Chapter 3

AGROBACTERIUM AND PLANT BIOTECHNOLOGY

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Abstract. *Agrobacterium*-mediated transformation has revolutionized agriculture as well as basic research in plant molecular biology, by enabling the genetic modification of a wide variety of plant species. Advances in binary vector design and selection strategies, coupled with improvements in regeneration technology and gene delivery mechanisms, have dramatically extended the range of organisms, including grains, that can be transformed. Recent innovations have focused on methods to stack multiple transgenes, to eliminate vector backbone sequences, and to target transgene insertion to specific sites within the host genome. Public unease with the presence of foreign DNA sequences in crop plants has driven the development of completely marker-free transformation technology and molecular strategies for transgene containment. Among the many useful compounds produced in genetically modified plants are biodegradable plastics, primary and secondary metabolites with pharmaceutical properties, and edible vaccines. Crop plant productivity may be improved by introducing genes that enhance soil nutrient utilization or resistance to viral,

bacterial, or fungal diseases. Other transgenes have been shown to confer increased tolerance to many of the environmental constraints, including drought, extreme temperature, high salinity, and heavy metal soil contamination, faced by resource-poor farmers attempting to cultivate marginally arable land. Early applications of plant biotechnology focused primarily on traits that benefit farmers in industrialized regions of the world, but recent surveys document the degree to which this pattern is changing in favor of modified crops that contribute to enhanced ecological and human health. Documented decreases in the use of pesticides attributable to genetically engineered plants are harbingers of the health and environmental benefits that can be expected from transgenic crop plants designed to decrease reliance on harmful agrochemicals. As one thread in a network that also includes integrated pest and soil fertility management, a reduced emphasis on monoculture, and traditional crop breeding, plant genetic modification has the potential to help those who currently suffer from inadequate access to a full complement of nutrients. The development of "golden rice" illustrates the possibility to imbue a plant with enhanced nutritional value, but also the challenges posed by intellectual property considerations and the need to introduce novel traits into locally adapted varieties. Implementation of plant genetic modification within a framework of sustainable agricultural development will require increased attention to potential ecological impacts and technology-transcending socioeconomic ramifications. Successful technology transfer initiatives frequently involve collaborations between scientists in developing and industrialized nations; several non-profit agencies have evolved to facilitate formation of these partnerships. Capacity building is a core tenet of many such programs, and new paradigms for incorporation of indigenous knowledge at all stages of decision-making are under development. The complex (and sometimes controversial) social and scientific issues associated with the technology notwithstanding, *Agrobacterium*-mediated enhancement of agronomic traits provides novel approaches to address commercial, environmental, and humanitarian goals.

1 INTRODUCTION

Plant biotechnology has had a dramatic impact on agriculture, and on public awareness of the role of the private sector in industrial-scale farming in developed countries. This chapter focuses on the seminal contributions of *Agrobacterium tumefaciens* to this technological revolution, and on the applications of genetic engineering that continue to expand the limits of plant productivity. *Agrobacterium*-mediated transformation has yielded a stunning array of transgenic plants with novel properties ranging from enhanced agronomic performance, nutritional content, and disease resistance to the production of pharmaceuticals and industrially important compounds. Many of these advances have been made possible by creative and elegant methodological innovations that have enabled gene stacking, targeted mutagenesis, and the transformation of previously recalcitrant hosts.

Transgenic plants are not a panacea for global food shortages, distributional failures, or other structural causes of poverty. They can, however, have a positive impact on both human and environmental health. Agricultural biotechnology's image has been tarnished by the perception that it fails to address the needs of the world's hungry, and indeed most of the commercial products to date represent technology that is inappropriate for subsistence farmers (Huang et al., 2002a). As this chapter documents, there is ample potential for genetically modified plants to ameliorate some of the constraints faced by resource-poor farmers. Even modest enhancements of agronomic traits have the potential to help farmers overcome endemic problems such as lack of food security, limited purchasing power, and inadequate access to balanced nutritional resources (Leisinger, 1999). Many of these innovations will come from public sector research, and the vast majority of the applications described herein have in fact emanated from basic investigations and collaborative product-oriented research originating in the non-profit realm. As plant biotechnology research moves forward and outward to include more stakeholders in developing countries, it will continue to complement, rather than to replace, plant breeding (Morandini and Salamini, 2003). Whether these applications will enjoy increased public acceptance depends in large part on whether they progress in a context of sustainable development that incorporates integrated natural resource management and understanding of the socioeconomic realities of small-scale farming (Serageldin, 1999).

2 THE DEVELOPMENT OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION

The first demonstration that *A. tumefaciens* could be used to generate transgenic plants (Barton et al., 1983 and see Chapter 2) heralded the beginning of a new era in agriculture as well as in plant molecular biology research. Plant transformation entails not only delivery and integration of engineered DNA into plant cells, but also the regeneration of transgenic plants from those genetically altered cells. Thus it was no accident that the earliest successes in plant genetic engineering occurred in species (e.g., tobacco, petunia, carrot and sunflower) that were both good hosts for *A. tumefaciens* and for which much was known about the conditions required to regenerate whole plants. Indeed, it has frequently been the plant tissue culture technology, rather than the transformation process itself, that has been the limiting step in achieving efficient genetic modification (Herrera-Estrella

et al., 2005). Through extensive experimentation, protocols have been established for *Agrobacterium*-mediated transformation and regeneration of many other host plants including cotton (Umbeck et al., 1987), soybean (Hinchee et al., 1988), sugarbeet (D'Halluin et al., 1992), rice (Hiei et al., 1994), maize (Ishida et al., 1996), sorghum (Nguyen et al., 1996; Zhao et al., 2000), wheat (Cheng et al., 1997), barley (Tingay et al., 1997), papaya (Fitch et al., 1993), banana (May et al., 1995), and cassava (Li et al., 1996). Generation of transgenic monocots using *Agrobacterium*, initially believed to be impossible, is now considered routine for particular cultivars of some monocot species. However, transformation of several agronomically important cereal genotypes still poses significant challenges and represents an area where considerably more research is needed (S.B. Gelvin, personal communication).

2.1 Requirements for generation of transgenic plants

Generally speaking, *Agrobacterium*-mediated transformation involves incubating cells or tissues with bacteria carrying the foreign gene construct of interest, flanked by border sequences. Plant cells in which the foreign DNA has integrated into the genome are selected and propagated via a callus stage before hormone-induced regeneration of a transgenic plant, in which each cell is derived from the genetically altered progenitor cell (Walden and Wingender, 1995). Over the past two decades, a number of techniques have been developed to improve the efficiency of *Agrobacterium*-mediated gene delivery: wounding the plant tissue by sonication of embryonic suspension cultures, by glass beads, or by particle bombardment; bombardment with microprojectiles coated with agrobacteria; and imbibing germinating seeds have all proven successful in at least one host species. Other approaches are summarized in Newell (2000). The totipotency of plant cells has allowed the transformation of many different cell types, although tissues from different plant species respond differently to culture conditions, so optimal culture and regeneration methods must be established for every host tissue and species (Walden and Wingender, 1995). Explants are often used as the target for transformation because they are less prone to changes in DNA methylation status, chromosomal rearrangements and other genetic and epigenetic alterations that occur in plant tissue culture and that result in somaclonal variation (Christou, 1996). Hormone-induced regeneration of transgenic plants from transformed explants can occur via organogenesis (the direct formation of shoots) or somatic embryogenesis (the generation of embryos that can

directly germinate into seedlings from somatic tissues). Most economically important plants, especially monocots, are regenerated using the latter approach, since callus is easily initiated from the scutellum of immature embryos (Hansen and Wright, 1999; Zuo et al., 2002). Delivery of the foreign DNA directly into meristematic tissue or immature embryos has also been found to limit somaclonal variation because it minimizes the amount of time in tissue culture (Walden and Wingender, 1995; Christou, 1996). A vacuum infiltration method, in which agrobacteria are applied to entire flowering *Arabidopsis*, was developed to avoid altogether the requirement for plant tissue culture or regeneration (Bechtold et al., 1993). More recently, this approach has been further simplified; in the "floral dip" process only the developing floral tissue is submerged into a solution of agrobacterial cells, and the labor-intensive vacuum infiltration step is eliminated (Clough and Bent, 1998).

In addition to susceptibility to *Agrobacterium* infection and the ability to regenerate whole plants from transformed cells, a third requirement for successful genetic modification is an efficient selection method for plant cells containing integrated trans-DNA (Chung et al., 2006). As described in Chapter 2, the first demonstration that the *Agrobacterium* lifestyle could be exploited to generate transgenic plants relied on a bacterial strain in which the T-DNA was still partially intact. Identification of transformed cells was achieved by screening for the production of nopaline (Barton et al., 1983). Published almost simultaneously, a number of other papers provided several key improvements on this initial transformation system. Foremost among these was the use of T-DNA-derived promoters and 3' regulatory regions (from the nopaline synthase gene) to drive *in planta* transcription of a bacterial antibiotic resistance gene such as chloramphenicol acetyltransferase or neomycin phosphotransferase (*nptII*). Expression of these chimeric genes in the plant allowed the selection of antibiotic-resistant transformed plant cells and hence the elimination of the opine synthesis genes from the transferred DNA (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983a; Herrera-Estrella et al., 1983b). Phenotypically normal and fertile plants were regenerated from the resistant calli, and the resistance trait was passed to the progeny in a Mendelian fashion (De Block et al., 1984; Horsch et al., 1984). Two innovations in vector design circumvented the difficulties associated with cloning into the very large Ti plasmid. Zambryski et al. (1983) replaced the entire oncogenic region of the Ti plasmid with the standard cloning vector pBR322; DNA sequences of interest cloned into a pBR vector could thus easily be introduced into the T-region by a single recombination event.

The labs of Schilperoort and Bevan designed binary vector strategies in which one broad-host range replicon carried the DNA to be transferred, while a second, compatible pTi-derived plasmid provided the *vir* functions required for DNA transfer (Hoekema et al., 1983; Bevan, 1984). Both of these systems provided enormous versatility because the DNA to be transferred could be easily manipulated in *E. coli*, and the demonstration that integration of these altered T-DNAs did not interfere with normal plant cell differentiation (Zambryski et al., 1983) opened the floodgates for the wave of plant genetic modifications that followed.

2.2 Binary vectors

In the two decades since their initial development, *Agrobacterium*-mediated transformation systems have undergone a number of refinements. Ease of DNA manipulation in *E. coli* has been achieved by modification of the replication functions on the binary vectors to enhance copy number, reduction in the size of the vectors (Hellens et al., 2000), and incorporation of convenient multiple cloning sites (Komari et al., 2006). The Overdrive sequence adjacent to the right border (RB) enhances T-DNA transfer (Peralta et al., 1986), and some binary vectors include this sequence (Hellens et al., 2000). In addition to the *nptII* gene originally used as the selectable marker, a variety of other selection schemes, including chimeric genes conferring resistance to methotrexate (Eichholtz et al., 1987) and hygromycin (Van de Elzen et al., 1985) have been developed, and several families of binary vectors now provide a choice of marker (Hellens et al., 2000). Many of the early binary vectors carried the selectable marker near the RB, where it would be transferred before the transgene of interest. In contrast, placement of the marker closest to the left border greatly diminishes the chance of selecting transgenic plants resulting from interrupted bacterium-to-plant DNA transfer that carry only the marker (Hellens et al., 2000). This strategy is especially important when introducing very large fragments of foreign DNA into plants. Binary bacterial artificial chromosomes (BIBAC) and transformation-competent bacterial artificial chromosomes (TAC) have been developed that allow the delivery of fragments of at least 80-150 kb (Hamilton et al., 1996; Shibata and Liu, 2000). Such large-capacity vectors are likely to prove particularly useful in identifying and confirming quantitative trait loci (QTLs) controlling agronomically significant characteristics such as crop yield, disease resistance, and stress tolerance (Shibata and Liu, 2000; Salvi and Tuberosa, 2005).

Binary vectors are typically used with so-called "disarmed" *Agrobacterium* strains, in which the virulence functions required for DNA processing and transfer are provided by a modified Ti plasmid lacking oncogenic DNA. Certain strains carrying the "supervirulent" Ti plasmid pTiBo542 exhibit greatly enhanced transformation efficiency (Jin et al., 1987), and the popular transformation strain EHA101 carries a disarmed version of pTiBo542 (Hood et al., 1986). Capitalizing on the discovery of a supervirulent pTi, super-binary vectors carry the *virB*, *virE,* and *virG* genes of pTiBo542 or the Ti plasmid from another supervirulent strain, Chry5 (Torisky et al., 1997). Super-binary vectors have provided critical improvements in transformation efficiency, and were a key factor in extending the host range of *Agrobacterium*-mediated transformation to the cereals in the 1990s (Komari et al., 2006). Practical technical information about binary and super-binary vectors and disarmed strains, along with email addresses and websites of contacts for those who wish to obtain these resources, has been compiled in two recent reviews (Hellens et al., 2000; Komari et al., 2006).

Many binary vectors use the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter to drive expression of the target gene (Chung et al., 2005), although the maize ubiquitin I promoter and the rice actin promoter/intron sequences are more frequently used for expression in monocots (Walden and Wingender, 1995). Alternative promoters exhibiting similarly high or even higher levels of constitutive transcription include a chimera derived from the octopine and mannopine synthase genes (Ni et al., 1995). Inducible and/or tissue-specific promoters provide the possibility of activating a transgene at the most favorable time of development or upon perception of certain environmental cues; use of such promoters can also prevent deleterious effects associated with constitutive production of a toxic product (reviewed in Gelvin, 2003b). A bidirectional promoter, permitting expression of a gene at either end, offers the potential to stack traits (Xie et al., 2001). A completely different strategy for the coordinated production of two proteins makes use of a virally derived polyprotein proteolytic processing peptide. A gene constructed from multiple coding regions separated by this 18-amino acid peptide gives rise to a polyprotein that is co-translationally self-processed to yield stoichiometric amounts of the individual proteins (de Felipe et al., 2006). This approach has even been used successfully to co-produce two proteins targeted to different subcellular compartments (François et al., 2004).

2.3 Transgene stacking

As researchers have moved beyond the introduction of simple traits conferred by a single gene, strategies have been developed that allow the coordinated manipulation of multiple genes in the same plant. The most basic approaches entail sequential sexual crossings or retransformation, and both of these have been used successfully, although they are time-consuming and prone to complications arising from independent segregation in subsequent generations if the introduced genes integrate at different loci (Halpin and Boerjan, 2003). Thus, methods that allow the introduction of multiple genes in one step and their co-integration are more desirable. Somewhat unexpectedly, co-transformation with two T-DNAs on the same or different plasmids within the same bacterium, or even in two different bacterial cultures that are mixed before co-cultivation, can yield remarkably high rates of co-transformation and even, on occasion, co-integration (Halpin and Boerjan, 2003). Such double-T-DNA systems have proven effective in manipulating two or more transgenes at a time in *Arabidopsis*, tobacco, rapeseed, rice, soybean and maize (Slater et al., 1999; Miller et al., 2002; Li et al., 2003). However, engineering of more complex metabolic pathways will require that even more transgenes be stacked. Recently, construction of transformation-ready cassettes was greatly simplified by the advent of binary vectors compatible with the GATEWAY technology, which is based on site-specific recombination between two DNA molecules carrying complementary recombination sites (Invitrogen; http://www.invitrogen. com). The first generation of GATEWAY-compatible destination binary vectors allowed overexpression of a gene, with or without a visible marker, construction of N- or C-terminal Green Fluorescent Protein fusions, or post-transcriptional gene silencing of a target gene (Karimi et al., 2002). This elegant system was subsequently extended to accommodate simultaneous assembly of up to three DNA fragments onto one binary vector (Karimi et al., 2005). Alternatively, as many as six genes can be inserted into a single binary vector containing sites for rare-cutting restriction enzymes (Goderis et al., 2002). Transfer of up to 10 genes into the rice genome has also been achieved using a TAC-based vector, with assembly of the various inserts mediated by the Cre/*lox*P recombination system and homing endonucleases (Lin et al., 2003). Like the GATEWAY-based system, both of these approaches rely on auxillary donor vectors. Perhaps the most advanced system currently available is the pSAT series of vectors, which offers unprecedented versatility in the choice of restriction sites, plant selectable markers, and the possibility of constructing fusions with any of six different autofluorescent tags (Tzfira

et al., 2005). An added benefit of the newest pSAT vectors is the opportunity to choose from among a variety of promoter and terminator sequences for combined expression of the target, selection and reporter genes. This is a critical advantage, since diversity among promoters and terminators can reduce the risk of transgene silencing in plants (see section 2.6 and Chung et al., 2005).

2.4 Marker genes and marker-free transformation

In response to concerns about the potential for transfer of antibiotic-resistance genes to gut microbes, a number of antibiotic-free marker systems have been developed. Herbicide resistance genes are also in widespread use as selectable markers [see Hare and Chua (2002) for examples], although they too pose perceived dangers to health and the environment (Hood, 2003). Rather than killing non-transformed cells (negative selection), one can also use a positive selectable marker that confers on transformed cells a growth or metabolic advantage (Hohn et al., 2001). For example, introduction of the phosphomannose isomerase gene rescues plants from the growth inhibition associated with mannose (Negretto et al., 2000). The desire to transform recalcitrant plant species has driven the development of other positive selection markers, including native plant genes conferring resistance to bacterial pathogens (Hood, 2003). Erikson et al. (2004) devised a clever scheme in which introduction of a single gene, encoding a D-amino acid oxidase, allows either positive or negative selection. Selection can be exerted by spraying certain D-amino acids onto soil-grown seedlings; transformed plants exhibit resistance to toxic D-amino acids (e.g. D-alanine or D-serine), whereas only wild-type plants survive exposure to innocuous D-amino acids (D-isoleucine or D-valine) that are converted by the enzyme to toxic keto acids. This dual selection scheme has the distinct advantage of permitting positive selection for transformation, followed by negative selection to identify desired plants that have lost the selectable marker gene (Scheid, 2004). Other positive selection markers, such as the agrobacterial or plant cytokinin synthesis isopentyl transferase (*ipt*) genes, promote regeneration of shoots from transformed calli or explants in the absence of critical growth regulators (Zuo et al., 2002). Inducible expression circumvents the developmental defects associated with constitutive overexpression of *ipt* (Kunkel et al., 1999). However, over-produced cytokinins can cause spurious regeneration of non-transformed neighboring cells. Thus, introduction of cytokinin signal transduction pathway genes may be a preferable selection scheme to avoid

non-transgenic escapes (Zuo et al., 2002). Finally, marker-free transformation was achieved in potato using a virulent *A. tumefaciens* and PCR screening for successfully altered shoots (de Vetten et al., 2003).

Non-antibiotic/herbicide resistance markers address concerns about potential health and ecological risks, but they still suffer from other shortcomings: the selection scheme can have negative consequences for plant cell proliferation and differentiation, and multiple transgenes cannot be stacked through sequential retransformations using the same marker gene (Ebinuma et al., 1997). These constraints have spurred the development of various methods to remove the marker gene after transformation. In one such strategy, the selectable marker is inserted into a transposable element, allowing transposition-mediated loss of the marker after selection of the transformed plants (Ebinuma et al., 1997). Alternatively, one can place the transgene of interest on the transposon; in this case, transposition to new sites not only separates the transgene from the marker gene, but also provides an opportunity to obtain a series of plants with varying transgene loci, and potentially differing expression levels, from a single transformant (Hohn et al., 2001). Excision of the marker gene can be achieved by flanking the marker gene with recombination sites and incorporating the cognate site-specific recombinase on the transgenic unit or crossing with a second plant carrying a recombinase-encoding transgene; in either case counter-selectable marker genes can be included within the "elimination cassette" to ensure excision (Hohn et al., 2001). Among the popular recombinase options are the bacteriophage P1 Cre/*lox* system (Dale and Ow, 1991) and the yeast Flp/FRT system (Hare and Chua, 2002). In the simplest case, the marker gene is excised in the F1 generation and the recombinase gene is removed through segregation in the subsequent generations (Gilbertson et al., 2003). Inducible (Zuo et al., 2001) or transient (Hare and Chua, 2002) expression of the recombinase, or transient exposure of the plants to agrobacteria that deliver the recombinase (Vergunst et al., 2000) avoids the need to eliminate the recombinase gene through genetic segregation. Marker excision through recombination has also been achieved using bacteriophage λ *attP* sequences as the flanking DNA (Zubko et al., 2000). Surprisingly, introduction of a recombinase was not required, making this strategy especially attractive for crops that are propagated vegetatively and for which it would therefore be difficult to eliminate the recombinase gene through subsequent crosses (Zubko et al., 2000). Finally, marker genes can be eliminated by co-transforming with tandem marker- and trans-genes, each flanked by its own border sequences, on a single binary vector (Matthews et al., 2001). *Agrobacterium*-mediated delivery of

such a construct can lead to independent integration events in the same plant cell, and the marker can therefore be segregated away from the transgene (Hohn et al., 2001).

2.5 Elimination of foreign DNA other than the transgene of interest

Agrobacterium-mediated transformation frequently results in the unintentional introduction of vector backbone sequences (Kononov et al., 1997; Wenck et al., 1997; see also Chapter 12 in this volume for a more detailed description of T-DNA integration patterns). Like marker genes, backbone sequences in a transformed plant are undesirable from a commercial perspective. Incorporation of a lethal gene into the non-T-DNA portion of a binary vector causes a dramatic decrease in the percentage of tobacco, tomato, and grape plants carrying a vector-borne reporter gene, without markedly reducing the overall transformation efficiency (Hanson et al., 1999). Thus, this strategy can be an efficacious way to enrich for T-DNA-only transformants in situations where the presence of vector backbone sequences would be problematic. Alternatively, a systematic comparison of multiple agrobacterial strains and T-DNA origins of replication revealed that integration of "backbone" sequences can almost be eliminated if the border-flanked transgene is located on the bacterial chromosome (H. Oltmanns and S.B. Gelvin, personal communication).

As the preceding discussion implies, the presence of foreign DNA (in addition to the desired transgene itself) may or may not increase health or environmental risks associated with a transgenic plant, but it frequently poses public relations problems, and in fact accounts for much of the dissatisfaction that has led to widespread public rejection of genetically modified crops (Rommens, 2004). In addition to the transgene and the selectable marker, other non plant-derived genetic elements needed for stable transgene expression frequently include promoters, transcriptional terminators, and of course the T-DNA borders. On average, genetically engineered plants approved for commercialization contain ten genetic elements from non-plant sources; typically these have come from bacteria or viruses, or are synthetic sequences. In an effort to decrease dependence on non-plant genetic material, researchers have identified a variety of plant genes associated with agronomically relevant traits such as disease resistance, insect resistance, herbicide tolerance, enhanced storage or nutritional characteristics, and stress tolerance. Additionally, hundreds of plant promoters, both constitutive and tissue-specific, and transcription termination sequences

for most important crop species are now available (Rommens, 2004). Rommens et al. (2004) used database searches and PCR to isolate plant sequences that resemble T-DNA borders. Strikingly, these "P-DNA" sequences function to mediate DNA transfer to potato. Using transient expression of a selectable marker carried on a conventional "Life-Support" T-DNA to block proliferation and regeneration of cells that had not received exogenous DNA, these authors were able to document integration events comprised of marker-free P-DNA. Such all-native transformations can be obtained by co-infecting with two *Agrobacterium* strains (one carrying the P-DNA binary and the other providing the Life-Support T-DNA vector), either simultaneously or sequentially, but the frequency of marker-free P-DNA insertion is as high or higher (depending on the host species) if both binaries are present in a single bacterial strain. By selecting against backbone integration events as described above, this approach can be used to generate completely marker-free transgenic plants at a frequency that is consistent with commercial scale production (Rommens et al., 2004).

2.6 Influence of position effects and gene silencing on transgene expression levels

The fact that *Agrobacterium*-mediated DNA integration into the host plant's genome occurs by illegitimate recombination (see Chapter 11) has profound implications for the generation of transgenic plants. Expression levels of the transgene can be dramatically affected by the chromosomal context of the integration site, and insertional disruption of an active host gene can have unintended phenotypic consequences on the resulting plant (Kumar and Fladung, 2001). Targeting the insertion event (see section 2.7) to a specific innocuous, yet transcriptionally active, locus could provide a way to circumvent this variability, particularly if insertions at the same genomic position routinely exhibit similar expression levels (Gilbertson et al., 2003). In at least one study, targeted insertions into the same site did result in reproducible transgene expression levels; however, in nearly half the insertion events, partial or complete silencing of the transgene was observed (Day et al., 2000). Such "position effects" are consistent with our growing appreciation for the striking variability and unpredictable nature of transgene expression levels, a ubiquitous phenomenon in almost all eukaryotes. In the face of repressive influences exerted on transgenes by neighboring genes or the surrounding chromosomal structure, the standard, albeit costly, approach has been to generate enough transgenic plants to find some with the desired level of expression (Hansen and Wright, 1999).

There is some hint that naturally occurring matrix attachment regions (MARs), sequences that associate with the nuclear matrix and mediate looping of DNA, may stabilize expression levels (Han et al., 1997; Iglesias et al., 1997), although the benefit of flanking *Agrobacterium*-delivered transgenes with MARs may be only marginal (Gelvin, 2003b).

In the context of plant transformation, transgene silencing also results from insertion of multiple copies or high-level expression from a constitutive promoter, and an introduced transgene can lead to silencing of a homologous host gene (Vaucheret et al., 1998). Multicopy transgenic loci, particularly those including binary vector sequences, appear prone to transcriptional silencing attributable to meiotically heritable epigenetic modifications, most often methylation and/or condensation of chromatin (Matzke and Matzke, 1998; Vaucheret et al., 1998). Silencing can also occur by a post-transcriptional mechanism termed "cosuppression," in which the formation of double-stranded RNA (dsRNA) results in sequence-specific degradation of homologous RNA molecules (Soosaar et al., 2005). The degree of cosuppression tends to correlate with the strength of the promoter driving the transgene, although reciprocal and synergistic silencing between host genes and transgenes can also result from production of aberrant RNA above a threshold level that activates the RNA degradation machinery (Vaucheret et al., 1998). Conversely, expression of heterologous genes can be stimulated by adjacent ribosomal DNA spacer regions, at least in transgenic tobacco. Strikingly, the enhancement is attributable to amplification of the gene copy number as well as increased transcription, and both changes are stably inherited (Borisjuk et al., 2000).

2.7 Targeting transgene insertions

Gene targeting after *Agrobacterium*-mediated transformation was initially demonstrated as recombination between endogenous or engineered tobacco protoplast sequences and a homologous incoming gene fragment; successful targeting restored a functional selectable marker gene (Lee et al., 1990; Offringa et al., 1990). However, the frequency of such homologous recombination events is relatively low. In contrast, efficient targeted transgene insertion can be achieved by first creating a plant line with a *lox* "target" site; in subsequent transformations of this plant line, incoming DNA carrying a *lox* sequence is specifically and precisely integrated at this chromosomal site via Cre-mediated recombination (Gilbertson et al., 2003). Inclusion of a promoter at the site of integration provides a simple selection scheme for successful insertion of a T-DNA carrying the marker

gene (Albert et al., 1995). Targeted transgene integration via site-specific recombination can be combined with a second recombination system that eliminates the selectable marker gene (Srivastava and Ow, 2004). The efficiency of the targeted integration reaction is enhanced when the T-DNA carries two *lox* sites, allowing for formation of the required circular integration substrate (Vergunst et al., 1998). A variety of approaches have been used to stabilize the insertion and prevent subsequent Cre-mediated excision (Gilbertson et al., 2003).

A second important application of homology-directed DNA insertion is gene inactivation via targeted disruption. Although large collections of random T-DNA insertions (e.g., Feldmann, 1991) have proven to be an immensely valuable tool for plant molecular biologists, not all genes are represented and not all alleles are null mutants (Britt and May, 2003). Disruption of a specified locus in *Arabidopsis* can be accomplished by flanking a selectable marker with two genomic fragments from the target gene and screening for a double cross-over event that eliminates another T-DNA-borne marker gene or other T-DNA sequences (Miao and Lam, 1995; Kempin et al., 1997; Hannin et al., 2001). Several refinements of this procedure enabled the first targeted disruption in a monocot, rice (Terada et al., 2002). Those improvements include optimizing the efficiency of the *Agrobacterium*-mediated transformation itself and the use of a stringent PCR screen for true recombinants. A third, and probably critical, factor was the inclusion of toxin-encoding genes at either end of the vector DNA to provide strong counter selection against random integration of the T-DNA elsewhere in the genome. Finally, it is plausible that recombination occurs more readily in the highly proliferative callus tissue typically used in rice transformation than in the plant tissues used in other transformations (Shimamoto, 2002). The gene targeted for disruption in this application was Waxy, which encodes granule-bound starch synthase. Lower Waxy mRNA abundance in Japonica rice accounts for its stickier nature as compared to Indica rice, in which the gene is expressed at higher levels (Hohn and Puchta, 2003). The success of this gene targeting process in rice paves the way for other gene knockouts in this important staple crop to study gene function or to alter nutritional or growth traits.

Conventional approaches to gene targeting appear to be limited by the preference in plants for non-homologous end-joining (NHEJ) over homologous recombination for DNA double-stranded break repair. Recent advances in enhancing targeted mutagenesis have focused on harnessing the NHEJ process and on stimulating homologous recombination by engineering plants to express a yeast recombination gene (Tzfira and White,

2005). NHEJ frequently introduces insertion and/or deletion mutations at double-stranded breaks, thus raising the possibility that targeted mutagenesis could be accomplished by inducing double stranded breaks at the desired locus. Successful implementation of this approach was achieved using *Agrobacterium*-mediated transformation to introduce a synthetic zinc-finger nuclease that then created the double-stranded break (Lloyd et al., 2005). These zinc-finger nucleases consist of custom-made C2H2 zinc fingers, with each finger recognizing a specified three-nucleotide sequence, fused to a non-specific restriction enzyme. Expression of this chimeric gene in a plant allows the targeted digestion of a specific and unique sequence of 18 nucleotides, which then becomes a substrate for error-prone NHEJ-mediated repair (Tzfira and White, 2005). Using a heat-shock promoter to drive production of the zinc-finger nuclease in *Arabidopsis*, Lloyd et al. (2005) demonstrated highly efficient mutagenesis and transmission of the induced mutations, and suggested on theoretical grounds that this technology should be applicable to most plant genes in most plant species. In a second approach to increasing the frequency of directed gene disruption or replacement, Shaked et al. (2005) introduced the yeast chromatin remodeling protein RAD54 into *Arabidopsis* and reported a 10-to-100 fold improvement in homology-based integration efficiency.

2.8 Extending the range of susceptible hosts for *Agrobacterium*-mediated transformation

A variety of factors have been shown to influence the range of hosts that can be transformed by *A. tumefaciens*. On the bacterial side of the interaction, certain virulence loci including *virC* and *virF* are considered host range determinants (Yanofsky et al., 1985; Jarchow et al., 1991; Regensburg-Tuink and Hooykaas, 1993), and constitutive transcription of the virulence genes improves the efficiency of plant transformation in both susceptible and recalcitrant species (Hansen et al., 1994). Genes within the T-region can also affect the range of susceptible host species (Hoekema et al., 1984). Overexpression of certain plant genes, particularly *HTA1* (encoding histone 2A) and *VIP1* (which may facilitate nuclear targeting of the T-complex) can also enhance plant susceptibility (Mysore et al., 2000; Tzfira et al., 2002). The manipulation of host genes to improve transformation frequency is the subject of two recent reviews (Gelvin, 2003a; Gelvin, 2003b). Bacterial and plant contributions to host range are discussed in more detail in Chapters 1 and 13, respectively, in this volume. It is worth noting that there are almost certainly more factors yet to be identified that

limit the interaction between *A. tumefaciens* and specific plant species. For example, maize root exudates contain a potent inhibitor of VirA/VirG-mediated signal perception, leading to the possibility that bacterial mutants with enhanced resistance to this inhibition may prove useful in extending the transformation efficiency of maize (Zhang et al., 2000). One approach to circumvent host range limitations involves the use of *Agrobacterium rhizogenes* to generate composite plants, comprised of transgenic roots on wild-type shoots. This system provides a useful method to study transgene activity in the root in the context of a wild-type plant, and has been used successfully in species such as soybean, sweet potato and cassava, that are recalcitrant to *A. tumefaciens* transformation (Taylor et al., 2006).

Somewhat ironically, of all the advances in plant transformation described in this chapter, some of the most pronounced long-term impacts on plant biotechnology may result from an innovation that has the potential to obviate the requirement for *Agrobacterium* as a gene delivery vehicle. Motivated by the desire to "invent around" the myriad intellectual property constraints that limit use of *Agrobacterium*-mediated transformation by the public and the private sector, Broothaerts et al. (2005) successfully modified several species outside the *Agrobacterium* genus to stably transform a variety of plants. (The complex issues surrounding intellectual property in agricultural biotechnology are developed more fully in Chapter 20.) *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* strains of bacteria endowed with a disarmed Ti plasmid acquired the ability to deliver DNA from a standard binary vector; the vector was modified with a unique tag to facilitate tracking of the provenance of the transferred DNA. Rice, tobacco and *Arabidopsis* were genetically modified to express an intron-containing beta-glucuronidase (GUS) gene, indicating that monocots as well as dicots can serve as recipients with non-*Agrobacterium* bacteria, albeit at frequencies that ranged from 1-40% of that observed with *Agrobacterium*-mediated transformation. (The presence of the intron prevents reporter gene expression in the bacteria, and thus ensures that any observed GUS activity results from expression in the plant cell; Vacanneyt et al., 1990). Various tissues, and hence transformation mechanisms (floral dip for *Arabidopsis*, somatic tissue for tobacco and rice), were utilized in these experiments, and stable integration was confirmed by Southern blotting, sequence analysis of the insertion junctions, and Mendelian transmission of the transgene to progeny. This alternative technology may have

profound implications for the plant biotechnology community for two reasons. First, this technology has been configured to be freely accessible and "open-source," with no commercial restrictions other than covenants for sharing improvements, relevant safety information, and regulatory data (http://www.bioforge.net). Second, the exceptionally broad host range of the *Rhizobium* strain used, and the potential to extend the technology to additional bacteria species, make it likely that previously recalcitrant plant species may become transformable. As a plant pathogen, *Agrobacterium* elicits a variety of defense responses that can block any step of the transformation process, thereby limiting its host range. While a better understanding of *Agrobacterium*-triggered defense responses may lead to methods to lower or subvert a plant's natural barriers (Zipfel et al., 2006), the use of non-*Agrobacterium* species as T-DNA delivery systems provides a way for plant biotechnologists to invent around the obstacles erected by both plant evolution and patent lawyers.

2.9 Alternatives to *Agrobacterium*-mediated gene delivery

In the 1980's, the apparent recalcitrance of several agronomically important crop plants, including maize, wheat, barley, and rice, to infection by *A. tumefaciens* drove the development of alternative methods of DNA delivery for genetic engineering. Protoplast transformation, although achievable through electroporation, microinjection, or polyethylene glycol fusion, proved to be inefficient because the regeneration of plants from protoplasts is time-consuming and non-trivial (Newell, 2000). Particle bombardment, in which tungsten or gold microprojectiles are coated with DNA and accelerated into the target plant tissue, has proven highly successful in a wide range of species (Klein et al., 1987), and is the most reliable method by which chloroplasts can be transformed. This biolistic approach presents certain advantages over *Agrobacterium*-mediated gene delivery; many types of explants can be bombarded and yield fertile plants, and the gene to be delivered need not be cloned into a specialized transformation vector (Herrera-Estrella et al., 2005). Nonetheless, particle gun delivery of DNA is generally not the method of choice for a plant species that can be transformed by *Agrobacterium*, as the bombardment process typically results in integration of multiple copies of the DNA, as well as rearranged and/or truncated DNA sequences (Newell, 2000). These complex integration patterns can lead to genetic instability, due to homologous

recombination among the identical copies, and/or epigenetic silencing of the transgene (see section 2.6). "Agrolistic" transformation was designed to mitigate these shortcomings by combining the high efficiency of biolistic DNA delivery with the simpler integration pattern characteristic of *Agrobacterium*-mediated DNA transfer. Particle bombardment of the *virD1* and *virD2* genes, under the control of the CaMV35S promoter, with a target plasmid carrying the transgene of interest flanked by T-DNA border sequences, allows transient expression of the *vir* genes in the plant. The insertion events resulting from *in planta* VirD1/2-mediated processing and integration resemble those generated by traditional *Agrobacterium*-mediated transformation (Hansen and Chilton, 1996).

Agrobacterium-mediated and biolistic delivery of foreign DNA are typically used to stably transform plants, although transient expression of genes delivered by *A. tumefaciens* on binary vectors can be used to produce recombinant proteins without the delays and technical barriers associated with stable integration (Chung et al., 2006). Heterologous genes can also be introduced into plants on viral vectors; because of the amplification associated with viral infection, transient expression of the transgenes can yield commercial-scale quantities of pharmaceutical proteins. In a novel hybrid technology, *A. tumefaciens* has been used to expedite the production process by circumventing the need for *in vitro* synthesis of the RNA viral vector. Building on the idea of "agroinfection," in which a viral genome is delivered as a cDNA inserted between border sequences (Grimsley et al., 1986; Grimsley et al., 1987), complete viral replicons have been assembled *in planta* through site-specific recombination among DNA modules delivered by *Agrobacterium* (Marillonnet et al., 2004). Additional refinements of the viral vectors further enhanced the efficiency of the system, which was limited by the low infectivity of viral vectors carrying larger genes and apparently by nuclear processing of a viral transcript that normally never experiences the nuclear milieu (Marillonnet et al., 2005). By infiltrating whole mature plants with a suspension of agrobacteria carrying the encoded viral replicons, the bacteria take on the viral infection function, while the viral vector mediates cell-to-cell dissemination, amplification, and high-level expression of the transgene (Gleba et al., 2005). This "magnifection" process is rapid and scalable; the modular nature of the viral components facilitates adaptation to new transgenes, and the yield can reach 80% of total soluble protein (Marillonnet et al., 2004).

3 APPLICATIONS OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION

3.1 Production of foreign proteins in plant cell cultures

Agrobacterium-mediated transformation has been extensively utilized to engineer plants producing a wide variety of useful, and in many cases clinically relevant, metabolites and exogenous proteins. Most applications to date have focused on field-grown plants, although recombinant proteins and metabolites can also be produced in plant cell cultures. Despite limited commercial use so far, cultured plant cells such as the tobacco-derived BY-2 and NT-1 lines offer several advantages over expression systems in intact plants: they can be maintained in simple media, and are not subject to variations in weather and soil conditions; products can be easily harvested, especially when secreted into the culture medium (Hellwig et al., 2004). In the future, functional genomics and combinatorial biochemistry are likely to increase dramatically the range of products that can be generated in genetically modified plant cell cultures (Oksman-Caldentey and Inze, 2004).

3.2 Genetic modification of plants to generate useful products

3.2.1 Biodegradable plastics

Among the more notable foreign products produced in plants are biodegradable plastics. Drawing on the natural ability of many bacterial species, including *Ralstonia eutropha*, to synthesize carbon storage products with plastic-like properties (Hanley et al., 2000), Chris Somerville's lab first demonstrated poly-3-hydroxybutyrate (PHB) synthesis in *Arabidopsis* by introducing biosynthetic genes from *R. eutropha* (formerly *Alcaligenes eutrophus*) (Poirier et al., 1992). Yields of this simple C4 polymer, which is synthesized from acetyl-CoA by the sequential action of the bacterial *phbA*, *phbB*, and *phbC* gene products, could be increased 100-fold by N-terminal addition of the pea small subunit RUBISCO-transit peptide, thereby targeting the three encoded enzymes to the chloroplast (Nawrath et al., 1994). Further increases in yield, from 14% to as much as 40% of the plant dry weight, were achieved by using gas chromatography-mass spectrometry to screen large numbers of transgenic *Arabidopsis* plants for high levels of production; however, the high producing lines exhibited

stunted growth, loss of fertility, and significant alterations in the levels of various amino acids, organic acids, sugars, and sugar alcohols (Bohmert et al., 2000).

Properties of PHB, including brittleness and low-temperature decomposition, preclude its use commercial use. In contrast, the co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is considerably more flexible and therefore useful (Hanley et al., 2000). Slater et al. (1999) successfully engineered both *A. thaliana* leaves and seeds of *Brassica napus* (oilseed rape) to synthesize PHBV at a significantly lower cost than the previous industrial-scale bacterial fermentation process (Poirier, 1999). Because PHBV synthesis requires not only the abundant metabolite acetyl-CoA, but also the relatively scarce propionyl-CoA, Slater et al. had to redirect the metabolic flow of two independent pathways to generate a pool of propionyl-CoA in the plastid (Slater et al., 1999).

Finally, Neumann et al. (2005) have recently reported the synthesis in transgenic tobacco and potato plants of cyanophycin, which can be hydrolyzed to yield the soluble, non-toxic, biodegradable plastic-like compound poly-aspartate. Although these transgenic plants exhibit morphological alterations in chloroplast structure and in growth rate, additional engineering of the amino-acid biosynthesis pathways may permit economically viable levels of biodegradable plastic production (Conrad, 2005). If successful, the substitution of a renewable process (solar-driven carbon fixation) for conventional petrochemically derived plastic production technologies would have substantial positive environmental consequences, decreasing our reliance on finite petroleum resources, while reducing the accumulation of indestructible plastics (Poirier, 1999; Conrad, 2005).

3.2.2 Primary and secondary metabolites with desirable properties

Considerable effort has been dedicated to metabolic engineering of terpenoids in plants. Terpenoids, also known as isoprenoids, are a family of more than 40,000 natural compounds, including both primary and secondary metabolites, that are critically important for plant growth and survival. Some of the primary metabolites produced by the terpenoid biosynthetic pathway include phytohormones, pigments involved in photosynthesis, and the ubiquinones required for respiration (Aharoni et al., 2005). Secondary metabolites, including monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), and triterpenoids (C30), also provide physiological and ecological benefits to plants. Some function as antimicrobial agents, thus contributing to plant disease resistance, while other terpenoid compounds serve to repel pests, attract pollinators, or inhibit

growth of neighboring competitor plant species. Additionally, many terpenoids have commercial value as medicinals, flavors, and fragrances. Interest in manipulating the inherent properties of plants (e.g., enhanced aromas of ornamentals, fruits, and vegetables), or in using plants as sources of pharmaceuticals and cosmetics, has driven the development of terpenoid metabolic engineering in a variety of species (Aharoni et al., 2005).

The terpenoid biosynthetic pathway and strategies for its manipulation have been reviewed recently (Mahmoud and Croteau, 2002; Aharoni et al., 2005). A comprehensive listing of transgenic plants with altered terpenoid biosynthetic properties is available elsewhere (Aharoni et al., 2005). Examples include expression of heterologous synthases in tomato, leading to enhanced aroma in ripening fruit (Lewinsohn et al., 2001), reduced production in mint of an undesirable monoterpenoid that promotes off-color and off-flavor (Mahmoud and Croteau, 2001), and the introduction of bacterial genes directing the production of keto-carotenoids, thought to have medicinal value, into tomato and tobacco (Ralley et al., 2004). Other endogenous, plant-derived terpenoids with demonstrated pharmaceutical properties include the anti-malarial agent artemisinin, the diuretic glycyrrhizin, and the cancer drugs Taxol and perilla alcohol. Several of these compounds are currently derived from endangered species in threatened ecosystems (Bouwmeester, 2006), while chemical synthesis of terpenes can be prohibitively costly and inefficient (Wu et al., 2006).

Plants contain two terpene biosynthetic pathways; the mevalonate pathway leads to the synthesis of sesquiterpenes and triterpenes at the level of the endoplasmic reticulum, while the methyl-D-erythritol-4-phosphate pathway functions in the chloroplast to produce monoterpenes, diterpenes, and carotenoids (Aharoni et al., 2005). Most attempts to manipulate the pathways involve introducing terpene synthase genes whose products could divert pathway intermediates towards the production of desired, and in some cases novel, terpenes (Chappell, 2004). To date, generating monoterpenes in transgenic plants has proven easier than modifying the metabolism of longer-chain terpenoids (Aharoni et al., 2005). The complexity of the biosynthetic pathway, giving rise to a vast number of natural products, and the subcellular compartmentalization of the processes pose challenges for terpenoid genetic engineering. Manipulating terpenoid biosynthetic pathways in plant species that produce the same class of terpenes is less problematic, because the plant already has the specialized structures necessary to carry out the storage and transport of volatile, hydrophobic compounds. In contrast, introducing novel pathways into species that lack the secretory structures required may prove to be far more difficult

(Mahmoud and Croteau, 2002). A recent comprehensive evaluation of the factors required for high-level terpene production in tobacco identified several effective strategies for enhancing synthesis as much as 1,000-fold (Wu et al., 2006). By over-producing, in the same subcellular compartment, an enzyme producing an isoprenoid substrate and a terpene synthase that rapidly incorporates this substrate, Wu et al. (2006) have advanced the technology necessary to achieve commercial-scale production of industrially or pharmaceutically relevant terpenes (Bouwmeester, 2006).

3.2.3 Commercially relevant traits in ornamentals and trees

In ornamental plants, flower color, architecture, and post-harvest life are all targets for transgenic modification (Mol et al., 1995). Commercially important traits in trees have also been a focus of recent *Agrobacterium*-mediated transformation (Tzfira et al., 1998). Tree improvement goals include increasing timber yield and decreasing generation time; together, these traits could pave the way for economically viable plantation forests, leading to decreased pressure on natural forests as sources of wood (Fenning and Gershenzon, 2002). In this regard, overexpression of a key enzyme in the gibberellin biosynthetic pathway resulted in enhanced biomass and accelerated growth rate in hybrid aspen, but had a negative effect on rooting. Interestingly, the transgenic trees also exhibited longer and more numerous xylem fibers that could be advantageous in producing stronger paper (Eriksson et al., 2000). Altering plant composition could also enhance the production of bioethanol, a renewable energy source for the transportation sector with substantial positive environmental impact (Boudet et al., 2003). Finally, in poplar and aspen, biotechnology has proven to be an effective way to manipulate levels of the undesirable cell wall component lignin by downregulating the last step of the lignin biosynthetic pathway through an antisense strategy (Baucher et al., 1996; Li et al., 2003); the transgenic trees required fewer chemicals for delignification and yielded more high-quality pulp (Pilate et al., 2002). Since removal of lignin in the paper and pulp industry is an energy-consuming process that requires large amounts of hazardous chemicals, the success of the antisense trees holds promise for more environmentally friendly processing in the future.

3.2.4 Biopharmaceuticals/edible vaccines

Using *Agrobacterium*-mediated transformation, transgenic plants have been engineered to express a wide variety of exogenous proteins, from

spider dragline silk (a fiber with high tensile strength and elasticity; Scheller et al., 2001) to vaccines, antibodies, and other life-saving biopharmaceuticals such as anti-coagulants, human epidermal growth factor, and interferon (Giddings et al., 2000). To date, most such clinically relevant proteins have been produced in tobacco, although potatoes, alfalfa, soybean, rice and wheat have also been used successfully. While green tissue has a distinct advantage in terms of productivity, seeds or tubers are most useful for delivery of an edible product such as a vaccine; they can be stored for long periods of time (Daniell et al., 2001) and shipped long distances at ambient temperature (Streatfield et al., 2001).

Edible vaccines may hold considerable promise for the developing world, where refrigeration, sterile syringes and needles, and trained health care personnel are frequently in short supply (Arntzen et al., 2005). Since many pathogens utilize mucosal surfaces as their point of entry, priming the entire mucosal immune system via oral stimulation is an especially attractive mode of immunization (Streatfield et al., 2001). Nonetheless, lack of a profit incentive for private industry, coupled with concerns about inadequate biosafety infrastructure in developing countries and the complexity of government-financed health care delivery systems, have resulted in the development of relatively few products (Ma et al., 2005b) in the 14 years since the first report of an antigen expressed in transgenic plants (Mason et al., 1992). Oral immunization has been achieved using transgenic potatoes expressing antigens including the heat-labile enterotoxin from *E. coli* (Haq et al., 1995; Mason et al., 1998), the Norwalk virus capsid protein (Tacket et al., 2000), and the hepatitis B surface antigen (Richter et al., 2000; Kong et al., 2001), as well as transgenic alfalfa expressing proteins from the foot and mouth disease virus (Dus Santos et al., 2005), among others. Despite these successes, it should be noted that there are no transgenic-plant-derived pharmaceuticals in commercial production (Ma et al., 2005a). This may change in the near future, as a large European consortium with collaborators in South Africa is actively engaged in developing plant-based production platforms for pharmaceuticals targeted to HIV, rabies, tuberculosis and diabetes. This group would be the first to carry out clinical trials of plant-derived candidate pharmaceuticals within the European Union regulatory framework (http://www.pharma-planta.org/).

Plant-derived pharmaceuticals have many potential advantages over those produced in animal cell culture or by microbial fermentation. High yields, favorable economics, existing technologies for harvesting and processing large numbers of plants, and the possibility of expressing proteins in specific subcellular compartments where they may be more stable,

all contribute to the choice of transgenic plants over bacterial expression systems for recombinant proteins (Daniell et al., 2001). Like animal cells, plants have the ability to carry out post-translational modifications, and can fold and assemble recombinant proteins using eukaryotic chaperones, but plant expression systems have the added benefit of minimizing the potential for contamination with human pathogens (Woodard et al., 2003; Arntzen et al., 2005). Finally, multimeric protein complexes may be reconstructed in transgenic plants by stacking transgenes through successive crosses among plants resulting from single transformation events (Hiatt et al., 1989; Ma et al., 1995; Ma et al., 2005a). This is a particularly important consideration when producing multimeric secretory antibodies to protect against microbial infection at mucosal sites (Giddings et al., 2000).

One concern about plant-based pharmaceuticals is the potential for non-mammalian glycosylation patterns that might result in immune sensitization or loss of function (Bardor et al., 1999; Giddings et al., 2000). However, at least one plant-derived monoclonal antibody was found to be functional despite differences in N-linked glycosylation (Ko et al., 2003), and stable expression of a human galactosyltransferase in plants has been shown to yield "plantibodies" with mammalian glycosyl modifications (Bakker et al., 2001). Other potential limitations of plant expression systems include low and/or variable yield (Chargelegue et al., 2001), unexpected localization of the expressed protein (Hood, 2004), and, for edible vaccines, induction of oral tolerance and/or gastrointestinal degradation of the antigen (Ma, 2000; Daniell et al., 2001). Finally, contamination of food and feed crops with pharmaceutical crops, either in the field or post-harvest, poses potentially serious health and public relations risks (Ma et al., 2005b).

3.3 Bioremediation

Two classes of transgenic plants have been developed to address the serious risks to human health posed by industrial and naturally occurring environmental pollutants: some serve as biomonitors, detecting the presence of toxic compounds in the environment, while others detoxify contaminated soils. By integrating an engineered marker gene, beta-glucuronidase, Barbara Hohn and coworkers have pioneered a strategy in which transgenic *Arabidopsis* has successfully been used to report enhanced rates of homologous recombination or point mutation due to heavy metal ions (Kovalchuk et al., 2001a; Kovalchuk et al., 2001b), and to ionizing radiation resulting from the Chernobyl accident (Kovalchuk et al., 1998).

Increasing levels of pollution resulting from global industrialization have focused attention on the possibility of phytoremediation: using plants to remove or inactivate pollutants from soil or surface waters. Factors that influence the utility of a plant in phytoremediation include (i) the availability of the trace element in a form that can be taken up by the plant's roots; (ii) the rate of uptake; (iii) the ability of the plant to transform the pollutant into a less toxic, and potentially volatile, compound; and (iv) the movement of the compound from the roots into the shoots (Kramer and Chardonnens, 2001). Theoretically, genetic manipulation of heavy metal accumulation in plants could be used to imbue a plant with any of these traits or to enhance an existing capability (Clemens et al., 2002). Introduction of bacterial genes has enabled the creation of transgenic *Arabidopsis* plants capable of converting the highly toxic contaminant methylmercury to the volatile and much less toxic elemental mercury (Bizily et al., 1999; Bizily et al., 2000). Similar modifications have resulted in *Arabidopsis* and poplar able to process and sequester mercury ion (Rugh et al., 1996; Rugh et al., 1998), Indian mustard that processes selenite (a common contaminant in oil-refinery wastewater) (Pilon-Smits et al., 1999), and tobacco engineered to facilitate degradation of the explosive trinitrotoluene (TNT) (Hannink et al., 2001). To deplete arsenic contamination from groundwater, researchers have introduced bacterial genes that confer on *Arabidopsis* the ability to extract and accumulate in the leaf levels of arsenic that would normally poison the plant (Dhankher et al., 2002). Second generation phytoremediating plants will likely capitalize on the finding that overexpression of a yeast vacuolar transporter in *Arabidopsis* leads to enhanced accumulation, and hence tolerance, of heavy metals such as cadmium and lead (Song et al., 2003b).

3.4 Increasing crop plant productivity by altering plant physiology and photosynthetic capacity

The Green Revolution succeeded in increasing net food productivity per capita in Asia, India, and Latin America by combining, through traditional breeding, high yield and dwarfing traits in several of the world's most important grain crops (Evenson and Gollin, 2003). The advent of basic plant molecular biology, made possible in large part by the availability of *Agrobacterium*-mediated techniques for introducing and knocking out plant genes, has dramatically augmented our understanding of how plant architecture and generation time are regulated, and these discoveries may enable further improvements in yield. For example, manipulating plant

brassinosteroid levels resulted in a more erect leaf structure in rice, increasing yield under dense planting conditions (Sakamoto et al., 2006). Tissue-specific modulation of the growth hormone gibberellin catabolism in transgenic rice led to a semi-dwarf phenotype without a loss in grain productivity (Sakamoto et al., 2003). In other cases, yield may be enhanced by decreasing the time required for the plant to produce the edible portion. Exogenous expression of the *Arabidopsis* flower initiation genes LEAFY or APETALA accelerated the generation time of citrus trees (Pena and Seguin, 2001). Dormancy in potatoes was controlled by expressing a bacterial gene that altered sprouting behavior (Farre et al., 2001), while tomatoes with prolonged shelf- and vine-life characteristics were created by manipulating the biosynthesis of the ripening-promoting hormone ethylene (Oeller et al., 1991), or by increasing levels of the anti-ripening polyamines (Mehta et al., 2002), respectively.

Other attempts to increase yield potential have centered on the photosynthetic process, and in particular the inefficiency of the carbon assimilation pathway in C_3 plants, a group that includes many agronomically important crop plants. The alternative C_4 pathway makes use of both altered biochemical pathways and spatial segregation within the plant to concentrate CO_2 for the crucial Calvin-cycle enzyme ribulose 1,5-bisphosphate carboxylase (Rubisco) (Edwards, 1999). Using *Agrobacterium*-mediated transformation, Matsuoka and co-workers have expressed three key C_4 enzymes in rice (a C_3 plant) (Ku et al., 1999; Ku et al., 2001), but it seems likely that successful enhancement of photosynthetic capabilities will require the specialized leaf anatomy of C_4 plants. Another strategy, involving expression in tobacco of a cyanobacterial enzyme, successfully improved photosynthetic capacity and concomitantly increased the plants' biomass (Miyagawa et al., 2001). However, grain production is tightly linked to nitrogen availability, and hence larger plants will not necessarily yield more grain unless soil nitrogen levels are sufficient (Sinclair et al., 2004).

3.5 Enhancing crop productivity by mitigating external constraints

A plant's physiology and its photosynthetic capacity are inherent characteristics, but crop yields can also be limited by many external factors, including inadequate soil fertility, disease, climatic stresses, and/or the presence of soil constituents (e.g., heavy metals) that compromise plants' growth and development. Among the approaches to mitigating these constraints are some that involve genetically modifying the crop plant. It is

important to stress that there are also many highly successful non-biotechnological practices that have been in use for centuries, including integrated pest and vector management, crop rotation, dissemination of pathogen-free plant material (Rudolph et al., 2003), and removal of weeds that can serve as reservoirs of infection (Wilson, 1993). Indeed, farming systems that combine careful land management with a diverse array of species and genetic backgrounds within a species can be highly productive even in the absence of modern varieties or biotechnology "improvements" (Brown, 1998). The lessons of such a holistic approach to agriculture are enjoying a resurgence of popularity among small and some medium-scale farmers in the industrialized world; for example, integrated production and organic farming guidelines are in practice on 85% of the farmland in Switzerland (Xie et al., 2002). Nonetheless, the predominant model of agriculture in much of the developed world is one of monocultures grown with high external inputs. At the other end of the spectrum, resource-poor farmers cultivating marginally arable land face myriad environmental constraints which, for a variety of reasons, have proven recalcitrant to the available integrated approaches. The following sections highlight some of the applications of *Agrobacterium*-mediated genetic modification of plants that may address these constraints and/or mitigate negative consequences of the conventional solutions. None of these biotechnological approaches is a panacea. On the other hand, although biotechnology is anathema to most proponents of organic farming practices, it is likely that our ability to meet the growing challenge of adequate food production may benefit from open-mindedness and creative approaches that incorporate the genetic modifications described below into sustainable, ecosystem-centered cultivation systems.

3.5.1 Enhanced nutrient utilization

The negative environmental impacts of inorganic, petroleum-based fertilizers are well-documented, as are the prohibitive costs that preclude their use by subsistence farmers attempting to cultivate depleted soils (Good et al., 2004). Engineering plants with enhanced capabilities to absorb micronutrients from the soil, by over-expressing nitrogen, potassium and phosphorus transporters and/or manipulating their regulation, could decrease the need for fertilizers (Hirsch and Sussman, 1999). For some nutrients, such as iron and phosphorus, the limiting factor is often solubility rather than abundance in the soil. Plants synthesize and secrete a variety of organic acids that can chelate insoluble compounds, allowing uptake of the complex (Guerinot, 2001). Several important grain crops such as rice,

maize and sorghum are particularly sensitive to low iron availability in alkaline soils, where iron is less soluble. *Agrobacterium*-mediated introduction of genes conferring enhanced biosynthesis of an iron chelator in rice resulted in improved growth and four-fold higher grain yields under conditions of low iron availability (Takahashi et al., 2001). Finally, it may be possible to engineer plants to secrete nutrients that specifically promote growth of beneficial microbes in the rhizosphere (O'Connell et al., 1996).

3.5.2 Enhanced tolerance to abiotic stress

Solubility of soil constituents is also an important factor influencing a plant's tolerance for metal ions. The abundant metal aluminum normally exists as harmless oxides and aluminosilicates, but in acidic soils it is solubilized into the toxic $Al^{3+,}$ which inhibits root growth. Plants that tolerate otherwise toxic levels of $Al^{3+,}$ do so by secreting organic acids such as citrate or malate at the root apex that chelate the $Al^{3+,}$ in the soil and prevent uptake (Ma et al., 2001). [Other plants accumulate aluminum in the leaves and detoxify it internally by forming organic acid-complexes; the characteristic variation in hydrangea sepals from pink to blue, for example, is determined by the pH-dependent aluminum concentration in the cell sap; Ma et al., (2001).] Attempts to engineer aluminum tolerance by introducing bacterial citrate synthase genes into tobacco and papaya were met with mixed success; enhanced tolerance was reported, but could not be reproduced by another group (de la Fuente et al., 1997; Delhaize et al., 2001). Improved tolerance of zinc in transgenic plants has also been observed (van der Zaal et al., 1999).

Metal contamination in the soil is but one of the abiotic stresses that constrain crop plant productivity. Growing global demand for food continues to force farmers onto marginally arable land where soil salinity, water deficits, and climatic challenges such as low or high temperatures limit cultivation (Bartels, 2001). Strategies to engineer enhanced tolerance to such adverse conditions fall into at least two categories: direct protection from the stressor(s), and enhanced resistance to the physiological damage caused by the stressor. In the latter category, a family of aldose-aldehyde reductases are activated in response to a wide variety of stresses (Bartels, 2001). Ectopic expression of an alfalfa aldose-aldehyde reductase gene via *Agrobacterium*-mediated transformation results in reduced damage upon oxidative stress, apparently by eliminating reactive aldehydes, and increased tolerance to salt, dehydration, or heavy metal stress (Oberschall et al., 2000). Several other transgenic improvements in stress tolerance [e.g., overexpression of glutathione peroxidase (Roxas et al., 1997) and

overexpression of superoxide dismutase (McKersie et al., 1996)] likewise function by providing oxidative protection (Zhu, 2001).

Osmolytes also confer stress tolerance by scavenging reactive oxygen species (Zhu, 2001). The non-reducing disaccharide trehalose stabilizes biological structures upon dessication in many bacteria, fungi, and invertebrates, but apparently does not accumulate naturally in plants (Penna, 2003). Transgenic tobacco and rice engineered to produce trehalose exhibit enhanced resistance to drought (Romero et al., 1997; Pilon-Smits et al., 1998), salt, and low-temperature stress (Garg et al., 2002). Production of mannitol results in tobacco with enhanced tolerance to high salinity (Tarczynski et al., 1993). Other low-molecular-weight compatible solutes that accumulate in some plants to protect proteins from stress-induced damage include glycinebetaine, polyols and amino acids. Glycinebetaine accumulation confers on transgenic *Arabidopsis* an increased ability to withstand high temperatures during germination and seedling growth (Alia et al., 1998).

In addition to small osmolytes, a number of proteins have also been shown to have stress protective activity, primarily in response to low temperature. A variety of plants produce antifreeze proteins, as do several fish and insects; these proteins function to inhibit growth of intercellular ice crystals (Griffith and Yaish, 2004). There have been a variety of attempts to introduce a gene encoding one of these anti-freeze proteins into tobacco, tomato, potato, and *Arabidopsis*, with the ultimate goal of lowering the freezing temperature, even by a few degrees, so that the plants could survive a light frost (Griffith and Yaish, 2004). At least one such experiment was successful; although the plant did not exhibit higher rates of survival upon freezing, the freezing temperature was indeed lowered (Huang et al., 2002c). Another transgenic strategy to achieve freezing tolerance involves the introduction of bacterial ice nucleation genes, which permit slow dehydration that minimizes tissue damage (Baertlein et al., 1992).

High soil salinity impedes plant growth, both by creating a water deficit in the soil and within the plant, as sodium ions impinge on many key biochemical processes. Strategies to increase salt tolerance involve limiting exposure of cytoplasmic enzymes to the salt and may include blocking Na^+, influx, increasing Na^+ efflux, and compartmentalizing Na^+ (Zhu, 2001). Successful transgenic approaches are described in detail in Yamaguchi and Blumwald (2005). Many of these entail over-expressing the *Arabidopsis* vacuolar Na^+/H^+ antiporter, which enhances tolerance to soil salinity, with few or no detrimental effects on seed quality or plant growth, in canola (Zhang et al., 2001), *Arabidopsis* (Apse et al., 1999), tomato

(Zhang and Blumwald, 2001), and wheat (Xue et al., 2004). Sequestration of cations in the *Arabidopsis* vacuole, resulting in enhanced salt and drought tolerance was also achieved by overexpressing the vacuolar H^+ pyrophosphatase (Gaxiola et al., 2001). Increased expression of a plasma membrane Na^+/H^+ antiporter augmented salt tolerance by limiting Na^+ accumulation (Shi et al., 2003). Finally, tolerance in rice, resulting from expression of a bacterial Na^+/H^+ antiporter, was accompanied by biosynthetic activation of the osmoregulatory molecule proline (Wu et al., 2004).

Both freezing and high temperature cause damage to plant tissues and proteins, leading to diminished crop yield. A comprehensive listing of attempts to enhance plant thermo-tolerance through genetic modification can be found in Sung et al. (2003). This chapter will highlight some of the most common approaches. One of the earliest reports of altered chilling sensitivity resulted from engineering the degree of fatty acid saturation in tobacco membranes (Murata et al., 1992). Another strategy stems from the identification of the low-temperature transcriptional activator CBF1, which induces expression of multiple cold-regulated (COR) genes associated with cold acclimation (Sarhan and Danyluk, 1998). Using *Agrobacterium*-mediated overexpression of CBF1, Jaglo-Ottosen et al. (1998) successfully mimicked an acclimated state and enhanced the freezing tolerance of *Arabidopsis*. The existence of a more universal transcriptional response that includes *cor* genes, induced by the DREB (dehydration-responsive element binding) transcription factor family (Smirnoff and Bryant, 1999), suggests that there is likely to be extensive cross-talk among the stress-responsive signal transduction pathways (Sung et al., 2003). Indeed, stress-inducible over-expression of DREB1A conferred enhanced tolerance to freezing, water stress, and salinity without affecting plant growth, while increased constitutive expression of DREB1A also caused a significant improvement in stress tolerance but at the expense of severe growth retardation under normal growing conditions (Kasuga et al., 1999). Although the biochemical functions of the encoded stress-induced proteins are unknown, it is worth noting that the effect of DREB1A on freezing tolerance was substantially greater than that of CBF1 (>10°C vs. 1°C) (Zhu, 2001).

In concluding this section on engineering tolerance to environmental constraints, it is important to recognize that reductions in crop viability and yield are compounded by combinations of abiotic stresses. Such combinations can elicit plant responses that are not easily extrapolated from the plant's response to each stress applied individually (Mittler, 2006). Strategies designed to mitigate the effects of combinations of environmental stress conditions might, for example, target stress-responsive signal

transduction pathways, which could exhibit synergistic or antagonistic cross-talk. Regardless of the approach taken, lab-based proof-of-concept experiments must be complemented by testing under conditions that mimic the field environment (Mittler, 2006).

3.5.3 Improved disease resistance

Crop productivity is limited by a variety of parasites and pathogens, including fungi, bacteria, viruses, and insects (Baker et al., 1997). In naturally occurring ecosystems, elaborate networks of defenses function at many levels to protect plants from disease (Abramovitch and Martin, 2004). Elucidation of these defense pathways has recently become a particularly active area of research in plant molecular biology, and has led to our growing appreciation for the complex interplay between basal defenses and specific disease resistance (Feys and Parker, 2000). A major contributor to disease susceptibility is the reliance of industrial-scale agriculture on monocultures. Cultivation of plant lines bred for resistance to one or a few pathogens, often conferred by so-called R genes, can lead to the emergence of pathogens that have undergone natural selection to overcome the resistance (Gurr and Rushton, 2005). Despite the potentially short-sighted nature of such agricultural practices, identification of R genes has been the focus of considerable effort over the past decade (Baker et al., 1997; Dangl and Jones, 2001). At least one such gene, the Bs2 gene from pepper, has been used successfully to engineer durable resistance to the agronomically significant bacterial spot disease in tomato (Tai et al., 1999). The Xa21 resistance gene from rice, which provides wide-spectrum resistance to the devasting bacterial blight caused by *Xanthomonas oryzae* pathovar *oryzae*, has been introduced into a variety of rice cultivars using *Agrobacterium*-mediated gene delivery (Wang et al., 2005). Likewise, broad spectrum resistance to potato late blight is conferred by one of four R genes cloned from a wild, highly resistant, potato species (Song et al., 2003a). Pyramiding of multiple R genes can confer resistance to a range of pathovars within a species (e.g., Li et al., 2001), but the introduction of R genes can also result in a substantial fitness cost to the plant (Gurr and Rushton, 2005).

More recently, attention has shifted to the basal or non-host resistance plant defenses, which tend to target entire classes of pathogens. These pathways are generally activated in response to common patterns shared by many pathogens, such as fungal cell walls or bacterial flagellin. Elicitation of defense-related signal transduction pathways can be achieved by introduction or overexpression of receptor-like kinases (Gurr and Rushton, 2005) such as the receptor responsible for perception of the

pathogen-associated molecule flagellin (Zipfel et al., 2004). A related strategy involves engineering a plant to express a pathogen-derived elicitor of specific or basal defense responses (Keller et al., 1999). In this case, limiting the expression to sites of infection using pathogen-inducible promoters (Rushton, 2002) is essential, since constitutive activation of defense pathways can lead to reductions in plant health and even cell death (Gurr and Rushton, 2005).

Generic plant defenses include antimicrobial compounds such as defensins and chitinases. Ectopic expression of plant-derived or synthetic antimicrobial peptides in transgenic potatoes provides robust resistance to bacterial and fungal pathogens (Gao et al., 2000; Osusky et al., 2000), although only the former study tested resistance in the more relevant field setting (van der Biezen, 2001). A variety of antibacterial proteins from sources other than plants have been used to confer resistance to bacterial diseases in several transgenic plants (reviewed in Mourgues et al., 1998). *Arabidopsis* plants expressing antifungal peptides fused to a pathogen-specific recombinant antibody derived from chicken exhibited resistance to the fungal pathogen (Peschen et al., 2004). Finally, plant-derived defense molecules including proteinase inhibitors (Urwin et al., 1997) and lectins (Jung et al., 1998) have potential as nematicidal agents.

A third approach to engineering enhanced disease resistance takes advantage of the rapid expansion in our understanding of the pathways downstream of the initial pathogen perception events. Here, targets for genetic manipulation include "master-switch" transcriptional regulators, particularly those that activate local or global resistance networks involving salicylic acid, jasmonate, pathogenesis-related proteins, and the systemic acquired resistance that primes defenses in uninfected areas of the plant (Gurr and Rushton, 2005). For example, overproduction of the transcription factor NPR1 (also known as NIM1) results in enhanced resistance to bacterial and fungal pathogens and enhances the efficacy of fungicides (Cao et al., 1998; Friedrich et al., 2001). Plants engineered to produce elevated levels of salicylic acid also exhibit enhanced disease resistance (Verberne et al., 2000). Finally, appreciation for the involvement of the iron-binding protein ferritin in the oxidative stress response and the central role of oxidative stress in plant defense responses led to the successful demonstration that ectopic expression of ferritin can enhance tolerance to viral and fungal pathogens (Deak et al., 1999). Given the explosion in knowledge of plant defense mechanisms over the past decade, as well as the continued reliance on approaches to industrial-scale cultivation that

foment rampant pathogen spread, genetic engineering for disease resistance promises to be a very active area of research in the near future.

The quest to engineer virus resistance in plants stems from the proposal that expression of pathogen-derived genes within a plant can induce resistance to the pathogen in question (Sanford and Johnston, 1985). The first successful validation of this concept was the creation of tobacco mosaic virus-resistant tobacco plants producing the virus coat protein (Powell-Abel et al., 1986). A multitude of virus-resistant plants have since been developed using the same strategy (reviewed in Lomonossoff, 1995 and Wilson, 1993). A markedly effective implementation of coat protein-mediated protection was instrumental in saving the Hawaiian papaya crop from the papaya ringspot virus; the transgenic papaya has been commercialized and efforts are underway to transfer the technology to developing countries, which produce 98% of the world's papaya crop (Gonsalves, 1998). Although this particular application made use of particle bombardment rather than *Agrobacterium* to deliver the transgene, it serves as a convincing illustration of the potential for achieving virus resistance in other highly susceptible crops.

Production of viral proteins generally provides moderate levels of protection to a relatively broad spectrum of related viruses (Lomonossoff, 1995). In several cases, *Agrobacterium*-mediated expression of a viral replicase gene (Baulcombe, 1994) or virus movement proteins (e.g., Beck et al., 1994), rather than the viral coat protein, effectively conferred resistance. Unexpectedly, a number of researchers discovered that in some instances, levels of resistance did not correlate with the amount of foreign protein produced; furthermore, translationally defective genes could also provide protection (reviewed in Lomonossoff, 1995). Taken together, these findings indicated that at least some component of the resistance was attributable to the transgenic RNA, not the protein itself (Lindbo et al., 1993; Pang et al., 1993; Goregaoker et al., 2000; Prins, 2003). These observations coincided roughly with the initial reports of cosuppression (see section 2.6), and contributed to the discovery of post-transcriptional RNA silencing (PTGS) in plants, as well as in fungi and animals (Hannon, 2002). The recognition that the observed RNA-mediated virus resistance was a manifestation of PTGS, in turn, led to the realization that homology-dependent gene silencing is responsible for much of the phenotypic variability observed in transgenic plants (Kooter et al., 1999).

The intracellular series of events by which dsRNA brings about gene silencing in plants has been extensively studied (reviewed in Tenllado et al., 2004), and it is now clear that the process functions as a naturally

occurring defense system in plants in response to dsRNA formed during virus replication (Tenllado et al., 2004; Soosaar et al., 2005). RNA-mediated protection tends to provide resistance even to high levels of viral infection, but, as might be expected given the mechanism, is usually very virus-specific (Lomonossoff, 1995). Engineering plants to produce a self-complementary hairpin RNA corresponding to a viral gene target confers virus resistance; notably, the percentage of virus-resistant plants can be increased to almost 100% by including an intron within the hairpin region (Smith et al., 2000). Using *Agrobacterium*-mediated infiltration to deliver hairpin loops of viral RNA, Diaz-Ruiz and colleagues have demonstrated that it is possible to induce virus resistance in plants simply by exogenous exposure to the dsRNA; this work opens the door for future development of field-scale approaches in which bacterial lysates containing dsRNA are sprayed directly on the plants to confer resistance (Tenllado et al., 2004). Endogenous microRNAs, important regulators of gene expression that cause translational repression or cleavage of their target mRNAs, can also be engineered to contain sequences complementary to particular plant viruses. Transgenic plants expressing precursors of these artificial microRNAs exhibit resistance to the targeted viruses, even at temperatures that compromise hairpin dsRNA–mediated silencing (Niu et al., 2006). In contrast with RNA-mediated resistance, artificial microRNAs do not run the risk of complementing or recombining with non-target viruses, and thus pose less of an environmental biosafety threat (Garcia and Simon-Mateo, 2006).

Generally speaking, the mechanism by which expression of viral proteins causes resistance in plants is not as well understood as the process of viral RNA mediated suppression (Uhrig, 2003), and is rather protein-specific (Lomonossoff, 1995). Nonetheless, recent attempts to improve virus resistance in transgenic plants have targeted both protein- and RNA-mediated mechanisms. In several instances, introduction of a defective or truncated protein-coding sequence has proven more effective than expression of an intact, functional version in inducing resistance (Uhrig, 2003). Rudolph et al., (2003) have demonstrated that transgenic expression of a dominant interfering peptide from a viral nucleocapsid protein, identified using the yeast dihybrid assay, is sufficient to bring about virus resistance. *Agrobacterium*-mediated delivery of a ribozyme, a small RNA molecule capable of cleaving RNA, has been successful in conferring at least partial resistance to viruses and viroids in tobacco and potato (de Feyter et al., 1996; Yang et al., 1997).

A significant shortcoming of RNA-mediated virus resistance is the high degree of sequence homology (>90%) required (Prins, 2003), limiting the

possibility of engineering resistance to multiple viruses with one transgene. Furthermore, virus resistance achieved in the lab does not always translate into the field, where added environmental stresses compound the plants' susceptibility (Wilson, 1993). With our growing appreciation of PTGS as a natural form of self-protection in plants came the predictable discovery that many viruses produce suppressors of PTGS as a counter-defense strategy (Rovere et al., 2002; Soosaar et al., 2005). This presents a potential problem for the use of silencing-based virus resistance in the field, where a secondary infection with a suppressor-carrying virus could allow the targeted virus to overcome the engineered resistance. Some attempts to stack viral genes have been successful in achieving resistance to multiple, related, viruses (Prins et al., 1995); one logical strategy would entail engineering resistance to possible co-infecting viruses that carry PTGS suppressors as well as the virus of interest (Rovere et al., 2002). Finally, expression of a viral transgene under the control of the 35S CaMV promoter can be substantially attenuated if the plants happen to become infected with CaMV, leading to silencing of the transgene and a loss of immunity (Mitter et al., 2001). Likewise, herbicide resistance, conferred by a 35S CaMV-driven transgene, was rendered ineffective upon CaMV infection (Al-Kaff et al., 2000). These observations suggest that virus-derived suppression of transgene expression, attributable to transcriptional or post-transcriptional gene silencing, may prove to be a significant limitation in maintaining engineered traits in a field setting.

3.6 Reduction in the use of harmful agrochemicals by enhancing plant resistance to herbicides and pests

3.6.1 Herbicide resistance

Much has been written in the popular press about the creation and marketing of herbicide resistant crop plants. The rationale is that these crops allow farmers to eliminate weeds with one broad-spectrum, somewhat less toxic, herbicide without damaging the crop. Two of the most common herbicide/herbicide resistant seed packages involve the herbicides glyphosate (inhibitor of the shikimate pathway for aromatic amino acid biosynthesis; marketed by Monsanto as RoundupTM) and glufosinate ammonium (glutamine synthase inhibitor; Hoechst's trademark BastaTM); others include sulfonylurea (acetolactate synthase inhibitor) and bromoxynil (Nottingham, 1998). In most cases, resistance is conferred by foreign

genes encoding enzymes that are not susceptible to the action of the herbicide (Comai et al., 1985), or by overproduction of the target enzyme (Dale et al., 1993). In addition, the bacterial *bar* gene product provides resistance to glufosinate ammonium by detoxifying it (De Block et al., 1987). The most widely planted herbicide-resistant crop plant is Monsanto's Round-Up Ready soybean; other glyphosate-resistant crops include maize, canola, oilseed rape, sugarbeet, tobacco, and cotton (Nottingham, 1998). Although many herbicide-resistant crops were initially developed using *Agrobacterium*-mediated gene delivery, the current method of choice is particle bombardment. For this reason, these plants will not be discussed further here; the reader is referred to Nottingham (1998) for a more complete discussion of the private sector interests responsible for the development of these crops.

3.6.2 Insect resistance

One of the early selling points of transgenic crop plants was the promise of a reduction in the use of hazardous pesticides. By far the most widely used insect resistance traits are conferred by the *cry* genes, encoding toxins derived from the soil bacterium *Bacillus thuringiensis*. Several different Bt toxin gene products have slightly different modes of action and target different orders of insects, but the general strategy is similar: the crystalline toxins bind to the membrane of the larval gut and prevent nutrient uptake (Nottingham, 1998). Bt toxins are considered particularly attractive because of their high specificity, biodegradable nature, and lack of toxicity for humans and other non-target animals. *Agrobacterium* was first used to introduce a Bt gene into tobacco and tomato in 1987 (Vaeck et al., 1987), and the first transgenic plant was commercialized in 1996. The most widely planted Bt crops include maize (resistant to the European corn borer and/or southern corn rootworm), cotton (resistant to the cotton bollworm and the tobacco budworm), and potato (target pest is the Colorado potato beetle) (Shelton et al., 2002). Several other Bt crops, including canola, soybean, tomato, apple, peanuts, and broccoli are under development (Bates et al., 2005). Bt rice may hold considerable promise for Asian agriculture (High et al., 2004). A substantial body of literature exists on the economic impact of Bt crops in industrial and developing countries [see, for example, Morse et al., (2004); for a comprehensive review of ecological, economic, and social consequences, together with risk assessment of Bt crops, the reader is referred to Shelton et al., (2002)]. It should be noted that yield increases due to genetic modifications such as Bt transgenes are likely to be much higher in developing countries than in industrialized

nations; this difference is attributable to high pest pressure, and low availability/adoption of chemical alternatives in areas such as south/southeast Asia and Africa, where farmers cannot afford chemical inputs (Qaim and Zilberman, 2003). In a clear validation of the original rationale for insecticide-producing transgenic crops, Huang et al. have documented impressive reductions in pesticide application and in pesticide-related poisoning among Chinese farmers cultivating Bt cotton and rice (Huang et al., 2002b; Huang et al., 2005). Similar decreases in the use of pesticides have also been reported among farmers planting Bt cotton in India (Qaim and Zilberman, 2003).

At the same time, effects such as the long-term regional declines in the pink bollworm population density attributed to the planting of Bt cotton (Carriere et al., 2003), suggest that this technology will have significant and lasting ecological impacts. Concern about the emergence of insect resistance to Bt has led to a variety of insect resistance management strategies including regulation of the toxin dosage, mandated planting of refuge regions, and temporal or tissue-specific toxin expression (Bates et al., 2005). Pyramiding two or more Bt toxin genes in the same transgenic plant has been demonstrated to delay the evolution of resistance (Zhao et al., 2003). However, selection for resistance will continue to occur even in plants with pyramided resistance genes as long as the transgenes are also used singly in other varieties; Pink and Puddephat (1999) have argued instead for plant "multilines" that are heterogeneous with respect to the resistance genes they carry, with the composition of the mixture commensurate with the frequency of the corresponding virulence alleles in the pathogen population. Additional non-Bt proteins that target non-Bt receptors under development include the Vip3A toxin (Moar, 2002) and toxin A from the bacterium *Photorabdus luminescens* (Liu et al., 2003). Other classes of insecticidal proteins are the protease inhibitors, produced by a wide variety of plants to inhibit animal or microbial digestive enzymes, and plant-derived lectins (Nottingham, 1998). *Agrobacterium*-mediated introduction of the cowpea trypsin inhibitor gene has been shown to provide tobacco with increased resistance to the tobacco budworm (Hilder et al., 1987); the same gene in rice also confers greatly enhanced resistance to the rice stem borer (Wang et al., 2005). Pyramiding *cry* genes with genes encoding lectins and/or protease inhibitors is an active area of research in many crops of import to developing world agriculture (see, for example, the report from the Indo-Swiss Collaboration in Biotechnology at http://iscb.epfl.ch).

3.7 Enhanced nutritional content in crop plants

In addition to increasing yields and reducing the use of inputs associated with negative environmental and/or health consequences, genetic modification of food crops offers the possibility of enhancing the nutritional content of the food (Huang et al., 2002a). In some cases, the goal is to improve nutritional value by removing naturally occurring, but harmful, substances. Perhaps the best-known cases are the toxic cyanogens found in the important staple food cassava. Labor-intensive processing is required to remove these cyanide precursors, which pose particular risks to individuals with protein-poor diets, from the tubers. By blocking the synthesis of the cyanogen precursors with antisense constructs, Siritunga and Sayre (2003) achieved a 99% reduction in root cyanogen levels, even though the *Agrobacterium*-mediated transgenic modification targeted the leaf-based biosynthetic pathway.

More frequently, however, nutritional enhancement entails increasing the content of relatively rare constituents and/or creating a more balanced amino acid complement. Rice, for example, is a staple crop for over half the world's population (Wang et al., 2005), yet lacks many essential nutrients (Ye et al., 2000), and loses more nutritional value during processing (Al-Babili and Beyer, 2005). The gene encoding a non-allergenic seed albumin protein with a well-balanced amino acid content was introduced into potato (Chakraborty et al., 2000), while canola and soybean have been modified to augment their notoriously low levels of lysine (Tabe and Higgins, 1998). Transgene-driven biosynthesis of naturally occurring or modified sulfur-rich proteins has been achieved in canola (Altenbach et al., 1992) and could be used to ameliorate low methionine levels in other edible plants; this deficiency is especially pronounced in legume seeds (Tabe and Higgins, 1998). Quantity and quality of starch are other targets of food-crop engineering (Slattery et al., 2000); manipulation of the adenylate pools in potato increased both the starch content and the yield of transgenic potatoes (Regierer et al., 2002). Successful production of health-promoting very long chain polyunsaturated (including omega-3) fatty acids in oilseed crops has recently been reported (Wu et al., 2005). Although accumulation of the desirable fatty acids in linseed is limited by the availability of biosynthetic intermediates, alternative strategies, including engineering fatty acid production in green vegetables, have been proposed (Abbadi et al., 2004). If successful, such genetic modifications hold promise as a sustainable alternative to fish, which are prone to problems including dwindling stocks and contamination with heavy metals and other pollutants (Qi et al., 2004).

As might have been predicted from their roles in cancer prevention, in promoting immunity, and in slowing the progression of several degenerative human diseases (Shintani and DellaPenna, 1998), augmentation of anti-oxidant levels in plants has been another attractive goal of food crop engineering. Biosynthesis of one such group of essential antioxidants, Vitamin E (Sattler et al., 2004), has been significantly enhanced in *Arabidopsis*, corn, and soybean using *Agrobacterium*-mediated redesign of the pertinent pathways (Shintani and DellaPenna, 1998; Cahoon et al., 2003; Van Eenennaam et al., 2003). Production of other potent anti-oxidants including lycopene has been increased through transgenic overexpression of relevant enzymes in tomatoes (Muir et al., 2001; Mehta et al., 2002; Niggeweg et al., 2004). Fruit-specific silencing of the photomorphogenesis gene *DET1* in tomato elevated flux through both the flavonoid and carotenoid biosynthetic pathways, increasing the content of beta-carotene as well as lycopene, without the use of exogenous genes and without negative impacts on fruit yield or quality (Davuluri et al., 2005).

Mineral fortification of crop plants through genetic alteration or selection has been envisioned as a way to address dramatic global dietary deficiencies in iron, zinc, iodine, selenium and several other essential minerals. Identifying genes and conditions that promote mineral accumulation in plants is the focus of the HarvestPlus program within the CGIAR (Consultative Group of International Agricultural Research). In the initial phase of this initiative, six crops (beans, cassava, maize, rice, sweet potato and wheat) are being targeted; an additional 11 subsistence crops will be added in phase 2 (http://www.harvestplus.org/about.html). Transgenic approaches to increase bioavailability have targeted mineral uptake, transport to edible tissues, and augmented levels of organic compounds, including ascorbate and beta-carotene, that promote mineral absorption in humans (White and Broadley, 2005). Expression of the soybean iron storage protein ferritin in rice, for example, resulted in a three-fold rise in seed iron content (Goto et al., 1999). Alternative strategies include engineering plants to express phytase, thereby removing a key impediment in most animals to mineral uptake (Brinch-Pedersen et al., 2002).

3.7.1 "Golden Rice"

Beta-carotene is an essential dietary constituent, required in vertebrates to synthesize Vitamin A, the key visual pigment retinal, and the morphogen retinoic acid (Giuliano et al., 2000). Beta-carotene and other carotenoids are synthesized in plants from phytoene, and introduction of a phytoene synthase or desaturase from bacteria dramatically increased flux

through the carotenoid pathway in canola (Shewmaker et al., 1999) and in tomato (Romer et al., 2000), respectively. Vitamin A deficiency is a significant health problem in much of the developing world, leading to an estimated 2 million deaths and 250,000 cases of childhood blindness each year (Ye et al., 2000). A public sector initiative to engineer the beta-carotene biosynthetic pathway into rice endosperm resulted in the development of "golden rice" in 1999. *A. tumefaciens* was used to deliver into rice phytoene synthase and lycopene cyclase genes from daffodil, along with a bacterial phytoene desaturase gene. All three encoded enzymes carried transit peptides targeting them to the plastid, the natural site of synthesis of the phytoene precursor geranylgeranyl diphosphate (Ye et al., 2000). This prototype golden rice contained one tenth of the recommended daily allowance (RDA) of beta-carotene per 300 grams of rice (Giuliano et al., 2000). This relatively low yield, coupled with concerns about the presence of an antibiotic selectable marker, led to the creation of second-generation golden rice in two agronomically important rice cultivars. The details of these modifications, carried out in parallel by both public and private sector researchers, have been summarized in an excellent review by Al-Babili and Beyer (2005). Dramatic improvement in the yield of beta-carotene was achieved by substituting a phytoene synthase gene from maize for that from daffodil (Paine et al., 2005). Using generally accepted conversion factors for bioavailability and processing within the human, it is estimated that this second generation golden rice can provide 50% of the vitamin A RDA for children in a 72 g serving (Al-Babili and Beyer, 2005). Ultimately, however, as with all transformed crop plants, the only relevant value will be the nutritional contribution provided by field-grown, locally adapted varieties. Additional goals for complementary rice improvement include increasing the content of vitamin E to stabilize the beta-carotene, and increasing iron accumulation to address the iron deficiencies often found in the same populations who would benefit from golden rice (Al-Babili and Beyer, 2005).

Golden rice serves as an excellent illustration of the challenges inherent in technology transfer to developing countries. Although the research and development was provided exclusively though the public sector, the project had drawn on a wide variety of patent-protected DNA fragments and technologies, and hence the modified plant was encumbered with no fewer than 70 patent constraints held by 32 different companies and universities (Potrykus, 2001). Through a series of complex negotiations, free licenses were eventually obtained for every intellectual and technical property component (see, for example, Normile, 2000). Current efforts are focused

on introducing the engineered traits into as many local adapted varieties and ecotypes as possible. The central player in this phase of the project is the Indo-Swiss Collaboration in Biotechnology, a program funded through the Indian Department of Biotechnology and the Swiss Development Corporation. This collaborative effort, which incorporates studies on biosafety, ecological impact, and socioeconomic considerations, and which is committed to ensuring that the technology reaches the target populations, should serve as a model for future technology transfers (Potrykus, 2001). Public adoption of golden rice will depend on many factors, but as Potrykus (2001) succinctly spells out, this product of *Agrobacterium*-mediated genetic modification fulfills each of the requirements for acceptability put forth by activists opposed to genetically engineered crops.

4 GENE FLOW AND MOLECULAR APPROACHES TO TRANSGENE CONTAINMENT/MONITORING

Despite the panoply of potential benefits associated with plant genetic modification, public enthusiasm for this technology has been far from universal. Concerns range from risks inherent in the technology-such as potential ecological damage resulting from transgene escape to wild plant relatives, or possible adverse health effects of consuming genetically modified (GM) food-to sociopolitical ramifications that transcend the technology. In the latter category, valid questions have been raised about inequitable access to the new crop varieties and the impact that this may have on the distribution of wealth within poor societies. On a global scale, growing disparities in wealth between North and South (industrialized and developing countries) may be exacerbated by the practice referred to as bio-prospecting or bio-piracy (depending on one's perspective), in which genes from landraces and traditional varieties found to confer desirable traits are utilized/appropriated to genetically modify crop plants (Leisinger, 1999). Like the dangers associated with monoculture discussed earlier, these technology-transcending risks are not specific to plant genetic engineering, but they should not be dismissed as irrelevant to the discourse on GM crops.

A detailed discussion of biosafety issues and regulatory considerations associated with agricultural biotechnology is beyond the scope of this chapter. However, in light of the serious nature of the concerns, and the widespread public mistrust of the technology (Kleter et al., 2001), it would

be irresponsible not to include a brief overview of the topic. For a more detailed analysis, the reader is referred to chapter 19 in this volume.

Most health-related concerns center on the possibility of transgene transfer to gut microbes. As alluded to in section 2.4, antibiotic-resistance marker genes have come in for special scrutiny in this regard. Many questions remain unanswered concerning the ability of ingested DNA to survive passage through the digestive tract in a biologically active form, the potential for gene flow during silage production using GM crops, and the significance of GM plant-derived antibiotic resistance marker genes in comparison with the rampant dissemination of bacterial resistance attributable to overuse of antibiotics in clinical and livestock settings (Heritage, 2005). The cultivation of plants producing pharmaceutical proteins also presents possible health risks including exposure of non-target organisms and of humans to potential allergens (Peterson and Arntzen, 2004).

Adverse environmental impacts of transgenic plants may arise from toxicity to non-target organisms or from increased selective pressure on target pests, although our ability to predict the evolution of resistance development is limited (Sandermann, 2004). Transgene contamination of plants can occur via cross-pollination or inadvertent dispersal of GM seeds during harvest, transportation, or planting (Smyth et al., 2002). Gene flow from transgenic plants to wild relatives and non-transgenic crop plants has been documented for both Bt and herbicide resistance traits (reviewed in Sandermann, 2004). Contamination of conventional varieties destined for "GM-free" or organic markets represents a serious concern to farmers who have chosen to abstain from cultivating genetically engineered crops (Smyth et al., 2002). Incidents that appear to threaten the livelihood of this cohort of producers or the integrity of the booming organic movement are likely to cause a substantial negative backlash in public perceptions of agricultural biotechnology.

Molecular strategies to limit gene flow include interfering with pollen production and interruption of seed formation; both approaches can rely on *Agrobacterium*-mediated delivery of exogenous genetic material. A third, non-*Agrobacterium* mediated approach- maternal inheritance-involves introducing the transgene into the chloroplast genome to avoid pollen-based gene dissemination (Daniell, 2002). Nuclear-encoded male sterility was first accomplished by Mariani et al. (1990), who expressed an RNase gene under the control of a promoter specific for the tapetum. RNase-induced destruction of the tapetum, one of the specialized tissues in the anther required for pollen development, prevents pollen formation. Restoration of male fertility can be achieved by crossing in the *barstar* gene, encoding an

inhibitor of the *barnase* RNase, also under tapetum-specific promoter control (Williams, 1995). Other approaches to conditional male sterility include engineering a plant with a gene or set of genes, under inducible control in male reproductive tissues, that poison the plant cells or that alter the levels of metabolites such as amino acids needed for the production of pollen (Perez-Prat and van Lookeren Campagne, 2002).

Genes required for seed formation have also been targets for gene containment strategies. In the infamous "terminator" technology, inducible expression of Cre recombinase results in the removal of a spacer sequence that otherwise prevents seed-specific production of a cytotoxic ribosome inhibitor protein; application of an exogenous stimulus relieves repression of the *cre* gene and leads to destruction of the seed tissue (Daniell, 2002). Although this technology has significant potential as a built-in safety mechanism to prevent unintended dispersal of GM seed, it gained notoriety as an impediment to growers wishing to save and replant harvested seed containing proprietary alterations. As such, it is perceived as exemplifying the insensitivity of the agricultural biotechnology enterprise to the needs of subsistence farmers, and winning the acceptance of biotechnology skeptics will be a challenge (Smyth et al., 2002).

Several recent reviews focus on monitoring gene flow and on mathematical modeling of risk assessment (Wilkinson et al., 2003; Heinemann and Traavik, 2004; Nielsen and Townsend, 2004; Lee and Natesan, 2006). Transgene presence in living plants can be monitored using fluorescent marker genes (Stewart, 2005). "Bio-barcodes," consisting of uniform recognition sequences flanking a unique variable sequence to facilitate PCR amplification and sequencing of the barcode, could be incorporated into all transgene events; comparison to a universal database of barcode sequences would provide information pertinent to liability claims, intellectual property violations, and dispersal tracing (Gressel and Ehrlich, 2002). Unfortunately, our ability to predict ecological consequences of transgenic crop cultivation still lags far behind the implementation of monitoring technology, and even further behind the development of the crops themselves (Snow, 2002). As the many emerging applications of plant genetic engineering described in section 3 are adapted for novel geographical locations, each will need to be assessed on a case-by-case basis, taking into consideration the particular ecological context in which the plants are to be grown (Dale et al., 2002).

5 GLOBAL STATUS OF AGRICULTURAL BIOTECHNOLOGY AND TECHNOLOGY TRANSFER

A concise summary of the global growth of commercialized GM crops and their economic impact, replete with graphs and figures, is compiled annually by the International Service for the Acquisition of Agribiotech Applications (ISAAA) and can be easily accessed via the internet at http://www.isaaa.org (James, 2005). Additional insights concerning future trends can be gleaned by examining data available on the internet regarding approved field trials, field trial applications, and patent applications in the U.S and internationally (http://www.aphis.usda.gov/brs/brs_charts.html; http://www.nbiap.vt.edu). An analysis of these data from 1987 through 1999 reveals that the early emphasis on single gene traits-primarily herbicide and insect resistance-has now given way to attempts to alter more complex traits, such as nutritional quality and the physiological characteristics that affect crop yield (Dunwell, 2000).

As of 2005, 90 million hectares in 21 countries were planted with approved GM crops; 11 of the 21 nations, producing 38% of the world's biotech crops, are in the developing world (James, 2005). With public funding levels that far exceed those in any other country, China accounts for over half of the plant biotechnology expenditures in lesser-developed nations, with Brazil and India trailing far behind (Huang et al., 2002a). Early claims that plant genetic engineering would help ameliorate food shortages among the world's poorest populations have led to sustained skepticism and even cynicism from biotechnology opponents, in part because the first transgenic crops to be commercialized appear to benefit primarily the agro-chemical industry and corporate-scale farmers in industrialized countries, rather than consumers or subsistence farmers (Vasil, 2003). This picture is changing, however; of the 8.5 million farmers cultivating genetically engineered crops in 2005, 7.7 million of them were poor subsistence farmers. The vast majority of those farmers (6.4 million) live in China (James, 2005), the world's largest producer of rice, and genetic modification of rice is the focus of considerable attention in China's program to develop more sustainable agriculture (Wang et al., 2005). Biosafety procedures in China require multiple levels of testing for environmental release, and rice engineered for resistance to lepidopteran insects or bacterial blight is currently in the final stages of safety trials prior to commercialization (Wang et al., 2005). In addition to rice, the Chinese have placed substantial emphasis on engineering a variety of fruit and vegetable crops in an effort to bolster food security (Huang et al., 2002b). Although herbicide tolerance

is still the most prevalent engineered trait worldwide (currently constituting 71% of the global area devoted to GM crops) (James, 2005), over 90% of the field trials in China target insect and disease resistance (Huang et al., 2002b). Pest and pathogen resistant plants are already starting to have a significant impact on productivity and on reducing the environmental impact of pesticide use in China (Huang et al., 2002b; Huang et al., 2005).

Over the past four decades, public sector research institutions in several regions of the developing world have played a pivotal role in the improvement of staple crops through conventional breeding. In addition to the 20 CGIAR centers (http://www.cgiar.org), national agricultural research agencies have contributed to introducing new traits into local varieties and to facilitating distribution and adoption of these varieties by farmers. Collaborations with both academic and corporate plant biotechnology programs in industrialized nations are now beginning to make biotechnology approaches available to these public sector institutions (Toenniessen, 1995). The most successful of these collaborations have as core tenets strong emphases on capacity building, and on sustainable cropping practices that incorporate indigenous knowledge at all levels of decision-making. The following section highlights the goals, participants, and innovative aspects of some of these programs. More information on national and international public-sector research stations, and on international organizations involved in facilitating biotechnology transfer is available elsewhere (Toenniessen, 1995). A detailed investigation of the capacity for biotechnology research in four developing countries-Mexico, Kenya, Indonesia, and Zimbabwe-together with policy recommendations arising from the study, has also been published (Falconi, 2002).

The resource- and knowledge-intensive nature of plant genetic engineering has precluded development of biotechnology research programs by many of the countries that face the most pressing food security issues. Furthermore, with a few notable exceptions (China, Brazil, India and South Africa), national government investment in agricultural research is generally insufficient to maintain programs that could address local constraints and/or transfer modifications developed elsewhere to locally favored varieties (Huang et al., 2002a). Several collaborative initiatives, some including private sector partners, have evolved to meet these challenges; most of the projects undertaken within these collaborations rely on *Agrobacterium*-mediated transformation of target plants. One of the oldest such partnerships is the Indo-Swiss Collaboration in Biotechnology (ISCB), which was established in 1974. During its first two decades, this long-term bilateral program focused on developing a cadre of highly trained Indian scientists

and establishing research capacity within the Indian academic sector. In the last few years, the ISCB has promoted research partnerships between Swiss and Indian institutions, with an emphasis on increased productivity of wheat and pulses through enhanced disease resistance; a parallel initiative centers on sustainable management of soil resources (http://iscb.epfl.ch). Other bilateral programs include the Peking-Yale Joint Center for Plant Molecular Genetics and Agrobiotechnology, established in 2000 (Yimin and Mervis, 2002), and a partnership between scientists in Bolivia and those at the University of Leeds, who are developing nematode-resistant potatoes by introducing proteinase-inhibitor genes (Atkinson et al., 2001).

For several years starting in 1992, the Dutch government-funded Special Programme on Biotechnology brought together scientists, farmers, and local leaders in Zimbabwe, India, Kenya, and Colombia to develop local biotechnology agendas that addressed the needs of small scale producers. This project-based program differed from most other collaborations in the primacy it placed on participatory technology development, developing new paradigms for integrating the perspectives of farmers, consumers, and socio-economic policy experts into the process of setting research priorities (Broerse, 1998). Specific research projects included the transformation of cassava, sweet potato, and cowpea to confer virus resistance (Sithole-Niang, 2001). In Zimbabwe, the Dutch program also funded capacity building through a Master's level training program in biotechnology and shorter local training workshops.

Complementing these bilateral models for technology transfer are networks of researchers focused on one crop, as exemplified by the Cassava Biotechnology Network (CBN). Founded in 1988 by two CGIAR centers, the Centro International de Agricultural Tropical (CIAT) and the International Institute of Tropical Agriculture (IITA), in collaboration with several small research institutes in North America and Europe, the goals of the CBN are to develop strategic biotechnology tools and appropriate biotechnology applications for cassava improvement. With support from the Dutch Special Programme for Biotechnology, the network has expanded to over 800 active researchers in 35 countries and includes collaborators focusing on needs assessment, anthropology, plant breeding, and post-harvest issues including market economics. Although cassava is not cultivated in industrialized nations and therefore has not been a target for improvement by the private sector, it is an important source of nutrition and food security in many of the world's least developed areas (Taylor et al., 2004). Several characteristics make cassava a staple for subsistence

farmers, a cash crop for local markets, and a reliable source of food and animal feed during periods of famine. It is drought tolerant and grows with low inputs in areas of marginal fertility. The edible roots can be left in the ground for one to two years without decay and the leaves are an important source of protein and vitamins in many parts of Africa (Siritunga and Sayre, 2003). Through direct participation by cassava farmers, the CBN has identified several targets for improvement: resistance to bacterial blight, viral disease, and insect-inflicted damage; reduction of toxic cyanogens; enhanced nutritional value including increased vitamin content, protein content, and quantity and quality of starch in the roots; and stress tolerance (Thro et al., 1999). A recent comprehensive review, describing how each of these goals is being addressed through *Agrobacterium*-mediated transformation of cassava (Taylor et al., 2004), serves to illustrate the potential for improvement in one key crop of central importance to resource-poor farmers.

Several collaborative ventures have involved significant contributions from the private sector. In 1991, the ISAAA was created to build partnerships and to broker transfer of proprietary technology from industrialized countries to developing nations. One model project praised for its inclusion of a substantial training component involved the donation by Monsanto of coat protein genes conferring virus-resistance to Mexican scientists working on potato. The technology was further disseminated to scientists from the Kenyan Agricultural Research Institute (Krattiger, 1999). ISAAA has centers on five continents and is funded by the McKnight Foundation, the Rockefeller Foundation, various bilateral agencies, and the private sector. Like ISAAA, the USAID-funded Agricultural Biotechnology Support Project, based at Michigan State University, was initiated to bring together public sector and commercial research efforts. Between 1991 and 2003 this program funded a number of plant genetic modification projects that were undertaken in collaboration with the Agricultural Genetic Engineering Research Institute in Egypt. Goals included development of resistance to potato tuber moth, drought- and salinity-tolerant tomato and wheat, stem borer resistance in tropical maize, virus resistant tomato and sweet potato, and micropropagation techniques for pineapple and banana (http://www.iia.msu.edu/absp/). As a third example, in 2005 the Bill and Melinda Gates Foundation provided funding through its Grand Challenges in Global Health initiative for the Kenyan-based food organization A Harvest to partner with Pioneer Hi-Bred International and the Council for Scientific and Industrial Research in South Africa to develop a more nutritious and easily digested variety of sorghum (http://www.gcgh.org/).

The future success of these collaborative programs will depend on sustained commitments to their funding, and on a continued recognition of the complementarity between biotechnology and traditional crop breeding programs (Huang et al., 2002a). A key component in many of the examples described above is the emphasis placed on transfer of the technological knowledge and the tools required for scientists in the developing countries to pursue future projects more independently. Such capacity building includes training of research personnel, but also requires the establishment of a regulatory framework that is sensitive to local ecological, legal, and cultural contexts. In contrast with commercial crops that have already been vetted by Western regulatory agencies, novel locally developed crops "pose unique challenges for institutes seeking regulatory approval" (Cohen, 2005). The Swedish Biotechnology Advisory Commission was formed to help developing countries meet the challenges of biosafety capacity building through training, advising, and information exchange (L. Paula, personal communication). Similarly, a core mission of the ISAAA is training, including institutional capacity building in biosafety regulation (Krattiger, 1999). The Biotechnology Service at the International Service for National Agricultural Research (ISNAR) has also provided training in agricultural biotechnology management and performed assessments on intellectual property issues as they related to agricultural biotechnology (http://www.isnar.cgiar.org). In 2004, ISNAR was folded into the International Food Policy Research Institute and is now located in Addis Ababa, Ethiopia (http://www.ifpri.org/divs/isnar.htm).

Finally, a number of public sector research institutes are dedicated to developing and transferring biotechnology knowledge and resources to developing countries. These include the Applied Biotechnology Center at CIMMYT in Mexico City, devoted to genetic engineering of wheat and maize (http://www.cimmyt.org/ABC); the Center for the Application of Molecular Biotechnology to International Agriculture (CAMBIA) in Canberra, focusing on rice transformation using *Agrobacterium* (http://www.cambia.org); and the International Laboratory for Tropical Agricultural Biotechnology, focusing on rice, cassava, and tomato (http://www.danforthcenter.org/iltab).

A recent and highly illuminating survey of the public-sector research pipelines for GM crops in 15 developing countries identified a number of key trends in research agendas and regulatory considerations (Cohen, 2005). In contrast with the worldwide situation, where three crops (soybean, maize, and cotton) account for 95% of the global land area devoted to commercialized GM crops (James, 2005), the 201 genetic transforma-

tion events in these 15 countries encompassed no fewer than 45 different crops important to local economies, including chickpeas, cowpeas, lupin, cacao and a wide variety of fruits and vegetables in addition to rice, potato and maize (Cohen, 2005). Somewhat surprisingly, given the potential of the collaborative ventures described in the preceding section, most of the research described in this survey was carried out by single institutions, and the partnerships that did exist most often involved only public-sector institutions within the same country (Cohen, 2005).

The vast majority of the projects surveyed target biotic or abiotic stresses, while others strive to achieve prolonged shelf life or nutritional enhancement; only 5% of the transgenic plants under development are being engineered for herbicide tolerance (Cohen, 2005). This, again, is in stark contrast to the situation in industrialized nations, as described above, and reflects a much more consumer-centric approach to genetic transformation that focuses on local needs in these predominantly poor countries. Indeed, one of the most important observations to be made from this survey is the degree to which the biotechnology research in these countries actually has the potential to realize the oft-touted promise of enhancing human health and reducing poverty. By substantially decreasing the use of pesticides, fungicides, and other harmful agrochemicals, these crops should provide significant environmental and health benefits. Reducing losses attributable to pests can result in less acreage devoted to a single staple or cash crop, thereby contributing to greater biodiversity in a given area (Atkinson et al., 2001)). Enhanced shelf life can diversify a farm family's diet and allow farmers to wait out a glutted supply stream before bringing crops to market, thus increasing the financial return on their investment. Likewise, higher yields due to improved disease, pest, salt and drought tolerance lead to increased food security and more purchasing power, with the potential for "spillover effects" in the local economies (Cohen, 2005). These effects include enhanced educational opportunities for female children, personal hygiene leading to less transmission of communicable disease, and reduced population growth (Rosegrant and Cline, 2003). These, then, are the applications of *Agrobacterium*-mediated transformation that truly reflect a "poverty focus" (Conway, 1997) and that give renewed life to the promise of benefits for resource-poor farmers.

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