1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
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4.0
4.5
5.0

1 of 1

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PROGRESS REPORT

Overview - The long term aim of this project is to assess the feasibility of increasing the conversion of photosynthate into starch via manipulation of the gene that encodes for ADPglucose pyrophosphorylase, a key regulatory enzyme of starch biosynthesis. In developing storage tissues such as cereal seeds and tubers, starch biosynthesis is regulated by the gene activation and expression of ADPglucose pyrophosphorylase, starch synthase, branching enzyme and other ancillary starch modifying enzymes, as well as the allosteric-controlled behavior of ADPglucose pyrophosphorylase activity. During the last two years we have obtained information on the structure of this enzyme from both potato tuber and rice endosperm, using a combination of biochemical and molecular biological approaches. Moreover, we present evidence that this enzyme may be localized at discrete regions of the starch grain within the amyloplast, and plays a role in controlling overall starch biosynthesis in potato tubers.

Structure of the Rice Endosperm ADPglucose Pyrophosphorylase Subunit - Before the commencement of this DOE grant, we had been successful in isolating a cDNA clone which encoded a rice endosperm tissue-specific subunit of ADPglucose pyrophosphorylase. Although the DOE grant was centered on the potato tuber ADPglucose pyrophosphorylase, initial efforts were directed at the rice endosperm enzyme, as we believed that comparison of the primary structures of ADPglucose pyrophosphorylases from different plants may provide some valuable information on structure-function relationships of this enzyme. Since the cDNA clone lacked information for a considerable portion of the amino terminus of the enzyme, a second cDNA library was constructed, using the approach developed by Heidecker and Messing (1), a method which is particularly useful in obtaining full-length cDNA inserts. Screening of this second cDNA library resulted in the isolation of pRSc6, which possessed about 1650 nucleotides. DNA sequence analysis of this clone showed that it contained a single, long open reading frame beginning with an AUG codon. As we had previously estimated that the mRNA transcript for ADPglucose pyrophosphorylase was about 1800 nucleotides in length (2), it was not clear whether this AUG represented the translation initiation site, or whether it was an internal methionine codon. The open reading frame encoded a polypeptide with a molecular weight of about 52,000, a size somewhat smaller than the estimated 56,000 product obtained by *in vitro* translation. To verify whether this AUG was the initiator codon, RNA sequencing studies were conducted. A synthetic 16mer oligonucleotide was prepared, annealed to poly(A+)-RNA and subjected to the chain termination reactions (3), using reverse transcriptase. An additional 27 nucleotides were obtained containing two nonsense codons, one located in-frame with the long open reading frame. The presence of an in-frame nonsense codon indicated that the long open reading frame of pRSc6 contains the complete coding sequence for ADPglucose pyrophosphorylase. Analysis of the primary sequence revealed some interesting findings on possible structure-function relationships of the plant enzyme, as summarized below. A more complete description can be obtained in an appended J. Biol. Chem. manuscript (Appendix 1).

1. A comparison of the N-terminal sequence of the rice endosperm subunit to the published primary sequence of the mature spinach leaf 51 kD subunit (4) suggested a leader sequence of about 24 residues, presumably needed for targeting and transport of this protein to the amyloplast. Other than a hydrophobic N-terminus and a highly basic region near the C-terminal end, the putative transit leader showed very little homology to the consensus transit peptide sequence conserved in chloroplast-localized, nuclear-encoded proteins (5).
2. A comparison of the primary sequence of the rice endosperm subunit to the *Escherichia coli* enzyme showed two regions, residues 48 to 227 and residues 297 to 395, displaying significant homology. About 40% identity was evident upon direct alignment of the plant and bacterial sequences. The conservation of residues in the peptide region 48 to 227 is noteworthy, as these contain lys_{68} and lys_{195}, residues required for allosteric and catalytic activity, respectively, in the bacterial enzyme (6).
3. Other than the relatively non-conserved peptide region between residues 228 to 296, the plant subunit contains an extended C-terminal peptide region not present in the bacterial enzyme. The C-terminal addition of the plant enzyme contains a lysine residue reactive to pyridoxal phosphate which mimics the allosteric activator 3-phosphoglycerate (7,8). This result suggests that at least a portion of the primary sequence responsible for regulation of the plant enzyme lies near the C-terminus.
4. In addition to an extended C-terminus, the plant enzyme displays at least two other structural changes from the bacterial enzyme. It has been shown that point mutations, $Lys_{296} \rightarrow Glu$ and $Gly_{336} \rightarrow Asp$, result in the formation of a bacterial enzyme which is less dependent on allosteric activation by fructose 1,6-bisphosphate (9). It is interesting to note that the plant enzyme has acidic residues at these positions, which may suggest that point mutations of specific residues to acidic amino acids and the C-terminal addition to the plant enzyme may have resulted in the loss of allosteric specificity to fructose 1,6-bisphosphate (the normal activator of the bacterial enzyme), but a gain in sensitivity to 3-phosphoglycerate.

Genomic Structure and Expression of the Rice ADPglucose Pyrophosphorylase Gene - The structure of the rice endosperm-specific ADPglucose pyrophosphorylase gene has been elucidated during this granting period. The gene spans over 6 kilobases of DNA and is very complex in structure, as it contains 10 exons and 9 introns. The first intron, which has a length of 1435 nucleotides, is much larger than typically seen for introns present in plant genes (11). All of the introns, except for intron 2, followed the typical GT/AG splice site rule. Intron 2, however, had CA/CC borders, but examination of the DNA sequence in the vicinity of the splice site showed similarity with the consensus border sequences of all exon-intron splice sites. This similarity suggests that the overall DNA sequence context, rather than the specific GT/AG sequence, determines the splice site. The transcriptional initiation site was determined by the primer extension technique (12) and is situated about 116 bp upstream from the initiator codon. The TATA and CAAT boxes, ubiquitous in almost all eucaryotic genes, are evident at -30 bp and -80 bp from the transcriptional start

site. These conserved elements of the ADPglucose pyrophosphorylase gene showed very high sequence similarity with analogous elements present in the promoter region of the rice glutelin storage protein genes (13). The 5' promoter region also contains a number of repeated sequences, several of which resemble the SV40 enhancer, a motif prevalent in plant genes. The developmental pattern of accumulation for ADPglucose pyrophosphorylase transcripts was determined by both Northern and dot blot analyses. This gene is activated very early during seed development, and its mRNA transcripts constitute about 0.2% of the total mRNA present in 5-7 days post-anthesis seeds. In subsequent periods of seed development, the mRNA levels steadily decline. A more detailed discussion of the structure and expression of the ADPglucose pyrophosphorylase gene is described in Appendix 2.

Biochemical Studies on the Potato Tuber ADPglucose Pyrophosphorylase - The subunit structure of the plant ADPglucose pyrophosphorylases has been re-examined during the past few years. Copeland and Preiss (14) were the first to purify the spinach leaf ADPglucose pyrophosphorylase to apparent homogeneity. The enzyme was found to have a native molecular weight of around 200,000, which dissociated into two dissimilar sized subunits of about 54 kD and 51 kD under denaturing conditions. As the bacterial enzyme is a homotetramer comprised of a single 49 kD subunit (15), it was thought that the smaller 51 kD subunit was a proteolytic product of the 54 kD subunit. Sowokinos and Preiss (16) later showed that the potato tuber enzyme, as detected earlier for the bacterial enzyme, appeared to be composed of a single subunit of 50 kD. More recent biochemical and genetic data (discussed in the proposal) supported the hypothesis, however, that at least the leaf-specific ADPglucose pyrophosphorylase was composed of dissimilar sized subunits and products of two different genes. In view of the apparent discrepancy of the subunit structure of the leaf and tuber forms of ADPglucose pyrophosphorylase, the purification and structural analysis of the potato tuber enzyme was undertaken (see Appendix 3). By modifying the purification protocol of Sowokinos and Preiss (16) and further employment of FPLC, the enzyme was purified to near homogeneity at a specific activity of 56 μmol/min/mg protein, a value about 4-fold greater than that obtained in an earlier study (16). As shown previously (16), only a single polypeptide of about 50 kD was evident on SDS polyacrylamide gels. Further examination of this purified enzyme fraction by two-dimensional polyacrylamide gel electrophoresis revealed, however, that the tuber enzyme was not composed of a single subunit which assembles to form a homotetramer. Under denaturing isoelectric focusing conditions in the first dimension and SDS polyacrylamide gel electrophoresis in the second dimension, two groups of nearly identical size polypeptides were clearly evident. The slightly more basic 51 kD subunits resolved into 3-5 components, whereas the somewhat smaller 50 kD subunits separated into 1-3 species. The degree of heterogeneity observed for both groups of subunits appeared to depend on the isoelectric focusing conditions and age of the protein sample. The identity of these two different groups of polypeptides was analyzed by Western blotting. Antibodies were raised against the purified tuber ADPglucose pyrophosphorylase and used to probe blots of the two-dimensional polyacrylamide gels. Reactivity to both groups of polypeptides by the anti-tuber enzyme was readily observed. In contrast, the spinach leaf anti-51 kD subunit (4) obtained from J. Preiss, recognized only the smaller 50 kD subunits; no reaction was apparent with

the slightly larger 51 kD species. These results support the hypothesis that the tuber ADPglucose pyrophosphorylase is composed of at least two distinct subunits with the tuber 50 kD subunits sharing structural homology to the 51 kD subunit of the spinach leaf enzyme. The results from this study do not prove that the slightly larger 51 kD subunits are part of the ADPglucose pyrophosphorylase holoenzyme, as it does not react with the anti-54 kD subunit of the spinach leaf enzyme (4). Both biochemical and genetic evidence, however, support a structural role for the 51 kD subunit of the potato tuber ADPglucose pyrophosphorylases, as discussed in the body of the proposal.

Immunocytochemical Localization of Tuber ADPglucose Pyrophosphorylase - ADPglucose pyrophosphorylase in photosynthetic tissue, along with the other enzymes involved in starch biosynthesis, is localized in the chloroplast (17,18). Information on the subcellular localization of this enzyme in non-photosynthetic tissue is less extensive, due to the extreme fragility of the starch-containing amyloplast. To verify the specific localization of ADPglucose pyrophosphorylase to amyloplast, an immunocytochemical approach was undertaken. Thin sections of tuber tissue were fixed with glutaraldehyde and paraformaldehyde and embedded in LR White resin, according to standard protocols in this laboratory. The thin sections were then incubated with antibodies prepared against the spinach leaf enzyme (4) or purified potato tuber enzyme, washed, and subsequently incubated with protein-A gold particles. When visualized by electron microscopy, all of the amyloplasts were disrupted and no evidence of protein-A gold particle labeling was evident. The disruption of the amyloplast was not due to the fixation conditions, as thin sections post-stained directly with 2% uranyl acetate and 2% lead citrate showed intact organelles. Because intact amyloplasts in ultra-thin sections could not survive the immuno-staining procedure, we resorted to the analysis of 1 μm thick sections at the light microscope level, using both fluorescence and silver enhancement localization techniques (Appendix 4). At the light microscope level, the amyloplasts could be readily distinguished by carbohydrate staining with periodic acid followed by Schiff base. Under these conditions, many of the amyloplasts had a sub-structure of concentric light and dark layers. Thick sections of these potato tuber cells were incubated with fluorescein-conjugated goat anti-rabbit IgG, after treatment with antibodies raised against either the spinach leaf holoenzyme or purified 51 kD subunit. Intense immunofluorescence was detected only in the amyloplasts. Similar results were also obtained with immunogold labeling followed by silver enhancement. Label was found distributed across the amyloplasts, although there was often an increase in the amount of labeling at the outer periphery of the grains. This likely reflects a higher concentration of the enzyme in the stromal layer surrounding the starch grain. In starch grains with highly defined contour patterns, labeling was often most intense along layers that stained darkest with safranin. ADPglucose pyrophosphorylase has been assumed to be a soluble enzyme, but our immunocytochemical observations suggest that this enzyme may be associated with the starch grain at specific regions.

The Role of Ortho-Phosphate in Starch Biosynthesis - In leaf tissue, substantial evidence has been obtained that starch biosynthesis is controlled via the allosteric regulation of ADPglucose pyrophosphorylase. Heldt *et al.* (19) have shown that the ratio of 3-

phosphoglycerate:Pi levels in leaf tissues or isolated chloroplasts, correlate well with the overall level of starch biosynthesis and, in turn, the catalytic activity of ADPglucose pyrophosphorylase. Moreover, sequestration of Pi by exogenously-applied mannose (which forms non-metabolizable mannose 6-P) in leaf tissue of C3 plants results in a significant stimulation of starch biosynthesis (20,21), presumably by removing the inhibitory effect of Pi on ADPglucose pyrophosphorylase activity and diverting carbon into starch. Although the *in vitro* activity of the tuber ADPglucose pyrophosphorylase is influenced by 3-phosphoglycerate and Pi, it is not at all clear whether the levels of these effector molecules oscillate in tuber tissue and affect starch biosynthesis, as they do in chloroplasts. To determine the role of Pi in affecting starch synthesis in potato tuber tissue, we studied the effect of exogenously-applied mannose on the capacity of tuber slices to synthesize starch from ^{14}C-sucrose (Appendix 5). Sucrose uptake by tuber slices was measured in the presence or absence of 10 mM mannose. Such analysis showed that 10 mM mannose had no significant effect on sucrose uptake. Higher mannose concentrations had a slightly inhibitory effect; at 50 mM mannose, about a 27% decrease in sucrose uptake was observed. Although mannose had very little effect on sucrose uptake, it did have a significant impact on the incorporation of sucrose into starch. About a 50% increase in starch synthesis was evident in tuber slices incubated with 10 mM mannose, as opposed to the untreated samples. The simplest explanation that may account for these observations is that metabolites formed from sucrose via glycolysis can enter the amyloplasts via the phosphate translocator (22). The reduction of cytoplasmic phosphate by mannose would enhance the import of triosephosphates into the amyloplasts at the expense of Pi. These events would lead to an increase in the ratio of 3-phosphoglycerate:Pi in the amyloplast compartment, resulting in enhanced ADPglucose pyrophosphorylase activity and, in turn, increased starch production.

Isolation and Characterization of Potato Tuber ADPglucose Pyrophosphorylases Genes- A major effort during the past few years of the DOE supported research was to elucidate the structure of the potato gene. With a cDNA probe for the rice endosperm ADPglucose pyrophosphorylase subunit in hand, we felt that the isolation of the potato gene would be a rather straightforward task. Antibodies to both the spinach leaf and potato tuber enzyme cross-reacted with the rice endosperm subunit, suggesting that the primary sequences of the smaller subunits have remain conserved. Indeed, screening of a potato genomic library resulted in the isolation of a lambda recombinant clone which reacted with the rice cDNA probe. DNA sequence analysis, however, quickly revealed that the potato DNA was a false clone. To assess the relatedness of the rice cDNA probe to transcripts coding for the potato ADPglucose pyrophosphorylase 50 kD subunit, Northern blot studies of developing tuber RNA were conducted. No hybridization signals, however, were evident, even when very low hybridization-wash conditions were employed. The lack of cross-reactivity suggests that the rice endosperm cDNA and tuber mRNA transcripts have diverged at the DNA sequence level.

In view of our unsuccessful attempt to isolate the tuber pyrophosphorylase gene or cDNA by using a heterologous rice cDNA probe, alternative approaches were taken. First, we had attempted to obtain sequence information of the N-terminus of the ADPglucose pyrophosphorylase protein. The purified potato tuber enzyme was subjected to Edman degradation, using an automatic peptide sequencer, but

it was readily apparent that the N-terminus of this protein was blocked. A second approach was the selection of the clone of interest, by screening of an expression library with the spinach leaf anti-51 kD subunit. This antibody reacted specifically with ADPglucose pyrophosphorylase 50 kD subunit, and showed very little cross-reactivity with other polypeptides present in crude extracts of potato tuber. In contrast, antibodies raised against the spinach leaf enzyme also recognized the tuber subunit but it also cross-reacted with other polypeptides. cDNA was synthesized, using developing tuber poly(A+)-RNA as a template, and inserted into either lambda gt11 or lambda zap. From two consecutive rounds of screening, using the anti-51 kD spinach leaf subunit as a probe, two cDNA clones were isolated. Both clones were shown to be homologous, as evidenced by the presence of common restriction enzyme sites and Southern blot hybridization analysis.

To further identify the nature of these recombinant cDNA clones, Western blot analysis was carried out. The anti-51 kD spinach leaf subunit was affinity-purified using cell lysates containing the fusion protein, and then used to probe a Western blot containing native tuber protein. The affinity-purified anti-51 kD spinach leaf subunit reacted specifically with a high molecular weight protein of about 200,000, whose migration coincided with a band stained for ADPglucose pyrophosphorylase enzyme activity. Despite this immunological evidence that the recombinant cDNA clone encoded at least a segment of ADPglucose pyrophosphorylase, DNA sequence analysis of this cDNA clone revealed it to contain only very short stretches of peptide homology to the primary sequence of the potato subunit and hence did not contain authentic coding sequences for ADPglucose pyrophosphorylase

The lambda zap cDNA library was re-screened using antiserum prepared against the purified potato tuber pyrophosphorylase. Because this antiserum binds to what we have postulated are the two different potato tuber pyrophosphorylase subunits, we hoped to isolate cDNA clones for both subunits. This antiserum, however, cross-reacted with other tuber proteins, although at much lower affinity (See Appendix 3). When screening the cDNA library with this antiserum, care was taken to choose recombinant plaques with a high-intensity radioactive signal to avoid isolating false positives. Twenty-four positives were isolated following three rounds of screening. These clones were also positive at the third round of screening with the anti-51 kD spinach leaf antiserum. These cDNA clones, representing the slightly smaller 50 kD potato tuber subunit were then excised from lambda DNA *in vivo*, according to the manufacturer's protocol, and were shown to be intact by Southern blot hybridization and restriction enzyme mapping. The sizes of the cDNA inserts ranged from 1.25 to 3.0 kb, with several being 1.6 kb. The terminal sequences of 4 of these cDNA clones representing the various size classes were determined, and they all contained sequences at the amino acid level which were very similar to the primary sequence of the rice ADPglucose pyrophosphorylase subunit. The sequence data also showed that all four clones contained the same 5' end, but varied at the 3' end. The largest clones were a result of a cloning artifact at the 3' end, whereas the 1.6 kb clone contained a poly(A) tail at the 3' end. All of these clones were truncated at the same point at the 5' termini. This cDNA library was prepared by protecting the internal EcoRI sites through methylation, ligating linkers containing an EcoRI site to the termini, digesting the cDNA with EcoRI to generate cDNA molecules with EcoRI cohesive ends, followed by ligation to the lambda zap vector. Apparently the internal EcoRI sites were not protected, and digestion of the

pyrophosphorylase cDNA generated recombinant clones truncated at an identical EcoRI site at the 5' end. We were unsuccessful in isolating cDNA clones for the 51 kD subunit. Examination of Western blots containing the tuber pyrophosphorylase indicated that this antiserum bound to both putative subunits with equal affinity (Appendix 3). We are unclear at this point as to the reason for our inability to isolate the slightly larger, more basic 51 kD subunit. One possible explanation is that the transcript for the 51 kD subunit has a very short poly(A) tail. Under these conditions it may have been lost during our selection of poly(A+)-RNA by oligo(dT)-cellulose chromatography and was therefore unrepresented in the cDNA library. Other explanations are that the 51 kD subunit expressed by the recombinant phage is unusually unstable or is not recognized by the potato antiserum because of conformational differences displayed by the fusion protein.

Sequence analysis of these cDNA clones revealed that the primary sequence of the potato 50 kD subunit, rice 50 kD subunit, and spinach leaf 51 kD subunit were almost identical, except at the 5' end which encoded the N-terminus of the mature polypeptide. However, at the DNA level, the wobble base for almost every codon was different between the potato and rice cDNAs. The differences in codon usage was the principal reason why the rice cDNA could not be used as a heterologous probe in cDNA and genomic library screening and Northern blot hybridization, even at low stringent hybridization and wash conditions.

Northern blot hybridization analysis, using the tuber 1.25 kb cDNA clone as a probe, showed that ADPglucose pyrophosphorylase mRNA was present in both leaf and tuber, with low amounts in stolon tissue (Fig. 1). It is not clear at present whether the leaf and tuber transcripts represented the same gene(s) or tissue-specific genes of close similarity. The size of the ADPglucose pyrophosphorylase mRNA was approximately 1800 bases. This indicates that the 1.6 kb cDNA clone is probably missing 200-300 bp at the 5' end and efforts are now directed at obtaining a full length cDNA.

In potato tubers, the central cortex cells accumulate less starch than the outer parenchyma cells (23). In order to determine whether this gradient of starch accumulation was a direct result of ADPglucose pyrophosphorylase mRNA and protein levels, tissue blot studies were initiated. Developing tubers were cut longitudally, blotted onto a nylon membrane and probed with radioactive cDNA probe. The results were negative, most likely due to the very low quantity of mRNA being transferred to the membrane. An alternative approach has been to determine the localization of the enzyme by Western blot (24) using potato tuber antiserum followed by protein-A (I^{125}). Our initial results showed non-specific binding with pre-immune serum and present efforts are directed at reducing the background binding. Additional work has been done to determine if the pyrophosphorylase mRNA levels fluctuate during tuber development. Total RNA fractions, isolated from tubers ranging in weight from 0.68 g to 21.6 g, were separated by formaldehyde-agarose gel electrophoresis, and the pyrophosphorylase mRNA detected by Northern blot hybridization. These results indicate that the ADPglucose pyrophosphorylase mRNA levels remain constant on a total RNA basis throughout tuber development (Fig. 2).

Eighteen genomic clones have been isolated from a *Solanum tuberosum* var. Russet Burbank genomic library provided by David Anderson (Phtytogen). Seven clones have been analyzed by restriction mapping and Southern blot hybridization, and four of these have different physical maps. Fine restriction endonuclease mapping and hybridization using

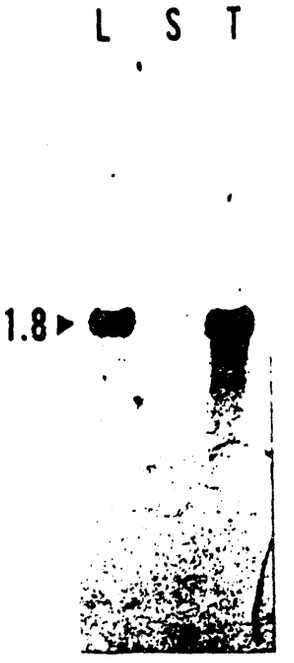


Figure 1. Tissue specific expression of the 50 kD potato ADPglucose pyrophosphorylase 50 kD subunit. Poly (A+) RNA (2 ug) iolsted from leaf (L), stolon (S), and tuber (T) tissue was fractionated on a 1.2 % agarose-formaldehyde gel, transferred to a nylon memebrane (Zetaprobe, BioRad) and hybridized with a potato ADPglucose pyrophosphorylase cDNA clone. The size of the pyrophosphorylase mRNA is approximetly 1.8 kilobases.

Figure 2. Developmental pattern of expression of ADPglucose pyrophosphorylase in developing potato tubers ranging in weight from 37 g to 0.65 g. Total RNA (30 ug) was fractionated on a 1.2 % agarose-formaldehyde gel, transferred onto a nylon memebrane (Zetaprobe, BioRad) and hybridized with a potato ADPglucose pyrophosphorylase cDNA clone. These results indicate that the level of pyrophosphorylase mRNA remains constant throughout tuber development

probes from various parts of the cDNA is currently underway to determine which part of the gene(s) these clones contain.

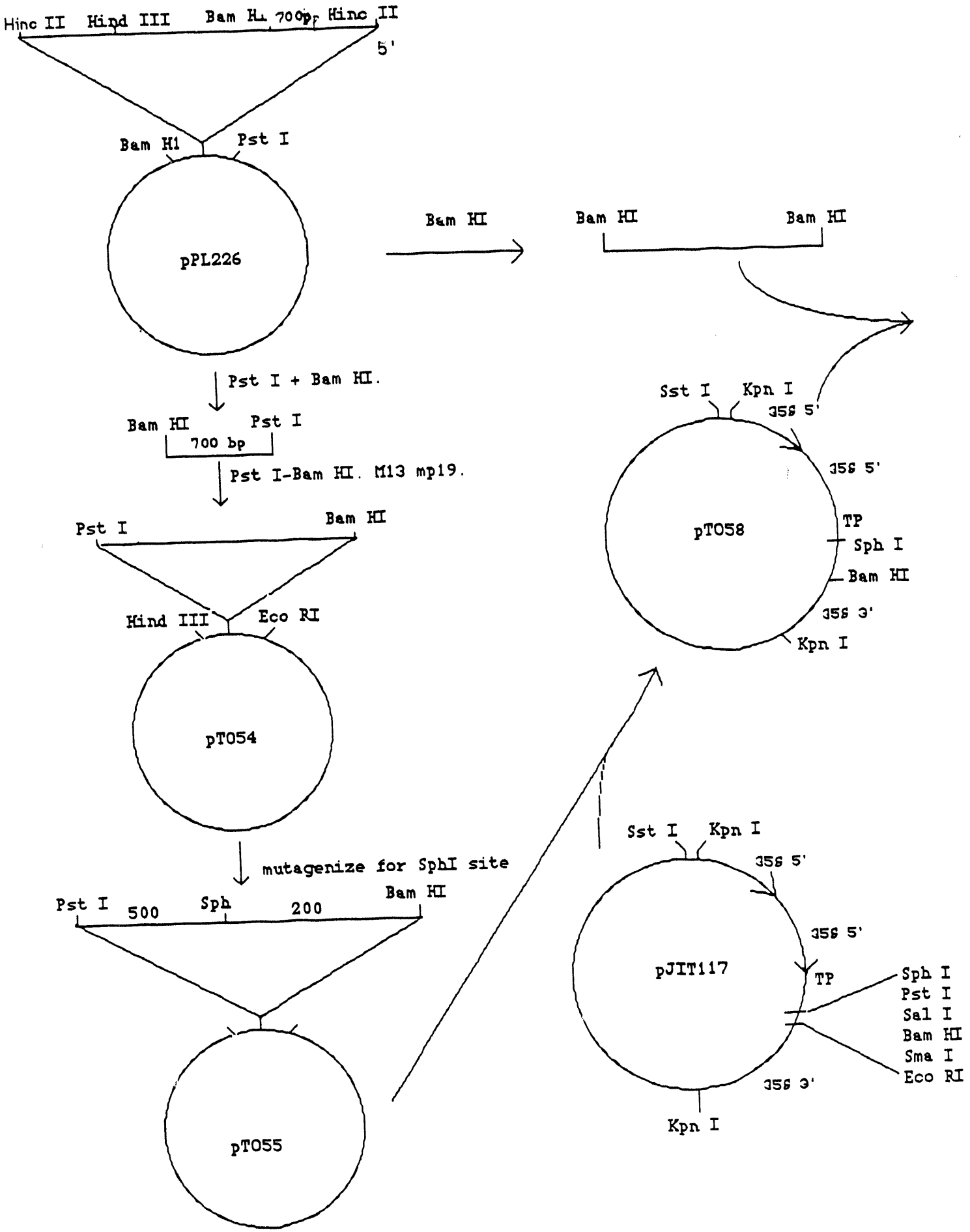
Construction of a Bacterial ADPglucose Pyrophosphorylase Expression Plasmid - In an effort to enhance the flow of carbon into starch via manipulation of the ADPglucose pyrophosphorylase activity, plasmid constructions have been made to express a bacterial gene for ADPglucose pyrophosphorylase in plant cells. In on-going collaborations with Jack Preiss (Michigan State University), our laboratory has received from him plasmid pPL226, which contains a mutated gene for the *E. coli* ADPglucose pyrophosphorylase. The enzyme activity from this mutant strain is abnormally high and does not respond in a normal fashion to allosteric control by small effector molecules, as compared to the wild-type enzyme. Therefore, we believe that a functional expression of this bacterial gene in plant cells may result in the accumulation of an active enzyme refractive to allosteric control which, in turn, may possibly enhance carbon flow into starch. Figure 2 depicts a diagram of the construction steps used to allow expression of the bacterial gene, using the appropriate plant transcriptional regulatory and intracellular targeting signals. To facilitate these constructions, pJIT 117 (obtained from J.F. Guerineau of the John Innes Institute, ref. 25) was utilized. This plasmid contains a double CaMV 35S promoter segment for enhanced transcription, a coding segment for the transit peptide for the small subunit of Rubisco to target polypeptides into the plastid organelle, multiple cloning sites from pUC9, and a CaMV polyadenylation signal. By taking advantage of the unique cloning sites, presumably any coding segment can be expressed in plant cells and localized within the plastid.

The bacterial gene lies within a HincII fragment which was cloned into pUC8. This HincII fragment contains a portion of the 3' end of *glgX* (amylase), an intergenic region, *glgC* (ADPglucose pyrophosphorylase) gene, and a portion of the *glgA* (glycogen synthase) gene. To facilitate the construction of an in-frame translational fusion of the bacterial enzyme to the small subunit transit peptide, site-specific mutagenesis experiments were carried out. The 700 bp PstI-BamHI fragment from pPL226 was sub-cloned into M13 mp19, and the resulting recombinant phage propagated with the *dut*$^-$ *ung*$^-$ strain CJ236. The uracil-containing single-stranded DNA was then annealed to a synthetic oligonucleotide, as shown below.

pyro sequence AAAAGGAGTTAGTC**ATG**GTTAGTTTAGAGAAGAACG

oligo TTTTCCTCAATCCG**TAC**GAATCAAATCTCTTCTTGC

Two point mutations were inserted into the bacterial sequence at position -2 and +4 from the initiator codon (bold letters), resulting in the production of a unique SphI site. The +4 point mutation also resulted in an amino acid substitution in the bacterial sequence from a Val to Leu. This amino acid replacement, however, is a neutral mutation with regard to polarity and net charge and should have little, if any, effect on enzyme assembly and function. Isolation of the 200 bp SphI-BamHI fragment from pTO55 and insertion into pJIT117 yielded pTO58, a plasmid containing a translational fusion between the transit peptide of the Rubisco small subunit and N-terminal region of the bacterial ADPglucose pyrophosphorylase. The 1100 bp Bam HI fragment encoding the C-terminal region of the bacterial gene was then cloned into pTO58 to give pTO59, a construct capable of expressing the bacterial ADPglucose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Hind III
Bam HI
700bp
Hinc II
5'
Bam HI
Pst I
pPL226
Bam HI
Bam HI
Bam HI
Pst I + Bam HI.
Bam HI
Pst I
700 bp
Pst I-Bam HI. M13 mp19.
Pst I
Bam HI
Hind III
Eco RI
pTO54
Sst I
Kpn I
35S 5'
35S 5'
pTO58
TP
Sph I
Bam HI
35S 3'
Kpn I
mutagenize for SphI site
Pst I
500
Sph
200
Bam HI
pTO55
Sst I
Kpn I
35S 5'
35S 5'
pJIT117
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Sal I
Bam HI
Sma I
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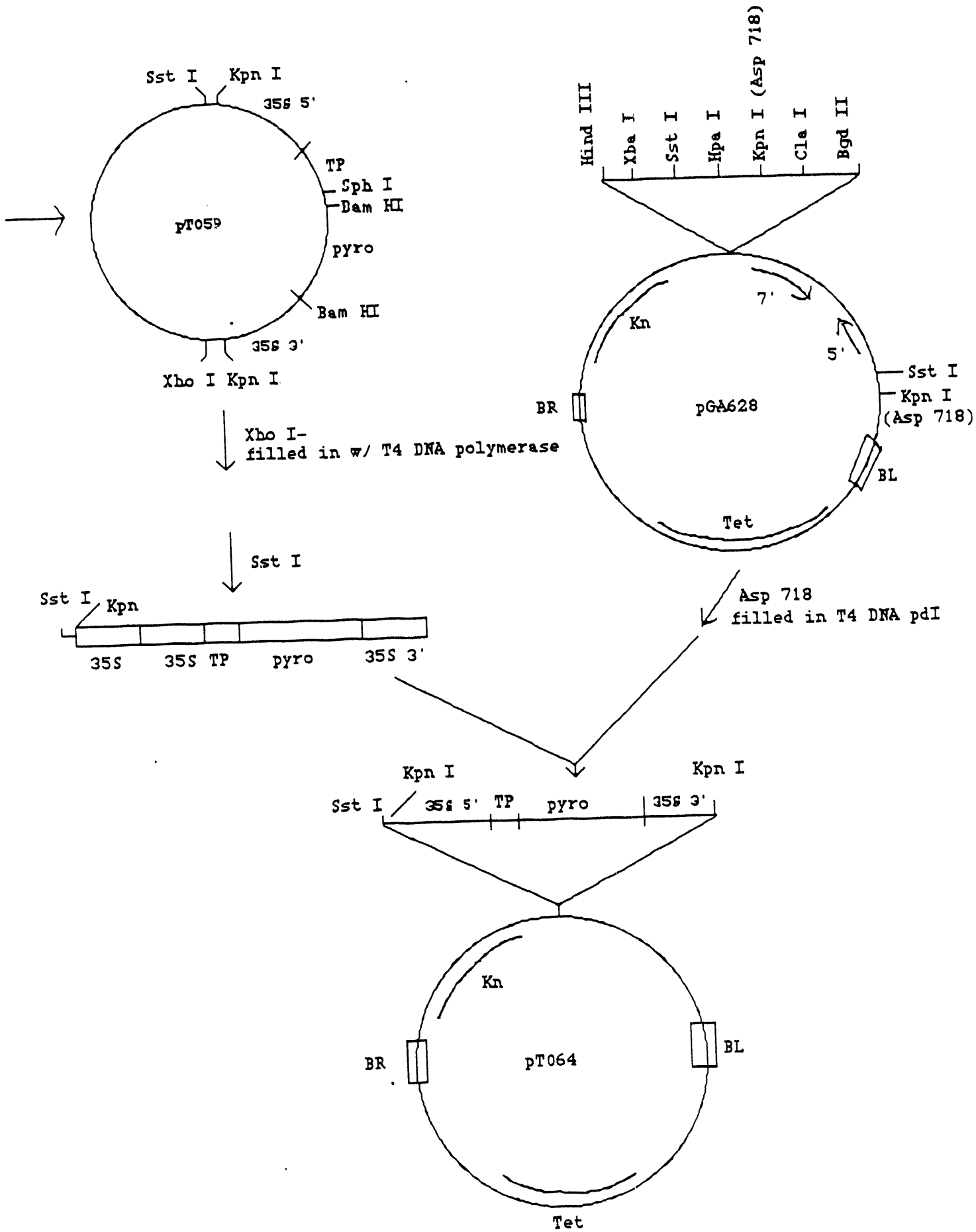
Kpn I


Sst I
Kpn I
35S 5'
TP
Sph I
Dam HI
pyro
Bam HI
35S 3'
Xho I Kpn I
pT059
Xho I-
filled in w/ T4 DNA polymerase
Sst I
Sst I
Kpn
35S
35S TP
pyro
35S 3'
Hind III
Xba I
Sst I
Hpa I
Kpn I (Asp 718)
Cla I
Bgd II
Kn
7'
5'
BR
pGA628
Sst I
Kpn I
(Asp 718)
BL
Tet
Asp 718
filled in T4 DNA pdI
Kpn I
Kpn I
Sst I
35S 5'
TP
pyro
35S 3'
Kn
BR
pT064
BL
Tet

pyrophosphorylase gene under the control of the double CaMV 35S promoters. Relevant portions of pTO59 were then inserted into pGA628 to yield pTO64, a T-DNA vector for *Agrobacterium tumefaciens*-mediated transformation of plant cells. At present, we have transformed tobacco with pTO64, and both transgenic callus tissue and regenerating shoots have been obtained. In the next 6 to 8 weeks, sufficient transgenic tissue will be obtained for analysis of bacterial ADPglucose pyrophosphorylase enzyme activity. With the assistance of Professor Clarence Ryan's laboratory, efforts are directed at transferring this DNA construct into potato and tomato.

INTRODUCTION

Most plant species store at least a portion of their photosynthetic assimilates as starch. In numerous cases, this macromolecule represents the principal energy reserve of plant storage organs. The production of starch is metabolically controlled by the activity of ADPglucose pyrophosphorylase, an allosterically-regulated enzyme which catalyzes the first committed step in the starch biosynthetic pathway. Although much is known about the enzymatic properties of this central enzyme, virtually nothing is known on why the plant ADPglucose pyrophosphorylase, unlike the bacterial enzyme, is composed of two distinct subunits and the molecular mechanisms by which the developmental, hormonal and environmental control of ADPglucose pyrophosphorylase genes are effected. Our research program is just beginning to provide information on the structure of the tuber ADPglucose pyrophosphorylase and the nature of the pyrophosphorylase gene, which may be used in efforts to enhance photosynthate into starch and, hence, improve overall plant productivity. The support requested in this proposal will allow us to continue these studies and will permit an investigation of the structure-function relationships of the potato tuber enzyme, and the identification of *cis*-acting regulatory sequences of the tuber ADPglucose pyrophosphorylase genes by *in vivo* plant transformation techniques. Moreover,it will provide information on the possible consequences in carbon metabolism and growth patterns of transgenic plants expressing an altered bacterial ADPglucose pyrophosphorylase gene.

LITERATURE REVIEW

Higher plants accumulate starch primarily in leaf and storage tissues. Leaf starch is transitory, being synthesized during the active periods of photosynthesis and catabolized to varying extents during the night. In storage tissue, on the other hand, the synthesis and degradation of starch occurs at specific stages of plant ontogeny. Irrespective of the site of deposition, starch normally consists of two components: amylose, a linear chain of α-1,4-linked glucosyl residues; and amylopectin, a branched polymer composed of both α-1,4- and α-1,6-linked residues. The linear chains of amylose are the result of starch synthase activity, whereas the branched points of amylopectin are generated by branching enzyme. Despite the simplicity of the above biochemical reactions, the mechanisms responsible for the unique morphology of the starch granules displayed by various plant species remain largely unknown (23).

Much of the information regarding the enzymology of starch biosynthesis has been gathered from studies of maize endosperm and spinach leaf tissue (4,7,8,16,26-38). Preiss and his associates (26,29,34-36) have shown based on extensive biochemical and immunological

evidence, that both starch synthase and branching enzyme exist as multiple activities in starch-bound and soluble forms. Both the bound and soluble starch synthases can be divided into two additional isozymic classes I and II, while branching enzyme (BE) can be resolved into enzyme activities BE I, BE IIa and BE IIb.

Despite the complexity of multiple starch biosynthetic enzymes, the assignment of specific functional roles for some of these activities is just beginning to emerge from biochemical analysis of maize endosperm starch mutants (30). *Waxy* maize endosperms are deficient in amylose, presumably due to a biochemical defect in bound starch synthases I and II (26,29-32). Likewise, analysis of *amyloextender* mutants containing a higher proportion than normal of amylose chains suggests a defect in BE II activities, particularly BE IIb (26,34,38). In contrast, the biochemical lesion in *dull* mutants, which also accumulate a higher level of amylose, appears to affect starch synthase II activity rather than branching enzyme (26).

Although variation in the isozymic forms of starch synthase and branching enzyme is an important factor in generating the unique morphologies of starch grains, other factors and enzymatic activities undoubtedly play a role. Pan and Nelson (39) have shown that a deficiency in debranching enzyme activity (α-1,6-glucosidase) was the primary lesion in *sugary* endosperms. The structure of amylopectin which contains asymmetric A and B chains is, therefore, the product of an equilibrium between branching and debranching enzyme activities. In light of this proposed function for an enzyme normally assigned a degradative role in starch metabolism, it is entirely possible that other enzymes (e.g., amylase, phosphorylases, etc.) and proteins, as well as other constituents (e.g., lipid), may also influence the overall architecture of the starch grain.

The Regulation of Starch Biosynthesis - Starch synthesis is controlled by the allosteric behavior of ADPglucose pyrophosphorylase, an enzyme which catalyzes the key regulatory step in this process (reviewed in ref. 26). ADPglucose pyrophosphorylase is subject to allosteric control by small effector molecules; it is allosterically activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Pi). Other glycolytic intermediates are also found to stimulate the pyrophosphorylase activity, but are not as effective as 3-PGA, although their total concentration may contribute to the allosteric activation of this enzyme (40). The spinach leaf enzyme has been extensively characterized by the Preiss laboratory, although isolates of this enzyme from other plant and algal species show similar responses of activation and inhibition (26,41-44). The presence of 3-PGA as low as 20 μM results in a 50% increase in pyrophosphorylase activity and, just as important, reduces the Km for its substrates by 2- to 10-fold. In the absence of activator, the spinach leaf pyrophosphorylase is extremely sensitive to Pi, with concentrations of 22 μM resulting in 50% inhibition of enzyme activity. In the presence of 1 mM 3-PGA, a 50% reduction of ADPglucose synthesis requires the presence of at least 1.5 mM Pi. It is therefore evident that the presence of the activator, 3-PGA, antagonizes Pi-mediated inhibition (4,26,41).

In contrast to the extensive studies on the leaf ADPglucose pyrophosphorylases, we know less about the physical and kinetic properties of this enzyme from storage tissues (i.e., seeds and tubers). Early observations of the enzymes from maize endosperm (42,45) and potato tuber (46-48) indicated that although ADPglucose pyrophosphorylase was

found to be allosterically-activated by 3-PGA and inhibited by Pi, the response to these effectors was not as large as that seen in leaf tissue. Maximal activation of these enzymes was observed at rather high concentrations of 3-PGA (2-10 mM), with only a 3- to 4-fold activation. Recent studies from the Preiss laboratory indicate that ADPglucose pyrophosphorylases from non-photosynthetic tissues are less stable than their counterparts in leaf tissue and, when appropriate precautions are used, the enzymes are fully responsive to allosteric regulation *in vitro*. Plaxton *et al.* (49) have shown that the endosperm-specific enzyme from maize tissue is highly sensitive to proteolysis, resulting in an enzyme less responsive to allosteric activation by 3-PGA. Inclusion of protease inhibitors during the isolation of this enzyme was shown to preserve the integrity of the enzyme and maintain allosteric control to an extent evident for the leaf-specific enzyme. Similarly, Sowokinos and Preiss (16) showed an absolute dependency on 3-PGA for ADPglucose synthesis by the purified potato tuber enzyme. An approximate 32-fold activation was detected with 3 mM 3-PGA, a response nearly identical to that detected with the enzyme from leaf tissue (24). This response was only achieved, however, when dithiothreitol (3 mM) was included in the assay.

Although ADPglucose pyrophosphorylases from leaf and non-photosynthetic tissue show similar responses to allosteric activation and inhibition by small effector molecules, an important difference in catalytic properties between these enzymes is their turnover rate for ADPglucose synthesis. The purified spinach leaf enzyme has a specific activity of about 150 units/mg protein (4), whereas the highly purified potato tuber enzyme is about 1/3 of this value (50). The lower specific activity of the tuber enzyme may be compensated for by the overall amounts of this enzyme during tuberization. Indeed, the most important aspect of starch biosynthesis in storage tissues such as potato tubers (46), maize seeds (51) and wheat seeds (52,53) may be the regulation of synthesis of the starch biosynthetic enzymes, including ADPglucose pyrophosphorylase. In most of these studies, ADPglucose pyrophosphorylase activity or antigen level increased in correlation with the amount of starch or dry weight during the early stages of development.

Several lines of evidence support the regulation of ADPglucose pyrophosphorylase as the dominant control of starch biosynthesis. Many of these studies have evaluated the effect of Pi levels on the rate of starch biosynthesis. Heber and his associates (54) have shown that the concentration of Pi in leaf chloroplasts ranges from 5-10 mM and decreases by 30-50% in the light. Assuming that the 3-PGA concentration remains unchanged at 5 mM under these light-dark conditions, *in vitro* kinetic data indicates that the rate of ADPglucose synthesis would increase 5- to 23-fold. Since other glycolytic intermediates and ATP undoubtedly increase during photosynthesis (70), the rate of ADPglucose synthesis is probably much higher. More recent studies (18,55,56) also support the role of Pi in regulating starch biosynthesis. Steup *et al.* (55) have demonstrated that low concentrations of Pi have little effect on the rate of CO_2 fixation in illuminated spinach leaf chloroplasts, whereas higher concentrations (1 mM) not only induced a long lag in starch biosynthesis, but also suppressed the maximum rate attained. Similar studies by the laboratories of Heldt, Heber and Walker (18) revealed that starch synthesis in illuminated chloroplasts was inhibited by 1 mM Pi, a phenomenon which could be reversed by the addition of 3-PGA in the medium. Through measurement of metabolite concentrations under such experimental conditions, these workers were able to show that the

rate of CO_2 fixation into starch corresponded directly with the ratio of 3-PGA:Pi in the stroma and, more importantly, agreed very well with the *in vitro* activity of ADPglucose pyrophosphorylase at the different 3-PGA:Pi ratios (4). The role of Pi in regulating starch biosynthesis via ADPglucose pyrophosphorylase can also be inferred from studies on starch accumulation in wheat leaves infected by *Puccinia striiformis* (57) and mannose feeding of leaf discs (20,21). In both instances, the sequestration of Pi by the pathogen (for utilization for its own metabolic needs), or through the formation of an unmetabolizable end product (mannose 1-P), resulted in elevated levels of starch biosynthesis. The overwhelming evidence supports the hypothesis that the allosteric behavior of ADPglucose pyrophosphorylase responds to fluctuating levels of 3-PGA:Pi *in vivo* and plays a dominant role in the regulation of starch biosynthesis.

The Structure of Higher Plant ADPglucose Pyrophosphorylases - Extensive genetic and biochemical studies from the Preiss laboratory have demonstrated that the bacterial ADPglucose pyrophosphorylase from both *Escherchia coli* and *Salmonella typhimurium* is encoded by a single gene locus, *glg*C (15). This gene encodes an ADPglucose pyrophosphorylase subunit of 48 kD, which aggregates to form a homotetramer of 200 kD. The higher plant enzyme was thought to have a structure similar to the bacterial enzyme, as the molecular weight of the holoenzyme was first estimated to be about 200 kD (58). A subsequent study by Copeland and Preiss (14), who purified the spinach leaf enzyme to apparent homogeneity, showed that under denaturing conditions, subunits of 54 kD and 51 kD at equimolar amounts were observed (4). It was not clear at that time whether the two subunits represented distinct polypeptides, or whether the smaller species was simply a product of proteolysis. Both subunits appear to be widely distributed in plants, as Western blot studies of maize, wheat, rice (52) and *Arabidopsis* (59-60) leaf extracts showed immuno-reactive bands co-migrating with the 54 kD and 51 kD subunits. More recent studies have now demonstrated that these subunits are non-identical, and are products of two different genes. Morell *et al.* (4) were able to purify both subunits and showed that these subunits could be distinguished on the basis of their N-terminal amino acid sequences, peptide mapping patterns, and cross-reactivity to different polyclonal antibodies to ADPglucose pyrophosphorylase. Moreover, mutants deficient in ADPglucose pyrophosphorylase have been obtained in *Arabidopsis* (59,60). In one mutant, *adg*2, leaf extracts only contained about 5% of the wild-type levels of ADPglucose pyrophosphorylase and were deficient in the 54 kD subunit when analyzed by Western blot analysis (60). A second mutation, *adg*1, affects the levels of both subunits, and genetic analysis suggests that the defective locus may be of a regulatory nature (59). Biochemical and genetic evidence indicates that the leaf-specific ADPglucose pyrophosphorylase from higher plants is composed of two distinct subunits, and both are required for maximum catalytic activity.

The structure of the enzyme from non-photosynthetic tissues is less clear, although evidence is accumulating that it is encoded by two distinct genes. Sowokinos and Preiss (16) showed that the purified ADPglucose pyrophosphorylase from tubers appeared to be composed of a single subunit of about 50 kD as viewed by single dimensional SDS polyacrylamide gel electrophoresis. More detailed analysis from my laboratory, however, suggests a more complicated structure. Two groups of isoforms of nearly identical molecular weight are evident when the

homogeneous purified tuber enzyme is analyzed on two-dimensional polyacrylamide gels. Only the smaller 50 kD isoforms cross-react with the spinach leaf anti-51 kD subunit, indicating that at least portions of the primary structure of these subunits have been conserved. Less clear is the nature of the slightly larger, more basic 51 kD isoforms of the tuber enzyme, as these polypeptide species did not cross-react with the spinach leaf anti-54 kd. A role for the tuber 51 kD subunits is thus circumstantial at this time, and studies proposed in the next granting period are directed at defining the structure of the tuber ADPglucose pyrophosphorylase.

Genetic and biochemical evidence in maize support the hypothesis that the endosperm-specific ADPglucose pyrophosphorylase is composed of two distinct subunits and products of unique genes. Two mutations, *Brittle* 2 and *Shrunken* 2, have been isolated which are defective in ADPglucose pyrophosphorylase activity and starch production (61-66). Putative cDNA clones have been isolated by differential screening methods and purported to encode polypeptides of 60 kD and 54 kD for *Shrunken* 2 and *Brittle* 2, respectively (Carolyn Barton, personal communication). Preliminary biochemical and molecular biological evidence support the assignment of these sized subunits to be components of the maize endosperm enzyme. Western blot analysis of the purified enzyme fraction with heterologous antibodies revealed that a prominent 54 kD polypeptide evident on polyacrylamide gels by Coomassie blue staining cross-reacts with the spinach leaf anti-51 kD subunit (49). Homology of the maize endosperm 54 kD polypeptide to the spinach leaf 51 kD subunit was confirmed by the ability of their respective cDNA clones to cross-hybridize (Barton and Preiss, personal communication). Moreover, slight cross-reactivity was also evident of the maize endosperm 60 kD polypeptide to the spinach leaf anti-54 kD subunit (26, Danner and Preiss, personal communication). The overall evidence suggests that the maize endosperm enzyme, like the leaf tissue-specific forms, is composed of two unique subunits, the smaller subunit displaying stronger conservation to the leaf 51 kD subunit than the larger molecular weight subunit.

Despite the wealth of information on the physical and kinetic properties of ADPglucose pyrophosphorylases, particularly from leaf tissue, very little is known about the molecular aspects of the genes that code for these proteins. Information on the nature of the pyrophosphorylase genes, however, is just beginning to unfold. Using antibodies raised against the spinach leaf ADPglucose pyrophosphorylase (a gift from Dr. Jack Preiss, Michigan State University), we were able to show that tissue-specific forms of this enzyme existed in several different plant species (52). SDS polyacrylamide gel electrophoresis of immunoprecipitated products from *in vitro* translation experiments revealed that the leaf subunits were synthesized as larger precursors of 73 and 76 kD, suggesting an additional leader sequence to facilitate their transport into the plastid (52). In contrast, endosperm poly(A+) RNA directed the synthesis of 56 and 50 kD subunits, with presumably the former representing the precursor form. The 50 kD polypeptide may have resulted from partial processing in the protein synthesizing extracts employed in this study. Construction of a cDNA library in λgt 11 (67) and screening by immunological methods (68) resulted in the isolation of several recombinant clones. Northern blot studies showed that the leaf ADPglucose pyrophosphorylase transcript was about 2,100 bases in length, while the endosperm species were about 300 bases shorter (52). It is not known, however, whether these mRNAs are products of different genes, or

products formed by different post-transcriptional processing events under developmental control. However, Southern blot reconstruction experiments suggest that at least 3 different genes are present in rice DNA, which may account for the different tissue-specific forms.

The protein structure of one of the rice endosperm subunits has been determined, as inferred from its cDNA sequence (8,69). The primary sequence shows a leader sequence on the N-terminus, presumably capable of targeting this protein to the amyloplast. The mature protein displays significant homology to the bacterial enzyme indicating that genes which encode these proteins have remained conserved. The structure of a genomic clone for this pyrophosphorylase subunit has also been elucidated. The gene is highly complex, as the coding sequences are present over 10 exons. The first intron is quite large and is over 1400 base pairs in length (70). It is interesting to note that the *Shrunken* locus of maize (71), which encodes sucrose synthase, also has a similar gene structure composed of multiple introns. As evident for the ADPglucose pyrophosphorylase gene, the first intron of *Shrunken* is unusually large for a plant gene and spans over 1040 base pairs in length. The presence of multiple introns in these starch biosynthetic genes, including *waxy* (72), differs considerably from the major storage protein genes found in cereals (reviewed in ref. 73) which have either few introns (e.g., 3 introns in the case for the rice glutelin), or lack this structural unit (e.g. maize zein or rice prolamine). Whether the presence of multiple introns plays a role in overall expression of these starch biosynthetic genes, as observed for the maize alcohol dehydrogenase (74), remains to be explored.

Alignment of the primary sequence of the rice ADPglucose pyrophosphorylase subunit to the bacterial enzyme suggests possible structure-function relationships of the plant enzyme (69). Both the bacterial and plant enzymes catalyze the same reaction, but are controlled by different effector molecules. The bacterial enzyme is activated specifically by fructose 1,6-bisphosphate and inhibited by AMP (15), whereas the plant enzyme, as previously mentioned, is controlled by 3-PGA to Pi levels (26). Such differences in the nature of the effector molecules may be reflected in the primary sequences of these proteins. As discussed in more detail in the Progress Report section of this proposal, we hypothesized that the differences in allosteric specificity between the plant and bacterial enzyme may be due to specific point mutations to acidic residues and extension of the C-terminal end of the plant enzyme.

The homology to the bacterial enzyme is presently limited to the smaller subunit of the plant ADPglucose pyrophosphorylase. Evidence, however, has been obtained to support a role for the larger subunit in enzyme function as it is specifically labeled with pyridoxal phosphate (8,75,76,79) and the substrate analog 8-azido-ATP (77). The presence of dissimilar subunits suggests additional functional and/or cellular features of the plant ADPglucose pyrophosphorylase, presently unknown and not required for the function of the procaryotic enzyme. The plant enzyme presumably exists freely in the stroma compartment (17,18) but immunocytochemical studies suggest that at least a portion of this enzyme lies at discrete regions on the starch grain (78). Moreover, the smaller unit is specifically phosphorylated by both a purified soybean protein kinase activity (79) and endogenous activities present in seed and leaf extracts (Krishnan and Okita, unpublished observations). The significance of these observations is not at all clear and further studies on the primary structures of these subunits is clearly needed.

SIGNIFICANCE AND OBJECTIVES

Many agronomically important crop plants are viewed as potential resources of renewable energy and, hence, improvements in crop yield remain an important consideration in this endeavor. For many crop plants, however, improvements in agricultural technology and the application of greater amounts of mineral nutrients no longer result in cost-effective enhancement of crop yield. The optimization of future crop yields will in all likelihood result from the genetic improvement of existing cultivars through increased efficiency of photosynthate yield and buttressing of the plant's defenses against pest and pathogens.

Because relatively minor increases in the efficiency of photosynthesis can result in dramatically increased crop yields, much current effort is directed toward an enhancement of photosynthesis. The bulk of this research has focused primarily on the photosynthetic organs and the photochemical-biochemical events that lead to carbon dioxide fixation (80). Some success has been achieved through the selective breeding of plants with an increased canopy area; however, the additional energy and nutrient investment (primarily in terms of nitrogen) required to support such structures may seriously limit the efficacy of these plants in terms of crop yield. Genetic alteration of the photosynthetic apparatus, and of ribulose bisphosphate carboxylase, remain attractive possibilities (80).

A second area of study through which the efficiency of photosynthesis could be increased is through the enhancement of photosynthate storage capacity. Dry-weight production in potato tubers and other storage plant organs is limited by a number of complex physiological processes, including net photosynthesis, as discussed above, translocation, unloading and sugar utilization in the synthesis of proteins, lipids and starch. In very general terms, plant productivity in storage organs is regulated by the rate and duration of carbon fixation in the leaf tissue (source) and the capacity of the developing storage tissue (sink) to assimilate photosynthate (80,81-83). Under environmental conditions where photosynthesis is not limiting, overall productivity appears to be regulated by the sink strength (i.e., the efficiency to utilize photosynthate in dry weight production). For example, the removal of storage organs from wheat (84), cotton (85) and potato (86) induces a general depression of photosynthetic activity in the leaves, whereas expansion of the sink tissue (by root-top grafts) stimulates photosynthesis (87). It is, therefore, likely that overall productivity could be enhanced if the plant possessed a greater capacity to store fixed carbon through increasing the efficiency of photosynthate utilization.

In potato plants, the major storage form of photosynthate is starch, which can represent over 70% of the storage organ dry weight (23). Although the rate of starch formation can be limited by the physiological processes of loading, transport and unloading of assimilate (88) as well as its conversion into glycolytic intermediates (89), it is definitely controlled by the catalytic activity of ADPglucose pyrophosphorylase (26). This enzyme, however, is allosterically-regulated by 3-PGA:Pi ratios. *In vitro* kinetic data of the potato tuber pyrophosphorylase indicates that at equal concentrations of 3-PGA and Pi (levels typically seen in almost all systems so far studied), the rate of ADPglucose formation would be only 1/5 as that in the absence of Pi (48). Moreover, our mannose feeding experiments with tuber discs support a role for Pi

levels in controlling starch synthesis presumably by influencing ADPglucose pyrophsophorylase actvity (90). Maximal carbon flow into starch has not reached its highest potential in the tuber. Because of the pivotal role of this enzyme in starch biosynthesis and the multiple levels at which this enzyme and catalytic activity is regulated, ADPglucose pyrophosphorylase is a prime target for the enhancement of photosynthate sink storage strength in potatoes. Furthermore, because the employment of the potato plant has certain advantages (e.g., amenable to transformation by *Agrobacterium* and plant regeneration), it could thus serve as a model system for the engineering of other crop plants that accumulate starch as a major constituent in their storage tissues.

In light of the above discussion, the sink strength of the potato tuber can potentially be increased through: (1) elevation of ADPglucose pyrophosphorylase gene expression; (2) introduction of a pyrophosphorylase gene that encodes for a more catalytically-active enzyme; and/or (3) introduction of a pyrophosphorylase gene that encodes an enzyme refractive to Pi inhibition. All of these approaches, however, require fundamental understanding of the structure of the ADPglucose pyrophosphorylase genes and how these genes may be controlled during tuber development, a process influenced by hormones and diverse environmental conditions (106-125). Moreover, the structural differences displayed by the plant and bacterial enzyme infer properties of the plant enzyme yet to be identified which may play a role in enzyme function. Clearly, information on the structure-function relationships of the plant enzyme and developmental regulation of this gene is required before rationale attempts to manipulate carbon metabolism in plants can be initiated. Therefore, to fill this gap in our knowledge on the biochemical and molecular aspects of ADPglucose pyrophosphorylase, we propose the following specific objectives:

1. Biochemical analysis of the tuber ADPglucose pyrophosphorylase enzyme structure.
2. Isolation and structural elucidation of the potato ADPglucose pyrophosphorylase genes.
3. Identification of regulatory elements controlling ADPglucose pyrophosphorylase gene expression.
4. Analysis of the bacterial ADPglucose pyrophosphorylase gene expression in transgenic plants.

PROPOSED RESEARCH

Objective 1. Biochemical Analysis of the Tuber ADPglucose Pyrophosphorylase Enzyme Structure.

Analysis of the purified ADPglucose pyrophosphorylase by two-dimensional polyacrylamide gel electrophoresis indicated the presence of two types of polypeptides, which differ only slightly in net charge and molecular weight (50). Both types of polypeptides react with antiserum prepared against the purified tuber enzyme, but only the smaller 50 kD subunit cross-reacted with the spinach leaf anti-51 kD subunit. The cross-reactivity indicates that this subunit is a component of the ADPglucose pyrophosphorylase, and this has been verified by the available DNA sequence data of a cDNA clone for this tuber subunit. Less clear is whether the more basic, slightly larger 51 kD polypeptides are essential components of the tuber enzyme, and what role these subunits may have in enzyme function.

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