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Transgenic Plants as Tools to Study the Molecular Organization of Plant Genes

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Transgenic plants are generated in nature by *Agrobacterium tumefaciens*, a pathogen that produces disease through the transfer of some of its own DNA into susceptible plants. The genes are carried on a plasmid. Much has been learned about how the plasmid is transferred, how the plasmid-borne genes are organized, regulated, and expressed, and how the bacteria's pathogenic effects are produced. The *A. tumefaciens* plasmid has been manipulated for use as a general vector for the transfer of specific segments of foreign DNA of interest (from plants and other sources) into plants; the activities of various genes and their regulation by enhancer and silencer sequences have been assessed. Future uses of the vector (or others like it that have different host ranges) by the agriculture industry are expected to aid in moving into vulnerable plants specific genes that will protect them from such killers as nonselective herbicides, insects, and viruses.

THE SOIL PHYTOPATHOGEN *Agrobacterium tumefaciens* IS A sophisticated parasite that uses genetic engineering processes to force infected plant cells to divert some of their organic carbon and nitrogen supplies for the synthesis of nutrients (called opines), which the infecting bacteria can specifically catabolize (*1*). The genetically engineered plant cells are also stimulated to proliferate and thus form typical tumor tissues called crown galls.

A detailed genetic and molecular analysis of this phenomenon became possible when it was discovered (*2*) that large extrachromosomal plasmids in agrobacteria carry the genes responsible for both tumorous growth and opine synthesis of crown gall tissues as well as for opine catabolism by agrobacteria. Genetic evidence thus pointed to specific transfer of genes from the Ti plasmids in agrobacteria to the genome of plant cells.

Transposon mutagenesis of Ti plasmids (*3, 4*) revealed that two segments of these plasmids were involved in oncogenicity and therefore, presumably, in the transfer of DNA from bacteria to plant cells. One of these segments, later called the *vir* region, contains genes whose inactivation leads to a loss of tumor-inducing capacity by the mutant strains. Insertions into the other segment, called the T region, produced mutant strains that could still transfer DNA into plant cells, but the cells then either lacked the capacity to synthesize opines or the tissues had aberrant morphologies (shoot-forming teratomas or root-forming calli). A striking observation made with these insertions was that a transposon within the T region of a Ti plasmid was physically integrated into the genome of transformed plant cells (*5*). This finding confirmed that the T region of Ti plasmids was physically transferred to plant cells and showed that Ti plasmids could be used to introduce foreign DNA into plant cells.

When small DNA fragments from Ti plasmids were hybridized with DNA from transformed plant cells a well-defined segment called transfer DNA (T-DNA), derived from the T region of these plasmids, was covalently integrated into the plant nuclear genome (*6*). RNA-DNA hybridizations showed that the T-DNA in plant cells was transcribed into a number of well-defined polyadenylated transcripts. Some of these transcripts correlated with the T-DNA functions genetically identified by transposon mutagenesis of the T region of Ti plasmids (*7*).

T-DNA Oncogenes Code for Enzymes

Plant cells harboring a T-DNA segment in their genome do not require supplementation with cellular growth factors, such as auxins

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and cytokinins, for continued growth and division in tissue culture. T-DNA genes 1, 2, and 4 (*7*) play the major role in the control of neoplastic growth. The function of individual T-DNA genes was determined by using clonal tobacco cell lines that contained either a single T-DNA gene or a defined combination of T-DNA genes. Tobacco cells containing only T-DNA genes 1, 2, and 4 produced undifferentiated tumors, indistinguishable from wild-type crown galls. Cells expressing only genes 1 and 2 produced root-sprouting tissues, and those expressing only gene 4 grew as shoot teratomas (*8*). These observations showed that the combination of genes 1 and 2 establishes a new pathway for auxin synthesis in transformed plant cells (*8, 9*), whereas gene 4 codes for an enzyme that catalyzes the synthesis of a cytokinin (*10*).

These observations largely explained the molecular mechanism of tumorous growth in plant cells and provided direct genetic evidence in favor of the long-held notion that the ratio of the concentrations of auxins and cytokinins is an important signal for the control of growth and differentiation in plant tissues (*11*). There is circumstantial evidence (*12*) that the T-DNA auxin gene 1 (*iaa*M) coding for a tryptophan 2-monooxygenase and the auxin gene 2 (*iaa*H) coding for an indole-3-acetamide hydrolase, as well as the gene 4 (*ipt*Z) coding for an isopentenyltransferase, are of prokaryotic origin and related to similar genes found in other soil bacteria that have symbiotic or parasitic interactions with plants.

The Mechanism Underlying T-DNA Transfer

Three genetic elements in *Agrobacterium* are essential for the transfer of T-DNA from Ti plasmids to plant cells: *vir* genes (*13*), T-DNA border sequences (*14*) located on Ti plasmids, and the chromosomal virulence genes *chv*A and *chv*B *10* (*15*). The *chv* loci mediate attachment of the bacterium to the plant cell. The expression of the *chv* loci is constitutive and these genes may, therefore, play a more general role in a mechanism allowing the agrobacteria to seek out plants. Target plant cells may also express functions that take part in the bacterium–host cell recognition mechanism.

Phenolic signal molecules such as acetosyringone (*16*) induce the Ti plasmid *vir* genes which, in turn, set in motion the molecular events leading to T-DNA transfer (*17–20*). The unregulated *chv* functions leading to recognition and attachment, and the specifically induced *vir* functions leading to T-DNA mobilization and transfer, therefore, combine to make the *Agrobacterium*–plant cell interaction an efficient and specific mechanism for (unilateral?) DNA transfer leading to the genetic colonization of plant cells by T-DNA genes.

On Ti plasmids, the T region is flanked by a 25–base pair (bp) border sequence directly repeated at both ends of the T region [summarized in (*21*)]. The right border sequence (but not the left) is essential for efficient T-DNA transfer (*4, 22, 23*). Genetic data indicated that the T-DNA transfer normally occurs in a polar or oriented fashion from the right toward the left border sequence (*22*). Activation of *vir* gene expression results in the generation of site-specific nicks in the bottom strands of T-region border sequences (*17–19, 24*) and also in the production of free, linear, single-stranded copies of the T region (*17, 18*). The *vir*D operon encodes the relevant site-specific endonuclease (*18–20*). The polarity of the transfer, as well as the occurrence of single-stranded copies of the T region, is reminiscent of bacterial conjugation (*17*).

Development of Ti Plasmid–Based Gene Vectors

Three fundamental observations formed the logical basis for the development of the vectors commonly used for transfer of genes to plants.

1) Foreign DNA sequences inserted within the T region of Ti plasmids are transferred to the plant genome (*5*).

2) None of the genes located within the T region is involved in the mechanism responsible for T-DNA transfer and integration in the plant genome (*25*). Thus a "disarmed" Ti plasmid vector, pGV3850, was constructed in which all of the oncogenic phytohormone biosynthetic genes were removed from the T region and replaced with the linearized plasmid pBR322. The pBR322 DNA provides homology for cointegration of the T region of pGV3850 with any pBR322 vector derivative carrying a cloned gene of interest. Various dominant selectable marker genes were introduced into pGV3850 in order to identify transformed plants readily (*26*). A triparental bacterial conjugation system was developed (*27*) to transfer any pBR322 derivatives from *Escherichia coli* to *A. tumefaciens* pGV3850 and to select for cointegrated pGV3850 containing the desired cloned genes. Various other disarmed Ti vectors have now been constructed and used successfully (*28*).

3) The T region does not have to be physically linked to the *vir* genes of Ti plasmids (*29*). In fact, any DNA segment flanked by the 25-bp sequences normally bordering T regions of Ti plasmids and carried by Ti plasmids or by other plasmids, or even by the bacterial chromosome, will be transferred from agrobacterial hosts to plant genomes, provided the *Agrobacterium* strain has functional *vir* and *chv* genes. This knowledge paved the way for the construction of small, so-called "binary" or "trans" Ti gene vectors (*30–33*). Highly developed binary systems have two elements: (i) A helper Ti plasmid from which the whole T region, including the border sequences, has been removed by deletion; this helper Ti plasmid provides trans-acting *vir* gene products. (ii) A broad-host-range plasmid that has cloning sites and marker genes for the identification or selection of transformed plants and is flanked in the proper polarity by right and left 25-bp T-region border sequences. An example (*32, 34*) of such a system has the plant marker genes, the multiple cloning sites, the appropriate bacterial marker genes, and the replication and mobilization functions of a broad-host-range plasmid, all united in a single small "vector cassette." The basic element of this cassette is a conditional mini-RK_2 replicon that is maintained and mobilized by trans-acting functions—derived from plasmid RK_2—independently introduced into appropriate *E. coli* and *Agrobacterium* hosts. This vector cassette can be inserted easily into various vector plasmids or into transposons and phage derivatives, which thereby acquire plant-gene vector functions. Genes introduced between the 25-bp repeats of the cassette become part of the T-DNA unit.

A further important expansion of the use of binary vector systems is based on the observation that *Agrobacterium* strains containing different T-DNA units—each flanked by 25-bp repeats—often transfer these T-DNA units independently of one another (*31, 35*). (Apparently the trans-acting *vir* functions can mobilize one or the other or both T-DNA units present in the same *Agrobacterium* host.)

Different *Agrobacterium* strains have different host ranges. A general strategy that can be used to develop the *Agrobacterium* vector strains best suited to transform a particular plant species or cultivar is the following. First, wild-type *Agrobacterium* strains are chosen that can efficiently transform the particular plant cultivar under study. A broad-host-range vector cassette is introduced into these strains. Plants carrying the selectable marker gene and capable of normal development are then used to produce transgenic plants harboring the T-DNA unit of the vector cassette but not the T region of the wild-type Ti plasmid. In this respect the use of *A. rhizogenes* as vector strains might be of particular importance (*35–37*). By applying these strategies, we find that many different plant species, including most of the major crop plants—with the possible

exception of some cereals—will lend themselves to genetic engineering via *Agrobacterium*-based Ti plasmid vectors. Transgenic plants obtained through the use of these plasmids can transmit the introduced genes to their offspring in a Mendelian fashion (*38*). A foreign gene introduced in this fashion into tobacco had high meiotic stability (*39*).

Chimeric Genes as Dominant Selectable Marker Genes

One of the first observations made after it had become clear that foreign DNA sequences, when inserted into the T region of Ti plasmids, were transferred to plant genomes was that, although the wild-type T-DNA genes coding for auxin and cytokinin or opine-synthesizing enzymes were functional in the plant genome, other bacterial genes were not transcribed (such as the Tn5 neomycin phosphotransferase II gene or the Tn7 methotrexate-resistant dihydrofolate reductase gene). A simple explanation for this discrepancy was that bacterial genes carried by wild-type T regions had acquired regulatory sequences specific for plant gene expression. Chimeric genes were constructed to test this idea and to provide efficient, dominant selectable marker genes for use with disarmed Ti plasmid vectors as well as to provide convenient reporter enzymes to facilitate the functional analysis of gene regulatory mechanisms in plants. These chimeric genes linked 5′ (upstream) and 3′ (downstream) sequences of opine synthase genes derived from Ti plasmid T regions with the coding sequences of bacterial genes coding for enzymes such as chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase II (NPTII), and methotrexate-resistant dihydrofolate reductase. All of these constructs were active in plant cells (*26, 40*), and it turned out that the NPTII enzyme was a good selectable marker for a number of plants as well as a good, but expensive and somewhat messy (high radioactivity), reporter enzyme. The CAT enzyme, in contrast, was a poor selectable agent but a convenient reporter enzyme, especially in tobacco, which has little or no background CAT activity.

Today other transcription signal sequences are commonly used to drive the expression of selectable marker genes such as the 35*S* and 19*S* RNA promoters from the cauliflower mosaic virus (*41*) and a small, dual-promoter fragment of 479 bp derived from the T_R-DNA of octopine Ti plasmids (*42, 43*). The latter expression system has the advantage that one side of the dual promoter can be used to drive a selectable marker gene, whereas the other side can be used to drive a test gene. Both transcriptional activities seem to be unresponsive to position effects (*43*).

In addition, new selectable marker genes as well as new and more powerful reporter enzymes have been developed. The most useful selectable marker genes, in addition to NPTII, appear to be hygromycin phosphotransferase (HPT) (*44*), mouse methotrexate-resistant dihydrofolate reductase (*45*), the bleomycin resistance gene from Tn5 (*46*), and a phosphinotricine acetyltransferase (*47*). Two types of particularly attractive reporter enzymes have recently been developed and tested: one is expressed by the *E. coli* β-glucuronidase gene (GUS) (*48*) and the other is luciferase, either from fireflies (*49*) or from *Vibrio harveyi* (*50*). Expression of GUS can be accurately measured in fluorometric assays on very small (milligram) amounts of transformed plant tissues.

Although expression of luciferase has not, thus far, yielded plants that shine brightly in the dark, this enzyme activity can be assayed accurately in different plant tissues by simple luminometer measurements. Of particular interest is the observation that the Lux alpha and the Lux beta polypeptide subunits from *V. harveyi*, which together catalyze the oxidation of long-chain fatty aldehydes, are functionally assembled in plant cells when expressed from "translational-transcriptional" cassettes based on the dual 1′2′ T_R promoters (*50*). A gene construct fusing the coding sequences of the alpha and beta subunits in a single translational unit yields an active luciferase (*51*).

Enhancer and Silencer Sequences

The next logical step was to dissect plant genes in order to assess the complexity of the cis-acting sequence motifs involved in the regulation of gene expression in plants. Some of the first genes to be analyzed at the molecular level were T-DNA genes that participate in opine synthesis (*52*). These studies showed why these genes, although of bacterial origin, were active in plants. They exhibited the common features of eukaryotic promoters such as TATA sequences approximately 30 bases and CAAT sequences 60 to 80 bp on the 5′ side of the start of transcription. These and perhaps an AGGA consensus sequence replacing the CAAT motif were later detected in most plant genes (*53*). The fact that the opine synthase genes and other T-DNA genes (*54*), as well as plant genes such as the maize zein genes (*55*), do not have introns indicates that intervening sequences, although occurring in many plant genes, are not essential for gene expression in plants. This also explains why chimeric genes lacking introns have been found to be functional in plants.

The consensus AATAAA sequence was found to be correlated with polyadenylation in plant genes (*54, 56*). Having established that the general structure of plant genes is essentially similar to that of other eukaryotic genes, the next goal was to identify plant-specific regulatory sequences. The first question asked was whether or not such regulatory sequences were located in the immediate upstream vicinity of a number of regulated plant genes and whether or not these regulatory sequences had the general properties of transcription "enhancers" or "silencers." Genes involved in photosynthesis, in stress response, and in organ specificity were considered in finding the answer to this question.

Photosynthetic genes. Most proteins that contribute to photosynthesis in chloroplasts are coded for by nuclear genes. Two typical and well-studied examples are genes coding for the small subunit of ribulose diphosphate carboxylase (SS *rbcS*) and genes coding for the chlorophyll a/b binding protein of the light harvesting complex (*LHCP* a/b). The proteins are synthesized on cytoplasmic ribosomes as precursor polypeptides with signal peptide sequences and are transported into chloroplasts (*57*).

These genes were particularly attractive as model systems to study regulation of gene expression in plants because several signal transducing systems appear to participate in their regulation. First, some of these genes react to changes in environmental conditions—that is, to fluctuations in ambient light quality and flux. Pigments like phytochrome and, in the *rbcS* gene of pea, also a blue light receptor, take part in the regulation of this light-mediated gene expression [for review see (*58*)]. Second, these genes are developmentally controlled, and it has been shown that the nuclear gene is expressed in response to changes in chloroplast structure or function [(*59*) and the work discussed here]. Sequences contained within the first few hundred base pairs upstream from the transcription initiation site of these genes are sufficient to control light-inducible phytochrome and green chloroplast–dependent gene expression, as demonstrated by using these upstream sequences to drive the expression of reporter enzymes from chimeric genes introduced into transgenic tobacco plants. Thus, it was first shown that a fragment

of 973 bp derived from a pea *rbcS* gene could drive the light- and chloroplast-dependent expression of a CAT reporter gene (*60*). In subsequent work (*61*), a 280-bp sequence, from −330 to −50 relative to the transcription start of a pea *rbcS*, was shown to participate in light inducibility.

That the light-inducible cis-acting upstream regulatory sequences of the pea *rbcS* have the general properties of transcription enhancers was demonstrated (*62*) by fusing a fragment derived from the upstream −90 to −973 region of the pea *rbcS ss*3.6 gene either to a truncated homologous promoter or to a truncated heterologous (nopaline synthase) promoter and driving the expression of CAT as a reporter enzyme. The regulatory upstream sequence was able to turn the homologous as well as the heterologous truncated promoter into a photoregulated unit. The light enhancement occurred equally well whether the regulatory sequence was fused in its original orientation (−973 to −90) or in the opposite (−90 to −973) orientation relative to the TATA box of the truncated promoters.

A weaker but clear-cut enhancement was also observed when the enhancer segment was fused in either orientation to the 3′ end of the reporter gene driven by the truncated homologous promoter. A similar analysis of the upstream regulatory sequences of the pea *LHCP* a/b gene not only confirmed the existence of light-regulated enhancer sequences but in addition demonstrated that a 247-bp segment of upstream flanking sequences (−347 to −100) of this gene combines enhancer and silencer properties (*63, 64*). Transgenic tobacco plants containing a chimeric gene, consisting of a promoter derived from the nopaline synthase gene and the Tn5 NPTII coding sequence (*pNOS-NPTII*), express this gene equally well in their leaves and in their roots and also equally well whether or not the plants have been illuminated or kept in the dark. In contrast, similar transgenic plants containing the same chimeric *pNOS-NPTII* gene, but with the 247-bp regulatory element of the pea *LHCP* a/b gene fused to the 5′ upstream end, express this chimeric gene in their leaves at a basal level when kept in the dark, but at a level five to eight times higher in light. However, in these plants the chimeric gene is silent in roots. The 247-bp regulatory element, therefore, functions as a light- and chloroplast-regulated enhancer in leaves and as a tissue-specific silencer in roots.

In order to test whether or not light-regulated enhancer elements are responsible for the light- and chloroplast-regulated expression of other nuclear genes as well, such a gene (of as yet unknown function) was isolated from a potato genomic library (*65*) and called ST-LSI. This is a light-inducible single-copy gene, expressed in photosynthetically active tissues only. A DNA segment consisting of the −334 to +11 upstream sequences of the ST-LSI gene was shown to be able to drive the expression of a chimeric CAT gene in transgenic potato and tobacco plants. This regulatory element was sufficient to confer all qualitative regulatory traits of ST-LSI to the corresponding chimeric genes (*66*). That this element has enhancer-like properties was shown by fusing a DNA fragment, containing this element, in both orientations to a truncated 35*S* cauliflower mosaic virus promoter. Other cis elements located farther upstream (up to position 1600) were needed in a quantitative way to achieve maximal induction levels.

Destruction of chloroplasts by photooxidation, after treatment with the herbicide norflurazon resulted in a drastic reduction in the activity of all three light-induced nuclear genes under study—*rbcS, LHCP* a/b (*64*), and ST-LSI (*66*). A signal monitoring the developmental and functional integrity of the chloroplasts must therefore be involved in the regulation of the expression of these genes. It is interesting that this signal must also interact with the upstream cis-regulatory elements shown to be involved in the phytochrome-mediated photoregulation. It is likely that separate sequence motifs are responsible for the interaction with the different regulatory signals, but in the case of these three genes, the dissection of the upstream cis elements has not yet allowed a definitive functional assignment to different sequence motifs.

The chalcone synthase gene. The chalcone synthase gene (*chs*) is of particular interest because it is the key enzyme of the flavonoid-glucoside pathway in higher plants and, as such, is involved in several biosynthetic pathways, including the anthocyanin pigment biosynthetic pathway.

In those cases in which pigment formation is primarily a reaction to ultraviolet (UV) stress, the *chs* gene is induced by strong and prolonged UV-B irradiation. The *chs* gene was isolated from *Antirrhinum majus* and characterized (*67*). In transgenic plants containing chimeric reporter genes, the cis-acting regulatory sequences were located in the upstream region of the gene (*68*). At least two distinct and separate cis-regulatory control regions were identified: (i) a region upstream from −357 that influences maximum expression after induction and (ii) a UV-B light–responsive sequence contained within a 318-bp fragment located immediately upstream from the TATA box (*69*).

With this and other systems (see later), work was initiated to identify the putative sequence-specific nuclear factors that interact with the cis regulatory sequences to initiate transcription (*70*). Gel retardation assays showed that crude nuclear extracts of tobacco seedlings contain one or more proteins that bind specifically to a 47-bp direct repeat located between positions −564 and −661. Whereas the binding proteins in tobacco seedlings that had not been irradiated with UV-B light were located primarily in the cytosolic fraction, binding proteins extracted from irradiated seedlings were associated primarily with the nuclear fraction. It is therefore conceivable that UV induction results in the transport of regulatory DNA-specific proteins from the cytoplasm into the nuclear compartment (*71*). Deoxyribonuclease I footprinting (*72*) plus binding competition experiments with synthesized oligonucleotides revealed three sequences within the 47-bp repeat sequence that appear to be directly involved in the binding. Binding to the UV-B light-responsive sequence was also observed. Oligonucleotides representing the binding sequences within the 47-bp repeat did not compete for this binding, indicating that a separate binding factor was involved.

Organ-specific and stress-regulated genes. The tuber of potatoes is a remarkable plant organ. Storage proteins in seeds or tubers often fulfill functions in addition to serving as a protein reserve [see (*73*)]. The study of the genes coding for storage proteins is of particular interest because developmental, organ-specific, and environmental factors must all play a role in their regulated expression. We studied the regulation of expression of patatin genes and proteinase inhibitor II genes, both of which code for storage proteins in potato tubers.

Patatin is a trivial name for a group of 40-kilodalton (kD) glycoproteins that are the major storage proteins of potato tubers. Patatin is encoded by a multigene family. Under normal circumstances these genes are expressed predominantly in tubers and occasionally also in stems and roots, but never in leaves (*74–76*). Regulatory sequences located in the 5′ upstream regions of some of the patatin genes are responsible for the tissue-specific expression of patatin (*77, 78*). Perhaps the most striking observation demonstrating that 5′ upstream transcription regulatory sequences play a determining role in the specific developmental regulation of plant genes was made in the following way (*79*). The transcribed, as well as the 3′ downstream region, of a cloned patatin gene (*76*) was fused to the 5′ upstream region of the tobacco leaf–specific gene ST-LSI. This chimeric ST-LSI-patatin gene was introduced into tobacco plants with appropriate Ti plasmid vectors. Transgenic tobacco

plants carrying the ST-LSI-patatin gene contained properly spliced messenger RNA (mRNA) (the patatin gene contains six introns) and a 42-kD stable protein that reacted with antiserum to patatin. The expression was tissue-specific—highest in leaves, lower in stems, and undetectable in roots—exactly as for the ST-LSI gene itself. Because the patatin transcript was properly spliced and processed in tobacco leaves (notwithstanding its six introns), it follows that no organ- and species-specific factors take part in the post-transcriptional events required for the expression of this gene. It had been speculated that patatin might have a lipid acyl hydrolase activity (*80*). Since tobacco leaves are completely devoid of patatin, it was a straightforward matter to prove that the presence of patatin in the leaves of transgenic tobacco led to the appearance of a lipid acyl hydrolase activity in these tissues (*79*).

Among tuber-specific complementary DNA (cDNA) clones derived from potato mRNA, some were found to correspond to proteinase inhibitor–II (PI2) mRNA (*75, 81*). Proteinase inhibitors are usually found in storage organs, such as seeds and tubers, of unwounded plants. Their presence in other tissues, such as leaves, is detected only when these tissues are severely damaged by chewing insects or by mechanical wounding (*82*). Wounding apparently releases a signal—probably oligosaccharides released from the plant's cell wall (*83*)—which in turn is responsible for the induction of the proteinase inhibitor gene, not only at the site of wounding but also systemically throughout the wounded plant. The proteinase inhibitor is supposed to be part of a defensive response of the plant against attacking insects (*84*). A copy of one of the potato PI2 genes was cloned (*81, 85*) and used to demonstrate that transcription of PI2 is under developmental (tuber-specific) as well as environmental (wound-inducible) control.

Tobacco apparently does not have genes homologous to the tomato or potato PI2 genes. It was, therefore, of interest to see whether the potato gene would be functional in transgenic tobacco plants. Whereas little or no RNA homologous to PI2 mRNA was detected in unwounded tissues, mechanical wounding or treatment of detached leaves with oligosaccharides led to the presence of high levels of PI2 mRNA in these tobacco tissues. Wounding of one particular leaf led to a systemic induction of PI2 mRNA in other unwounded leaves, as well as in unwounded stem and roots (*86*). These observations demonstrated that tobacco, though lacking genes homologous to PI2, nevertheless has the capacity to regulate the expression of the potato gene in the same complex manner as potato. Apparently wound-induced signals are similar in different plants and probably serve to regulate the expression of different genes in different plants. In order to define the regulatory sequences controlling the transcription of PI2 genes, a chimeric gene, with CAT as a reporter enzyme–coding sequence, was fused at its 5′ upstream end to a 1-kilobase-pair (kpb) fragment derived from the 5′ region of a potato PI2 gene and at its 3′ end to a 260-bp fragment derived from the 3′ region of the same PI2 gene. Transgenic tobacco plants containing this chimeric gene were shown to exhibit a wound-inducible CAT activity (*87*). Replacing the 3′ end with sequences unrelated to PI2 abolished the wound-inducible expression, indicating that in this instance regulatory sequences might be located both 5′ and 3′ to the PI2 coding sequence.

Although the PI2 gene is expressed as a result of wounding in most tomato and potato tissues, it is also expressed in unwounded potato tubers. The question, therefore, arises whether gene expression in general is affected by the wounding of potato tubers. Although the steady-state pattern of proteins in tubers was not markedly affected 18 hours after mechanical slicing, a different picture emerged when proteins made in vitro by mRNA from wounded versus unwounded potato tubers were examined. Patatin disappeared and prominent ~20-kD proteins were apparently induced. By differential hybridization two different cDNA clones (wun 1 and wun 2) were obtained representing mRNAs specifically induced by wounding of potato tubers (*78, 88*). No expression of these clones could be detected in unwounded tubers. However, 30 minutes and 4 hours after wounding, respectively, mRNA from wun 1 and wun 2 became detectable, with maximum expression after 8 to 14 hours. Preliminary evidence suggests that wun 1 and wun 2 code for the previously observed prominent 20-kD proteins. In sharp contrast, the steady-state level of patatin mRNA was drastically reduced and barely detectable as little as 30 minutes after wounding. The induction of wun 1 and wun 2 mRNA and the suppression of patatin mRNA by wounding were shown to be due, respectively, to induction and suppression of the initiation of transcription. It is likely that wun 1 and wun 2 correspond to single genes in the haploid potato genome, and the corresponding genes have been isolated; wun 1 and wun 2 are also expressed in wounded stems and to a lesser extent in leaves and roots. Whereas in the case of PI2 gene, the wound-induced signal appears to be a polysaccharide, this does not seem to be so for wun 1 and wun 2. The suppression of patatin seems to be mediated through ethylene (*88*).

Heat-inducible genes. The structural analysis of heat-shock genes from animals and plants has revealed that the 5′ region of these genes contains a conserved heat-shock consensus sequence or heat-shock element (HSE) [for review see (*89*)]. These HSEs contain the cis-regulatory sequences responsible for the heat-inducible regulation of gene expression (*90*). A chimeric gene was constructed with HSEs from the *hsp*70 gene of *Drosophila* linked to the coding sequence of a reporter enzyme sequence. Transgenic tobacco plants expressed this chimeric gene, and its expression was shown to be heat-regulated in callus tissue, roots, stems, and leaves, but not in pollen (*91*). For these and other parameters, the *Drosophila* HSE regulates expression in much the same way as for endogenous plant heat-shock genes (*92*). Apparently a tobacco heat-shock activator factor is capable of properly recognizing the *Drosophila* HSE cis-acting regulatory element. The mechanism underlying this type of stress response must, therefore, have been tightly conserved throughout evolution.

Genes induced by fungal elicitors. Treatment of cultured parsley cells with a hyphal cell wall preparation (elicitor) from a plant pathogenic fungus leads to the excretion of coumarin derivatives with antifungal activity (*93*). This reaction is regarded as typical for some of the mechanisms used by plants in defense against pathogen attack. Several of the genes involved in the synthesis of the antifungal products (phytoalexins) have been isolated (*94*). The analysis of signal transduction and gene activation in this system will benefit greatly from the demonstration (*95*) that protoplasts of a parsley cell culture have retained the capacity to react specifically to elicitors by inducing the transcription of genes involved in phytoalexin synthesis. Such parsley protoplasts can also be used to study the regulation of chimeric genes in transient expression assays after DNA uptake.

Genes induced by symbiotic bacteria. The symbiotic association between soil bacteria such as *Rhizobium* and plants belonging to the family of Leguminosae leads to the formation of very specialized organs known as nodules. Approximately 30 different plant-encoded proteins (nodulins) are specifically synthesized during the development of root nodules. The best known of these nodulins is leghemoglobin. Different genes coding for four different leghemoglobin proteins have been cloned and found to be organized in two clusters (*96*). When a chimeric gene that linked the 5′ and 3′ regions of the leghemoglobin *lbc*3 gene from soybean to the coding sequence for a CAT reporter enzyme was introduced into an heterologous legume plant (*Lotus corniculatus*), no expression could be detected in any tissue of uninfected transgenic plants. When, however, roots of transgenic lotus plants were infected with *Rhizobi-*

um loti, a high level of CAT expression was detected at the precise stage of nodule development at which leghemoglobin is normally induced (*97*). The trans-acting regulatory factors responsible for this nodule-specific induction were identified by performing gel-retardation assays with different sequences derived from the 5′ region of the soybean *lbc*3 gene. Nodule extracts, but not root extracts, contained proteins that bound specifically to the previously determined cis-acting regulatory elements within the 5′ upstream region of *lbc*3. Two AT-rich sequence-motifs were shown, by binding competition experiments with defined oligonucleotides, to be directly involved in the binding of nodule-specific proteins (*98*).

Enhancer- and silencer-type cis-regulatory sequences were detected and partially characterized in all of the genes under study. In all cases it has been possible to construct expression vectors in which these regulatory elements were used to drive the regulated expression of foreign genes in plants.

Targeting of Foreign Proteins for Transport into Chloroplasts

A large fraction of the proteins found in chloroplasts are encoded by nuclear genes and synthesized on free cytoplasmic ribosomes as larger precursor proteins. The precursor proteins have an amino-terminal extension or transit peptide [for review see (*99*)]. The transit peptide is cleaved from the mature protein concomitant with translocation into chloroplasts. An effort was made to determine the relative role of transit peptide and mature protein in the efficiency and mechanism of translocation. Sequences encoding only the transit peptide of the SS *rbcS* gene (*100*) or sequences encoding this transit peptide as well as sequences encoding amino acids from the amino-terminal end of the mature protein (*101, 102*) were fused to NPTII as a reporter enzyme. Transgenic plants harboring these chimeric genes (*100, 101*) or fusion proteins made in vitro and combined with purified intact chloroplasts (*102*) were used to demonstrate that, whereas the transit peptide alone is sufficient for translocation of the fusion proteins, the presence of amino-terminal residues of the mature protein is essential for high-efficiency translocation.

Isolation of Plant Genes by Gene Tagging

Transposable elements, best known in plants through the work of Barbara McClintock (*103*) have been cloned and used as probes to isolate genes reversibly mutated by insertion of these transposable elements (*104*). The time-consuming step in this approach is the genetic analysis of the mutant plants. The molecular isolation of the tagged locus, however, is straightforward.

This approach was made available in plants for which no transposable elements are known or cloned when the maize controlling element *Ac* was transferred into tobacco by means of a Ti plasmid vector and shown to excise from its original location in the T-DNA and to integrate in the tobacco genome (*105*). By constructing an NPTII gene whose expression is prevented by the insertion of an *Ac* element, a convenient system for the phenotypic assay of *Ac* transposition activity in foreign host plants was developed (*106*).

Agrobacterium-mediated transformation itself, leading to random integration of T-DNA segments, is also useful as a gene tag. Some Ti plasmid vectors have been specifically designed for this purpose (*34, 107*). The potential advantage of T-DNA–based tags is that in most transgenic plant cells the T-DNA is inserted at a single locus. The disadvantage, relative to transposable elements, is the lack of reversion of the mutant phenotype, which is the most convenient way to demonstrate that the mutant phenotype is the direct consequence of insertion of the gene tag.

The most important gene that has, thus far, been isolated via the gene-tagging approach is the C1 locus of *Zea mays* (*108*). This is a regulatory gene controlling anthocyanin biosynthesis. The C1 gene encodes two overlapping transcripts, one of which was cloned as a complete cDNA. Its sequence (*109*) revealed a protein sequence that is homologous to the DNA-binding *myb* proto-oncogene product and having a domain structure resembling the yeast GAL4-encoded transcriptional activator (*110*).

Gene Transfer as an Additional Tool in Plant Breeding

Although transgenic plants actually expressing chimeric genes were first reported in 1983, we are already witnessing the practical use of these methods for agriculturally relevant plant breeding. Not surprisingly, all of these early examples have to do with the transfer and expression of single genes and several of these are derived from bacterial genes. Probably the most advanced examples involve genes protecting crop plants against nonselective herbicides (*111*). Other examples are relevant for insect control (*112*) or tolerance to viral infections (*113*). These are but the first examples of major applications, but they demonstrate the validity and the potential of this approach. It might be argued that the host range of *Agrobacterium* is limited and that some of the most important crops, such as cereals, are not amenable to the described gene transfer techniques. However, in the near future, techniques will be available to introduce genes into almost any crop, including the major cereals.

It appears that some of these techniques will be fairly simple and perhaps somewhat mundane when compared to the refined mechanisms used by *Agrobacterium* itself. Indeed direct injection, with a hypodermic needle, of DNA coding for a selectable marker gene (NPTII) into the tillers of rye just underneath a developing inflorescence, apparently led to the uptake of the DNA in the genome of developing germ cells. From among 3000 seeds obtained from a few hundred injected plants, three independent transgenic plants that contained and expressed the transferred kanamycin-resistant gene were obtained (*114*). An even more direct way to introduce genes into cereals is suggested by the observation that mechanically isolated mature wheat embryos derived from dry seeds are able to take up DNA by imbibition of a DNA solution and express a chimeric NPTII gene transiently. These DNA-treated embryos can readily be cultured into full plants (*115*). Whether offspring from plants derived from these DNA-treated embryos have inherited integrated copies of the introduced DNA remains to be determined.

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Migration and Western Europe: The Old World Turning New

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The 1960s meant a historical turn of Western Europe, becoming an immigration area. Net immigration has been concentrated to some of the prosperous Western European countries and has been mainly determined by the demand of their particular national labor regimes. The size of alien employment has been very differently affected by the 1973 crisis, but a multiethnical society will remain a novel feature of most Western European countries. Political abdication from full employment and technological change makes a ghetto of un(der)employment a likely prospect of a large part of the second generation of recent immigrants into Western Europe.

POST–WORLD WAR II MIGRATIONS HAVE TRANSFORMED Western Europe to an extent and a depth which Europeans—citizens, politicians, official statisticians, scholars—are still only beginning to cope with. Currently there are more foreign-born residents of Sweden (7.8% in 1985) or of the United Kingdom (7% in 1983) than of the United States (6.0%, 1981–1985) (*1*). The proportion of resident aliens at the end of 1984 or 1985 was 9.1% in Belgium, 8.1% in France, 14.4% in Switzerland, and 7.1% in West Germany (*2*).

Recent migrations have changed the position of Europe in the world and inter-European relations as well as the internal structure of Northern and Central European societies. Of old, at least since the beginning of the conquest of the Americas, Europe was a continent of emigration. Between 1850 and 1960 it has been estimated that about 55 million people, equivalent to about 18% of the Western European population in 1910, left Western Europe for other continents (*3*). However, after the end of World War II, Western Europe became a region of immigration.

If we disregard the force migration of Germans from Eastern Europe into West Germany in the aftermath of the defeat of the Nazis—an exodus of massive proportions, landing 8 million people in the Federal Republic by 1950, 16% of the total population of the country—the historical turn took place in two stages. The first one occurred in the 1950s, resulting in a migration surplus in Western Europe (including Greece) of almost 500,000 for the decade (*4*). But this first wave of net immigration had a rather special character that might be thought of as temporary. It was heavily dominated by East Germans moving into West Germany, 3 million between 1950 and 1960 (*5*).

The second phase, however, showed decisively that fundamental structural changes were taking place. Counting in decades, the key period runs from 1964 to 1973. Then migration for all the countries of the area (including Greece) taken together showed a surplus of 2,314,000 (*6*). In France and Germany the demographic impact of immigration was quite dramatic: 37% of French population growth between 1964 and 1973 was due to immigration, and at its recent height, in 1970, net immigration meant a population increase of 0.35%. At its peak in Germany, in 1968–69, net immigration each year added 0.9% to the total population, and for the years 1964 to 1973, immigration accounted for 90% of population growth (*6*). As a yardstick for comparison, take a figure from U.S. immigration at its peak. In 1913, net arrivals of immigrants from overseas corresponded to 0.9% of the then American population (*7*), the same as net migration into West Germany in 1968 and 1969.

The turn of Western Europe from a people-exporting to a people-importing area was the product of two migratory changes. One, and the more important one, was the opening of immigration routes from outside Western Europe. By the mid-1980s, there were from major ethnic groups about 6.7 million registered non–Western European resident aliens and ex-colonial immigrants in the Western European countries of significant gross immigration, about 2.2% of the total population (*8*). This opening was above anything else a reversal of the old colonial relationship of European settlement. Modernizing social changes combined with little or truncated development, after as well as before independence, turned the old colonizing countries into ex-colonial labor markets. Of the 6.7 million mentioned, 3.9 million are ex-colonials. (Returned European settlers are not counted here.)

The second process involved has been a redirection of Western European emigration from intercontinental to intracontinental migration. Finnish emigration was redirected already after World War II to Sweden. But for Greece, Italy, Portugal, and Spain the turn

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