GENETIC ENGINEERING, PLANTS

1. Introduction

Several discoveries in the 1980s and 1990s permitted the transition of plant molecular biology from a fledgling science to commercial reality. These discoveries ranged from the identification of biologically important genes to the development of methods to introduce new genes into plants and regulate gene

expression. The former process is commonly referred to as transformation. Nearly five dozen plant species have been transformed and the list of plant species subject to transformation include principal field crops such as corn, cotton (qv), rape, rice, soybean, and wheat (see SOYBEANS AND OTHER OILSEEDS; WHEAT AND OTHER CEREAL GRAINS). In addition, several horticultural species such as tomato, potato, petunia, chrysanthemum, apple, walnut, melons, etc, have been subject to transformation. More than 500 field tests have been conducted and transgenic plants such as transgenic tomato, soybean, corn, rape, potato, petunia, melons, and cucumbers are in the advanced stages of commercial development and regulatory process.

Four methods have been extensively investigated for the introduction of *transferred deoxyribonucleic acid (T-DNA)* into plants. These include agrobacterium mediated T-DNA transfer (1,2), direct uptake of DNA by protoplasts (3), particle acceleration techniques such as electrostatic discharge or biolistics gun technology (4–7), and DNA uptake into partially digested immature embryos (8). By far the most commonly used method for gene introduction into dicotyledonous plants is the agrobacterium technology. This bacterium delivers genes contained in the T-DNA region of the Ti plasmid to the nucleus of several dicotyledonous species. Within the nucleus, the T-DNA is randomly inserted into the chromosome of the recipient cell. The clonal progenies of the cell containing the inserted gene show a high degree of stability. The gene is transmitted in a Mendelian fashion during sexual stages of cell division and development (1,2,9).

Although agrobacterium mediated gene introduction into plants is highly efficient and routinely used, its primary limitation is that several plant species are recalcitrant to transformation via this bacterium. This is particularly so for monocotyledonous species such as corn, rice, and wheat. In these instances, particle gun technology is routinely used for the introduction of genes. Whereas most genes introduced into plants via the gun technology appear to be nuclear localized, this technology also has been reported to be useful in transforming the chloroplast of plant cells (10). Several reviews documenting the progress in plant transformation during the early 1990s are available (6,11,12).

Expression of genes that have been introduced into plants is regulated by promoters, although the extent of regulation of gene activity by the promoter is influenced at least to some extent by the insertion site of the gene within the chromosome. As of this writing methods for DNA transfer cause random insertion of the DNA into the chromosome. Techniques for precise introduction of the transgene to specific sites with the plant genome are being developed. Numerous promoters have been used for gene expression in plants. The choice of promoters is dictated by the tissue and developmental specificity required for gene expression. By far the most commonly used promoter for constitutive gene expression in both mono- and dicotyledonous plants is the Cauliflower mosaic virus (CaMV) 35S promoter. This promoter appears to be expressed in several plant organs and cell types; however, it is not truly constitutive in that it is not uniformly expressed in all plant tissues. DNA elements within the 35S promoter, which cause tissue specific expression of genes, have been described (13,14). The activity of the 35S promoter may be enhanced by use of multiple copies of enhancer elements located within the 35S promoter (15).

For tissue regulated gene expression, promoters have been described which are expressed in a tissue specific manner (16). Examples of such promoters include the promoter for patatin which causes tuber specific expression of genes in potato (17–20), the 7S promoter of soybean (21), or the napin promoter of *Brassica* (22), which cause seed specific expression of genes, and the RB7 promoter which causes root specific expression of genes (23). These promoters may not only be spatially regulated in terms of cell and tissue specificity but may also be temporally regulated in that the promoters are active only at certain developmental stages of the cells and tissues in which the promoters are expressed.

In order to determine which plant cells have been transformed, selectable marker genes are introduced during transformation. These marker genes permit selective growth of transgenic cells on the medium used for tissue propagation whereas the nontransgenic cells are killed. Examples of selectable marker genes include antibiotic resistance genes such as neomycin phosphotransferase (NPT-II), hygromycin phosphotransferase, and chloramphenicol acetyl transferase, as well as herbicide resistance genes such as phosphinothricin acetyl transferase, bromoxynil nitrilase, 2,4-D-oxygenase, etc. Plant cells expressing the NPTII gene are able to survive kanamycin [8063-07-8] and addition of kanamycin permits selection of those cells receiving and expressing the NPTII gene during transformation (see ANTIBIOTICS; HERBICIDES).

Herein two specific applications of plant biotechnology are discussed. The first is concerned with 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), the enzyme which is the target for the widely used herbicide glyphosate [1071-83-6], $C_3H_8NO_5P$. The second is directed toward a discussion of increasing starch biosynthesis in plants. The first application deals with a trait which directly impacts the farmer during the production phase of agriculture; the second application deals with a trait that impacts the consumer of agricultural products. These traits may be referred to as agronomic and quality traits, respectively.

A number of other agronomic and quality traits are being investigated. These include insect, virus, disease, and nematode resistance, fertilizer-use efficiency, ripening control, fruit firmness, etc. Of these traits the most advanced agronomic trait for bioengineering is insect resistance. Insect resistant cotton and corn have been obtained by introduction and expression of a *Bacillus thurigiensis* kurastaki gene (BtK gene) (24). The BtK protein encoded by this gene is selectively toxic to the lepidopteran pests, ie, cotton boll worm, pink boll worm, and European corn borer, which attack these crops. Insect-resistant potato has been obtained by expression of a BtT gene which encodes a protein, selectively toxic to the Colorado potato beetle, a principal pest of potato (25,26). This topic has been reviewed (26–28) (see also INSECT CONTROL TECHNOLOGY). Virus resistance, conferred by expression of the viral coat protein (CP) gene in transgenic plants, has also received considerable attention. Products such as potato, squash, melons, etc, based on this technology are in advanced stages of development and commercialization (29).

Both tomato fruit ripening and fruit firmness are among the advanced quality traits that are being investigated. A variety of approaches, based on inhibition of ethylene production (30,31) are being pursued for enhancement of shelf life of tomato. For enhancing fruit firmness, cell wall hydrolytic enzymes such as polygalacturonidase and pectin methylesterase are being investigated (32–34).

2. Bioengineering of Glyphosate Tolerance

The enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase catalyzes the transfer of a carboxyvinyl moiety of phosphoenol pyruvate (PEP) to shikimate 3-phosphate (S3P), yielding inorganic phosphate and EPSP as reaction products. EPSPS has received considerable attention in recent years, in view of the demonstration that glyphosate (*N*-(phosphonomethyl)glycine [1071-83-6]), the active ingredient of the herbicide Roundup, kills plants by inhibition of this enzyme. EPSPS catalyzes the sixth reaction during aromatic amino acid biosynthesis via the shikimate pathway which exists only in plants and microorganisms. EPSP is the immediate precursor of chorismate, the first important branch point during aromatic amino acid and vitamin biosynthesis.

Perhaps the most important inhibitor of the EPSPS reaction is glyphosate which inhibits the EPSPS reaction via formation of a ternary complex with either S3P or EPSP and enzyme. Glyphosate is a competitive inhibitor with respect to PEP and an uncompetitive inhibitor with respect to S3P. Glyphosate, however, is not a structural analogue of PEP because the glyphosate does not inhibit any other PEP-dependent reaction. Whereas it has been suggested that glyphosate may be a transition-state analogue of the carbonium ion intermediate of PEP formed during catalysis, the bulk of the evidence suggests that this is unlikely. Nevertheless, glyphosate inhibits a wide range of EPSPS enzymes of bacterial, fungal, and plant origin. A number of structural analogues of glyphosate have also been tested for the ability of inhibit EPSPS. Only a few such analogues, eg, *N*-amino and *N*-hydroxy glyphosate, have been found to be inhibitors.

Roundup is a nonselective, post-emergent herbicide having activity against a wide range of annual and perennial grasses as well as broadleaf weeds. Because Roundup has no selectivity for weeds, use for weed control during active growth period of crops is fairly limited. Despite its nonselectivity, glyphosate, is extensively used in weed management because of broad-spectrum, systemic herbicidal activity; rapid inactivation in the soil (does not sterilize the soil); decomposition in the soil to the natural products, ie, carbon dioxide (qv), ammonia (qv), and phosphate; no toxicity to animal, aquatic, and avian species; it binds tightly to soil and does not contaminate ground water; and its cost effectiveness in weed control. In view of all the desirable features of glyphosate, the engineering of glyphosate tolerance in crop plants has the potential to open up new frontiers in weed management during cultivation. A substantial effort has been directed toward introducing Roundup tolerance to crop plants (35–38).

2.1. Engineering Roundup Tolerance. Knowing that the mode of herbicidal action of glyphosate is mediated via inhibition of EPSPS, at least two mechanisms can be considered for the introduction of Roundup tolerance to plants. The first option is to simply overproduce EPSPS so as to leave sufficient EPSPS enzymatic activity within the plant cells to satisfy the flux through the shikimate pathway. Alternatively, a gene encoding a glyphosate tolerant EPSPS enzyme can be used so that the EPSPS reaction is unaffected even in the presence of glyphosate. In addition, other approaches which are not related to the mode of action of glyphosate, such as glyphosate inactivation and inhibition of uptake, can be considered. These last are not discussed herein.

Overproduction of EPSPS. Overproduction of EPSPS has been demonstrated to confer glyphosate tolerance to both bacteria (39) and plant cells (40–42). Glyphosate tolerant plant cells have served as an excellent starting material for the isolation and purification of the EPSPS protein to homogeneity. *N*-Terminal amino acid sequence of the resulting protein provided the requisite information for synthesis of oligoprobes which were used for screening a complementary DNA (cDNA) library of petunia cells tolerant to glyphosate. From the library, the cDNA encoding petunia EPSPS was isolated and sequenced. The protein encoded by the cDNA had an *N*-terminal extension of 72 amino acids compared to the protein sequence obtained from the purified EPSPS enzyme. This *N*-terminal extension is necessary and sufficient to direct the EPSPS protein into the chloroplasts of plant cells (43). These studies also led to the conclusion that aromatic amino acid biosynthesis occurred primarily in the chloroplast of plant cells.

Whereas plant cells overproducing EPSPS could be generated by stepwise selection on glyphosate and the cells were glyphosate tolerant, these cells could not be regenerated into intact plants. Availability of the cDNA clone for EPSPS, however, provided a convenient tool for generating trangenic plants capable of overproducing EPSPS. Using an *Agrobacterium tumefaciens* transformation system, the EPSPS gene was introduced into both petunia and tobacco plants. Petunia plants overproducing EPSPS were thus produced and shown to be tolerant to Roundup (44). However, the extent of tolerance was not adequate for commercial use.

Glyphosate-Tolerant EPSPS. Several groups have tried to introduce Roundup tolerance into plants using genes encoding glyphosate-tolerant EPSPS enzymes. A mutant glyphosate-tolerant EPSPS enzyme, fivefold less sensitive to glyphosate, was isolated from *Salmonella typhimurium* (45). The introduction of the S. typhimurium mutant EPSPS gene into tobacco plants resulted in expression of the mutant gene such that plants were tolerant to glyphosate, but the extent of tolerance was not commercial (46).

Other bacterial mutants, such as a mutant *Escherichia coli* enzyme tolerant to glyphosate, have been described (47). The *E. coli* mutant had a single amino acid change from the wild type, resulting in substitution of glycine 96 with alanine. An identical mutation was reported in glyphosate-tolerant *Klebsiella pneumoniae* (48). The nature of changes in the kinetic constants of the *K. pneumoniae* enzyme is similar to that of the *E. coli* enzyme.

The *E. coli* mutant EPSPS was fused to the chloroplast transit peptide (CTP) sequence of petunia EPSPS in order to target the bacterial protein to the chloroplast (49). *In vitro* uptake experiments confirmed that the bacterial enzyme could indeed be imported and processed to a mature protein by chloroplast preparations. Introduction into petunia and tobacco plant cells led to regenerated plants expressing the bacterial gene either targeted to chloroplast or the cytosol. Tobacco plants containing the *E. coli* mutant EPSPS targeted to the chloroplast had higher levels of Roundup tolerance compared to either plants overproducing wild-type EPSPS or the control nontransgenic plants, but the level of tolerance was not sufficient for commercial use. The level of Roundup tolerance of plants having the *E. coli* enzyme targeted to the cytosol was only slightly higher than that of control plants, suggesting that the cytosolic EPSPS reaction was unable to complement the chloroplastic deficiency of EPSPS.

The glycyl 96 (G96) and prolyl 101 (P101) residues occur in a conserved region of EPSPS which is present in bacterial, fungal, and plant EPSPS enzymes. Replacement of G96 with an amino acid other than alanine (A) results in an inactivation of the EPSPS activity of the protein. However, the G96 to A mutation can be transferred to other bacterial and plant EPSPS enzymes, and in every case the alanyl enzyme has a higher glyphosate tolerance compared to the glycyl enzyme (50). This suggests that there is a high degree of conservation of the active site of EPSPS between bacterial, fungal, and plant enzymes.

Mutation of the conserved P101 to a serine residue also results in glyphosate tolerance of the EPSPS enzyme. This mutation was introduced into petunia EPSPS by site-directed mutagenesis and the seryl enzyme was demonstrated to be glyphosate tolerant. Analogous to the *S. typhimurium* enzyme, this mutation confers only marginal (sevenfold) glyphosate tolerance and no significant changes in the kinetic constants for the substrates. The petunia cDNA containing the prolyl to seryl mutation and the targeting sequence was introduced into tobacco plants. The Roundup tolerance of the tobacco plants expressing the seryl mutant was intermediate between plants expressing the wild-type and alanyl mutant enzymes.

Numerous bacteria which utilize glyphosate as a growth substrate were screened for the presence of EPSPS enzymes having binding constants for PEP close to those of the wild-type enzyme, but at least a 100–10,000-fold increase in affinity for glyphosate (37). The EPSPS from agrobacterium CP4 is perhaps the best studied. These enzymes are referred to as class II in order to distinguish them from the class I enzyme already described. The class II EPSPS enzymes have natural resistance to glyphosate and a low binding constant for PEP whereas the class I EPSPS enzymes are highly sensitive to glyphosate. The agrobacterium CP4 enzyme has 28% identity to the *E. coli* enzyme and has the conserved glycyl to alanyl change. Antibodies which recognize the *E. coli* EPSPS recognize petunia EPSPS but do not recognize agrobacterium CP4 EPSPS. Similarly antibodies reacting with the agrobacterium CP4 EPSPS do not show immune reaction with either *E. coli* or petunia EPSPS.

Transgenic soybean plants expressing the CTP-CP4 EPSPS display commercial levels of Roundup tolerance. These results validate the importance of substrate kinetics of EPSPS in order to maintain adequate rates of aromatic biosynthesis. Furthermore, the fact that glyphosate tolerance can be obtained by expression of a glyphosate-tolerant EPSPS illustrates that the herbicidal mode of action of glyphosate is related solely to inhibition of the EPSPS reaction.

As described earlier, translation of the EPSPS mRNA of plants results in the formation of a protein which has an *N*-terminal extension. The *N*-terminal extension, referred to as the chloroplast transit peptide, is necessary and sufficient for the import of the preprotein by the chloroplast. Once imported by the chloroplast, the transit peptide is cleaved releasing the mature enzyme. As expected, introduction of the EPSPS transit peptide to other protein sequences results in the importation of the fusion protein by the chloroplast.

The three-dimensional structure of EPSPS from *E. coli* has been established by crystallographic techniques (51). A number of amino acid residues have been modified to establish the necessity of these residues for enzymatic activity. At its *N*-terminus, the lysyl residue at position 22 of the *E. coli* enzyme

has been shown to be highly reactive and essential for enzymatic activity (52,53). It is likely that the lysyl residue is involved in substrate recognition. In addition to the lysyl residue at position 22, the arginyl residue at position 28 of EPSPS is conserved in all EPSPS enzymes studied to date. This arginyl residue is highly reactive, and its reaction with arginine reagents is inhibited by S3P and to a higher extent by a mixture of S3P and glyphosate. By site-directed mutagenesis, the arginyl residue has been replaced by lysyl, histidinyl, and glutaminyl residues. The latter two replacements appear to be detrimental for EPSPS activity, whereas the lysyl enzyme retains substantial activity (54). The roles of histidinyl, glutamyl, and cysteinyl residues of EPSPS have been probed by reaction with chemical modification reagents. These studies suggest that a glutamyl and histidinyl residue are critical for EPSPS activity (55). Similar studies with cystein modification suggest that cys-408 of *E. coli* EPSPS, although in a conserved region, is not essential for activity but is proximal to the active site (56).

3. Bioengineering of Increased Starch Content

The primary form of carbohydrate reserve in plants is starch (qv), entirely composed of the six-carbon sugar (qv) glucose. Starch typically is deposited in the form of water-insoluble granules, and is synthesized and stored in chloroplasts in photosynthetic tissues or in amyloplasts. Starch is a generic term used to describe a very heterogeneous class of molecules which differ in size and structure between different plants, different tissues within a plant, and at different stages of plant development. The heterogeneity of starch has proven useful in a number of different applications; for example, pea starch is widely used as a sizing agent in paper (qv) manufacture, and corn and potato starch are widely used to give viscosity, freeze-thaw tolerance, and body to a number of processed foods (see CARBOHYDRATES; FOOD PROCESSING).

The primary and likely sole pathway of starch biosynthesis is the adenosine diphosphate (ADP) glucose pathway (57). In this pathway the first enzyme, ADPglucose pyrophosphorylase (ADPGPP), catalyzes the conversion of glucose-1-phosphate to ADPglucose. In plants, it has been proposed that sucrose synthase is involved in the production of the ADPglucose used in starch biosynthesis (58). This model is not considered to be accurate given a number of mutants characterized affecting both starch and sucrose biosynthesis, and this topic has recently been reviewed (59). Another route for starch biosynthesis is through the action of starch phosphorylase. This enzyme is involved in the degradation of starch, forming glucose-1-phosphate from successive removal of glucose units from the polymer. The reaction is reversible *in vitro*; thus this enzyme potentially plays a role in the formation of starch. Through expression of antisense RNA, this enzyme has been eliminated in the amyloplast of potatotubers with no effect on starch content; thus any role in biosynthesis is proposed to be very minor (60).

3.1. Enzymes Involved in Starch Biosynthesis. Much of the early data dealing with starch biosynthesis in plants are derived from the study of various mutants. The shrunken-2 and brittle-2 mutants of maize have greatly reduced levels of ADPGPP activity owing to the absence of one of the two

subunits of this enzyme, and result in a shrunken seed appearance. Mendel's early work on inheritance of traits was performed with a pea mutant deficient in branching enzyme activity (61). Mutations in plants affecting starch biosynthesis can have severe results to plant morphology and viability.

ADP Glucose Pyrophosphorylase. The rate-limiting reaction in both bacterial glycogen and plant starch biosynthesis is the first step, catalyzed by the enzyme ADPGPP. In bacteria the enzyme functions as a homotetramer subject to tight allosteric regulation by effector molecules that reflect the energy state of the cell, and is the only enzyme in the pathway of glycogen biosynthesis subject to such regulation. The enzyme is activated by glycolytic intermediates and inhibited by adenosine monophosphate (AMP), ADP, and/or inorganic phosphate (Pi). Fructose 1,6-bisphosphate is typically the primary activator and AMP the primary inhibitor (57,62,63). The role of the activator is to increase the affinity of the enzyme for its substrates, adenosine triphosphate (ATP) and glucose-1-phosphate, and increasing amounts of the activator relieves inhibition caused by AMP, ADP, or Pi. The allosteric regulation of this enzyme has been shown to regulate the flux of carbon through this pathway and control the level of glycogen that is produced. Much of this work has been performed with mutants of *E. coli* and *S. typhimurium* affected in their ability to accumulate glycogen.

The bacterial ADPGPP enzymes each have subunits that contain allosteric activator and inhibitor binding regions, substrate binding sites, and a site for binding Mg^{2+}. A series of chemical modification experiments lead to the elucidation of amino acid residues responsible for interacting with the various effector and substrate molecules (64,65). The ADPGPP enzymes in plants function as heterotetramers consisting of two distinct subunits encoded by two different genes (57). These subunits differ in molecular weight, amino acid composition and sequence, and antigenic properties. Antibodies made against the large subunit only weakly react with the small subunit from a given plant (and vice-versa); but antibodies against the large (or small) subunit recognize the corresponding subunit from different plant species (66), ie, certain sequences are conserved between widely devergent plant species. As in bacterial glycogen biosynthesis, ADPGPP catalyzes the rate-limiting step in starch biosynthesis. The levels of control are primarily via allosteric regulation, but regulation of gene expression also plays a role in controlling ADPGPP activity. The primary effector molecules differ from those in bacteria. For every plant system studied, the plant enzymes are activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Pi). One possible exception is the wheat endosperm enzyme which appears not to be activated by 3-PGA (67). However, this enzyme is inhibited by Pi, and the presence of 3-PGA overcomes the inhibition.

The importance of allosteric regulation to *in vivo* ADPGPP activity and starch content in plants has been demonstrated (68). The gene encoding the ADPGPP enzyme from *E. coli* strain 618, which is relatively insensitive to allosteric control, was isolated and inserted into transgenic potato plants via *Agrobacterium tumefaciens* transformation. The gene was designed to express the active protein only in the potato tuber, and such expression resulted in a 25–50% increase in starch content. In contrast, expression of the ADPGPP gene from a wild-type *E. coli* K12 strain, which encodes an enzyme subject to normal allosteric regulation, had little effect on starch content. These results showed the

importance of allosteric control to ADPGPP activity, and circumvention of this control increases the flux of carbon through this pathway and results in an increase in starch biosynthesis and composition.

It is of interest to determine why the plant enzyme is composed of two distinct subunits and the bacterial enzymes only one. Because the enzyme must have binding sites for the allosteric activator and inhibitor, the substrates, and a catalytic site, it is possible that these sites are located on different subunits. The shrunken-2 and brittle-2 mutants of maize endosperm lack the large and small subunits, respectively, of the ADPGPP enzyme. These mutants have 12% and 17% of the wild-type ADPGPP activity and about 25% of wild-type levels of starch (69), demonstrating that both subunits are required for normal levels of enzyme activity and starch content, but that a single subunit by itself can form an active enzyme. This is supported by a starch-deficient mutant of *Arabidopsis* which lacks the large subunit, has about 5% wild-type levels of ADPGPP activity, and about 40% wild-type levels of starch (70). In addition, elimination of one of the ADPGPP subunits in transgenic potato through expression of antisense RNA results in a reduction in ADPGPP activity to 1.5–17% of wild type, and starch content to 4–35% of wild type (71). These results suggest that allosteric, substrate, and catalytic sites reside on each of the subunit types.

ADPGPP genes in plants are also controlled at the level of gene expression. In potato, the transcripts corresponding to the large and small subunits differ in their accumulation profiles in different organs (72). The steady-state levels of transcripts corresponding to the large subunit of ADPGPP are highest in tubers and stolons and are inducible by sucrose. In contrast, the steady-state levels of transcripts corresponding to the small subunit of ADPGPP are relatively equivalent in tubers, stolons, and aerial portions of the plant and are not strongly influenced by carbohydrates. Why the gene encoding the large subunit of ADPGPP is more tightly regulated than that encoding the small subunit is unknown.

Starch Synthase. In contrast to the bacterial systems where a single synthase is responsible for the elongation of the glucose chain, in plants several synthases are involved in building the starch granule. These synthases are either soluble or granule-bound. The soluble synthases are divided into two forms, designated as Type I and Type II, distinguished by size, kinetic properties, and immunological properties (63). These forms are encoded by separate genes which may show tissue and developmental regulation. Given these differences, the two types of enzymes likely play distinct roles in the formation of the starch granule, although this role is thought to be primarily involved in the synthesis of amylopectin, the branched form of starch. The granule-bound starch synthases are immunologically, physically, and kinetically distinct from the soluble synthases, and are encoded by one or more distinct genes. In maize endosperm, two forms of granule-bound synthase have been identified, bringing the total number of synthases identified in this tissue up to four. Unlike the situation for the ADPGPP gene in potato, the potato granule-bound starch synthase gene has been shown to be regulated solely at the level of gene expression (73).

The primary role of granule-bound starch synthase may be in the formation of amylose, the linear fraction of starch. Waxy-like mutations which are devoid of amylose and granule-bound starch synthase have been characterized in a number of plant systems, including maize, rice, barley sorghum, and potato (74). The

waxy mutation was obtained in transgenic potato through expression of antisense RNA to granule-bound starch synthase (75) providing strong evidence that the waxy locus encodes the granule-bound starch synthase enzyme, and that this enzyme is responsible for the synthesis of amylose *in vivo*.

Branching Enzyme. Multiple forms of branching enzyme have been found in a number of plant species. These enzymes are all soluble and catalyze essentially the same reaction, but differ in physical, immunological, and kinetic properties, and like the synthases probably play different functional roles in the synthesis of the starch molecule (57). Branching enzymes are also encoded by multigene families which may show developmental and tissue-specific expression profiles (76,77). The most detailed studies involve the isoforms from maize endosperm (78), where three different forms of branching enzyme have been purified and designated BEI, BEIIa, and BEIIb. Polyclonal antibodies against BEI do not react against either form of BEII, and vice-versa, but forms BEIIa and IIb appear to be closely related. Monoclonal antibodies have been produced which react with all three isoforms, showing that the enzymes share a few common epitopes but are otherwise divergent (79). Each endosperm-branching enzyme has been highly purified and the branching characteristics studied (80). BEI was found to have high activity on amylose but little on amylopectin, and was found to preferentially transfer long chains. These chains would represent the B chains in the cluster model proposed for the structure of amylopectin (81). BEIIa and IIb were found to have low activity on amylose and high activity on amylopectin, and transferred preferentially short, or A chains. Differences between these two isoforms in the types of branches produced were not noted, and these enzymes appear to be very similar (80).

Branching enzymes have been characterized from a variety of other plant tissues. Only a single isoform has been detected in potato tubers. The gene for potato branching enzyme is regulated in a manner similar to the potato large subunit ADPGPP gene and is expressed most abundantly in the potato tuber (82). Antisense RNA expression in transgenic potatoes has resulted in a 90% decrease in branching enzyme activity, but with no discernable effect on starch content or structure (60). This implies that either branching enzyme activity is present in vast excess, or a second enzyme indeed exists. The former seems to be the case. In pea, the wrinkled seed phenotype has been linked to the *r* locus and results in a 66–75% reduction in total starch, and an increase in amylose from 33% in wild-type pea up to 60–70% in the mutant (83). Branching enzyme activity is reduced to 14% of wild-type levels because of the complete absence of one isoform of branching enzyme. The decrease in total starch levels is caused by a similar mechanism as in bacteria lacking branching enzyme activity, ie, as the glucose chain is elongated, it becomes a poorer substrate for the synthase enzyme.

One function of branching enzymes is to clip the elongating chain and provide additional substrate to the synthase enzymes. In this model, the synthase and branching enzyme work in concert, whereas the synthase elongates the chain, the branching enzyme cleaves, transfers a maltodextrin, and forms a new branch, which is then further elongated by the synthase. The dependence of starch synthase on branching enzyme has been shown in *in vitro* systems where the activity of starch synthase is observed to be greatly enhanced by the

addition of branching enzyme (84). This model of concerted activity also provides the rationale for the existence of multiple isoforms of starch synthase and branching enzyme in plants. Amylopectin is an asymmetric molecule formed of both short (12–42 residues) and long (>49 residues) glucose chains (85). Synthesis of such an asymmetric structure requires starch synthases and branching enzymes having different specificities for elongation and for insertion of branch points at different distances along A- and B-chains. Further evidence for this comes from the study of a low starch mutant of *Chlamydomonas* that lacks soluble starch synthase II and shows a decrease in intermediate length chains in the amylopectin fraction (86). Thus the structure of the starch granule can be influenced by the properties of both starch synthases and branching enzymes, and further controlled by regulation of gene expression in different tissues or during plant organ development.

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