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New Opportunities to Dissect and Manipulate Plant Processes

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New opportunities to dissect and manipulate plant processes

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SUMMARY

The use of transgenic plants has become a standard tool of experimental plant biology and is changing many approaches to plant improvement. The technology has greatly expanded the range of methods available to isolate and identify new plants genes, and has permitted great strides in understanding the mechanisms which regulate gene expression. In addition, the ability to use cloned genes to alter the functional expression of the gene in transgenic plants has created entirely novel opportunities to examine the biological role of virtually any cellular constituent.

1. INTRODUCTION

In the decade since the first reports of the production fertile transgenic plants, it has become routinely possible to produce large numbers of transformants of many plant species. For easily transformed species, such as tobacco, the methods for producing transgenic plants are technically simple and inexpensive. Thus, in spite of the fact that several months are usually required to obtain transformed differentiated tissue, production of transgenic plants has become an indispensable component of the methodological repertoire in virtually all aspects of experimental plant biology. For instance, because many methods of gene isolation are based on the use of indirect genetic criteria, the final proof that a gene corresponds to a particular mutation is ultimately the ability of a cloned gene to complement the mutation. Once a gene is cloned, most methods of examining the function of the gene now depend upon the production of transgenic plants.

In this brief overview I have attempted to summarize the various ways in which the methodology is currently exploited, and to speculate on additional possibilities.

2. ISOLATING GENES

(*a*) *Gene-product based cloning*

Insights into many aspects of plant biology can be gained by isolating the genes which control the process of interest. Thus, for instance, in order to understand the mechanisms which regulate the response of plants to light, it has been essential to have access to genes for proteins which regulate (Wagner *et al.* 1991) or are regulated by light (Green *et al.* 1988). However, the utility of the approach is proportional to the ease with which a gene of interest can be isolated. Figure 1 shows schematically most of the methods which are currently employed to isolate plant genes. Until recently, the most widespread method of gene isolation was 'gene product based'. This is typically accomplished by isolating sufficient quantities of a gene product so that an antibody can be raised or so that enough peptide sequence can be obtained to synthesize oligonucleotide probes or polymerase chain reaction (PCR) primers. These materials can then be used to screen appropriate cDNA or genomic libraries, or to amplify target sequences by PCR. The limitation of this approach is that for many genes, the product is not known or cannot be purified in sufficient quantities.

(*b*) *Insertional inactivation*

The currently most popular alternative to product-based cloning is transposon or T-DNA tagging. In those species such as *Antirrihnum majus* or *Zea mays* where several well-characterized transposable elements are known, many important genes have been isolated by tagging (Balcells *et al.* 1991). The limitation of this approach is that it is usually necessary to screen very large numbers of individuals in order to identify a mutation in a particular gene. For instance, in the isolation of the *opaque-2* gene by transposon tagging with the *Spm* element, approximately 500 000 kernels were examined in order to find a mutation. Thus, this approach has been most useful in identifying mutations which result in readily scored phenotypes such as alterations in colour or morphology (Carpenter & Coen 1990).

Since transposable elements are not well characterized in many other important plant species, there has been widespread interest in introducing a well-characterized transposable element into these species. Most of the effort has been focused on the maize Ac/Ds system which appears to retain transposon characteristics when transferred to dicot species such as tobacco

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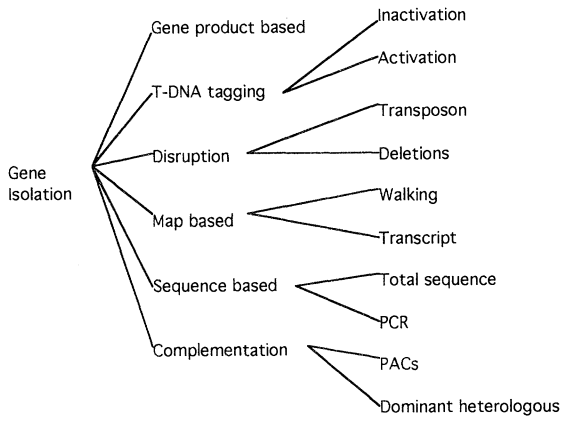

Figure 1. The various methods used in plant gene isolation.

and *Arabidopsis* (Dean *et al.* 1992). Although these heterologous systems have not as yet been sufficiently refined so that they are generally useful, the general approach seems very promising in the long term.

A variant of transposon tagging has been developed to exploit the fact that when transgenic plants are produced, there is inevitably a disruption of the genome by the insertion of ectopic DNA into the chromosome (Feldman 1991; Koncz *et al.* 1990). If the disruption caused a detectable mutation, the disrupted DNA can be readily cloned by using the ectopic DNA as a probe for the flanking sequences. Because it is somewhat tedious to produce transgenic plants on a large enough scale to use T-DNA as an insertional mutagen, the utility of this method has been largely restricted to *Arabidopsis* because it has a small genome which is almost devoid of repetitive DNA. Thus, it appears that a very large proportion of T-DNA insertions generate mutations. Indeed, approximately 19% of all T-DNA insertions produce a visible phenotype (Feldman 1991). In addition to the small genome size, a facile method has been developed for transformation of *Arabidopsis* which has permitted the production of more than 13 000 transgenic lines, many of which are available from the *Arabidopsis* Resource Centers at Ohio State University and the University of Nottingham. In this method, seeds are briefly germinated in a culture of *Agrobacterium tumefaciens* then grown to maturity. By some unknown mechanism, a small proportion of the progeny are found to be transformed and can be identified by selection for a kanamycin resistance marker on the T-DNA. Presumably, the plants arising from the seed inoculation become systemically infected and are occasionally transformed by the intracellular population of *A. tumefaciens*. Although the method has led to the isolation of many interesting genes, the approach is inherently limited by the fact that it is opportunistic rather than systematic. That is, if a mutation in a particular gene is not represented in the existing collection, the method is not applicable.

Another promising variant of T-DNA tagging is based on the concept of enhancer trapping. In this method a promoterless gene encoding a reporter gene (e.g. GUS) or selectable marker (e.g. kanamycin resistance) is placed adjacent to a T-DNA border so that if the T-DNA inserts into the genome adjacent to an active promoter, the gene is expressed and the transformed cell survives exposure to the selective agent or expresses the reporter gene (Kertbundit *et al.* 1991; Topping *et al.* 1991). The complementary approach is to place a strong enhancer adjacent to the T-DNA border so that a quiescent gene is activated by the proximity of the enhancer (Walden *et al.* 1991). The large numbers of independent transformation events required for this approach are obtained by protoplast transformation. The key to the successful use of this strategy seems to be in designing screens which will narrow the number of surviving colonies to those in which a particular subset of genes has been targeted. In the first successful application of this method, antibiotic resistant tobacco cells which grow in the absence of exogenous auxin have been identified following transformation with an enhancer. If major regulatory genes control the production of secondary metabolites (see Ludwig *et al.* 1990), this approach might prove very useful for the identification of genes which are required for the production of secondary metabolites such as taxol or other complex plant metabolites of medicinal value. For instance, insertion of an enhancer near the regulatory locus might activate high level expression of the pathway in cultured cells. Many plant-derived drugs are sufficiently complex so that antibodies can be raised against the compounds and these might be used to screen the transformants for the presence of cells which express high levels of the compounds.

(*c*) *Subtractive methods*

The conceptual complement to the isolation of genes on the basis of a transposable element or T-DNA insertion is to exploit the existence of a deletion of a target gene. The utility of this approach was demonstrated by the isolation of a gene which complemented a mutation of the *ga1* locus of *Arabidopsis* (Sun *et al.* 1992). The mutation had previously been suggested to be a deletion on the basis of conventional genetic criteria. A portion of the gene was highly enriched by several successive rounds of hybridization of small fragments of DNA from the wild type with biotinylated DNA of the mutant, followed by removal of DNA hybrids by chromatography on an avidin matrix. The main limitation to the utility of this approach is the difficulty associated with producing and identifying small deletions in higher plants. A variant of the method can, however, be exploited to enrich for genes which are more highly expressed under one condition than another.

(*d*) *Map-based cloning*

The ideal method for gene isolation should be capable of isolating any gene for which a phenotypically detectable recessive variant allele exists. Map-based cloning methods such as chromosome walking are theoretically suitable in that they permit the isolation of any allele which can be genetically mapped. This effectively excludes only quantitative

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trait loci (QTLs). However, these methods may be considered less than ideal because of the amount of effort required to implement map based cloning in higher plants.

The strategy in map-based cloning is to genetically map an allele of a target gene near previously isolated DNA fragments, typically restriction fragment length polymorphism (RFLP) markers. Then, the region of the genome containing the gene is isolated, and subfragments are used to complement the mutation. *Arabidopsis* is the most suitable plant for the application of map-based cloning methods because, in addition to suitable genetic characteristics, it has a uniquely small nuclear DNA content which is almost devoid of interspersed highly repetitive DNA (Meyerowitz 1989). Several RFLP maps have been constructed (Chang *et al.* 1988; Nam *et al.* 1989), which now contain approximately 312 markers distributed over the five chromosomes. As the total DNA content has been estimated to be 70 000 kb, the average distance from any gene to the nearest RFLP marker is about 225 kb. This is comparable to the average insert size of about 160 kb in the several yeast artificial chromosome (YAC) genomic libraries which are available for *Arabidopsis* (Grill & Somerville 1991; Ward & Jen 1990).

The general utility of these resources was recently demonstrated by the map-based isolation of a gene which complements a mutant (*fad3*) of *Arabidopsis* deficient in omega-3 fatty acids (Arondel *et al.* 1992). In this instance, a YAC covering the region of the *fad3* gene was used to probe a cDNA library prepared from a tissue in which the biological information had suggested the *fad3* transcript would probably be moderately abundant. The YAC encoded only one moderately abundant cDNA. Production of transgenic plants which expressed this cDNA resulted in genetic complementation of the *fad3* mutation, thereby establishing the identity of the gene. In those instances where the target gene is weakly expressed, it is necessary to subclone the YACs and transform the mutant with a series of overlapping subclones to find the complementing fragment. This was essentially the approach which was used to clone the *ABI3* gene from *Arabidopsis* by map-based methods (Giradaut *et al.* 1992). Although it remains somewhat tedious to isolate genes by this approach, it seems likely that with the development of an ordered set of YACs for the *Arabidopsis* genome it will become much easier to implement this approach. At present, more than one third of the genome has been covered by identified YACs of known map position (Hwang *et al.* 1991).

Although *Arabidopsis* has unique advantages for map-based cloning, it may be possible to implement the related method of 'chromosome landing' in other plant species, or in *Arabidopsis*, to accomplish similar ends. The idea is to bypass the construction of a very high density RFLP map and to focus instead on identifying polymorphic markers which are very tightly linked to a target gene. This is possible because of the observation that if random 10 base pair (b.p.) oligonucleotides are used as primers in PCR using genomic DNA from polymorphic individuals, the PCR products frequently exhibit polymorphisms designated RAPDs (randomly amplified polymorphic DNA) (Reiter *et al.* 1992). The central concept underlying the use of RAPDs for chromosome landing is that if one pools DNA from a number of phenotypically identical F2 progeny from a cross between two polymorphic parental lines which carry different alleles for a gene of interest, the pooled progeny will show the polymorphisms of both parents for all regions of the genome except the small region surrounding the target gene (Michelmore *et al.* 1991). In this region, the pooled progeny will necessarily have the same chromosomal composition as the mutant parent. It is possible to identify closely linked DNA probes (i.e. RAPDs) by preparing DNA from each parent and from the pooled F2, then screening these three DNA samples for polymorphisms by the PCR-based method. Polymorphic bands which are present in the pooled F2 and the mutant parent represent fragments of DNA which have a high probability of being closely linked to the mutation. Tight linkage can be verified by scoring additional F2 progeny and tightly linked polymorphic fragments can then be used to screen a YAC library. Because of the relative ease with which a thousand or more different primers can be screened, this method has the inherent potential to generate very tightly linked markers even for species with relatively large genomes.

(*e*) *Large-scale sequencing*

Although not, as yet, widely employed in plant biology, the recent improvements in instruments for large-scale automated DNA sequencing promise to significantly change many current practices. It has now become possible for one laboratory to obtain partial nucleotide sequence (ESTs, expressed sequence tags) for many thousands of cDNA clones in a period of a few months (Adams *et al.* 1991, 1992). Furthermore, because of the large amount of sequence information available in public nucleotide and protein sequence databases, a significant proportion of randomly sequenced cDNA clones can be assigned probable function by comparison to the sequence databases. In the case of human cDNA clones, approximately 18% of the first 5000 ESTs were assigned probable function (Adams et al 1991). Similarly, of the first 1000 ESTs from rice, approximately 20% were assigned probable function: mostly by reference to non-plant genes (Uchimiya *et al.* 1992). In view of the efficiency of this approach as a mechanism for connecting plant biology to the large amount of sequence information available in other orders, it is apparent that very large numbers of ESTs will soon be available for several plants such as rice, maize and *Arabidopsis*.

For the foreseeable future, it will not be possible to assign function to most ESTs by reference to the databank, or the proposed homology will be of marginal statistical significance, or the correspondence between the function of the gene product in one organism and another may be obscured by species specific differences. In these instances, the ESTs will

serve essentially as stimuli for hypotheses about the existence of a previously unidentified gene. These hypotheses may be expected to become increasingly important leads into novel aspects of plant biology as more and more genes of readily evident function are characterized. The ability to analyse these ESTs in transgenic plants will be the essential technique for testing these hypotheses.

(*f*) *Cloning by complementation*

Much of the power of yeast genetics is based on the ease with which a gene which complements a mutation can be isolated. Because of the technical difficulties associated with producing large numbers of transformed plants, it is unlikely that the isolation of genes by complementation of plant mutations, per se, will be implemented in higher plants. However, the use of map-based cloning methods is a compromise solution in which the amount of DNA which must be introduced into the mutants is reduced so that fewer transgenic plants need to be produced.

An increasingly important method of cloning plant genes is the use of plant cDNA clones to complement microbial mutants. Many biochemical pathways are similar or identical in plants, bacteria and fungi, and functional complementation of auxotropic mutations is not uncommon (Minet *et al.* 1992). An informative example of the possible opportunities was the recent cloning of two different genes encoding potassium channels by the complementation of the same yeast mutant (Anderson *et al.* 1992; Sentenac *et al.* 1992).

Many of the traits, for which genes are sought, occur in plants in which conventional genetic methods are not likely to be implemented. For example, if one wanted to identify the genes for the synthesis of a particular secondary metabolite in an exotic plant species, it is unlikely that it would be possible to justify the expenditure of time and effort required to establish a genetic system in a new species even if it was amenable to the approach. A possible solution to this problem may be to develop a facile system for the development of PACs (plant artificial chromosomes). This is not possible at present because no plant centromere has yet been isolated. If it were possible to produce libraries of PACs containing foreign DNA in a readily transformable species such as tobacco or *Arabidopsis*, it may be possible to isolate genes by a kind of 'dominant heterologous complementation' in which genes for particular traits are identified by their expression in a distantly related host. This might be a particularly interesting approach to the isolation of indispensable genes which regulate aspects of growth and development. This might eventually also be a useful approach in the isolation of genes for enzymes involved in the synthesis of complex secondary metabolites.

3. GENES AND BIOLOGY

Cloned plant genes are generally used to examine some aspect of genetic mechanisms, to examine the role of the gene product in the life of the organism

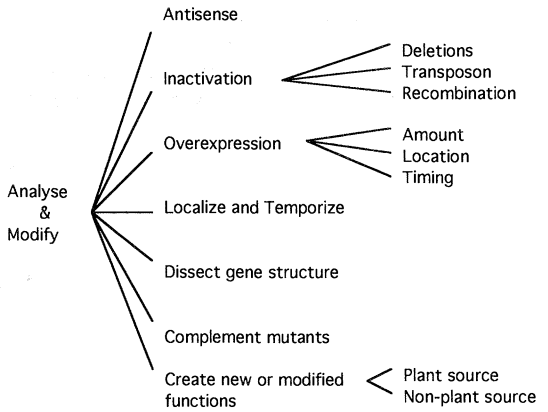

Figure 2. Schematic outline of the ways in which transgenic plants are used to examine the structure and function of plant genes.

or to add, alter or remove a trait of practical importance. The methods by which these objectives are realized are summarized in figure 2 and described below.

(*a*) *Reducing gene expression by antisense or cosuppression*

One of the simplest methods of experimentally reducing the level of accumulation of a gene product is to produce a transgenic plant in which some portion of the gene transcript is expressed in an antisense orientation from an ectopic copy of the gene (Gogarten *et al.* 1992; van der Krol *et al.* 1988). As no firm rules have emerged for the optimal design of an antisense construct, the usual approach is to use the entire transcript, the 5′ half and the 3′ half in three parallel experiments. It is not uncommon to find that all three constructs produce a similar range of effects. As in most experiments with transgenic plants, because of position effects, it is generally necessary to produce a minimum of several dozen independent transgenic plants to as to survey the full range of possible levels of suppression.

Although the mechanism of antisense suppression is not understood, it is possible to imagine mechanisms which account for the effect. By contrast, the observation that expression of ectopic copies of part of a homologous sense transcript may also cause loss of expression of both the ectopic and the native copy of the gene is mysterious. This phenomenon, termed cosuppression, has only been reported in higher plants and has been observed for only a few genes such as chalcone synthase, which is involved in flower pigment formation (Napoli *et al.* 1990; van der Krol *et al.* 1990). In this case the tissue specificity of gene expression can be observed by observing the pattern of pigment formation on the flower. It is apparent from the patterns seen in plants exhibiting cosuppression that the effect may vary depending upon the developmental state of the tissue. The occurrence of colour variegation also indicates that the effect can be unstable as might be expected for an epigenetic phenomenon. The eventual resolution of the mechanism for this interesting effect may eventually suggest novel mechanisms of directed gene control.

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(*b*) *Gene inactivation*

Although there have been several reports of homologous replacement of an endogenous gene with a cloned gene, the frequency is too low to be directly useful at present (Halfter *et al.* 1992; Lee *et al.* 1990; Paszkowski *et al.* 1988). However, it has only been several years since similar problems with directed gene replacement in cultured animal cells were overcome by methodological improvements (Capecchi 1989; Mansour *et al.* 1988). The most important innovation was the development of methods for selecting against ectopic integration of exogenous DNA so that only homologous integrations survived selection. It seems likely that the implementation of similar techniques will result in similar improvements in homologous recombination in plant cells. In many plants it is now possible to transform protoplasts at high efficiency and to select rare recombinants. Thus, there seems no intrinsic limitation to the application of this method in easily cultured plant species.

An alternate possibility for obtaining gene disruption (although not gene replacement) may arise from the fact that the Ac/Ds transposon system from maize has been shown to be active in all other plant species tested (see, for example, Dean *et al.* (1992)). The Ac element has the property that it preferentially transposes to a nearby site. This property can, in principle, be exploited by creating a series of transgenic lines of a plant species in which Ac or Ds elements are scattered around the genome. To inactivate a cloned gene, one would first map the cloned gene by RFLP-mapping. In the next stage, a family of Ac/Ds transposition events is produced using an Ac or Ds element which is inserted near the mapped gene. The presence of an individual in the population in which Ac or Ds has jumped into the target gene can be assessed by using the PCR reaction to test for an Ac/Ds insertion in or near the target gene. This can, in principle, be done by preparing total DNA from subpools of all the plants which experienced transpositions, and then using one PCR primer based on the sequence of the Ac/Ds element, and one based on the sequence of the target gene. If the Ac/Ds element lands sufficiently near the target gene, the PCR with produce a new band. The individual transposition event can be identified by successive subdivisions of the population of transposition events.

A similar use of the PCR reaction to isolate genes which have been deleted or disrupted by other mechanisms may also be imagined. For instance, there are approximately 13 000 lines of *Arabidopsis* carrying random T-DNA insertions. If DNA were made from these lines in pools of 50 or 100 plants, it would be possible to screen the entire population for insertion in a target gene by scoring only a few hundred PCR reactions using one or more primers based on the target sequence and a primer based on the T-DNA sequence. Similarly, one might screen pools of mutagenized plants for a specific deletion by using primers which are derived from sequences which are normally too far apart in the wild-type to give rise to a PCR product.

(*c*) *Altered expression of cloned genes*

In some cases the best way to examine the function of a cloned gene may be to increase the amount of expression or to alter the location or timing of expression of the gene. A beautiful example of the utility of the approach was the demonstration that the *Lc* gene of maize was an autonomous transcriptional activator (Ludwig *et al.* 1990). Introduction of the coding sequence of the *Lc* gene under control of a constitutive promoter into various tissues such as roots which are not normally pigmented resulted in expression of the entire pathway of anthocyanin production. This result indicated that the product of the *Lc* gene is a transcriptional regulator which is able to induce transcription of all the genes in the pathway in cells representing many different developmental states.

In some instances a fresh insight into some aspect of the biology of an organism may be gained by introducing genes from other organisms. For instance, in order to alter the composition of the sugars in the vacuole or the apoplast of photosynthetic tissues, Sonnewald *et al.* (1990) used a yeast invertase gene to target abnormal accumulation of invertase to these cellular locations. The invertase hydrolysed sucrose which was normally present in these compartments. This, in turn, caused informative perturbations in the transport of assimilates and in overall photosynthetic activity.

(*d*) *Localizing and dissecting gene expression*

At present, the standard method for identifying where and when a plant gene is expressed is to fuse the promoter sequence of the gene to the coding sequence for a bacterial β-glucuronidase (GUS) and produce transgenic plants (Jefferson *et al.* 1987). The presence of GUS activity can be conveniently detected by histochemical staining of microscopic sections. The widespread and enthusiastic application of this method has led to the accumulation of a vast amount of information about the cell and tissue specificity of gene expression. In many instances, the application of this method has resolved longstanding problems which could not be resolved by other methods. For instance, it had been known for many years that the leaves of dicots contained a cytoplasmic and a chloroplast isozyme of glutamine synthase, but the physiological significance of these isozymes was unknown. Brears *et al.* 1991 resolved this by using GUS fusions of the two promoters to demonstrate that the two isozymes were expressed in distinct cell types with different physiological functions within the leaf.

In the absence of the ability to produce transgenic cells, the study of the relationship between gene structure and function would necessarily be a kind of archeological discipline in which the wonders of extinct creatures are imaginatively reconstructed from bits of fossilized bone. It has been suggested that shooting gold particles coated in DNA into cells at several thousand feet per second and examining the average activity of a reporter gene is only a small step

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forward in this respect. Fortunately, it is now routinely possible to produce transgenic plants of many species in which the hypotheses derived from such transient expression assays or other criteria are routinely tested (Schell 1987). Although it is not convenient to produce transgenic plants of certain species, the fact that promoters from one species of plant are generally expressed at the correct place and time in distantly related species has permitted the widespread use of a few species as 'universal' recipients of genes from other species.

(*e*) *Introducing new or improved traits*

The potential applications of genetic engineering methods to the improvement of economically important plant species are too vast to enumerate. Many of the goals of traditional plant breeding seem likely to be accelerated by the application of molecular genetics when the relevant genes are available (Gasser & Fraley 1989). For instance, durable disease resistance is consistently ranked as one of the highest priorities of many breeding programs. Substantial progress towards achieving virus resistance has been achieved by the expression of viral coat proteins in transgenic plants (Beachy *et al.* 1990). Similarly, expression of anti-insecticidal proteins in transgenic plants has provided new mechanisms for resistance to insect pests (Williams *et al.* 1992). This latter application is expected to have a very beneficial effect upon the environment by leading to reduced use of insecticides. A creative application of detailed knowledge of tissue specific gene expression has been the use of a pollen-specific promoter to express ribonuclease in developing pollen (Mariani *et al.* 1990). This causes male sterility, a trait which is very useful in the large-scale production of hybrid seed.

Many of the conceivable applications involve altering the quality or quantity of the economic product in ways that cannot be accomplished by conventional plant breeding. Thus, for instance, potatoes which produce very high levels of starch were produced by introducing a bacterial gene for ADPglucose pyrophosphorylase under control of a tuber specific promoter (Stark *et al.* 1992). Unlike the plant enzyme, which is subject to allosteric regulation by intermediates of the starch biosynthetic pathway, the in vivo activity of the bacterial enzyme was not subject to any form of feedback regulation. Thus the bacterial enzyme was not inhibited by the high concentration of biosynthetic intermediates and, as a result, greater metabolic flux of photosynthate toward starch synthesis was possible. Many other modifications of starch, oils or storage proteins can be envisioned by the similar introduction of one of a few genes.

In addition to providing new tools to facilitate conventional breeding, the ability to move genes from one organism to another provides entirely new possibilities to use plants as sources of new biomaterials. An example is the recent production of biodegradable plastics such as poly-β-D-hydroxybutyric acid (PHB) in plants. This compound, which serves as a storage material in many species of bacteria, normally accumulates as small intracellular granules. PHB is made from acetoacetyl-CoA in two enzymatic steps. When the two bacterial genes involved in the conversion of acetoacetyl-CoA to PHB were expressed in transgenic *Arabidopsis* plants, PHB accumulated in small granules within the plant cells and appeared to have the same properties as PHB which normally accumulates in bacteria (Poirier *et al.* 1992). If adequate levels of production of PHB or related polyalkanoates can be produced in transgenic field crops it might be feasible to satisfy a significant proportion of our needs for this class of materials from a renewable source. Furthermore, the availability of an alternate outlet for agricultural production would have a beneficial effect on the farm economy in the developed world and could reduce or eliminate the need for agricultural subsidies.

4. CONCLUDING REMARKS

Experimental plant biology is in the midst of a scientific and technical revolution which has been made possible by the development of methods for isolating genes and analysing their structure and function in transgenic plants. The explosive growth of information which has resulted is fueling a secondary revolution in the ways in which plant improvement is undertaken. More importantly perhaps, the ability to move genes from other orders of organism into plants and to precisely control the expression of foreign genes will expand the ways in which we utilize plants. However, virtually all of the knowledge which has been gained to date concerns temperate plants. To exploit the full potential of this technology, it is essential that work begin on developing the methods for producing transgenic plants of many of the important tropical species upon which much of the worlds population depends for food and fibre. Some of these species, such as oil palm and coconut, pose technical challenges because of long life cycles, but tremendous opportunities because of the very high productivity of these and related species.

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