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(54) **Title**: MATERIALS AND METHODS FOR ENHANCED HEAT STABILITY AND KINETIC PARAMETERS OF ADP-GLUCOSE PYROPHOSPHORYLASE

(57) **Abstract**: Materials and methods for enhancing heat stability of AGPase by mutating amino acids within the large subunit of AGPase are disclosed. Polynucleotides that encode a mutant large subunit of AGPase provide for increased heat stability when expressed with AGPase small subunit. In one embodiment, the mutant large subunit has a single amino acid mutation. Exemplified mutant large subunits include those of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO:16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, and SEQ ID NO:52. In further embodiments, the mutant large subunit comprises two or more amino acid mutations. An exemplified mutant large subunit comprising two or more amino acid mutations is designated as SH2N131R:C424V having an amino acid sequence shown in SEQ ID NO:23. In another exemplified embodiment, a mutant large subunit of the invention, designated herein as SH2-E, comprises 8 amino acid mutations.

DESCRIPTION

MATERIALS AND METHODS FOR ENHANCED HEAT STABILITY AND KINETIC PARAMETERS OF ADP-GLUCOSE PYROPHOSPHORYLASE

CROSS-REFERENCE TO RELATED APPLICATION

The present application is a claims the benefit of U.S. Provisional Application Serial No. 61/857,546, filed July 23, 2013, which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

GOVERNMENT SUPPORT

This invention was made with government support under grant numbers IBM 0444031 and IOS 0815104 awarded by the National Science Foundation and grant numbers 2006-35100-17220, 2008-35318-18649, and 2010-04228 awarded by the United States Department of Agriculture. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

ADP-glucose pyrophosphorylase (AGPase), a critical enzyme in the starch biosynthetic pathway for plants, catalyzes the formation of ADP-glucose from ATP and glucose-1-phosphate (G-1-P). Regulation of this rate limiting step is controlled by a series of complex checkpoints including transcription, posttranslational modification, heat stability and allostery (Reviewed in Ballicora *et al.*, 2003; Hannah, 2007; Hannah and Greene, 2008; Hannah and James, 2008; and Preiss, 2009). The relative importance of each type of control is specific to the organism and tissue of expression; however, allosteric regulation by 3-phosphoglyceric acid (3-PGA) and inorganic phosphate (Pi) is common to most plant AGPases.

Most bacterial AGPases exhibit a homotetrameric structure, while the plant enzymes consist of two large and two small subunits, leading to a heterotetramer arranged in an $\alpha 2\beta 2$ structure (Reviewed in Keeling and Myers 2010). The two plant subunits were derived from

gene duplication (Bhave *et al.*, 1990, and Bae *et al.*, 1990). The maize endosperm subunits show 43.2% identity and 61% similarity, and loss of the large subunit *shrunken-2* (*Sh2)* or the small subunit *brittle-2* (*Bt2)* function abolishes >90% of endosperm AGPase activity (Hannah and Nelson, 1976). Rather than interchangeable subunits, the plant AGPase subunits have undergone sequence divergence leading to two different subunits; however, both are required for allosteric and catalytic properties of the enzyme (Cross *et al.*, 2004). Throughout the plant kingdom, the small subunits of AGPase are strikingly conserved compared to the large subunits. Using an evolutionary approach as well as measurement of the effect of amino acid changes on the activity of maize endosperm AGPase expressed in *E. coli*, the AGPase small subunit was shown to be more conserved because it was less tissue specific, less redundant, and had to form functional enzyme complexes with different large subunits expressed both in the cytosol and in the plastid (Georgelis *et al.*, 2007 and 2008).

The importance of AGPase to plant agriculture is evident from the many studies in which altered or over-expressed AGPases were placed in plants and yields were increased (Stark *et al.*, 1992, Giroux *et al.*, 1996, Smidansky *et al.*, 2002 & 2003, Sakulsingharoj *et al.*, 2004, Obana *et al.*, 2006, Wang *et al.*, 2007, Lee *et al.*, 2009 and Hannah *et al.*, 2012). Heat stability of the endosperm enzyme is particularly important as evidenced by placement of a moderately heat stable AGPase into some cereals. This conditioned yield increases of 38% wheat (Smidansky *et al.*, 2002), 23% in rice (Smidansky *et al.*, 2003) and up to a 68% in maize (Hannah *et al.*, 2012).

Previously, phylogenetic analyses were used to predict amino acid positions that could lead to the functional divergence of the AGPase large subunit (Georgelis *et al.*, 2008). Some of these amino acid residues were conserved within, but variable among AGPase large subunit families (type-II functional divergence; Gu, 2006). Others were positively selected at some point during the evolution of the AGPase large subunit families. Site-directed mutagenesis was used to alter several of these amino acid positions in SH2 (Georgelis *et al.*, 2009).

BRIEF SUMMARY OF THE INVENTION

The subject invention concerns materials and methods for enhancing heat stability of AGPase by mutating amino acids within the large subunit of AGPase. A series of type II and positively selected amino acid residues in SH2 were substituted with amino acids found in

other AGPase large subunit families and their effect on heat stability and kinetic parameters was determined. Selection was practiced for alterations enhancing k_{cat}, decreasing K_m and K_a values for substrates and the activator 3-PGA, 3-PGA independent activity and heat stability in the presence and absence of bound substrates and effectors. Variants were identified for each selected trait. These altered phenotypes were combined successfully into a single gene as gleaned from studies of an enzyme variant containing all the selected amino acids. One aspect of the present invention concerns polynucleotides that encode a mutant large subunit of AGPase that provides for increased heat stability when expressed with AGPase small subunit. In one embodiment, the mutant large subunit has a single amino acid mutation. In one embodiment, the mutant large subunit comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V. Exemplified mutant large subunits include those comprising the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, and SEQ ID NO:27. In further embodiments, the mutant large subunit comprises two or more amino acid mutations. An exemplified mutant large subunit comprising two or more amino acid mutations is designated as SH2N131R:C424V having an amino acid sequence shown in SEQ ID NO:23. In a specific embodiment, a mutant large subunit of the invention, designated herein as SH2-E, comprises 8 amino acid mutations: N131R, T142F, A160T, Q261S, A396S, V416I, S444A, and C424V. In an exemplified embodiment, the SH2-E mutant subunit comprises the amino acid sequence shown in SEQ ID NO:25. In a specific embodiment, the polynucleotide comprises the nucleotide sequence shown in any of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45,

SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, or SEQ ID NO:51.

The subject invention also concerns methods for increasing heat stability and/or increasing starch biosynthesis, and/or increasing AGPase enzymatic activity, and/or increasing crop yield of a plant or plant tissue. Increased resistance of a plant to heat conditions provides for decreased yield losses that are generally observed at elevated temperatures. In one embodiment, a method of the invention comprises introducing one or more polynucleotides of the present invention into a plant.

The subject invention also concerns mutant AGPase large subunit polypeptides encoded by the polynucleotides of the invention. The subject invention also concerns mutant plant AGPase enzymes comprising one or more mutant AGPase large subunit polypeptides of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1. Summary of the characteristics of SH2-E the individual mutants pyramided in SH2-E along with the 3-PGA independent MP + SH2-E construct. White bars: activity at 37°C; Gray bars: activity at 55°C. Black bars: activity in the absence of 3-PGA. Striped bars: $T_{1/2}$ at 37°C. The high K_{cat} mutant, SH2C424V, published previously (Georgelis *et al.*, 2009) was also included in *Sh2-E*.

Figure 2. Amino acid tree of AGPase large subunits in angiosperms. The tree was constructed in (Georgelis *et al.* (2008)) and presented in (Georgelis *et al.* (2009)). Boxes at branch junctions indicate duplication events. Positively selected amino acid sites (261, 142, 131, 155, 160, 198, 341, 364) detected in the thick branches (Georgelis *et al.* (2008)) were utilized as candidate sites for functional divergence. Bootstrap values >50% of the branches leading to the large subunit groups are shown. Accession numbers are provided by Georgelis *et al.* (2008).

Figure 3. Purity of evolutionary mutants. Each purified enzyme preparation was electrophoresed on an SDS-PAGE gel and stained with COOMASSIE brilliant blue. The arrow indicates the location of the large and small subunits. The numbers listed in each lane

refer to the % purity of each sample. The % of AGPase (small and large subunits) in each sample was estimated using IMAGEJ imaging analysis software as outlined in rsb.info.nih.gov/ij/docs/menus/analyze.html#gels. (U.S. National Institutes of Health, Bethesda, Maryland, USA). The % purity was then used to correct the *k*cat values presented in Tables 2-5.

Figures 4A and 4B. Potato small subunit AGPase tetramer crystal structure (Jin *et al.* (2005)). (Figure 4A) The active (with ADP-glucose) and effector sites are indicated. The amino acids of interest are colored: green = SH2T142 (potato ss A79), yellow = SH2V416 (potato ss L351) and orange = SH2R381 (potato ss R316). In Figure 4B, several amino acid side chains have been removed to allow SH2V416 and SH2R381 to be visible.

Figure 5. Potato small subunit AGPase tetramer crystal structure (Jin *et al.* (2005)). SH2A160 (potato ss N98) is highlighted in green. The missing amino acid sequence (disordered region) is indicated by the straight green to light blue line.

Figure 6. Two subunits of the potato small subunit AGPase tetramer crystal structure (Jin *et al.* (2005)). SH2A396 (potato ss S331) is highlighted in green on the light blue subunit and BT2V347 (potato ss M323) is highlighted in blue on the pink subunit.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is an amino acid sequence of a wild type maize endosperm large subunit AGPase polypeptide.

SEQ ID NO:2 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2N131R.

SEQ ID NO:3 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T142A.

SEQ ID NO:4 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T142F.

SEQ ID NO:5 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T151A.

SEQ ID NO:6 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2G155N.

SEQ ID NO:7 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2A160G.

SEQ ID NO:8 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2A160T.

SEQ ID NO:9 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Y198A.

SEQ ID NO:10 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Q261H.

SEQ ID NO:11 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Q261S.

SEQ ID NO:12 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Q336A.

SEQ ID NO:13 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T341N.

SEQ ID NO:14 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T341R.

SEQ ID NO:15 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2P364F.

SEQ ID NO:16 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T374K.

SEQ ID NO:17 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2P380R.

SEQ ID NO:18 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2A396S.

SEQ ID NO:19 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2V416E.

SEQ ID NO:20 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2V416I.

SEQ ID NO:21 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2E425H.

SEQ ID NO:22 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2S444A.

SEQ ID NO:23 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2N131R:C424V.

SEQ ID NO:24 is an amino acid sequence of a mutant AGP small subunit polypeptide (designated as TI).

SEQ ID NO:25 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2-E.

SEQ ID NO:26 is the amino acid sequence of a chimeric AGP small subunit protein designated herein as MP.

SEQ ID NO:27 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2-E-F142T.

SEQ ID NO:28 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2N131R.

SEQ ID NO:29 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T142A.

SEQ ID NO:30 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T142F.

SEQ ID NO:31 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T151A.

SEQ ID NO:32 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2G155N.

SEQ ID NO:33 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2A160G.

SEQ ID NO:34 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2A160T.

SEQ ID NO:35 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Y198A.

SEQ ID NO:36 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Q261H.

SEQ ID NO:37 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Q261S.

SEQ ID NO:38 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2Q336A.

SEQ ID NO:39 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T341N.

SEQ ID NO:40 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T341R.

SEQ ID NO:41 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2P364F.

SEQ ID NO:42 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2T374K.

SEQ ID NO:43 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2P380R.

SEQ ID NO:44 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2A396S.

SEQ ID NO:45 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2V416E.

SEQ ID NO:46 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2V416I.

SEQ ID NO:47 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2E425H.

SEQ ID NO:48 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2S444A.

SEQ ID NO:49 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2N131R:C424V.

SEQ ID NO:50 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2-E.

SEQ ID NO:51 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2-E-F142T.

SEQ ID NO:52 is an amino acid sequence of a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2C424V.

SEQ ID NO:53 is a nucleotide sequence encoding a mutant maize endosperm large subunit AGPase polypeptide designated herein as SH2C424V.

SEQ ID NO:54 is an amino acid sequence of a mutant chimeric maize small subunit AGPase polypeptide designated herein as MP-TI.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention concerns materials and methods for enhancing heat stability of AGPase by mutating amino acids within the large subunit of AGPase. One aspect of the present invention concerns polynucleotides that encode a mutant large subunit of AGPase that, when expressed with AGPase small subunit, provides for increased heat stability of the enzyme AGPase. In one embodiment, the mutant AGPase large subunit has a single amino acid mutation. In one embodiment, the mutant large subunit of the invention comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be one or more of N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V (wherein the first letter represents the original amino acid at the position, the number represents the position corresponding to the position of wild type AGPase maize endosperm large subunit, and the last letter represents the amino acid that replaces the original amino acid at the position). Exemplified mutant large subunits include those comprising the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof. In one embodiment, a mutant large subunit of the invention comprises the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In further embodiments, the mutant large subunit comprises two or more separate amino acid mutations. An exemplified mutant large subunit comprising two or more amino acid mutations, and is designated as SH2N131R:C424V, comprises the amino acid sequence shown in SEQ ID NO:23, or a biologically active fragment or variant thereof. In a specific embodiment, a mutant large subunit of the invention, designated herein as SH2-E, comprises 8 amino acid mutations: N131R, T142F, A160T, Q261S, A396S, V416I, S444A, and C424V. In an exemplified embodiment, the SH2-E mutant subunit comprises the amino acid sequence shown in SEQ ID NO:25, or a biologically active fragment or variant thereof. In a specific embodiment, the

polynucleotide comprises the nucleotide sequence shown in any of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, or SEQ ID NO:53, or a fragment or variant thereof. In a specific embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:30, or a fragment or variant thereof. In a further specific embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:50, or a fragment or variant thereof.

The subject invention also concerns polynucleotides encoding mutant plant AGPase large subunits that when combined with a AGPase small subunit to form a mutant AGPase enzyme, the enzyme exhibits increased heat stability and/or decreased K_m for G-1-P and/or ATP, and/or the enzyme exhibits decreased K_a for 3-PGA when compared to a wild type plant AGPase or a control AGPase, *e.g.*, an AGPase that does not comprise a mutant AGPase large subunit of the present invention. In one embodiment, a polynucleotide of the invention encodes a mutant large subunit of AGPase that comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be one or more of N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V. In certain embodiments, the polynucleotides introduced into the plant encode one or more polypeptides comprising the amino acid sequence shown in any of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof. In one embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In another embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:25, or a biologically active fragment or variant thereof. In a more specific embodiment,

the polynucleotide comprises the nucleotide sequence shown in any of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, or SEQ ID NO:53, or a fragment or variant thereof. In a specific embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:30, or a fragment or variant thereof. In another embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:50, or a fragment or variant thereof.

The subject invention also concerns methods for increasing heat stability and/or increasing starch biosynthesis, and/or increasing AGPase enzymatic activity, and/or increasing crop yield and/or increasing 3-PGA independence of AGPase and/or altering plant or seed component composition of a plant, plant part, or plant tissue relative to a control plant. A control plant can include wild type plants and plants that do not comprise or express a mutant polynucleotide or polypeptide of the invention. Increased resistance of a plant to heat conditions provides for decreased yield losses that are generally observed at elevated temperatures. Crop yield includes seed yield, *e.g.*, increased seed mass or seed number from a plant. Crop yield also includes whole plant yield, *e.g.*, increased total plant mass. Plant and seed composition alterations include altered "starch to protein" and "starch to lipid" ratios, as well as altered "amylopectin to amylose" ratios. In one embodiment, the starch to protein or starch to lipid ratio is increased relative to ratios observed in wild type or non-mutant plants. In a further embodiment, amylose levels in a plant or seed are increased relative to wild type or non-mutant plant or seed. In one embodiment, a method of the invention comprises introducing one or more polynucleotides of the present invention into a plant, plant part, plant tissue, or plant cell, and/or expressing the polynucleotide in the plant, plant part, plant tissue, or plant cell. In one embodiment, the mutant AGPase large subunit encoded by the polynucleotide comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be one or more of N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V. In certain embodiments,

the polynucleotides introduced into the plant encode one or more polypeptides comprising the amino acid sequence shown in any of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof. In one embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In another embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:25, or a biologically active fragment or variant thereof. In a more specific embodiment, the polynucleotide comprises the nucleotide sequence shown in any of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, or SEQ ID NO:53, or a fragment or variant thereof. In a specific embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:30, or a fragment or variant thereof. In another embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:50, or a fragment or variant thereof. In one embodiment, the AGPase large subunit encoded by the polynucleotide is heterologous to the plant, plant part, plant tissue, or plant cell. In one embodiment, the method further comprises introducing and/or expressing in a plant, plant part, plant tissue, or plant cell a polynucleotide encoding a mutant or wild type small subunit of AGP, such as the mutant small subunit comprising the amino acid sequence of SEQ ID NO:24, or a biologically active fragment or variant thereof, or a chimeric small subunit of AGP, such as the chimeric subunit having the amino acid sequence of SEQ ID NO:26 or SEQ ID NO:54, or a biologically active fragment or variant thereof. In one embodiment, the polynucleotide is stably incorporated into the genome of the plant, plant part, plant tissue, or plant cell. The polynucleotide can comprise regulatory elements, such as a promoter and/or enhancer sequences, that provide for increased expression of the polynucleotide and/or the polypeptide encoded thereby. In a specific embodiment, the promoter sequence is one that provides for constitutive or tissue-specific (*e.g.*, endosperm) expression. Plants, plant parts,

plant tissues, or plant cells containing the polynucleotide, or progeny of the plants, optionally can be screened for increased expression of a polynucleotide or polypeptide of the invention. Two or more generations of the plants can be grown and screened for expression to ensure that expression of the polynucleotide and/or polypeptide is stably maintained and inherited. The invention can provide for overexpression of the polynucleotide and/or polypeptide in the plant. In one embodiment, multiple copies of one or more polynucleotides of the invention are introduced into a plant, plant part, plant tissue, or plant cell and stably incorporated into the genome of the plant. In another embodiment, successive generations of a plant are transformed with one or more copies of a polynucleotide of the invention. In one embodiment, a polynucleotide of the invention is provided in an expression construct as described herein.

The subject invention also concerns methods for providing a plant, plant part, plant tissue, or plant cell with an AGPase enzyme that is 3-PGA independent, wherein the methods comprise introducing one or more polynucleotides of the present invention into a plant, plant part, plant tissue, or plant cell and/or expressing the one or more polynucleotides therein. In one embodiment, the polynucleotide is heterologous to the plant, plant part, plant tissue, or plant cell. In one embodiment, the polynucleotide encodes an amino acid sequence of SEQ ID NO:4, SEQ ID NO:8, or SEQ ID NO:25, or a biologically active fragment or variant thereof. In a specific embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:30, SEQ ID NO:34, or SEQ ID NO:50, or a fragment or variant thereof. In one embodiment, the method further comprises introducing and/or expressing in a plant, plant part, plant tissue, or plant cell a polynucleotide encoding a mutant or wild type small subunit of AGP, such as the mutant small subunit comprising the amino acid sequence of SEQ ID NO:24, or a biologically active fragment or variant thereof, or a chimeric small subunit of AGP, such as the chimeric subunit having the amino acid sequence of SEQ ID NO:26 or SEQ ID NO:54, or a biologically active fragment or variant thereof.

The subject invention also concerns mutant AGPase large subunit polypeptides encoded by the polynucleotides of the invention. In one embodiment, the mutant large subunit comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be one or more of N131R, T142A, T142F,

T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V. In one embodiment, the polypeptide comprises the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof. In one embodiment, a mutant large subunit of the invention comprises the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In further embodiments, the mutant large subunit comprises two or more amino acid mutations. An exemplified mutant large subunit comprising two or more amino acid mutations is designated as SH2N131R:C424V having an amino acid sequence shown in SEQ ID NO:23, or a biologically active fragment or variant thereof. In a specific embodiment, a mutant large subunit of the invention, designated herein as SH2-E, comprises 8 amino acid mutations: N131R, T142F, A160T, Q261S, A396S, V416I, S444A, and C424V. In an exemplified embodiment, the SH2-E mutant subunit comprises the amino acid sequence shown in SEQ ID NO:25, or a biologically active fragment or variant thereof.

The subject invention also concerns mutant plant AGPase large subunits that when combined with a AGPase small subunit to form a mutant AGPase enzyme, the enzyme exhibits decreased K_m for G-1-P and/or ATP, and/or the enzyme exhibits decreased K_a for 3-PGA when compared to a wild type plant AGPase or a control AGPase, *e.g.*, an AGPase that does not comprise a mutant AGPase large subunit of the present invention.

The subject invention also concerns mutant plant AGPase enzymes comprising one or more mutant AGPase large subunit polypeptides of the invention. The mutant plant AGPase can also comprise one or more wild type or mutant and/or chimeric AGPase small subunit polypeptides and/or one or more wild type or mutant AGPase large subunit polypeptides. In one embodiment, the mutant large subunit comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be one or more of N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A,

T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V. In specific embodiments, a mutant plant AGPase enzyme comprises one or more mutant AGPase large subunit polypeptides any of which can comprise the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant of any such sequence, wherein the mutant AGPase enzyme exhibits increased heat stability relative to a wild type AGPase enzyme or a control AGPase. In one embodiment, the mutant plant AGPase enzyme comprises one or more AGPase large subunits comprising the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In another embodiment, the mutant plant AGPase enzyme comprises one or more mutant AGPase large subunits comprising 8 amino acid mutations: N131R, T142F, A160T, Q261S, A396S, V416I, S444A, and C424V. In an exemplified embodiment, the mutant AGPase large subunit comprises the amino acid sequence of SEQ ID NO:25, or a biologically active fragment or variant thereof. In one embodiment, the mutant plant enzyme comprises two mutant AGPase large subunits of the invention, wherein the mutant polypeptides can have the same mutation(s) or can have different mutation(s). In one embodiment, the mutant or chimeric plant AGPase enzyme comprises one or more AGPase small subunits comprising the amino acid sequence of SEQ ID NO:24, SEQ ID NO:26, or SEQ ID NO:54, or a biologically active fragment or variant thereof.

The subject invention also concerns mutant plant AGPase enzymes comprising one or more mutant AGPase large subunit polypeptides of the invention and one or more mutant AGPase small subunit polypeptides. In one embodiment, a mutant small subunit AGPase is a chimeric plant AGPase small subunit comprising sequences from two different plants (as described in U.S. Patent No. 7,173,165) and/or a plant AGPase small subunit that comprises an amino acid mutation wherein the threonine amino acid corresponding to amino acid position 462 of wild type maize endosperm AGPase small subunit is substituted with an amino acid that confers increased heat stability (as described in U.S. Patent No. 8,536,407). In a specific embodiment of the latter, the amino acid substituted for threonine is an isoleucine; in an exemplified embodiment, the mutant plant AGPase small subunit comprises

the amino acid sequence shown in SEQ ID NO:24, or a biologically active fragment or variant thereof. A chimeric AGPase small subunit that can be used in the present invention can comprise a C-terminal portion from one plant and an N-terminal portion from another plant. In one embodiment, a chimeric AGPase small subunit comprises an N-terminus sequence having approximately the first 150 to 250 amino acids of the N-terminus of a first plant AGPase small subunit and a C-terminus sequence comprising approximately the terminal 300 residues or less of the C-terminus of a second plant AGPase small subunit. Thus, the C-terminus of the chimeric small subunit can comprise the terminal 300, or 299, or 298, or 297, or 296, or 295, *etc.*, residues of the C-terminus of the second plant. The small subunit sequences can be from an AGPase of a monocot or dicot plant, or both a monocot and a dicot. Monocotyledonous plants, such as, for example, rice, wheat, barley, oats, sorghum, maize, lilies, and millet are included within the scope of the invention. Dicot plants can include, for example, tobacco, soybean, potato, sweet potato, radish, cabbage, rape, apple tree, and lettuce. In one embodiment, the first 200 or so amino acids of the N-terminus of the chimeric AGPase small subunit are from the N-terminus of maize endosperm AGPase small subunit and the C-terminus amino acids are from the C-terminus of potato tuber AGPase small subunit plus the mutation corresponding to amino acid position 462 of the present invention. In a specific embodiment, the C-terminus region of a chimeric AGPase small subunit of the present invention comprises the terminal 276 amino acids of the AGPase small subunit of potato tuber. In an exemplified embodiment, the chimeric AGPase small subunit comprises a portion of the small subunit of maize endosperm AGPase and a portion of the small subunit of potato tuber AGPase. In a specific embodiment, the chimeric AGPase small subunit contains a) the first 199 amino acids (*i.e.*, amino acids 1 through 199) from the small subunit of maize endosperm AGPase and the carboxyl terminal end of the small subunit of potato tuber AGPase, starting at amino acid 246 (*i.e.*, amino acids 246 through 521) using the amino acid sequence shown for the protein deposited as Genbank accession number X61186 (or, alternatively, starting at amino acid 175 using the numbering system for the potato AGPase subunit as in Hannah *et al.*, 2001) and b) the mutation wherein the threonine amino acid corresponding to amino acid position 462 of wild type maize endosperm AGPase small subunit is substituted with an amino acid that confers increased heat stability, such as an isoleucine. In an exemplified embodiment, the plant chimeric AGPase small subunit comprises the amino acid sequence shown in SEQ ID NO:26 or SEQ ID NO:54, or a

biologically active fragment or variant thereof. In one embodiment, one of the mutant AGPase large subunit polypeptides can be any of those as described in any of U.S. Patent Nos. 5,589,618; 5,650,557; 5,872,216; 6,069,300; 6,184,438; 6,403,863; 6,809,235; 7,173,165; 7,312,378; and 6,969,783. In one embodiment, a mutant AGPase large subunit polypeptide comprises a Rev6 mutation. In another embodiment, a mutant AGPase large subunit polypeptide comprises one or more heat stable (HS) mutations, as described in U.S. Patent Nos. 6,069,300; 6,403,863; 6,809,235; 7,312,378; and 6,969,783, and published International patent application nos. WO 99/58698; WO 2003/0070901; WO 98/22601; and WO 02/072784, such as, for example, the HS33 mutation. In one embodiment, the mutant plant AGPase enzyme comprises two mutant AGPase large subunit polypeptides of the invention, wherein the mutant AGPase large subunit polypeptides can have the same mutation(s) or can have different mutation(s), as described herein. In another embodiment, the mutant plant AGPase enzyme comprises two mutant AGPase small subunit polypeptides wherein the mutant AGPase small subunit polypeptides can have the same mutation(s) or can have different mutation(s). In a further embodiment, the mutant plant AGPase enzyme comprises two mutant AGPase small subunit polypeptides of the invention and two mutant SH2 polypeptides, wherein the mutant AGPase small subunit polypeptides can have the same mutation(s) or can have different mutation(s) and the mutant AGPase large subunit polypeptides can have the same mutation(s) or can have different mutation(s), as described herein.

The subject invention also concerns methods for providing for a mutant plant AGPase enzyme having increased heat stability relative to wild type plant AGPase. In one embodiment, the method comprises incorporating or providing one or more mutant AGPase large subunit polypeptides of the present invention with wild type or mutant AGPase small subunits in an AGPase enzyme. In one embodiment, the AGPase enzyme comprises a tetramer of polypeptide subunits, wherein one, two, or more of the subunits is a mutant polypeptide of the present invention. In one embodiment, the AGPase enzyme can also comprise a mutant AGPase large subunit polypeptide subunit, such as a mutant large subunit comprising a Rev6 and/or another heat stability mutation, such as HS33.

The subject invention also concerns plants, plant parts, plant tissue, and plant cells of the invention that comprise a polynucleotide or the protein encoded by the polynucleotide of the invention, or that express a mutant polypeptide of the invention, or a biologically active

fragment or variant thereof, or that comprise or express a mutant plant AGP enzyme of the present invention. In one embodiment, a large or small AGPase subunit of the invention is heterologous to the plant, plant part, plant tissue, or plant cell. Plant parts include, but are not limited to, fruit, seed, flowers (including floral organs and structures such as sepals, petals, stamens, carpels, anthers, and ovules), leaf, roots, stem, scion, tubers, and rootstock. Plant tissue includes, but is not limited to, vascular tissue, dermal tissue, and ground tissue. Plant genera contemplated within the scope of the invention include, but are not limited to, Agrotis, Allium, Ananas, Anacardium, Apium, Arachis, Asparagus, Athamantha, Atropa, Avena, Bambusa, Beta, Brassica, Bromus, Browaalia, Camellia, Cannabis, Carica, Ceratonia. Cicer, Chenopodium, Chicorium, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Coix, Cucumis, Cucurbita, Cynodon, Dactylis, Datura, Daucus, Dianthus, Digitalis, Dioscorea, Elaeis, Eliusine, Euphorbia, Festuca, Ficus, Fragaria, Geranium, Glycine, Graminae, Gossypium, Helianthus, Heterocallis, Hevea, Hibiscus, Hordeum, Hyoscyamus, Ipomoea, Lactuca, Lathyrus, Lens, Lilium, Linum, Lolium, Lotus, Lupinus, Lycopersicon, Macadamia, Macrophylla, Malus, Mangifera, Manihot, Majorana, Medicago, Musa, Narcissus, Nemesia, Nicotiana, Onobrychis, Olea, Olyreae, Oryza, Panicum, Panicum, Panieum, Pannisetum, Pennisetum, Petunia, Pelargonium, Persea, Pharoideae, Phaseolus, Phleum, Picea, Poa, Pinus, Pistachia, Pisum, Populus, Pseudotsuga, Pyrus, Prunus, Pseutotsuga, Psidium, Quercus, Ranunculus, Raphanus, Ribes, Ricinus, Rhododendron, Rosa, Saccharum, Salpiglossis, Secale, Senecio, Setaria, Sequoia, Sinapis, Solanum, Sorghum, Stenotaphrum, Theobromus, Trigonella, Trifolium, Trigonella, Triticum, Tsuga, Tulipa, Vicia, Vitis, Vigna and Zea. Plants within the scope of the present invention include monocotyledonous plants, such as, for example, rice, wheat, barley, oats, rye, sorghum, maize, sugarcane, pineapple, onion, bananas, coconut, lilies, turfgrasses, and millet. Plants within the scope of the present invention also include dicotyledonous plants, such as, for example, tomato, cucumber, squash, peas, alfalfa, melon, chickpea, chicory, clover, kale, lentil, soybean, beans, tobacco, potato, sweet potato, yams, cassava, radish, broccoli, spinach, cabbage, rape, apple trees, citrus (including oranges, mandarins, grapefruit, lemons, limes and the like), grape, cotton, sunflower, strawberry, lettuce, and hop. Herb plants containing a polynucleotide of the invention are also contemplated within the scope of the invention. Herb plants include parsley, sage, rosemary, thyme, and the like. In one embodiment, the plant, plant part, plant tissue, or plant cell is *Zea mays*. In one embodiment, a plant, plant part, plant tissue, or plant

cell is a transgenic plant, plant part, plant tissue, or plant cell. In another embodiment, a plant, plant part, plant tissue, or plant cell is one that has been obtained through a breeding program.

A control plant, plant part, plant tissue, or plant cell or a control enzyme provides for a comparison for evaluating changes or differences in a plant, plant part, plant tissue, or plant cell or enzyme of the subject invention. Thus, a plant, plant part, plant tissue, or plant cell or enzyme of the subject invention can be compared to a control plant, plant part, plant tissue, or plant cell or control enzyme to evaluate the extent of differences or changes (*e.g.*, increased enzymatic activity) exhibited by the subject plant, plant part, plant tissue, or plant cell or enzyme relative to the control. A control plant can be, for example, a plant of the same genotype as a plant of the subject invention but that does not comprise and/or express a mutant polynucleotide or polypeptide of the invention. A control enzyme can be, for example, an enzyme that is identical to an enzyme of the invention but that does not comprise a mutant polypeptide of the invention.

Polynucleotides useful in the present invention can be provided in an expression construct. Expression constructs of the invention generally include regulatory elements that are functional in the intended host cell in which the expression construct is to be expressed. Thus, a person of ordinary skill in the art can select regulatory elements for use in bacterial host cells, yeast host cells, plant host cells, insect host cells, mammalian host cells, and human host cells. Regulatory elements include promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements. In one embodiment, a regulatory element is heterologous to an AGPase large subunit of the invention (*e.g.*, a promoter that is not normally associated with a plant AGPase large subunit gene). As used herein, the term "expression construct" refers to a combination of nucleic acid sequences that provides for transcription of an operably linked nucleic acid sequence. As used herein, the term "operably linked" refers to a juxtaposition of the components described wherein the components are in a relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation.

An expression construct of the invention can comprise a promoter sequence operably linked to a polynucleotide sequence encoding a mutant polypeptide of the invention. Promoters can be incorporated into a polynucleotide using standard techniques known in the art. Multiple copies of promoters or multiple promoters can be used in an expression

construct of the invention. In a preferred embodiment, a promoter can be positioned about the same distance from the transcription start site in the expression construct as it is from the transcription start site in its natural genetic environment. Some variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the expression construct.

If the expression construct is to be provided in or introduced into a plant cell, then plant viral promoters, such as, for example, a cauliflower mosaic virus (CaMV) 35S (including the enhanced CaMV 35S promoter (see, for example U.S. Patent No. 5,106,739)) or a CaMV 19S promoter or a cassava vein mosaic can be used. Other promoters that can be used for expression constructs in plants include, for example, prolifera promoter, Ap3 promoter, heat shock promoters, T-DNA 1'- or 2'-promoter of *A. tumefaciens*, polygalacturonase promoter, chalcone synthase A (CHS-A) promoter from petunia, tobacco PR-1a promoter, ubiquitin promoter, actin promoter, alcA gene promoter, pin2 promoter (Xu *et al.*, 1993), maize WipI promoter, maize trpA gene promoter (U.S. Patent No. 5,625,136), maize CDPK gene promoter, and RUBISCO SSU promoter (U.S. Patent No. 5,034,322) can also be used. Tissue-specific promoters, for example fruit-specific promoters, such as the E8 promoter of tomato (accession number: AF515784; Good *et al.* (1994)) can be used. Fruit-specific promoters such as flower organ-specific promoters can be used with an expression construct of the present invention for expressing a polynucleotide of the invention in the flower organ of a plant. Examples of flower organ-specific promoters include any of the promoter sequences described in U.S. Patent Nos. 6,462,185; 5,639,948; and 5,589,610. Seed-specific promoters such as the promoter from a β-phaseolin gene (for example, of kidney bean) or a glycinin gene (for example, of soybean), and others, can also be used. Endosperm-specific promoters include, but are not limited to, MEG1 (EPO application No. EP1528104) and those described by Wu *et al.* (1998), Furtado *et al.* (2002), and Hwang *et al.* (2002). Root-specific promoters, such as any of the promoter sequences described in U.S. Patent No. 6,455,760 or U.S. Patent No. 6,696,623, or in published U.S. patent application Nos. 20040078841; 20040067506; 20040019934; 20030177536; 20030084486; or 20040123349, can be used with an expression construct of the invention. Constitutive promoters (such as the CaMV, ubiquitin, actin, or NOS promoter), developmentally-regulated promoters, and inducible promoters (such as those promoters than can be induced by heat,

light, hormones, or chemicals) are also contemplated for use with polynucleotide expression constructs of the invention.

Expression constructs of the invention may optionally contain a transcription termination sequence, a translation termination sequence, a sequence encoding a signal peptide, and/or enhancer elements. Transcription termination regions can typically be obtained from the 3' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. A signal peptide sequence is a short amino acid sequence typically present at the amino terminus of a protein that is responsible for the relocation of an operably linked mature polypeptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Targeting gene products to an intended cellular and/or extracellular destination through the use of an operably linked signal peptide sequence is contemplated for use with the polypeptides of the invention. Classical enhancers are cis-acting elements that increase gene transcription and can also be included in the expression construct. Classical enhancer elements are known in the art, and include, but are not limited to, the CaMV 35S enhancer element, cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element. Intron-mediated enhancer elements that enhance gene expression are also known in the art. These elements must be present within the transcribed region and are orientation dependent. Examples include the maize *shrunken-1* enhancer element (Clancy and Hannah, 2002).

DNA sequences which direct polyadenylation of mRNA transcribed from the expression construct can also be included in the expression construct, and include, but are not limited to, an octopine synthase or nopaline synthase signal. The expression constructs of the invention can also include a polynucleotide sequence that directs transposition of other genes, *i.e.*, a transposon.

Polynucleotides of the present invention can be composed of either RNA or DNA. Preferably, the polynucleotides are composed of DNA. In one embodiment, the DNA is complementary DNA (cDNA) synthesized from or based on a messenger RNA (mRNA) template sequence. The subject invention also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein. Polynucleotides and polypeptides of the invention can be provided in purified or isolated form.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode mutant polypeptides of the present invention. A table showing all possible triplet codons (and where U also stands for T) and the amino acid encoded by each codon is described in Lewin (1985). In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, mutant polypeptides of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional activity of the polypeptide encoded by the polynucleotides of the present invention. Allelic variants of the nucleotide sequences encoding a wild type or mutant polypeptide of the invention are also encompassed within the scope of the invention.

Substitution of amino acids other than those specifically exemplified or naturally present in a wild type or mutant polypeptide and/or AGPase enzyme of the invention are also contemplated within the scope of the present invention. For example, non-natural amino acids can be substituted for the amino acids of a mutant AGPase large subunit polypeptide, so long as the mutant polypeptide having the substituted amino acids retains substantially the same functional activity as the mutant polypeptide in which amino acids have not been substituted. Examples of non-natural amino acids include, but are not limited to, ornithine, citrulline, hydroxyproline, homoserine, phenylglycine, taurine, iodotyrosine, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ-amino butyric acid, ε-amino hexanoic acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, norleucine, norvaline, sarcosine, homocitrulline, cysteic acid, τ-butylglycine, τ-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, C-methyl amino acids, N-methyl amino acids, and amino acid analogues in general. Non-natural amino acids also include amino acids having derivatized side groups. Furthermore, any of the amino acids in the protein can be of the D (dextrorotary) form or L (levorotary) form. Allelic variants of a protein sequence of a wild type or mutant AGPase small or large subunit polypeptide of the present invention are also encompassed within the scope of the invention.

Amino acids can be generally categorized in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby a wild type or mutant

AGPase large subunit polypeptide of the present invention and/or a wild type or mutant AGPase small subunit polypeptide having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the polypeptide having the substitution still retains substantially the same functional activity (*e.g.*, increased heat stability of an AGPase enzyme) as the polypeptide that does not have the substitution. Polynucleotides encoding a wild type or mutant AGPase large subunit polypeptide and/or a wild type or mutant AGPase small subunit polypeptide having one or more amino acid substitutions in the sequence are contemplated within the scope of the present invention. Table 7 below provides a listing of examples of amino acids belonging to each class.

Table 7.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

The subject invention also concerns variants of the polynucleotides of the present invention that encode functional wild type or mutant AGPase large or small subunit polypeptides of the invention. Variant sequences include those sequences wherein one or more nucleotides of the sequence have been substituted, deleted, and/or inserted. The nucleotides that can be substituted for natural nucleotides of DNA have a base moiety that can include, but is not limited to, inosine, 5-fluorouracil, 5-bromouracil, hypoxanthine, 1-methylguanine, 5-methylcytosine, and tritylated bases. The sugar moiety of the nucleotide in a sequence can also be modified and includes, but is not limited to, arabinose, xylulose, and hexose. In addition, the adenine, cytosine, guanine, thymine, and uracil bases of the nucleotides can be modified with acetyl, methyl, and/or thio groups. Sequences containing nucleotide substitutions, deletions, and/or insertions can be prepared and tested using standard techniques known in the art.

The subject invention also contemplates fragments of large and small AGPase subunits of the invention, and the use thereof, so long as the fragment retains functional and/or biological activity substantially the same as the full length polypeptide. Fragments and variants of a mutant polypeptide of the present invention can be generated as described herein and tested for the presence of enzymatic, heat stability, and other functions using standard techniques known in the art. Thus, an ordinarily skilled artisan can readily prepare and test fragments and variants of a mutant polypeptide of the invention and determine whether the fragment or variant retains functional or biological activity (*e.g.*, enzymatic activity, increased heat stability of an AGPase enzyme, *etc.*) relative to full-length or a non-variant mutant polypeptide.

Polynucleotides and polypeptides contemplated within the scope of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those sequences of the invention specifically exemplified herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. *See* NCBI/NIH website.

The subject invention also contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the polynucleotide sequences exemplified herein so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis *et al.*, 1982). As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at

20-25 C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature, Tm, is described by the following formula (Beltz *et al.*, 1983):

Tm=81.5 C+16.6 Log[Na+]+0.41(%G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20 C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

As used herein, the terms "nucleic acid" and "polynucleotide" refer to a deoxyribonucleotide, ribonucleotide, or a mixed deoxyribonucleotide and ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides. The polynucleotide sequences include the DNA strand sequence that is transcribed into RNA and the strand sequence that is complementary to the DNA strand that is transcribed. The polynucleotide sequences also include both full-length sequences as well as shorter sequences derived from the full-length sequences. Allelic variations of the exemplified sequences also fall within the scope of the subject invention. The polynucleotide sequence includes both the sense and antisense strands either as individual strands or in the duplex.

Techniques for transforming plants, plant parts, and plant cells with a polynucleotide or gene are known in the art and include, for example, *Agrobacterium* infection, biolistic methods, electroporation, calcium chloride treatment, PEG-mediated transformation, *etc.* U.S. Patent No. 5,661,017 teaches methods and materials for transforming an algal cell with a heterologous polynucleotide. Transformed cells can be selected, redifferentiated, and grown into plants that contain and express a polynucleotide of the invention using standard methods known in the art. Plants expressing a polynucleotide of the invention can also be regenerated from plant callus, explants, organs and parts thereof using methods known in the art. The seeds and other plant tissue and progeny of any transformed or transgenic plant cells or plants of the invention are also included within the scope of the present invention.

The subject invention also concerns methods for producing a plant that exhibits increased heat stability relative to a wild type plant, or producing a plant expressing an AGPase that exhibits increased heat stability relative to wild type enzyme, wherein a polynucleotide encoding a mutant AGPase large subunit polypeptide of the present invention is introduced into or provided in a plant cell and the polypeptide(s) encoded by the polynucleotide(s) is expressed. In one embodiment, the AGPase large subunit is heterologous to the plant. In one embodiment, the plant cell also comprises non-mutant genes encoding wild type AGPase small subunit polypeptide. In another embodiment, the plant cell comprises at least one polynucleotide encoding a mutant AGPase small subunit polypeptide. In a further embodiment, a polynucleotide encoding a mutant AGPase large subunit polypeptide is also introduced into a plant cell along with the polynucleotide encoding the mutant AGPase small subunit polypeptide. In one embodiment, the polynucleotide or polynucleotides is incorporated into the genome of the plant cell and a plant is regenerated or grown from the plant cell. In a preferred embodiment, the plant grown from the plant cell stably expresses the incorporated polynucleotide or polynucleotides.

The subject invention also concerns a composition comprising one or more polynucleotides that encode one or more mutant large subunit of AGPase of the present invention and one or more polynucleotides that encode one or more mutant, chimeric and/or wild type small subunit of AGPase, including the mutant and chimeric small subunits described herein. In one embodiment, the mutant or chimeric small subunit comprises the amino acid sequence of SEQ ID NO:24, SEQ ID NO:26, or SEQ ID NO:54, or a biologically active fragment or variant thereof.

The subject invention also concerns oligonucleotide probes and primers, such as polymerase chain reaction (PCR) primers, that can hybridize to a coding or non-coding sequence of a polynucleotide of the present invention. Oligonucleotide probes of the invention can be used in methods for detecting and quantitating nucleic acid sequences encoding a mutant AGPase large subunit polypeptide of the invention. Oligonucleotide primers of the invention can be used in PCR methods and other methods involving nucleic acid amplification. In a preferred embodiment, a probe or primer of the invention can hybridize to a polynucleotide of the invention under stringent conditions. Probes and primers of the invention can optionally comprise a detectable label or reporter molecule, such as fluorescent molecules, enzymes, radioactive moiety (*e.g.*, ^{3}H, ^{35}S, ^{125}I, *etc.*), and the like.

Probes and primers of the invention can be of any suitable length for the method or assay in which they are being employed. Typically, probes and primers of the invention will be 10 to 500 or more nucleotides in length. Probes and primers that are 10 to 20, 21 to 30, 31 to 40, 41 to 50, 51 to 60, 61 to 70, 71 to 80, 81 to 90, 91 to 100 or more nucleotides in length are contemplated within the scope of the invention. Probes and primers of the invention can have complete (100%) nucleotide sequence identity with the polynucleotide sequence, or the sequence identity can be less than 100%. For example, sequence identity between a probe or primer and a sequence can be 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75%, 70% or any other percentage sequence identity so long as the probe or primer can hybridize under stringent conditions to a nucleotide sequence of a polynucleotide of the invention. In one embodiment, a probe or primer of the invention has 70% or greater, 75% or greater, 80% or greater, 85% or greater, 90% or greater, or 95% to 100% sequence identity with a nucleotide sequence of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, or SEQ ID NO:53, or the complement thereof.

The subject invention also concerns isolated mutant AGPase large subunit polypeptides. In one embodiment, the mutant AGPase small subunit polypeptide is an AGPase large subunit polypeptide of *Zea mays*. In a specific embodiment, an AGPase large subunit polypeptide of the invention comprises an amino acid sequence as shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof. In one embodiment, a mutant large subunit of the invention comprises the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In further embodiments, the mutant large subunit comprises two or more amino acid mutations. An exemplified mutant large subunit comprising two or more amino acid mutations is designated as SH2N131R:C424V having an amino acid sequence shown in SEQ ID NO:23, or a

biologically active fragment or variant thereof. In a specific embodiment, a mutant large subunit of the invention, designated herein as SH2-E, comprises 8 amino acid mutations: N131R, T142F, A160T, Q261S, A396S, V416I, S444A, and C424V. In an exemplified embodiment, the SH2-E mutant subunit comprises the amino acid sequence shown in SEQ ID NO:25, or a biologically active fragment or variant thereof. An AGPase large subunit polypeptide or enzyme of the invention can be purified using standard techniques known in the art. In one embodiment, a polynucleotide of the invention encoding an AGPase large subunit polypeptide is incorporated into a microorganism, such as *E. coli*, and the AGPase large subunit polypeptide expressed in the microorganism and then isolated therefrom.

In certain embodiments, polypeptides of the invention, and functional peptide fragments thereof, can be used to generate antibodies that bind specifically to a polypeptide of the invention, and such antibodies are contemplated within the scope of the invention. The antibodies of the invention can be polyclonal or monoclonal and can be produced and isolated using standard methods known in the art.

Fragments of a mutant AGPase large subunit polypeptide of the invention or an AGPase small subunit polypeptide, as described herein, can be obtained by cleaving the polypeptides of the invention with a proteolytic enzyme (such as trypsin, chymotrypsin, or collagenase) or with a chemical reagent, such as cyanogen bromide (CNBr). Alternatively, polypeptide fragments can be generated in a highly acidic environment, for example at pH 2.5. Polypeptide fragments can also be prepared by chemical synthesis or using host cells transformed with an expression vector comprising a polynucleotide encoding a fragment of an AGPase large subunit polypeptide of the invention, for example, a mutant polypeptide that is a fragment of the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52. Fragments of a mutant large or small subunit AGPase polypeptide of the invention also contemplated herein include fragments of the polypeptide wherein all or a part of a transit or signal sequence of the polypeptide is removed.

The subject invention also concerns cells transformed with a polynucleotide of the present invention encoding a mutant AGPase large subunit polypeptide of the invention. In

one embodiment, the mutant AGPase large subunit comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V. In one embodiment, the cell is transformed with a polynucleotide sequence encoding a sequence comprising the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof. In one embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In another embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:25, or a biologically active fragment or variant thereof. In a specific embodiment, the cell is transformed with a polynucleotide sequence comprising the nucleotide sequence shown in any of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, or SEQ ID NO:53, or a fragment or variant thereof that encodes a AGPase large subunit that is biologically active. In a specific embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:30, or a fragment or variant thereof. In another embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:50, or a fragment or variant thereof. In one embodiment, a cell is also transformed with a polynucleotide encoding a wild type or a mutant or chimeric AGPase small subunit polypeptide as described herein. In one embodiment, the mutant or chimeric small subunit comprises the amino acid sequence shown in SEQ ID NO:24, SEQ ID NO:26, or SEQ ID NO:54, or a biologically active fragment or variant thereof. In one embodiment, the polynucleotide sequence of the invention is provided in an expression construct of the

invention. The transformed cell can be a prokaryotic cell, for example, a bacterial cell such as *E. coli* or *B. subtilis*, or the transformed cell can be a eukaryotic cell, for example, a plant cell, including protoplasts, or an animal cell. Plant cells include, but are not limited to, dicotyledonous, monocotyledonous, and conifer cells. In one embodiment, the plant cell is a cell from a *Zea mays* plant. Animal cells include human cells, mammalian cells, avian cells, and insect cells. Mammalian cells include, but are not limited to, COS, 3T3, and CHO cells.

The subject invention also concerns methods for increasing starch synthesis in a plant or plant tissue (such as a plant seed or endosperm tissue) and methods for increasing AGPase enzymatic activity of a plant and methods for increasing resistance of a plant to heat stress conditions and methods for increasing crop yield of a plant. In one embodiment, a method of the invention comprises introducing one or more polynucleotides of the present invention into a plant. In one embodiment, the mutant large subunit comprises one or more amino acid mutations at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit amino acid sequence. In a specific embodiment, the amino acid mutations can be N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V. In certain embodiments, the polynucleotides introduced into the plant encode one or more polypeptides comprising the amino acid sequence shown in any of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof. In one embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof. In another embodiment, the polynucleotide encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:25, or a biologically active fragment or variant thereof. In a specific embodiment, the polynucleotide comprises the nucleotide sequence shown in any of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45,

SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, or SEQ ID NO:53, or a fragment or variant thereof. In a specific embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:30, or a fragment or variant thereof. In another embodiment, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:50, or a fragment or variant thereof. In one embodiment, the polynucleotide is stably incorporated into the genome of the plant or plant tissue. The polynucleotide can comprise regulatory elements, such as a promoter and/or enhancer sequences, that provide for increased expression of the polynucleotide and/or the polypeptide encoded thereby. In a specific embodiment, the promoter sequence is one that provides for constitutive or tissue-specific (*e.g.*, endosperm) expression. Plants or plant tissues containing the polynucleotide, or progeny of the plants, optionally can be screened for increased expression of a polynucleotide or polypeptide of the invention. In one embodiment, multiple copies of one or more polynucleotides of the invention are introduced into a plant or plant tissue and stably incorporated into the genome of the plant. In one embodiment, a polynucleotide of the invention is provided in an expression construct as described herein.

Single letter amino acid abbreviations are defined in Table 8.

Table 8.

Letter Symbol	Amino Acid	Letter Symbol	Amino Acid
A	Alanine	M	Methionine
B	Asparagine or aspartic acid	N	Asparagine
C	Cysteine	P	Proline
D	Aspartic Acid	Q	Glutamine
E	Glutamic Acid	R	Arginine
F	Phenylalanine	S	Serine
G	Glycine	T	Threonine
H	Histidine	V	Valine
I	Isoleucine	W	Tryptophan
K	Lysine	Y	Tyrosine
L	Leucine	Z	Glutamine or glutamic acid

MATERIALS AND METHODS

Amino acid selection and preparation of enzyme variants were described by Georgelis *et al.*, 2009.

Plasmid preparation. The plasmids, pMONcBt2 and pMONcSh2, containing the small and large subunits of maize endosperm AGPase, respectively, (Giroux *et al.*, 1996) were expressed in *E. coli* AC70R1-504 cells (Iglesias *et al.*, 1993) as previously described (Boehlein *et al.*, 2008). Evolutionary mutants were prepared by QuikChange site-directed mutagenesis (Agilent) using pMONcSh2 as template.

Sh2-E was prepared synthetically by GenScript. The insert was subcloned into the pMONcSh2 vector using standard cloning methods and T4 DNA Ligase (Invitrogen).

The small subunit mosaic AGPase, referred to as MP, was constructed by Cross *et al.*, 2005. The N-terminal 199 amino acids were derived from the maize endosperm with the remaining 277 amino acids derived from the potato tuber AGPase small subunit. This chimeric enzyme was co-expressed with the maize large subunit.

Protein expression and purification. The evolutionary-based variants of the large subunit were co-expressed in *E. coli* AC70R1-504 with the wildtype maize small subunit and purified according to standard procedures (Boehlein *et al.*, 2008). SDS–PAGE was used to determine the purity of the enzymes. Since many of the enzymes did not reach homogeneity (~95%) the amount of AGPase contained in each sample was corrected by determining the percent purity of each preparation. This was accomplished by running each purified enzyme preparation on SDS–PAGE followed by analysis with IMAGEJ imaging analysis software (U.S. National Institutes of Health, Bethesda, Maryland, USA; Figure 3). The k_{cat} values presented here are based on the corrected amount of the AGPase in each purified preparation.

Determination of kinetic constants, K_m, k_{cat} and K_a. The rate of the AGPase reaction was determined from a coupled assay in which the amount of pyrophosphate (PPi) formed during the reaction was coupled to a decrease in NADH concentration (Boehlein *et al.*, 2008; Boehlein *et al.*, 2009). Each reaction was developed by adding 200 µl of coupling reagent (25 mM imidazole pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA, 0.2 mM NADH, 0.725U aldolase, 0.4U triose phosphate isomerase, 0.6U glycerophosphate dehydrogenase, 1 mM fructose 6-phosphate and 0.8 µg purified PPi-PFK per reaction) to each tube and determining the absorbance at 340 nm. Blank samples contained complete reaction mixtures without enzyme. The amount of pyrophosphate (PPi) produced was determined from a standard curve using PPi in complete reaction mixtures lacking AGPase. Change in absorbance between the blank and the reaction was used to calculate the amount of PPi produced. Each reaction was linear with time and enzyme concentration. Saturation plots to determine kinetic parameters were

hyperbolic in all cases. Cooperative effects were not observed as a component governing substrates/activators/inhibitor kinetics. When held constant, reaction mixtures contained 50 mM HEPES pH 7.4, 15 mM $MgCl_2$, 2.0 mM ATP, and 2.0 mM G-1-P and 5 mM 3-PGA in a total volume of 300 µl. The Michaelis constants for the substrates of the various proteins were determined by incubating the purified AGPase with a varying level of substrate (ATP or G-1-P) at a constant saturating level of co-substrate. Likewise, the activation constant (K_a) was determined by varying the activator at fixed constant levels of both substrates.

Kinetic constants in the absence of 3-PGA were determined by varying one substrate at several constant concentrations of the co-substrate. The reactions were pre-warmed to 37° C, initiated by addition of the appropriate enzyme, and terminated by boiling for 1.5 min. Reactions were then developed with coupling reagent as described above.

Catalytic activity at 37° C and 55° C. Activity was measured using the method for determining the kinetic constants as described above, with the following exceptions. All reactions had constant concentrations of substrates (2.0 mM) and activator (2.5 mM). Reactions mixes (1300 µl) were pre-warmed to either 37° C or 55° C and the assay was started with the addition of the appropriate enzyme. Assay time points (300 µl) were removed every 2.5 min and boiled, for a total of 10 min. All reactions were linear for 10 min at 37° C. For those reactions at 55° C that were linear with time for 10 min, a rate was calculated.

Resistance to thermal denaturation of purified maize AGPase and evolutionary mutants. Resistance to thermal denaturation in the absence of substrates or activators was determined using desalted enzymes supplemented with 0.5 mg/mL BSA in a total volume of 10 µl. Samples were incubated at 37° C for 0–7.5 min, then immediately cooled with ice. The remaining catalytic activity of each sample was determined from the standard assay (forward direction) in the presence of 2.5 mM 3-PGA. Reactions were initiated by adding AGPase (36 ng) to the reaction mixture and incubating for 10 min at 37° C. Reactions were linear with respect to time. Data were plotted as log % activity vs time and the inactivation constant $T_{1/2}$ was calculated as follows: slope = –k/(2.3). $T_{1/2}$ is calculated from the equation k = $0.693/T_{1/2}$.

Phosphate inhibition. Standard reaction mixtures contained 50 mM HEPES pH 7.4, 15 mM $MgCl_2$, in a total volume of 300 µl. When held constant in the absence of 3-PGA, the following substrate concentrations were used: ATP, 1.0 mM; G-1-P, 5.0 mM. In the absence of 3-PGA, ATP and G-1-P were varied from 0 to 3 mM and 0 to 2 mM, respectively. Pi was

varied from 0 to 20 mM when ATP was varied and 0–0.5 mM when G-1-P was varied. When 3-PGA was present (5 mM), ATP and G-1-P concentrations were fixed at 0.4 mM and 0.2 mM, respectively. When ATP and G-1-P were the variable substrates, their range was from 0 to 1.5 mM. Pi was varied from 1.0 to 10 mM. Reactions were performed as explained above.

Data analysis. The Michaelis constants were obtained by nonlinear regression using the following equation, $v = V_{max}S/(K_m + S)$ where v is the measured velocity, V_{max} is the maximum velocity, S is the substrate concentration. The activation constant (K_a) was obtained using the following equation, $V = V_{min} + V_{max}(X)/(K_a + X)$, where V_{min} is the velocity in the absence of activator and V_{max} is the change in activity from V_{min} to the total velocity. X is the activator concentration, K_a is the activation constant. All linear regression was carried out using the software program Prism (Graph Pad, San Diego CA).

Data analysis for inhibition data. Inhibition data were fitted to Eqs. (1) and (2), which correspond to partial mixed type inhibition (Eq. (1)) or noncompetitive inhibition (Eq. (2)) using GraphPad Prism software. v is the measured velocity, V_m is the maximum velocity, S is the substrate concentration, K_i is the inhibition constant, K_s is the dissociation constant for the ES complex, I is the inhibitor concentration, α is the factor by which K_i changes when the inhibitor is present and β is the factor by which k_p (rate constant for the breakdown of the enzyme:substrate complex to enzyme + products) changes when the inhibitor is present.

$$(1)\ v = V_m\,(S/K_s + (\beta SI)/(\alpha K_i K_s))/(1 + S/K_s + I/K_i + SI/\alpha K_i K_s) \qquad \text{P-MT}$$

$$(2)\ v = V_m\,(S/K_s)/(1 + S/K_s + I/K_i + SI/K_i K_s) \qquad \text{NC}$$

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1—SH2 mutants

SH2 amino acids selected for mutagenesis are shown in Table 1. Type II amino acid positions are conserved within AGPase large subunit groups but variable between groups

(Georgelis *et al.*, 2008). Positively selected amino acid positions were detected by branch-site models within the PAML software by Georgelis *et al.*, 2008 and are shown in Figure 2. Both positively selected and type II amino acid sites are candidates for functional divergence among different large subunit groups and could have important roles in catalytic and allosteric properties as well as protection from heat denaturation of AGPase (Georgelis *et al.*, 2009). Site-directed mutagenesis was used to replace SH2 amino acids with ones in the other clades. More than one amino acid was substituted in some of the individual positions as detailed in Table 1. Each variant large subunit was expressed in *E. coli* with the maize wildtype small subunit, BT2, and purified according to standard procedures (Boehlein *et al.*, 2008) utilizing the following steps: protamine sulfate and ammonium sulfate precipitations, ion exchange chromatography and hydroxyapatite chromatography and concentration.

Example 2—Kinetic constants of evolutionary mutants

Apparent K_m values for ATP and G-1-P and the K_a values for 3-PGA were determined to establish if the evolutionary changes synthesized in the maize endosperm AGPase impacted kinetic constants (Table 2). All reactions were linear with time and enzyme concentration and the average of two experiments are presented. Saturation plots to determine kinetic parameters were hyperbolic in all cases, and no cooperative effects were observed. Interestingly many of the variants did not yield homogenous preparations following standard purification protocols. Thus, the purified preparations were electrophoresed on SDS–PAGE, stained with COOMASSIE brilliant blue, and enzyme concentration estimated in relation to other bands representing impurities (Figure 3). The apparent K_m values were assayed by varying one substrate at a constant saturating concentration of the co-substrate at saturating levels of the activator, 3-PGA. As shown in Table 2, amino acid changes resulted in up to a 3-fold difference in the kinetic constants for ATP or G-1-P were observed. The catalytic constants presented in Table 2 were corrected based on the purity of the individual AGPase preparation. Even with this correction, several variants had substantially impaired catalytic rates. Mutation to asparagine or arginine in the threonine 341 position yielded enzymes with 2.7- and 6-fold lower activity, while changes in glutamine 261 to histidine or serine yielded enzymes with 9.5- and 4-fold lower activities. While decreased catalytic activity occurred with some mutations, several changes enhanced the catalytic rate. These included mutations to alanine 160, glutamate 425 and serine 444. Thus, while these variants had little or no

impact on the Michaelis constants, several changes had a significant effect on the catalytic rate at 37° C.

The 3-PGA activation constant (K_a) value for each of the variants was determined by varying the concentration of 3-PGA in the presence of 2 mM ATP and G-1-P (Table 2). The K_a for 3-PGA is greatly decreased (>6-fold) in the T142F variant, thus binding appears to be greatly enhanced. This variant also has approximately 30% activity in the absence of 3-PGA, when compared to its presence, as can be seen by the first entry in the k_{cat} column of Table 2.

The other single amino acid change at this position, T142A did not confer this attribute, thus the T142F variant is the only single amino acid variant that had this effect. The 3-PGA independent activity is noteworthy since MP, a small subunit variant (Cross *et al.*, 2004; Boehlein *et al.*, 2009), also has 3-PGA independent activity and confers enhanced seed number in maize plants grown at relatively high temperatures (Hannah, unpublished). Conversely, two mutants, T341R and V416E, had significantly weaker (5- to 10-fold) 3-PGA binding or response to 3-PGA.

The kinetic parameters in the absence of 3-PGA were measured for the T142F mutant (Table 3), because it had approximately 30% activity in the absence of this effector under the specified conditions. Two fundamentally different mechanisms have been reported for the activator 3-PGA depending on the isoform studied (Boehlein *et al.*, 2013a). 3-PGA binding was accompanied by a decrease in the K_m for both ATP and G-1-P in the wildtype maize endosperm enzyme (Tables 2 and 3) and the MP mosaic (Boehlein *et al.*, 2009). In contrast, for the oxidized and reduced potato tuber enzymes, the binding of the first substrate, ATP, was unaffected by the binding of 3-PGA, while the binding of the second substrate, G-1-P, was significantly affected. The K_m for G-1-P in mutant T142F is only slightly increased (~2-fold) by the presence of 3-PGA (Tables 2 and 3). However, the K_m for ATP is almost an order of magnitude higher in the absence of activator and, thus, the K_m of the first substrate, ATP, is still adversely affected in this mutant.

Accurate estimates of the ATP K_m in the absence of 3-PGA could not be made for the maize and MP AGPases, because saturating concentrations of G-1-P could not be reached. Thus, the values presented here were obtained from velocity vs ATP plots at the highest G-1-P concentration (15 mM) and are taken from Boehlein *et al.*, 2010a. It has been previously proposed (Boehlein *et al.*, 2010a) that G-1-P has two roles in the absence of 3-PGA. G-1-P acts as both a substrate, at low to moderate concentrations, and an activator at high G-1-P

concentrations. Thus, in the absence of 3-PGA, G-1-P not only binds as a substrate for the reaction, but can also bind at a separate activator site. This activation site can also bind 3-PGA; hence, G-1-P activation is not seen in the presence of 3-PGA and G-1-P substrate saturation for the maize and MP enzymes does not occur in the absence of 3-PGA. In contrast, the potato enzyme does not bind G-1-P at the activation site and G-1-P readily saturates the enzyme in the absence of 3-PGA, albeit at a higher concentration. The T142F mutant shows a behavior similar to that of the potato enzyme. G-1-P readily saturates T142F in the presence (Table 2) or absence (Table 3) of 3-PGA with K_m values of 0.042 and 0.09 mM, respectively. Thus, if G-1-P binds to a second site in this variant, it is not accompanied by an increase in activity.

As gleaned from the crystal structure of the homotetrameric potato enzyme (Jin *et al.*, 2005), T142 is part of an alpha helical structure that is very proximal to the sulfate binding sites and is at the interface between two subunits (Figures 4A and 4B). It has been proposed that the activator site for the maize endosperm AGPase lies within this interface (Boehlein *et al.*, 2010b). If all activators bind at this interface and the T142F change prevents G-1-P from activating the enzyme, perhaps other larger, more bulky activators no longer stimulate this variant as well. Accordingly, the activation constants (K_a) for the larger activators of the maize enzyme, fructose-6 phosphate (F-6-P) and glucose-6-phosphate (G-6-P; (Boehlein *et al.*, 2005)), were determined for the T142F variant in the presence of 5 mM ATP and G-1-P (Table 4). These increased substrate concentrations were used to induce a higher activity in the absence of activators so a more accurate rate could be obtained. Here again it is shown that the T142F variant has considerable activity in the absence of activator and, although it is not activated by G-1-P, the comparably-sized F-6-P and G-6-P activate quite effectively (Table 4). F-6-P and G-6-P not only activate T142F, but they also possess lower K_a values than in wildtype. Why G-1-P saturates the mutant enzyme T142F and not the wildtype maize enzyme is unclear. It may bind to the activator site, but not be effective in activation similar to the mechanism demonstrated in an *E. coli* mutant (Figueroa *et al.*, 2011).

We next examined the effect of ADP-Glc on the T142F mutant. ADP-Glc activates the maize enzyme in the absence of 3-PGA by increasing the affinity of the enzyme for the substrates, while in the presence of 3-PGA it acts as an inhibitor (Boehlein *et al.*, 2013a). Here we determined the apparent K_m for ATP in the presence and absence of ADP-Glc (data not shown). ADP-Glc activation was seen in both T142F and the wildtype maize enzyme.

When 0.75 mM ADP-Glc was added to the T142F and wildtype maize AGPases an increase in velocity (2.5- and 12-fold, respectively) and a decrease the K_m for both enzymes (approximately 2.5-fold) were seen. Thus for T142F, although G-1-P does not have a dual role (substrate and activator) in the absence of 3-PGA, the bulkier activators, F-6-P, G-6-P and ADP-Glc all stimulate the enzyme activity.

Example 3— Enzyme denaturation and catalytic activity at 55°C

We next focused on how temperature affects the behavior of the enzyme at two main levels: influencing the enzyme stability (protein unfolding) and having an effect on the reaction rate. First, the half-life of each variant enzyme was determined in the absence of any effector molecules known to bind to the enzyme. This assay assessed thermal denaturation of the unbound (naked) enzyme. Purified samples were placed at 37° C for varying times and then cooled on ice, followed by conventional assays. Second, activity assays were performed at 55° C, a temperature that completely inactivates the wildtype maize enzyme. Here, all assay components were pre-warmed at 55° C and started with the addition of enzyme. Enzyme activity was then monitored at several time points between 2.5 min and 10.0 min. For those reactions that were linear with time for 10.0 min a rate was calculated. Hence, heat denaturation and activity at increased temperatures were monitored (Table 5).

SH2 amino acid changes fall into four categories (Table 5): (i) increased $T_{1/2}$ of the naked enzyme at 37° C, (ii) detectable activity at 55° C, (iii) increased $T_{1/2}$ of the naked enzyme at 37° C as well as detectable activity at 55° C and (iv) no effect on either parameter. These data point to the fact that there must be fundamentally different mechanisms for heat denaturation and interactions with effector molecules have a significant stabilizing effect on the enzyme. Importantly, of the 10 changes having activity at 55° C, six exhibited an enhanced $T_{1/2}$ as compared to the wildtype enzyme. Some amino acid changes were targeted for additional experiments as detailed below. Interestingly, eight of the 10 amino acid changes exhibiting activity at 55° C also had less than wildtype activity at 37° C. Hence, the majority of the changes enhancing stability have detrimental effects on the turnover of the enzyme at lower temperatures.

While many SH2 changes had some catalytic activity at 55° C, none had activity levels at 55° C equaling activity at 37° C. The exception is the small subunit variant MP-T462I. This variant contains the MP change described in (Cross *et al.*, 2004; Boehlein *et al.*,

2009; Boehlein *et al.*, 2013a) and the T462I change described in Georgelis and Hannah, 2008. It was included here for comparison, because MP is an enzyme that is more stable to heat denaturation than wildtype and less dependent on 3-PGA. And the T462I variant is even more resistant to thermal denaturation. When expressed with the wildtype Sh2 gene, this variant has the same amount of activity at 37° C and 55° C (Table 5). Not only does this variant have the greatest activity at 55° C, it also exhibited the greatest resistance to thermal denaturation in the absence of effector molecules.

Example 4—Formation of a superior large subunit

All evolutionary variants were screened for qualities envisioned to be important in grain yield (activity at increased temperatures, decreased thermal denaturation, lower 3-PGA K_a and higher k_{cat}). Each of these parameters was altered among the phylogenetically-selected amino acid changes. Several variants had both activity at 55° C and an increased $T_{1/2}$ (Table 5), while changes at three positions (160, 425, and 444) conferred enhanced k_{cat} values (Table 2). Additionally, one mutation, T142F, yielded an enzyme with higher activity in the absence of 3-PGA (Table 2), a lower 3-PGA K_a (Table 3) and elevated activity at 55° C (Table 5), although the k_{cat} for this variant was diminished (Table 2). Based on these data, an "evolutionary" mutant was made, by pyramiding all of these changes into a single gene. A summary of each individual mutation used and the parameters associated with them can be found in Figure 1.

This new gene, Sh2-E, was then synthesized, purified, characterized and compared to the individual mutations. As shown in Tables 2 and 5, SH2-E retained both activity at 55° C and activity in the absence of 3-PGA, but did not retain increased resistance to thermal denaturation. Furthermore, while most of the individual changes of this enzyme had activity at 55° C, k_{cat} was diminished in those individual mutations. By combining two high k_{cat} mutants with several mutants with activity at 55° C an enzyme was created which had an activity at 55° C equal to its activity at 37° C (Table 5). This high rate at 55° C is similar to what is seen with the MP small subunit, our most interesting small subunit variant to date. Interestingly, when the T142F mutation is removed from Sh2-E over 90% of the activity at 55° C is lost and the enzyme no longer has increased activity in the absence of 3-PGA (data not shown). Thus T142F seems to be critical for both properties.

While variations in the kinetic constants in the presence of 3-PGA were minimal, some noteworthy changes occurred in the absence of 3-PGA (Table 3). As described previously, G-1-P was able to readily saturate the T142F mutant in the absence of 3-PGA. This was also the case for the SH2-E mutant. The G-1-P K_m is identical in the presence or absence of 3-PGA. Additionally, the k_{cat} for the reaction increased from 53.8 s^{-1} in the T142F mutant to 67.6 s^{-1} in SH2-E. Therefore, the SH2-E mutant has retained many of the individual properties of the selected mutants with T142F being the most favorable individual change.

Example 5—Pi inhibition

Another property of ADP-glucose pyrophosphorylases with likely important agricultural implications (Giroux *et al.*, 1996) is phosphate (Pi) inhibition. Hence, the Pi inhibition patterns exhibited by the SH2-E variant were examined (Table 6). Previously, a detailed study of Pi inhibition for the maize endosperm, oxidized potato tuber, reduced potato tuber and MP AGPases in the absence of 3-PGA was carried out (Boehlein *et al.*, 2013b). Here we obtained similar data for SH2-E (Table 6). The Pi inhibition data were obtained by varying one substrate at several fixed concentrations of Pi, while keeping the co-substrate at constant and sub-saturating levels. This was performed both in the absence and the presence of saturating 3-PGA. These data were fit to various mixed type inhibition equations as described in Materials and Methods. In a mixed type inhibition model, which includes competitive, noncompetitive and uncompetitive models as special cases (Segel, 1975), an α value of 1 indicates that the effector does not alter the binding of the substrate and the mechanism reduces to a noncompetitive model. However, if α is large, it is indicative of a competitive inhibitor, whereby the inhibitor prevents binding of the substrate. If the inhibitor enhances the binding of the substrate, α is very small, and the model reduces to an uncompetitive model. An additional term (β) is added to the equations if the inhibition is not complete (high concentrations of the inhibitor do not drive the reaction rate to zero) and reflects the degree of inhibition. If both substrate binding (mixed type) and product formation (partial) are altered by the inhibitor, it is classified as partial mixed type inhibition. Special cases include pure noncompetitive inhibition, where $\alpha = 1$ and $\beta = 0$ and partial noncompetitive inhibition whereby $\alpha = 1$ and $0 < \beta < 1$.

A non-competitive pattern was obtained with the SH2-E enzyme when Pi was varied in the absence of 3-PGA and ATP was the varying substrate. An extremely high K_i was

obtained for this mutant enzyme, Ki = 20.9 mM (Table 6). The inhibition appeared to be linear up to 20 mM, the highest concentration of Pi tested. A dramatically different pattern was obtained when G-1-P was the varying substrate. Here, only partial noncompetitive inhibition was seen, the K_i was very low (0.05 mM), but inhibition was incomplete, as observed by the β value of 0.44 (Table 6). Thus, although inhibition begins at low Pi concentrations, inhibition only proceeds until about half of the activity is inactivated and then the enzyme is not susceptible to further inhibition. These results are significantly different from those seen with the wildtype maize AGPase, where the presence of Pi at low concentrations had a positive effect on substrates binding (Table 6 and (Boehlein *et al.*, 2010a)).

Inhibition in the presence of 3PGA yielded simple noncompetitive patterns when either ATP or G-1-P was varied (Table 6). The K_i for Pi was approximately 7 mM when either substrate was varied. Thus, the addition of Pi to this variant enzyme did not affect substrate binding (α = 1 and inhibition was complete, β = 1). These relatively simple Pi inhibition patterns differ appreciably from those exhibited by the wildtype maize enzyme in which the Pi patterns are classified as partial mixed type inhibition.

Example 6—Preparation of a double mosaic enzyme

The combination of the most promising individual subunits, MP (small subunit) and SH2-E (large subunit), was assembled and characterized (Tables 2-5, Figure 1). Also characterized were the SH-E variant with a wildtype BT2 protein, MP with a wildtype SH2 protein and several of the single amino acid changes in SH2 expressed with a wildtype BT2 protein. Importantly, the SH2-E:MP variant retained high activity at 55° C and had an increased $T_{1/2}$ at 37° C (Table 5) as judged by comparison with the single MP and SH2-E variants. The kinetic parameters in the absence of 3-PGA were examined since both of these individual proteins yielded enzymes with increased heat stability and substantial activity in the absence of 3-PGA (Table 3). Tables 2 and 3 show that the 3-PGA independent activity rises markedly in theSH2-E:MP double variant. Additionally, an unexpected finding was that the K_m for ATP decreased ~7-fold for the SH2-E:MP variant. This was not a property of either MP or SH2-E, although only the apparent K_m for ATP could be determined for the MP mutant due to the high K_m for G-1-P. Since the SH2-E variant has a decreased K_m for G-1-P, the SH2-E:MP variant can now function at wildtype levels independent of 3-PGA. The K_m

values for both substrates are similar to the maize enzyme in the presence of 3-PGA. Thus, the binding of 3-PGA, once required by the maize AGPase to align and efficiently bind the substrates, is no longer required for this variant. The k_{cat} in the absence of 3-PGA is greater than in its presence and the substrates can bind to the enzyme easily. A summary of the individual amino acid changes in SH2-E can be found in Figure 1.

Example 7

Protein sequences necessary for basic functions of an enzyme are conserved throughout evolution and it is this conservation that is used for phylogenetic (evolutionary) analysis of enzymes. It is shown herein that alteration of amino acids identified through phylogenetic analysis is a powerful and efficient method for creating new, potentially superior variants of enzymes from naturally occurring sequences. The phylogenetic tree of angiosperm AGPase large subunits has four major clades, or phylogenetic groups (Georgelis *et al.*, 2007). These clades are: (1) leaf tissue from eudicots and monocots, (2) leaf and sink tissue from eudicots, (3) eudicot sink tissue and (4) monocot sink tissue. Here, we varied amino acid positions that were conserved within, but varied between clades (type II sites) and amino acid positions that were under positive selection after the duplications that led to the generation of the four clades (positively selected sites) to create a selected variant of the maize endosperm AGPase large subunit, SH2. Initially, sixteen sites were altered; seven of which were eventually combined to make the synthetic large subunit, *Sh2*-E. This synthetic gene also contains an eighth change identified in previous studies. All four clades contributed to the alterations in *Sh2*-E. Two replaced amino acids are found in clade 3, three are in clade 1, two are in all clades except 4 and the final replacement is only in clade 4, but not in SH2. Five of the eight changes in SH2-E are in positively selected sites and three are in type II sites. Four sites chosen for SH2-E had two amino acids initially changed and analyzed. Two amino acid mutations were studied at sites that had more than one alteration. For example, maize SH2 has a threonine at site 142. Clade 2 has phenylalanine at this site and the other clades have alanine. Both changes were analyzed and found to have effects on enzyme parameters; however, the phenylalanine change significantly improved the enzyme Five of the sixteen amino acid sites studied had two changes made and four of these conditioned enhanced characteristics in the resulting large subunit. Eight amino acid changes with desirable enzymological characteristics were pyramided into the new large subunit gene

Sh2-E. When combined with the wild-type *Bt2*, the resulting enzyme has all of the selected properties of the individual mutants. Furthermore, when *Sh2*-E is combined with the chimeric small subunit, MP, that is more heat stable and less dependent on 3-PGA, the resulting enzyme is far superior and it has activity that is now independent of the activator 3-PGA.

A possible explanation for some of the observed changes can be gleaned from examination of the crystal structure of the homotetrameric potato AGPase (Jin *et al.*, 2005). The amino acid equivalent to SH2T142F lies at the bottom of the cleft formed by two subunits where three sulfates bind. This cleft is the location for the binding of the activator 3-PGA (Boehlein *et al.*, 2010b). The presence of the larger, hydrophobic phenylalanine yields an enzyme that is active in the absence of the activator, reduces the 3-PGA K_a and slightly increases the K_m for ATP. Although SH2T142F is not adjacent to the ATP binding site, it is on the side of the effector cleft nearest the active site. Alanine was also substituted for threonine at this site and had no effect on 3-PGA activation. However, both the alanine and phenylalanine changes resulted in enzyme with activity at 55°C.

Changes of SH2A160 to glycine or threonine produced enzymes that had a significant increase in the catalytic rate. This amino acid is located on the inside of the homotetrameric structure along a stretch of 5-9 amino acids that were disordered in the crystal structure. One reason amino acids are not resolved in structures is due to intrinsic flexibility within the structure. These observations suggest that internal movement within this region may be required for catalysis.

Alanine 396 of the large subunit is located along the SS:LS interface and appears to make contact with V347 of the small subunit, BT2. As shown here, when SH2A396 is changed to serine, a substantial increase in thermo-stability is seen (Table 5). Previously, this amino acid was changed to a valine, and again, this mutant was more heat stable (Burger 2001). Interestingly, the binding interaction partner from the small subunit, V347, is one of five amino acids important for the heat stability of the MP small subunit - maize large subunit AGPase (Cross *et al.*, 2005 and Boehlein *et al.*, 2008).

Changes to SH2V416 yielded interesting results. This residue is found near the effector site, above sulfate 2 (Jin *et al.*, 2005) and adjacent to SH2R381 (Boehlein *et al.*, 2010b). Changing this amino acid to a charged residue (glutamate) greatly impairs 3-PGA binding (Table 2). However, substituting it with an isoleucine leads to heat stability with little effect on the kinetic constants.

Noteworthy is the fact that the MP/SH2-E enzyme is 3-PGA independent. Here, it is shown that in the absence of 3-PGA, BT2/SH2-E has a G-1-P K_m value comparable to that observed in its presence; whereas, the ATP K_m is high. The opposite is true for MP/SH2; in the absence of 3-PGA, ATP binds with high affinity, but the G-1-P K_m is extremely high (Table 3). If the MP enzyme affects two 3-PGA sites in the small subunit and the SH2-E mutant affects the two 3-PGA sites in the large subunit, then all the 3-PGA sites may be affected in the MP/SH2-E double mutant, allowing all substrates to bind efficiently in the absence of 3-PGA.

It is fascinating to note that the ATP and G-1-P K_m values of the MP/SH2-E AGPase in the absence of 3-PGA are some 10 fold lower than those of wildtype. Since the slow step in catalysis of the maize endosperm AGPase is substrate binding or product release (Boehlein *et al.*, 2010a), variants enhancing substrate binding can be beneficial to plant agriculture.

It is also interesting to note that the Pi inhibition patterns exhibited by Sh2-E enzyme stands in stark contrast to the patterns exhibited by the wildtype enzyme. While complex patterns of Pi inhibition were observed for the wildtype maize enzyme, relatively simple patterns were observed for the Sh2-E enzyme. In the absence of 3-PGA, Pi can be classified as a linear non-competitive inhibitor when varying ATP for this enzyme. The only other AGPase isoform displaying this pattern is the oxidized potato tuber enzyme. Even though the patterns are similar, the SH2E mutant has a Ki ~150x greater the potato enzyme. The maize AGPase has a Ki 3.4mM but is recalcitrant to additional Pi after reaching about 50% inhibition. On the other hand, when G-1-P is the variable substrate, inhibition only reaches about 50%, and Pi slightly increases the substrate affinity. In the presence of 3-PGA, Pi displays a non-competitive pattern when ATP or G-1-P is varied. *Sh2-E* is similar to the maize enzyme, whereby no cooperativity is seen, but inhibition appears to be complete with *Sh2-E*. Finally it should be noted that the extremely large Ki for Pi can negate Pi inhibition in the maize endosperm, thereby making this beneficial for agriculture.

Heat stability of AGPase is important for plant yield as evidenced by placement of a moderately heat stable AGPase into rice, wheat and maize. Given the importance of heat stability of AGPase to the yield of the agriculturally important cereals, rice, wheat and maize (Smidansky *et al.*, 2002 and 2003; Hannah *et al.*, 2012), a phylogenic approach was used to identify amino acids important in this parameter. As can be gleaned from these data, this approach is quite efficient. Seven important sites were identified in a starting population of

16 amino acid positions. Not only were variants found that affect heat stability, but also variants were identified that altered kinetic parameters of the enzyme. Also noteworthy is the fact that the changes identified appear to be additive and can be pyramided into one form of the enzyme.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

TABLES

Table 1. Evolutionary mutants.

Site	# changes	Type
*Sh2*N131	1	Positively Selected
*Sh2*T142	2	Positively Selected
*Sh2*T151	1	Type II
*Sh2*G155	1	Positively Selected
*Sh2*A160	2	Positively Selected
*Sh2*Y198	1	Positively Selected
*Sh2*Q261	2	Positively Selected
*Sh2*Q336	1	Type II
*Sh2*T341	2	Positively Selected
*Sh2*P364	1	Positively Selected
*Sh2*T374	1	Type II
*Sh2*P380	1	Type II
*Sh2*A396	1	Type II
*Sh2*V416	2	Type II
*Sh2*E425	1	Type II
*Sh2*S444	1	Type II

Table 2. Kinetic constants for the *Sh2* evolutionary mutants and the MP-T462I small subunit variant.

Mutant	ATP K_m (mM)	G-1-P K_m (mM)	3-PGA K_a (mM)	k_{cat} (s^{-1})
Maize wildtype	0.13 ± 0.011	0.040 ± 0.005	0.19 ± 0.009	84.0 ± 2.4
N131R[b]	0.10 ± 0.020	0.051 ± 0.009	0.53 ± 0.07	16.5 ± 0.8
T142A	0.10 ± 0.006	0.042 ± 0.006	0.21 ± 0.02	66.9 ± 2.56
T142F	0.20 ± 0.008	0.040 ± 0.004	0.03 ± 0.006	17.8 ± 2.6[a]: 62.2 ± 4.1
T151A	0.12 ± 0.017	0.14 ± 0.062	0.45 ± 0.043	32.2 ± 0.93
G155N	0.14 ± 0.015	0.08 ± 0.012	0.63 ± 0.08	75.3 ± 2.1
A160G	0.13 ± 0.02	0.06 ± 0.007	0.63 ± 0.012	131.3 ± 5.4
A160T	0.09 ± 0.007	0.06 ± 0.002	0.09 ± 0.017	124.6 ± 3.35
Y198A	0.08 ± 0.01	0.05 ± 0.006	0.54 ± 0.071	27.7 ± 1.1
Q261H	0.07 ± 0.018	0.033 ± 0.011	0.34 ± 0.039	8.8 ± 0.28
Q261S	0.07 ± 0.013	0.10 ± 0.011	0.12 ± 0.019	20.7 ± 0.61
Q336A	0.09 ± 0.011	0.045 ± 0.009	0.15 ± 0.015	23.1 ± 0.47
T341N	0.19 ± 0.014	0.034 ± 0.006	0.17 ± 0.03	31.2 ± 1.2
T341R	0.15 ± 0.018	0.059 ± 0.001	0.93 ± 0.21	14.0 ± 1.5
P364F	0.08 ± 0.014	0.04 ± 0.004	0.20 ± 0.027	48.3 ± 6.1
T374K	0.19 ± 0.012	0.041 ± 0.002	0.13 ± 0.014	38.2 ± 0.56
P380R	0.05 ± 0.01	0.09 ± 0.008	0.11 ± 0.015	79.5 ± 2.2
A396S	0.07 ± 0.008	0.060 ± 0.006	0.14 ± 0.024	38.5 ± 1.1
V416E	0.32 ± 0.01	0.03 ± 0.009	1.75 ± 0.25	22.4 ± 2.1
V416I	0.13 ± 0.066	0.032 ± 0.004	0.27 ± 0.03	50.8 ± 1.4
E425A	0.11 ± 0.019	0.09 ± 0.012	0.37 ± 0.067	94.5 ± 4.5
S444A	0.06 ± 0.008	0.05 ± 0.005	0.31 ± 0.067	105.4 ± 6.0
N131R:C424V	0.08 ± 0.008	0.05 ± 0.005	0.32 ± 0.02	38.9 ± 0.67
MP + SH2	0.10 ± 0.004	0.05 ± 0.003	0.10 ± 0.01	32.5 ± 2.1: 116 ± 10.1
MP-T462I[c]	0.16 ± 0.012	0.03 ± 0.002	0.07 ± 0.02	13.7 ± 1.4: 50.1 ± 3.5
SH2-E	0.10 ± 0.01	0.03 ± 0.003	0.07 ± 0.006	14.7 ± 0.87: 58.1 ± 1.46
MP + SH2-E	0.07 ± 0.003	0.03 ± 0.003	0.05 ± 0.016	29.8 ± 1.9: 55.3 ± 4.8

When held constant, ATP and G-1-P were at 2.0 mM, 3-PGA was held constant at 5.0 mM.

[a]When activity is detected under these conditions in the absence of 3-PGA, the amount of activity in the absence of 3-PGA is presented first, followed by the amount of activity in the presence of 3-PGA.

[b]All entries contain mutant large subunit variants expressed with a wildtype BT2 subunit except entries where the MP small subunit is specified.

[c]MP-T462I is a small subunit variant containing the MP change described in Cross *et al.* (2004) and Boehlein *et al.* (2005 and 2009) and the T462I change described in Georgelis and Hannah (2008). This variant was expressed with the wildtype large (SH2) subunit.

All other entries contain mutant large subunit variants expressed with a wild-type BT2 subunit.

Table 3. Kinetic constants in the absence of 3-PGA.

Enzyme	ATP K_m (mM)	G-1-P K_m (mM)	k_{cat} (s^{-1})	K_{ia}
Maize[a]	4.0 ± 0.35[c]	2.8 ± 2.1	35.2 ± 2.7	ND[e]
MP[b]	0.9 ± .01[c] 0.43 ± .04[d]	11.9 ± 3.0	67.6 ± 8.3	ND[e]
T142F	1.73 ± 0.25	0.09 ± 0.017	53.8 ± 2.6	1.5 ± 0.7
Pot red[b]	0.31 ± 0.052	1.07 ± 0.18	13.7 ± 1.1	1.07 ± 0.2
SH2-E	2.53 ± 0.43	0.03 ± 0.005	67.6 ± 4.5	5.4 ± 5.6
MP + SH2-E	0.32 ± 0.021	0.09 ± 0.008	193.2 ± 4.2	0.59 ± 0.09

[a]Data taken from Boehlein *et al.*, 2010.
[b]Data taken from Boehlein *et al.*, 2013a.
[c]ATP K_m could not be determined because G-1-P was not saturating. Since G-1-P saturation was not reached, value reported is the apparent K_m, in the presence of 15 mM G-1-P.
[d]ATP K_m in the presence of 25 mM G-1-P.
[e]K_{ia} could not be calculated because G-1-P was not saturating.

Table 4. Activation by F-6-P and G-6-P.

Enzyme	3-PGA K_a(mM)	Activation fold	F-6-P K_a(mM)	Activation fold	G-6-P K_a(mM)	Activation fold	k_{cat} (activator) (s^{-1})
wt.	0.15 ± 0.03	7.4	4.6 ± 1.5	14.2	1.6 ± 0.47	9.8	5.4
T142F	0.09 ± 0.01	2.4	0.9 ± 0.26	2.9	0.4 ± 0.09	2.3	18.6 ± 1.2

ATP and G-1-P were held constant at 5 mM.

Table 5. Temperature dependence of selected mutants.

SEQ ID NO.	Mutant	$T_{1/2}$ 37°C	[a]Activity 37°C	37°C rate as % of wt	Activity 55°C	% Activity remaining at 55°C
	wt	4	20000	100	0	
2	SH2N131R	14.9	4819	24	1075	22
3	SH2T142A	3.1	16611	83	1533	9
4	SH2T142F	4.6	21850	109	5980	27
5	SH2T151A	7.4	10514	53	1095	10
6	SH2G155N	3.8	26108	131	0	
7	SH2A160G	4.5	44160	221	0	
8	SH2A160T	5.8	37442	187	2282	6
9	SH2Y198A	4.2	12413	62	535	4
10	SH2Q261H	5.0	2509	13	0	
11	SH2Q261S	10.5	6192	31	855	14
12	SH2Q336A	14.4	6237	31	0	
13	SH2T341N	3.8	9070	45	0	
14	SH2T341R	9.0	4313	22	0	
15	SH2P364F	4.3	14375	72	0	
16	SH2T374K	9.0	11672	58	732	6
17	SH2P380R	3.8	24605	123	0	
18	SH2A396S	11.4	12545	63	3655	29
19	SH2V416E	4.0	10952	55	0	
20	SH2V416I	4.8	15123	76	3108	21
21	SH2E425H	7.8	26286	131	0	
22	SH2S444A	4.7	29870	149	0	
23	SH2N131R:C424V	5.1	14493	72	1512	10
54	[b]MP-T462I	32	20220	101	20000	99
25	SH2-E	3.5	22400	112	24300	108
	MP (SEQ ID NO:26) + SH2-E (SEQ ID NO:25)	46	18400	92	23600	128
	MP	15	23000	115	13600	59

$T_{1/2}$ is calculated in minutes. Activity is in nmol/min/mg

[a]Rates were normalized based on protein concentration as determined by ImageJ software

[b]MP-T462I is a small subunit variant containing the MP change described in Cross *et al.*, 2004 and Boehlein *et al.*, 2005 and 2009 and the T462I change described in Georgelis and Hannah, 2008. This variant was expressed with the wildtype large (SH2) subunit.

All other entries contain mutant large subunit variants expressed with a wildtype BT2 subunit.

Table 6. Pi inhibition in the absence and presence of 3-PGA.

Enzyme	Varied Substrate	3-PGA	[b]Pattern	K_m (mM)	K_i (mM)	α	β	k_{cat} (sec^{-1})	V_{max}
[a] Maize	ATP	-	P-MT	20.4+/-10.1	3.4+/-1.1	0.063+/-0.034	0.52+/-0.17	23.5 ± 7.7	6.7+/-2.2
	G-1-P	-	P-MT	22.2+/-9.0	5.1+/-2.1	0.095 +/-0.065	0.92 +/-0.31	14.7 ± 3.8	4.2+/-1.1
SH2E	ATP	-	NC	0.97+/-0.081	20.9+/-1.6			38.5 ± 4.9	11.0+/-1.4
	G-1-P	-	P-NC	[c] 0.20+/-0.02	0.055+/-0.013	0.85 +/-0.17	0.44 +/-0.03	19.6 ± 5.2	5.6+/-1.5
Maize	ATP	+	P-MT	0.11+/-0.0057	0.37+/-0.053	4.61 +/-0.70	0.83+/-0.037	109.5 ± 1.4	31.3+/-0.4
	G-1-P	+	P-MT	0.091+/-0.0042	1.00+/-0.18	0.90 +/-0.012	0.52 +/-0.023	87.1 ± 1.1	24.9+/-0.3
SH2E	[c] ATP	+	NC	0.21/-0.01	7.8+/-0.21			52.8 ± 1.1	15.1+/-0.3
	G-1-P	+	NC	0.063+/-0.003	6.62+/-0.39			35.0 ± 0.7	10.0+/-0.2

[a] data is taken from Boehlein *et al.*, 2013a and presented here for comparison

[b] P-MT partial mixed type inhibition, NC noncompetitive inhibition, P-NC partial noncompetitive inhibition

the units for the K_i value and K_m are in mM and V_{max} is presented in μmol/min/mg

[c] ATP was held constant at 1 mM

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CLAIMS

We claim:

1. A polynucleotide encoding a mutant plant AGPase large subunit comprising one or more amino acid mutations, wherein the one or more amino acid mutations are at positions corresponding to positions 131, 142, 151, 155, 160, 198, 261, 336, 341, 364, 374, 380, 396, 416, 424, 425, or 444 of wild type AGPase maize endosperm large subunit.

2. The polynucleotide according to claim 1, wherein said one or more amino acid mutations are selected from N131R, T142A, T142F, T151A, G155N, A160G, A160T, Y198A, Q261H, Q261S, Q336A, T341N, T341R, P364F, T374K, P380R, A396S, V416E, V416I, E425H, S444A, or C424V.

3. The polynucleotide according to claim 1, wherein said mutant plant AGPase large subunit comprises the mutation T142F.

4. The polynucleotide according to claim 1, wherein said polynucleotide is provided in an expression construct.

5. The polynucleotide according to claim 1, wherein said polynucleotide encodes said mutant plant AGPase large subunit comprising the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:52, or a biologically active fragment or variant thereof.

6. The polynucleotide according to claim 1, wherein said polynucleotide comprises the nucleotide sequence of one or more of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID

NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, or SEQ ID NO:53, or a fragment or variant thereof.

7. The polynucleotide according to claim 1, wherein said polynucleotide comprises the nucleotide sequence of SEQ ID NO:30 or SEQ ID NO:50, or a fragment or variant thereof.

8. The polynucleotide according to claim 1, wherein said polynucleotide encodes the amino acid sequence of SEQ ID NO:25, or a biologically active fragment or variant thereof.

9. The polynucleotide according to claim 1, wherein said polynucleotide encodes the amino acid sequence of SEQ ID NO:4, or a biologically active fragment or variant thereof.

10. The polynucleotide according to any of claims 1 to 9, wherein said polynucleotide further comprises a nucleotide sequence encoding a mutant plant AGPase small subunit that comprises a mutation wherein the threonine amino acid corresponding to amino acid position 462 of wild type maize endosperm AGPase small subunit is substituted with an amino acid that confers increased heat stability.

11. The polynucleotide according to claim 10, wherein said mutant plant AGPase small subunit comprises the amino acid sequence shown in SEQ ID NO:24, or a biologically active fragment or variant thereof.

12. The polynucleotide according to any of claims 1 to 9, wherein said polynucleotide further comprises a nucleotide sequence encoding a chimeric plant AGPase small subunit that comprises amino acid sequences from two different plant AGPase small subunits.

13. The polynucleotide according to claim 12, wherein said mutant plant AGPase small subunit comprises the amino acid sequence shown in SEQ ID NO:54, or a biologically active fragment or variant thereof.

14. A polynucleotide encoding a mutant plant AGPase large subunit comprising one or more amino acid mutations, wherein when said mutant plant AGPase large subunit is combined with a plant AGPase small subunit to form a mutant AGPase enzyme, said mutant AGPase enzyme exhibits a decreased K_m for glucose-1-phosphate (G-1-P) and/or ATP; and/or said mutant AGPase enzyme exhibits a decreased K_a for 3-phosphoglyceric acid (3-PGA) relative to a control or wild type plant AGPase that does not comprise said one or more amino acid mutations.

15. The polynucleotide according to any of claims 1 to 14, wherein said polynucleotide is DNA.

16. The polynucleotide according to claim 15, wherein said DNA is cDNA.

17. The polynucleotide according to any of claims 1 to 16, wherein said polynucleotide is a recombinant polynucleotide.

18. An expression construct comprising a polynucleotide of any of claims 1 to 17.

19. The expression construct according to claim 18, wherein said expression construct comprises one or more regulatory elements selected from promoters, transcription termination sequences, translation termination sequences, enhancers, and/or polyadenylation elements.

20. A transformed or transgenic plant, plant part, plant tissue, or plant cell comprising a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 17 to 19.

21. The plant, plant part, plant tissue, or plant cell according to claim 20, wherein said mutant large subunit further comprises a mutation that confers increased seed weight and/or seed yield relative to a control or wild type plant.

22. The plant, plant part, plant tissue, or plant cell according to claim 20, wherein said plant, plant part, plant tissue, or plant cell is monocotyledonous or dicotyledonous.

23. The plant, plant part, plant tissue, or plant cell according to claim 22, wherein said monocotyledonous plant, plant part, plant tissue, or plant cell is selected from the group consisting of rice, wheat, barley, oats, sorghum, maize, lilies, and millet.

24. The plant, plant part, plant tissue, or plant cell according to claim 20, wherein said plant is *Zea mays* or said plant part, plant tissue, or plant cell is from *Zea mays*.

25. The plant, plant part, plant tissue, or plant cell according to claim 22, wherein said dicotyledonous plant, plant tissue, or plant cell is selected from the group consisting of peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, and lettuce.

26. The plant, plant part, plant tissue, or plant cell according to claim 20, wherein said plant part is a seed.

27. A polypeptide encoded by the polynucleotide of any of claims 1 to 17.

28. A cell transformed with a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 18 to 19.

29. The cell according to claim 28, wherein said cell is a plant cell.

30. The cell according to claim 29, wherein said plant cell is from *Zea mays*.

31. A method for increasing heat stability of a plant, plant part, plant tissue, or plant cell, comprising expressing in said plant, plant part, plant tissue, or plant cell a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 18 to 19, whereby said plant, plant part, plant tissue, or plant cell exhibits increased heat stability relative to a control plant, plant part, plant tissue, or plant cell or a wild type plant, plant part, plant tissue, or plant cell in which said polynucleotide or expression construct has not been introduced.

32. A method for increasing starch biosynthesis of a plant, plant part, plant tissue, or plant cell, comprising expressing in said plant, plant part, plant tissue, or plant cell a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 18 to 19, whereby said plant, plant part, plant tissue, or plant cell exhibits increased starch biosynthesis relative to a control plant, plant part, plant tissue, or plant cell or a wild type plant, plant part, plant tissue, or plant cell in which said polynucleotide or expression construct has not been introduced.

33. A method for increasing crop yield of a plant, plant part, plant tissue, or plant cell, comprising expressing in said plant, plant part, plant tissue, or plant cell a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 18 to 19, whereby said plant, plant part, plant tissue, or plant cell exhibits increased crop yield relative to a control plant, plant part, plant tissue, or plant cell or a wild type plant, plant part, plant tissue, or plant cell in which said polynucleotide or expression construct has not been introduced.

34. A method for altering plant or seed composition of a plant, plant part, plant tissue, or plant cell, comprising expressing in said plant, plant part, plant tissue, or plant cell a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 18 to 19, whereby said plant, plant part, plant tissue, or plant cell exhibits altered plant or seed composition relative to a control plant, plant part, plant tissue, or plant cell or a wild type plant, plant part, plant tissue, or plant cell in which said polynucleotide or expression construct has not been introduced.

35. A method for increasing AGPase enzymatic activity of a plant, plant part, plant tissue, or plant cell, comprising expressing in said plant, plant part, plant tissue, or plant cell a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 18 to 19, whereby said plant, plant part, plant tissue, or plant cell exhibits increased AGPase enzymatic activity relative to a control plant, plant part, plant tissue, or plant cell or a wild type plant, plant part, plant tissue, or plant cell in which said polynucleotide or expression construct has not been introduced.

36. A method for providing a plant, plant part, plant tissue, or plant cell with an AGPase enzyme that is 3-PGA independent or that exhibits decreased K_a for 3-PGA, comprising expressing in said plant, plant part, plant tissue, or plant cell a polynucleotide of any of claims 1 to 17 or an expression construct of any of claims 18 to 19, whereby said plant, plant part, plant tissue, or plant cell exhibits 3-PGA independent AGPase enzymatic activity or AGPase with decreased K_a for 3-PGA relative to a control plant, plant part, plant tissue, or plant cell or a wild type plant, plant part, plant tissue, or plant cell in which said polynucleotide or expression construct has not been introduced.

37. The method according to any of claims 31 to 36, wherein said plant, plant part, plant tissue, or plant cell is monocotyledonous or dicotyledonous.

38. The method according to claim 37, wherein said monocotyledonous plant, plant part, plant tissue, or plant cell is selected from the group consisting of rice, wheat, barley, oats, sorghum, maize, lilies, and millet.

39. The method according to any of claims 31 to 36, wherein said plant, plant part, plant tissue, or plant cell is *Zea mays*.

40. The method according to claim 37, wherein said dicotyledonous plant, plant part, plant tissue, or plant cell is selected from the group consisting of peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, and lettuce.

41. The method according to claim 34, wherein said altered plant or seed composition comprises altered starch to protein ratio and/or altered starch to lipid ratio of said plant or seed.

42. The method according to claim 34, wherein said altered plant or seed composition comprises altered amylopectin to amylose ratio of said plant or seed.

43. The method according to claim 41, wherein starch levels are increased relative to protein and/or lipid levels of said plant or seed.

44. The method according to claim 42, wherein amylose levels are increased relative to amylopectin of said plant or seed.

45. The method according to any of claims 31 to 44, wherein said method further comprises expressing in said plant, plant part, plant tissue, or plant cell a polynucleotide or expression construct comprising a nucleotide sequence encoding a mutant plant AGPase small subunit that comprises a mutation wherein the threonine amino acid corresponding to amino acid position 462 of wild type maize endosperm AGPase small subunit is substituted with an amino acid that confers increased heat stability.

46. The method according to claim 45, wherein said mutant plant AGPase small subunit comprises the amino acid sequence shown in SEQ ID NO:24, or a biologically active fragment or variant thereof.

47. The method according to any of claims 31 to 44, wherein said polynucleotide further comprises a nucleotide sequence encoding a chimeric plant AGPase small subunit that comprises amino acid sequences from two different plant AGPase small subunits.

48. The method according to claim 47, wherein said mutant plant AGPase small subunit comprises the amino acid sequence shown in SEQ ID NO:26 or SEQ ID NO:54, or a biologically active fragment or variant thereof.

49. A mutant plant AGPase enzyme comprising a polypeptide of claim 27.

50. The mutant plant AGPase enzyme according to claim 49, wherein said enzyme further comprises a mutant plant AGPase small subunit that comprises a mutation wherein the threonine amino acid corresponding to amino acid position 462 of wild type maize endosperm AGPase small subunit is substituted with an amino acid that confers increased heat stability.

51. The mutant plant AGPase enzyme according to claim 50, wherein said mutant plant AGPase small subunit comprises the amino acid sequence shown in SEQ ID NO:24, or a biologically active fragment or variant thereof.

52. The mutant plant AGPase enzyme according to claim 49, wherein said enzyme further comprises a chimeric plant AGPase small subunit that comprises amino acid sequences from two different plant AGPase small subunits.

53. The mutant plant AGPase enzyme according to claim 52, wherein said mutant plant AGPase small subunit comprises the amino acid sequence shown in SEQ ID NO:26 or SEQ ID NO:54, or a biologically active fragment or variant thereof.

54. A composition comprising one or more polynucleotides of any of claims 1 to 9, and one or more polynucleotides that encode one or more mutant, chimeric, or wild type small subunit of AGPase.

55. The composition according to claim 54, wherein the mutant or chimeric small subunit comprises the amino acid sequence of SEQ ID NO:24, SEQ ID NO:26, or SEQ ID NO:54, or a biologically active fragment or variant thereof.

56. The composition according to claim 54, wherein said polynucleotide is DNA.

57. The composition according to claim 56, wherein said DNA is cDNA.

58. The composition according to claim 54, wherein said polynucleotide is a recombinant polynucleotide.

59. The composition according to claim 54, wherein the polynucleotides are provided in or are part of an expression construct.

60. The composition according to claim 59, wherein said expression construct comprises one or more regulatory elements selected from promoters, transcription termination sequences, translation termination sequences, enhancers, and/or polyadenylation elements.

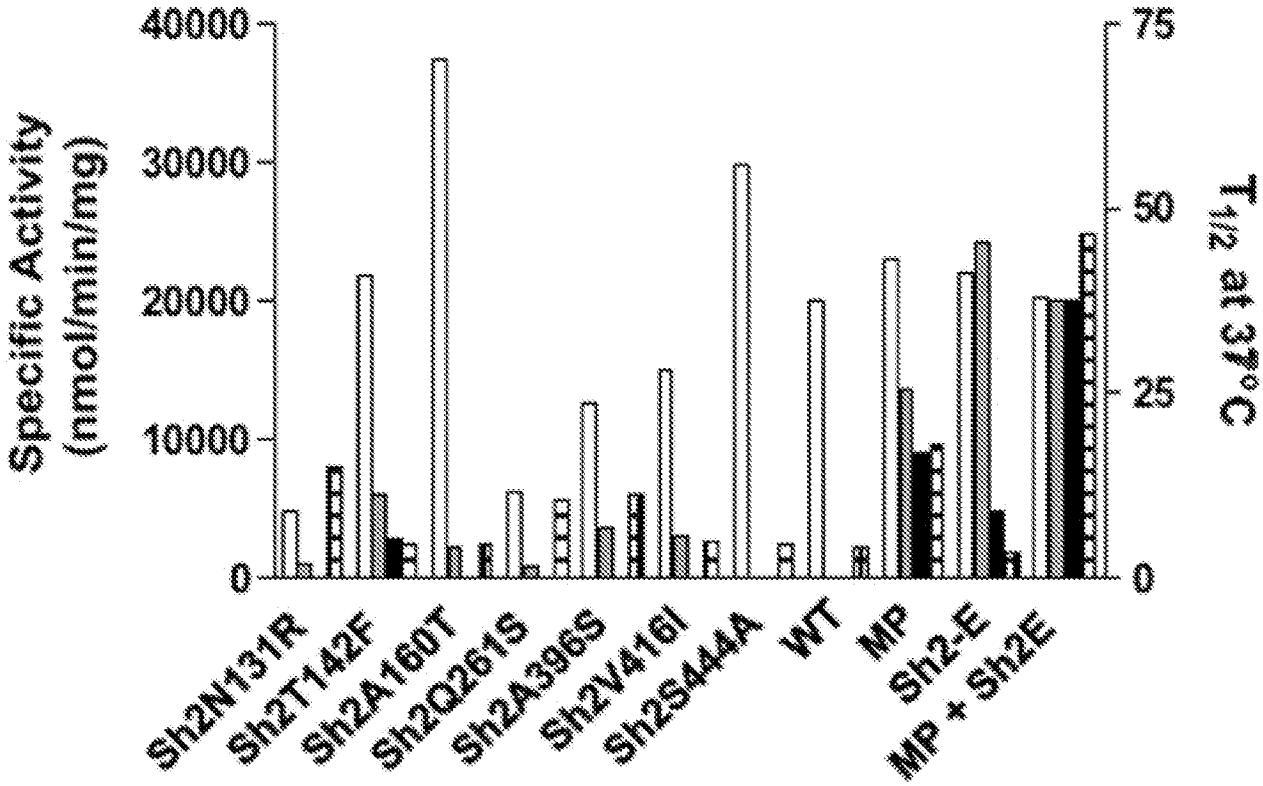
Specific Activity (nmol/min/mg)
40000
30000
20000
10000
0
75
50
25
0
T1/2 at 37°C
Sh2N131R
Sh2T142F
Sh2A160T
Sh2Q261S
Sh2A396S
Sh2V416I
Sh2S444A
WT
MP
Sh2-E
MP + Sh2E

FIG. 1

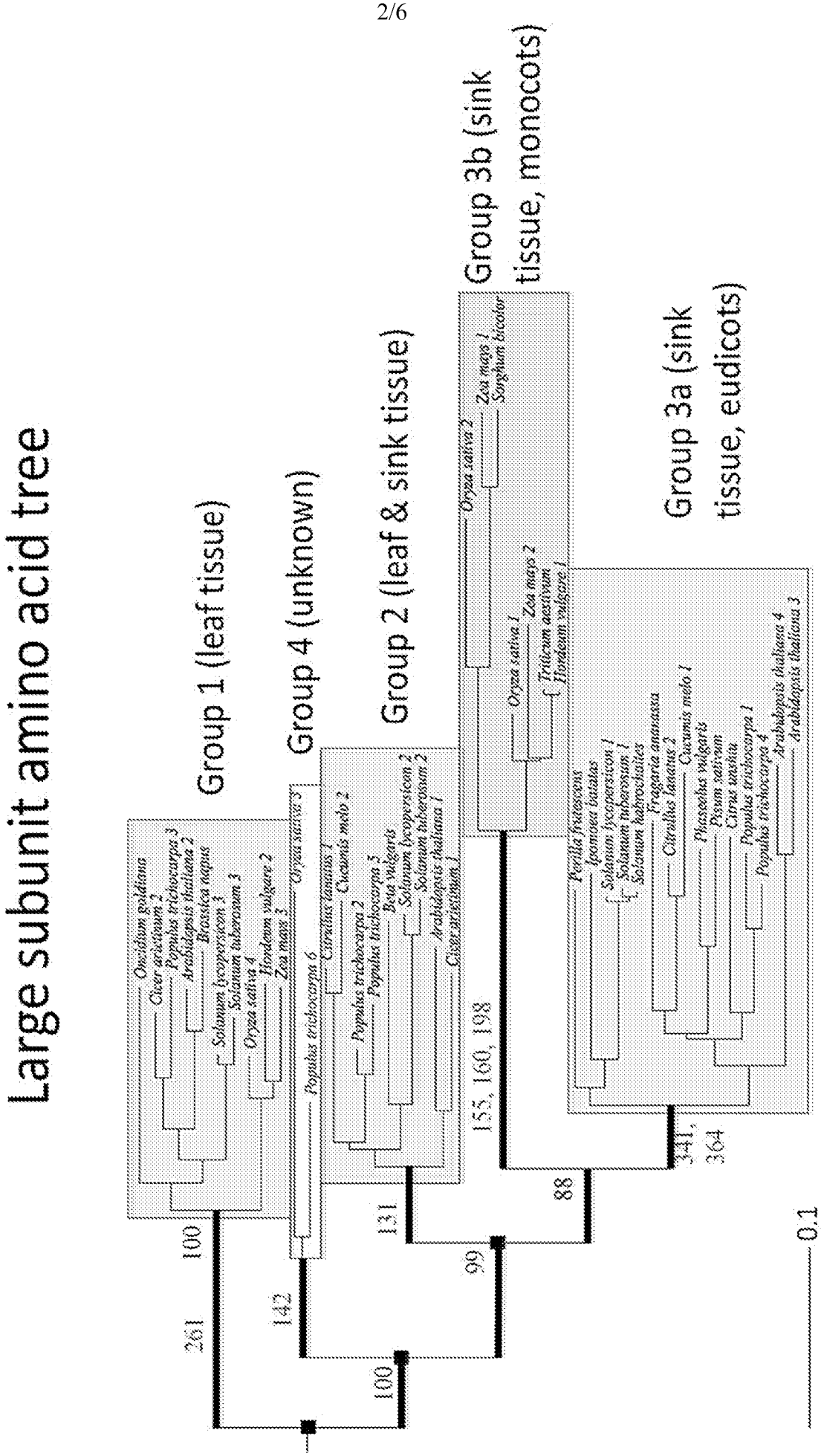
Large subunit amino acid tree
Group 1 (leaf tissue)
Group 4 (unknown)
Group 2 (leaf & sink tissue)
Group 3b (sink tissue, monocots)
Group 3a (sink tissue, eudicots)
Oncidium goldiana
Cicer arietinum 2
Populus trichocarpa 3
Arabidopsis thaliana 2
Brassica napus
Solanum lycopersicum 3
Solanum tuberosum 3
Oryza sativa 4
Hordeum vulgare 2
Zea mays 3
Oryza sativa 3
Populus trichocarpa 6
Citrullus lanatus 1
Cucumis melo 2
Populus trichocarpa 2
Populus trichocarpa 5
Beta vulgaris
Solanum lycopersicum 2
Solanum tuberosum 2
Arabidopsis thaliana 1
Cicer arietinum 1
Oryza sativa 2
Zea mays 1
Sorghum bicolor
Oryza sativa 1
Zea mays 2
Triticum aestivum
Hordeum vulgare 1
Perilla frutescens
Ipomoea batatas
Solanum lycopersicum 1
Solanum tuberosum 1
Solanum habrochaites
Fragaria ananassa
Citrullus lanatus 2
Cucumis melo 1
Phaseolus vulgaris
Pisum sativum
Citrus unshiu
Populus trichocarpa 1
Populus trichocarpa 4
Arabidopsis thaliana 4
Arabidopsis thaliana 3
261
100
142
100
131
99
155, 160, 198
88
341, 364
0.1

FIG. 2

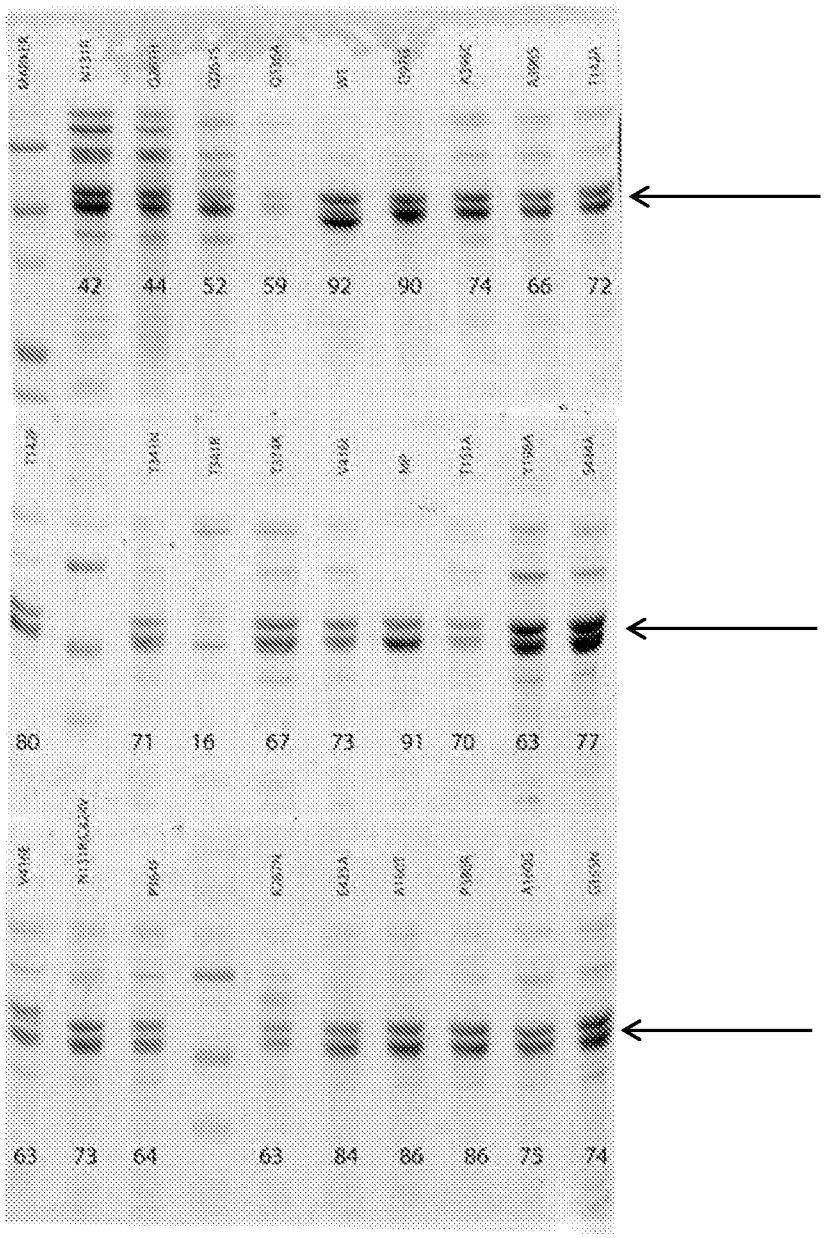
42 44 52 59 92 90 74 66 72
80 71 16 67 73 91 70 63 77
63 73 64 63 84 86 86 73 74

FIG. 3

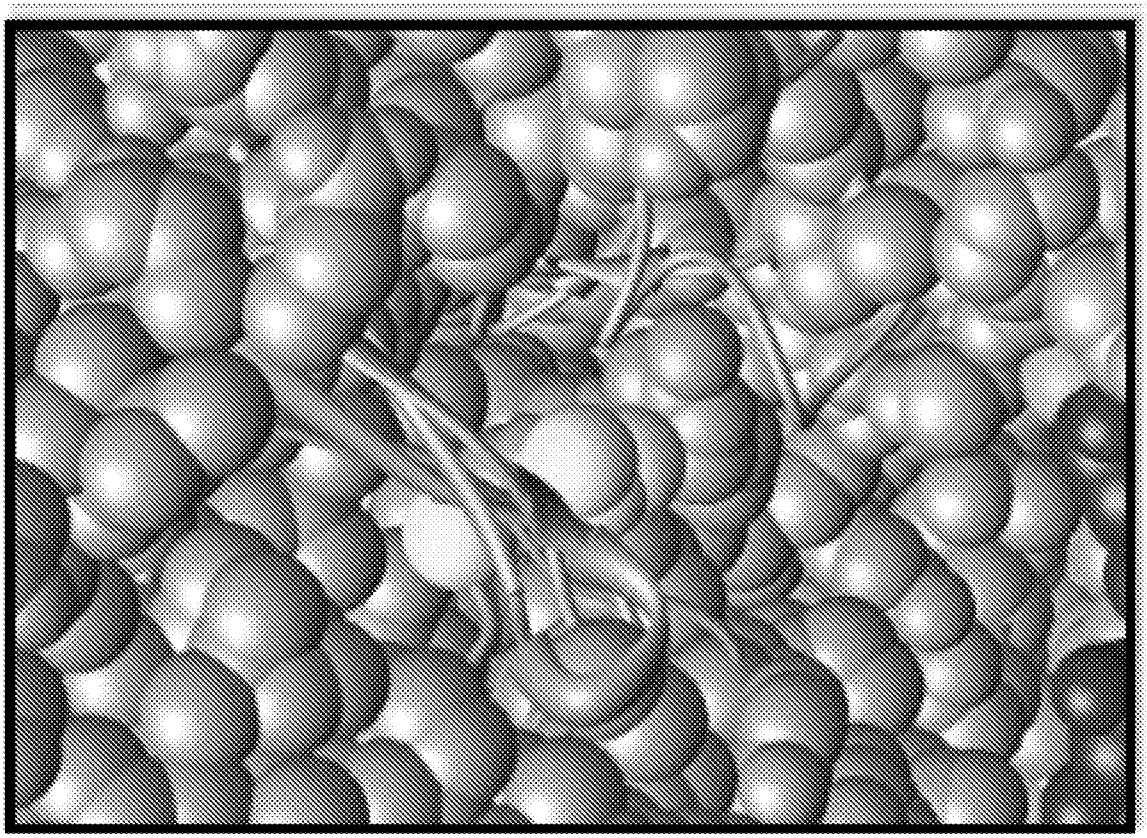

FIG. 4B

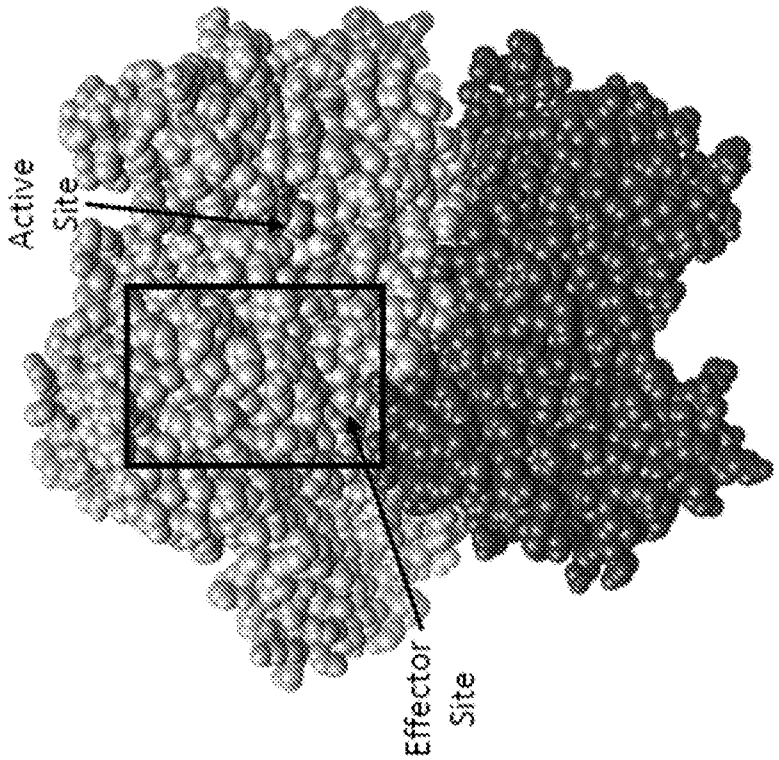

FIG. 4A

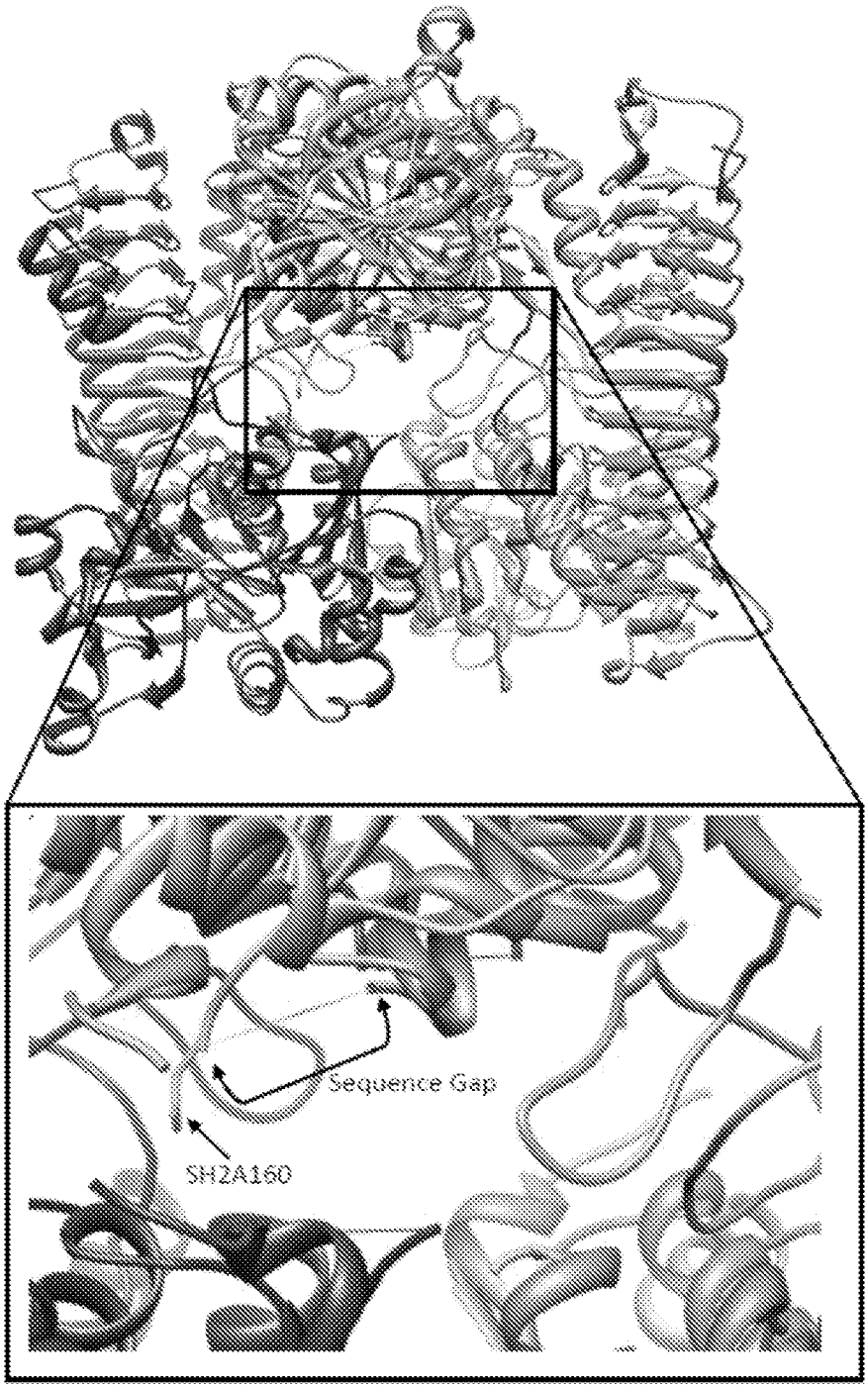

FIG. 5

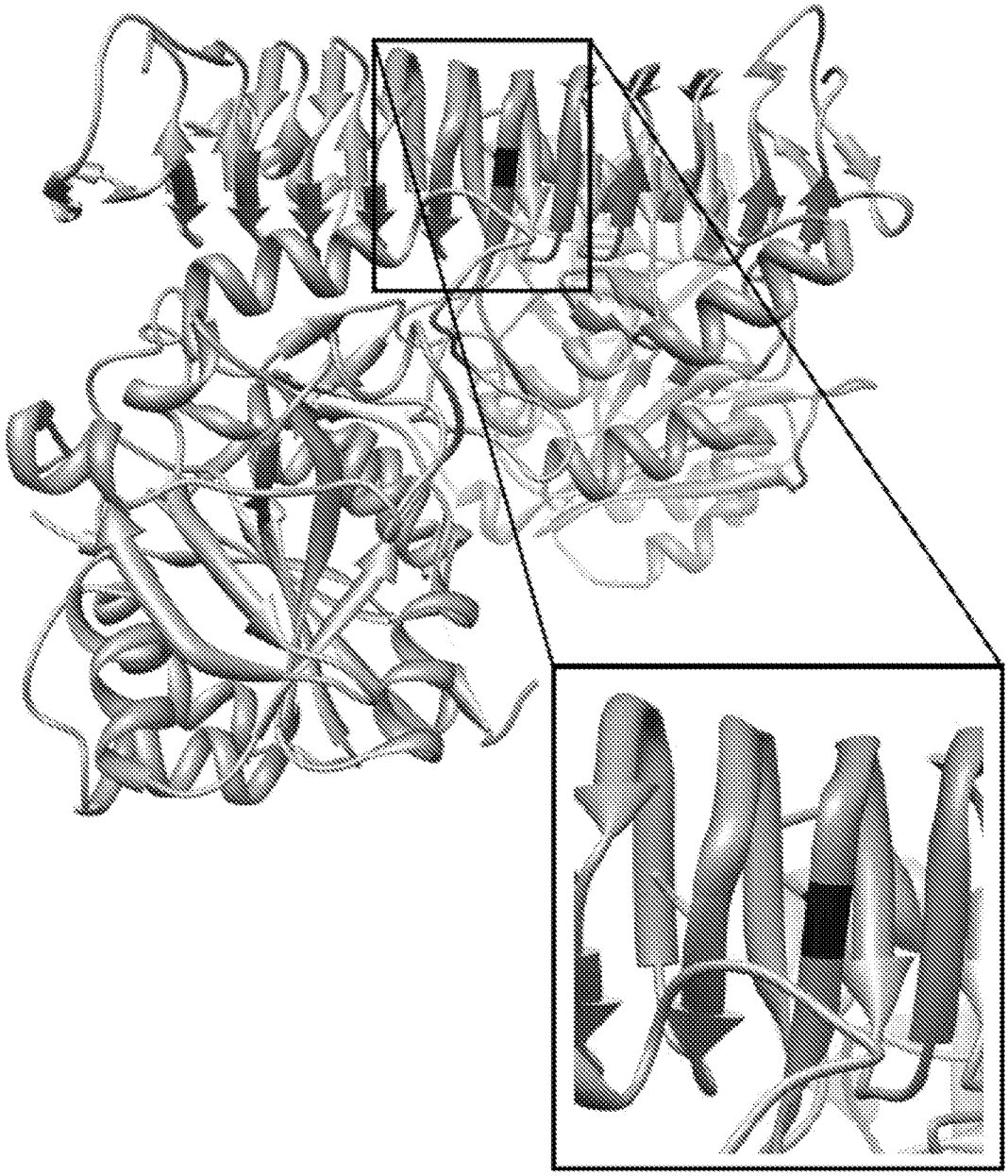

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/047879

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/54(2006.01)i, C12N 15/82(2006.01)i, A01H 5/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/54; C12N 15/29; A01H 5/00; C12N 15/52; A01H 5/10; C12N 15/82

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:ADP-glucose pyrophosphorylase, mutant, heat stability, maize endosperm

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 8362321 B2 (HANNAH, L. CURTIS et al.) 29 January 2013 See abstract; column 5, lines 1-11; column 6, lines 45-46; column 10, lines 14-15; claims 1-2, 5; SEQ ID NO: 13; table 4.	1,2,4,10-12,14,54-60
A		3,5-9,13
A	US 2009-0260101 A1 (HANNAH, L. CURTIS et al.) 15 October 2009 See abstract; claims 1-2.	1-14,54-60
A	GREENE, THOMAS W. et al., 'Enhanced stability of maize endosperm ADP-glucose pyrophosphorylase is gained through mutants that alter subunit interactions', Proceedings of the National Academy of Sciences of the United States of America, 27 October 1998, Vol. 95, No. 22, pp. 13342-13347. See the whole document.	1-14,54-60
A	LINEBARGER, CARLA R. et al., 'Heat stability of maize endosperm ADP-glucose pyrophosphorylase is enhanced by insertion of a cysteine in the N terminus of the small subunit', Plant Physiology, December 2005, Vol. 139, No. 4, pp. 1625-1634. See the whole document.	1-14,54-60

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 November 2014 (17.11.2014)	**17 November 2014 (17.11.2014)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/047879

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 16,19,21-26,29-30,38,40-44,46,48-53
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 16, 19, 21-26, 29-30, 38, 40-44, 46 and 48-53 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 15,17-18,20,27-28,31-37,39,45,47
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/047879

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOEHLEIN SUSAN, K. et al., 'Heat stability and allosteric properties of the maize endosperm ADP-glucose pyrophosphorylase are intimately intertwined', Plant Physiology, January 2008, Vol. 146, No. 1, pp. 289-299. See the whole document.	1-14,54-60

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2014/047879

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 8362321 B2	29/01/2013	US 2010-199385 A1	05/08/2010
US 2009-0260101 A1	15/10/2009	AR 071583 A1	30/06/2010
		AU 2009-234464 A1	15/10/2009
		CA 2720726 A1	15/10/2009
		CN 102203256 A	28/09/2011
		EP 2265719 A2	29/12/2010
		EP 2265719 A4	28/09/2011
		JP 2011-519270 A	07/07/2011
		MX 2010011102 A	22/02/2011
		US 2011-0078821 A1	31/03/2011
		WO 2009-126208 A2	15/10/2009
		WO 2009-126208 A3	18/03/2010
		WO 2009-126208 A8	18/11/2010