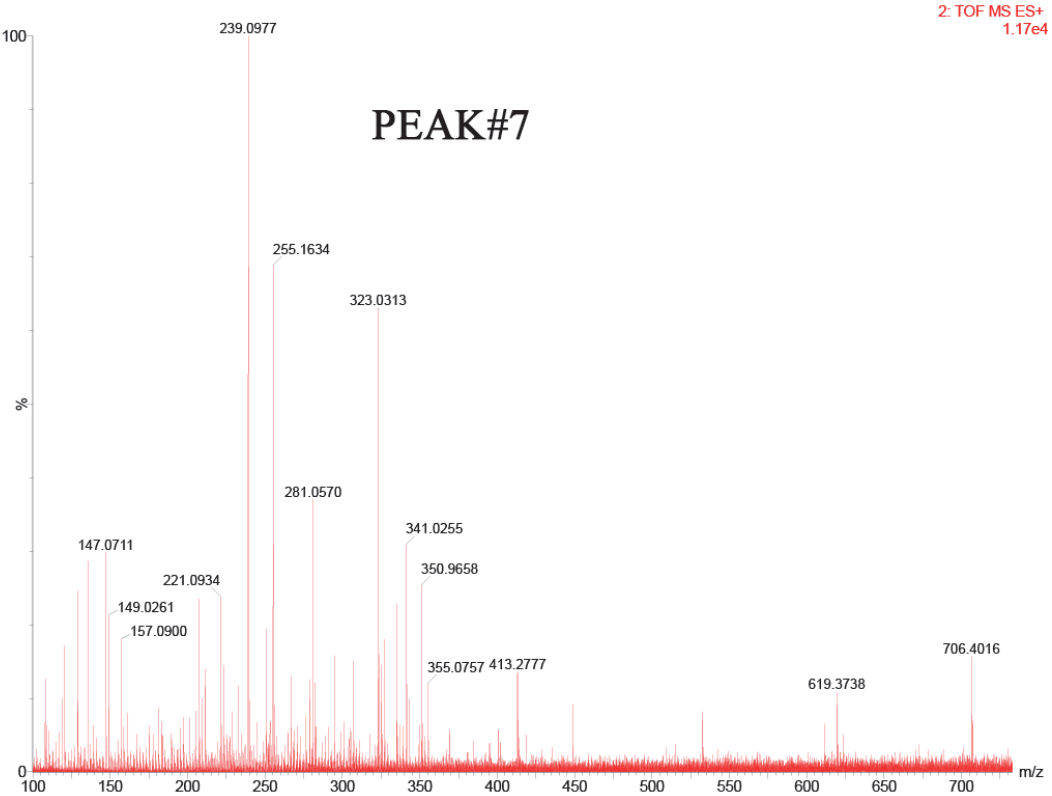
%
m/z

M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%
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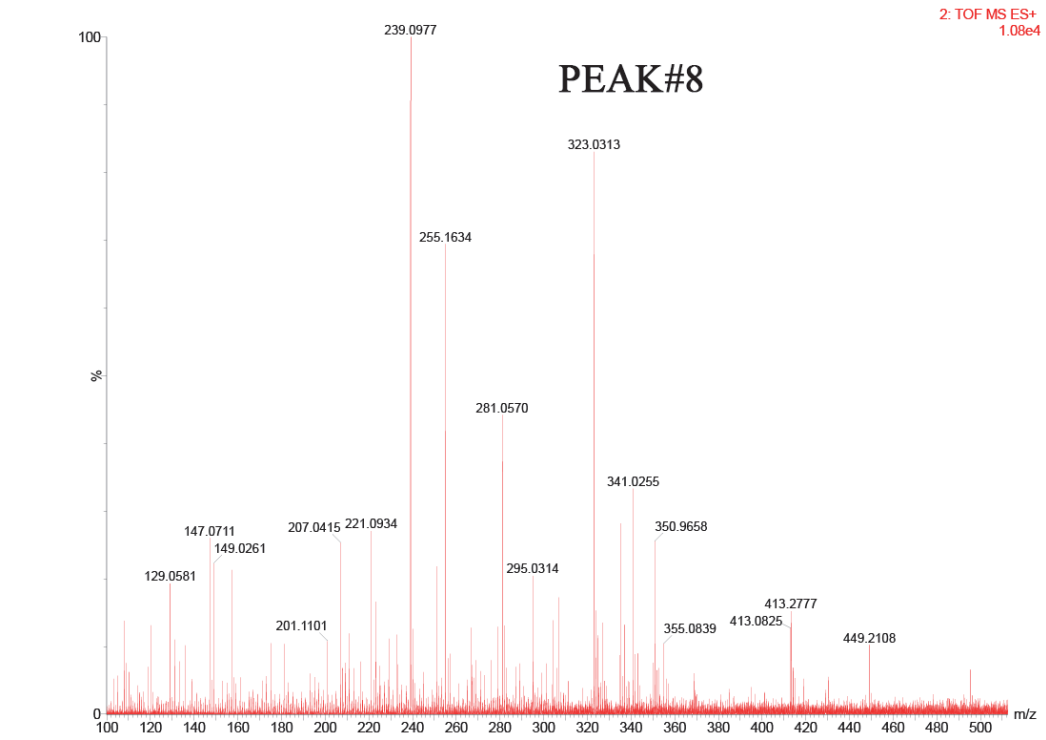

Figure S3-4 Mass spectra of dissociated smaller charged fragments in positive peaks under high energy mode. (A-G) pW64Ao2, (H-N) pK0326Y.

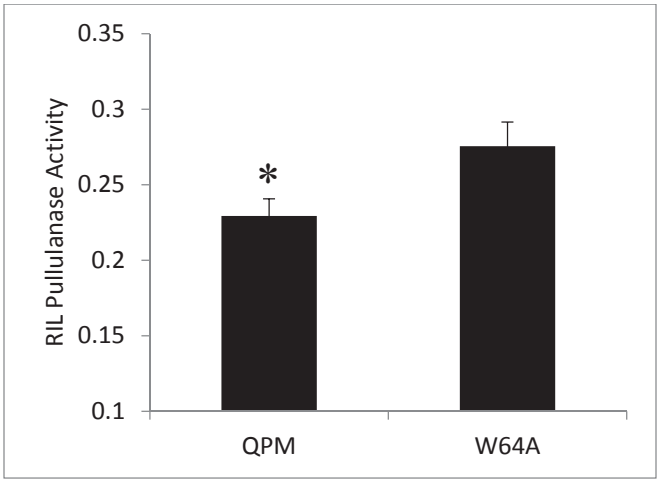

Figure S3-5 Pullulanase activity of between RILs homozygous for QPM-derived *Zpu1* alleles and W64A alleles. Pullulanase activity was measured by the spectrophotometer at 490 nm absorbance (y-axis). Each column represents mean pullulanase activity of all RILs with the corresponding genotype. The asterisk represent statistically significant differences among the lines with $p<0.05$ by two-tailed t-test. The error bars represent the standard error.

Table S3-1 Information of pullulanase amino acid sequences from 46 species

Abbreviation	Accession #	Amino Acid #	Species	Domain/ kingdom	Note
Ot	CAL50215	1057	Ostreococcus tauri (green algae)	Plant	Putative
Amt	XP_006837939	887	Amborella trichopoda	Plant	Putative
Ca	XP_004496127	953	Cicer arietinum (chickpea)	Plant	Putative
Cs	XP_004142505	960	Cucumis sativus (Cucumber)	Plant	Putative
Jc	XP_012080994	962	Jatropha curcas	Plant	Putative
Nt	XP_009587789	952	Nicotiana tomentosiformis	Plant	Putative
Pm	XP_008233250	967	Prunus mume	Plant	Putative
Sob	EES10414	966	Sorghum bicolor	Plant	Putative
St	XP_006361707	951	Solanum tuberosum (Potato)	Plant	Putative
At	NP_196056	965	Arabidopsis thaliana	Plant	Characterized
Hv	AAD34348	962	Hordeum vulgare (Barley)	Plant	Characterized
Os	ACY56113	918	Oryza sativa (Rice)	Plant	Characterized
Pt	EEF01505	943	Populus trichocarpa (California poplar)	Plant	Characterized
So	CAA58803	964	Spinacia oleracea (Spinach)	Plant	Characterized
Tc	EOY18848	965	Theobroma cacao (cocoa tree)	Plant	Characterized
Tu	EMS57285	866	Triticum urartu (Wheat)	Plant	Characterized
Zm (K0326Y)	KP872822	963	Zea Mays (Maize K0326Y)	Plant	Characterized
Zm (W64Ao2)	KP872821	963	Zea Mays (Maize W64Ao2)	Plant	Characterized
Al	GAC14300	1463	Aliiglaciecola lipolytica E3	Bacteria	Characterized
Am	WP_044583853	1443	Alteromonas macleodii	Bacteria	Characterized
As	WP_026375076	1446	Aestuariibacter salexigens	Bacteria	Characterized
Ba	GAE33974	2162	Bacillus akibai JCM 9157	Bacteria	Characterized
Bc	AKE17112	852	Bacillus cereus	Bacteria	Characterized
Bs	KJJ43347	718	Bacillus subtilis	Bacteria	Characterized
Bw	AIW82864	713	Bacillus weihenstephanensis	Bacteria	Characterized
Cm	WP_039912609	1483	Cellvibrio mixtus	Bacteria	Characterized
Cp	WP_033092782	1429	Colwellia psychrerythraea	Bacteria	Characterized
Fp	EET21947	1070	Francisella philomiragia	Bacteria	Characterized
Ga	GAC07232	1450	Glaciecola agarilytica NO2	Bacteria	Characterized
Gm	GAC24413	1450	Glaciecola mesophila	Bacteria	Characterized
Kp	WP_046654683	1102	Klebsiella pneumoniae	Bacteria	Characterized
La	AJP47013	1185	Lactobacillus acidophilus	Bacteria	Characterized
Pd	KID32704	1434	Pseudoalteromonas distincta	Bacteria	Characterized
Pl	WP_046395570	1052	Photorhabdus luminescens	Bacteria	Characterized
Px	CEG50831	1039	Pseudomonas xanthomarina	Bacteria	Characterized
Rt	WP_045859784	1090	Raoultella terrigena	Bacteria	Characterized
Sap	WP_024266499	930	Salinispora pacifica	Bacteria	Characterized
Sb	WP_006085096	1440	Shewanella baltica	Bacteria	Characterized

Table S3-1 Continued

Abbreviation	Accession #	Amino Acid #	Species	Domain/ kingdom	Note
Sd	WP_011467045	1432	Saccharophagus degradans	Bacteria	Characterized
Se	KFN88274	2218	Streptococcus equinus JB1	Bacteria	Characterized
Sm	KJQ77065	761	Streptococcus mitis	Bacteria	Characterized
Sp	CKH14036	1165	Streptococcus pyogenes	Bacteria	Characterized
Sw	WP_02877276	1443	Shewanella waksmanii	Bacteria	Characterized
Tm	AKE31465	843	Thermotoga maritima	Bacteria	Characterized
Vc	CPR23059	1201	Vibrio cholerae	Bacteria	Characterized
Xp	WP_045959066	937	Xenorhabdus poinarii	Bacteria	Characterized

Table S3-2 Primers used to clone and sequence *Zpu1* gene fragments

Name	**Sequence**
*Zpu1*_F_XhoI	ACTAATCTCGAGCGAATCCAAACGCGGACGCA
Zpu1_1R	CACCCAGTAAGCCCTCGCATCCA
Zpu1_2F	GCCCAAAGGGTGCGTCCCGT
Zpu1_2R_BstBI	TAATAATTCGAAGCCGCCAATCCATCCCAGGCA
Zpu1_3F	TCGCCTACACTGGACCGCTTG
Zpu1_3'R_BstBI	ATTTATTTCGAATGCCGAATGCACTTGCTCGCT
T7 Promoter	TAATACGACTCACTATAGGG
T7 Terminator	GCTAGTTATTGCTCAGCGG

Note: All of them but *Zpu1*_F_XhoI were used for sequencing the full length *Zpu1* clone, and first 6 primers were used to clone the *Zpu1* gene fragments.

CHAPTER FOUR

SSIII is One of the Factors that Influence the Pullulanase Activity

Abstract

Prior studies showed that pullulanase encoded by W64A-derived *Zpu1* allele might have higher activity than the one encoded by QPM-derived *Zpu1* allele. These data were contradictory to the finding that the pullulanase from W64A*o2* had lower activity than from K0326Y. Therefore, we proposed that there might be other influencing factor which may segregate during the formation of gametes. In this study, the *Zpu1*-204 mutant line (null mutant of pullulanase) and the *du1-M4* mutant line (null mutant of SSIII) were used to analyze possible interaction between pullulanase and SSIII. The pullulanase activity assay of W64A+ (wild type background), *Zpu1*-204 and *du1-M4* showed that *du1-M4* had significantly lower pullulanase activity compared with W64A+, indicating that the SSIII deficiency might inhibit the activity of pullulanase. But the deficiency of pullulanase may not have noticeable effect on SSIII abundance. SSIII had a unique long N-terminal extension that could bind numbers of other starch biosynthetic enzymes that form a large complex, so the decrease of SSIII abundance might affect other enzymes in starch biosynthetic pathway, which in turn influences the starch thermal properties and the surface characteristics of starch granules.

Introduction

Starch comprises 70% of dry weight of cereal seeds, serving as a major carbohydrate storage reservoir for plants and a main energy source for germination (Jobling

2004, Hannah and James, 2008). Starch also plays an essential role in human feed and nutrition, food and pharmaceutical industries, renewable energy development, as well as biodegradable material productions (Slattery et al, 2000; Rivard et al., 1995; Copeland et al., 2006). Studies of starch and starch biosynthetic enzymes could provide deep insight and practical solutions to meet future challenges of rapidly increasing of world population, shortening of arable land on earth and reducing of traditional energy sources.

Starch is composed of two categories of glucan polymers: amylose and amylopectin. 99% of amylose molecules are linear glucan polymers of α-D-glucopyranosyl units joined by α-1,4-glycosidic linkages, whereas amylopectin molecules are highly branched glucan polymers and branches are interlinked by α-1,6-glycosidic linkages. The ratio between amylose and amylopectin may influence the waxiness of starch. Normal maize starch has an amylose: amylopectin ratio of 23:77, whereas waxy starch has very low proportion of amylose (less than 15%), and high amylose starch (lowest waxiness) contains up to 70% of amylose (Liu et al., 2006; Tester et al., 2004; Shi et al., 1998)

Starch biosynthesis is a dynamic and complicated process in which four types of enzyme are involved, ADP-glucose pyrophosphorylase (AGPase), starch synthases (SSs), starch branching enzymes (SBEs) and starch debranching enzymes (DBEs). AGPase produces ADP-glucose (ADPG) from glucose-1-phosphate (G1P), and ADPG is a major glucosyl donor for the elongation of glucan chains with α-1,4-glycosidic linkages (Denyer et al., 1996; Ballicora et al., 2004). In higher plants, there are two categories of starch synthases: 1) granule bound starch synthase (GBSS) mainly responsible for the biosynthesis of amylose, and also associated with final editing of amylopectin (Mason-Gamer et al., 1998; Merida et al., 1999); 2) soluble starch synthases are involved in

elongation of glucan chains of amylopectin. In rice, wheat and Arabidopsis, four isoforms of soluble starch synthases were characterized, SSI, SSII, SSIII and SSIV, however, SSIV has not been identified in maize (Dian et al., 2005; Leterrier et al., 2008; Roldan et al., 2007). SBE was also called Q-enzyme previously in rice (Yamanouchi and Nakamura, 1992). Its major role in starch biosynthesis is to attack an α-1,4-glycosidic bond on a glucan chain and transfer it to C6 position on another glucosyl residue, forming an α-1,6-glycosidic bond (Zeeman et al., 2010). In maize three isoform of SBE were isolated, SBEI, SBEIIa and SBEIIb (Fisher et al., 1993). On the contrary, DBE is to attack α-1,6-glycosidic bond to break branch points, in order to remove misplaced branches to help organize the structure of starch granule (Zeeman et al., 2010). Two categories of DBEs have been identified: isoamylase and pullulanase. Some plants such as Arabidopsis, wheat, and Brachypodium, contain three isoamylase isoforms, designated ISA1, ISA2 and ISA3 (Zeeman et al., 2007; Kang et al., 2013; Chen et al., 2014), whereas in maize only one form of isoamylase was identified in studies of sugary-1 mutants, but maize heteromeric isoamylase may contain two subunits, encoded by ISA1 and ISA2 (Pan and Nelson 1984; Lin et al., 2012). Pullulanase is named limit dextrinase in some plants, such as barley and cacao, because it has relatively higher specificity to hydrolyze β-limit dextrins than other long glucan chains (Burton et al., 1999; Motamayor et al., 2013). Pullulanase also cleaves α-1,6-glycosidic bond of pullulan (a polymer consisting of multiple maltotriose units generated during starch metabolism) and produces free maltotriose which contains three glucose molecules joined with α-1,4 glycosidic bonds (Beatty et al., 1999).

Starch biosynthetic enzymes are interrelated and their functions are coordinated with one another. One form of interaction is the physical association of those enzymes with

starch granule. Addition to GBSS, early studies in maize revealed that proportion of SSI and SBE II could also bind to starch granule (Mu-Forster et al., 1996). Then proteome and phosphoproteome studies identified more starch biosynthetic enzymes, such as SSIIa, SSIII, BEI and starch phosphorylase (SP) (Grimaud et al., 2008). And the deficiency of one component may influence the abundance and activities of others (Grimaud et al., 2008). Starch granules function as a medium to bring many components in starch biosynthetic pathway together. The second form of interaction is the formation of heteromeric enzyme complexes. In maize, yeast two-hybrid and immuno-precipitation studies detected eight pairwise interactions between SSI, SSIIa, SSIII, SBEIIa and SBEIIb. And an approximately 600 kD complex consisting of SSIIa, SSIII, SBEIIa, and SBEIIb was discovered through gel permeation chromatography (Hennen-Bierwagen et al., 2008). Besides, the heteromeric enzyme complexes may contain some other factors, such as pyruvate orthophosphate dikinase (PPDK), ADPase, and sucrose synthase etc, which may participate in starch metabolic pathways (Hennen-Bierwagen et al., 2009). So far, in wheat and barley, researchers also identified and characterized the protein complexes (Tetlow et al., 2008; Ahmed et al., 2015). The protein-protein interactions could be regulated by phosphorylation which helps recruit and stabilize components in the complexes (Liu et al., 2009; Tetlow et al., 2004). The third form is the functional interaction. Some starch biosynthetic enzymes and other related protein factors may not have direct interactions between one another, but they might still interdependent functionally. In Arabidopsis, mutational study of four isoforms of DBEs (ISA1, ISA2, ISA3 and Pullulanase) showed partially functional redundancy, and the deficiency of ISA1 and ISA3 increased the activity of pullulanase, suggesting that those enzymes can partially compensate one another in

situations of stress when the expression of one or two genes decreased (Wattebled et al., 2008). In maize, SSIII could interact with isoamylase to repress the accumulation of phytoglycogen and to maintain the normal crystallization within the starch granule (Lin et al., 2012). Pullulanase activity is also influenced by several factors. Prior studies discovered that thioredoxin h, a small protein involved in cell redox regulation, could increase the pullulanase activity (Wu et al., 2002; Gelhaye et al., 2004). Also, MacGregor reviewed that a proteinaceous inhibitor in barley could reduce the pullulanase activity (MacGregor, 2004). According to the amino acid sequence alignment, barley pullulanase inhibitor is homologous to maize bifunctional Hageman factor/amylase inhibitor (MacGregor, 2004; Behnke, et al, 1998), but more studies are required to test if it can reduce the activity of pullulanase in maize.

Previous studies showed that there was no significant correlation between *Zpu1* gene expression (encoding pullulanase) and pullulanase activity, suggesting that there could be other factors post-transcriptionally affecting pullulanase activity. Also, SSIII may functionally influence the activity of pullulanase according to previous data in Chapter 2. Therefore, in this study, *Zpu1-204*and *du1-M4* mutant were analyzed in order to further confirm the functional interaction between pullulanase and SSIII. However, we were still not clear if there was a physical interaction between the two enzymes.

Materials and Methods

Genetic Materials

All maize lines in the study were grown and harvested in summer, 2013 in Elm Mott, TX. W64A+ (inbred line from Madison, WI), *Zpu1-204*(*Zpu1* null mutant) and *du1-*

M4 (SSIII null mutants) were harvest at 18 DAP. They all have W64A genetic background. All developing kernels were stored at -80oC. The Escherichia coli strain used to maintain the recombinant plasmid was NEB 10-beta Electrocompetent E. coli, and its genotype was *Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC)* (NEB, Ipswich, MA). The yeast strain PJ69-4A was obtained from Dr. Bessie Kebaara's Lab (Baylor University), and its genotype was *MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, MET2::GAL7-LacZ* (Nazarenus et al., 2005).

Pullulanase Activity Assay

Kernel proteins were extracted according to the method described previously (Chapter Two). The extracts were diluted with sodium acetate buffer (200 mM sodium acetate, pH 5.0 adjusted by glacial acetic acid) to make the final concentration of total water-soluble proteins 10 mg/mL. For pullulanase zymogram assay, 10 μL of the diluted extracts were loaded on 7.5% native polyacrylamide gel with 6% [w/v] red-pullulan (Megazyme International. Wicklow, Ireland). Native PAGE was performed at 4° C for 4 hours at 80V. The gel then was incubated in pullulanase activation buffer (25mM Tris, pH 8.2, 192 mM Glycine, 1 mM Dithiothreitol) for 36 hours at room temperature, and photographed on a light box. For quantitative activity assay, 50 μl of diluted extracts were mixed with same volume of 2% [w/v] red-pullulan solution with 50 mM KCl followed by 20 min of incubation at 40 ˚C for pullulanase digestion. The reaction was terminated with 100 μL of 100% ethanol for 10 min, and undigested red-pullulan molecules were precipitated by centrifugation at 16,000Xg, room temperature. The supernatant, containing the ethanol-soluble small dyed oligosaccharides, was collected and 80 μL of the

supernatant was transferred into a 96-well plate, and absorption measured at a wavelength of 490 nm.

SDS-PAGE and Western Immunoblotting

Kernel protein extracts were diluted with distilled water to total protein concentration of 12 mg/mL before being loaded onto a 7.5% acrylamide denaturing gel with 0.4% [w/v] SDS. SDS-PAGE was performed at room temperature for 1.5 hours at 150V. Then the gel was transferred onto nitrocellulose sheets using standard methods (Towbin et al., 1979). Western Immunoblotting was performed following the conventional procedure described at Chapter Two.

Maize Starch Isolation and Differential Scanning Calorimetry

Starch from mature maize seeds was isolated using a small-scale wet milling procedure (Gutierrez et al., 2002) and purified following the procedure described at Chapter Two. Scanning samples were processed according to the protocol of previous study (Chapter two). Gelatinization of each sample was performed using a heating rate of 5°C/min from 35-95°C. The onset, peak endotherm and total enthalpy of melting were determined using the DSC built-in analysis software (TA Instruments, New Castle, DE). Statistical analysis of the data was performed using JMP (Version Pro 9.0, SAS Institute Inc., Cary, NC).

Scanning Electron Microscopy

Maize endosperms were cut medial-longitudinally with a razor blade and mounted on SEM pedestals with double-sided carbon tapes. After sputter-coating with gold, the

samples were observed with a JEOL JSM5410 scanning electron microscope at 25mm working distance and 10kV.

Construct of Recombinant Yeast Plasmids

The plasmid vectors used in this study were pGAD-C1 and pGBD-C1 (pBK110 and pBK113 from Dr. Kebaara's Lab, Baylor University, Waco, TX). The genotype of pGAD-C1 is *LEU2, 2μ*, *ADH1* promoter::*GAL4* (codon 768-881, encoding activation domain)::polylinker::*ADH1* terminator. And the genotype of pGBD-C1 is *TRP1, 2μ, ADH1* promoter::*GAL4* (codon 1-147, encoding DNA-binding domain, encoding activation domain)::polylinker::*ADH1* terminator. Both of them have ampicillin resistance gene. The insert genes were W64A-derived *Zpu1* (2956 bp) and SSIII (5062 bp) allele. They were amplified through 50 μL PCR system with 10 μL of Phusion® HF Buffer, 1 μL of dNTP, 1 μL of each primer (Listed at Table S4-1), 0.5 μL of Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, MA), 1.5 μL of 100% DMSO, 2 μL of cDNA and up to 50 μL diH_2O. The reaction condition was set as pre-denaturation 98ºC 30s, denaturation 98 ºC 10s, annealing 69 ºC (for *Zpu1*)/60 ºC (for SSIII) 25s, extension 72 ºC 1 min/1kb, final extension 72 ºC 10min, and the cycle number was set as 35. The target bands were gel purified using Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, United Kingdom), following by restriction digestion with SalI and XmaI at 37 ºC for 1 hour. The two vectors were also digested with same pair of restriction enzymes used for the inserts, following by ligation at 16 ºC overnight with a vector: insert molar ratio of 3:1. The constructs were shown at Figure S4-1. The recombinant plasmids, including pGAD-C1-*Zpu1*, pGBD-C1-*Zpu1*, pGAD-C1-SSIII, and pGBD-C1-SSIII, were transformed into 10-beta Competent *E. coli* (NEB, Ipswich, MA)

through electroporation, followed by overnight incubation on LB plates with 100 μg/mL of ampicillin at 37ºC. Positive colonies were screened through colony PCR using same 25 μL EconoTaq System on ligation test, and were stored at -80 ºC as glycerol stocks.

Results

Pullulanase Activity and SSIII Abundance between W64A+, du1-M4 and Zpu1-204

The pullulanase activities of W64A+, *du1-M4* and *Zpu1-204*were tested by quantitative enzyme activity assay and zymogram based on Native-PAGE (Figure 4-1 A and B). The result showed that *Zpu1*-204, as a null mutation of *Zpu1* gene, had very low pullulanase activity. And *du1-M4*, a SSIII null mutation, had significantly lower pullulanase activity (approximately three-fold decrease) than its wild type background, indicating that the absence of SSIII may decrease the activity of pullulanase. The SSIII abundance was assayed by Western Immunoblotting (Figure 4-1C). The data showed that the *Zpu1-204*did not have significant change in SSIII abundance, suggesting that the decrease of pullulanase may not influence the SSIII abundance.

Thermal Properties of W64A+, du1-M4 and Zpu1-204

The thermal properties of W64A+, *du1-M4* and *Zpu1-204*were tested via DSC. W64A+ had significantly lower onset and maximum temperature than did *Zpu1*-204, which was significantly lower than *du1-M4* (Figure 4-2 A and B), whereas no significant difference was observed in enthalpy values between W64A+, *du1-M4* and *Zpu1-204*(Figure 4-2C). The data indicated that the mutants could influence the starch fine structures, such as amylopectin glucan chain length distribution, resulting in varied onset

and maximum temperature. However, the overall starch crystallinity between those three lines may not change.

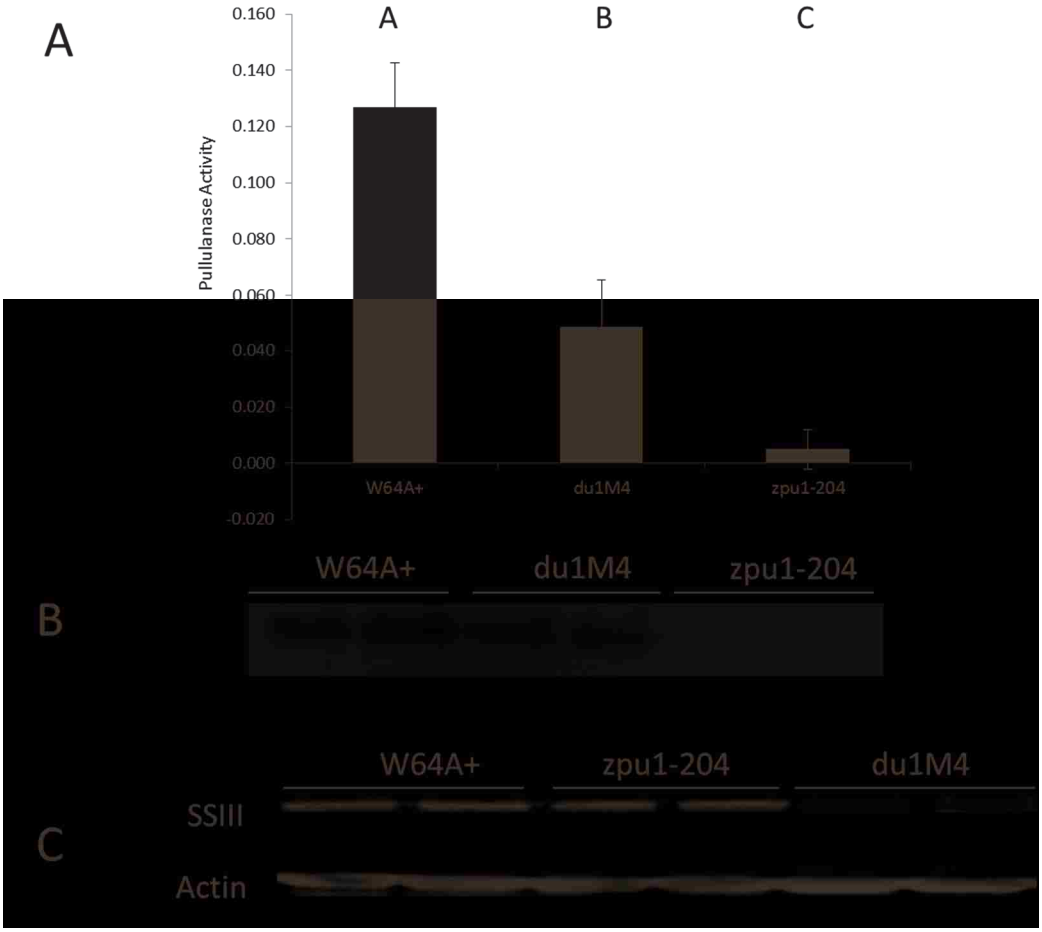

Figure 4-1 Pullulanase activity and SSIII abundance between W64A+, *Zpu1-204*and *du1-M4*. (A) Pullulanase activity was measured quantitatively by the spectrophotometer at 490 nm absorbance (y-axis). The letters above each column represent statistically significant differences among the lines with $p<0.05$ by pairwise two-tailed t-test. The error bars represent standard error. (B) Pullulanase zymogram assay was performed based on native-PAGE with red pullulan. Endosperm extracts with same total protein concentration were loaded onto the gel. The band clearness is proportional to the pullulanase activity. (C) Western blot analysis of SSIII abundance controlled with the house keeping protein actin.

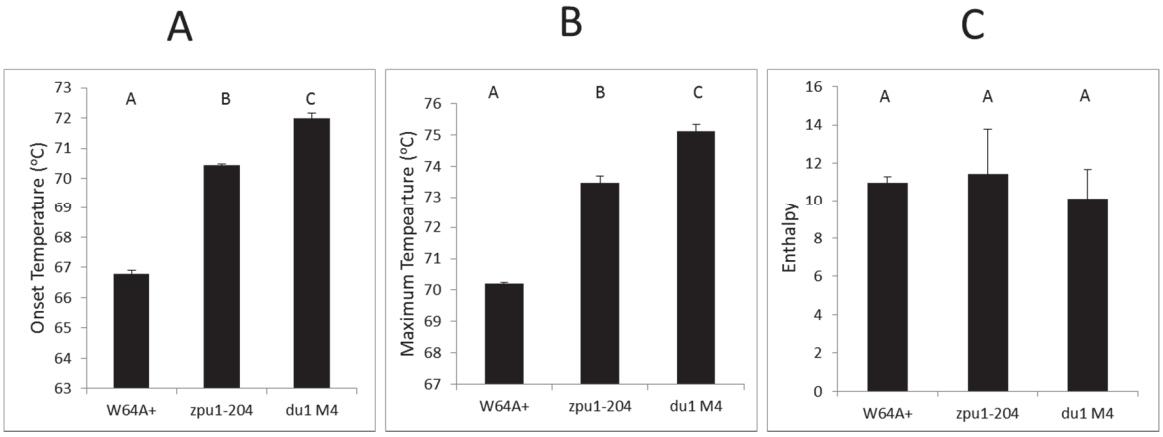

Figure 4-2 Starch thermal properties between W64A+, *Zpu1-204*and *du1-M4*. The measurement includes onset temperature (A), maximum temperature (B) and enthalpy (C). The letters above each column represent statistically significant differences among the lines for $p<0.05$ by pairwise two-tailed t-test. Columns sharing the same letter are not significantly different from one another. The error bars represent standard error.

Scanning Electron Microscopy

In order to explore the phenotypic characteristic of starch granule of the mutants, scanning electron microscopy was performed for sputter coated mature endosperm of W64A+, *du1-M4* and *Zpu1*-204. Some starch granules in W64A+ showed wrinkled surface (Figure 4-3A), which might be part of proteinaceous matrix or substances for interconnecting starch granules with one another. *Zpu1-204*starch granules had relatively smooth surface and no interconnection substances was observed (Figure 4-3B). *du1-M4* starch granules had large amount of interconnection substances between one another (Figure 4-3C). Those data suggested that the mutants may alter the starch structure and interaction between the starch and matrix proteins.

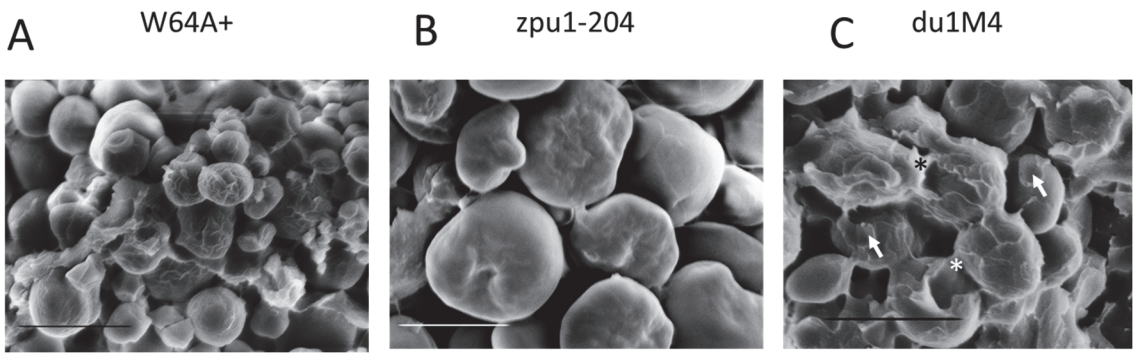

Figure 4-3 Scanning electron microscopy of starch granules between W64A+, *Zpu1*-404 and *du1-M4*. (A) Wrinkled starch granules in W64A+ endosperm. (B) Smooth starch granules in *Zpu1-204*endosperm. (C) Contacts (arrows) and interconnections (asterisks) formed between adjacent starch granules in *du1-M4* endosperm. Scale bars represent 10 um.

Discussion

Previous studies revealed that pullulanase and SSIII could be two of essential factors responsible for the formation of vitreous endosperm phenotype in quality protein maize (QPM) (Chapter 2). And *Zpu1* cloning experiment at Chapter 3 showed that W64Ao2 (homozygous for W64A-derived *Zpu1* allele) had higher pullulanase activity than did K0326Y (homozygous for QPM-derived *Zpu1* allele), which was consistent with the pullulanase activity between RILs but discrepant with the pullulanase activity between parent, suggesting that pullulanase activity could be affected by multiple factors, but some factors might be segregated during the formation of gametes. In this study, we would discuss possible factors that influence the pullulanase activity.

The SSIII null mutant could largely decrease the pullulanase activity, but pullulanase null mutant did not affect the SSIII abundance (Figure 4-1), suggesting that SSIII played an important role in pullulanase functioning. Prior studies reported that SSIII had longest N-terminal extension (769 amino acids) compared with other starch

biosynthetic enzymes (Gao et al., 1998; Zhang et al., 2005). Prior yeast two-hybrid assay revealed that SSI and SBEIIa could bind to the N-terminal extension of SSIII (Hennen-Bierwagen et al., 2008). Also, the deficiency of SSIII in du1 mutant may cause the increase of SSI activity (Figure S2-2B), which may function as a compensation to the loss of SSIII activity. Therefore, SSIII could not only function as a starch synthase to catalyze the elongation of glucan chains, but also regulate the activity of other starch biosynthetic enzymes. In this study, SSIII could be one of the important candidate regulators of pullulanase.

The *Zpu1-204*and *du1-M4* mutant altered the thermal properties of their genetic background (W64A+), indicating that the loss of function of pullulanase and SSIII may change the starch fine structure. Prior studies showed that pullulanase did not cause significant change in glucan chain length distribution of developing endosperms (Dinges et al., 2003). However, the endosperms used in this study were from mature kernels, the change of starch structure caused by pullulanase may occur in later stages of endosperm development. Also, as a starch debranching enzyme, pullulanase itself may only affect the starch branching pattern, but not the glucan chain length. du1 mutants increased proportion of DP 11-15 and DP 21-33 glucan chains and increase average number of building blocks in cluster (Lin et al., 2012; Zhu et al., 2013), which may contribute to the increase of onset and maximum temperatures (Figure 4-2 A and B). Note that building block number reflects the number of branches in a cluster (Zhu et al., 2013), so du1 mutants may produce greater number of branches in a cluster than W64A+, which may result from the decreased activity of pullulanase. There was no significant difference in enthalpy value between W64A+, *Zpu1-204*and *du1-M4* (Figure 4-2C), but the increase of SSIII abundance (with QPM-

derived *SSIII* allele) given the W64A-derived *Zpu1* allele could significantly increase the enthalpy value (Figure 2-3F), indicating that pullulanase and SSIII were not the key determinants of starch crystallinity which may be balanced by some redundant factors, but the increase of the SSIII abundance could enhance the starch crystallinity.

The *Zpu1-204*mutants accumulated branched maltooligosaccharides, but did not cause distinguishable phenotype on the surface of endosperm. The SEM of *Zpu1-204*endosperms showed that the surface of their starch granule was relatively smooth compared with their wild type counterpart (Figure 4-3 A and B), which was similar to the opaque endosperms (Figure 2-5 A, C, E and F). However, *Zpu1-204*did not form opaque endosperms, so this data suggest that lack of contacts and interconnections on the surface of starch granule may not necessarily result in opaque kernels, and those accumulated malto-oligosaccharides in matrix might be involved to compensate light transmission. Also du1 mutants were partially opaque, but the SEM showed contacts and interconnections between starch granules (Figure 4-3C), which were similar to the vitreous kernels. One possible reason was that the du1 mutant altered the activity of many starch biosynthetic enzymes, resulting in different starch granule structure that may block the light transmission. However, so far the chemical components of the contacts and interconnections and the mechanism(s) regarding their physical interaction with starch granules still remained unclear.

In summary, SSIII is a unique starch synthase with long N-terminal extension, which makes SSIII a scaffolding protein that could bind to multiple enzymes in starch biosynthetic pathways. Therefore, based on the data in this study and prior reports, SSIII could be a potential candidate that regulates the pullulanase activity.

Authors' Contributions

Hao Wu Writing, experimental design and data (All figures and tables included). Bryan C. Gibbon Experimental design and writing. Bernd Zechmann, SEM technical support.

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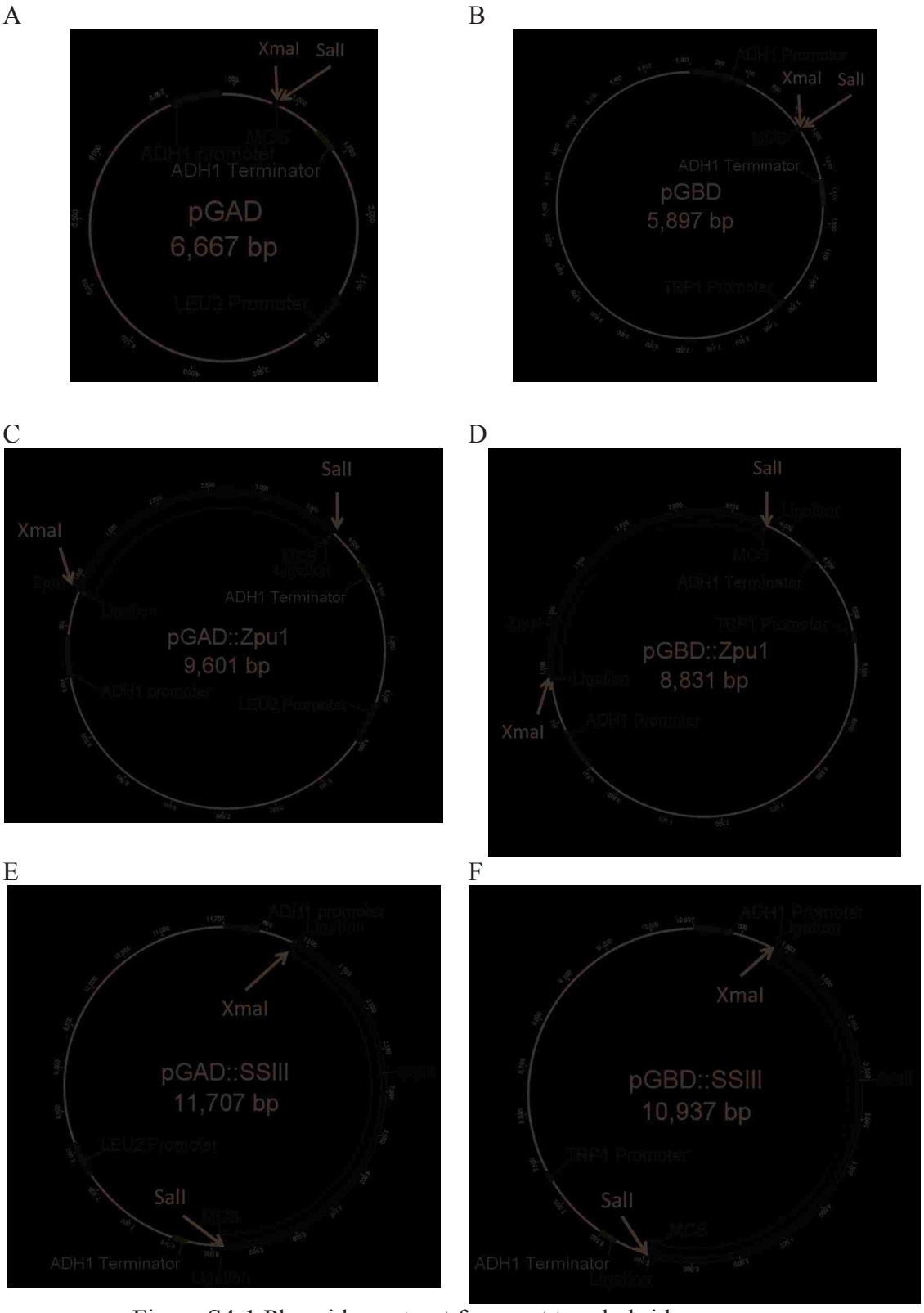

Figure S4-1 Plasmid construct for yeast two-hybrid assay.

Table S4-1 Primer sequences for *Zpu1* and *SSIII* gene cloning

Name	Sequence
*Zpu1*_F_XmaI	TCAATTCCCGGGCGAATCCAAACGCGGACGCA
*Zpu1*_3'R_SalI	ATTTATGTCGACTGCCGAATGCACTTGCTCGCT
SSIII_F_XmaI	TCTATACCCGGGGACCCTTCTTTTCTTCCCCTTC
SSIII_R_SalI	AACACTGTCGACTTACAATTTGGACGCTGAAC

CHAPTER FIVE

Conclusion and Perspective

Due to hard endosperm texture and relatively high lysine content, QPM has great potential to be commercially developed on a large scale, and benefits both human and animal diets. However, the gene make-up of o2 modifiers in QPM still needs to be further characterized. This study revealed that pullulanase and SSIII were two important factors that influence the kernel vitreousness.

The parental lines, W64Ao2 and K0326Y, had different *Zpu1* and *SSIII* alleles that may give rise to difference expression levels and enzyme activities. Pullulanase activity was dependent on both *Zpu1* alleles and *SSIII* alleles. Compared with W64Ao2 pullulanase, the one amino acid substitution (T→P) in K0326Y decreased the pullulanase activity, probably because the protein tends to form tight turn in 3D structure, so that the substrate binding might be inhibited. However, more studies are needed to elucidate the specific function of C-terminal region of pullulanase. We proposed that the *SSIII* alleles might regulate the pullulanase activity based on the evidence in RILs that different *SSIII* alleles could result in different pullulanase activity given the same *Zpu1* alleles; also in mutant lines that the deficiency of SSIII could reduce the pullulanase activity. But the underlying mechanism for pullulanase-SSIII interaction was still unknown.

As important components in starch biosynthetic pathway, change of pullulanase and SSIII caused the difference in starch fine structure. We chemically processed the isolated starch using isoamylase, followed by FACE assay, and the output data showed varied glucan chain length distributions and polydispersity indices, which had significant

non-linear relationship with vitreousness. The differences in starch fine structure also reflected by thermal properties measured by DSC during starch gelatinization. The onset temperature, maximum temperature and enthalpy were also dependent on *Zpu1* alleles and *SSIII* alleles. In addition, the diverse surface characteristics of starch granule might be caused by different starch structure influenced by pullulanase and SSIII. Prior studies showed that the granule surface pattern might be associated with the kernel vitreousness (Gibbon et al., 2003), but the granule surface patterns in *Zpu1-204*and *du1-M4* endosperm might not match well with their kernel vitreousness, suggesting that kernel vitreousness is a complicated issue. To solve this problem, starch structures and starch-protein interactions need to be further investigated.

A model of pullulanase and SSIII affecting the kernel vitreousness is illustrated in Figure 5-1. In the future, transcriptomic and proteomic studies need to be done to explore if there is any transcription factor that controls the expression of starch biosynthetic enzymes as well as other endosperm proteins. Also, *Zpu1*, SSIII double mutant line will be developed to further investigate the effect of the two enzymes on starch structure and kernel phenotypes.

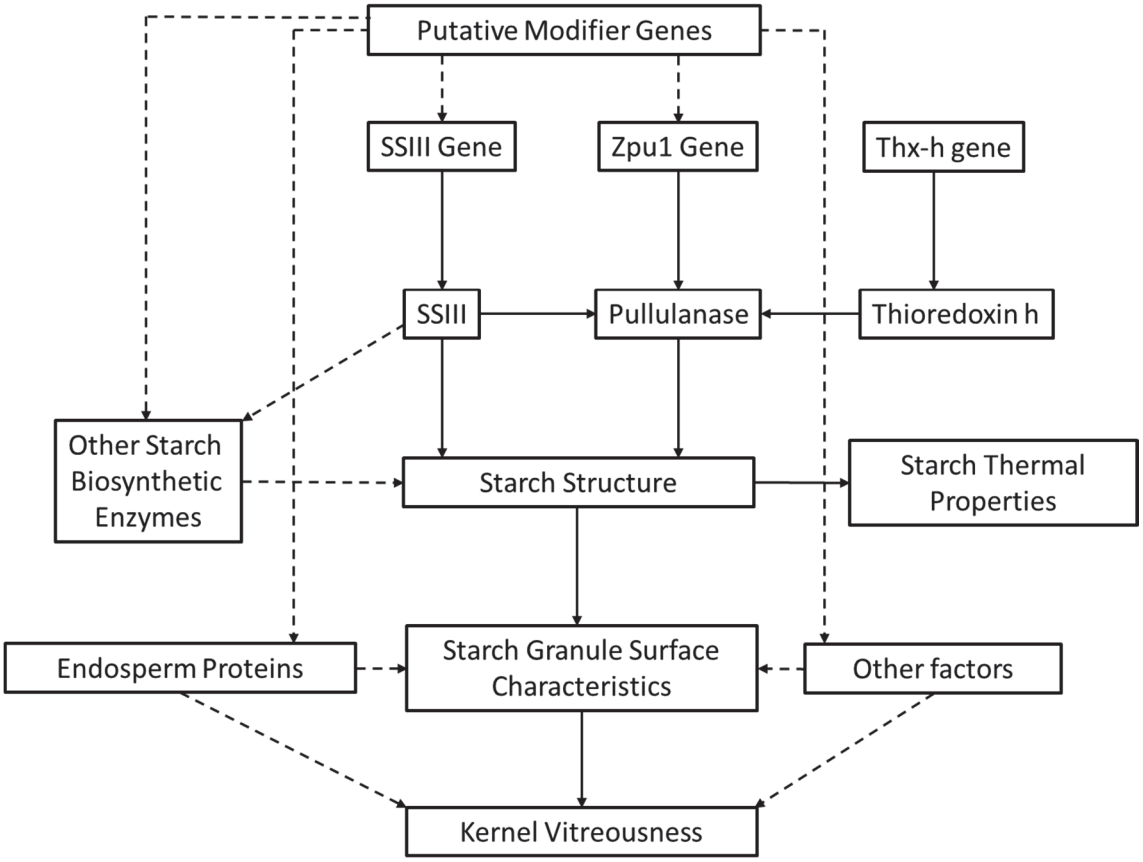

Figure 5-1 model of pullulanase and SSIII affecting the kernel vitreousness. The solid lines represent the pathways that have already been well-characterized, whereas the dash lines represent the pathways that need to be further characterized.

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