Regulation of starch accumulation by granule-associated plant 14-3-3 proteins

Paul C. Sehnke, Hwa-Jee Chung*, Ke Wu†, and Robert J. Ferl‡

Program in Plant Molecular and Cellular Biology, Department of Horticultural Sciences, University of Florida, Gainesville, FL 32611

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In higher plants the production of starch is orchestrated by chloroplast-localized biosynthetic enzymes, namely starch synthases, ADP-glucose pyrophosphorylase, and starch branching and debranching enzymes. Diurnal regulation of these enzymes, as well as starch-degrading enzymes, influences both the levels and composition of starch, and is dependent in some instances upon phosphorylation-linked regulation. The phosphoserine/threonine-binding 14-3-3 proteins participate in environmentally responsive phosphorylation-related regulatory functions in plants, and as such are potentially involved in starch regulation. We report here that reduction of the ε subunit of Arabidopsis 14-3-3 proteins by antisense technology resulted in a 2- to 4-fold increase in leaf starch accumulation. Dark-grown starch breakdown was unaffected in these "antisense plants," indicating an unaltered starch-degradation pathway and suggesting a role for 14-3-3 proteins in regulation of starch synthesis. Absorption spectra and gelatinization properties indicate that the starch from the antisense plants has an altered branched glucan composition. Biochemical characterization of protease-treated starch granules from both Arabidopsis leaves and maize endosperm showed that 14-3-3 proteins are internal intrinsic granule proteins. These data suggest a direct role for 14-3-3 proteins in starch accumulation. The starch synthase III family is a possible target for 14-3-3 protein interaction involving four types of enzymes: ADP-glucose pyrophosphorylase (AGP), starch synthases (SSs), starch-branching enzymes (SBEs), and starch-debranching enzymes (DBEs) (1). AGP forms ADP-glucose from glucose 1-phosphate. SSs add ADP-glucose to the elongating end of an α(1→4)-linked glucan chain, whereas SBEs cut α(1→4) links and rejoin them as α(1→6) branches that are subsequently trimmed by DBEs to yield short chains for further synthetic extension. However, different isoforms of SSs (soluble and granule-associated SSI, SSII, and SSIII) can participate in the production of branched glucans. For example, the granule-bound SSI, the waxy-encoded protein in maize, is directly and perhaps exclusively involved in producing amylose, an α(1→4) glucan polymer with little branching. In contrast, SSII participates in the synthesis of amylopectin, an α(1→6) branched glucan polymer that typically is found together with amylose to form starch granules. The ratio of these two glucans affects the physical characteristics of starch such as gelatinization and the absorption spectra of iodine-complexed starch. The alternation or absence of certain starch biosynthetic enzymes (3–5) has a dramatic effect on the physical characteristics of starch, as well as the level of starch accumulated by the plant. In a similar, yet opposing, manner dark-regulated starch degradation occurs by means of catabolic enzymes such as amylose, α-glucosidase, and starch phosphorylase. The resulting starch stasis is the consequence of the metabolism and catabolism orchestrated by the respective enzymes.

Regulation of some enzymes involved in major resource allocation is affected by allosteric effectors, substrate levels, and product levels, as well as by phosphorylation (6–8). For several key enzymes, regulation of activity is a two-step process involving phosphorylation of the enzyme, followed by formation of a complex with 14-3-3 proteins to complete the regulatory transition (9, 10). For example, the assimilation of nitrogen for production of amino acids or nucleotide bases is tightly controlled by nitrate reductase (NR). NR responds to environmental signals, such as light and metabolite levels, by phosphorylation and interacts with 14-3-3 proteins (11, 12), thereby rapidly altering nitrogen flux according to the plant's metabolic requirements. This phosphorylation-dependent interaction of NR with 14-3-3 proteins has become a paradigm for posttranslational regulation of metabolic enzymes (9). Recently, 14-3-3 proteins have been identified inside plastids (13), thereby implicating a potential role in starch regulation.

We have investigated the biological role of chloroplast-localized 14-3-3 proteins in carbon partitioning, namely starch accumulation. We found that reduced levels of starch granule-associated 14-3-3 proteins result in a dramatic increase in starch accumulation. On the basis of the presence of 14-3-3 consensus binding domains and biochemical experiments, one target of the granule 14-3-3 proteins appears to be the SSIII family.

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Abbreviations: SS, starch synthase; NR, nitrate reductase; Zm-, Zea mays.
*Present address: Kumho Life and Environmental Science Laboratory, Kwangju, Korea.
†Present address: Department of Anatomy and Cell Biology, University of Florida, Gainesville, FL 32611.
‡To whom reprint requests should be addressed. E-mail: robferl@ufl.edu.

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Materials and Methods
Antisense GF14 Vector Construction and Transformation into Arabidopsis. Clones for the Arabidopsis 14-3-3 proteins GF14 ε and GF14 μ, from yeast two-hybrid vectors (14), were used as templates for PCR to produce XbaI cassettes that were subsequently subcloned into the binary plant transformation vector pBI121 (CLONTECH). Gene orientation was determined by automated DNA sequencing on a Perkin–Elmer ABI 373A. Clones containing the antisense GF14 gene orientation were amplified in Escherichia coli INVαF’ and used to transform competent Agrobacterium tumefaciens strain EHA105 by the freeze–thaw method (15). The vector-harboring Agrobacterium was used to transform Arabidopsis ecotype WS seedlings by using vacuum infiltration, essentially as described by Bechtold and Pelletier (16). Transformants were screened on germination media plates using 40 μg/ml kanamycin selection as described previously (17). Seed from positive transformants were selected through three successive generations to ensure homozygous transgenic lines. A minimum of 12 antisense lines were generated for both GF14 ε and GF14 μ.

Plant Growth. Arabidopsis plants were grown in constant light at 22°C on germination media plates oriented in a vertical position or in flats of Transplant mix A (Vergro, Tampa, FL). Starch degradation experiments were done by transferring the plants to dark and samples taken at three hour intervals.

Starch Analysis. Starch was visualized by Lugol’s iodine staining reagent (Sigma). Leaves from 10-day-old plants were harvested and blanched in 80% (vol/vol) ethanol. After rinsing with double-distilled water the leaves were stained with Lugol’s reagent and briefly destained with water. Stained plants and leaves were photographed with an Olympus SZH10 stereo dissecting microscope and DP10 digital camera.

Enzymatic measurement of starch in leaves was performed by using a method adapted from Zeeman et al. (18). Rosettes were harvested and weighed, then boiled in 80% ethanol. After clearing, the samples were ground in a mortar and pestle in 80% ethanol and the crude starch pellet was recovered by centrifugation at 5,000 rpm for 5 min in a Beckman JA20 rotor and J2–21 centrifuge. The crude starch was resuspended in 80% ethanol and repelleted two more times. The final pellet was dried and resuspended in double-distilled water, then placed at 85°C for 10 min. The starch solution was then digested with 3 mg/ml amylase and 3 mM CaCl2 for 24 h at 37°C. The final digested starch solution was treated with thermolysin to remove surface-associated proteins (21), then washed and digested at 25°C with α-amylase and amyloglucosidase in 100 mM Tris-acetate buffer, pH 7.5, containing 100 mM KCl, 2.5 mM DTT, 10% (vol/vol) glycerol, 25 mM NaF, 3 mM CaCl2, and 0.1% BSA for 3 h by using a protocol adapted from MacDonald and Preiss (23). Undigested material was removed by ultracentrifugation in a Beckman SW55 Ti rotor at 4°C at 50,000 rpm for 30 min. Supernatant was transferred to a plastic conical tube and BSA was added to a final concentration of 0.1%. The supernatant was passed over anti-14-3-3 ε- and μ-conjugated Sepharose made from CNBr-activated Sepharose (Amersham Pharmacia Biotech) and the 14-3-3 protein antiserum IgG fractions (13). A control column containing antibodies raised against the transcriptional cofactor GIP1 (unpublished data) was used as a negative control. The columns were loaded with the starch-derived protein extract, then washed three times with phosphate-buffered saline (PBS), pH 7.6, containing 25 mM NaF. The processed beads were boiled for 1 min in 2× SDS/PAGE sample buffer. The beads were removed by centrifugation and supernatant was loaded onto 10% polyacrylamide gels before SDS/PAGE. The proteins were separated by nitrocellulose and blocked overnight with Blotto Tween (24). The membranes were probed with antiserum to the Zea mays (Zm)SSIII DU1 (25). The membrane was washed and incubated with horseradish peroxidase-conjugated antibodies to rabbit IgG. Labeled bands were identified by the process of chemiluminescence, using SuperSignal West Pico Chemiluminescent Substrate according to the supplier’s instructions (Pierce).

Biotinylated 14-3-3 Protein Overlay Experiments. To identify corn starch proteins that are potential targets for 14-3-3 protein binding, proteins from corn starch were separated by electrophoresis and assayed by using a blot overlay procedure with biotinylated recombinant 14-3-3 Zm GF14–12. Zm GF14–12 was expressed in E. coli and purified by nickel-Sepharose chromatography as described previously (26). The protein was dialyzed against 100 mM sodium borate, pH 8.8, overnight before addition of bovine serum protein N-hydroxysuccinimide ester in DMSO at a ratio of 50 μg of ester per mg of protein. After 4 h at room temperature, the reaction was terminated by the addition of 1 M ammonium chloride, pH 8.0. The biotinylated 14-3-3 protein was dialyzed exhaustively against PBS over the course of 2 days at 4°C. Proteins from 10 mg of corn starch boiled in SDS/PAGE sample buffer were separated by PAGE and transferred to nitrocellulose, then incubated overnight at 4°C with biotinylated 14-3-3 protein in PBS containing 1% BSA. The blot was washed three times with PBS/1% BSA and incubated for 30 min with streptavidin-conjugated horseradish peroxidase diluted in PBS/1% BSA. The blot was washed three additional times and the 14-3-3-bound protein was identified by using chemiluminescence as described above.

Results and Discussion
Transgenic Arabidopsis plants expressing antisense cDNA of At 14-3-3s GF14 ε and μ, two members of the ε subgroup of 14-3-3 proteins, displayed normal growth behavior but demonstrated phenotypic changes relative to wild-type plants with regard to starch accumulation in leaves. Although the absolute level of starch present in the leaves of Arabidopsis depended upon
culture conditions and the lines examined, the leaves of plants from all 12 GF14 ε and GF14 μ antisense lines consistently accumulated increased starch levels relative to leaves of wild-type plants. Iodine staining indicated that the increased starch accumulation was equally distributed throughout the leaves of the antisense plants (Fig. 1 A–C). Quantitative measurements of the starch present in the leaves of plants grown in constant light revealed an approximately 2-fold increase in total starch content in antisense plants over wild-type plants (28 ± 7 mg of starch per g fresh weight in transgenic plants vs. 15 ± 3 mg of starch per g fresh weight in wild-type plants). The extractable starch from antisense plants was approximately 4-fold higher than that from wild-type plants (43 ± 5 mg of starch per g fresh weight vs. 9 ± 2 mg of starch per g fresh weight, respectively). Isolated starch granules from antisense plants were used to evaluate the absorption spectra of the iodine/starch complex, as an indicator of unbranched and branched glucan ratios. The absorption spectrum of the iodine/starch complex from antisense plants (Fig. 2, spectrum B) was blue-shifted relative to the absorption spectrum of the iodine/starch complex from wild-type plants (Fig. 2, spectrum A), suggesting that the starch from antisense plants has an increase in branched glucan content. This premise is further supported by the observation that the percentage of gelatinizable starch from antisense plants was reduced relative to that found in wild-type plants (data not shown).

To determine whether altered degradation rates might be responsible for the elevated starch accumulation in 14-3-3 antisense plants, plants were grown in constant light and harvested after a dark period of 18 h. Iodine staining of leaves at the end of the dark period was indistinguishable between wild-type (Fig. 1 D) and antisense plants (Fig. 1 E and F). To measure the rate of starch breakdown, leaf samples were taken every 3 h after the plants were placed in the dark. Wild-type plants degraded starch at a rate of approximately 1 mg of starch per g fresh weight per h, whereas the antisense plants cleared starch from their leaves at rates of approximately 1.3 to 1.5 mg of starch per g fresh weight per h. This result indicates that the starch degradation pathway is fully functional in the antisense plants and suggests that reduced negative regulation of starch biosynthesis is responsible for increased starch in the 14-3-3 antisense plants. The 14-3-3 proteins would therefore appear to function as inhibitory proteins in starch metabolism by normally shutting down starch biosynthesis, thereby playing a key regulatory role in carbon allocation that is similar to their role in nitrogen fixation.

Antibodies to 14-3-3 proteins were used in an immunolocalization electron microscopy experiment looking at starch granules in the leaves of wild-type Arabidopsis. The inside of chloroplast starch granules was densely decorated by antibodies that recognize eight non-ε subgroup members (Fig. 3B). Antibodies specific to GF14 ε also decorated the inside of starch granules, but more sparsely (Fig. 3C). This limited amount of ε in the starch granules of wild-type plants may explain why the antisense plants displayed reduced levels of starch-associated GF14 ε, whereas the cytoplasmic levels of ε remained reasonably normal (data not shown). These data also indicate that non-ε 14-3-3 proteins may be involved in starch biosynthesis, although no
phenotypic data yet exist to support this conclusion. The relationship among the 14-3-3 isoforms present in starch grains, as well as the question of whether active forms of 14-3-3 proteins exist as homodimers or heterodimers, is not well established and therefore will need to be addressed in future studies.

To confirm that 14-3-3 proteins are present within chloroplast starch granules and that increased starch production is a result of decreased 14-3-3 proteins, starch granules from wild-type, GF14 \( \varepsilon \), and GF14 \( \mu \) antisense plant leaves were biochemically analyzed for the presence of 14-3-3 proteins. Purified starch granules were incubated with the protease thermolysin to remove external proteins, washed, boiled in SDS/PAGE sample buffer, and analyzed on SDS/PAGE by Western analysis with antibodies specific to 14-3-3 proteins GF14 \( \varepsilon \) or \( \mu \) (13). Wild-type starch contained both GF14 \( \varepsilon \) and \( \mu \) (Fig. 4 lanes 1 and 2), whereas antisense starch did not contain detectable amounts of either (Fig. 4 lanes 3 and 4). This coregulated suppression is not surprising, as the identity between cDNAs is \( \sim 70\% \) and therefore both mRNAs are presumably reduced by antisense regulation in planta. Western analysis of whole-leaf extracts did not demonstrate a pronounced decrease in GF14 \( \varepsilon \) and \( \mu \) proteins (data not shown). Starch granule-specific reduction of GF14 \( \varepsilon \) and \( \mu \) 14-3-3 proteins in starch granules is significant in that they appear essential for proper regulation of leaf starch biosynthesis in Arabidopsis. In addition, commercial starch from maize also possesses 14-3-3 proteins (Fig. 4 lane 5), suggesting that 14-3-3 protein regulation of starch synthesis is used by crops and occurs in other plastids, such as amyloplasts, and is not limited to photosynthetically active plastids.

Although a chloroplast-localized 14-3-3 protein partner in starch synthesis has not been reported, a search of all available starch-related enzyme sequences for the consensus 14-3-3-binding motif revealed the SSIII family as an obvious potential target within the plastid (Fig. 5). SSIII members from potato, Arabidopsis, Vigna unguiculata, Aegilops tauschii, Triticum aestivum, and maize all contain a conserved hexapeptide motif very similar to the 14-3-3 protein binding site of NR. This is the only example of an entire family sharing such a highly conserved potential binding site among the plastid enzyme sequences currently available. It is interesting to note that SSIII is directly involved in the production of amyllopectin and has significant control over other SS isoforms (4), perhaps explaining both starch accumulation and the qualitative shift in branched glucan content observed in 14-3-3 antisense plants.

Immunocapture experiments with anti-GF14 column and proteins isolated from processed corn starch were used to experimentally determine whether starch granule 14-3-3 proteins associate directly with SSIIIs. Commercial corn starch was chosen as a source of proteins because of its bulk availability and antibodies to the maize SSIII enzyme were available (25). SDS/PAGE and Western

![Fig. 3. Immunolocalization of 14-3-3 proteins in starch granules. Arabidopsis leaves were processed for electron microscopy (20) and immunolabeled with GF14 antibodies. Control antibodies to Dictyostelium spores (A) did not immunodecorate the granules; however, antibodies that recognize both \( \varepsilon \) (C) and non-\( \varepsilon \) (B) 14-3-3 proteins were concentrated inside the starch granules.](image)

![Fig. 4. Reduction in GF14 \( \varepsilon \) and \( \mu \) protein levels in the starch granules of antisense plants and presence of 14-3-3 proteins in commercial corn starch. Isolated starch granules from wild-type and antisense Arabidopsis were treated with thermolysin to remove externally attached proteins and subjected to SDS/PAGE Western analysis with 14-3-3 protein antibodies (29). Protein extracts from 3 mg of starch from wild-type (lanes 1 and 2), GF14 \( \varepsilon \) antisense (lane 3), and GF14 \( \mu \) antisense (lane 4) plants were probed with antibodies recognizing GF14 \( \varepsilon \) (lanes 1 and 3) and \( \mu \) (lanes 2 and 4). A clear reduction of these 14-3-3 isoforms is observed in the starch-granule proteins of antisense plants. A 3-mg sample of commercial corn starch was processed as described above and the blot was probed with antibodies that recognize maize 14-3-3 proteins (lane 5), indicating the presence of 14-3-3 proteins in starch grains from maize.](image)
14-3-3 CONSENSUS BD  RXRSP

NR 14-3-3 BD  540 1kRTASTPf
Potato SSIII  1333  amRGSP1Pvv
Dull1 SS  1578  amRGTYIPv
Vigna SSIII  1050  amRGSP1Pv
Triticum SSIII  1530  amRGSP1Pv
Aegilops SSIII  1513  amRGSP1Pv
At SSIII  943  amRGSP1Pv

Fig. 5. Consensus 14-3-3-binding sites in SSIII coding sequences. The phosphoserine/threonine-containing binding sequence for 14-3-3 proteins is present in all known members of the SSIII family listed in GenBank: SSIII from Vigna unguiculata (Vigna SSIII, AJ225088), SSIII from Solanum tuberosum (Potato SSIII, X94400 and X95759), SSIII Dull1 from Zea mays (Dull1 SS, AF023159), SSIII from Triticum aestivum (Triticum SSIII, AF258608), SSIII from Aegilops tauschii (Aegilops SSIII, AF258609), and a predicted SSIII from Arabidopsis thaliana (At SSIII, AL021713). The 14-3-3 protein consensus binding domain (BD) and the NR 14-3-3 binding domain are shown for comparison.