Kasi Azhakanandam · Aron Silverstone
Henry Daniell · Michael R. Davey *Editors*

Recent Advancements in Gene Expression and Enabling Technologies in Crop Plants

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Foreword by Mary-Dell Chilton, PhD, Syngenta Biotechnology, Inc.

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This book is dedicated to people who have died of starvation

Foreword

In the following pages, some of the world's most renowned researchers take a look at the state of the art and science of introducing novel genes into plant cells and plants. The various chapters deal with a wide range of products, from genetically modified seeds and plants to commodities made by such transgenic plants, including enzymes or vaccines. One important consideration is where and how the new genes are integrated into the host plant. The donor DNA may be inserted into the plant chromosome at random places or targeted to a specific location, by recombination or by employing site-specific nucleases. A future targeting technology may employ a minichromosome, an artificial vector assembled from parts of a normal chromosome (Chapter 13). A minichromosome is actually a megavector, which will be especially attractive for the introduction of a block of genes, for example those encoding an entire biochemical pathway for production of a valuable metabolite. At the other extreme of size, free replicons such as a (modified) plant DNA viral genome might be the most useful vector for some traits. Whatever the form and location of the vector, the DNA construct itself must mimic the plant's strategy for dictating quantity, timing, and location for the encoded protein to be made. In Chapter 2, Dr. Nuccio et al. provides a wellspring of information on plant trait gene design and approaches that have worked.

This book addresses many of these issues and will be useful to the plant genetic engineer, whether student or accomplished professional. I found new ideas and information in each chapter. I skipped around as my curiosity led me, and was excited to discover how many different types of challenges plant genetic engineering has posed, and how many creative solutions have been devised. I found the book quite readable for a technical work, with a refreshing honesty about the sometimes halting progress of scientific research.

While we are on the topic of honesty, I must confess to a motive underlying my writing of this foreword. I wanted to reach you, readers of this book, with one more message. Let me begin with a brief story: When my sons were quite young, we subscribed to a journal about the environment called Ranger Rick. One month it carried a story about insect galls, describing how the mother insect uses chemical signals to stimulate growth of the plant cells into a gall at the site where she deposits her eggs. When the insect larvae hatch, the gall serves her babies as a nice source

of food. By coincidence, my colleagues and I at the University of Washington had recently begun a research project on crown gall tumors, induced by *Agrobacterium* in plants. The insect gall story, aimed at children, made me think. Crown galls were known to produce new metabolites—octopine or nopaline, depending on the *Agrobacterium* strain that incited the gall. Could octopine and nopaline be baby food for *Agrobacterium*? When it was my turn to talk at our weekly research group meeting, I reported on the Ranger Rick article, and proposed that *Agrobacterium*, like the mother insect, might be producing the crown gall as a means of feeding its progeny. I can well recall the laughter and ridicule that ensued. The concept was named the Ranger Rick Hypothesis, and I was teased mercilessly about it for many months, until our competitors in France, Australia, and Belgium announced this very same concept as the "rationale of the gall" (in three languages). It became a respectable idea, eventually supported by increasing amounts of evidence.

There are several potential morals to this story, and I invite you to consider any of them that interest you. For me, the moral is that *Agrobacterium* truly was a genetic engineer before my colleagues and I ever thought of the possibility. The process that we now use to make genetically modified plants, the topic of this volume, is a natural one at core, invented first by a microbe and only refined by *Homo sapiens*. *Agrobacterium* worked out a way to transfer its desirable genes to the host plant cells, genes that caused abundant growth (the gall) and delicious (we suppose) meals for future generations. I hope that you who take a serious interest in the contents of this book will take equally seriously the need to inform the public that gene transfer is a natural and normal process. The products made by genetic modification of plants are more precise and predictable than those made by plant breeding, especially plant breeders use of wide crosses for introduction of new traits from wild relatives of crop plants.

By the year 2050, the world's population is expected to grow from its current 7 billion to 9 billion, a 30 % increase in the number of people. A distressing number of our present population is already hungry, even starving. Biotechnology alone cannot solve this problem, but it certainly has the potential to be an important part of the solution. Unless people accept foods produced through biotechnology, progress in food security will be slow. I believe that the principal risk of genetically modified crops is public perception, not the safety of the products themselves, which are thoroughly tested. If you share my view, I hope that you will not keep it a secret. Seek opportunities to speak to school children, garden clubs, church groups, or anyone who will listen. Tell them that there is nothing unnatural about gene transfer to plants by *Agrobacterium*. I believe that the success of genetically modified plant products depends upon the efforts of scientists like you and me to communicate to the public the safety and sanity of biotech plants.

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Preface

When we decided to edit a book on gene expression in plants, we realized that the most valuable contribution would be to combine reports from the biotech industry, and academic and research institutes that would focus on gene expression studies with economically important crops and related enabling technologies. Such a volume should be useful for students and researchers at all levels. Tremendous progress has been made in introducing novel genes and traits into plant genomes since the first creation of transgenic plants 30 years ago, and the first commercialization of genetically modified maize in 1996. Consequently, cultivation of biotech crops with useful traits has increased more than 100-fold from 1.7 million ha in 1996 to over 175 million ha globally in 2013. This achievement has been made possible by continued advances in understanding the basic molecular biology of regulatory sequences to modulate gene expression, enhancement of protein synthesis, and new technologies for transformation of crop plants.

In this book, authors who are experts in their fields describe current advances on commercial crops and key enabling technologies that will underpin future advances in biotechnology. They discuss state-of-the-art discoveries as well as future challenges. This book has three parts that encompass knowledge on genetically modified (GM) food crops that are currently used by consumers, those that are anticipated to reach the market place in the near future and enabling technologies that will facilitate the development of next generation GM crops. Part I focuses only on genetically modified maize and soybean (three chapters each), while Part II discusses the GM food crops rice, wheat, sorghum, vegetables, and sugarcane. Part III covers exciting recent developments in several novel enabling technologies, including gene targeting, minichromosomes, and *in planta* transient expression systems.

In the first chapter, Lu et al. provide a detailed overview of fascinating aspects of maize protein expression. This chapter reviews current understanding and future perspectives on key aspects that affect recombinant protein expression in this crop. These authors have summarized various factors that control gene expression, including promoters, subcellular targeting, and different regulatory elements, including introns, 5′ and 3′ untranslated regions (UTRs), spacers and insulators. In Chapter 2, Nuccio et al. present a detailed understanding on transgene design with

plant trait gene expression cassette design. The authors characterized several native maize promoters, and used the structure of these promoters to design constructs that deliver high-level gene expression/accumulation in maize. Chapter 3 is also devoted to maize. Howard and Hood review different strategies to maximize recombinant protein expression in kernels and discuss the characteristics that make maize a popular choice for recombinant protein production. These authors also assess various factors that contribute to high-level expression of heterologous proteins, together with examples of successful approaches.

In Chapter 4, Ramachandra et al. outline the breeding and biotech approaches to improve yield in soybean. The use of transgenes to complement traditional breeding through "gene stacking" will be important to further increase soybean yield and overcome biotic and abiotic stresses. One of the most successful innovations of biotech that had a major impact on farming is the introduction of herbicide tolerance in plants. Consequently, Huang et al. in Chapter 5 discuss the details of genes/traits, which have been exploited to make plants tolerant to herbicides. Tolerance to broad-spectrum herbicides makes weed control more efficient, which greatly assists the farming community. However, the increase of resistant weeds is creating new challenges for the biotech industry. In order to address this concern, authors discuss the use of trait stacking to manage hard-to-control and resistant weeds. They also describe the development of a new herbicide trait system for dicamba tolerance. Herman and Schmidt (Chapter 6) have focused on modification of soybean seeds for their use as protein bioreactors. Soybean seeds have high protein content and are used as a protein source in animal feed. These authors present the success and limitations of different approaches to produce heterologous proteins in seeds. They describe a protein rebalancing approach that increases expression of a model protein (green fluorescent protein) from 1.5 to 8 % of the total soy seed protein.

Significant progress has been made in cereal biotechnology. Many traits have been engineered into the rice genome to protect against biotic and abiotic stress or to improve grain and nutritional quality. In Chapter 7, Nandi and Khush review strategies to increase heterologous protein expression in rice grains. These authors summarize key factors responsible for controlling expression, including regulatory sequences, translational efficiency, posttranslational modifications, and compartmentalization of foreign proteins. They also discuss strategies to down-regulate endogenous protein expression in order to boost heterologous protein accumulation. In Chapter 8, Jones summarizes current advances in wheat biotechnology, particularly methods adopted for wheat transformation. He also summarizes progress in enhancing tolerance to biotic stress and to improve quality traits such as those for breadmaking. Biotechnology plays an important role in meeting the global demand for wheat, which is anticipated to increase more than 50 % by 2050. Recent advances in sorghum biotechnology are outlined by Do and Zhang (Chapter 9), with the challenges related to the tissue culture and transformation of this crop. The biotech approaches for insect pest management in vegetable crops are featured in Chapter 10 by Sreevathsa et al. The Bt protein was tested in vegetable crops to control insect pests, with discussion of different promoters used to achieve high-level expression, conferring greater resistance against target pests. The authors also discuss other

strategies, including the use of inhibitors of insect digestive enzymes, or engineering secondary metabolism of volatile communication compounds to combat pests. In recent years, there has been more biotechnology research directed to sugarcane not only for sugar production, but also for its use as biofuels. In Chapter 11, Wu discusses techniques for boosting sugar content through genetic engineering, including the expression of novel sugars.

As the opportunities of biotechnology increase, more complex tools are needed to deliver desired targets. In addition, newly acquired plant genomes' sequences provide a wealth of data that can be exploited. A key to understanding the functions of specific genes is the ability to rapidly overexpress or turn them off. Part III explores these enabling technologies. In Chapter 12, Petolino et al. describe gene targeting in plants by using Zinc Finger Nucleases (ZFNs). These authors explain how ZFNs are exploited for target mutagenesis, gene deletion and site-specific transgene integration. They also discuss other nuclease technologies, such as TALENs, meganucleases, and CRISPRs, as well as the relative advantages and limitations of these procedures. Minichromosomes combine native chromosome structural elements, like centromeres, along with transgenes for introduction into crop plants. Birchler (Chapter 13) reviews the status of "Minichromosome" technology in plants. One of the key advantages of artificial chromosomes is that multiple genes of interest could be stacked into plant genomes as a single entity without linkage to other chromosomes. Birchler also discusses both the challenges and opportunities associated with this novel technology.

Studies on gene function(s) utilizing stable transformation is time consuming and expensive. However, *in planta* transient sytems, using viral vectors developed in recent years, make it possible to study gene function by knocking down target genes or overexpression of genes of interest, although this approach has been limited to small genes (< 1.5 kb) in crop plants. There are efforts to build viral vectors, which can accommodate larger inserts. In Chapter 14, Lee et al. review various *in planta* transient expression systems for both RNAi-mediated down-regulation and over expression of target genes in monocotyledonous plants. These authors discuss the increasing use of transient *in planta* expression systems, such as virus-induced gene silencing (VIGS), virus-mediated overexpression (VOX), and cell culture-based transient approaches, as well as the advantages and disadvantages associated with each transient system. Chapter 15 by Whitham et al. presents recombinant plant viruses that are capable of carrying genetic payloads of whole genes or gene fragments that provide convenient platforms as vectors for transient gene expression and silencing in soybean. These authors focus on seven viral vector systems that have been used in this leguminous crop for VOX and/or VIGS applications. They discuss key features of the viral genomes, and future prospects to exploit viral vectors for soybean improvement.

In summary, this volume highlights a wide range of research tools, current methods, and future enabling technologies to improve crop plants to meet the ever increasing global demand for food, feed, and fuel. The editors believe that this book will be an excellent reference source for the scientific community interested in extending model plant systems into valuable applications in crop plants. We sincerely

thank all the authors for their hard work and valuable contributions, and colleagues at Springer for the invitation to edit this unique contribution to the literature for the scientific community.

Kasi Azhakanandam
Aron Silverstone
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Abstract

In the past two decades, agricultural biotechnology has had a major impact on farming, with genetically modified (GM) crops grown on more than 175 million ha globally. Although plant biotechnology has exploited model systems to gain fundamental knowledge, parallel research on field-grown plants has facilitated the development of GM crops that are used by consumers today. Biotechnology has also helped to create a rich pipeline of future products. This volume focuses on the innovations in both applied and basic research that are advancing our ability to deliver more complex multigene traits into plants. Although much of the work to date has been done on corn and soybean, other plants that are the subject of active transgenic development include rice, wheat, sorghum, sugarcane, and vegetable crops. There is a progression from the use of constitutive promoters and single traits to gene stacking, the design of transgene cassettes to more resemble native genes, the subcellular location of recombinant proteins, and manipulating storage tissues to achieve optimal performance. Herbicide tolerance and insect control have been and will continue to be highly desired traits. The future holds promise for novel modes of action to overcome current limitations. Targets for engineered recombinant proteins go beyond agronomic traits and focus on industrial or pharmaceutical uses, yield, and nutritional enhancement. Undoubtedly, future farming will advance from food/feed to industrial products, making crops more rewarding with value-added traits. Soon, even more sophisticated tools, including precision insertion or editing of genes and building novel chromosomes, will increase our ability to overcome current barriers in gene expression technology and facilitate rapid regulatory approval. The use of transient expression systems for crop plants will facilitate rapid evaluation of transgenes in crop plants. This book highlights a wide range of current research tools and enabling technologies to improve crop plants, with special emphasis on next generation approaches for engineering complex traits and value-added products that will revolutionize the future of agriculture to meet the ever increasing global demand for food, feed, fuel, and industrial products.

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Dr. Michael R. Davey has a BSc Honours degree in Botany from University College, Swansea, Wales, and a PhD from the University of Leicester, UK. In 1970, he was appointed to a research position at the University of Nottingham, UK where he continued his work on plant ultrastructure and gene transfer techniques. He has published extensively on plant cell culture and genetic engineering, and holds an Honorary Lectureship in the School of Biosciences, University of Nottingham.

Part I
Corn and Soybean

Chapter 1
Maize Protein Expression

Albert Lu, Scott Diehn and Mark Cigan

Introduction and Perspectives

Maize has been and will continue to be an important global food source with 857 million metric tons of corn produced in 2012–2013 for human and livestock consumption (USDA 2013). In addition to food and feed, industrial applications for maize extend into biofuel and starch production. Approximately 88 % of the maize acreage in the USA is transgenic, with insect resistance (IR), and/or herbicide resistance (HR) being the most prominent traits (Table 1.1). These traits improve yield and yield stability as a result of reducing stresses to the plant due to insect feeding or competition for essential nutrients by weeds. As a result of this success, companies involved in agricultural biotechnology, such as DuPont Pioneer, Monsanto, Syngenta, Bayer, and Dow AgroSciences, continue to perform research and develop new traits directed at maize crop improvement with the objective to increase grower's productivity and sustainably produce food to help feed a growing world population. In addition to productivity gains offered by transgenic traits, transgenic maize has been deployed as a cost-effective platform for expression of recombinant proteins on an agricultural scale (Table 1.2). The success of these applications is dependent on the ability to express effectively a single or multiple proteins in transgenic events.

Today's generation of transgenic maize events involves a routine process utilizing either particle bombardment- or *Agrobacterium*-based technologies. In either

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Table 1.1 Proteins expressed in commercial maize events for insect resistance and/or herbicide resistance[a]

Event name	Year	Protein(s) expressed	Trait	Expression elements			Gene design
				Promoter	5′ UTR/intron	Terminator/3′ UTR	
MON810	1996	Cry1Ab	IR	E35S CaMV	Hsp70 intron	no terminator (due to deletion)	Optimized
BT11	1996	Cry1Ab	IR	35S CaMV	ZM-ADH1gene IVS6 intron	NOS	Optimized
TC1507	2001	Cry1Fa2	IR	ZM Ubi	Zm-Ubi 5′ UTR-intron1	ORF25	Optimized
MON863	2002	Cry3Bb1	IR	35S CaMV		Tahsp17	Optimized
59122	2005	Cry34Ab1	IR	ZM Ubi	Zm-Ubi 5′ UTR-intron1	PINII	Optimized
		Cry35Ab1		Ta Peroxidase		PINII	Optimized
MON88017	2005	Cry3Bb1	IR	2XE35S CaMV		Tahsp17	Optimized
MIR604	2007	mCry3Aa	IR	MTL		NOS	Optimized
MON89034	2008	Cry1A.105	IR	35S CaMV	Cab-5UTR OsActin1 intron	Tahsp17	Optimized
		ZM-RBC SSU-CTP-Cry2Ab		FMV	hsp70 intron	NOS	Optimized
MIR162	2010	VIP3Aa20	IR	ZM Ubi	Zm-Ubi 5′ UTR-intron1	35S	Optimized
5307	2013	eCry3A.1Ab	IR	CMP		NOS	Optimized
DP4114	2013	Cry1Fa2	IR	ZM Ubi	Zm-Ubi 5′ UTR-intron1	ORF25	Optimized
		Cry34Ab1		ZM Ubi	Zm-Ubi 5′ UTR-intron1	PINII	Optimized
		Cry35Ab1		Ta Peroxidase		PINII	Optimized
T25	1995	PAT	HR	35S CaMV		35S	Optimized
GA21	1997	ZM-RBC-SSU CTP-MEPSPS	HR	OsActin	OsActin intron	NOS	Native
NK603	2000	AtCTP2-CP4EPSPS	HR	OsActin	OsActin-intron	NOS	Native

[a] Compiled from GM crop database (CERA 2012)
IR insect resistance, *HR* herbicide resistance, *CaMV* cauliflower mosaic virus, *FMV* figwort mosaic virus, *MTL* maize metallothionein, *PIN* II protease inhibitor II

Table 1.2 Examples of industrial and nonpharmaceutical applications in transgenic maize using constitutive promoters

Protein expressed	Expression elements			Targeting	Gene design	Reference
	Promoter	Intron	Terminator			
E1 endo-glucanase (*Acidothermus cellulolyticus*)	35S CaMV		Nos	PR1A SS	Native	Biswas et al. 2006
Avidin (chicken)	Zm-Ubi	Ubi intron	Pin II		Optimized	Hood et al. 1997
Beta- glucuronidase (*E. coli*)	Zm-Ubi	Ubi intron	Pin II		Native	Witcher et al. 1998
Aprotinin (Bovine)	Zm-Ubi	Ubi intron	Pin II	BAA SS	Optimizes	Zhong et al. 1999
Mn peroxidase (*Phanerochaete chrysosporium*)	Zm-Ubi	Ubi intron	Pin II	+/- BAA SS	Native	Clough et al. 2006
Laccase I (*Trametes versicolor*)	PGNpr1	Ubi intron	Pin II	+/- BAA SS; KDEL	Native	Hood et al. 2003
Xylanase bsx (*Bacillus sp.* NG-27)	Rubi3	Rubi intron	Nos	BAASS	Optimized	Gray et al. 2011
Xylanase xynB (*Clostridium stercorarium*)	Rubi3	Rubi intron	Nos	BAASS	Optimized	Gray et al. 2011

BAASS Barley alpha amylase signal peptide, *Pin* II protease inhibitor II, *CaMV* cauliflower mosaic virus

case, transgenic events result from the integration of the foreign DNA that contains a gene or genes of interest to be expressed, as well as a marker gene (such as an herbicide resistance gene) for selection and identification of transgenic events. The components or genetic elements within the integrated DNA can originate from multiple and diverse sources such as different plant and microbial species; all of which can be engineered to function in combination to contribute to effective expression of those genes and accumulation of the gene products within the correct tissue, at the right level, and at the right developmental stage(s) in maize plants. The fact that the genes to be expressed or genetic elements involved in expression may come from different species, genera, or even kingdoms, also presents a major challenge for finding ways to ensure that these elements work effectively together in a different host organism that results in the required level of protein expression. In this area, optimization of the coding region, choice of promoter, and other regulatory

elements (such as introns, untranslated regions, and terminators) can contribute to successful protein expression. Subcellular targeting can also be beneficial to protein expression by sequestering the protein in compartments where the turnover rate of the protein may be reduced, or the protein is prevented from exerting an effect that negatively impacts agronomic performance due to high expression of the foreign protein. In agricultural production, yield parity between nontransgenic and transgenic products plays a role in trait development, whereas cost and high protein yield is more of a factor in those applications where transgenic maize is used as a recombinant protein production vehicle.

In addition to the importance of genetic elements to protein expression, both integration site and the copy number of the insert can influence the level and consistency of protein expression. Generally, integration of the foreign DNA is difficult to control and genome-based effects may have significant impacts on expression levels. Efforts to target DNA to very precise locations in the maize genome are being developed to reduce positional effects, and the discovery of genetic elements that can buffer integrated DNA from surrounding influence has provided strategies that may ensure more consistent (maybe even more predictable) expression in maize.

The efforts to develop transgenic maize for input traits and as platforms for recombinant protein expression have resulted in the development of strategies to maximize transgene expression. This chapter explores the influence on, and contribution of, several of these strategies to the optimization of transgenic maize protein expression as well as providing knowledge of elements that have been tested or developed for this purpose.

Applications for Proteins Expressed in Maize

Insect Resistance and Herbicide Resistance

Commercial events expressing insecticidal proteins and/or enzymes conferring resistance to herbicides account for a large percentage of the transgenic acreage for maize. A summary of those events and their traits can be found in Table 1.1 along with the details of the various expression elements that were used to achieve levels of expression needed for trait efficacy.

Maize events with insect-resistance traits express one or more insecticidal proteins that are derived from the soil bacterium *Bacillus thuringiensis (Bt). Bt* has been exploited not only as a natural pest control agent but also as a source of insecticidal proteins that can be expressed in maize (and other crops) for the purpose of plant protection against a spectrum of lepidopteran and coleopteran insects (Szekacs and Darvas 2012) that can damage plants and reduce yield without chemical pesticide intervention. Since 1996, when the first commercial product was approved, nine maize events have been authorized by US regulatory agencies and eight of those continue to be available commercially in the USA. Recently, Event

5307 (Agrisure® Duracade™) and DP4114 maize have been deregulated by the United States Department of Agriculture (USDA; APHIS 2013). The experience gained by the process of optimization involved in the commercialization of insecticidal and herbicide traits has facilitated current understanding of what strategies may be important for protein expression in maize.

Expression of insecticidal genes derived from *Bt* in different crop species has been challenging due to the significant differences in GC nucleotide content between *Bt* and plant species. However, gene optimization to reduce the AT nucleotide content of *Bt* genes has been a contributing factor that may allow *Bt* genes to be expressed successfully at levels sufficient for plant protection in maize (Koziel et al. 1993; De la Riva and Adang 1996). An increase in GC content (with a concomitant reduction in AT content) generally reduces the presence of known or cryptic processing or instability signals that are AT-rich by nature, allowing for improved *in planta* expression (see gene optimization section). From Table 1.1, all IR transgenic events express *Bt* proteins that have been modified from their native (*Bt*) coding sequences for improved expression as indicated by "optimized" in the gene design column. Consistent with the strategy used for *Bt* gene expression, successful use of the phosphinothricin N-acetyltransferase (PAT) gene from the bacterium *Streptomyces viridochromogenes* to confer herbicide resistance to glufosinate (T25) required plant optimization of the coding sequence. In contrast, glyphosate resistance was achieved in maize through the use of essentially the native (plant) versions of the maize 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) gene with specific amino acid mutations (GA21), or the EPSPS gene from *Agrobacterium tumefaciens* CP4 (NK603). Today, commercial products often express one or more IR and HR trait genes, increasing the complexity of the optimization process required to provide expression levels to meet commercial trait efficacy.

Promoter selection is also a factor that contributes to the ability to express genes at efficacious levels in the necessary tissues at the correct developmental stages in maize. Promoters that are seed-specific, for example, are preferred for expression of proteins that have pharmaceutical and industrial value when using maize as protein production platforms. These promoters allow for high and stable accumulation of functional protein in the natural storage organs, kernels, of maize (Stroger et al. 2002; see also Chap. 3). Promoters that facilitate strong constitutive expression of proteins throughout different developmental stages of maize are useful for IR and HR applications. In these cases, high levels of protein expression of IR or HR genes are needed for protection against insect pests at multiple feeding sites (e.g., leaf, sheath, stalk, root, silk, and ears), or in the tissues that are sensitive to the action of herbicides, respectively. Most commercial events expressing *Bt* genes have used either the maize polyubiquitin 1 promoter (Ubi-1;Christensen and Quail 1996) or a plant viral promoter derived from the caulimovirus family (35S of cauliflower mosaic virus or figwort mosaic virus; Odell et al. 1985; Bhattacharyya et al. 2002). Root-preferred promoters such as a maize metallothionein (MTL) or a wheat peroxidase (Ta-Peroxidase) have been used to express corn rootworm insecticidal proteins in MIR604 and 59122 (Table 1.1). Resistance to the herbicide glyphosate in GA21 and NK603 has been achieved by constitutive expression of EPSPS genes

using a rice actin (Os-Actin) promoter (McElroy et al. 1990). The inclusion of a native intron that is naturally associated with the promoter, or the introduction of a heterologous plant intron within the 5′ untranslated leader sequence (UTR) of a gene, is a common strategy that has been used to enhance maize protein expression. (See section in this chapter on intron-mediated enhancement of gene expression.) This strategy has been effective particularly in combination with plant viral promoters such as c*auliflower mosaic virus* 35S promoter (CaMV 35S) and figwort mosaic virus (Table 1.1).

Industrial Enzymes and Nonpharmaceutical Protein Reagents Produced in Maize

Several proteins with industrial or reagent-based applications have been expressed in maize due to the competitive opportunity for large-scale protein production (Table 1.2). The advantages of using maize as a plant-based platform for protein production include a well-established system for genetic transformation, an established toolbox of regulatory elements, and targeting signals to help maximize transgene expression and accumulation, high yield in the field, infrastructure for field production and harvest, and relatively large grain size compared to other plant species (Ramessar et al. 2008). Maize as an expression platform can provide for the correct folding of complex proteins such as antibodies, posttranslational modification, scale of expression, and absence of human pathogens (Naqvi et al. 2011). The ability to express proteins in selective tissues like kernels offers flexibility for storage over long periods of time before protein extraction without significant loss in protein activity. Kernels may also be a means for delivery in feed applications. Grain size is an important factor when considering the often successful strategy of accumulation of recombinant protein in grain. Ramessar et al. (2008) and Hood and Howard (2009) provided an excellent overview of the range and purpose of proteins expressed in maize plants (particularly using seed-specific promoters) and are not covered extensively in this chapter.

Strategies that improve the expression and accumulation of heterologous proteins in maize for recombinant protein expression platforms have been developed with the emphasis on maximizing the yield of recombinant proteins per unit biomass to be as economically feasible as possible. Reduction of any potential negative impact of high protein expression on plant health, agronomics, and yield is also desirable. The need to satisfy both high yield per unit biomass and minimize effects on yield and agronomics has led to one strategy that combines the use of strong constitutive promoters, such as maize ubiquitin (Ubi1), rice ubiquitin (rUBi3), and CaMV 35S, in combination with subcellular targeting (Table 1.2).

High expression of proteins throughout the plant can be achieved by the use of these strong constitutive promoters. However, in several cases, aberrant plant phenotypes have been observed such as early senescence, male sterility, and low/no seed set (Clough et al. 2006), stunting and plant mortality (Hood et al. 2003),

and stunting, reproductive development problems, and shriveled grain (Gray et al. 2011). In some cases, constitutive expression resulted in high expression and normal plant phenotype (Hood et al. 1997; Witcher et al. 1998; Zhong et al. 1999). Whether a protein has an effect on plant health can be related to a combination of the properties of the overexpressed protein (e.g., enzyme, solubility, capability of interaction with plant proteins) and how well maize cells or tissues tolerate its expression. In several cases, depending on the types of genes that were expressed, subcellular targeting signals designed to sequester the proteins in different subcellular compartments (e.g., cell wall, endoplasmic reticulum, vacuole, cytoplasm) have been used to achieve high expression without observable aberrant plant phenotypes (Zhong et al. 1999; Hood et al. 2003). In other cases, confining expression of the heterologous protein to kernels using seed-specific promoters has been an effective strategy (see Chap. 3 in this book from Howard and Hood).

Influence of Gene Optimization on Protein Expression Levels

One factor in the successful expression of proteins in maize (and any other heterologous expression system) is the coding sequence. The nucleotide sequence can impact expression due to multiple factors that may affect how well a gene is expressed and translated in plant cells. In the majority of commercial products, gene optimization is a part of the process to maximize expression of heterologous proteins for different applications (see Table 1.1 and 1.2), especially if the gene is derived from phylogenically different sources (e.g., bacteria, animals). The increasingly low cost of gene synthesis provides the opportunity to back translate a protein and modify its nucleotide coding sequence to optimize expression without changing the protein sequence. In fact, many gene synthesis companies independently provide codon optimization services based on different algorithms that have been designed to improve expression. Most of these algorithms adapt the codon usage of a gene of interest to the typical codon usage of the intended host as one component of the design process, and generally take into account several other parameters including mRNA secondary structure.

A benefit of the genomics revolution has been the exposure of codon biases for many different plant species. This has led to codon counting to decipher which codons are favored in high expressing genes from an organism of interest. Adaptation of codon bias (Sharp and Li 1987; Carbone et al. 2003; Jansen et al. 2003) is usually a primary consideration for gene optimization in plants with the intention of mimicking a well-expressed host gene. Selecting the most frequently used codon for each amino acid allows the use of the most abundant tRNAs and minimizes effects on expression due to the presence of rare codons. The Codon Adaptation Index (CAI; Sharp and Li 1987) is one of several statistical approaches that have been developed that compares a designed gene with host codon bias. Genes that

maximize the CAI have expressed well in many instances, although the tested gene set is small. Maize has an overall G+C content of about 55 % (Nakamura 2000; www.kazusa.or.jp/codon) with a preference for a G or a C nucleotide in the third or wobble position of the codon (Fennoy and Bailey-Serres 1993; Liu et al. 2010). Koziel et al. (1993) constructed a synthetic version of a Cry1Ab gene for transgenic maize expression by increasing G+C content to 65 % that reflected a maize-preferred codon usage. This study reported expression of Cry1Ab protein in transgenic maize events at levels insecticidal to European corn borer. Improvements in the expression of heterologous genes as a result of maize codon optimization have been reported for blue fluorescent protein (BFP), green fluorescent protein (GFP), yellow fluorescent protein (YFP; Sattarzadeh et al. 2010) and xylanase bsx (Gray et al. 2011). Whether improved expression is due directly to the codon bias, or to other factors is difficult to differentiate. Increasing G+C content may inherently remove potential elements such as cryptic splicing sites, premature polyadenylation sites, RNA instability motifs (Murray et al. 1991; van Aarssen et al. 1995; Christov et al. 1998; Diehn et al. 1998; De Rocher et al. 1998), and other elements that may lead to reduced transcriptional and translational efficiency. The intentional elimination of several polyadenylation signals and instability motifs improved expression of a *Bt* gene in maize (De la Riva and Adang 1996).

Frequently, gene optimization is performed in the absence of experimentally testing expression of the native gene sequence in maize. This is done *a priori* based on a general assumption that an improvement in expression will be the likely outcome (Hood et al. 1997; Zhong et al. 1999; Gray et al. 2011). Optimization may be particularly beneficial if a gene to be expressed in maize originates from a bacterial species such as *B. thuringiensis* (Table 1.1) where its G+C content (35.5 %) is significantly lower compared to maize (55 %; De la Riva and Adang 1996). The lower G+C content increases the probability that multiple deleterious sequence motifs may be present since several of these sequence motifs (described above) frequently contain A+T rich sequences. A very low preference for G+C (24.6 %) at the wobble position, in the case of *B. thuringiensis* genes, compared to maize (64 %; www.kazusa.or.jp/codon) may result in the presence of maize rare codons in the native sequence.

Optimization may not be necessary to achieve good expression for every heterologous gene. There are several examples of native genes from fungi and animals that express well in maize and achieve their intended functionality (Hood et al. 2003; Woodard et al. 2003; Clough et al. 2006; Biswas et al. 2006) (Tables 1.1 and 1.2). In these cases, the genes have maize-like characteristics. Overall G+C content and preference for G+C in the wobble position is comparable to, or greater than, maize and deleterious sequences such as cryptic splicing sites, premature polyadenylation sites, and RNA instability motifs are rare or absent. The presence of rare codons is also minimal in these sequences. However, strict adherence to these characteristics may not always be required to obtain desired expression levels.

Control of Protein Expression

Promoters

A consideration to achieving the desired levels of expression in maize and other plant species is the choice of promoter. Promoters direct expression of transgenes in plants quantitatively, spatially, and temporally. Proper selection of a promoter is reflected by the specific end-use application of the transgene, most commonly recombinant protein production or crop protection. In transgenic maize plants generated for the purpose of recombinant protein expression, the latter may be targeted specifically in the seed. Applications directed toward IR or HR commonly focus on constitutive expression throughout most developmental stages of the plant. In both cases, optimizing protein expression and accumulation can require a balance between maximizing expression in the tissues of interest and minimizing negative impacts on the plant in the form of agronomic or yield penalties. How well a gene can be expressed (e.g., gene design), how potent the gene product is (e.g., efficacy, enzymatic activity), and the inherent level of plant toxicity caused by overexpression of the recombinant protein influences promoter selection. In most cases, optimization of expression (and phenotype) will be empirical, requiring the careful evaluation of multiple promoters to identify those that function effectively to achieve a desired outcome. This empirical approach requires the availability of alternative promoter choices that can be tested with each transgene.

The need for alternative promoters also plays a role in the ability to effectively coexpress multiple genes in a molecular stack configuration (Peremarti et al. 2010). Multigene transformation continues to increase in plant biotechnology in order to generate complex trait stacks or pyramids that satisfy future needs for transgenic maize products. These products may include different trait package combinations of HR, IR, improved agronomic characteristics, improved nutritional value, and recombinant protein production. The versatility to deploy different promoters can be beneficial for coexpression of multiple genes but also can increase construct integrity and reduce the potential for gene silencing. In the last 5 years, about 120 maize promoters have been patented (Fig. 1.1) primarily by commercial entities to provide promoters with different strengths and specificities to help meet the expression challenges needed for various transgenic applications.

Application of Expression Profiling Technology to Promoter Discovery

Previous methods used to identify promoters with desirable expression patterns relied primarily on information generated from the libraries of expressed sequence tags (ESTs) and microarrays which identified promoter candidates based on the

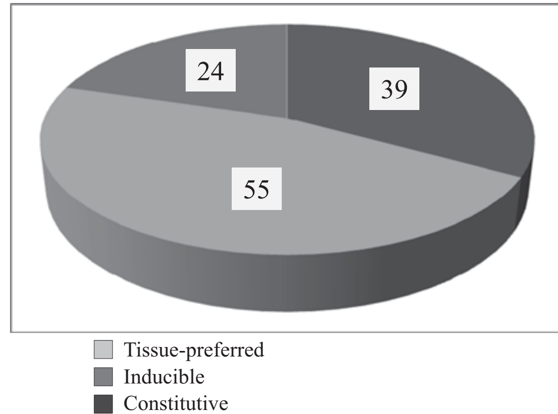

Fig. 1.1 The number of maize promoter patents issued from 2009 to 2013 for constitutive, tissue-preferred, and inducible promoters

expression profile of their coding regions. Knowledge of the coding region sequence then became the basis of isolating the corresponding promoter sequence by multiple PCR-mediated techniques including Genome Walking. Today, advances in next-generation sequencing and DNA synthesis technologies have improved the ability to profile mRNA expression and isolate promoter sequences that can subsequently be tested for their performance in plants. Transcriptome libraries provide a tool to develop "electronic" expression profiles of genes with respect to tissue preference, strength and timing of expression, and either induction or suppression of expression in response to abiotic and/or biotic stimuli. The availability of multiple plant genomic sequences may be used to identify the upstream regions of those genes (or orthologs) that were chosen based on their expression profile, and the candidate promoter regions synthesized and tested in transgenic plants to evaluate functionality and corresponding expression characteristics.

The tools available in the postgenomic era are creating opportunities to explore related plant species as an alternative source for promoters and other regulatory elements. *Brachypodium distachyon* (family Poaceae, subfamily Poodieae) is phylogenetically related to wheat and barley and is emerging as a model system for grasses. A diploid variety of *Brachypodium,* Bd21, was among the first of the Poodieae subfamily to have its genome sequenced (Coussens et al. 2012). Comparison of its genome sequence with rice and sorghum, which belong to two other Poaceae subfamilies, indicates that gene content and gene family structure are colinear between these species despite the large differences in genome size. This provides an initial step to identify not only constitutive promoters but also tissue-preferred promoters based on *in silico* analysis of transcriptome profiling data and genomic sequence information. Promoters from *Brachypodium* have been shown to function in a comparable way in maize (Coussens et al. 2012). Similar strategies have been used to identify Sorghum promoters for use in maize expression (Sivasudha and Kumar 2008; Ahmad et al. 2012).

Constitutive Promoters

Promoters are traditionally divided into three categories, namely constitutive, tissue-preferred, and inducible. Constitutive promoters are the most prevalent among all these categories and most are derived from plant viruses or plant housekeeping genes. Early work based on understanding plant virus infection and replication identified long intergenic regions that functioned as promoters in plant cells. These intergenic regions drive expression of viral replication, movement, and capsid proteins and are dependent on plant *trans*-acting factors for transcriptional regulation. The most recognized member of this group of promoters is the CaMV 35S, which controls the synthesis of the viral 35S major transcript (Odell et al. 1985; Kay et al. 1987). Although used widely, the CaMV 35S promoter performs poorly in monocotyledons such as maize (Goddijn et al. 1993; Urwin et al. 1997), but expression is enhanced by the addition of a downstream intron within the 5′ UTR (see intron-mediated enhancement section) (Morita et al. 2012). A range of promoters from other caulimoviridae members have been characterized in maize, including figwort mosaic virus (FMV; Bhattacharyya et al. 2002), Cestrum yellow leaf curling virus (CmYLCV; Stavolone et al. 2003), and Cassava vein mosaic virus (CsVMV; Verdaguer et al. 1996). Many of these promoters direct high-level expression of heterologous genes in transgenic maize plants. Expression using the CmYLCV promoter in maize was found to be twofold greater than either the CaMV 35S or maize ubiquitin 1 promoters (Stavolone et al. 2003). CaMV 35S, FMV, and CmYLCV have successfully been used to direct efficacious levels of expression of insecticidal genes in commercial products. These promoters provide the additional benefit of low or no pollen expression to minimize risks and concerns on the impact of insecticidal protein expression on nontarget/beneficial insects (Table 1.1). Plant-derived promoters have been preferred over promoters of viral origin due to the potential that viral promoters can be silenced in plant cells (Potenza et al. 2004).

The promoters from housekeeping genes have been utilized quite extensively in maize. Well-known examples of these types of promoters include the maize ubiquitin 1 (*Ubi-1*) promoter and the rice actin promoter (*actin1*). The *Ubi-1* promoter is more than tenfold stronger than the CaMV 35S promoter in maize protoplasts when combined with the first intron originating from Ubi-1 (Norris et al. 1993). The expression pattern in transgenic maize plants using this promoter is ubiquitous with high levels of expression in pollen, leaves, stalks, kernels, roots, and silks (Christensen and Quail 1996). The rice *actin1* promoter drives strong transgene expression in rice protoplasts transiently expressing gusA (McElroy et al. 1990) and in most tissues of transgenic rice plants (Zhang et al. 1991) and maize. The first intron of the *actin1* gene is required for promoter function (McElroy et al. 1990) and inclusion of this intron in a chimeric CaMV 35S promoter resulted in a 40-fold enhancement of activity in transgenic rice and corn (McElroy et al. 1991). The maize Ubi-1 promoter has found broad application for expression of heterologous proteins in IR and recombinant proteins (Tables 1.1 and 1.2). As a result of the wide spread use of this promoter in maize, much attention has been focused on identification and

functional testing of promoters from polyubiquitin orthologs from other monocotyledon species such as rice (Lu et al. 2008; Gray et al. 2011), Brachypodium (Coussens et al. 2012), switchgrass (Mann et al. 2011), and sugarcane (Wei et al. 2003) to provide useful alternatives for maize heterologous gene expression.

The maize histone H2B promoter has also been shown to drive strong constitutive expression in transgenic maize plants, particularly in metabolically active tissues (Rasco-Gaunt et al. 2003). Strong expression was dependent on the inclusion of either the maize Ubi-1 intron or the maize Adh1 intron immediately downstream of the promoter. Promoters from other housekeeping genes that have been demonstrated to provide constitutive expression include eukaryotic initiation factor alpha (EF1A) and S-adenosylmethionine decarboxylase (SAMDC; Coussens et al. 2012). In general, constitutive promoters isolated from one monocotyledonous species may show functionality in other monocotyledons and provide effective strategies for identification of promoters useful for maize transgene expression. In many cases though, the expression profile of promoters from an orthologous gene may be conserved, but strength of expression can be variable when tested in maize.

Tissue-Preferred Promoters

Multiple classes of tissue-preferred promoters have been described (Potenza et al. 2004). These types of promoters allow refined expression of a transgene in the tissues important for achieving the overall goal for heterologous expression in transgenic events. Tissue-preferred expression can be an effective approach to mitigate or reduce phytotoxic or negative agronomic issues related to overexpression using a constitutive promoter. While constitutive promoters remain the dominant promoter type for maize transgene expression, seed-preferred promoters have been used for production of proteins for industrial and nonpharmaceutical applications (see Chap. 3) and more applications for tissue-specific promoters are being realized in commercial products for IR.

Seed-preferred promoters are the most abundant class of tissue-preferred promoters that have been characterized due to their utility for improvement of oil and nutritional quality in grain and for the accumulation of foreign proteins in an environment favorable for long term stability and storage. Multiple promoters have been identified and characterized that direct expression in specific tissues within the seed. Storage proteins such as corn zein (Schernthaner et al. 1988), rice glutein (Leisy et al. 1989; Takaiwa et al. 1991; Zheng et al. 1993), barley hordein (Marris et al. 1988), rice prolamin (Qu and Takaiwa 2004) and wheat glutenin (Colot et al. 1987) have been the sources for seed-specific promoters, predominantly directing expression in the endosperm (Wobus et al. 1995). Promoters are available that direct gene expression in the embryo or in the aleurone (Opsahl-Sorteberg et al. 2004; Qu and Takaiwa 2004; Furtado and Henry 2005). Recently, several embryo-preferred promoters, a strong oleosin (OLE) promoter, a weaker early embryo protein (EAP1) promoter, and an aleurone-specific lipid transfer protein promoter (LTP2),

were used to overexpress transcription factors to significantly increase oil content in maize seeds (Shen et al. 2010). The OLE promoter has also been used to express the rice Giant Embryo gene (GE) in maize embryos to affect embryo to endosperm ratios (Zhang et al. 2012).

Anther-specific promoters, such as the maize 5126 promoter, and pollen-specific promoters, such as PG47 derived from a pollen-specific polygalacturonase gene, have important utility in the production of transgenic male sterile maize (Cigan et al. 2001). Numerous anther-specific and pollen-specific promoters from other plant species have been identified, including the RA8 promoter from rice (Jeon et al. 1999), the TA29 promoter from tobacco (Koltunow et al. 1990), and the A9 promoter from *Arabidopsis* (Paul et al. 1992).

Promoters for expression in vegetative tissues have been largely derived from leaf or green tissues primarily taking advantage of photosystem genes as a rich source of promoters (Gotor et al. 1993;Matsuoka et al. 1993; Orozco and Ogren 1993; Kwon et al. 1994; Yamamoto et al. 1994; Yamamoto et al. 1997). Rubisco small subunit (RbcS1) and Phosphoenolpyruvate carboxylase (PepC) promoters provide strong expression in bundle sheath and mesophyll cells, respectively (Sattarzadeh et al. 2010). A promoter controlling expression of the pyruvate orthophosphate dikinase gene (C4Pdk) showed exclusive expression in leaf blade mesophyll cells, but less expression in mesophyll cells in sheath and not in other tissues (Taniguchi et al. 2000). Promoters from this class have found application in development or testing of insect-resistant traits for maize (Event *Bt*176–CERA 2012) and rice (Datta et al. 1998).

Information on tissue-specific promoters that drive expression in nongreen maize tissues such as stalk, silk, and root is limited primarily due to the lack of need for such promoters in maize. Traditionally, constitutive promoters have been able to provide strong expression in these tissues particularly for current transgenic maize applications. However, increased efforts related to finding transgenic solutions for drought tolerance, nitrogen utilization, and the continuous need for new rootworm traits have provided an impetus to identify, characterize, and evaluate root promoters that can be used in these areas to help reduce expression of heterologous proteins in parts of the plant where it is not necessary to achieve a desired phenotype, and to help reduce pleiotropic effects on the plant. Root-preferred promoters, such as those driving expression of a maize root metallothionein gene (MTL) or a wheat peroxidase gene (Ta-Perox), have been effective in controlling the expression of corn rootworm insecticidal actives in commercial products (see Table 1.1). Other metallothionein gene promoters have been found to provide similar expression profiles when evaluated in transgenic plants (Fordham-Skelton et al. 1997; Dong et al. 2010) and are important sources for root promoters due to their important role in root development. A root-specific promoter from rice, RCc3 (Xu et al. 1995) was used to improve drought tolerance by overexpression of a transcription factor, OsNAC10, in transgenic rice (Jeon et al. 2010). Although the selection of available root promoters from maize appear to be limited, rice has been a valuable source of such promoters (Xu et al. 1995; Iwamoto et al. 2004; Yao et al. 2008;

Dong et al. 2010; Li et al. 2013) that can be tested in maize to determine if these promoters provide adequate expression required for specific traits.

Inducible Promoters

Inducible promoters can be useful due to their ability to respond to a changing environment. This characteristic can offer distinct advantages over constitutive and tissue-specific expression of a transgene, particularly when there is a need for regulated expression that is limited to specific tissues and/or at specific times (e.g., during insect feeding, infection by pathogens). Multiple inducible promoters have been identified in plants and they generally fall into two categories (1) those responsive to endogenous signals (e.g., plant hormones) and (2) those responsive to exogenous physical stimuli (abiotic and biotic stresses) such as light (Gilmartin and Chua 1990a, 1990b), temperature (Prandl et al. 1995; Kirch et al. 1997), and nutrient deficiency (Zhang and Forde 1998). The most widely studied class of inducible promoters is arguably the hormone-responsive promoters that are regulated by auxins, gibberellins, and abscisic acid. The molecular mechanism involved in regulation of these genes is well understood based on extensive studies in *Arabidopsis* (Abel et al. 1996; Busk and Pagès 1998; Chapman and Estelle 2009; Peremarti et al. 2010; Davière and Achard 2013).

Another class of inducible promoters is derived from the genes involved in plant defense from insect pests and pathogens. Wounding or pathogen infection can induce localized expression of sets of genes at the wound/colonization site as well as the induction of genes systemically in the plant. Several reviews have been published describing the advancements in understanding the induction pathway, promoter elements involved in induction, and the application of these promoters for controlling expression (Gatz 1997; Corrado and Karali 2009; Pauw and Memelink 2004; Potenza et al. 2004; Memelink 2009). Wound-inducible promoters have been characterized from a variety of plants including the potato wun1 and proteinase inhibitor II (pin2) genes. These promoters have been shown to direct high wound- and pathogen-inducible expression, but no or low basal expression in the absence of stimuli (Logemann and Schell 1989; Logemann et al. 1989; Siebertz et al. 1989; Keil et al. 1990; Xu et al. 1993). One of the uses of wound-inducible promoters is for the expression of insecticidal proteins during periods of insect pressure. However, a significant challenge with this strategy is the response time of the promoter in reaching the expression levels needed for plant protection. This is in contrast to constitutive or tissue-specific promoters where an insecticidal protein can accumulate in tissues and act immediately upon insect feeding. Even so, plant efficacy has been demonstrated against striped stem borer in transgenic rice events expressing Cry1B under control of a wound-inducible maize proteinase inhibitor (MPI) gene promoter (Breitler et al. 2004). Although expression was both local and systemic, better protection was observed with Cry1B driven by the maize Ubi-1 promoter compared to the MPI promoter, presumably due to a delay in expression from the

latter as a result of the need for activation of signal transduction and events leading to transcription (Breitler et al. 2004).

Inducible promoters may be more effective with stationary effectors that generally fall into the abiotic category which are characterized by persistence, where the plant is exposed to an extended condition that occurs over days and weeks rather than hours (like in insect feeding). This type of prolonged stress is more compatible with the induction time required for inducible promoters, and traits involved in nitrogen utilization or drought tolerance.

Chemical Switch Systems

Promoters that can respond to external chemical compounds provide an attractive strategy for more precise control of gene expression in plants. These promoters employ a combination of regulatory elements that may consist of *cis*-acting or both *cis*- and *trans*-acting factors that function with the transcriptional system of the host plant to respond to application of chemical agents not normally found in plants (Gatz 1997; Gatz and Lenk 1998; Zuo and Chua 2000; Corrado and Karali 2009). Conditional regulation of transgenes in plants fall into two general classes, these being promoters and receptors. Inducible expression systems which incorporate promoters have been developed that increase reporter gene transcription upon application of herbicide safeners, plant hormones, and heat shock (Severin and Schoeffl 1990; Jepson et al. 1994; Suehara et al. 1996; De Veylder et al. 1997). Although the rapid induction of specific reporter genes has been described, a concomitant increase in expression of native plant genes that naturally respond to these signals also occurs and can lead to phytotoxicity problems, particularly if high levels of inducer are required. In certain instances, induction of host genes can complement heterologous gene expression such as induction of plant defense pathways in combination with insecticidal gene expression (Cao et al. 2006).

Multigene systems consisting of regulatory proteins that facilitate the transcription or derepression of a cognate reporter gene have also been developed to respond to a variety of ligands (Padidam 2003; Borghi 2010). In dicotyledons, for example, induction of gene expression has been reported using the native yeast ACE1 and the *Aspergillus* alcR activators through the application of copper and ethanol, respectively (Caddick et al. 1998; McKenzie et al. 1998; Mett et al. 1993), while the derepression of reporter gene activity has been demonstrated using bacterial regulators, TetR and lacI, in the presence of the corresponding ligands (Gatz et al. 1992; Wilde et al. 1992). Synthetic tripartite transcriptional activators have also been developed for use in plant systems to evoke gene induction in response to mammalian steroid hormones (dexamethasone and estradiol), and steroidal and nonsteroidal agonists of the insect hormone, 20-hydroxyecdysone (Schena et al. 1991; Lloyd et al. 1994; Aoyama and Chua 1997; Martinez et al. 1999a; Martinez et al. 1999b; Bruce et al. 2000; Zuo and Chua 2000).

While these systems are useful for the regulation of a variety of genes for basic research, the majority of these approaches would be either impractical or inappropriate for the regulation of a gene in commercial crop applications. In contrast, the nonsteroidal ecdysone agonists, tebufenozide, and methoxyfenozide, are available commercially and have potential for field applications (Dhadialla et al. 1998; Martinez and Jepson 1999). A chemical switch system that has shown promise in agriculture relies on the application of a methoxyfenozide ligand to restore male fertility to genetically male sterile maize for hybrid seed production. In maize, the production of hybrid seed is divided into two stages, parent inbred maintenance and hybrid seed production. Hybrid seed production requires the female inbred parent to be male sterile. Male inbreds planted in adjacent rows are used to pollinate the female inbreds. Presently, male sterility is achieved genetically, using cytoplasmic male sterility (CMS), or by removing the developing tassels from the female inbreds (detasseling).

Regulating male fertility conditionally in female inbreds is an attractive alternative as it would overcome CMS germplasm conversion limitations and eliminate the cost of manually detasseling large acreages of hybrid production fields. In this system, a chemically regulated fertility gene would restore male fertility following chemical application to allow for self-pollination during inbred increase. However, in hybrid production fields, chemical application is unnecessary as the female inbreds are genetically male sterile. Thus, conditional complementation of a recessive male fertility gene in inbred production fields results in chemical application to limited acreages, and due to the recessive nature of the mutation, any inbred used as a male pollen donor in hybrid seed production will result in genetically fertile progeny in the farmer's field.

The components of this system consist of a mutant male fertility gene in maize known as *ms45* (Albertsen et al. 1993). Mutations at *Ms45* are recessive and homozygous mutants are male sterile due to the inability of pollen grains to develop normally and function. Male fertility can be restored in maize plants containing homozygous *ms45* by molecular complementation using a transformed copy of the wild-type MS45 gene expressed from its native as well as anther-specific and constitutive plant promoters (Cigan et al. 2001). Based on these observations, an Ms45 complementation assay was used to develop a chemical switch to conditionally express *Ms45* during the inbred increase portion of the corn production cycle allowing self-pollination of these otherwise male sterile plants (Unger et al. 2002).

Unger et al. (2002) evaluated the conditional regulation of *Ms45* by the nonsteroidal ecdysone agonist methoxyfenozide by modifying the transcriptional activator VP16-GAL4 with the addition of 340 amino acids of the European corn borer ecdysone receptor which included the hinge and ligand-binding domains. This receptor configuration, VP16-GAL4-EcR (VGEcR), was placed under the transcriptional regulation of the constitutive maize Ubi-1 promoter or the anther-preferred 5126 promoter with gal:MS45 and introduced into *ms45* maize. Unger et al. (2002) found that in contrast to untreated plants, methoxyfenozide-treated plants extruded anthers and shed pollen. Subsequent expression analysis of Ms45 protein in anthers from methoxyfenozide-treated plants demonstrated ligand-dependent expression of

MS45 protein with microspore development observed to proceed beyond the point typically associated with microspore abortion in *ms45* mutant maize.

The successful deployment of this approach to achieve male sterility provides one of the first examples where a chemical switch promoter was developed successfully for commercial use. This success also demonstrates that chemical switch promoters may be as effective as native promoters in meeting temporal and spatial expression needs in biotechnology.

Subcellular Targeting

Targeting of proteins to different subcellular compartments can have beneficial effects on protein accumulation, functionality, and plant health. The biochemical environment within a compartment may have significant influence on maximizing heterologous protein expression, stability, proper protein folding and posttranslational modification (e.g., glycosylation), while minimizing any detrimental effects that the expressed protein can have on plant phenotype and agronomics. The latter is most likely due to prevention of interactions of heterologous protein with plant cellular proteins or membranes and interference with important plant processes. In most cases, evaluating the effect of targeting a protein to different compartments is necessary to identify the optimal combination that provides high (or acceptable) yield of functional protein without penalty to plant health. In certain cases, such as with the expression of some insect-resistant or herbicide-tolerant traits, accumulation of protein in compartments can reduce the risk of allergenicity issues by avoiding unwanted protein glycosylation. Choices of subcellular compartments include cytosol, chloroplast, vacuole, apoplast/extracellular matrix, and endoplasmic reticulum (ER). Several effective targeting peptides that have been used in maize are summarized in Table 1.3.

One of the most prevalent peptide sequences used to target proteins to the extracellular matrix or apoplast is the Barley alpha amylase signal peptide (BAA SS). Targeting to the apoplast is a strategy that has been very effective for high-level accumulation of heterologous proteins in maize using either constitutive or seed-specific promoters. In the case of fungal Laccase I (Hood et al. 2003), targeting this enzyme to the extracellular matrix by addition of the BAA SS targeting peptide to the N-terminus was more effective at achieving high expression than cytosolic or ER accumulation of the protein. In fact, ER retention of laccase I under the control of the Ubi-1 promoter control resulted in plant mortality. Instances where using BAA SS was not successful include Mn peroxidase (Clough et al. 2006) and Xylanase bsx and xynB (Gray et al. 2011) where targeting resulted in poor plant health and reduced reproductive viability. Hood et al. (2007) demonstrated a differential impact of targeting E1 cellulase and cellobiohydrolase to the extracellular matrix (BAA SS), the ER (KDEL) or the vacuole (proaleurain vacuolar targeting sequence) for protein accumulation, stability and functionality. These studies show

Table 1.3 Commonly used subcellular targeting signals for maize protein expression

Subcellular compartment	Signal peptide	Protein targeted	Reference
Chloroplast	Maize rubisco SSU TP	mzYFP	Sattarzadeh et al. 2010
		ZmEPSPS	CERA 2012
	Pea rubisco SSU TP	mzGFP	Sattarzadeh et al. 2010
		mzBFP	Sattarzadeh et al. 2010
	At CTP2	CP4 EPSPS	
Extracellular matrix/ apoplast	Barley alpha amylase SS	Aprotinin	Zhong et al. 1999
		Manganese peroxide	Clough et al. 2006
		Laccase I	Hood et al. 2003
		Trypsin	Woodard et al. 2003
		Xylanase bsx and xynB	Gray et al. 2011
	PR1 SS	E1 endoglucanse	Biswas et al. 2006
Vacuole	Proaleurain	Cellobiohydrolase	Hood et al. 2007
Endoplasmic reticulum	KDEL	E1 cellulase	Hood et al. 2007
		Cellobiohydrolase	Hood et al. 2007

the importance of evaluating the effect of protein targeting in developing an effective strategy for protein production

Effective accumulation of heterologous proteins in maize chloroplasts has been facilitated by the maize and pea RUBISCO small subunit (SSU) chloroplast transit peptides and *Arabidopsis* CTP2 peptide for a variety of proteins with different applications. The maize RUBISCO SSU has been used to evaluate cell-type specific expression of YFP in transgenic maize lines (Sattarzadeh et al. 2010), as well as to facilitate targeting of proteins for insect-resistant and herbicide-tolerant traits. In the latter case, EPSPS is naturally expressed in plant chloroplasts as a component of the shikimate pathway. Targeting in event GA21 (Table 1.1) is necessary in order to achieve resistance to glyphosate. Similarly, the CTP2 chloroplast transit peptide from the *Arabidopsis* EPSPS gene was utilized in event NK603 to target the EPSPS gene, CP4, derived from *Agrobacterium tumefaciens*. Chloroplast accumulation using the maize RUBISCO SSU CTP has also been used with a Bt insecticidal protein, Cry2Ab, in event MON89034 (Table 1.1). Rawat et al. (2011) reported that chloroplast targeting of Cry1Ac significantly improved expression of the protein and reduced detrimental effects on regeneration and development of cotton and tobacco transgenics. Both maize and nonmaize derived targeting peptides have been used effectively for subcellular targeting in maize. Although only a few targeting peptides have been incorporated into constructs that have been successfully de-

veloped for insect-resistant, herbicide-resistance, and recombinant protein production, the opportunity exists to identify and evaluate alternative targeting peptides for functionality in maize. Several publicly accessible programs have been utilized to predict the presence of sorting signals/peptides in sequences derived from plants (e.g., PSORT (Yu et al. 2010) (http://www.psort.org/psortb/index.html), SignalP (Peterson et al. 2011) (http://www.cbs.dtu.dk/services/SignalP/), SLP-local (Matsuda et al. 2005) (http://sunflower.kuicr.kyoto-u.ac.jp/~smatsuda/slplocal.html)). Peptides obtained through this strategy can be evaluated in combination with various reporter genes or directly with genes of commercial value to determine their effectiveness for subcellular targeting.

Regulatory Elements That Can Influence Protein Expression

Intron-Mediated Enhancement of Gene Expression

The ability to achieve the desired level of transgene expression is often dependent on the choice of promoter and its strength, and spatial and temporal profile. The inclusion of an intron within the 5′ UTR of a transgene can provide an enhancing effect on gene expression; a phenomenon known as intron-mediated enhancement or IME (Mascerenhas et al. 1990). This increase in gene expression can be directly correlated with an increase in mRNA accumulation (Callis et al.1987; Mascerenhas et al. 1990; Luehrsen and Walbot 1991; Koziel et al. 1996; Rethmeier et al. 1997), suggesting that the mechanism of enhancement is related to increased RNA polymerase processivity. Since not all introns are capable of IME, the process of splicing and the formation of a splicing complex is itself insufficient for IME. However, splicing is an absolute prerequisite for IME (Morello et al. 2011). Enhancing introns have been identified in genes that are constitutive and highly expressed in different plant species (Parra et al. 2011). IME is more prevalent in monocotyledons than dicotyledons and has often been associated with the first intron of a gene, generally the longest intron and proximal to the promoter either within the 5′ UTR or close to the transcriptional start site within the coding sequence (Rose et al. 2008; Parra et al. 2011; Morita et al. 2012). The degree of IME has been observed to decrease with increased distance from the promoter (Rose 2004). Specific determinants within introns exhibiting IME are likely to be involved in enhancement of expression although the nature of these determinants has been elusive. Computational analyses of introns that demonstrate IME have identified several highly functional and conserved motifs, GATCTG (Morita et al. 2012) and CGATT (Parra et al. 2011). In the latter case, the addition of multiple copies of this pentamer to a poorly enhancing intron can transform it into a highly enhancing intron. IMEter v2.0 (Parra et al. 2011), (http://korflab.ucdavis.edu/cgi-bin/web-imeter2.pl), a word-based algorithm predictive of the ability of an intron to stimulate gene expression was developed based

on an Arabidopsis training set but is widely applicable to multiple plant species including maize. The relative level of IME is promoter dependent and in general the stronger the promoter the less impact on enhancement is observed (Mascerenhas et al. 1990) Several groups have identified introns that can be useful for enhancing gene expression with twofold to over 100-fold stimulation of gene expression over intronless versions of promoters (Table 1.4). Although many of these introns have been shown to provide IME in their species of origin it is clear that IME signals are conserved across species by their ability to provide the same function in another species. For example, the RpoT-i4 intron from the maize T3/T7-like DNA-dependent RNA polymerase (RpoT) gene and the UBQ10-i1 from the *Arabidopsis* Ubiquitin 10 gene enhanced accumulation of luciferase mRNA by 5.1-fold and 9.6-fold in barley, respectively, when included in the coding region of luciferase under Ubi-1 promoter control (Bartlett et al. 2009). An intron from the leader region of a putative *Arabidopsis* ortholog of OsTub6, AtTub6L1, provide similar levels of GUS enhancement (about tenfold) when used to replace the endogenous OsTub6 intron in transient transformation experiments in rice calli (Morello et al. 2010). The first intron of the rice superoxide dismutase gene (sodCc2), found in the 5′ UTR, was effective in elevating GUS and LUC expression levels in rice (~5–20-fold), wheat

Table 1.4 Selected introns with demonstrated IME function in monocotyledon species

Intron	Fold enhancement	Reference
ZM-Ubi1	35–110	Ueki et al. 2004
ZM-Adh1	5–22	Callis et al. 1987
ZM-Sh1	43	Vasil et al. 1989
ZM-RpoT-i4	10	Bartlett et al. 2009
Os-Actin1	2	McElroy et al. 1990
Rubi3	20	Sivamani and Qu 2006
OsTub6L1	9	Morello et al. 2011
OsTub4LL1	13	Morello et al. 2011
OsTub4SL1	4	Morello et al. 2011
OsTua2F1	12	Morello et al. 2011
OsTua3F1	6	Morello et al. 2011
OsCpk2LL1	17	Morello et al. 2011
OsCpk2CL1	11	Morello et al. 2011
Ostua1	34	Jeon et al. 2000
Ostub16	5	Morello et al. 2002
OsGAMyb	2	Washio and Morikawa 2006
OsRPBF	30	Washio and Morikawa 2006
OsPLD1	4–7	Ueki et al. 1999
OsSodCc2	5–20	Morita et al. 2012
AtUBQ10-i1	5	Bartlett et al. 2009
AtTub6L1	10	Morello et al. 2011

(8-fold) and maize (6-fold) but not in *Arabidopsis* or tobacco (Morita et al. 2012) when used in combination with the CaMV 35S promoter. In monocotyledons, the CaMV 35S promoter is weaker in comparison to its strength in dicotyledons. However, the addition of introns derived from either monocots or dicots to this promoter within the 5′ UTR significantly increases expression of reporter genes as high as 92-fold (Vain et al. 1996) in maize. Therefore, the utility of introns with IME properties can extend from dicotyledons to monocotyledons (Vain et al. 2004; Morello et al. 2010) and between monocotyledons (Table 1.4).

With the exception of the maize Ubi-1 intron 1, the maize hsp70 intron, the rice actin intron 1 (OsActin1), and the rice Ubi3 intron (rUbi3) very few IME type introns have been applied successfully to elevate foreign gene expression in transgenic maize for commercial use (Tables 1.1 and 1.2). The knowledge that IME functions are conserved across species, particularly in monocotyledons, opens up excellent opportunities to evaluate and identify a new suite of introns that can be used for plant biotechnology. While it may be best practice to incorporate introns derived from maize into the transgene cassette to avoid potential differences in intron recognition and splicing efficiency, today's tools (e.g., transient expression and molecular analysis) offer a way to evaluate multiple intron candidates to identify the best promoter–intron combination for optimization of protein expression.

5′ and 3′ Untranslated Regions

The 5′ UTR is located directly upstream of the translational initiation codon in the mRNA and can play a significant role in transcription and translation. Inclusion of an intron with properties of IME into the 5′ UTR of a transgene can have a positive impact on mRNA accumulation as outlined in the previous section. 5′ UTRs derived from high-expressing genes such as maize Ubi1, rice Ubi3, and rice Actin are commonly used for maize heterologous gene expression particularly in combination with their native promoters (Tables 1.1 and 1.2). Aside from the influence of 5′ UTRs on transcription, these regions can also include translational enhancers that lead to higher levels of protein accumulation. The mechanism for this enhancement is related to improved translational initiation and directly related to the efficiency at which ribosomes are able to scan and recognize the translational (ATG) start codon. This is influenced by secondary structure as well as the context surrounding the ATG start codon. A Kozak consensus sequence for plants has been described, 5′-ACN$_2$AAN$_3$(A/T)T(A/C)AACAATGGC-3′, that is present immediately flanking the ATG translational start codon of highly expressed genes from monocotyledonous and dicotyledonous plants (Sawant et al. 1999). This sequence is very similar to that described by Joshi et al. (1997), consisting of 5′-AAAAACAA(A/C)AATG-GCG-3′. Both sequences are distinguished by their high A/C richness upstream of the ATG which is expected to form less stable secondary structure and facilitate more efficient ribosome scanning through the UTR (Sawant et al. 1999).

Leader sequences from plant viruses have often been used as a source for translational enhancers. These sequences are generally associated with promoters such as CaMV 35S and tobacco etch virus (TEV) that control expression of viral capsid/coat proteins which are abundantly expressed in plants using host translational machinery. The 73 nt long CaMV 35S 5′ UTR provided a 35-fold increase in luciferase activity compared to the native luciferase leader in combination with the CaMV 35S promoter in transient assays (Rothstein et al. 1987). The combination of CaMV 35S with the 5′ UTR derived from TEV was effective in directing the expression of a selectable marker gene in maize plants (Frame et al. 2002). The demonstration that a plant caulimoviral promoter derived from the Cestrum yellow leaf curling virus (CmYLCV; Stavolone et al. 2003) is functional in maize and can provide strong constitutive expression of a transgene (see Promoter section) suggests that it may also possess translational enhancer elements. Combining IME or 3′ UTRs with translational enhancers can be an effective strategy for maximizing expression of a foreign protein in maize as demonstrated by Mitsuhara et al. (1996) using tobacco and rice and Nagaya et al. (2010) in *Arabidopsis*.

The 3′ UTR generally functions to provide signals for polyadenylation and mRNA stability and is a basic prerequisite for transgene expression. Choice of 3′ UTRs can affect the steady state level of mRNA depending on whether these regions contain A/U- rich destabilization signals (De Rocher et al. 1998) that lead to rapid degradation of mRNA or whether they are capable of forming strong stem-loop structures that protect the RNA from degradation by 3′ RNAses. Three basic *cis*-acting elements within the 3′ UTR are necessary for efficient termination of transcription and polyadenylation; far upstream elements, near upstream elements and cleavage sites (Shen et al. 2008). 3′ UTRs from genes that are highly expressed and accumulate to abundant levels in various plant species are effective for transgene expression in maize. Commonly used 3′ UTRs in maize include Pin II from potato protease inhibitor II, Nos (Nopaline synthetase) terminator from *Agrobacterium,* and the 35S terminator derived from the CaMV 35S transcript (Tables 1.1 and 1.2). In addition, the ORF25 terminator from *Agrobacterium* and the wheat (Ta) hsp17 terminator have been used as components of gene cassettes designed for IR. The pinII terminator has been demonstrated to contribute to increased mRNA stability (An et al. 1989). The ability of a terminator to increase mRNA levels of a foreign gene expression cassette has been correlated to their ability to efficiently terminate transcription via the presence of strong poly(A) sites (Gil and Proudfoot 1987). Enhancement of heterologous gene expression by 3′ UTRs has been demonstrated in multiple plant species (Ingelbrecht et al. 1989; Knirsch and Clerch 2000; Ali and Taylor 2001; Nagaya et al. 2010).

Spacers and Insulators

The need to express multiple trait genes composed of several transcription units in transgenic maize is becoming a standard approach today. As mentioned earlier in

this chapter, strong constitutive promoters are often used to achieve high levels of heterologous protein expression. Transcriptional termination in plants is not a very efficient process and poor termination can result in transcriptional read-through that negatively impacts expression of a downstream neighboring transcriptional cassette, a phenomenon known as transcriptional interference (TI; Shearwin et al. 2005). TI has been shown to decrease expression of the downstream gene in a tandem head to tail cassette configuration by as much as 70–80 % in tobacco protoplasts (Padidam and Cao 2001) and also by similar levels in tetracycline-activated expression in transgenic tomatoes (Thompson and Myatt 1997). The mechanisms of TI are not well understood. However, promoter occlusion, promoter competition, sitting duck, collisions and roadblock mechanisms have been proposed (Shearwin et al. 2005). TI needs to be taken into consideration for multigene cassettes where plant transcriptional units are in tandem or convergent orientations relative to each other, such as the case when there is a gene for overexpression and one for a selectable marker. This effect is more pronounced when the upstream transcription unit is controlled by a strong promoter. Depending on the promoter used and the location of the insert in the genome transcriptional read-through has been detected in several endogenous and transgenic plant genes within about 500 nt downstream of the poly(A)processing site and generally has been shown to be undetectable by about 1–1.3 kb downstream of this site (Xing et al. 2010). Strategies using terminators with strong poly(A) sites or transcriptional blockers (TBs), extending the distance between transcriptional units, and orienting transcription units in a head to head direction have been effective at reducing or eliminating TI (Ingelbrecht et al. 1991; Eggermont and Proudfoot 1993; Padidam and Cao 2001), so upstream and downstream genes can be expressed in an independent manner. In the case of TBs, a strong poly(A) signal in combination with a downstream pause site provided the most effective protection against the effects of TI (Eggermont and Proudfoot 1993; Padidam and Cao 2001). Given the observation that transcriptional read-through is limited to between 1 and 1.3 kb downstream of the cleavage site the use of different intergenic DNA lengths was shown to work well to prevent TI. In this case, different lengths of λ DNA were used to separate the transcription units and TI was reduced with fragment sizes of 0.7 and 1.5 kb and eliminated with a ~2.3 kb fragment (Padidam and Cao 2001). These results suggest that TI should be addressed to maximize levels of expression in transgenic maize or at least ensure that expression of transgenes reach levels necessary for efficacy.

In addition to unexpected changes in expression due to transcriptional interference between transgene cassettes, the impact of enhancer elements within the expression cassette(s) and positional effects from the insertion site may affect protein expression levels. The variability in transgene expression is often attributed to the influence of transcriptional activity within the region of insertion and the effects of enhancer or silencing elements in close proximity to the insertion site. These factors may result in changes to the strength and specificity of transgene expression and/or transgene silencing (Francis and Spiker 2005). Several strategies have been developed that can minimize these positional and regulatory element effects including site-specific integration (SSI; Albert et al. 1995; Ow 2007; Vega et al.

2008; Li et al. 2009; Nandy and Srivastava 2011; Nandy and Srivastava 2012) and addition of matrix attachment regions (MARs) flanking the transgene cassette that is integrated into the genome (Singer et al. 2012). The SSI strategy targets insertion of transgene cassettes into relatively benign regions of the genome that have been characterized experimentally to have minimal impact on transgene expression while the use of MARs elements work to prevent the spread of transcriptionally silenced heterochromatin into the region of insertion and preserve active transcription at the insertion site.

Since enhancers act over large distances and in an orientation independent context, enhancers located in the endogenous DNA can influence the strength and specificity of promoters contained within a transgene insert. Likewise, enhancers included within a transgene insert can impact plant endogenous promoters located near the insertion site or adjacent promoters contained in the insert in the case of a multigene cassette. In either case, the strong influence of enhancers can be problematic when precise control of transgene expression is required. Applying the same strategies used to reduce or eliminate transcriptional interference (e.g., spacer elements or relative promoter orientation) is not effective at preventing enhancer effects on mis-expression (Hily et al. 2009; Singer et al. 2011a). Alternatively, the use of promoters that achieve the necessary levels of expression in the absence of enhancers (e.g., maize Ubi-1 or rice actin1 promoters) can be one strategy to avoid mis-expression. An effective method to prevent mis-expression is needed in cases where a known enhancer element is used in a transgene construct or cryptic enhancers exist in plant viral promoters used in transgene constructs. The identification and use of insulators in metazoan and mammalian cell systems suggests that genetic elements exist that are capable of blocking the interaction between enhancer and promoter when positioned in between (West et al. 2002). The use of these specific elements or insulator sequences derived from plant species has the potential to be an effective strategy to minimize enhancer-mediated promoter modulation in transgenic maize. Several insulators with lengths ranging from 0.25 to 5 kb have demonstrated function using the 35S enhancer and different promoter types in various plant model systems such as tobacco, *Arabidopsis,* and *Brassica juncea* (Singer et al. 2011b), but so far none have been tested in maize or other crop plants.

Gene Position Effects

Fundamental to the production of proteins in plants is the ability to transfer and insert genetic elements into locations in the genome which do not disrupt native plant gene function and are competent to consistently express the protein over multiple generations. While *Agrobacterium*-mediated gene transfer has advantages over direct DNA transfer as transgene insertion is often less complex, variation in transgene expression and gene silencing have been reported for both approaches. Given the random nature of transgene insertion in the plant genome, common factors most credited to participate in expression variation are rearranged or repeated transgenes,

and the influence of neighboring DNA sequences or chromatin adjacent to the transgene insertion site. While variation is observed among rearranged and truncated transgenes, studies are limited which examine the relationship of single-copy nontruncated transgenes and high stable expression in plants. Two studies in *Arabidopsis* demonstrated that single complete copies of identical transgenes showed similar levels of gene expression, suggesting that transgene position may not be the major cause of variability (Buck et al. 2004; Nagaya et al. 2005). In the absence of accounting for copy-number and TDNA integrity, gene expression was observed to vary over 10,000-fold across uncharacterized primary transformants (Buck et al. 2004). However, when the single-copy intact TDNAs within this population were examined, variation of GUS expression across the majority of this class of TDNAs was less than fourfold. The authors suggest that, while at a low frequency, TDNA integration position may influence gene expression due to unidentified characteristics of the flanking sequence, integration position has a minor effect on transgene expression, and screening for single-copy TDNA insertions strongly enriches for uniform transgene expression.

Similar observations have been demonstrated in maize (Cigan et al. 2001; Unger et al. 2001). Molecular examination of maize transgenic events generated by either biolistic- or *Agrobacterium*-mediated transformation also reveal that screening for and identifying single-copy intact transgenes is an important step to eliminate the large variations observed in transgene expression. Given the randomness of gene insertions and the potential for large variation in gene expression reported, elimination of these truncated, multi-copy and rearranged gene cassettes is a critical first step to afford the researcher to focus on a small subset of molecularly characterized primary events for systematic evaluation of transgene design. When identical transgene cassettes are used to deliver a transgenic copy of the maize MS45 gene, the significance of this strategy is borne out. Primary events generated by particle gun bombardment identified wide variations (nearly 20-fold) in MS45 protein expression and did not correlate with the number of integrated Ms45 transgene copies. In contrast, when single-copy-complete TDNAs were compared to random biolistically generated events, Ms45protein was more uniform across the different TDNA insertions correlated with transgene copy-number and, most importantly, mirrored the spatial, temporal and steady-state expression of the endogenous Ms45 gene (Cigan et al. 2001). Additional studies which incorporated examining only single-complete-copies of TDNAs, were crucial for the development of more complicated gene designs focused on regulating Ms45 protein expression using chemical switches (discussed in this chapter).

The importance of establishing routine rigorous molecular analysis for evaluating transgene design was also uncovered while developing dominant male sterility strategies in maize. It was observed that the male fertility phenotype associated with specific transgene designs were influenced by the choice of the promoter used to express the linked herbicide resistance gene (Unger et al. 2001). In these studies, the maize anther preferred promoter, 5126, was used to transcribe *Escherichia coli* DNA (Adenosine-N6-)-Methyltransferase (DAM) gene (Zm5126:DAM) and placed upstream of either the CAMV35S:PAT or the ZmUbi-1:PAT herbicide

resistance genes which confer resistance the bialophos. In the absence of molecular analysis to correlate transgene copy-number with phenotype, biolistically generated transgenic events containing these sterility cassettes linked to either selectable marker conferred male sterile phenotypes to these otherwise morphologically normal plants. Closer inspection of these events by DNA hybridization studies revealed that a large proportion of the sterility cassettes containing the juxtaposed CAMV35S:PAT marker were male sterile independent of transgene copy-number. In contrast, only multi-copy events containing Ubi-1:PAT were male sterile. This suggested that, while anther-expressed DAM could confer male sterility to maize, placement of an adjacent gene strongly influenced the frequency of recovering this class of events. An identical study was conducted with the exception that sterility: herbicide gene combinations were introduced by *Agrobacterium*-mediated transformation to increase the number of low-copy events for herbicide gene expression. In addition, a third selectable marker gene (Rice Actin1 promoter transcribing PAT gene; rACTIN:PAT), was linked to the sterility cassette and included in this study. Similar to the previous experiments, only 4 of the 63 single-copy-complete Ubi-1 and rACTIN: PAT containing events were male sterile, while 7 of the 30 multicopy events were male sterile. In contrast, more than 80 % of the single-copy-complete CAMV35S:PAT events were sterile. In the absence of discriminating the single-copy-complete from the multicopy by relatively simple molecular studies, understanding the influence of adjacent promoter: gene combinations on overall transgene performance are left to speculation. This study was also used to more closely examine PAT protein expression across the different promoter: herbicide gene combinations. While a small number of outliers were observed, the majority of the single-copy-complete events revealed PAT expression varied less than 20 % across each construct examined. Distilling the large number of primary events to this subset class, clearly demonstrates the utility of fundamental molecular analysis as a foundation for refined transgene function studies in plants.

In summary, in conflict to much of the early literature which documented large variation in protein expression, these examples in *Arabidopsis* and maize support the concept that minimal protein expression variation is observed when single-complete-copies are used for gene expression studies. As transgene cassettes become more complex, routine application of existing and future molecular tools and approaches will be required in order to develop crops for the purpose of increasing yields on reduced acreages to feed an ever growing population while embracing environmental stewardship.

Transient Expression in Maize

Transient expression systems can provide an initial evaluation of promoter, gene design, expression, subcellular targeting and transgene cassette effectiveness prior to the generation of transgenic maize plants. This can reduce significantly the need to test all options available to maximize protein expression in stable events. The

predictability of how one or a combination of these elements affect protein expression in maize plants will depend on the availability of transient expression systems that are based directly on maize cells/tissues or on plant cells/tissues derived from related monocotyledon species such as rice, *Setaria,* and *Brachypodium*. Particle bombardment of maize tissues (Oard et al. 1990; Schenk et al. 1998) or PEG-mediated transformation of maize protoplasts or suspension cultured cells (reviewed in Sheen 2001) have been used routinely to elucidate gene functions, study of gene expression and silencing, and evaluation of various biochemical processes. Kirienko et al. (2012) improved the transient expression efficiency of particle bombardment in maize leaf tissue from 1 transformant per cm^2 to over 21 transformants per cm^2 of leaf tissue. In these experiments, fluorescent marker gene expression was observed and analyzed across five discontinuous regions along an expanding adult (50 cm) leaf blade. Transformation frequency was highest in the basal region from 0 to 3 cm from the ligule and the transformation efficiency decreased toward the leaf tip. Differences in transformation efficiencies were attributed to the developmental state of the cells in the basal region.

Agrobacterium tumefaciens -based transient expression (Fischer et al. 1999; Kapila et al. 1997; Komarova et al. 2010) is another approach that has been applied to monocotyledons. In these plants, poor *Agrobacterium* infiltration by simple pressure has been attributed to differences in leaf architecture and structure of the epidermis (Andrieu et al. 2012). Recently, *Agrobacterium*-mediated transient expression has been achieved in rice leaf tissue which facilitated a rapid analysis of rice genes based on RNA-interference (Andrieu et al. 2012). In this case, the use of a surfactant (Silwet L-77) improved *Agrobacterium* infiltration and enhanced transgene expression. *Agrobacterium*-mediated transient transformation has also been successfully reported in *Setaria viridis,* a C_4 grass, that demonstrated plastid localized yellow fluorescent protein (YFP) in leaf cells (Brutnell et al. 2010). The demonstration of transient expression and protein targeting in this species provides optimism that *Setaria* has the potential to become a model system for C_4 species. Hosein et al. (2012) optimized a transient system using *Agrobacterium* for gene expression studies in *Anthurium andraeanum,* a member of the family Araceae. Transformation efficiency of a GUS reporter gene was found to be dependent on developmental stage and tissue type with highest efficiency found in fully expanded young ("floppy") leaves. In addition, transient transformation efficacy was influenced by the cultivar tested varying from 0 to 83 % (Hosein et al. 2012).

Transient expression mediated by viral expression systems has also been an effective strategy for protein production that has the advantages of speed and versatility coupled to the ability to achieve high levels of expression. Plant viruses multiply within infected cells and any foreign gene that has been engineered into the viral genome can also be amplified. The increase in copy number and systemic spread of the virus infection results in high transient expression of the foreign protein. Although plant viral vectors have been used extensively in dicotyledonous systems for the production of heterologous proteins (Gleba et al. 2004), this strategy has been less effective in maize and other monocotledons primarily due to a lack of vectors that

are competent to spread systemically. Several vectors based on RNA plant viruses have been evaluated for expression including brome mosaic virus (BMV; French et al. 1986), barley stripe mosaic virus (BSMV; Joshi et al. 1990), and the DNA plant geminiviruses, wheat dwarf virus and maize streak virus (Shen and Hohn 1994; Shen and Hohn 1995; Palmer et al. 1999). Although expression of foreign genes are possible, expression is limited to the primary "infected" cells due to the elimination of coat protein or movement protein coding sequence(s) to accommodate the insertion of the foreign gene. Advances in these viral vectors for monocotyledonous systems have been made using Wheat streak mosaic virus (WSMV; Choi et al. 2000) and Foxtail mosaic virus (FoMV; Liu et al. 2010) to allow for either improved systemic spread of infection or enhanced level/uniformity of initial infection in maize. A WSMV vector was created with the insertion site for a foreign gene at the junction between the N1b (nuclear inclusion b) and CP (coat protein), with flanking viral protease cleavage sites to excise the heterologous protein from the viral polyprotein. Expression of either NPTII or GUS reporter protein was detected in the leaves of *in vitro* transcribed RNA -inoculated seedlings although instability of GUS was observed (Choi et al. 2000). The use of *Agrobacterium as* a delivery system to introduce an engineered viral vector into plant tissue was used successfully with FoMV, which possesses a broad host range infecting 56 species of Poaceae and about 35 species of dicotyledons (Short and Davies 1987). This system of protein expression addresses previous limitations related to the lack of systemic spread of monocotledon vector systems. In this agro-inoculation system, viral replication and expression will occur in each infiltrated cell resulting in more uniform and higher expression opportunities. In this process, agro-infiltration replaces primary infection and systemic movement (Gleba et al. 2007). The viral vector containing the FoMV proviral replicon with the GFP gene replacing the CP and TGB1 genes, was delivered into cells as a T-DNA using the CaMV 35S promoter to drive expression of the viral transcript. This system demonstrated accumulation of GFP up to 40% of the total extractable protein in *Nicotiana benthamiana* when coexpressed with an RNAi silencing suppressor. Unfortunately, expression of GFP in maize seedling leaves or other monocotyledons using this system was low and patchy, and was attributed to poor efficiencies inherent to agroinfiltration of monocotyledon tissues. However, there is continued interest to develop a similar system for monocotyledons based on expression systems that offer an attractive alternative to transgenic plant strategies (see Chap. 14).

Future Prospects

A key to success for maize is the concentration of protein that is expressed and accumulated in transgenic plants. Optimization of protein expression in maize is dependent on multiple factors that contribute to the strength and stability of expression,

while minimizing the impact on plant health. This optimization is never routine, often it is empirical, and requires testing of different construct configurations. Construct design may include choice of the right regulatory elements, gene optimization, subcellular targeting, and addition of elements that reduce negative position effects as a result of integration site or target-specific integration sites. An optimized construct would ensure that these types of elements work effectively together to deliver the overall goal; expression of foreign proteins at levels needed for basic or applied research, and development programs directed at crop improvement (IR, HR, agronomic traits, nutritional traits) and large-scale production of therapeutic, industrial and pharmaceutical-based proteins.

The understanding of how to optimize expression in maize and what factors have impact on protein expression continues to improve as information becomes available on the performance of different elements in transgenic events. However, only a small set of elements have been tested and used to date. The ability to evaluate a greater diversity of elements in different combinations may someday allow greater precision in the control of quantitative, temporal, spatial expression and accumulation that can be tailored individually based on the properties of the protein and the intended application. In the case of using transgenic maize for large-scale recombinant protein production, maximizing the protein production potential is an important consideration. However, for crop improvement traits like HR or IR, maximizing expression and accumulation may not be necessary in order to achieve optimal field performance.

The availability of emerging genomic sequences from a large diversity of plant species, including new potential model systems such as *Brachypodium* and *Setaria,* technologies that provide global expression profiles for genes in different tissues and environments, and improved bioinformatics capabilities that facilitate identification of new targeting peptide sequences and optimize genes for expression, can change fundamentally the way expression optimization is accomplished. Today, gene synthesis provides a greater opportunity to test heterologous elements derived from mining plant genomes and combining large number of these elements into a matrix to evaluate rapidly their effects on expression in transient-based systems. A more systematic approach to optimization may eventually help improve *in silico* predictions on how effective different element combinations work towards achieving the expression goal for any particular protein.

The ability to optimize expression of individual genes is an important step to the successful development of complex molecular stacks that could involve coexpression of multiple genes for multiple traits. The capability to ensure that each transcriptional cassette expresses the gene at the desired levels, interference between cassettes is eliminated, and the inserted DNA is insulated from negative positional effects from flanking plant genomic sequences, will contribute immensely to being successful to achieving this goal for a new generation of traits and commercial products in maize.

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Chapter 2
Plant Trait Gene Expression Cassette Design

Michael Nuccio, Xi Chen, Jared Conville, Ailing Zhou and Xiaomei Liu

Introduction

Plant genetic engineering relies on promoters to develop trait genes. Many early promoters were modeled on plant pathogens, such as the cauliflower and figwort mosaic virus (CaMV, FMV; Benfey and Chua 1990; Sanger et al. 1990) and *Agrobacterium tumefaciens* (An 1986). Their activity is regarded as "constitutive" because they contained all the necessary information to produce mRNA in most plant cells. In addition, they are generally active across plant species, although quantitative performance or the amount of transgene activity produced can be variable. The basic elements required to successfully produce mRNA in plant cells are a promoter, a coding sequence, and a terminator. The promoter contains the necessary information to recruit the transcriptional machinery and initiate transcription. The coding sequence encompasses the desired trait which can take the form of a protein or RNA. The terminator provides information to end transcription and signal polyadenylation (Birch 1997). This basic structure has been in use since the inception of modern plant genetic engineering more than 25 years ago.

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Transgene expression challenges were encountered as more scientists experimented with plant genetic engineering. These include transcriptional and posttranscriptional silencing, low or no protein accumulation, targeting transgene expression to specific cells, and enabling transgene expression under specific conditions. The transcriptional challenges were met by sourcing promoters from plants (Christensen and Quail 1996; McElroy et al. 1990) and incorporating enhancers found in certain plant pathogens (Gallie and Walbot 1992). Expression problems were addressed by introducing heterologous introns into 5′-untranslated regions (UTRs) or the trait gene coding sequence (Rose 2004), incorporating a Kozak sequence (Kozak 2002), altering codon usage (Koziel et al. 1996), introducing matrix attachment regions (Allen et al. 2000; Butaye et al. 2004), and altering terminators (Ingelbrecht et al. 1989). All of these innovations expanded the trait gene expression control toolkit, giving practitioners more flexibility (Lessard et al. 2002; Potenza et al. 2004).

The early days of plant genetic engineering included work to identify and characterize the basic plant-gene sequences required to initiate transcription (Katagiri and Chua 1992). Examples of this work include chalcone synthase, a gene in flavonoid biosynthesis pathway (Schulze-Lefert et al. 1989), the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO; Giuliano et al. 1988) in the Calvin cycle, and seed storage proteins (Jordano et al. 1989; Nunberg et al. 1994). Investigators identified regions that were responsible for environmental and hormone regulation, among other inputs. In many cases, fine-mapping work demonstrated the interaction of a specific transcriptional regulator with a specific sequence in a promoter (Gruissem 1990). This body of work defined numerous *cis*-regulatory elements, some of which have been shown to functionally enhance transcription from a given promoter (Chang and Sun 2002; Rombauts et al. 2003).

Other work focused is on the contributions of introns to trait gene expression control (Luehrsen and Walbot 1991). For example, the maize Shrunken-1 intron greatly improves trait protein production when incorporated into its corresponding promoter (Maas et al. 1991). Some plant-gene promoters require at least the first intron in order to function correctly in transgenic plants (Rose et al. 2008; Sieburth and Meyerowitz 1997). More recent work revealed a correlation between the physical properties of introns and protein production from transgenes (Korf and Rose 2009). Now, there is evidence that introns with specific properties can be used in a heterologous context to increase protein production from transgenes (Bartlett et al. 2009; Emami et al. 2013; Parra et al. 2011).

Terminators have received much less attention with respect to control of trait gene expression because the role of a gene's 3′-sequences in expression control has not been investigated extensively (Hunt 2008; Rothnie 1996). Investigations on sequences that contribute to the formation of 3′-end of mRNAs (Hunt 1994) and sequences that contribute to mRNA stability (Lidder et al. 2005) demonstrate that elements associated with plant transcriptional terminators contribute to overall gene activity. Another group demonstrated that a plant mRNA's 3′-UTR interacts with a metabolite, influencing its stability and ability to recruit ribosomes (Wachter et al. 2007). There has been an effort to map functional sequences within 3′-UTRs (Wachter et al. 2007), and the basic properties of transcription terminators have

been described (Xing et al. 2010). Much more work has been done in yeast and animal systems. For example, evidence shows that a gene's 3′-sequence functions in the formation of transcription loops that lead to the production and processing of mRNA (Moore and Proudfoot 2009).

The transcriptional terminator derived from the *Agrobacterium* nopaline synthase (NOS) gene was among the first used in plant transgenes, and remains in wide use today (Gleave 1992). Some investigators have shown that substituting a different terminator for the NOS terminator can influence trait protein production (Ingelbrecht et al. 1989). In one case, an *Arabidopsis* embryo-specific promoter was found to be nonfunctional when coupled to the NOS terminator, and functional when used with the gene's corresponding terminator (Nuccio 1997). These individual cases suggest that terminators potentially contribute far more gene expression control information than currently understood. The influence terminators exert on overall gene activity needs more attention. New methods should facilitate this work (Zhao et al. 2011).

Much of the early plant genetic engineering work revealed ways to manage individual trait gene expression problems (Koziel et al. 1996). More recent demands require multiple transgenes. Investigations over the years indicate that repeated use of a specific expression cassette, like the CaMV35S/NOS cassette, may not be an ideal or workable solution (Kebeish et al. 2007). In addition, trait gene stacking reveals a distinct shortage of reliable expression cassettes. There are two important aspects to this problem. The first is physically assembling multigenic vectors (Gibson et al. 2008). The second is identifying trait gene expression cassettes that effectively co-operate to enable the trait (Peremarti et al. 2010). Work to facilitate multigenic trait construction at the industry level is well underway (Que et al. 2010).

The trait gene assembly problem can be managed to some extent with current recombinant DNA methodology (Sambrook and Russell 2001), although efficiencies suffer as vector size extends beyond 20 kb. Large DNA molecules are sheared more easily when handled using common manual techniques. The chromatography kits routinely used to isolate DNA molecules do not work well with molecules larger than 20 kb. Furthermore, the DNA ligases used in early recombinant DNA work do not efficiently join large DNA molecules to produce the intended products. Ultimately, these early recombinant DNA methods fail with vectors larger than 50 kb. DNA recombination systems like Gateway™ are very useful in producing large DNA assemblies (Chen et al. 2006). More recently, a combination of DNA synthesis and *in vivo* homologous recombination demonstrated assembly of very large DNA molecules, including a complete microbial genome (Gibson et al. 2010).

Several prototype multigenic expression-control systems have been described for plant applications (Halpin 2005; Jiang et al. 2013; Zhu et al. 2008). Early work sought to string CaMV35S/NOS expression cassettes in tandem. This rarely worked well if the repetitive unit was viral in origin. This is not well understood. One theory suggests that sequence homology of more than 90 bp between two promoters in a transgenic plant leads to transcriptional gene silencing (Flavell 1994). This indicates that using the same promoter many times may lead to homology-based transgene silencing (Vaucheret and Fagard 2001). Another possibility is that the

CaMV35S promoter contains a recombination hotspot that can lead to unintended trait gene rearrangement (Kohli et al. 1999).

Work was more successful with plant gene-based expression cassettes (Naqvi et al. 2009). Another approach incorporated a protease signal, enabling construction of polycistronic protein coding sequence (Halpin et al. 1999). This enabled multiprotein production from a single promoter. More recent work combines unique expression cassettes together to form multigenic trait constructs, and utilizes well-characterized promoters (Fujisawa et al. 2009). Even here, some promoters are used more than once. It is easy to see that global expression profiling data might be leveraged to identify genes that share an activity profile. While promising, this area requires more investigation.

A simplified expression cassette development strategy for trait work is needed to meet today's trait expression control requirements. The focus here will be to use native plant-gene transcription units as a source for applications in maize. Global transcription profiling data simplify the identification of plant genes that possess desirable expression profiles (Wolfinger et al. 2001; Zimmermann et al. 2004). Furthermore, the ever-increasing availability of plant genome data provides the basic information required to design effective expression cassettes.

A Plant-Gene-Based Expression Cassette Design Strategy

The challenge is that little is known about how specific gene regulatory sequences work, or what sequences are necessary and sufficient to recapitulate a gene's expression profile. A method is necessary to leverage poorly characterized plant genes for expression of cassette development. An approach to address this issue (Nuccio et al. 2012) is described below. We elected to simplify plant-gene annotation into five basic units. They include the promoter which is 1.0–2.0 kb of sequence upstream of the transcription start site, the 5′-UTR or the sequence from the transcription start site to the translation start codon, the coding sequence which comprises most of the exons and introns, the 3′-UTR or the sequence from the translation stop codon to the end of the transcript, and 3′-downstream sequence which extends up to 1.0 kb past the translation stop codon. In isolation, these components have been shown to contribute trait gene expression control. The hypothesis is that these components possess the majority of a given plant gene's expression-control information, and could be combined to form robust and reliable expression cassettes without any direct knowledge of the exact sequences that regulate the donor gene's activity. The effectiveness of this approach is illustrated in the subsequent sections of this chapter.

Accurate sequence of both the gDNA and cDNA of a donor plant gene is required to be useful for expression cassette development. This information is widely available for many plant species. Gene annotation may also be available in public databases or genome browsers (Duvick et al. 2008; Karolchik et al. 2003; Liang et al. 2008; Ouyang et al. 2007). If not, the sequence data can be generated from donor plant tissue. The largest open reading frame in an mRNA sequence typically

defines the gene's protein-coding sequence, as identified by the translation start and stop codons. The protein sequence can support the accuracy of the sequence data. If necessary, techniques such as 5′- and 3′-rapid amplification of cDNA ends (RACE) can identify the mRNA's termini, which represent the transcription start and stop sites (Das et al. 2001). The gDNA and cDNA sequences can be aligned in several software tools to define the gene's basic architecture as outlined above (Wheelan et al. 2001). This information is sufficient to design expression cassettes based on most plant genes.

The objective is to develop expression cassettes that comprise the components listed above and are simple to use. In order to do this, a two-component regulatory system consisting of a gene's 5′- and 3′-regulatory sequence is defined. The 5′-regulatory sequence contains the promoter exon 1, intron 1, and part of exon 2. The 3′-regulatory sequence contains sequence downstream of the translation stop codon and 3′-nontranscribed sequence. From this point forward, these will be referred to as the promoter and terminator, respectively. This approach casts a wide net to capture most, if not all, regulatory sequence necessary to recapitulate a gene's expression profile.

Natural gene sequences present several challenges that limit their direct use in expression cassettes. First, they likely contain restriction endonuclease sites that prevent manipulation by standard recombinant DNA methodology. To address this, a standard restriction endonuclease profile for each expression cassette is defined. The promoter is flanked by SanDI on the 5′-end and NcoI on the 3′-end. The terminator is flanked by SacI on the 5′-end and RsrII on the 3′-end. Expression cassettes are designed to be assembled in an intermediate vector, and this configuration enables the cassette's mobilization into other vectors, such as binary vectors, as SanDI/RsrII fragments which can be ligated into either a SanDI or RsrII site. SanDI (aka. KflI) recognizes GG^GWCCC and RsrII recognizes CG^GWCCG. To produce compatible ends, W needs to be either A in both sites, or T in both sites. The SanDI site remains intact when a SanDI/RsrII fragment is ligated to a SanDI site. This enables a subsequent SanDI/RsrII flanked expression cassette to be ligated adjacent to the previous insert.

Furthermore, this configuration provides NcoI/SacI sites to insert a gene of interest. These sites are added to the plant-gene sequence. Internal restriction sites that interfere with the standard sites are eliminated by a single-point mutation in each site. This is easily done by substituting G for C, and A for T, and vice versa. Point mutations can be introduced using polymerase chain reaction (PCR) methods such as Stratagene's QuikChange® Site-Directed Mutagenesis Kit. Point mutations may disrupt the functionality of the target gene regulatory elements, but this is the least-invasive approach available at the moment. The terminator generally does not require more than this for incorporation into expression cassettes.

Fig. 2.1 outlines several additional changes to the promoter sequence necessary to make it useful in expression cassettes. The engineered NcoI site is the 5′-CDS ligation site and it provides the translation start site. This is located after the first 10–15 nucleotides of exon 2. The reason is that sequence proximal to the intron/exon junction may be required for proper intron excision. Also a maize-preferred

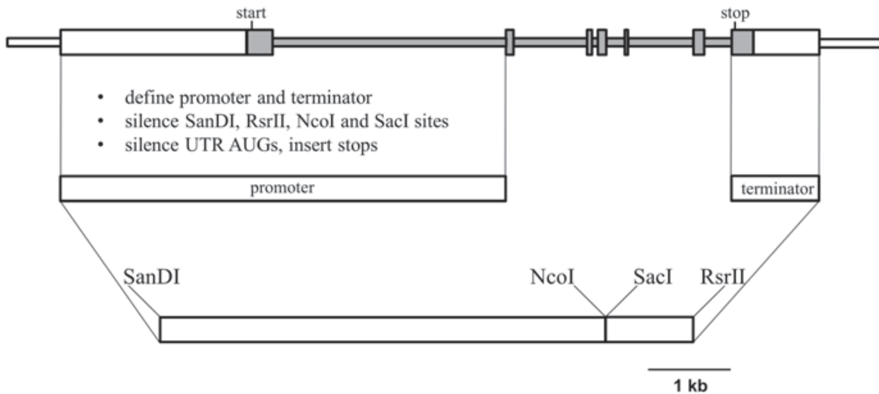

Fig. 2.1 The expression cassette design process. The gDNA at the top is minimally modified to define a promoter and a terminator. The design process captures as much regulatory sequence as possible and supports a standardized recombinant DNA framework. The primary considerations include location of the expression cassette translation initiation codon at the *NcoI* site and a restriction endonuclease arrangement to support industrial applications. In an assembly (or intermediate) vector, each expression cassette is flanked by *SanDI/RsrII* sites that can be mobilized into either a *SanDI* or *RsrII* site. This enables expression cassettes to be stacked into a binary vector. The coding sequence is directionally inserted into the *NcoI/SacI* sites. In the gDNA, the open boxes represent nontranscribed sequence, *gray* is transcript, *large gray* boxes are exons, *narrow gray* boxes are introns. The translation start and stop codons are also indicated

Kozak site, which is defined as AAAACCATGG is typically incorporated. In most cases, the promoter will possess codons that need to be eliminated. Exon 1 and exon 2 are examined for methionine codons in the three possible sequence contexts. The ATG codons are altered by a single point mutation that does not introduce an unwanted restriction endonuclease site. The same approach is used to ensure that at least one translation stop codon is in each frame upstream of the NcoI site. These manipulations ensure that translation initiates at the ATG in the NcoI site.

A final consideration for expression cassettes is proximity of donor genes to other genes. This is relatively straight forward for donor genes derived from plants with well-annotated genomes like *Arabidopsis,* rice, or sorghum. The purpose is to determine if the nontranscribed promoter and terminator sequence overlaps with an adjacent gene. This is common in compact genomes like that of *Arabidopsis*. If genome information is not available, simply BLAST (Altschul et al. 1990) the nontranscribed sequences against transcript databases. Investigate all high-quality hits. If overlap is found, it should be eliminated.

This strategy is designed to develop trait gene expression control technology with minimal effort. It is made possible by public and private investment in plant genome information. It enables expression cassette development in the absence of specific details regarding elements that control plant-gene expression. Syngenta applied this approach to many trait gene expression control problems over the years and found it to be reliable and robust (Lee et al. 2013; Nuccio 2013; Nuccio and

Richmond 2013; Nuccio et al. 2012). Several examples from work in maize are outlined below to illustrate the utility of this approach.

Ear-Specific Expression Cassettes Based on Rice MADS Genes

Trait development may require trait gene expression to be limited to specific cells at specific times. One challenge was to target trait gene expression to pedicel tissue in early development. The MADS (MCM1, AGAMOUS, DEFICIENS, SRF) transcription factor gene family was targeted because the plant-gene family members are often active in early flower development (Alvarez-Buylla et al. 2000; De Bodt et al. 2003). There is evidence that an orchid MADS gene, DoMADS3 (Yu and Goh 2000), was active in pedicels. Corresponding gDNA sequence was not available, so the DoMADS3 protein sequence was used to screen rice genome data (Goff et al. 2002) for candidates. This genomics approach identified candidate rice genes as the basis for expression cassettes that express transgenes in young developing ears. The rationale was that the rice DoMADS3 ortholog's expression profile would reflect that of DoMADS3. This led to the identification and annotation of 34 rice MADS genes, which are rank-ordered by similarity to DoMADS3 in Table 2.1.

OsMADS5, -6, -7, -8, -13, and -14 were selected for expression cassette development based on their rank in Table 2.1. The uncertainty with respect to the location of critical regulatory elements led to the development of the cassette-design strategy outlined in the previous section. Fig. 2.2 illustrates the structure of each gene. Thick lines below each annotation depict sequence incorporated into expression cassettes. Each expression cassette was fused to the β-glucuronidase (GUS) reporter gene for evaluation in transgenic maize.

Each expression cassette was characterized by histochemical localization of GUS activity in transgenic maize. Fig. 2.3 illustrates that each expression cassette produces a unique profile in developing ears. The OsMADS5 cassette is active in vasculature traversing the spikelet and the cob. OsMADS8 is active in the lemma and the nonvascular cob cells. OsMADS6 is active in the cob and spikelet vasculature and, to some extent, the glume. Fig. 2.3c shows that OsMADS6 is also active in ear node, shank vasculature, the inner bundle of the internode extending above the ear node, and basal shank vasculature. OsMADS13 is active in the central spikelet tissues including the vasculature and most likely the carpels. It is slightly active in cob vasculature. Fig. 2.3d shows that OsMADS13 is also slightly active in the ear node and discrete files within the shank. OsMADS7 is active in the embryo sac. The difference in histochemical deposition between Fig. 2.3g and 2.3h delineates the contribution of OsMADS7's first intron to its expression pattern. Inclusion of the intron (Fig. 2.3h) limits activity to the embryo sac. OsMADS14 (specifically OsMADS14 l) is active in the cob and cob vasculature as well as the spikelet vasculature. It is also active in the embryo sac. The lack of histochemical deposition in

Table 2.1 Identification of OsMADS genes using the DoMADS3 protein sequence

Locus[a]	Representative cDNA	SwissProt ID	Study name	Protein size (AAs)	Comparison to DoMADS3 (percentage)				
					Whole protein			*MADS domain*	
					Identity	Similarity	Gaps	Identity	Similarity
LOC_ Os08g41950.2	U78891	MADS7_ORYSJ S	OsMADS7	236	62	75	4	94	99
LOC_ Os03g11614.1	AF204063	MADS1_ORYSJ S		257	60	72	3	91	98
LOC_ Os09g32948.1	U78892	MADS8_ORYSJ S	OsMADS8	248	60	73	6	94	99
LOC_ Os03g54170.1	AB003324	MAD34_ORYSJ S	OsMADS14	239	59	74	3	80	95
LOC_ Os02g45770.1	U78782	MADS6_ORYSJ S	OsMADS6	250	58	69	4	91	99
LOC_ Os06g06750.1	AF141967	MADS5_ORYSJ S.	OsMADS5	225	57	72	2	92	97
LOC_ Os04g49150.1	AF095646	MAD17_ORYSJ S		254	55	67	6	94	99
LOC_ Os10g39130.1	AF141965	MAD56_ORYSJ S		233	51	67	0	84	91
LOC_ Os03g54160.2	AF139664	MAD14_ORYSJ S		246	50	66	1	78	95
LOC_ Os07g01820.3	AF345911	MAD15_ORYSJ S		267	50	68	5	80	95
LOC_ Os07g41370.1	AF139665	MAD18_ORYSJ S		249	48	66	0	80	95
LOC_ Os03g03100.1	AB003328	MAD50_ORYSJ S		230	48	64	0	77	91

Table 2.1 (continued)

Locus[a]	Representative cDNA	SwissProt ID	Study name	Protein size (AAs)	Comparison to DoMADS3 (percentage)				
LOC_Os12g10540.4	AF151693	MAD13_ORYSJS	OsMADS13	270	46	67	1	84	94
LOC_Os08g33488.1	AY177694	MAD23_ORYSJS		159	46	66	0	66	87
LOC_Os01g10504.3	L37528	MADS3_ORYSJS		276	45	68	1	84	94
LOC_Os12g31748.1	AY250075	MAD20_ORYSJS		233	42	67	5	64	88
LOC_Os02g52340.1	AB003322	MAD22_ORYSJS		228	42	58	0	68	78
LOC_Os06g45650.1	AY174093	MAD30_ORYSJS		221	42	63	0	63	87
LOC_Os02g07430.1	AY177697	MAD29_ORYSJS		260	41	62	2	66	87
LOC_Os04g52410.1	AY177698	MAD31_ORYSJS		178	41	61	3	68	87
LOC_Os12g10520.1	AY177700	MAD33_ORYSJS		202	41	61	0	66	87
LOC_Os06g49840.2	AF077760	MAD16_ORYSJS		224	40	59	2	64	80
LOC_Os01g66030.1	AF095645	MADS2_ORYSJS		209	40	61	5	64	90
LOC_Os01g69850.1	AF141964	NP_001045235.1		164	40	60	11	66	86
LOC_Os06g49840.2	AF424549	MAD16_ORYSJS		224	39	59	2	63	87

Table 2.1 (continued)

Locus[a]	Representative cDNA	SwissProt ID	Study name	Protein size (AAs)	Comparison to DoMADS3 (percentage)				
LOC_Os01g66290.2	AY177693	MAD21_ORYSJS		265	39	60	7	80	95
LOC_Os04g23910.1	AY177695	MAD25_ORYSJS		227	39	58	0	66	87
LOC_Os08g02070.1	AY115556	MAD26_ORYSJS		222	39	61	1	60	85
LOC_Os02g36924.1	AY177696	MAD27_ORYSJS		240	38	62	4	61	87
LOC_Os02g49840.1	AY224482	MAD57_ORYSJS		241	38	59	5	70	89
LOC_Os08g41960.1	AY177701	BAD11644.1		203	37	56	5	72	90
LOC_Os01g52680.1	AY177699	MAD32_ORYSJS		196	37	59	3	63	78
LOC_Os05g34940.2	L37527	MADS4_ORYSJS		210	37	60	5	63	85
LOC_Os03g08754.2	AJ293816	MAD47_ORYSJS		237	35	52	8	65	79

[a] Each gene is identified by its standard locus, a representative cDNA, and its SwissProt ID. The Study name is an internal designation. The rice MADS protein sequence was compared along the entire protein or just at the MADS domain. The order is by similarity to DoMADS3

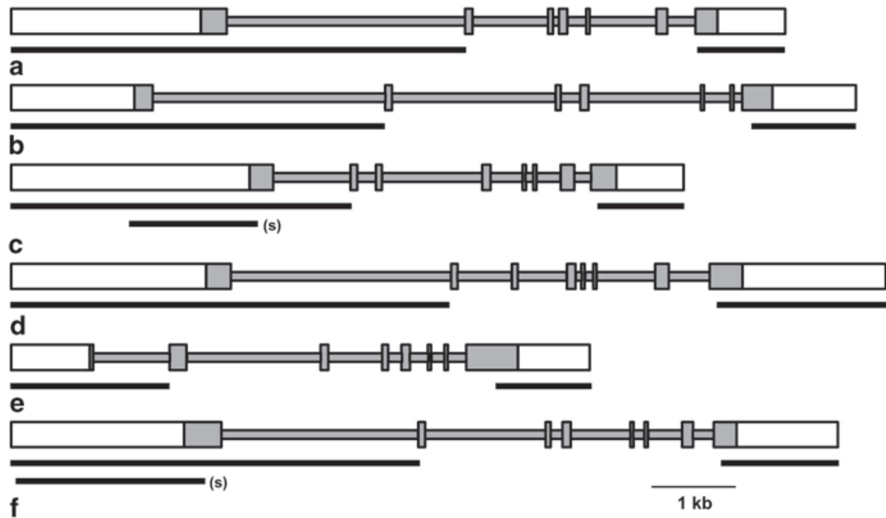

Fig. 2.2 OsMADS donor-gene annotation and expression cassette components. The genomic DNA sequence representing (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7, (**d**) OsMADS8, (**e**) OsMADS13 and (**f**) OsMADS14 was annotated by cDNA/gDNA alignment. These data were used to design trait gene regulatory components, depicted by the solid *black* lines. Promoters are on the *left* and terminators on the *right*. The size and orientation of each *line* indicates gDNA incorporated into each component. The gDNA components are labeled as in Fig. 2.1, except the translation start and stop codons are not indicated. The promoters designated with (s) are truncated and lack the intron

Fig. 2.3i indicates that lack of the OsMADS14 intron (in OsMADS14s) results in an inactive expression cassette.

Fig. 2.3 shows that each OsMADS expression cassette produces a unique activity profile in developing ears. Further characterization presented in Fig. 2.4–2.15 indicates that these OsMADS expression cassettes are active primarily in ears. No significant activity was detected in leaf, tassels, silk, and seedlings. Also, no significant activity was detected in embryo or endosperm. Activity extends from silk emergence (approximately 5 days before pollination) to kernel maturation. While histochemical data are not considered quantitative, it is worth noting that for OsMADS6, 8, 13, and 14, color development was evident 30 min after initiating the histochemical reaction.

These cassettes represent a collection of tools for targeting transgene expression to early developing ears. They were used to explore manipulation of ear sink strength as a strategy to improve yield in maize subject to an early reproductive water deficit. There is more information on the OsMADS expression cassettes elsewhere (Nuccio et al. 2012).

The data show that all the expression cassettes were active in young developing ears. Some were active to a much lesser extent in anthers. Some important observations were that the intron we included in each promoter was excised exactly as

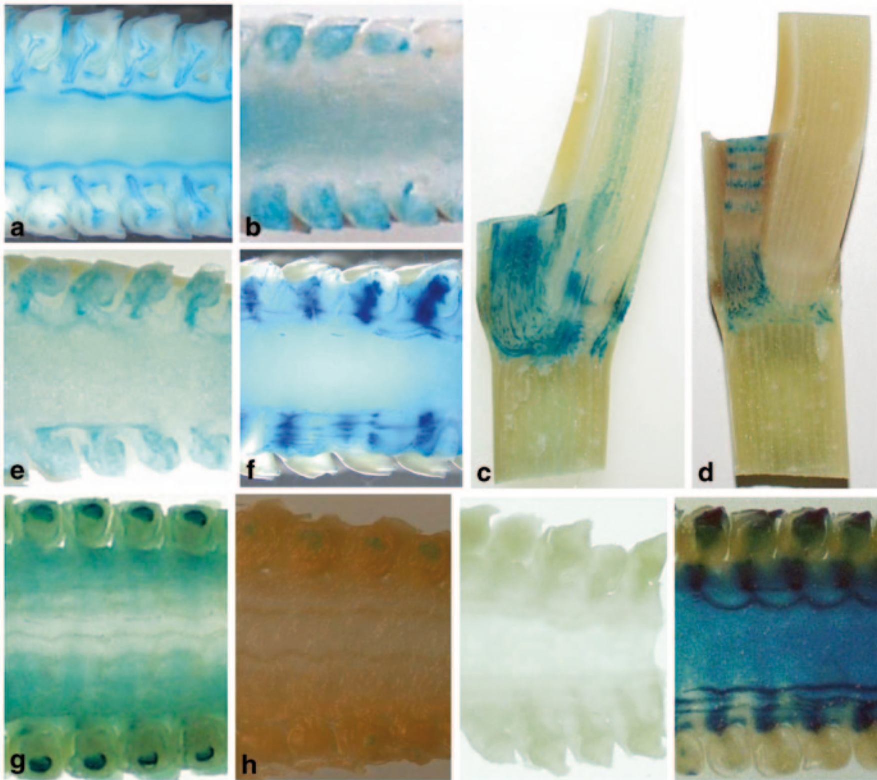

Fig. 2.3 Histochemical localization of GUS protein produced by OsMADS expression cassettes in T1 maize Tissue sections from plants expressing GUS from the (**a**) OsMADS5, (**b**) OsMADS8, (**c**) OsMADS6, (**d**) OsMADS13, (**e**) OsMADS6 (**f**) OsMADS13, (**g**) OsMADS7(s), (**h**) OsMADS7, (**i**) OsMADS14(s), and (**j**) OsMADS14 expression cassettes. (**a**, **b** and **e–j**) are central, longitudinal ear sections, (**c-d**) are stem sections taken at the ear node with the ear removed. Samples were harvested 5 days before pollination, and were incubated in the histochemical reagent for different times at 37°, then cleared with ethanol and photographed. *GUS* β-glucuronidase

expected. No evidence of unspliced sequence was found. Furthermore, the GUS coding sequence we used contained an intron derived from the *Arabidopsis thaliana* At5G14170 gene, which was also excised exactly as expected. The evidence shows that maize possesses the necessary machinery to recognize and process some heterologous or foreign introns. The data show that exclusion of the donor gene's first intron can affect expression cassette behavior. In the case of OsMADS7, the expression profile differed slightly depending on the presence of OsMADS7 intron 1. In the case of OsMADS14, GUS protein is not produced when OsMADS14 intron 1 is absent.

This illustrates the utility of the expression cassette design strategy. In neither OsMADS7 nor OsMADS14 case did the primary sequence data suggest the first intron in each gene contributes to gene activity. In addition, the evidence suggests intron excision information captured in the expression cassette design and RNA

Fig. 2.4 OsMADS expression cassette activity in T1 maize seedlings. β-Glucuronidase (GUS) activity in T1 seedlings from plants transformed with (**a**) OsMADS5, (**b**) OsMADS13, (**c**) OsMADS8, (**d**) OsMADS6, and (**e**) OsMADS14 was assessed by histochemical localization. Seeds were germinated and grown for 10 days in axenic culture. Seedlings were vacuum infiltrated with histochemical reagent and incubated at 37° for 24 h, then cleared with ethanol. There is very slight staining in the aerial tissue in (**a**), (**b**), and (**c**). Otherwise, GUS activity was not detected. GUS activity is not apparent in roots

splicing activity is conserved sufficiently to enable processing of at least these rice and *Arabidopsis* introns in maize. Furthermore, while expression profiles illustrate that we did not achieve a pedicel-specific expression pattern *per se*, most cassettes were active in pedicel tissue in maize ears prior to fertilization. This is close to the intended outcome, and not on expected given the strategy used in this example. The latter illustrates the risks associated with a sequence homology-based approach to developing a pedicel-specific expression cassette. The DoMADS3 (Yu and Goh 2000) regulatory sequence would have been the best choice for this work,

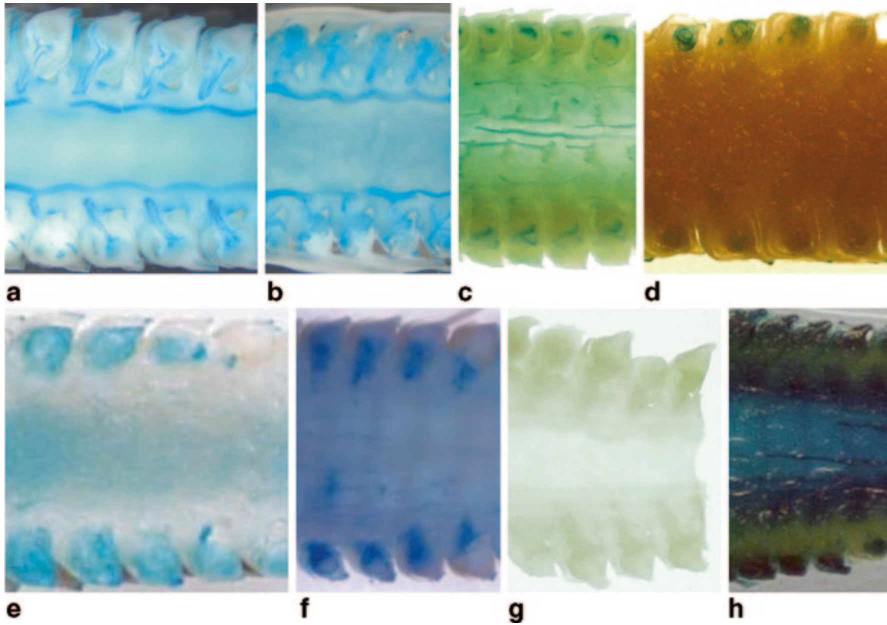

Fig. 2.5 OsMADS expression cassette activity in T1 maize ears at silk emergence. Longitudinal view of β-glucuronidase (GUS) activity in T1 ears from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS7 l, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Ears were excised from plants at silk emergence, sectioned with a razor, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at 37°, (**b**) 4 h at room temperature, (**c**) 18 h at 37°, (**d**) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (**h**) 16 h at 37°

but the DoMADS3 gDNA sequence was not available. We could have identified and worked with regulatory sequence based on the maize ortholog to DoMADS3, but the maize gDNA sequence was not widely available at the time. We elected to work with the rice MADS gene family because gDNA sequence was readily obtainable. The primary risk is that protein sequence conservation does not necessarily imply expression profile conservation. We attempted to manage this by focusing on several candidate genes. It did not work out exactly as planned. The examples below demonstrate more conservative approaches to identify candidate genes.

The Maize ABP3 Gene Is Transcribed in All Tissues but Pollen

Another trait expression control strategy focused on insect control protein accumulation in all maize tissues except pollen. There is some concern that insecticidal toxin accumulation in the pollen of transgenic maize harms nontarget pests (Pimen-

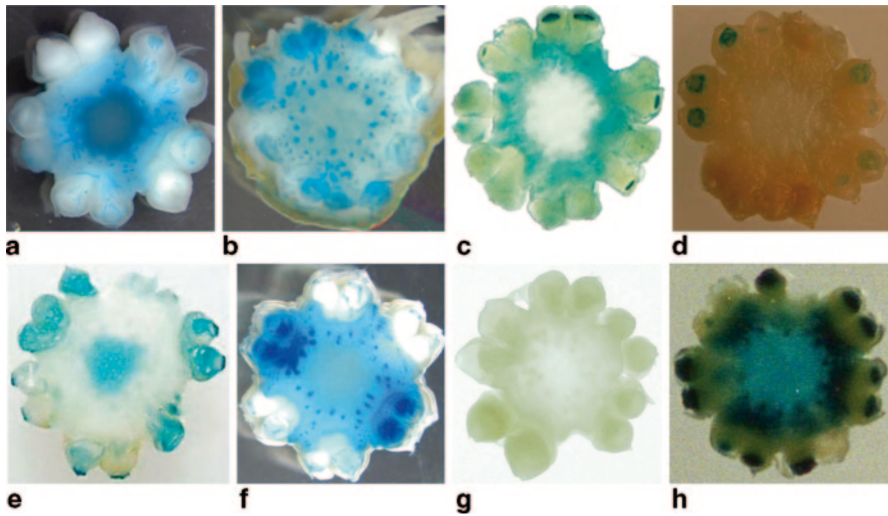

Fig. 2.6 OsMADS expression cassette activity in T1 maize ears at silk emergence. Cross sections showing β-glucuronidase (GUS) activity in T1 ears from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS7 l, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Ears were excised from plants at silk emergence, sectioned with a razor, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at 37°, (b) 4 h at room temperature, (**c**) 18 h at 37°, (**d**) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (h) 16 h at 37°

tel and Raven 2000). This expression-control technology was designed to address those concerns. The maize actin-binding protein 3 (ZmABP3) gene (Lopez et al. 1996) was identified as a donor for this purpose. The ZmABP3 gene was annotated and an expression cassette based on this gene was designed and built as described earlier. The promoter included a 5′-nontranscribed sequence, exon 1, intron 1, and part of exon 2. The terminator included the 3′-UTR and 3′-nontranscribed sequence. Several tests to characterize the expression cassette were conducted. The first iteration of this expression cassette expressed the AmCyan reporter gene. The AmCyan protein coding sequence was replaced with an insect control protein-coding sequence in the next variant. The final variant was built to measure the influence of a transcriptional enhancer complex derived from the CaMV35S/FMV34S (Lee et al. 2013) promoters on protein production. In this case, the transcriptional enhancers were located upstream of the ZmABP3 promoter. All three variants were characterized in transgenic maize.

Several transgenic maize events containing the ZmABP3-AmCyan expression cassette were produced. Those containing a single copy of the transgene and no unintended vector sequence were analyzed. All transgenic events accumulated AmCyan transcript in leaf tissue. Several tissues from a representative event were examined for AmCyan transcript accumulation by northern blot. The results in Fig. 2.16a show that the ZmABP3 expression cassette had strong expression in

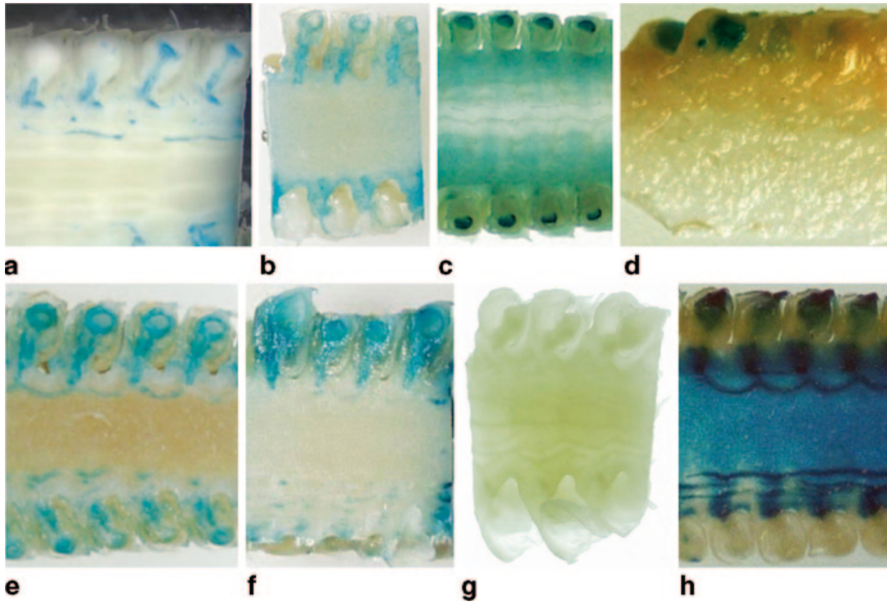

Fig. 2.7 OsMADS expression cassette activity in T1 maize ears shortly after pollination. Longitudinal view of β-glucuronidase (GUS) activity in T1 ears from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS7 l, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Ears were excised from plants 3–5 days after pollination, sectioned with a razor, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 15 h at 37°, (**b**) 2 h at room temperature, (**c**) 18 h at 37°, (**d**) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (**h**) 16 h at 37°

tassel, leaf, silk, ear, and root tissues, but does not produce detectable mRNA in pollen. Fig. 2.16b compares the integrity and relative RNA quantity in each sample. This result agrees perfectly with the natural ZmABP3 expression profile (compare Fig. 2.16 with Fig. 2.3 in (Lopez et al. 1996)). The data show that the ZmABP3 expression cassette had the necessary gene regulatory information to recapitulate the natural expression profile of the ZmABP3 gene.

In a second set of expression cassettes, the Cry1ABG6 protein-coding sequence was used (Lee et al. 2013). One expression cassette was as described for ZmABP3-AmCyan. The other contained a transcriptional enhancer complex derived from the CaMV35S/FMV34S promoters, in addition to the ZmABP3-Cry1ABG6 cassette. Hybrid maize seed for several events, representing each construct, were produced at a Syngenta field station in Bloomington, IL. Several seed were germinated in 5 cm pots. Seedlings were tested for transgene zygosity, and only hemizygotes were retained. A minimum of eight plants per event were transplanted to 11 L pots and grown in a temperature-controlled glasshouse. Leaf tissue from each plant was sampled and assayed for Cry1AbG6 protein at five stages of development, namely

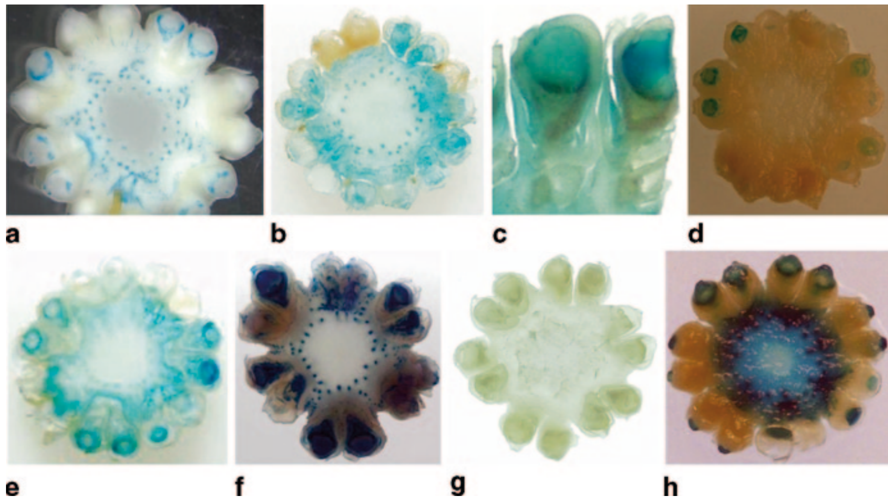

Fig. 2.8 OsMADS expression cassette activity in T1 maize ears shortly after pollination. Cross sections showing β-glucuronidase (GUS) activity in T1 ears from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS7 l, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Ears were excised from plants 3–5 days after pollination, sectioned with a razor, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at 37°, (**b**) 2 h at room temperature, (**c**) 18 h at 37°, (d) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (**h**) 16 h at 37°

V5-V6, V8, V10, R1, and R3-R4 (Ritchie et al. 1992). Pollen was also collected and assayed for Cry1AbG6 protein.

Samples were normalized for protein content and Cry1AbG6 was quantified by enzyme-linked immunosorbent assay (ELISA) using fully truncated Cry1Ab as a standard (Walschus et al. 2002). Results in Fig. 2.17 show that the ZmABP3-Cry-1AbG6 cassette produced a steady level of Cry1AbG6 protein in leaf tissue throughout development. Comparing events 5, 12, 15, and 16, some reduction in CryAbG6 protein was evident as the vegetative tissue began to senesce (R3-R4). Also evident was the three- to fivefold increase in Cry1AbG6 accumulation in events (events A-D) that also had the CaMV35S/FMV34S transcriptional enhancer complex. Finally, the data showed very low Cry1AbG6 protein accumulation in pollen. In all events, Cry-AbG6, on average, accumulated to less than 1.5 ng/ mg total soluble protein in pollen. Furthermore, the transcriptional enhancer complex did not influence Cry1AbG6 accumulation in pollen; the results are consistent in all events. This agreed with the data in Fig. 2.16, showing that ZmABP3 was not very active in pollen.

In addition, expression profiling analysis identified eight additional maize genes that possessed the ZmABP3 activity profile (data not shown). Characterization of expression cassettes based on these genes produced a similar trait gene expression outcome, demonstrating the robustness of this approach.

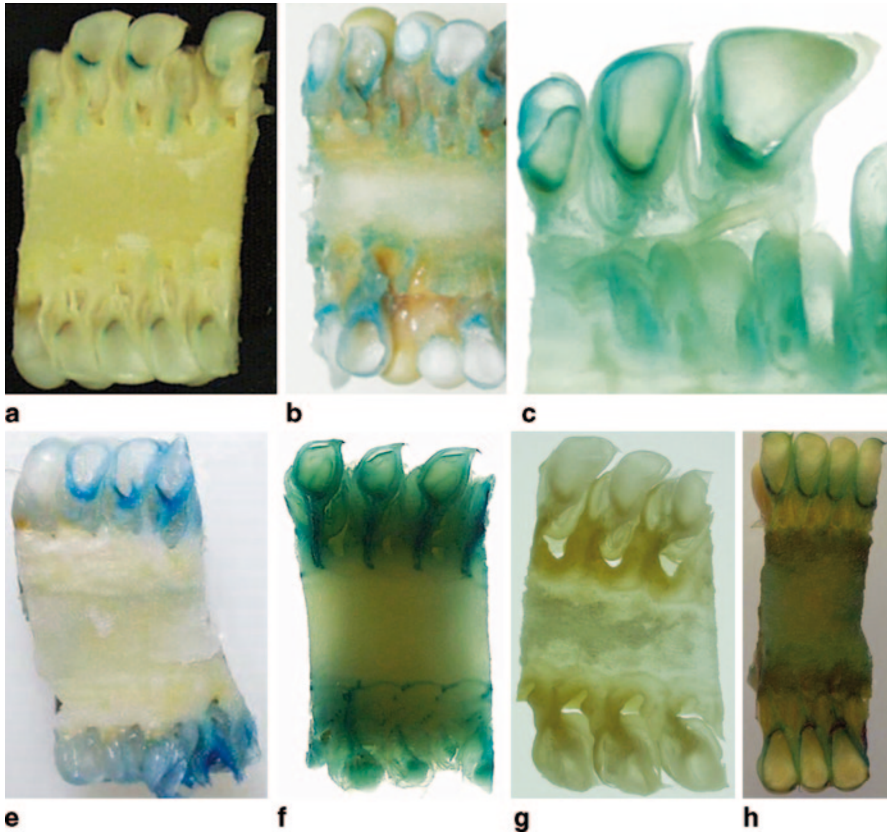

Fig. 2.9 OsMADS expression cassette activity in T1 maize ears 10–15 days after pollination. β-Glucuronidase (GUS) activity in T1 ears from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS8, (**e**) OsMADS13, (**f**) OsMADS14s, and (**g**) OsMADS14 l was assessed by histochemical localization. Ears were excised from plants, sectioned with a knife, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 8 h at room temperature, (**b**) 2 h at room temperature, (**c**) 18 h at 37°, (**d**) 5 h at room temperature, (**e**) 16 h at room temperature, (**f**) 18 h at 37°, and (**g**) 6 h at 37°

Development of an Epidermal-Specific Expression Cassette for Maize

Strategies to develop drought tolerance traits for maize using genetic engineering technology require both robust trait gene(s) and accurate trait gene expression control. Many trait genes implicated in drought tolerance redirect metabolic energy to survival mechanisms and, therefore, tend to carry a yield penalty (Cattivelli et al. 2008). Effective deployment of these trait genes depends on expression control. The trait gene should be active when and where it has the greatest positive effect and inactive elsewhere.

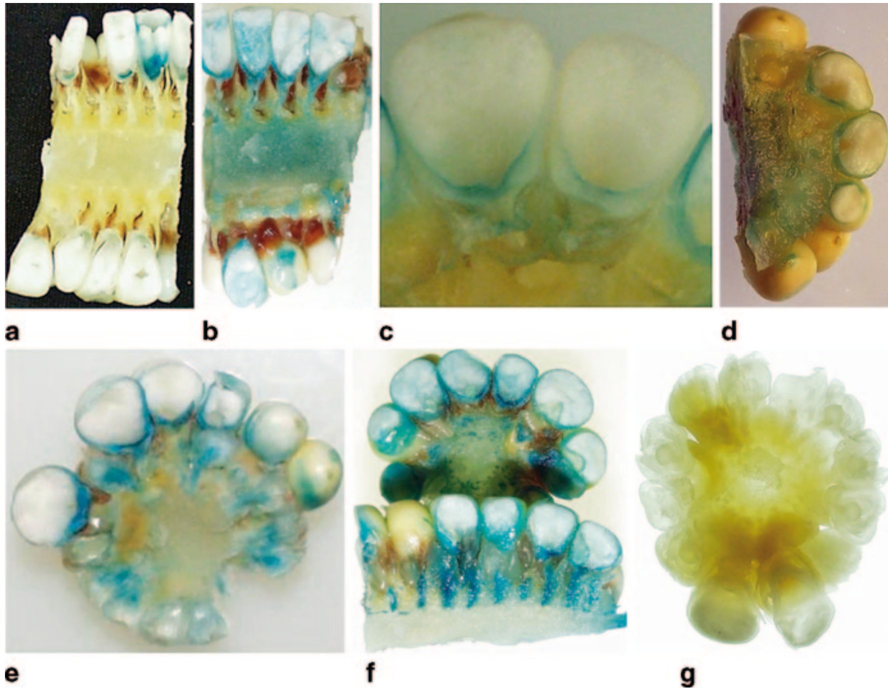

Fig. 2.10 OsMADS expression cassette activity in T1 maize ears 20–25 days after pollination. β-Glucuronidase (GUS) activity in T1 ears from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS14 l, (**e**) OsMADS8, (**f**) OsMADS13, and (**g**) OsMADS14s was assessed by histochemical localization. Ears were excised from plants, sectioned with a knife, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at room temperature, (**b**) 2 h at room temperature, (**c**) 18 h at 37°, (**d**) 17 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, and (**g**) 18h at 37°

One strategy to effectively deploy a drought tolerance trait is to limit expression to the L1 or epidermal cell layer in aerial tissue. There are no known monocotyledon promoters that deliver this type of expression control, but one has been described for dicotyledons, derived from the potato chitinase 2 gene (Ancillo et al. 2003). While it is not common to use dicotyledon promoters in maize or any other monocotyledon, there is evidence to suggest a potato promoter could function in monocotyledons. Studies show that a dicotyledon promoter will work in a monocotyledon and vice versa (Koyama et al. 2005; Liu et al. 2003). This is not universal, but supports investigating the activity of the potato chitinase 2 gene in maize.

The inclusive design strategy described above was used to ensure that all the requisite regulatory sequence was captured in the expression cassette. This began with base-level annotation of the potato chitinase 2 gDNA sequence which can be found in GenBank (AF153195). The promoter contains 1.310 kb of 5′-nontranscribed sequence, the 467 bp exon 1, the 82 bp intron 1, and 25 bp of exon 2. The 488 bp 5′-UTR represents exon 1 and part of exon 2. The expression cassette also contains the

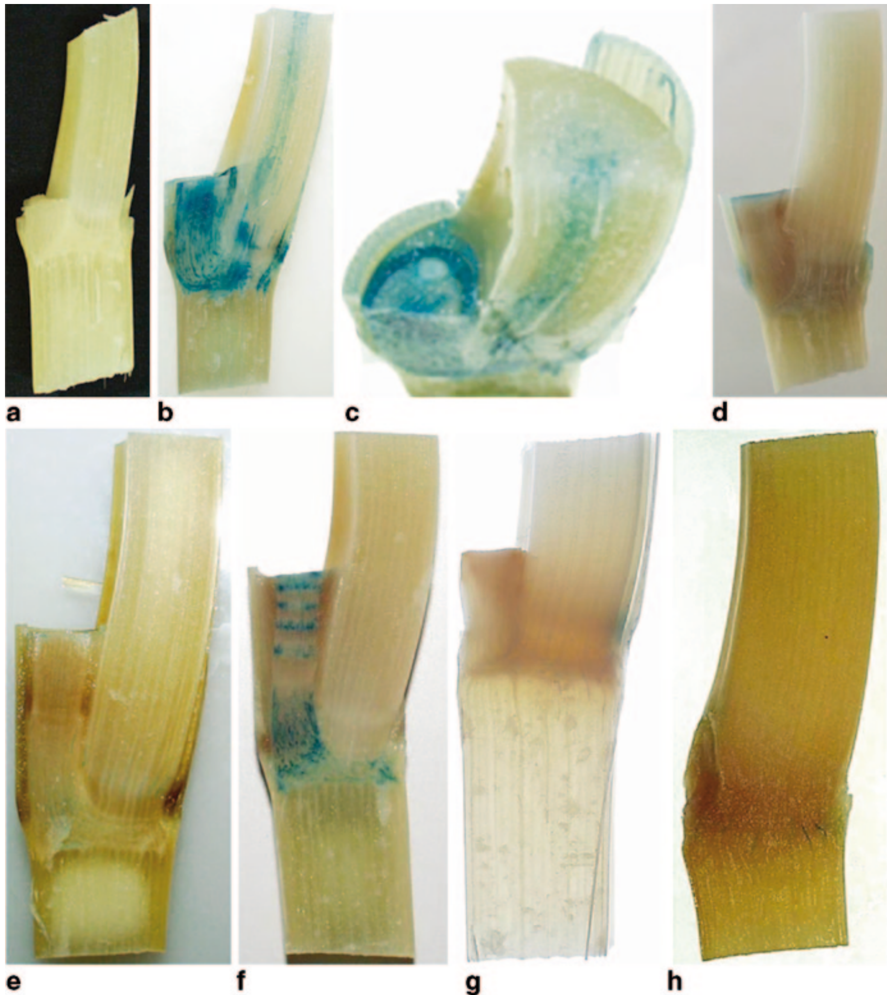

Fig. 2.11 OsMADS expression cassette activity in T1 maize ear node shortly before pollination. β-Glucuronidase (GUS) activity in T1 ear nodes from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS6-top view, (**d**) OsMADS7s, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Ear nodes were excised from plants just prior to silk emergence and ears were removed. Nodes were sectioned with a knife, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at 37°, (**b**) 14 h at room temperature, (**c**) 14 h at room temperature, (**d**) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (**h**) 16 h at 37°

terminator which is 638 bp of 3′-sequence that begins just past the translation stop codon. This includes about 81 bp of 3′-UTR and 557 bp of nontranscribed sequence. The StChiC expression cassette was fused to the GUS reporter protein-coding sequence and transformed into maize. Several independent events were produced.

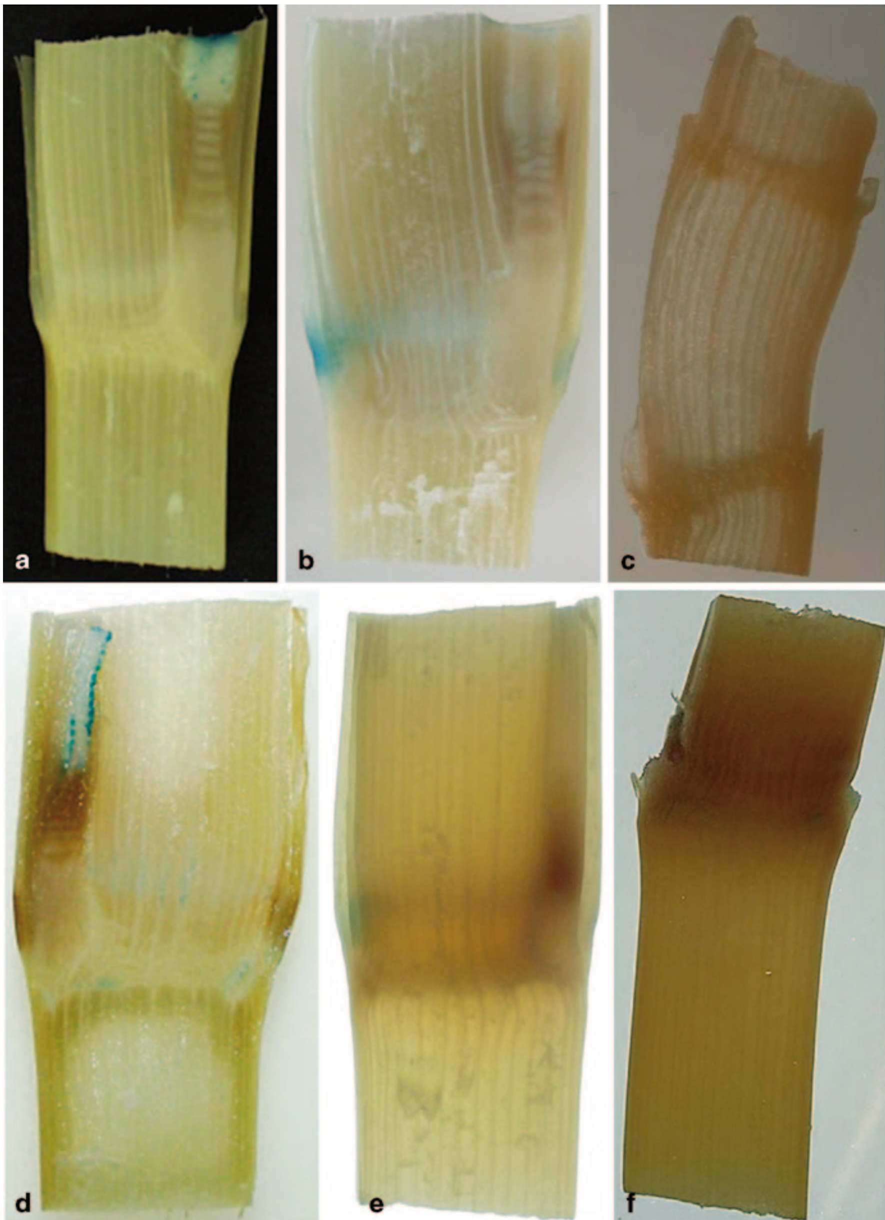

Fig. 2.12 OsMADS expression cassette activity in T1 maize node beneath the ear node shortly before pollination. β-Glucuronidase (GUS) activity in T1 tissue from plants transformed with (**a**) OsMADS5, (**b**) OsMADS7s, (**c**) OsMADS7 l, (**d**) OsMADS8, (**e**) OsMADS14s, and (**f**) OsMADS14 l was assessed by histochemical localization. Nodes were excised from plants just prior to silk emergence. Nodes were sectioned with a knife, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at 37°, (**b**) 18 h at 37°, (**c**) 18 h at 37°, (**d**) 5 h at room temperature, (**e**) 18 h at 37°, and (**f**) 16 h at 37°

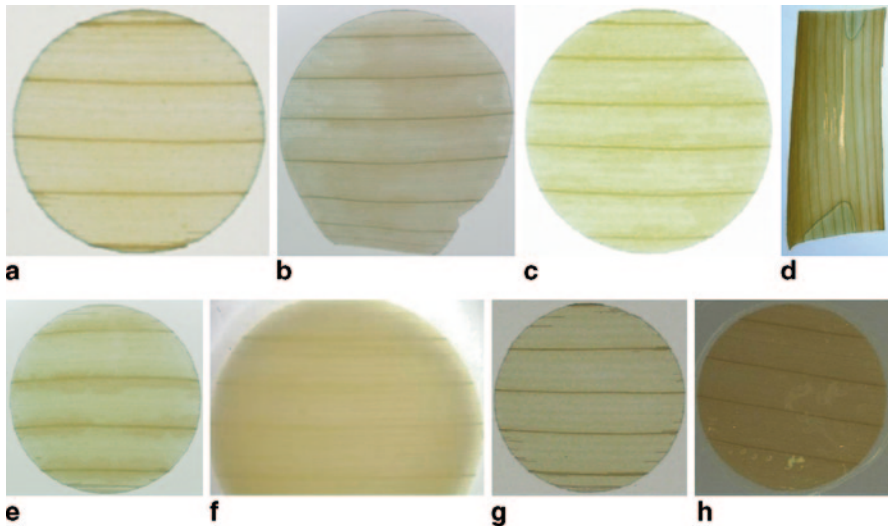

Fig. 2.13 OsMADS expression cassette activity in T1 maize leaf shortly before pollination. β-Glucuronidase (GUS) activity in T1 leaf punches from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS7 l, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Leaf samples were excised from plants just prior to silk emergence using a punch or scissors, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at 37°, (b) 14 h at room temperature, (c) 18 h at 37°, (d) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (**h**) 16 h at 37°

T0 plants were analyzed for GUS protein and transcript accumulation in leaf tissue, and three events, 11, 34, and 40, were selected for T1 analysis. Twenty-four seeds per event were planted, with germination rates of 42, 46, and 88 %, respectively. The seedlings were genotyped by zygosity TaqMan assay (Ingham et al. 2001). Histochemical localization of GUS activity and GUS ELISA were performed on young leaf tissue. The data are summarized in Table 2.2 and Fig. 2.18. In events 11 and 34, the GUS expression data are consistent with the zygosity data. The homozygotes accumulate GUS protein to a significantly greater concentration compared to heterozygotes. The azygotes (null segregants) did not accumulate GUS protein.

Two events were used to determine whether the GUS protein accumulated in a cell-layer specific manner. The data in Fig. 2.18 demonstrate this for event 40, showing the GUS activity is present in the outer layer of the leaf. Similar results were observed for event 11. Collectively, the evidence supports that the StChiC expression cassette functions to express GUS protein in the epidermal tissue of transgenic maize. This example illustrates the potato chitinase C regulatory sequence is recognized and functions correctly in transgenic maize. It suggests that encoding and decoding of gene regulatory information may be conserved in plants. Some evidence supports this (Khurana et al. 2013), but other evidence does not (Schünmann et al. 2003). However, not much work has been done in this area, so we do not know the extent to which this might be true.

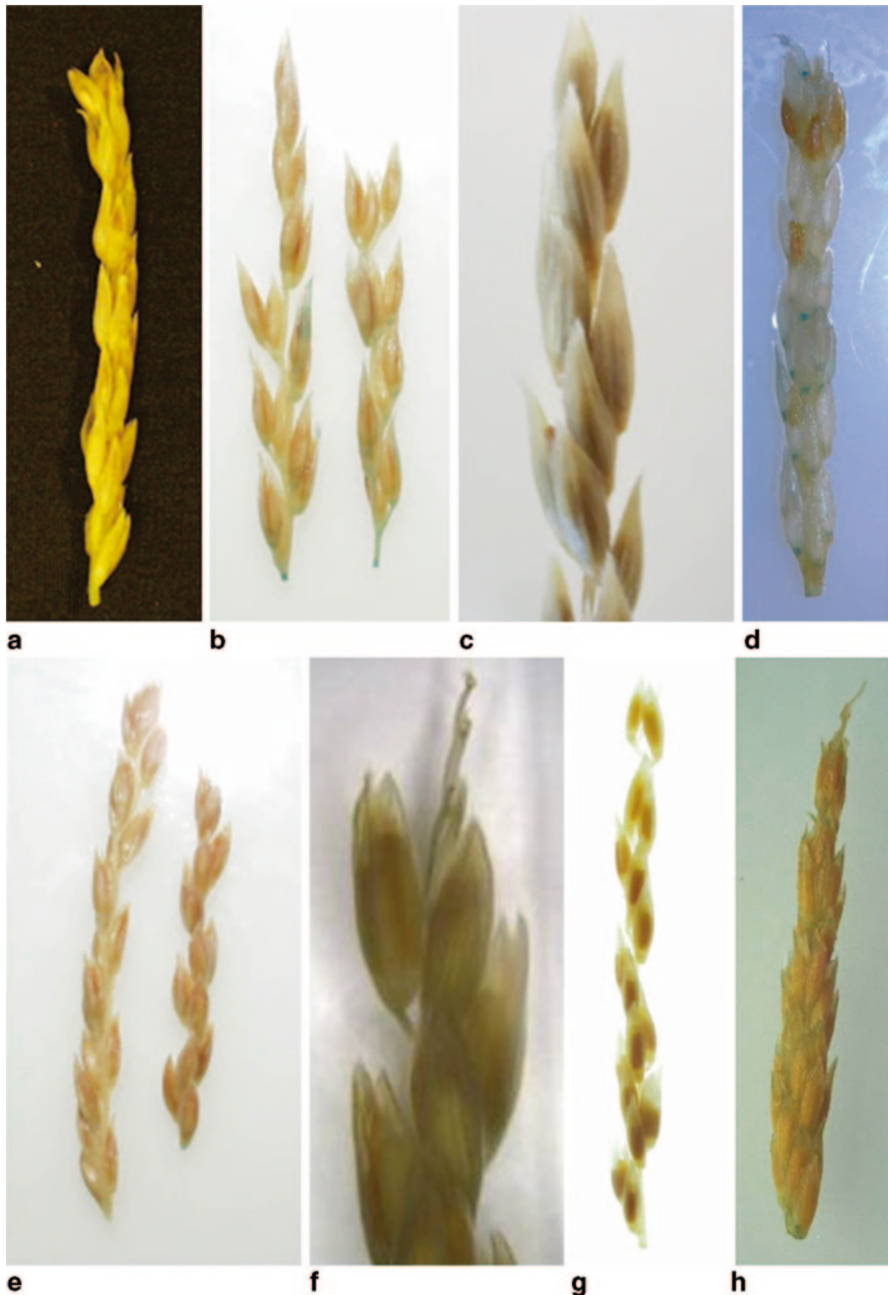

Fig. 2.14 OsMADS expression cassette activity in T1 maize tassel shortly before pollination. β-Glucuronidase (GUS) activity in T1 tassel from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS7 l, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Tassel samples were excised from plants just prior to pollen shed, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (**a**) 22 h at 37°, (**b**) 14 h at room temperature, (**c**) 18 h at 37°, (**d**) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (**h**) 16 h at 37°

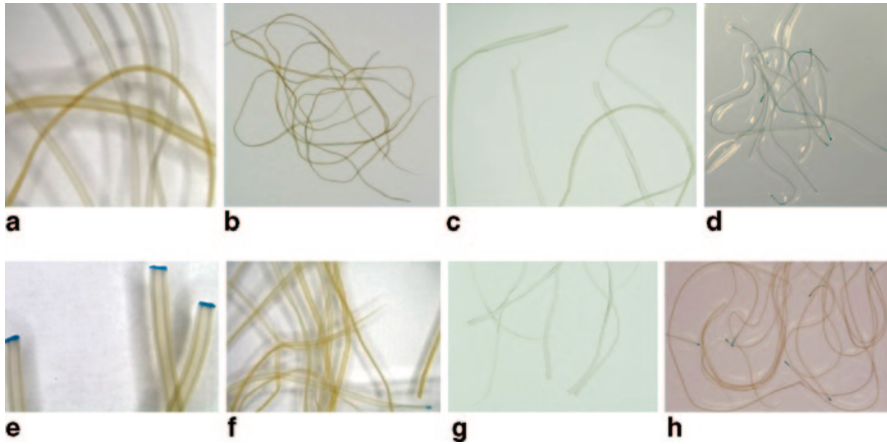

Fig. 2.15 OsMADS expression cassette activity in T1 maize silk shortly before pollination. β-Glucuronidase (GUS) activity in T1 silk from plants transformed with (**a**) OsMADS5, (**b**) OsMADS6, (**c**) OsMADS7s, (**d**) OsMADS7 l, (**e**) OsMADS8, (**f**) OsMADS13, (**g**) OsMADS14s, and (**h**) OsMADS14 l was assessed by histochemical localization. Silk samples were excised from plants just prior to silk emergence, vacuum infiltrated with histochemical reagent, and incubated at 37° for various times, then cleared with ethanol. The incubation conditions were (a) 22 h at 37°, (**b**) 14 h at room temperature, (**c**) 18 h at 37°, (**d**) 18 h at 37°, (**e**) 5 h at room temperature, (**f**) 16 h at room temperature, (**g**) 18 h at 37°, and (**h**) 16 h at 37°

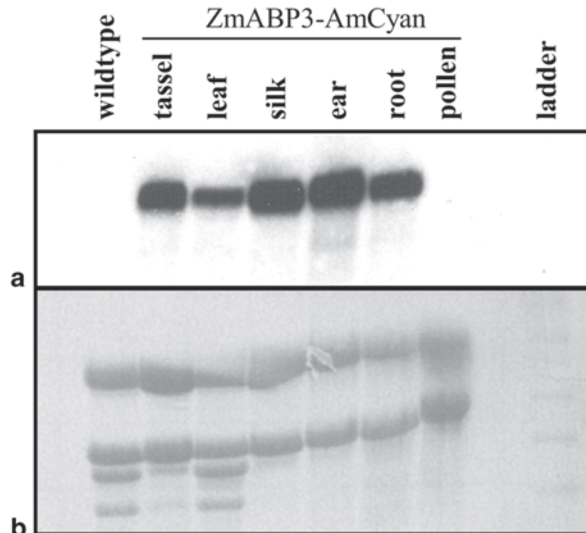

Fig. 2.16 Transcription from the ZmABP3-AmCyan expression cassette (**a**) Northern analysis of AmCyan transcript accumulation in maize. Samples are nontransformed leaf (AX5707) and tassel, leaf, silk, ear, root, and pollen from a representative T0 event. Each lane contains 10 µg of total RNA. The blot was hybridized to a ^{32}P-probe generated against the AmCyan sequence using high-stringency conditions. (**b**) Visualization of total RNA on the northern blot membrane using methylene blue

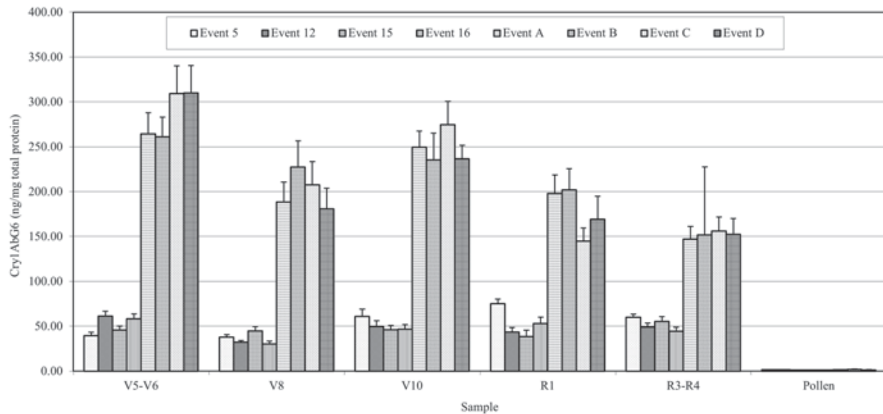

Fig. 2.17 Cry1AbG6 protein in transgenic maize tissue. The youngest developing leaf was tested for Cry1AbG6 by ELISA at five developmental stages (*V5-V6, V8, V10, R1, R3-R4*) for each plant. Cry1AbG6 was also measured in pollen. *Events 5, 12, 15,* and *16* express the ABP3-Cry1AbG6 construct, and *Events A-D* express the enhanced ABP3-Cry1Ab construct. Data shown are the mean ± SD (n = 8–10). ELISA enzyme-linked immunosorbent assay

A Guard Cell-Specific Expression Cassette Derived for the *Arabidopsis* At1G22690 Gene

Another trait development problem required that trait gene expression be directed to guard cells. Identification of guard cell-specific genes is not straightforward. Two *Arabidopsis* genes have been described; one encodes an MYB transcription factor (Cominelli et al. 2005) and the other an uncharacterized protein (Yang et al. 2008). Promoters based on both genes have been shown to be guard cell-specific in *Arabidopsis*. Could a functional expression cassette based on one of these genes be developed for applications in maize? Work below focused on At1G22690 (Yang et al. 2008).

A series of expression cassettes based on At1G22690 were designed and tested. As outlined in Table 2.3, these variants differed with respect to the promoter. Vector 19711 contained the original 1 kb component that includes 5′-nontranscribed sequence and the 5′-UTR (Yang et al. 2008). This promoter also contained the At1G22680 promoter, which initiated transcription on the opposite DNA strand. To create 19710, a total of 384 bases were removed from the 5′-end of the 19711 promoter to eliminate the At1G22680 promoter. The 19678 promoter has the same 5′-nontranscribed region as 19710 and is modeled on the design strategy outlined above, in that it includes exon 1, intron 1, and part of exon 2 from At1G22690. The 18620 promoter adds a tobacco mosaic virus Ω-translational enhancer (Gallie and Walbot 1992) to the 19678 cassette. The promoter in vector 19738 is similar to that in 19678, except the natural At1G22690 intron was replaced by the maize ubiquitin intron (Christensen and Quail 1996). All variants were characterized in stable, transgenic maize plants.

Table 2.2 Histochemical and ELISA assay of StChiC-driven GUS expression in T1 maize leaf tissue

Event ID[a]	Zygosity	GUS histochemical analysis	GUS ELISA (ng/ mg soluble protein)
11–2	Hom	Blue	965
11–3	Null	No activity	45
11–6	Hom	Blue	1199
11–7	Het	Blue	321
11–8	Het	Blue	394
11–9	Hom	Light blue	421
11–17	Het	Light blue	244
11–18	Het	Blue	376
11–24	Het	Light blue	289
34–3	Null	No activity	49
34–7	Null	No activity	37
34–10	Null	No activity	38
34–14	Het	Blue	243
34–18	Het	Blue	409
34–21	Null	No activity	32
34–22	Hom	Blue	1547
34–23	Hom	Blue	510
40–3	Het	Blue	204
40–4	Hom	Blue	1537
40–5	Hom	Blue	1811
40–6	Hom	Blue	1339
40–8	Hom	Blue	1130
40–9	Het	Blue	529
40–10	Het	Blue	318
40–11	Hom	Blue	1116
40–12	Hom	Light blue	152
40–15	Het	Light blue	326
40–16	Hom	Blue	369
40–17	Het	Blue	971
40–18	Het	Light blue	267
40–20	Het	Blue	405
40–21	Het	Light blue	484
40–22	Hom	Blue	1541
40–23	Hom	Blue	1226
40–24	Hom	Blue	838

[a] Segregating T1 siblings representing three events were evaluated for expression cassette activity. Trait gene zygosity was established by TaqMan. The histochemical reaction to detect GUS activity was in standard reagents. The tissue was incubated at 37° for 12 h, then cleared with ethanol

ELISA enzyme-linked immunosorbent assay, *GUS* β-glucuronidase

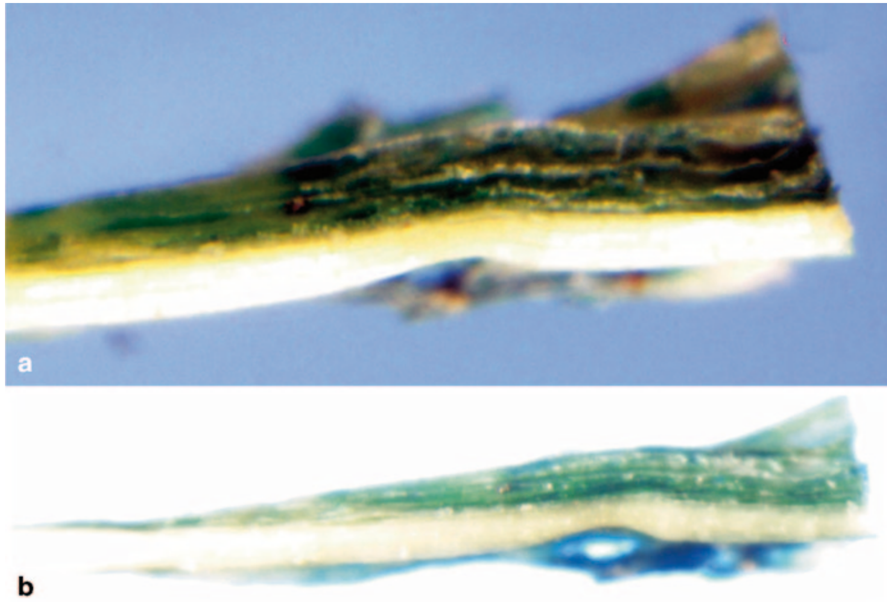

Fig. 2.18 Epidermal-specific localization of StChic-driven GUS expression in maize. Leaf tissue from the 40 to 20 T1 seedling was incubated with GUS histochemical reagent overnight at 37°, cleared with ethanol, dissected and examined in a low-power *light* microscope. (**a**) A representative image of the observed histochemical staining pattern as viewed under high magnification with high contrast. (**b**) The same sample as (**a**) but viewed at *lower* magnification and with low contrast. The adaxial surface is oriented up. *GUS* β-glucuronidase

Table 2.3 Expression cassettes based on the *Arabidopsis* GC1 gene and their activity in transgenic maize

Vector[a]	Enhancer	Promoter	Reporter	Terminator	Activity in maize	Guard cell specific
19711	FMV34S/ CaMV35S	Original AtGC1	GUS	AtGC1	None	
19710	FMV34S/ CaMV35S	Modified AtGC1	GUS	AtGC1	None	
19678	FMV34S/ CaMV35S	Modified AtGC1 + intron 1 + partial exon 2	GUS	AtGC1	Modest	Yes
18620	FMV34S/ CaMV35S	Modified AtGC1 + intron 1 + partial exon 2 + TMV-Ω	GUS	AtGC1	Low	Yes
19738	FMV34S/ CaMV35S	Modified AtGC1 + Zm Ubi1 intron + partial exon 2	GUS	AtGC1	High	No

[a] B1 maize lines with a single-copy, backbone-free T-DNA insert were analyzed. Representative observations based on qRT-PCR, ELISA, and histochemical localization data are presented

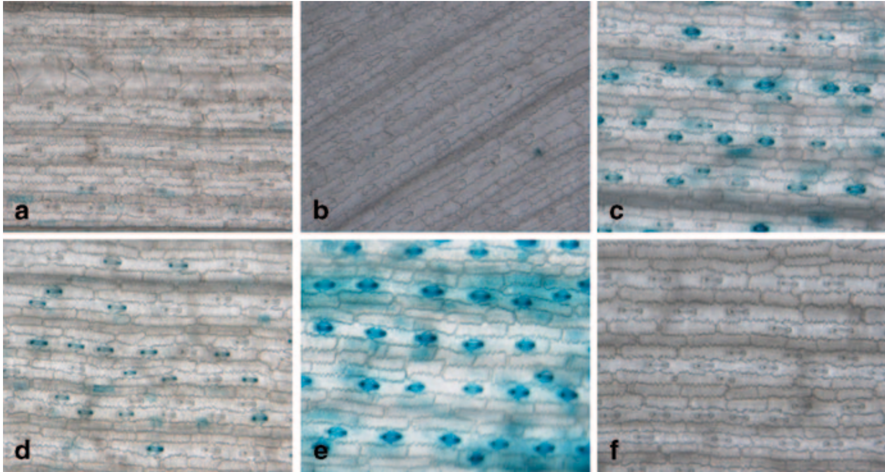

Fig. 2.19 Histochemical localization of GUS activity driven by various guard cell expression cassettes in T1 maize leaf tissue. Samples are from maize transformed with the (**a**) 19711, (**b**) 19710, (**c**) 19678, (**d**) 18620, (**e**) 19738, and (**f**) 19738 null plants was excised and vacuum infiltrated with the histochemical reagent to detect GUS enzyme activity. All tissue is from the R1 tassel leaf, except (**b**) which is from the first fully expanded leaf harvested at V4. Samples were incubated in the *dark* at 37° for 48 h, then cleared with ethanol. GUS β-glucuronidase. Construct information is in Table 2.3

Only single-copy, backbone-free events were analyzed. In each case, backcrossed (B1) seed for three events were germinated, and the presence of the guard cell expression cassette was established by zygosity TaqMan (Ingham et al. 2001). Six trait positive siblings were retained and assayed for GUS activity at various stages of development. Reporter gene activity was assessed by qRT-PCR, ELISA, and histochemical localization. The quantitative data are reported elsewhere (Nuccio 2013). Representative histochemical results are shown in Fig. 2.19.

Histochemical localization data from 19711 leaf tissue in Fig. 2.19a indicated little detectable GUS protein accumulation in guard cells or other cells. Histochemical localization was also used to investigate activity in different B1 tissues such as husk, cob, stem, root, tassel, and kernel. GUS enzyme activity was detected to some extent in stem, the pedicel region of the cob, and the epithelial layer surrounding the embryo (Nuccio 2013). The evidence shows that this promoter, which works well in *Arabidopsis* and tobacco (Yang et al. 2008), does not function well in maize.

Histochemical data from 19710 leaf tissue in Fig. 2.19b reveal no detectable GUS protein accumulation in guard cells or other cells. Further histochemical analysis of husk, cob, stem, root, tassel, and kernel revealed no GUS enzyme activity (Nuccio 2013). The evidence indicated that this promoter does not function in maize.

GUS data from 19678 leaf tissue in Fig. 2.19c indicated that the reporter protein was present in guard cells. The activity level was much greater than that in 19711. Similar data for different B1 tissues such as husk, cob, stem, root, tassel, and kernel showed the GUS enzyme activity accumulated just as it does in 19711. It was pres-

ent in the stem, the pedicel region of the cob, and the epithelial layer surrounding the embryo (Nuccio 2013). The data show that extending the 19711 5′-UTR to include exon 1, intron 1, and part of exon 2, enables high expression in maize guard cells.

The data from 18620 leaf tissue in Fig. 2.19d showed that adding the TMV-Ω sequence to the 19678 cassette reduced GUS protein accumulation by two- to threefold, but did not change the promoter's spatial activity. Thus, TMV-Ω reduces, rather than enhances reporter protein accumulation.

GUS data from 19738 leaf tissue in Fig. 2.19e indicated the reporter protein accumulated in guard cells, as well as other cells. The activity level was greater than that in 19678. Similar data for different B1 tissues, such as husk, cob, stem, root, tassel, and kernel, showed GUS enzyme activity was present in most plant parts (Nuccio 2013). The quantitative evidence suggested that substituting the maize ubiquitin 1 intron for the At1G22690 intron increased GUS protein accumulation by about sevenfold. It also contributed to a broader expression profile.

The data show that At1G22690 promoter variants lacking an intron have no significant activity in maize. This included the 19711 variant that was active in *Arabidopsis*. The variants with the first At1G22690 intron were active in maize guard cells, suggesting that it is required to for At1G22690-based expression cassette activity in maize. However, the variant with the TMV-Ω sequence was much less active. This suggested that the TMV-Ω sequence negatively influences trait protein accumulation in maize. In dicotyledons like tobacco and *Arabidopsis,* the TMV-Ω functions as a translational enhancer capable of increasing protein production by several fold (Gallie and Walbot 1992; Koziel et al. 1996). In general, it has been found that the TMV-Ω sequence reduces protein production in maize. Other evidence suggests it can improve trait protein production in maize in some contexts (Palmer et al. 1999).

Replacement of the first At1G22690 intron with a maize ubiquitin intron greatly increased GUS protein accumulation in guard cells, and other cells. This supports the concept that introns contribute to gene activity by influencing protein production, and suggests that introns possess some degree of autonomy, i.e., they can function in heterologous contexts. Some groups report similar results, and researchers are now beginning to explore these properties (Rose et al. 2008). In some cases, intron substitution simply increases expression cassette output. Here, intron replacement also altered the expression cassette's spatial activity profile.

The objective was to produce an expression cassette with activity limited to guard cells. Arguably, the expression cassettes were, at best, guard-cell preferred. The activity observed in other cells was not expected. The AtGC1 promoter is known to produce some non-guard cell expression in certain circumstances (Yang et al. 2008). However, a global activity profile of AtGC1 has not been reported. The results here could be due to both the low non-guard cell activity and the FMV34S/CaMV35S transcriptional enhancer complex (Lee et al. 2013). They could also be due to incomplete conservation of gene regulatory information between maize and *Arabidopsis*. Similar observations were reported for rice promoters evaluated in

rice and *Arabidopsis* (Khurana et al. 2013). Overall, the evidence shows the potential to produce expression cassettes with distinct properties, by including or substituting introns and enhancers.

Summary

Trait gene expression control is moving beyond the initial gene models that consist of a promoter-gene of interest terminator. These early expression cassettes were based and largely on the gene structure found in plant pathogens, and were able to address several important biotechnology problems. Trait gene expression cassettes began to incorporate plant sequences, specifically promoters, as they became available. Many studies have shown that the promoter is necessary and sufficient to drive transgene expression in plants. New information regarding plant-gene regulatory sequence emerged as exceptions to this concept were investigated. The complexity of expression cassette design evolved, but was constrained by the recombinant DNA technology tools that were widely used. More often than not, the drive was to recycle DNA molecules rather than rebuild them. Advances in chemical DNA synthesis technology have already changed this early paradigm. It is now faster and less expensive to have an expression cassette synthesized than to stitch it together from various components stored in a freezer.

Chemical DNA synthesis technology enables further refinement of the sequences that comprise trait gene expression cassettes. The occurrence or availability of specific endonuclease sites is no longer a driver. More regulatory components can be incorporated seamlessly into expression cassettes. For example, it is now possible to contemplate codon replacement, instead of a continuous coding sequence derived from cDNA, as an approach to plant expression cassette design. Finally, it is now routine to resynthesize expression cassettes rather than reuse previous cassettes. This enables a simple framework to incorporate new biological information as it becomes available.

The postgenomic era offers tremendous opportunity to expand the repertoire of trait gene expression tools. The approach outlined here is one way to exploit the wealth of information in plant genome databases. It does not require that regulatory DNA sequences be dissected into core elements, nor is it likely to produce new knowledge addressing plant-gene expression control. It simply provides an organizational context to facilitate trait expression cassette development. The basic premise is that natural plant-gene structure provides the best template for expression cassette design. In effect, it is prudent to copy nature until we more fully understand DNA structure/function relationships. It is expensive and time consuming to create and analyze transgenic plants, but transgene analysis remains the gold standard for establishing the efficacy of new gene expression-control tools. The basic information to produce virtually any gene expression pattern in plants is available and represents a new paradigm for expression cassette design. Effective expression cassette development can now be an information-driven process.

Outlook

Much of the work to develop trait gene expression cassettes occurred at a time when recombinant DNA technology relied on biological tools, such as restriction endonucleases, DNA phosphatases, and DNA ligases to assemble DNA molecules. Likewise, DNA sequence editing was limited to chemical or PCR-based mutagenesis tools. Skill and experience were required to effectively and efficiently use these tools. This is no longer the case. Chemical DNA synthesis technology replaced much of the need for laboratory work to support recombinant DNA technology. It offers single-nucleotide level resolution and molecules up to 20 kb are synthesized routinely. The length of chemically synthesized DNA molecules continues to increase and the unit cost for chemical DNA synthesis continues to decline. This advancement alone revolutionized expression cassette design and construction. Now the critical skills include analytical and computational skills to translate basic DNA sequence information into useful biotechnology tools.

This is an age where synthetic expression cassettes are quite possible (Mehrotra et al. 2011; Venter 2007). These tools consist of artificial sequences that are designed to function just like, or better than, naturally occurring sequences. However, few examples of functional, fully synthetic expression cassettes exist. Despite considerable effort to identify regulatory elements with specific properties, robust rules that define how these elements might be used outside their original context remain elusive. Plant biologists face a similar problem when characterizing trait genes that contribute to plant response to the environment. In many cases, the results apply only to the environment from which the data were acquired (Skirycz et al. 2011). Similar, but different environments, tend to elicit a different response (Barker et al. 2005). Plant response to the environment is often described as a complex process, resulting from the integration of many inputs by many cellular networks. Gene regulation likely reflects a similar system that integrates multiple inputs to trigger a response, primarily transcription initiation. Experience to date suggests that we do not fully understand how plant-gene regulatory information is organized. The nucleotide sequence is one layer of information.

Biological information, like that which contributes to control of gene expression, continues to advance. Coupled with chemical DNA synthesis, we can expect improvement in trait gene expression cassettes. Whole genome data provide not only the basis for plant-gene structure, but also are beginning to inform plant-gene organization. This will become increasingly important as biotechnology seeks to create multigenic products.

Our understanding of epigenetic mechanisms, such as DNA and histone methylation, on plant-gene regulation is also advancing (Law and Jacobsen 2009; Springer 2013). New studies show that transgenes are influenced by plant epigenetic machinery (Dalakouras et al. 2012). The mechanisms involved in DNA sequence-specific processes that contribute to transgene regulation (Voinnet et al. 1998) are much better understood. For example, small RNAs contribute to both transcriptional and posttranscriptional trait gene silencing (Brodersen and Voinnet 2006; Vaucheret

2006). Epigenetic processes will need to be accounted for in future expression cassette design strategy.

New biological information that we might expect to be incorporated into future expression cassettes takes many forms. It includes data describing nucleosome structure (Kaplan et al. 2009) and its role in plant-gene expression control (Chodavarapu et al. 2010). The occurrence and placement of insulator sequences (Raab and Kamakaka 2010) will influence the design and organization of multigenic traits. Empirical evidence, showing that protein coding sequence has a profound effect on protein production, reveals new opportunities to regulate trait gene expression (Kudla et al. 2009). While plant mRNA synthesis and processing remains poorly understood, work in yeast and animal systems shows that it is highly regulated and significantly contributes to overall gene activity (Moore and Proudfoot 2009). There is little doubt that expression cassette design will continue to evolve.

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Chapter 3
Strategies to Maximize Recombinant Protein Expression in Maize Kernels

John A. Howard and Elizabeth E. Hood

Introduction

Maize is a major cereal food crop worldwide, and most of its nutritive value is localized in the kernel. Historically, plant breeders and agronomists have increased the productivity of corn to keep pace with the demand for its traditional dietary uses. Over the past three decades, recombinant DNA technology has also been employed to increase yields by improving performance with respect to drought tolerance, pest resistance, and weed management (Kasuga et al. 1999; Kasuga et al. 2004; Funke et al. 2006; Morran et al. 2010b; Jouanin et al. 1998).

Recently, attention has turned to using plants as an alternate energy source to help supplement traditional fossil fuels and to provide a clean, indigenous, and renewable fuel source. Interest in biofuels has focused on the abundant and readily available starch obtained from the corn kernel, a precursor that can be converted easily to ethanol. Cornstarch accounts for the vast majority of biofuel in the USA today, and this alternate market has increased the demand for corn grain, such that biofuels now account for 40 % of corn production (http://www.ers.usda.gov/data-products/us-bioenergy-statistics.aspx).

Corn grain is a safe, inexpensive, and stable product that has prompted many additional applications that take advantage of these intrinsic properties, as well as the establishment of specialized processing methods to increase its functionality. Corn has been developed with altered kernel composition such as high lysine (Vasal 1994), high oil (Lambert 1994), and high protein (Dudley and Lambert 1992). These are exceptions, however, to the vast majority of the past work on corn which

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was focused on increasing yields without significantly changing the nature of the crop itself, or altering its composition.

With the advent of recombinant DNA technology, commodity corn is now being used as a starting point to add completely new functionalities to the grain itself (Ramessar et al. 2008; Naqvi et al. 2011). Many of these new functionalities are conferred by the overexpression of specific proteins. Corn grain has key characteristics that offer benefits to overexpress proteins that include high protein content (Shewry 2007), high levels of protease inhibitors (Habib and Fazili 2007), high carbohydrate content, and low water content, all of which aid accumulation of specific proteins in a stabilized form (Stoger et al. 2004; Lamphear et al. 2005).

New functionalities in corn grain can be achieved by adding specific recombinant proteins to exploit attributes for various outcomes. Some of these functions include (1) enhancing nutrition, by increasing the lysine content in corn seed using several methods, including the expression of lysine metabolic pathway genes, expression of high-lysine proteins, inhibiting lysine catabolic enzymes through RNA interference (RNAi) mechanisms, reducing lysine-poor zein protein levels, or combinations of these procedures (Frizzi et al. 2008; Houmard et al. 2007; Chiang et al. 2005); (2) expressing a protein that provides a high-intensity sweetener (e.g., brazzein) in the grain as a low-cost alternative to high-sugar snacks and cereals (Lamphear et al. 2005); and (3) expressing a vast array of industrial (Khan et al. 2013) and pharmaceutical proteins that promise to provide a low cost, animal-free source for applications in biofuels (Torney et al. 2007; Shetty et al. 2005), vaccines, and therapeutics (Daniell et al. 2001; Streatfield and Howard 2003b, 2003a; Ma et al. 2005; Ramessar et al. 2008; Boothe et al. 2010; Naqvi et al. 2011).

The common principle in these new applications is the reliance on the accumulation of specific proteins. This promise of increased functionality is only theoretical unless these proteins can accumulate at concentrations that are high enough to allow for economically viable products. Protein accumulation is inversely proportional to the cost of production and, therefore, one of the most critical factors leading to commercialization. Several reviews highlight a range of techniques to increase expression and accumulation of proteins in plants (Padh et al. 2010; Streatfield 2007; Mullis et al. 2012; Egelkrout et al. 2012; Hood et al. 2012; Table 3.1) including the various attributes that different host plants offer (Howard and Hood 2005b). This chapter focuses on strategies that have been used for the overproduction of recombinant proteins in maize grain.

Protein Accumulation

The basic principles of protein accumulation can be accounted for by comparing the rate of recombinant protein expression to the rate of degradation. In practice, however, there are many reasons that make this much more complicated than a simple subtraction problem. Many of these factors have been described previously

Table 3.1 Promoters used for protein accumulation in various plant species. (Reproduced with permission from Egelkrout et al. 2012)

Promoter	Expression (tissue)	Host species	Reference
Monocotyledon constitutive			
Os actins	Constitutive	Rice, wheat	McElroy et al. (1990); McElroy et al. (1991); Khanna and Daggard (2006); Xiao et al. (2007); Primavesi et al. (2008); He et al. (2009)
Os APX	Constitutive	Rice	Park et al. (2010)
CaMV 35S	Constitutive	Wheat, maize	Ransom et al. (2007b); Xiao et al. (2007); Cong et al. (2009)
At carotene hydroxylase	Constitutive	Rice	Liang et al. (2009)
Os Cc1	Constitutive	Rice	Jang et al. (2002); Park et al. (2010)
Zm histone H2B	Constitutive	Wheat and maize	Rasco-Gaunt et al. (2003)
Os PGD1	Constitutive	Rice	Park et al. (2010)
Os R1G1B	Constitutive, drought-stress induced	Rice	Park et al. (2010); Yi et al. (2010)
Os RUBQ1	Constitutive	Rice	Wang and Oard (2003)
Os RUBQ2	Constitutive	Rice	Wang and Oard (2003)
Os Tub A1	Constitutive	Rice	Jeon et al. (2000)
Zm ubiquitin	Constitutive	Wheat, maize, rice, barley	Christensen et al. (1992); Cornejo et al. (1993); Anand et al. (2003); Breitler et al. (2004); Streatfield et al. (2004a); Roy-Barman et al. (2006); Guerrero-Andrade et al. (2006); Zhang et al. (2010); Yang et al. (2007a); Eskelin et al. (2009)
Os ubiquitin (rubi3)	Constitutive	Rice	Lu et al. (2008a); Lu et al. (2008b)
Monocotyledon tissue-specific			
Zm ABA-inducible	Embryo	Maize	Streatfield et al. (2010a)
Zm globulin-1	Embryo	Maize, rice	Belanger and Kriz (1989); Kriz (1989); Kriz et al. (1990); Claparols et al. (2004b); Lee et al. (2004); Chen et al. (2008b); Streatfield et al. (2010a)

Table 3.1 (continued)

Promoter	Expression (tissue)	Host species	Reference
Zm globulin-2	Embryo	Maize	Streatfield et al. 2010a)
Os globulins	Embryo	Rice	Qu and Takaiwa (2004); Furtado et al. (2008)
Zm novel	Embryo	Maize	Streatfield et al. (2010a)
Os 18 kDa oleosin	Embryo, aluerone	Rice	Qu and Takaiwa (2004)
Os glutelins	Endosperm	Rice, barley	Qu and Takaiwa (2004); Hennegan et al. (2005); Yang et al. (2006, 2007c); Furtado et al. (2008); Qu et al. (2008); Lee et al. (2008); Eskelin et al. (2009)
Ta HMW-glutenin 1Dx5	Endosperm	Wheat	Tosi et al. (2004); Brinch-Pedersen et al. (2006b); Cong et al. (2009); Harholt et al. (2010)
Ta HMW-glutenin Bx17	Endosperm	Wheat	Oszvald et al. (2008); Tamas et al. (2009)
Ta LMW glutenin	Endosperm	Maize	Naqvi et al. (2009)
Ta HMW glutenin	Endosperm	Wheat, barley, rice	Furtado et al. (2008)
Hv hordein B1	Endosperm	Wheat, barley, rice	Weichert et al. (2010); Furtado et al. (2008, 2009)
Hv hordein D	Endosperm	Maize, wheat, barley, rice	Naqvi et al. (2009); Furtado et al. (2008)
Os prolamins	Endosperm	Rice	Qu and Takaiwa (2004); Shin et al. (2006); Lee et al. (2008)
Os PRO223	Endosperm	Rice	de Wilde et al. (2008b)
Ta puroinoline b	Endosperm	Rice	Hennegan et al. (2005)
Zm gamma zein	Endosperm	Raize	Zhang et al. (2009)
Zm Super gamma zein	Endosperm	Maize	Aluru et al. (2008)
Ta GstA1	Epidermis	Wheat	Altpeter et al. (2005); Schweizer (2008)
Os Leaf panicle (LP2)	Leaves, photosynthetic tissue, light inducible, very low in seed	Rice	Thilmony et al. (2009)
Zm p1 R2R3-MYB	Silk, pericarp, cob, husk, tassels	Maize	Cocciolone et al. (2005)
Zm P19z storage protein	seed	Maize	Yu et al. (2005)

Table 3.1 (continued)

Promoter	Expression (tissue)	Host species	Reference
Zm Phosphoenolpyruvate carboxylase	Mesophyll-specific	Maize	Sattarzadeh et al. (2010)
Agro. Rhizogenes Rolc	Phloem-specific	Rice, tobacco, chickpea	Saha et al. (2007)
Os RSs1 sucrose synthase	Phloem-specific	Rice, tobacco, Chickpea	Saha et al. (2007)
Zm Rubisco small subunit 1	Bundle-sheath specific	Maize	Sattarzadeh et al. (2010)
Zm "Silk-specific promoter"	Silk	Maize	Johnson et al. (2007)
Os starch-branching enzyme	Scutellum	Rice	Qu and Takaiwa (2004)
Os ADP glucose pyro-phosphoylase small sububit/large subunit	Seed, phloem of vegetative tissues	Rice	Qu and Takaiwa (2004); Takaiwa et al. (2007)
Os pyruvate orthophos-phate dikinase	Seed, phloem of vegetative tissues	Rice	Qu and Takaiwa (2004)
Hv bifunctional alpha amylase/subtilisin inhibi-tor (Isa)	Pericarp in barley	Wheat, barley	Furtado et al. (2008)
Os Glutamate synthase	Scutellum	Rice	Qu and Takaiwa (2004)
Ta Tapetum-specific	Tapetum	Wheat	Kempe et al. (2009)
Monocotyledon inducible			
Ta stress-induced pro-moter complex AIPC	Stress induced	Wheat	Vendruscolo et al. (2007)
Hv alpha-amylase	Germination-specific	Barley	Rogers (1985); Khursheed and Rogers (1988); Eskelin et al. (2009)

Table 3.1 (continued)

Promoter	Expression (tissue)	Host species	Reference
Ta PRPI defensins	Various tissues, wound-inducible	Rice, wheat	Kovalchuk et al. (2010)
Os PRPI defensins	Various tissues, wound-inducible	Rice, wheat	(Kovalchuk et al. 2010)
Os Dip1	Drought-stress induced	Rice	Yi et al. (2010)
Sugarcane dirigent	Stem-preferred, stress-induced	Sugarcane, rice, maize, sorghum	Damaj et al. (2010)
Os LEA3–1	Drought inducible	Rice	Xiao et al. (2007)
Os Lea3	Drought-stress induced	Rice	Yi et al. (2010)
Sugarcane O-methyltransferase	Stem-preferred, Stress induced	Sugarcane, rice, maize, sorghum	Damaj et al. (2010)
Zm P(SEE1)	Enhanced on senescence	Maize	Robson et al. (2004)
Zm proteinase inhibitor (mpi)	Wound inducible	Maize, rice	Breitler et al. (2004); Vila et al. (2005)
Zm Rab17	Drought inducible	Wheat, barely	Morran et al. (2010a)
Os Rab21	Drought-stress induced	Rice	Yi et al. (2010)
At SAG12	Senescence induced	Wheat	Sykorova et al. (2008)
Os Uge1	Drought-stress induced	Rice	Yi et al. (2010)
Os Wsi18	Drought-stress induced	Rice	Yi et al. (2010)
Dicotyledon constitutive			
CaMV 35S	Constitutive	*Arabidopsis*, tobacco, *Brassica*	Odell et al. (1985)
Agrobacterium opine synthase	Constitutive	*Arabidopsis*, tobacco	Bevan et al. (1983)
At ACT2	Constitutive	*Arabidopsis*	An and Meagher (2010)
At actin	Constitutive	*Arabidopsis*	An et al. (1996)

Table 3.1 (continued)

Promoter	Expression (tissue)	Host species	Reference
At ubiquitin	Constitutive	Tobacco	Callis et al. (1990)
Nt Ne1F-4A	Constitutive	Tobacco	Mandel et al. (1995)
Nt tCUP	Constitutive	Tobacco	Foster et al. (1999); Wu et al. (2001); Malik et al. (2002)
Dicotyledon tissue-specific			
Pv arcelin	Seed specific	*Arabidopsis*, tobacco, bean	Goossens et al. (1999); Downing et al. (2006); Kermode et al. (2007)
Bn napin	Seed specific	*Brassica*, *Arabidopsis*, tobacco	Broun and Somerville (1997); Dehesh et al. (2001); Vigeolas et al. (2007)
Pv phaseolin	Seed specific	Tobacco, soybean	Bustos et al. (1989); Keeler et al. (1997); Naoumkina et al. (2008)
Dicotyledon inducible			
Ps Rubisco small subunit	Light inducible	Tobacco	Barna et al. (2008)
Parsley Chalcone synthase	Light inducible	Parsley	Weisshaar et al. (1991a, b)
At Rd29A	Stress inducible	*Arabidopsis*, tobacco	Kasuga et al. (1999)
XVE Txn factor system	Various promoters and tissue specificities	*Arabidopsis*	Brand et al. (2006)
Pep25 system	Pathogen induced	*Arabidopsis*, tobacco	Mazarei et al. (2008)
pOp	Activated with LhG4 Txn factor	Tobacco	Moore et al. (1998)

CaMV cauliflower mosaic virus, *Zm* Zea mays, *Os* Oryza stiva, *HMW* high molecular weight, *LMW* low molecular weight, *Hv* Hordeum vulgare, *Ta* Triticum aestivum, *ADP* adenosine diphosphoglucose

(Streatfield 2007; Egelkrout et al. 2012), and the intent of this chapter is not to repeat these general rules, but, instead, to focus on aspects specific to corn and to cite examples wherever possible.

Protein of Interest

A critical factor for accumulation of a protein in any host is the makeup of the protein itself. While this consideration holds true for the accumulation of proteins in any host, corn kernels have shown advantages for the expression of, otherwise, recalcitrant proteins. One general class of proteins known for poor expression are membrane proteins (Bernaudat et al. 2011). Membrane proteins are not only critical for cellular functions and cell recognition but are also of practical importance in some medically related products, such as subunit vaccines, and for structural analysis. Thus, they are a target for overexpression in many types of recombinant hosts (Mason et al. 2002; Bernaudat et al. 2011; Mus-Veteau 2010; D'Aoust et al. 2008; Ahmad et al. 2012).

While membrane proteins are not among the most highly expressed proteins in any system, they have accumulated much better in maize than when expressed in other recombinant hosts. An example is the hepatitis B surface protein, HBsAg, which has been commercialized as a subunit vaccine. HBsAg has been expressed in many recombinant systems including the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, in cell cultures infected with recombinant baculovirus, vaccinia virus, and adenovirus (Cregg et al. 1987; Takehara et al. 1988; Davis et al. 1985; Mason et al. 1992), and in several plant hosts (Mekala et al. 2008; Guan et al. 2010; Pniewski 2013). One goal that has been undertaken to combat hepatitis is the development of an effective oral vaccine with this antigen. This could have dramatic outcomes, but a rate-limiting aspect has been the ability to express the antigen at the high concentrations required in an edible tissue for the oral vaccine to be administered in a food product. There are orders of magnitude differences in expression levels obtained using the different plant systems with the highest levels being reported in corn kernels (Hayden et al. 2012). This demonstrates the host advantage that corn can bring compared to some other plant tissues. Furthermore, this level was accomplished in non-optimized maize germ tissue, leaving great potential for even higher levels in the future (see discussion on optimization of germplasm).

The example above is dramatic for the high-level expression of a membrane-bound protein, but it is still at relatively low levels compared to results obtained with less refractory proteins. By contrast, thermostable proteins, such as cellulase and xylanase, have been shown, in general, to accumulate well in many plant systems (Herbers et al. 1995; Hyunjong et al. 2006; Xue et al. 2003; Jensen et al. 1996b; Ziegler et al. 2000a; Austin-Phillips et al. 1999b; Ruggiero et al. 2000). This generalization holds true for maize, and, as an example, the thermostable cellulase E1, an endo-β1,4-glucanase, has been shown to accumulate at 0.13 % dry

Table 3.2 Examples of high-level protein accumulation in maize kernels

Type	Promoter	Gene	Protein level	Reference
Constitutive	*Zm* ubiquitin *Zm* ubiquitin	GUS Avidin	0.7% TSP 0.27% dry weight	Witcher et al. (1998) Masarik et al. (2003)
Endosperm	Os glutelin	Cel6A	30%TSP	Devaiah et al. (2012)
Embryo	Zm globulin Zm globulin	E1 CBHI	0.13% dry weight 0.4% dry weight	Hood et al. (2012)
Pericarp	ZM *azs22.12* *ZM* GS_{1-2}	GUS GUS		Egelkrout et al. (2013) Muhitch et al. (2002)

TSP total soluble protein

weight in grain (Hood et al. 2012), among the highest concentrations known to accumulate in any plant. Some representative examples of high levels of recombinant proteins are shown in Table 3.2. The values given represent expression based on the whole kernels. However, the tissue specificity of the promoters would imply that the embryo promoters provide a tenfold higher concentration of protein if this is based solely on the germ tissue. Protein levels in the relatively small amount of pericarp tissue in the kernel were not quantified. However high protein concentrations in the pericarp together with high expression levels could indicate significant accumulation.

These examples illustrate not only that the nature of the protein of interest is critical in determining the expectations for overproduction but also the potential for high levels of accumulation, and the reason that the maize kernel is rapidly becoming a host of choice to overexpress many proteins (Ramessar et al. 2008; Naqvi et al. 2011). It is difficult to predict the specific reasons why some proteins have shown greater accumulation in maize grain because there are few studies by which direct comparisons can be made. The most likely reasons for high protein accumulation include an abundance of protease inhibitors, ample chaperones to ensure correct folding, high carbohydrate concentrations to stabilize protein, the large size of the kernel, and low water content, all which have been discussed elsewhere (Streatfield 2007; Naqvi et al. 2011). From a pragmatic perspective, it is apparent that many proteins do express better in grain than in other systems. There are many specific strategies used to overexpress proteins, and the discussion below is focused on illustrating examples where specific strategies for maize grain have shown benefit. A partial listing of proteins produced in plants can be found in Khan et al. (2013; see Table 3.3).

Location, Location, Location

The real-estate mantra of *location, location, location* applies to accumulation of recombinant proteins in grain. With the aim to accumulate as much of the specific protein in the kernel as possible, the obvious choice is to obtain a promoter that would express in all tissues throughout the whole seed. If there is no reason to be

Table 3.3 Partial list of industrial proteins expressed in plants. (Modified with permission from Khan et al. 2013)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
α-Amylase	Starch degradation; food and beverages, biofuels, textiles and paper industries	*Bacillus licheniformis*	*Nicotiana tabacum* SR1	0.3 % total soluble protein (TSP) in leaf	Unaltered plant phenotype secreted into intercellular space; extra complex sugar chains added; degradation products identical to native protein	Pen et al. (1992)
α-Amylase		*Bacillus licheniformis*	*Nicotiana tabacum* SR1	0.4 % TSP in seed	Constitutive expression; hydrolysis products identical to purified *B. licheniformis* α-amylase	Pen et al. (1991) NEuropean Patent 0449376
Thermostable α-Amylase		*Bacillus licheniformis*	*Vicia norbonensis* L.	1 mU/mL seed supernatant	Seed-specific USP promoter; accumulation in cotyledon protein bodies; posttranslationally modified	Czihal et al. (1999)
α-Amylase OS103		*Oryza sativa* cDNA	*Nicotiana benthamiana*	5 % TSP in leaf	Viral infection causes mild chlorosis and stunting; moderate glycosylation of protein in plants	Kumagai et al. (2000)
α-Amylase		*Bacillus licheniformis*	*Medicago sativa* Regen-SY-27	0.01 % TSP in leaf	Unaltered phenotype	Austin et al. (1995)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Bifunctional thermostable Amylopullula-nase (APU)	Pullulan and amylose degradation; detergent industry	*Thermoan-aero-bacter ethanolicus39E* (ATCC53033)	*Oryza sativa* L. cv Tainung 67	5.7 % total soluble protein in seed	Unaltered phenotype; elevation in pullulanase correlates with decrease in amylose; amyloplast location; starch completely hydrolyzed upon heating	Chiang et al. (2005)
Aprotinin	Inhibitor of trypsin and proteases; medical and research uses	Optimized bovine aprotinin sequence	*Zea mays* Hi-II	0.4 % TSP in seed	Multiple copies in genome; ubiquitin promoter; protein accumulation in seed embryo; transgenic protein biochemically identical to native protein	Zhong et al. (1999)
Aprotinin cDNA fusion with extension signal		*Synthetic bovine*	*Nicotiana benthamiana*	7100 trypsin inhibitory units/mg extract protein	Transient TMV virion transfection; product biochemically similar to native protein; large-scale production on 1.5 acres open field or 2500 ft^2 greenhouse yields 1 kg purified enzyme	Pogue et al. (2010)
Arginine decarboxylase (adc) cDNA	Degradation of arginine; medical and research uses	*Datura stramonium*	*Oryza sativa* L.	2-fold increase in putrescine levels following stress removal	Under drought conditions, wildtype plants are severely affected, whereas transgenic plants have normal phenotype	Capell et al. (2004)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Avidin	Irreversibly binds biotin; research uses	Chicken egg white	*Zea mays* L.	2.3 % TSP in seed; 230 mg/kg seed	Partial-to-complete male sterility in high-expressing plants; similar to native glycoprotein; stable during storage for over 3 months	Hood et al. (1997)
Endochitinase (ech42) cDNA	Chitin degradation; research and agricultural uses	*Trichoderma atroviride*	*Medicago sativa* Regen-SY	Up to 2650-fold higher than control plants	Unaltered phenotype; high-expression levels do not correlate with higher resistance to pathogen challenge	Samac et al. (2004)
Endocellulase E1	Cellulose degradation; biofuels and paper industries	*Acidothermus cellulolyticus*	*Zea mays* L.	Higher levels in ER than mitochondria; max 2.0 % total soluble protein	Targeted to ER and mitochondria; ER targeted E1-cellulase called "Spartan Corn 1"	Mei et al. (2009)
Endo-1,4-β-D-glucanase (E1 cellulase)	Cellulose degradation; biofuels and paper industries	*Acidothermus cellulolyticus*	*Zea mays* Hi-II	6.1 % (ER) and 5.6 % (vacuole) TSP in seed	No apparent effect on growth; truncated catalytic domain accumulates in ER and vacuole; 16 % TSP in single seed indicates high accumulation potential	Hood et al. (2007)
Endoglucanase E1		*Acidothermus cellulolyticus*	*Lemna minor* 8627	0.24 % TSP; 0.2 U/mg protein in fresh tissue	Unaltered phenotype	Sun et al. (2007)
Endoglucanase E1		*Acidothermus cellulolyticus*	*Nicotiana tabacum* L. cv. petit Havana SR1	0.25 % total soluble protein in leaf (apoplastic targeting)	Unaltered phenotype; stored seeds had 45 % more activity after 1 year	Dai et al. (2005)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
E1 endoglucanase		*Acidothermus cellulolyticus*	*Nicotiana tabacum* L. cv petit Havana SR1	1.35 % TSP in leaf	Chloroplast targeting; normal growth and development; activity decreases with leaf age and upon dehydration	Dai et al. (2000a)
Cellobiohydrolase I (CBHI)	Cellulose degradation; biofuels and paper industries	*Trichoderma reesei*	*Zea mays* Hi-II	3.2 % (cell wall) and 4.1 % (ER) TSP in seed	Holoenzyme in cell wall; single seed levels of 17.9 % indicate high accumulation potential	Hood et al. (2007)
Exo-cellobiohydrolase I (CBHI)	Cellulose degradation; biofuels and paper industry	*Trichoderma reesei*	*Nicotiana tabacum* L. cv petit Havana SR1	0.11 % TSP in leaf and 66.1 µmol/h/g total leaf protein activity; 0.082 % TSP in callus and 83.6 µmol h/g total callus protein activity	Unaltered phenotype	Dai et al. (1999)
Thermostable (1-3, 1-4) β-glucanase (codon adapted)		*Bacillus spp.*	*Hordeum vulgare* Golden Promise	40 ng enzyme/2 × 105 protoplasts for codon-modified constructs compared to none for unmodified constructs	Biolistic transformation; codon usage important for expression; unaltered phenotype; germination-induced expression of enzyme in grain	Jensen et al. (1996a)
β (1–3, 1–4)-glucanase		*Bacillus spp.*	*Hordeum vulgare* Golden Promise	1.29 g/mg TS; 5.4 % TSP in grain endosperm	Large variations in enzyme levels between transformants; levels stable for 3 years	Horvath et al. (2001)
Endo-1,4-β-glucanase (EGI cellulase)		*Trichoderma reesei egl1*	*Hordeum vulgare* Kymppi and Golden Promise	0.025 % TSP in seed	Plant morphology normal but reduced seed setting in transgenic plants	Nuutila et al. (1999)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Endoglucanase holoenzyme (E1) and catalytic domain (E1cd)		*Acidothermus cellulolyticus*	*Nicotiana tabacum* L.	1.6 % TSP in leaf	Unaltered phenotype; apoplast targeting of catalytic domain achieves 500-fold greater expression than cytosolic full length E1	Ziegelhoffer et al. (2001)
1,4-β-D-endoglucanase (E1)		*Acidothermus cellulolyticus*	*Solanum tuberosum* L.	2.6 % TSP in leaf	Unaltered phenotype; dual crop applications: Leaf targeting allows tubers to be used for culinary applications	Dai et al. (2000b)
Thermostable endo-1,4-β-D-glucanase		*Acidothermus cellulolyticus*	*Nicotiana tabacum* Wisconsin 38	Not quantified	Targeting to chloroplast in vitro and in vivo	Jin et al. (2003)
Modified endoglucanase cellulase (egl)		*Ruminococcus albus*	BY-2 tobacco suspension cells	0.1 % TSP; 30-fold greater truncated form activity than endogenous cellulase	Unaltered phenotype; three forms (preform; mature form; truncated form); truncated form has the highest expression	Kawazu et al. (1996)
Hybrid (1,4)-β-glucanase (cel-hyb1)		*Neocallimastix patriciarum*	*Hordeum vulgare* cv Golden Promise	1.5 % total grain protein	Endosperm targeted; codon optimization leads to 527-fold increase in expression levels; stable during post-harvest storage	Xue et al. (2003)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Catalytic domain endo 1,4-β-D-glucanase (E1cd)		*Acidothermus cellulolyticus*	*Zea mays* Hi-II	2.1 % TSP in leaf and 0.845 nmol/μg/min activity; 2.08 % TSP in root and 0.835 nmol/μg/min activity	Set seeds at maturity	Biswas et al. (2006)
Catalytic domain 1,4-β-endoglucanase E1		*Acidothermus cellulolyticus*	Zea mays Hi-II	1.13 % TSP	Unaltered phenotype; apoplast targeted; successful conversion of corn stover into glucose following AFEX pretreatment	Ransom et al. (2007c)
Thermostable catalytic domain endo-1,4-β-glucanase		*Acidothermus cellulolyticus*	Oryza sativa L. Japonica cv. Taipei 309	4.9 % TSP	Unaltered phenotype; constitutive promoter; capable of hydrolyzing AFEX-treated stover	(Oraby et al. 2007)
Truncated endoglucanase (t-egl)		*Ruminococcus albus*	Tobacco BY-2 suspension cells	0.5 % TSP	Unaltered phenotype; cell disruption allows cell-wall digestion to occur	Kawazu et al. (1999)
Thermostable 1,4-β-D-endoglucanase catalytic domain		*Acidothermus cellulolyticus*	*Arabidopsis thaliana* *L*. Heynh. Columbia	26 % TSP in leaf	No abnormal phenotype; apoplast targeting; activity and immunochemically similar to native enzyme	Ziegler et al. (2000b)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Thermo-stable 1,4-β-D-Endoglucanase E2 and E3		*Thermomonospora fusca*	*Medicago sativa* L. *Nicotiana tabacum L.* Solanum.tuberosum L.	E2-0.1 % TSP E3 0.02 % TSP	Unaltered phenotype	Ziegelhoffer et al. (1999)
Thermostable cellulases (Cel6A, Cel6B)		*Thermobifida fusca*	*Nicotiana tabacum* L. Samsun and 22X-1	4 % TSP	Homoplasmic, transplastomic plants using plastid-directed vector; not optimized	Yu et al. (2007)
Recombinant hyper-thermostable endo-glucanase Cel5A		GenBank accession number At3g4890	*Nicotiana tabacum* cv. Petite Havana	5.2 % TSP in leaf	Unaltered phenotype; chloroplast targeted; stable active enzymes	Kim et al. (2009)
Chimeric chymosin (rennin)	Milk curd formation; dairy industry	Bovine	*Brassica napus*	0.5 % (w/w) TSP	Seed targeted	Rooijen et al. (2008) US Patent 7,390,936
Coumarate-3-hydroxylase (C3H)	Lignin modification; biofuels	*Medicago sativa*	*Medicago sativa* cv. Regen SY	C3H levels 5 % of wild type levels	No serious phenotypic impairment	Ralph et al. (2006)
ADP-glucose pyrophosphorylase modified (Sh2r6hs)	Starch synthesis; agricultural and research uses	*Zea mays*	*Triticum aestivum* Hi-Line	5-fold greater protein accumulation	Modified large subunits permit greater stability and yield; endosperm-specific promoter	Meyer et al. (2004)
ADP-glucose pyrophosphorylase modified large subunit (Shrunken 2 gene Sh2r6hs)		*Zea mays L.*	*Triticum aestivum* L.	91 % more activity in the presence of 10 mM Pi	Transgenic wheat plants produced 38 % more seed weight; 31 % higher biomass; transgene stable after five generations	Smidansky et al. (2002)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Human placental β-glucosidase (GCase)	Degradation of glycosidic bonds; biofuels and research uses	Human placenta	Nicotiana tabacum L., cv. Xanthi	750 U/kg seed	Seed viability totally impaired above 500 U/kg; taken up by human fibroblasts; free from immunogenic xylose and fucose	Reggi et al. (2005)
β-glucuronidase (GUS) with αAmy8 regulatory and signal sequence		αAmy8 sequences from rice	*Nicotiana tabacum* L. cv. petit Havana SR1; *Oryza sativa* L. cv. Tainung 62: *Oryza sativa* L.cv. Tainan 5; *Solanum tuberosum* L. cv. ADH69	40 % total secreted proteins	Fusion to β-glucuronidase (GUS); inducible by sugar; tunicamycin causes ER accumulation	Chan et al. (1994)
Laccase I	Lignin degradation; biofuels, wood and paper industry	*Trametes versicolor*	*Zea mays* L.	0.55 % TSP 4 ng/mg dry weight (T2) to 70 ng/mg dry weight (T6)	Variable expression levels; breeding and selection increased levels 20-fold in five generations; embryo-preferred promoter with cell-wall targeting supports highest expression; germplasm background affects germination frequency	Hood et al. (2003)
Laccase		*Trametes versicolor*	*Zea mays* L.	0.20 % of dried, defatted corn germ	Contains both water soluble and immobilized laccase; some laccase is inactive apoenzyme form	Bailey et al. (2004)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Laccase		*Melanocarpus albomyces* *Pycanoporus cinnabarinus*	*Oryza sativa* Anjungbyeo	13 ppm (riceMaL) 39 ppm (ricePycL)	Endosperm targeted; seed production was normal; recombinant protein is biochemically similar to native proteins, but had lower kinetic parameters	de Wilde et al. (2008a)
Lipase	Lipid breakdown; dairy, food, biofuels, and detergent uses	Recombinant dog gastric lipase	*Nicotiana tabacum* cv. PBD6 and cv. Xanthi	5 % (vacuolar retention signal) and 7 % (secretion signal) of acid extractable protein	Active glycosylated protein with similar properties to native protein; specific activity dependent on subcellular compartment; Normal leaf morphology	Gruber et al. (2001)
Lipase		Dog gastric lipase	Tobacco (species not specified)	360 U/mg protein	Impact of subcellular targeting on glycosylation; transient expression system	Mokrzycki-Issartel et al. (2003)
Manganese-dependent lignin peroxidase (MnP)	Lignin degradation; biofuels, wood and paper industry	*Phaenerochaete chrysosporium*	*Medicago sativa* L.	0.5 % TSP in leaf	Reduction in dry matter and height related to expression levels; yellow foliage; MnP expression segregates in sexual progeny	Austin et al. (1995)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Manganese peroxidase (MnP)	Lignin degradation; biofuels, wood and paper industry	*Phanerochaete chrysosporium*	*Zea mays* (not specified)	15 % TSP in seed 3 % TSP in leaf	Cell-wall targeting yields full-length MnP; cytoplasmic targeting produces truncated products; seed-targeted promoter has higher expression levels and improved plant health outcomes over constitutive promoter	Clough et al. (2006)
Anionic peroxidase cDNA	Lignin structural modification; biofuels, wood and paper industry	*Nicotiana tabacum* L.cDNA of isozyme	*Nicotiana sylvestris*; *Nicotiana tabacum* var Xanthi nc	> 10X higher peroxidase activity compared to wildtype	CMV35S promoter; chronic severe wilting through loss of turgor in leaves initiated at the time of flowering	Lagrimini et al. (1990)
Phytase phyA2	Phytic acid breakdown; animal feed uses	*Aspergillus niger*	*Zea mays* Hi-II	2200 U/kg of seed	Embryo-specific globulin-1 promoter; different glycosylation pattern; stable over four generations; normal transgenic seed germination	Chen et al. (2008a)
Phytase		*Schwanniomyces occidentalis*	*Oryza sativa* (not specified)	4.6–10.6 U/g fresh weight in leaves	Stable in silage for 12 weeks	Hamada et al. (2006)
Rationally designed phytase		*Aspergillus fumigatus*	*Triticum aestivum* L.	4777 FTU/kg seed flour	Vacuole accumulation despite apoplast targeting; unaltered phenotype	Brinch-Pedersen et al. (2006a)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Secretory phytase (PHY)		Synthetic gene	*Solanum tuberosum* L. cv. Désirée	40 % more phosphate in transgenic plants	Trichoblast-specific promoter; healthy plants, but with altered leaf shape	Zimmermann et al. (2003)
Chimeric phytase ex::phyA		*Aspergillus niger*	*Nicotiana tabacum* W38	3.7-fold more phytase secretion and 52 % higher P accumulation in transgenic plants	Presence of soil phytate essential	George et al. (2005)
Phytase *phy*A		*Aspergillus niger*	*Triticum aestivum* L	4-fold increase in plants with constructs with a-amylase signal peptide; 56 % increase in plants with constructs without signal peptide; Phytase activity 3000 FTU/kg	Endosperm, but not embryo accumulation; gene stability over three generations	Brinch-Pedersen et al. (2000)
Phytase gene		*Aspergillus fumigatus*	*Japonica* rice var. Taipei 309	130-fold increase in grain phytase level	Unchanged phenotype; coexpressed in endosperm with *Phaseolus vulgaris* ferritin gene and overexpressed endogenous cysteine-rich metallothionein	Lucca et al. (2001)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Phytase MtPHY1		*Medicago truncatula*	*Arabidopsis thaliana* C58 and ecotype Columbia	12.3- to 16.2-fold higher levels in root apoplast	Dry weight of transgenic plant upto 4.0 times higher than control and P content up to 5.5-fold higher; root-specific and constitutive promoters used	Xiao et al. (2005)
Phytase GmPhy		*Glycine max* L. Merr.	Glycine max L. Merr. cv Williams 82 tissue culture	2- to 3-fold higher than controls	Novel phytase similar to purple acid phosphatases	Hegeman and Grabau (2001)
Phytase cDNA		*Aspergillus niger*	*Nicotiana tabacum* cv Petit Havana SR1	26 % dry weight of leaves 14.4 % TSP in leaf	Constitutive expression with secretion signal from tobacco pathogen-related protein S; differences in glycosylation compared with native protein	Verwoerd et al. (1995)
Phytase phyA		*Aspergillus niger*	*Brassica napus* cv. Jet Neuf	600 U/g of multi-copy T1seed; 103 U/g in single copy line	Unchanged morphology; seed-specific CruA promoter used; gene-dosage related expression; stable over three generations; no correlation between high expression and seed germination	Ponstein et al. (2002)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Phytase phyA		*Aspergillus niger*	*Glycine max* L. Merr. cv. Williams 82	920 pKat M/g total soluble protein	Secretion and glycosylation may be necessary for activity; transgenic protein smaller than native protein, but has similar biochemical profile	Li et al. (1997)
Phytase SrPf6 (*S. ruminatum*) appA (*E. coli*)		*Selenomonas ruminantium Escherichia coli*	*Oryza sativa* L. cv. Tainung 67	0.6 U/mg (appA); 1.4 U/mg (SrPf6) of TSP in seed; up to 60 times activity of control	No adverse effects; germination-inducible Amy8 promoter and Amy8 signal peptide; multiple copy number	Hong et al. (2004)
Phytase cDNA		*Aspergillus niger*	*Medicago sativa* L.	2.0 % TSP	Un-glycosylated, but stable	Austin-Phillips et al. (1999a)
Transglutaminase (rTGp)	Formation of peptide bonds; food industry	Rat prostate	*Oryza sativa* var. EYI 105	0.15 U/mg h leaf	Ca^{2+}-dependent enzyme	(Claparols et al. 2004a)
Trypsin	Protein hydrolysis; medical and research uses	Bovine pancreas	*Zea mays L.*	0.025 % seed dry weight	Equivalent to native enzyme levels suitable for commercial production; produced as zymogen	Woodard et al. (2003) US Patent 6,087,558
Xylanase B *xyn*B	Hemicellulose degradation; biofuels, wood and paper industry	*Streptomyces olivaceoviridis*	*Solanum tuberosum L.*	5 % TSP in leaf	Stable gene expression for several generations	Yang et al. (2007b)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Xylanase A xynA thermostable catalytic domain xynA1		*Clostridum thermocellum*	*Oryza sativa* L. subsp. *Japonica cultivar Notohikari*	Not quantified	Normal plant phenotype; stable expression in seed and straw; activity in desiccated seed	Kimura et al. (2003)
Xylanase *xyn*C-oleosin fusion		*Neocallimastix patriciarum*	*Brassica napus* L.	2000 U/kg seed (oil body of seed)	Fusion protein retains optimal temperature, Km, and specificity, but has reduced pH sensitivity	Liu et al. (1997)
Xylanase Modified *xyn*C		*Neocallimastix patriciarum*	*Hordeum vulgare* L. cv. Golden promise	0.004 % dry weight of seed endosperm	GluB-1 promoter better than Hor2–4 promoter; protein stable during seed maturation, desiccation and storage; 40 % low fertility in one line	Patel et al. (2000)
Xylanase *xyn*II		*Trichoderma reesei*	*Arabidopsis thaliana* ecotype Columbia	1.2 % (cytosol); 3.0 % (chloroplast); 1.7 % (peroxisome); 4.8 % (chloroplast + peroxisome) total soluble protein	Unaltered phenotype; levels highest at flowering; dual targeting to chloroplasts and peroxisomes causes much higher levels than either compartment alone, although RNA levels are similar	Hyunjong et al. (2006)
Xylanase xynII		*Trichoderma reesei*	*Arabidopsis thaliana* ecotype Columbia	3.2 % TSP in leaf	Chloroplast expression exhibit normal growth, but cytosolic accumulation affected transgenic plant growth	Bae et al. (2008)

Table 3.3 (continued)

Enzyme (gene)	Enzyme function; application in industry	Gene source	Host plant	Maximum expression level	Comments	Reference
Xylanase Thermostable, truncated *xynZ*		*Clostridum thermocellum*	*Nicotiana tabacum* L. cv. Samsun NN	4.1 % TSP in leaf	Unaltered phenotype; proteinase II signal peptide used; enzyme enrichment following heat treatment	Herbers et al. (1995)
Xylanase Thermostable, truncated xyn2		*Clostridum thermocellum*	*Nicotiana tabacum* L. cv. Wisconsin	Not quantified	Clearance zone develops at 3 h	Komarnytsky et al. (2000)
Xylanase (XYLD-A) and β (1–3, 1–4) glucanase (XYLD-C)		*Ruminococcus flavefaciens*	*Nicotiana tabacum* L. cv. Samsun NN	170 μM/min/m2 xylanase and 2000 μM/min/m2 glucanase in leaves	Unaltered phenotype; separate constructs; apoplast targeting; glucanase-accumulated higher-protein levels than xylanase	Herbers et al. (1996)

ER endoplasmic reticulum, *USP* ultra stable promoter, *AFEX* ammonia fiber expansion, *TMV* tobacco mosaic virus

concerned about toxicity in other plant tissues due to high expression (see discussion on protein toxicity, below), a strong constitutive promoter that expresses in all parts of the plant should work well. This strategy has been shown to be extremely efficient for the protein avidin when using the constitutive ubiquitin promoter, leading to some of the highest levels of expression reported in the kernel (Hood et al. 1997). Not all constitutive promoters are alike. Both CaMV and ubiquitin (Christensen and Quail 1996) promoters drive high expression in leaves, but very low levels of protein accumulate in the seed with the CaMV promoter (Stoger et al. 2005), while high levels were demonstrated in seed with the ubiquitin promoter (Witcher et al. 1998).

Accumulation in the kernel may be desired, but overexpression in other tissues may be detrimental to the plant (see discussion on protein toxicity, below). Most enzymes will alter significantly the metabolism of the cell when overexpressed. Therefore, it can be greatly advantageous, and in some cases essential, to have high expression in the kernel with little or no expression in other parts of the plant. Regarding the kernel, the endosperm accounts for the vast majority of the biomass, with the embryo (~10%) and the pericarp or seed coat (~5%) making up the remainder. In theory, a promoter is possible that could drive expression specific to the kernel in all three of these tissues, but there have been no natural promoters identified to date with this feature, nor have synthetic promoters been created. This may be possible in the future, but presently reliance must be on promoters that drive expression preferentially in one of these tissues.

At first glance, it would seem that the endosperm would be the best tissue for protein accumulation since it has the most biomass to store the protein. Strong endosperm-preferred promoters have been used and do show great utility (Schernthaner et al. 1988; Russell and Fromm 1997; Streatfield et al. 2004b). Interestingly, however, when the constitutive ubiquitin promoter was used, the majority of the recombinant protein accumulated in the embryo rather than the endosperm (Hood et al. 1997; Witcher et al. 1998; Zhong et al. 1999). One could argue that this is a specific feature of the ubiquitin promoter and would not hold true when strong endosperm promoters are compared to strong embryo promoters. However, the greatest accumulation of recombinant proteins in the seed, to date, has been achieved using embryo-preferred promoters (Stoger et al. 2005; Streatfield et al. 2010b; Egelkrout et al. 2012; Hood et al. 2012).

Promoters are not only responsible for tissue specificity; they are one of the most important factors driving the level of expression. A partial list of some maize promoters, along with other components that modulate expression, such as codon usage, terminator, and leader sequences, has been presented (Egelkrout et al. 2012; see Table 3.1). One aspect that modulated the levels of protein expression, which is favored in monocotyledons compared to dicotyledons, is intron-mediated enhancement (IME). This phenomenon was first discovered in cultured maize cells (Callis et al. 1987). The first intron in many plant genes has been shown to increase accumulation up to tenfold through posttranscriptional mechanisms (Rose 2008). The enhancing effect of introns in plants was identified initially in *Arabidopsis*, but studies have shown that the first intron is the only one that shows this effect,

and that no specific sequence appears to be responsible. Other researchers have found that certain introns function in monocotyledons, but not dicotyledons (Morita et al. 2012), although all introns that show the effect have the conserved motif "GATCTG." The use of introns to provide an IME needs to be tested empirically.

Intracellular Targeting

Proteins within each tissue can be targeted to specific subcellular locations using well-characterized targeting sequences (Kermode 1996; Lau and Dale 2009). Chloroplasts in the leaves of plants have shown great potential for protein accumulation (Chebolu and Daniell 2009; De Marchis et al. 2012), but there are no functional chloroplasts in the kernel. While the cytoplasm would appear to have the advantage of a large volume for protein accumulation, this site has only provided modest expression levels at best (Hood et al. 2003). The most consistent intracellular targets for high-level expression in the seed have been the cell wall, vacuole, and endoplasmic reticulum. This was illustrated initially with laccase (Hood et al. 2003) and confirmed with several other proteins (Woodard et al. 2003; Clough et al. 2006; Hood et al. 2007). Each of these sites also permits glycosylation, which can be essential for correct folding and biological activity (Gomord et al. 2010; Solá and Griebenow 2010), or used to reduce clearance rates in pharmaceutical proteins (Doran 2000; Solá and Griebenow 2010).

However, in rare cases, such as when a protein of bacterial origin has an inadvertent glycosylation site in a particularly strategic position like the catalytic site, glycosylation can cause inactivation of the protein. The popular marker protein, GUS, beta-glucuronidase is inactivated by glycosylation (Iturriaga et al. 1989; Farrell and Beachy 1990), thereby limiting the native protein's use as marker, when targeted to intracellular sites that glycosylate the protein. Thus, proteins targeted for expression should be scanned for potential sites of glycosylation.

Protein Toxicity

Many proteins possess biological activity that can interfere with metabolic processes in the host cell. This turns out to be one of the major limitations for high accumulation of many recombinant enzymes in foreign hosts. Even proteins that are not considered detrimental to metabolism can interfere when they accumulate at high concentrations. Some of the more obvious examples of proteins that can interfere with metabolism include proteases, glycosidases, phosphatases, and redox enzymes. Strategies to overexpress these proteins without causing toxicity have led to several options to sequester the activity of the protein and prevent it from interfering with the plant's metabolism.

Avidin is a protein that binds tightly to biotin, an important vitamin and enzyme cofactor, and an example of a protein that can cause toxicity by depleting biotin

when accumulated at high concentrations in foreign host tissue. However, when sequestered in the apoplast, it can accumulate to concentrations with few complications (Hood et al. 1997). At very high concentrations, however, it causes male sterility, so even this sequestration is not sufficient when a constitutive promoter is used. Another example of enzyme toxicity is illustrated by the protein laccase. In this case, free radicals are formed that, presumably, are detrimental when the enzyme is present at high concentrations. Protein accumulation was increased greatly by targeting the enzyme to the embryo, whose high oil and low water content retards radical formation (Galuszka et al. 2005; Riva 2006). Although embryo expression showed great promise, higher concentrations of laccase in seeds were inhibitory to germination. High-oil germplasm was used to overcome this damaging activity, with improved germination rates from 40 to 75%. Furthermore, this germplasm also provided an increase in accumulation due to the increase in the ratio of the germ size to the kernel (Hood et al. 2003; Hood et al. 2007).

Manganese peroxidase (MnP) is another example of an enzyme whose expression at high levels had a detrimental effect on the health of the plant. In particular, leaves and stems showed browning and compromised growth (Austin et al. 1995; Clough et al. 2005). Cofactor availability can be modulated in such cases to allow the expression of proteins that potentially interfere with cell metabolism, while limiting their activity (Hofrichter 2002). MnP was successfully accumulated in maize kernels by restricting expression to the seed. When the protein was subsequently extracted, there was only a low level of activity in the extract. However, when the cofactor, Mn, was added exogenously, protein activity was greatly increased, indicating that cofactor was required for optimal activity and was limiting in the plant (Clough et al. 2005). A similar situation was found to be the case for organophosphate hydrolase, which requires cobalt as its cofactor (Pinkerton 2004).

An alternate technology to accumulate enzymes that interfere with metabolism is to express the zymogen form of the enzyme that would be inactive in the plant but could be activated at a later time. Trypsin is an example of a protease that is very difficult to express at high levels in recombinant hosts because of its broad specificity to cleave proteins. However, expression was accomplished in maize kernels by expressing the zymogen (Woodard et al. 2003; Király et al. 2006). In addition to expressing the proenzyme trypsinogen, rather than the active enzyme, the protein was also targeted to the kernel where there is an abundant supply of protease inhibitors (Woodard et al. 2003). The combination of these strategies was needed to reach high levels. Other approaches to expressing zymogens may include intein technology which would allow for an inactive enzyme to accumulate in the plant tissue. Then, under the appropriate conditions, it would self-cleave to release the active protein (Raab 2010).

One tactic to limit toxicity in the plant is to use heat-activated enzymes. Many thermostable proteins only have activity at high temperatures not experienced during normal plant development. An example is a thermophilic cellulase, which would degrade the cell wall if it were active in the cell. At ambient temperatures, however, it is innocuous, and the enzyme can accumulate without any apparent effect on the plant (Ransom et al. 2007a; Biswas et al. 2006; Hood and Woodard 2002).

Another potential strategy to express a toxic protein is to place the gene under the control of a chemically induced promoter, and to initiate expression shortly before harvest to moderate adverse effects on the host plant (Corrado and Karali 2009). Promoters have been used that are induced by physiological stress (Yi et al. 2010), or pathogen infection (Rana et al. 2012). This strategy was explored for enzymes such as cellulase (Lebel et al. 2005). While this method has considerable potential, this has only provided moderate levels of enzyme accumulation. Future efforts may require the use of a synthetic promoter that fuses high-expression promoters with inducible promoters.

Gene Silencing

A major concern limiting gene expression in plants has been the phenomenon known as gene silencing (Meister and Tuschl 2004; Moazed 2009; Huntzinger and Izaurralde 2011). This has not been a major problem in the case of seed-specific expression in maize. A lack of gene silencing effects may be due, in part, to the fact that the DNA sequence is known to play a large role, and the majority of gene-silencing events utilize the viral promoter, CaMV, which may be particularly prone to silencing. As noted earlier, seed-specific and endogenous promoters are used for high accumulation, which may alleviate much of the gene-silencing effects.

Multiple copies of the same gene can be introduced by the biolistic process and can also jumble sequences when inserted. This was the case for aprotinin when expressed using a constitutive promoter. In some of these cases, variable levels of expression from the multiple copy inserts also indicated that gene silencing was occurring (Zhong et al. 1999). Increased protein accumulation was usually observed when multiple copies were inserted in a more precise manner using *Agrobacterium*-mediated transformation. However, in one case, using a gene for cellulase, there was evidence for lower expression when four identical copies of the gene and promoter were used, possibly due to recombination in the host (Egelkrout et al. 2013). Thus, copy number effects can be unpredictable and must be determined empirically.

Protein Stability

The ability to accumulate protein in a tissue is not only related to its expression but also to its degradation. The environment of the protein can be critical for this, and is presumably one of the main reasons different intracellular compartments can accumulate different amounts of the same protein. In the context of protein stability, it is pertinent to discuss posttranslational modifications. This begins with the presence of molecular chaperones and disulfide isomerase in maize seed to help fold the protein appropriately, since proteins that are inappropriately folded, or modified, may be targeted preferentially for degradation. Low proteolytic activity and desiccation of the seed also protects proteins from degradation (Naqvi et al. 2011).

Proteolytic activity can be further minimized by removing known protease sites, or using plants expressing cathepsin D protease inhibitor. Protease inhibitors may serve a dual purpose by inhibiting the digestive proteases of insects that consume the seeds, as well as inhibiting endogenous proteases in the seed (Goulet et al. 2010; Schlüter et al. 2010).

Whole-Plant Genetic Strategies to Maximize Protein Concentrations in Seeds

Breeding and Selection

When molecular strategies for optimal protein expression in maize seed are satisfied, genetic means are then employed for increasing target protein accumulation. The transformation of foreign genes is normally not site specific in plant chromosomes, and, therefore, multiple high-expressing T1 lines from several independent events are usually screened to ensure recovery of high grain-yielding lines with high expression. One of the most interesting phenomena observed in the past several years is the ability to increase heterologous protein accumulation in grain through breeding and selection from plants derived from an initial transformation event. It is unclear what exact mechanisms are responsible or how applicable this is to other species, but, doubtless, it is a major strategy for increasing heterologous protein accumulation in maize seed.

When genes are transformed into corn, first-generation plants with the best recombinant protein levels are chosen for further breeding. Figure 3.1 illustrates the breeding scheme. As shown in Fig. 3.1a, 10–15 plants from the T1 generation representing several independent transgenic events from each transformation vector are propagated in the T2 generation. These plants are chosen because some of the seeds analyzed showed high expression (Fig. 3.1b). For example, plants CDN0201 and CDN0202 are better choices than CDN0303 and CDN0304 because each has seeds with really high expression levels, whereas CDN03 plants have much lower expression in their top seeds. Each T1 ear produces 20–50 seeds, in general. It was determined statistically that analyzing six individuals of that group of seeds would be representative of the range and variation of all seed from each plant. Thus, the remaining seed from each of these analyzed plants will reflect the same range and variation in expression as the six individuals analyzed. The "low-expressing" individuals in Fig. 3.1b (less than 2% total soluble protein; TSP) represent background noise of null segregants. If single insertions are recovered, only one copy of the transgene is found on one chromosome without a duplicate on the paired chromosome. Therefore, when pollinated with a wildtype inbred plant, only half of the progeny will express the transgene. Thus, because T1 seeds segregate 1:1 for the transgene, when these seeds are planted, they must be screened for nulls so that only transgenic plants are propagated. Selection is accomplished by spraying plants

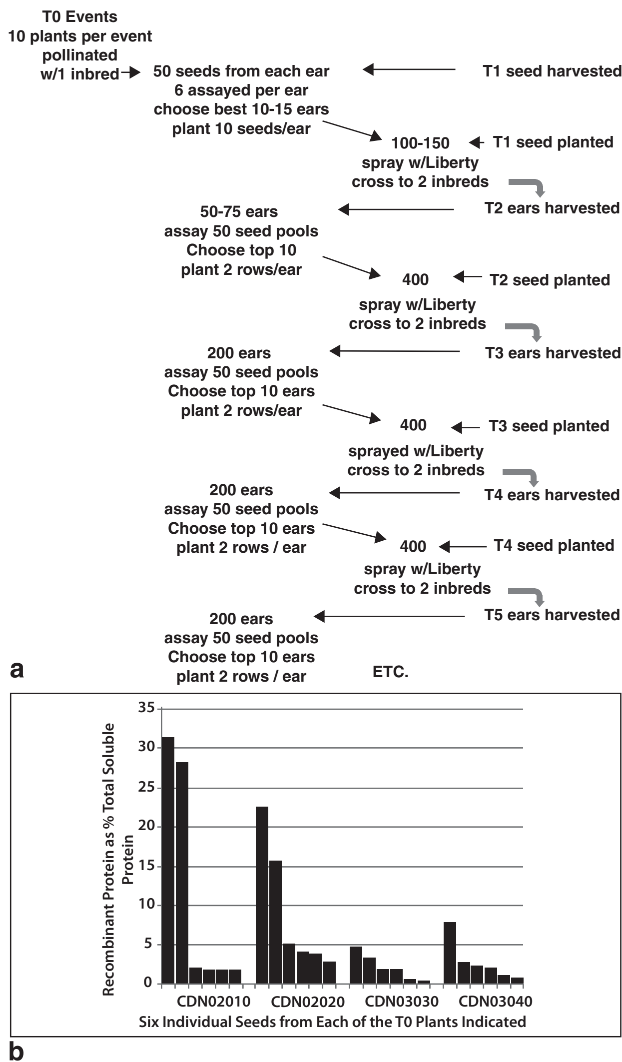

Fig. 3.1 Breeding scheme for selecting for higher target protein accumulation from first-generation independent transgenic events in corn. **a**. First-generation plants (T0) are regenerated from tissue culture. Each of the ten plants from each independent transgenic event is pollinated with an elite inbred in the glasshouse and seeds are collected. An average of 50 seeds per ear is recovered. Six individual seeds are analyzed singly for protein concentrations. The highest-expressing ears (10–15) are chosen from each vector, representing several events, and planted for continuing in the backcross program. T1 seed are planted and young plants screened for the transgenic trait by resistance to the herbicide, Liberty®. Half of the plants should be resistant. Some T1 plants are pollinated with the original inbred, and equal numbers are pollinated with an inbred that is compatible to produce a hybrid. This process is continued for six generations until sufficient elite germplasm is present in the transgenic line. **b**. Variability is observed in the single-seed analyses of T1 seed. Averages of seeds from T1 lines would mask the potential of the high-expressing lines. CDN02010 and CDN02020 are plants from a single event. CDN03030 and CDN03040 are plants from a second event. Values below 2 % TSP in these lines indicate background activity in the assay and are not transgenic. *TSP* total soluble protein

with the herbicide, Liberty®, to which the transgenic plants are resistant. Transgenic plants remain green, while null segregants show extensive leaf damage or death. It is important in the early breeding generations to have more than one event represented because insertions can affect agronomic performance, including yield, in subsequent hybrids. When surviving plants are pollinated with either of two inbreds, they produce T2 generation seed. The two inbreds are the complementary parents of a high-producing hybrid, and both inbreds must carry the transgene for maximum protein production in grain.

Each T2 ear recovered is analyzed individually using a random selection of 50 seeds. Each generation of plants produces ears with variable protein accumulation levels that cover a broad range of values (see Fig. 3.2). Although the amount of protein recovered per ear covers a broad range of values (Fig. 3.2a), the highest values in each generation increase (Fig. 3.2b; Hood et al. 2012). Additional seed from these highest-expressing ears is replanted the following season, screened for herbicide resistance, and crossed again to the elite inbred for the backcross program.

By the fourth or fifth generation, the breeding program selects one or two events for production. From the protein expression levels illustrated in Fig. 3.2, the top eight to ten ears would be chosen for replanting. Choices are also based on yield and field performance of the plants. Unfortunately, yield cannot be predicted before the hybrid lines are generated from the inbreds and grown for grain production as illustrated in Fig. 3.3. Thus, it is useful to have more than one event or line in the breeding program, even at this late stage of development. Six generations of inbred germplasm are generally used to move the transgenic event into elite lines. After the backcrossing is finished, the transgenic lines are self-pollinated twice to generate inbred lines that are homozygous for the transgenic trait.

Some observations that are encountered in the breeding process are segregation of the Hi-II parental germplasm, the high variability of expression in each ear, and a decrease in expression levels from T1 to T2 generations. Thus, the highest-expressing seeds should be carefully selected for breeding in the T1 and T2 generations. The cellobiohydrolase I (an exocellulase) and E1 (an endocellulase) in Table 3.2 are examples that illustrate the result of moving from generation T1, first-generation seed from the tissue culture-derived plants, to generation T2. T1 seed is analyzed singly, using six randomly chosen seeds from each recovered ear. As was seen in this example, tremendous seed-to-seed variability is always observed in the first generation, presumably because of the hybrid transformation host Hi-II. Hi-II is a cross between A and B parents (Armstrong et al. 1991) that segregates in the ovules of first generation reproduction. This segregating variability is compounded by pollination of the Hi-II ovules with an elite inbred to begin the movement of the transgene into production germplasm. The best T1 seed expression recovered from all T1 seed analyzed is illustrated in Table 3.4. However, T2 lines, in contrast to T1 lines, are screened using 50 seed pools from each ear, meaning that each sample comprises equal numbers of transgenic and null seeds, and that variably expressing seeds are mixed in this population. Thus, often in T2, the recovered expression value drops below the first-generation average seed values. Nevertheless, this result shows that improved protein accumulation is occurring because the average expression includes null seeds. Choosing the

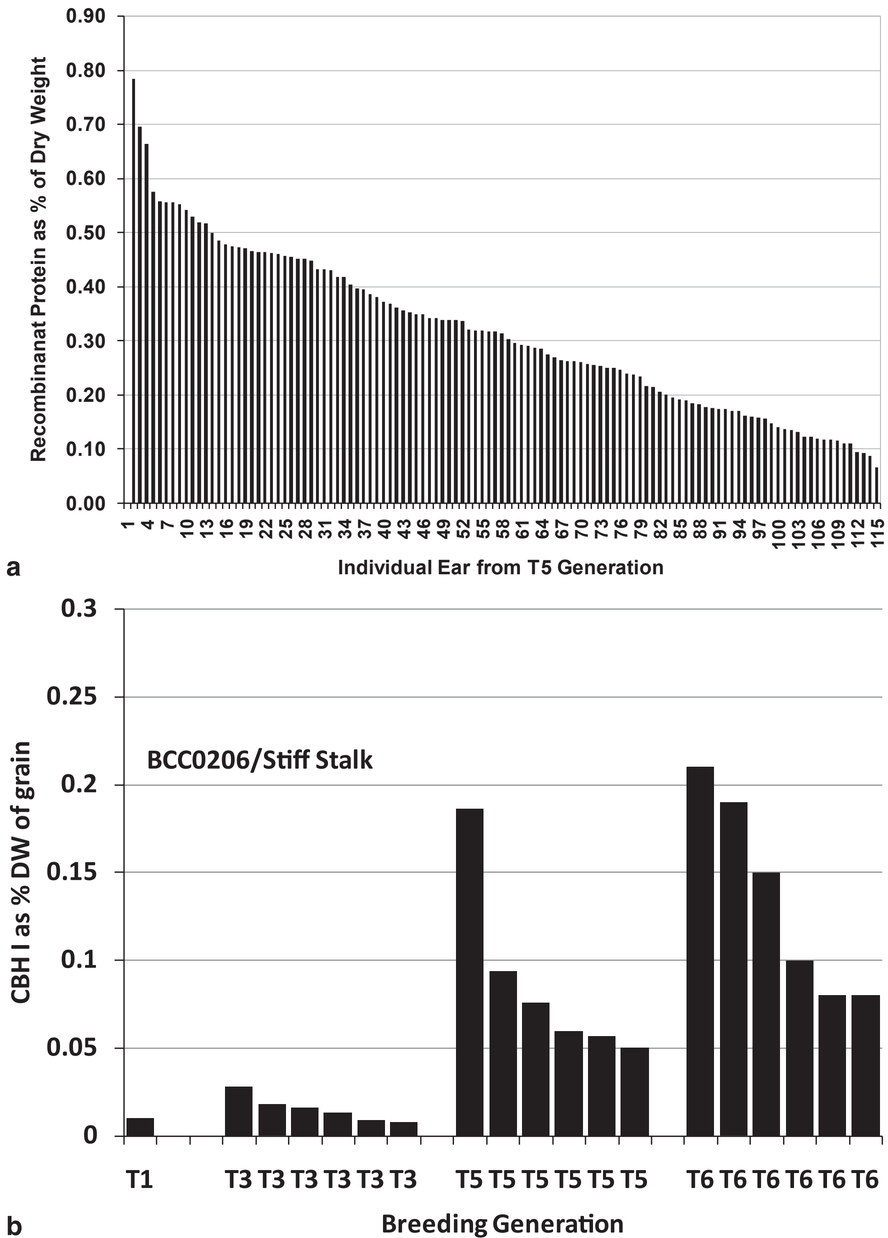

Fig. 3.2 **a** Range of values of recombinant protein accumulation in individual ears from a single backcross generation. Each bar is the value for a single 50-seed pool from an individual ear. The variation from 0.08–0.8 is tenfold. All ears are derived from a single transgenic event. **b** By planting only the highest-expressing ears from each generation, significantly higher expression levels can be achieved in subsequent generations, reaching equilibrium by generation T6

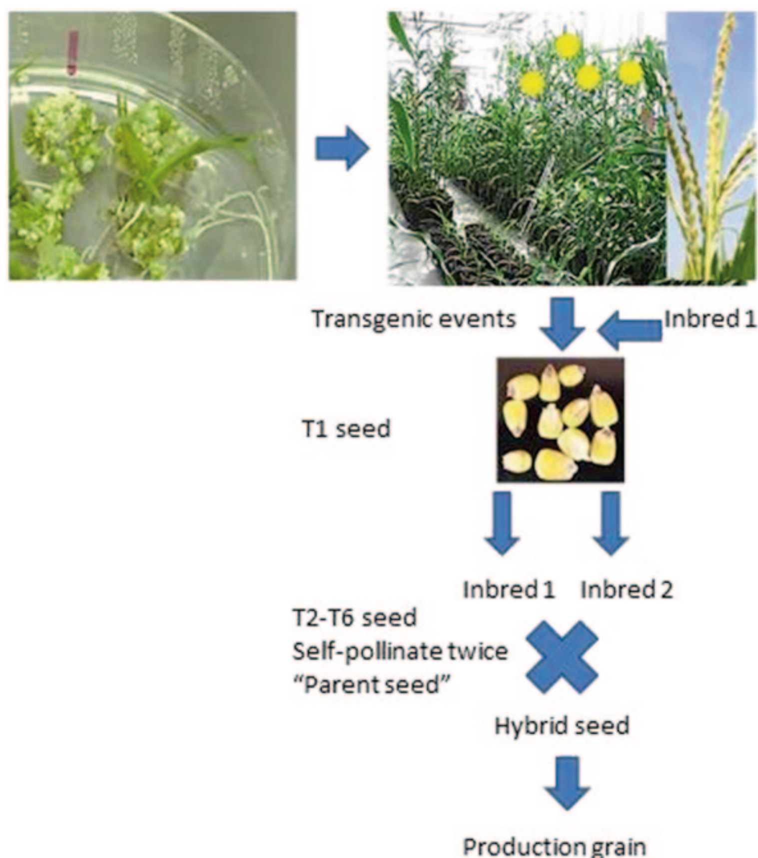

Fig. 3.3 The breeding program is important to recover high-yielding plants for production in the field. Tissue culture-derived plants are grown in the glasshouse and pollinated separately with an inbred. In subsequent generations, the plants are pollinated with two inbreds to generate homozygous parent seed. The compatible parent inbreds are crossed to generate hybrid seed that then can be planted to produce grain for protein production

Table 3.4 E1 and CBHI transgenic events and level of enzyme accumulation in the average of all positive T1 seeds, and highest T2 ear from each event. Six seeds were used separately for enzyme assay from each T1 transgenic plant and 50 seed bulks were analyzed for T2 ears

Gene	Target organelle	T1 Mean %TSP	T2 %TSP
E1	ER	6.1	3.5–4.5
	Vacuole	5.6	8–9
CBHI	Cell wall	3.2	3.7–5.1
	ER	4.1	3.2–3.8

TSP total soluble protein, *ER* endoplasmic reticulum

highest-expressing ears in T2 for replanting allows recovery of higher-expressing ears in subsequent generations. This strategy, while more complex than that used with many other plants, has been successful for more than 12 genes and, in each case, resulted in expression levels greater than tenfold higher than the initial level in the T1 seed.

Germplasm Pools

Types of corn produced include sweet corn, popcorn, and dent corn, with various minor types such as waxy corn and colored corn. Dent corn has, by far, the largest acreage and is used for ethanol, animal feed, and processed corn products. A wide array of varieties and stocks of germplasm pools are available representing the genetic diversity of dent corn available for current breeding (Mikel and Dudley 2006; Mikel 2011), including Oh43, Lancaster, Oh07-Midland, Iodent, the commercial hybrid-derived Maiz Amargo, and Stiff Stalk varieties. Combining germplasm from different groups allows strong heterosis for commercial hybrids. B73, a Stiff Stalk variety, and Mo17, a Lancaster variety, are the most frequently used germplasm backgrounds for generating commercial hybrids. They are often crossed with other germplasm pools to create a unique material that is used subsequently in commercial hybrids (Mikel and Dudley 2006). The take-home lesson is that corn germplasm is extremely diverse, and current hybrids have only begun to tap into the possibilities to enhance recombinant protein.

Specialized germplasm with specific characteristics that allow high protein accumulation are of interest for breeding programs. Examples of germplasm groups with valuable traits include high-oil phenotypes with large embryos, high-protein phenotypes with reduced endosperm volume (Dudley and Lambert 1969), and opaque-2 mutants with reduced zein (Puckett and Kriz 1991). Each of these genotypes has a mechanism that allows maximizing embryo-localized protein recovery on a weight basis (Hood and Howard 2009). Several recombinant proteins in maize, i.e., laccase, avidin, MnP, brazzein, aprotinin, and trypsinogen, were tested with these germplasm pools. All crosses yielded a significant increase in recombinant protein accumulation in either high oil or opaque-2 backgrounds. When laccase lines were crossed to high-oil lines, improvements were seen in germination as well as protein accumulation (Hood et al. 2003). High oil also improves protein accumulation above what would be expected from the increase in germ size. The high-oil crosses could be particularly interesting from a production standpoint because they are commercial lines with high yields. Other specialized pools, e.g., high protein and opaques, may have limited utility because of lower yields from those lines. Nevertheless, as is true for elite germplasm, the possibilities are vast for genetic manipulation to maximize recovery of traits of interest.

The sequence of the B73 maize genome was published in 2009 (Schnable et al. 2009), providing a powerful tool for understanding much of the molecular and genetic variation among varieties and germplasm pools by providing a basis for

comparison across genetic lines (Lai et al. 2010). Indeed, with the cost of DNA and RNA sequencing declining rapidly, detailed comparisons can be made among similar genetic lines to identify variations in coding loci, insertions and deletions, and single-nucleotide polymorphisms (SNPs), as well as low-sequence-diversity intervals (Lai et al. 2010). These comparisons can inform genome dominance in crosses and inheritance of variability that may be associated with particular traits of interest, such as high-protein accumulation in seed.

To date, the generational increases in protein accumulation have been determined empirically. Identification of high- and low-expressing lines per generation is determined only through quantification of the recombinant protein in each ear recovered in each generation; often as many as 3000–4000 analyses from a backcross nursery of 500 rows. Molecular markers that identify relevant loci could be used in earlier generations to select promising lines to continue breeding into elite or preferred specialty germplasm, potentially eliminating the time-consuming protein analysis on each progeny ear.

In an effort to identify the factors that contribute to the increase in protein accumulation during breeding and selection, transcriptome sequencing of high-and low-expressing lines was conducted. High and low lines recovered from the same generation were analyzed for differences in gene expression. Those differences would potentially be the basis for the genetic factors that determine the ability to increase gene expression and protein accumulation at each generation. Current transcriptome sequencing experiments have described embryos at 15, 21, and 27 days after pollination (DAP; Teoh et al. 2013). In these experiments, an unidentified storage protein gene in the *cupin* family is expressed at higher levels than *globulin-1*, the protein previously determined to be present at the highest concentrations in maturing embryos (Belanger and Kriz 1991). Data such as these could yield new regulatory sequences that could change the methods and level of recovery of recombinant proteins. Mining the genome will yield many new tools, but will require a great deal of effort to identify the genes or sequences of interest.

Additional studies of messenger RNA (mRNA) sequences between isogenic high- and low-protein accumulation lines from the same generation at 15, 21, and 27 DAP show some interesting differences in abscisic acid synthesis genes as well as increases in a number of unannotated genes. It is planned to continue this analysis to identify loci and alleles that account for the majority of the high-accumulation phenotype, similar to quantitative trait loci (QTLs), and also determine if SNPs can be associated with those loci. The SNPs would be convenient tools for early selection during breeding.

Containment Principles

Many proteins being expressed in maize are intended for industrial and pharmaceutical purposes. Additional regulatory requirements above those, for input traits, must be addressed to avoid intermixing with food/feed corn. Regulatory guidelines

outline containment management practices to prevent the inadvertent introduction of these proteins into the food chain that follow the same principles used for other food organisms (e.g., bacteria, yeast, and eggs) and have proven to be very effective. In addition, United States Department of Agriculture (USDA) has added regulatory guidelines for containment management practices as they relate specifically to plants. Maize pollen is relatively heavy and does not survive long under desiccation nor travel far, so physical isolation is a viable strategy (Luna et al. 2001; Ma et al. 2004). Genetic strategies to prevent intermixing may be desirable to complement physical isolation (Lee and Natesan 2006; Al-Ahmad et al. 2004; Daniell 2002) to alleviate some of these onerous requirements and provide greater confidence to the public.

Male sterile corn is an obvious method to prevent inadvertent pollen transfer. Methods for this are well established using a cytoplasmic male sterility system (Dewey et al. 1987). In addition, other systems have been proposed that rely on the preferential expression of proteins in the anther and pollen that devitalize the pollen. Several methods have been described that allow for restoration of viable pollen (Schnable and Wise 1998; Weider et al. 2009).This has the added benefit of being linked to the foreign gene of interest and may be a useful tool in the future.

Another example of containment is to control germination. Systems, such as terminator technology and controlled germination, have been proposed that manipulate the germination of seeds (Lee and Natesan 2006; Schernthaner et al. 2003; Oliver and Hake 2012). These approaches could increase flexibility in production of selected products, but a practical system is not currently available.

One recommendation that often comes up in relation to genetically engineered (GE) plants that express pharmaceutical proteins, vaccines, or industrial enzymes, i.e., nonfood traits in a food crop, is having some visual marker that allows identification of the transgenic lines. For maize, the most obvious way to track a GE crop with proteins in the seed is to mark the seed coat with a color. An obvious choice for driving expression of a visual marker is the use of the promoter for the extensin gene in maize because it is highly expressed in silk and pericarp (Hood et al. 1993). Two series of experiments have failed subsequently to demonstrate that this promoter is active in pericarp, one using an 840 bp region upstream of the extensin gene, and a 1978 bp region upstream of the extensin gene that contains several repeated regions that could account for differential expression in multiple tissues of this single-copy gene. An independently identified pericarp promoter actively promotes expression of beta-glucuronidase in pericarp tissues at relatively high levels. This promoter could be coupled with a reporter gene that would allow field identification of GE plants by cursory examination rather than by molecular analysis.

Reporter genes are needed in combination with seed coat-preferred promoters. For example, a fluorescent protein could be detected in the field or storage bin using a hand-held ultraviolet light source, although in bright sunlight the detection would be difficult. Alternatively, flavonoids, carotenoids, or xanthophylls could be used as long as they are active in the germplasm of interest. These genes often require

activation loci which are not present in all germplasm sources, for example, the *b1* locus in maize (Selinger and Chandler 2001).

Summary and Conclusions

Maize has been manipulated for centuries in order to improve its ability to provide a reliable supply of food and feed. This highly efficient production platform is now being developed as a source for industrial products, as well as for new uses that are continuing to emerge. The most common approach to increase the crop's utility for new products relies on the high level of expression of novel proteins in the kernel. Maize has proven to be one of the most useful crops to meet this need for several reasons, including its low cost of production, its inherent safety as a food and feed product, its demonstrated ability to express novel genes at high concentrations, the diverse germplasm available to customize the novel protein expression, and its ability to integrate the novel proteins directly into food, feed, and industrial applications without the need for purification of the protein.

Genetic manipulation both at the molecular and whole-plant level can help maximize protein accumulation. The technology is well suited for cost-effective production of large volumes and low-cost proteins and/or avoiding human pathogens in the final product. Because of this potential, a number of studies are underway with the aim to produce new foods, feeds, vaccines, pharmaceuticals, and industrial products.

This potential for making new products has led researchers to investigate novel ways of increasing expression. The kernel has proven to be a very effective site for overaccumulation of proteins that is aided by its inherent qualities of sequestering active proteins in the kernel, a relatively low metabolically active tissue, reduced concerns over gene silencing and proper folding, high protease inhibitors to limit degradation, and multiple methods to restrict gene flow to address regulatory concerns. With these advantages, the maize seed will continue to be the system of choice for high-volume output traits until such time that a customized plant can be generated without the concern for food/feed intermixing (Howard and Hood 2005a).

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Chapter 4
Breeding and Biotech Approaches Towards Improving Yield in Soybean

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Introduction

Soybean, the fourth most important crop in the world, is a key component of global food security. Soybean serves as feed for livestock and aquaculture and as a biofuel, forms a great part of oil and protein in the human diet, and contributes to cropping systems as a valuable contributor of soil nitrogen. Soybean production has increased steadily due to demand for protein products for animal feed and a significant increase in the meat-intensive food consumption. Increased production can be achieved by new cultivars with greater harvest index (HI) and adopting best management practices. Further increase in the genetic potential is challenging as there is little room to increase the HI (Fischer and Edmeades 2010). With pressure increasing to meet the demands of the growing population and with the limited availability of arable land, realizing soybean yield potential in the field represents a path forward.

Yield is a multigenic trait determined by physiological, genetic, abiotic, and biotic factors and the cross talk between these factors. Yield potential (Yp), also called potential yield, is the yield of a crop cultivar when grown under optimal conditions of water and nutrient availability coupled with effective control of biotic stress (Evans 1993; Van Ittersum and Rabbinge 1997). Yield potential studies estimate yields

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at figures ranging from 60 to 80% of the optimal level (Foulkes et al. 2009). This yield gap is a result of suboptimal environmental conditions and agronomic practices. It is estimated that, in the USA alone, adoption of improved management practices is expected to increase yield between 25 and 66% (Board and Kahlon 2011).

This chapter focuses on the history of soybean production and the yield gain over the last 50 years. It presents strategies to enhance yield by: (a) managing biotic and abiotic factors and (b) altering the source capacity and/or sink strength of the plant. In addition, it discusses potential candidate genes that can be targeted to enhance tolerance to suboptimal conditions with focus on drought and heat tolerance.

Global Soybean Production

Domestication of soybean is thought to have taken place in China during the Shang dynasty (approximately 1500–1027 BC) or earlier (Hymowitz 1970). However, historical and geographical evidence could only be traced back to the Chou dynasty (1027–221 BC) where soybean was utilized as a domesticated crop in the northeastern part of China. By the first century AD, soybean probably reached central and southern China as well as peninsular Korea. The movement of soybean germplasm was probably associated with the development and consolidation of territories and the disintegration of Chinese dynasties (Hymowitz and Newell 1981).

From the first century AD to approximately the fifteenth and sixteenth centuries, soybean was introduced into several countries, with land races eventually developing in Japan, Indonesia, the Philippines, Vietnam, Thailand, Malaysia, Myanmar, Nepal, and northern India. The movement of soybean throughout this period was due to the establishment of sea and land trade routes, the migration of certain tribes from China, and the rapid acceptance of harvested seeds as a staple food by other cultures (Hymowitz and Newell 1981). Beginning in the late sixteenth century and throughout the seventeenth century, soybean was used by the Europeans and, in the seventeenth century, soy sauce was a common item of trade from the east to the west (Hymowitz and Newell 1981).

Soybean was introduced into North America in the eighteenth century by Samuel Bowen in the colony of Georgia in 1765 (Hymowitz and Shurleff 2005). In 1851, soybean was introduced in Illinois and subsequently throughout the Corn Belt. In 1853, soybean seeds were deposited at the New York State Agricultural Society, the Massachusetts Horticultural Society, and the Commissioner of Patents. The two societies and the Commissioner of Patents distributed soybean seeds to growers throughout the USA. Soybean has been cultivated extensively and improved through conventional breeding following its introduction into the USA and subsequently has become a key source of nutrients for food and feed (Singh and Hymowitz 1999).

Soybean is now the most widely grown oilseed in the world, with approximately 268 million metric tons (MMT) of harvested seed produced in 2012, which rep-

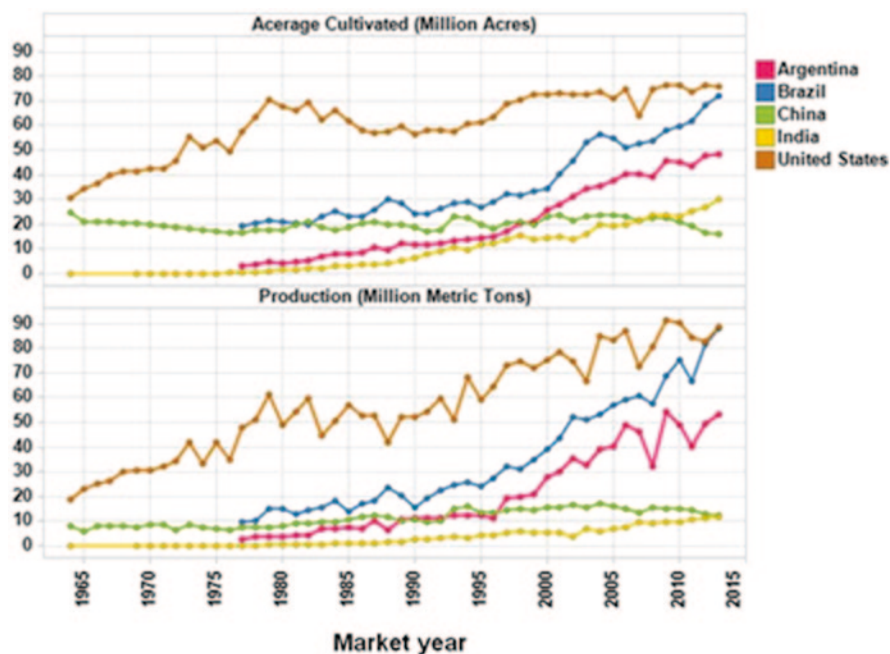

Fig. 4.1 Acreage and production of soybean by major producers the USA, Brazil, Argentina, China, and India. (Data sourced from USDA Foreign Agricultural Service's Production, Supply and Distribution (*PSD*) online database; http://www.fas.usda.gov/psdonline/psdHome.aspx)

resented 57% of the world's oilseed production that year (ASA 2013). Soybean is grown as a commercial crop in more than 35 countries. The major producers of soybean are (in order of production) the USA, Brazil, Argentina, China, India, Paraguay, and Canada, accounting for approximately 95% of the global soybean production in 2012 (ASA, 2013; Fig. 4.1).

Soybean has been the second largest field crop in the USA after corn. According to data from USDA-NASS (2013), soybean was planted on approximately 77.2 million acres in the USA in 2012, producing 3 billion bushels of seed with an estimated value of more than US$ 43.2 billion (USDA-NASS 2013). Soybean crop yields have risen consistently in North America since the 1920s. In the USA, the average annual yield increased from approximately 11 to 39.6 bushels/acre (bu/a), during the period from 1924 to 2012, equivalent to a yield increase of 360% (Fig. 4.2). Annual improvement in soybean yields attributable to rapid producer adoption of cultivars resulting from continuous improvement of agronomic or management practices (see the section "Yield Improvement Through Management of Biotic and Abiotic Factors") and genetic improvements (see the section "Genetic Improvement of Soybean Varieties") enhanced yield by reducing "on-farm" yield constraints (Specht et al. 1999).

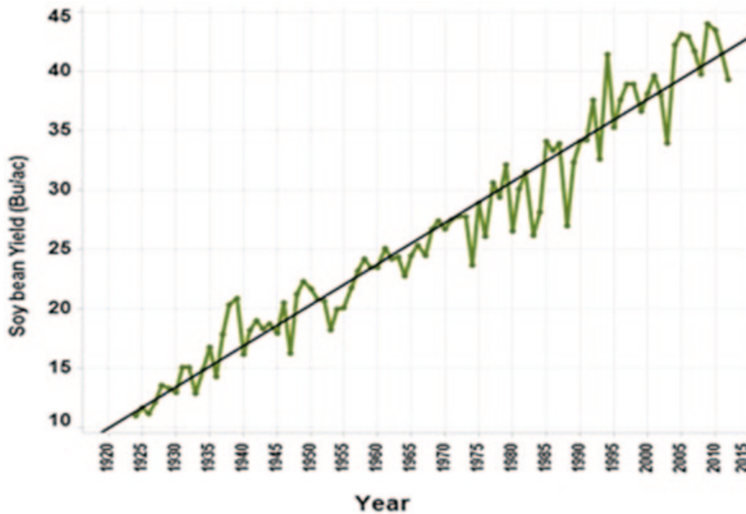

Fig. 4.2 US soybean yield 1924–2012. (Data sourced from USDA-NASS 2012; http://www.nass.usda.gov)

Yield Improvement Through Management of Biotic and Abiotic Factors

Several agronomic and management practices have contributed to greater soybean yields. Specific grower decisions that positively impact on yield in recent years include elimination of weed competition, management of insect pests and disease, and irrigation. These factors are discussed in the following sections.

Weed Management

Weed control in soybean is essential to optimizing yields because weeds compete with soybean for light, nutrients, and soil moisture (see Chap. 5). The primary factors that affect potential yield loss in soybean from weed competition are the invasive species, their density, and the duration of the competition. When weeds are left to compete with soybean for the entire growing season, yield losses can exceed 75 % (Dalley et al. 2001). Weeds can also harbor insects and diseases, and interfere with harvest, causing extra wear on equipment (Pedersen 2008). Herbicide-tolerant soybean was introduced to provide growers with additional options to improve crop safety and control weeds. The Roundup Ready® soybean system (planting Roundup Ready® soybean and applying glyphosate in crop to provide primary weed control) was introduced in 1997 and has become the conventional weed control program in the USA, Argentina, and Brazil (Fig. 4.3).

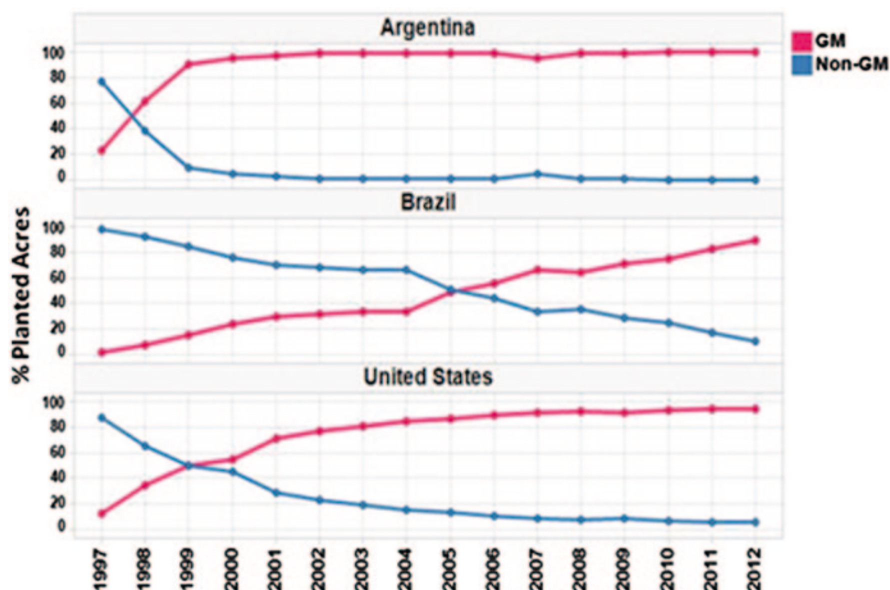

Fig. 4.3 Genetically modified (*GM*) soybean adoption by growers in the USA, Argentina, and Brazil (1997–2012). (Data sourced from ASA 2012)

Management of Insect Pests

The area under soybean cultivation worldwide is 90 million ha of which 69 million ha is contributed by genetically modified soybean (James 2009). The cultivation of soybean restricted to specific regions (mainly the USA and South America) renders it highly susceptible to a range of insect pests and diseases across different development stages. The prevalence of specific insect pests is not homogeneous across all soybean-growing areas; i.e., the insect spectrum can vary between countries. Insect injury can impact yield, plant maturity, and seed quality in soybean (Aref and Pike 1998). The insect pests attack all parts of the plant, but each pest is tissue specific (Grossi-de-Sa et al. 2011). Lepidopterans, primarily species of the family Noctuidae, are the major insect pests attacking above-ground plant parts, specifically, the leaves and pods (Harding 1976). Other secondary insect pests include the soybean aphid (*Aphis glycines* Matsumura), which originates from North Asia and has now spread across many parts of the USA and Canada (Li et al. 2010).

Leaf-feeding insects comprise the biggest group of soybean pests. Research on defoliation has determined that a major effect of leaf injury is to reduce light interception by the soybean canopy (Board et al. 2010) which, in turn, can have a significant effect on yield (Higley and Boethel 1994). Soybean has the capacity to withstand considerable defoliation early in the season without significant yield loss. Hunt and Baldin (2012) recommended treating against defoliating insects in vegetative stages if defoliation reaches 40 %. In contrast, defoliation during repro-

ductive stages poses a greater threat to yield, because the soybean plant has less time to compensate for injury compared to vegetative growth stages. Defoliation during pod development has the most impact on yield (McWilliams et al. 2004; see the section “Soybean Development and Yield Potential”). During these stages, treating against insects is recommended when defoliation reaches 20 % (Hunt and Baldin 2012). This suggests an opportunity for developing insect-resistant soybean, translating potentially to increased economic welfare.

Biotech approaches are being investigated to address this challenge. The most successful attempt has been the use of cry1Ac-like genes from *Bacillus thuringiensis*. Transgenic soybean expressing these genes has shown resistance to soybean podworm (*Helicoverpa zea*), soybean looper (*Chrysodeixis includens*), velvet bean caterpillar (*Anticarsia gemmatalis*), and tobacco budworm (*Heliothis virescens*; Stewart et al. 1996; McPherson and MacRae 2009). MacRae et al. (2005) generated soybean plants expressing a synthetic Cry1Ac-like protein (TIC107) from *B. thuringiensis* and evaluated the plants under controlled environment and field conditions in the USA and Argentina. Transgenic plants showed complete efficacy against *A. gemmatalis* and *C. includens* (Walker et al. 2000) in the USA and against *A. gemmatalis, Crocidosema aporema, Rachiplusia nu,* and *Spilosoma virginica* in Argentina. Under laboratory conditions, larvae fed on an artificial diet containing Bt soybean leaf tissue showed complete mortality, whereas when fed with control leaves larval mortality was less than 10 %. Similar results were obtained by Miklos et al. (2007). Expression levels of Cry toxin in the transformed plant lines was very high (6.12 µg/mg of total extractable protein) and the phenotypes of the high-expressing lines were indistinguishable from controls. Insect bioassay data demonstrated complete protection against soybean looper, soybean podworm, and velvet bean caterpillar, whereas negative controls exhibited defoliation as much as 98 %. These results demonstrate that the expression of TIC107 in soybean is highly efficacious in the control of multiple lepidopterans under laboratory and field conditions, and also suggest high-dose expression of TIC107 for effective insect resistance management.

In addition to Cry toxins, other proteins have shown potential for efficacy against insect pests of soybean. Quantitative trait loci (QTLs; 229-H, 229-M, QTL-G) have been used in combination with Cry1Ac and shown resistance to an array of lepidopteran insects (Walker et al. 2000, 2004; Zhu et al. 2008). Trypsin inhibitors, lectins, and enzymes have also been evaluated (Sharma et al. 2000). Transgenic tobacco plants expressing a cowpea trypsin inhibitor showed resistance to *H. virescens* larvae as well as *Diabrotica* spp. and *Spodoptera* spp. (Gatehouse et al. 1993; Hilder et al. 1987). Studies with lectins have shown that insect damage is reduced in plants overexpressing lectins. Expressing rice cystatin I in potato enhanced larval mortality by 53 % (Lecardonnel et al. 1999) and expression of concanavalin A in potato retarded larval development (Satyendra et al. 1998). Among enzymes, chitinase has been the most studied. Chitinase from various sources when expressed in tobacco increased resistance to lepidopteran insects (Ding et al. 1998; Gatehouse 1995).

Disease Management

Bacteria, fungi, and nematodes account for 11 % of economic yield loss (Hartman et al. 1999). Pathogens can affect all parts of the plant and the extent of damage is dependent on the kind of pathogen, the tissue in question, plant development stage, host susceptibility, and the environmental condition (Hartman and Hill 2010).

Soybean rust caused by *Phakopsora pachyrhizi* is a major disease accounting for yield losses ranging from 55 % in the USA and South America (Mueller et al. 2009) to 80 % in Taiwan (Hartman et al. 1991). Fungicides have been used in the management of rust (Mueller et al. 2009). Recent approaches like host-plant resistance have been employed but their success is limited as new isolates of *P. pachyrhizi* have arisen that are virulent to a soybean cultivar with such genetic resistance (Paul and Hartman 2009; Pham et al. 2009). To date, there are no biotech approaches for the management of soybean rust. However, identification of potential novel genes in *Glycine tomentella* may provide an opportunity to combat soybean rust (Soria-Guerra et al. 2010).

Another important constraint to soybean productivity in the USA is the damage due to soybean cyst nematode (SCN; Hartman et al. 2011). Agronomic practices like crop rotation and host resistance appear to be the best measures for controlling SCN (Niblack and Chen 2004; Schmitt et al. 2004). However, populations of *Heterodera glycines* have adapted to the resistance genes and hence this technology is not long lasting. New methods to control SCN using the RNA interference (RNAi) technology by host-induced gene silencing in the parasite have been demonstrated in experimental systems (Sindhu et al. 2009). Some target genes in *H. glycines* that have been silenced encode proteinase (Urwin et al. 2002), aminopeptidase (Lilley et al. 2005), cellulase pectate lyase, chorismate mutase, and secretion peptide SYV46 (Bakhetia et al. 2007). Resistance can also be obtained by expressing double-stranded RNA (dsRNA) of the nematode target genes in plants. Steeves and coworkers (2006) expressed dsRNA of the major sperm protein and have shown that soybean plants are resistant to SCN. Recently, it was reported that the use of four different RNAi gene-silencing constructs was able to decrease cyst nematodes in transformed soybean roots (Klink et al. 2005, 2009). Results using RNAi technology are promising and appear to be a potential strategy to generate plants resistant to phytopathogenic nematodes.

Irrigation

The productivity of soybean is highly dependent upon soil and climatic conditions. In the USA, the soil and climatic requirements for growing soybean are very similar to corn. The soils and climate in the midwestern, eastern, and parts of the Great Plains regions of the USA provide sufficient water under typical climatic conditions to produce a soybean crop. The general water requirement for a high-yielding soybean crop is approximately 20 in. during the growing season (Hoeft

et al. 2000). Soil texture and structure are key components determining water availability in soils, where medium-textured soils hold more available water, allowing soybean roots to penetrate deeper compared to clay soils. In the western and southern soybean-growing regions of the USA, irrigation is used on approximately 9% of soybean acreage to supplement the water supply during dry periods (ASA 2013).

Efficient utilization of available water resources for crop production is essential for agriculture in regions where water is limited. Under normal growth conditions, soil water depletion by soybeans is generally confined to the upper 0.6–1.3-m soil depth and the crop could effectively utilize all water in the upper 1.8 m of the soil depth under periods of drought (FAO 2013, http://www.fao.org/nr/water/cropinfo_soybean.html). One method to calculate water use efficiency (WUE) for soybeans is to divide seed yield by total water used throughout the growing season. Based on the yield component analyses, soybean cultivars show differential yield response to irrigation timing during their vegetative and reproductive development. The sensitivity to water stress (measured by yield reduction) tended to increase dramatically as the crop advanced through its natural sequence of reproductive development (Shaw and Laing 1966). Generally, soybeans produce flower buds in abundance, but 43–81% of the buds abscise during development without producing mature seeds (Schaik and Probst 1958). Water deficit/stress during flowering and pod development is considered to be a dominant environmental factor accelerating the rate of abortion (Westgate and Peterson 1993). Water stress during reproductive development reduces photosynthesis and the amount of photoassimilates partitioned to support the developing reproductive structures, thereby accelerating the rate of abortion (Raper and Kramer 1987). A study by Kadhem et al. (1985) suggested that the timing of irrigation during reproductive development has a significant impact on the yield response. Multiple irrigation régimes throughout reproductive development maximized seed return per plant. Therefore, the final seed yield at the end of the growing season is determined, to a large extent, by rainfall during reproductive development.

Climatic conditions, including rainfall, temperature, atmospheric CO_2 concentration, and ozone levels, are projected to change over the next century (Karl et al. 2009). The increase in extreme temperature during the day, warm nights, and more variable rainfall will continue to impact soybean production. In addition, interactions between these environmental conditions during critical developmental stages could result in changes in ecosystems (increased competition by weeds, pests, and pathogens), leading to significant reductions in seed yield. Given that field-grown soybean frequently encounters suboptimal conditions during critical growth periods, such as pod formation and seed filling, there is an opportunity to further stabilize yield through enhancing drought tolerance or WUE of the crop plant.

Genetic Improvement of Soybean Varieties

From the beginning of the twentieth century to the 1970s, the annual genetic gain represented 45–50% of the realized yield gain for that period (Luedders 1977; Specht and Williams 1984). Breeding for higher-yielding varieties resulted in

changes associated with plant architecture, plant physiology, seed properties, and disease resistance. The average plant height of modern soybean cultivars decreased compared to the soybean introductions grown at the beginning of the twentieth century (Specht and Williams 1984; Boerma 1979). This was achieved mostly by shortening of the internodes rather than reducing their number. Several studies reported improved lodging resistance (Luedders 1977; Wilcox et al. 1979, Voldeng et al. 1997), which facilitated harvesting and contributed to reduced harvest losses (Luedders 1977). Some researchers observed that the yield increase was associated with more pods per plant (Boerma 1979); others reported that it was due to more seeds per plant (Morrison et al. 2000; De Bruin and Pedersen 2009).

Soybean Development and Yield Potential

Soybean plant development can be separated into two major, generally overlapping, vegetative and reproductive developmental phases. The time of onset and the duration of the various growth stages in soybean are highly dependent on photoperiod (hours of daylight and darkness) and temperature (Major et al. 1975). Therefore, for the same soybean plant grown at different latitudes, the onset and duration of the growth stages and the time from planting to maturity may be different. Also, in contrast to most other temperate-season crops, soybean is a "short-day" plant, meaning that maturity is delayed by longer day length (Major et al. 1975). In soybeans, flowering is initiated only after the night is longer (and days shorter) than a critical length (Holshouser 2010). Once flowering begins, temperature controls the duration of flowering time (Heatherly and Elmore 2004). The duration of these phases is controlled primarily by genetics, temperature, and day length (Pedersen et al. 2007). Soybean producers influence the duration of these phases through variety selection, geographic location, and planting date.

Yield potential is initially determined by vegetative growth when the formation and development of organs for nutrient absorption and photosynthesis provides the machinery to produce yield. The reproductive phase is typically the most important for yield determination (Fehr and Caviness 1981). The period from R1 to R6 stages is critical for yield, because this is when both pod and seed number are set. The period between the R5 stage and onset of the R7 stage is important in setting seed weight. Because pod development begins at the R3 stage and seed growth ends at the R7 stage, conditions that limit growth during this period can impact yield by limiting seed number, seed weight, or both (Pedersen et al. 2007). During the reproductive phase, the number and size of seeds are limited by the capacity and efficiency of the soybean canopies to produce and translocate assimilate (Egli 1999). Canopy-level photosynthetic rates provide the best estimate of assimilate availability at a given time (Long et al. 2006b). The rate of canopy photosynthesis is determined by Leaf Area Index (LAI; Westgate 2001), the photosynthetic capacity of the leaves, and environmental conditions.

It is necessary to optimize parameters that play a critical role in yield in every production environment. Yield potential is the maximum yield (seed dry matter) of

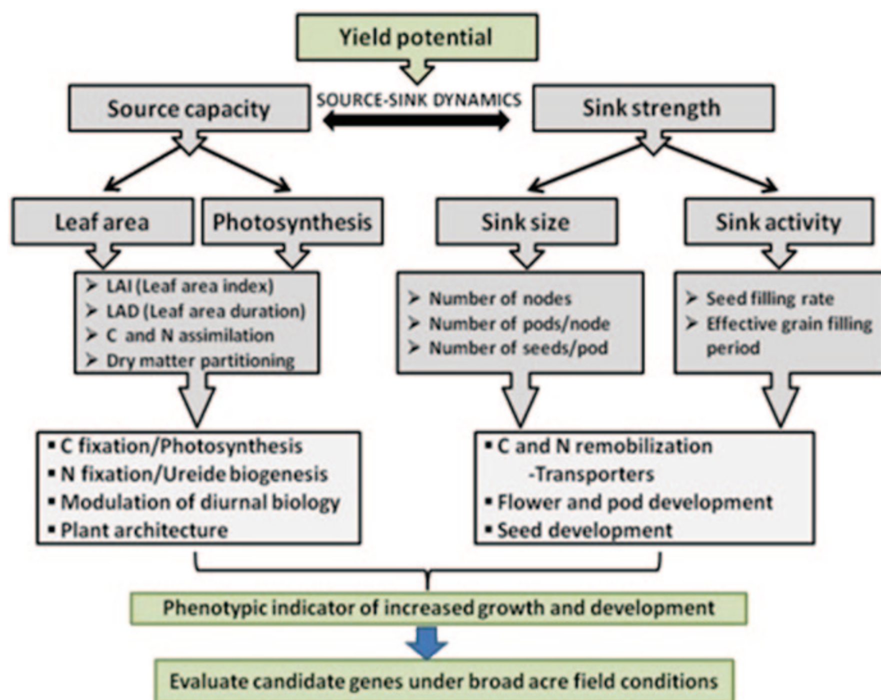

Fig. 4.4 Source capacity and sink strength drive yield potential in soybean

a cultivar grown in an environment with all conditions optimal for growth (Evans and Fischer 1999). Maximizing yield potential can be achieved by manipulating the relative source capacity/assimilatory capacity and the potential sink strength of a crop. Source capacity in soybean is defined as a function of photosynthesis/net assimilation rate and leaf area, which includes LAI, leaf area duration, and specific leaf area (Fig. 4.4). Sink strength is the competitive ability of a sink to attract assimilates and is a product of sink size and sink activity (Fig. 4.4; Marcelis 1996).

Soybean is a Source-Limited Crop

Efforts by various researchers to increase soybean yield have focused mainly on enhancing source capacity such that it can provide the required assimilates for utilization by the developing sink. A dynamic relationship exists between the source and the sink tissues (Egli and Bruening 2001). Various agronomic techniques have been employed to alter source activity and the developmental stage at which these variations are done is important. The critical stage at which soybean is responsive to source alterations has been identified to be a 2-week period between late flowering and early pod formation in which source strength differences have the greatest

effect on pod number and yield (Schou et al. 1978). The position of the source leaves that supply assimilates to the developing sink also plays a crucial role. Pods have the potential to form five seeds, but typically only three develop. Pods that form at a particular node receive a major portion of their assimilates from the subtending leaf with minor contribution from the leaves located two nodes above and two nodes below (Carlson and Brun 1984).

Early attempts were promising to increase photosynthesis by either increasing the atmospheric CO_2 concentration or increasing irradiance. Various authors have shown an increase in photosynthetic rate (Jones et al. 1984; Rogers et al. 1984) and leaf area (Ziska and Bunce 1995) as a result of enrichment in CO_2 concentration, suggesting that the stimulation of vegetative growth could increase seed yield. However, other researchers have demonstrated that enriching CO_2 during the reproductive growth period rather than at the vegetative growth period has a profound positive effect on seed yield (Hardman and Brun 1971; Ackerson et al. 1984). Elevating CO_2 concentrations from 384 to 550 µmol/mol in field conditions increased yield by 15 % (Long et al. 2006a). The response to CO_2 enrichment is associated with an ability to set additional pods on branches (Ziska et al. 2001). Nakamoto et al. (2004) reported that CO_2 enrichment increases seed yield by improving pod set on the higher-order racemes that opened later during the flowering period.

In field-grown soybean, most photosynthesis occurs in the upper 20 % of the canopy that makes one third of the LAI and intercepts 90 % of incident photosynthetic active radiation (PAR; Zhu et al. 2010). Supplemental light (cool white and red fluorescent) given to the lower canopy during the day for 3 weeks during flowering reduced floral abscission and increased the number of mature pods and seed yield per node. However, the number of flowers produced per node, individual seed weight, and seeds per pod were not affected by light. Conversely, reducing photosynthesis by shading reduced the amount of radiation intercepted by 45 %, affecting the number of pods on the main stem, thereby resulting in fewer pods per reproductive node. Significant reduction in dry matter accumulation was also observed (Board et al. 1995; Andrade and Ferreiro 1996). The most pronounced effect of shading was seen when applied at the early stages of reproductive development. In a study with radiolabeled carbon (14 C), shading the flowers and young pods reduced the relative amount of radiolabel accumulated by 30 % and also increased abscission. Light perceived by soybean flowers and young pods probably has a role in regulating both abscission as well as the capacity to accumulate photoassimilates (Heindl and Brun 1983).

Limiting the source by defoliation impacts yield, but the extent and the developmental stage at which defoliation is carried out is an important aspect to consider (see the section "Management of Insect Pests"). Defoliation at the vegetative stage did not result in yield loss whereas defoliation at the reproductive stage led to a progressive reduction in yield by reducing photosynthesis, light interception, amount of stored dry matter, and reduction of the pod-filling period (Hanway and Thompson 1967; Todd and Morgan 1972; Hinson et al. 1978; Ingram et al. 1981). The most sensitive stages for defoliation were R5 and R6 (Caviness and Thomas 1980; Goli and Weaver 1986; Board et al. 1994). Defoliating less than one third of the total leaf

area or up to 100 % at V5 did not reduce yield (Turnipseed 1972; Pickle and Caviness 1984). Yield loss was 9 % with 40 % defoliation at R5, and increased to 32 % with 80 % defoliation (McAlister and Krober 1958).

Strategies to Improve Source Capacity in Soybean

The positive correlation between photosynthesis and yield suggests that targeting the components of the photosynthetic machinery could be promising. This includes strategies to improve the efficiency with which the intercepted photosynthetically active radiation is converted to biomass and assimilates partitioned to the seed. In addition, increasing assimilate availability through an extended period of photosynthetic activity may also provide the opportunity to further enhance soybean yield. Examples that leverage each approach are described in the following section.

Increasing photosynthesis means increasing the efficiency of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), for carbon fixation in C3 plants. RuBisCO has dual specificity to CO_2 and oxygen; hence, increasing the specificity for CO_2 relative to oxygen is useful (Zhu et al. 2004; von Caemmerer and Evans 2010). Considerations need to be addressed like the specificity, the catalytic rate of RuBisCO and the fact that the large and small subunits need to come together to form the holoenzyme (Parry et al. 2007; Peterhansel et al. 2008). A better RuBisCO alone is not sufficient. RuBisCO activase, a key enzyme in the activation and stability of RuBisCO, is also important. Yin et al. (2010) cloned and characterized soybean RuBisCO activase and showed a positive correlation between RuBisCO activase, RuBisCO, photosynthetic rate, and yield (Spreitzer and Salvucci 2002; Yin et al. 2010).

In addition to altering RuBisCO and RuBisCO activase, other enzymes of the Calvin cycle can be targeted for enhancing yield. Using tobacco as a model system, various authors have overexpressed Calvin cycle enzymes and shown a positive correlation with biomass and yield. Tobacco plants expressing plastid fructose 1,6-bisphosphate aldolase (aldolase, EC 4.1.2.13) showed increased biomass in a CO_2-enriched environment, enhanced carbon fixation, and improved ribulose biphosphate (RuBP) regeneration (Uematsu et al. 2012). Overexpression of sedoheptulose-1,7-bisphosphatase (SBPase) resulted in higher photosynthetic rates, greater concentrations of sucrose and starch accumulation, and increase in leaf area and biomass up to 30 %. The photosynthetic capacity per unit leaf area and plant biomass increased (Lefebvre et al. 2005). Tobacco plants expressing fructose bisphosphatase (FBPase) from cyanobacteria or SBPase from *Chlamydomonas* had greater dry matter, enhanced photosynthetic CO_2 fixation and growth rate, RuBP regeneration capacity, and RuBisCO activation state. This study suggested that SBPase is the most important factor for RuBP regeneration in the Calvin cycle and FBPase is important for partitioning the fixed carbon towards RuBP regeneration or starch synthesis (Tamoi et al. 2006).

Components of the photosynthetic electron transport chain can also be modified in an attempt to increase the yield potential. This involves two aspects: the light-harvesting chlorophyll complexes and chlorophyll fluorescence. Researchers believe that, in soybean, having smaller antenna complexes at the upper canopy could help mitigate the losses associated with overexcitation and induction of nonphotochemical quenching (Melis 2009; Ort et al. 2011; Zhu et al. 2010).

In C3 plants, a major amount of energy is spent on photorespiration. Decreasing photorespiration can help by improving the conversion efficiency. The higher photosynthetic efficiency in C4 plants is due to the intrinsic CO_2-concentrating mechanism. There are efforts to engineer a C4 pathway in C3 plants (Edwards et al. 2001; Hibberd et al. 2008; Zhu et al. 2010). Transgenic *Arabidopsis* and *Nicotiana tabacum* plants engineered with the ictB gene (involved in bicarbonate accumulation) from *Synechococcus* showed reduced photorespiration and increased rates of carbon dioxide uptake (Lieman-Hurwitz et al. 2003). In another attempt, the key genes from *Escherichia coli* for metabolism of glycolate to phosphoglycerate were expressed in *Arabidopsis*. Transgenic plants showed reduced metabolite flow through photorespiration, enhanced carbon assimilation, and better growth (Kebeish et al. 2007; Peterhansel et al. 2008). However, these efforts have not been very successful and constant improvements are being made to address this challenge.

The timing and duration of soybean development is influenced by the photoperiod. For example, floral induction is usually suppressed under long days (LD) but induced when day length is shorter than a critical length. The sensitivity to photoperiod varies among cultivars. In soybean, the post-flowering photoperiod has been identified as critical as it regulates both reproductive development and affects vegetative growth (Han et al. 2006). Enhanced vegetative growth during post-flowering (R3–R5 stages) increases photosynthetic capacity translating into an increased level of photoassimilate that can be transported to the developing sinks (Preuss et al. 2012).

Photoperiod signals are mediated by phytochromes which serve as the functional receptors. Genes required for the day length response have been identified to be either regulatory or those involved in circadian regulation. Circadian rhythms synchronize biological events with daily environmental changes. Plants measure changes in day length to precisely control flowering time to maximize reproductive success. This necessitates the abundance of clock gene transcripts to change during the transition from dusk to dawn (Song et al. 2010; Imaizumi 2010). The complex interaction between the major genes of the circadian clock CCA1 (circadian clock associated 1) and LHY (late elongated hypocotyl) and the photoperiod genes CO (constans) and FT (flowering locus T) regulates the transcriptional expression of other genes such as PRR5 (pseudo-response regulator 5), TOC1 (timing of CAB expression), CHE (CCA1 hiking expedition), GI (gigantea), LUX (LUX arrhythmo), and ELF4 (early flowering 4), thereby influencing flowering time (Mizoguchi et al. 2005; Imaizumi et al. 2003; Yanovsky and Kay 2002; Doyle et al. 2002).

Recently, Preuss and others (2012) have shown that the expression of *At* BBX32 (B-box 32) induces changes in clock gene transcripts during transition from dark to light which could be a result of modulation of endogenous diurnal processes. In soybean plants expressing BBX32, floral initiation was unaltered, but timing of the later stages of development was altered suggesting a role for *At* BBX32 in regulating the duration of post-flowering reproductive phase. The authors hypothesize that *At* BBX32 alters the expression of circadian clock genes specifically at dawn, modifies the input pathway of the clock, and dampens the clock rhythms at dawn. This modification extends the duration of pod development and seed-filling stages (R3–R5), indicative of an extended period of photosynthetic activity, resulting in increased pod number, seed number, and individual seed weight. This corroborates with previous studies which have indicated a relationship between developmental timing and yield in soybeans (Egli and Donald 2004).

Collectively, all the literature evidence suggests that soybean source capacity could be improved by multiple approaches by manipulating photosynthetic capacity, biomass production, photorespiration, flowering time, and duration of pod filling to increase the supply of assimilates to developing pods. However, excessive biomass production would reduce the HI and, therefore, well-balanced control of source capacity and sink potential is essential for increasing grain yield.

Sink Strength in Soybean

Sink strength is the competitive ability of a sink to attract assimilates and is a product of sink size and sink activity. Sink activity is dependent on sink age and proximity of the sink to the source. In soybean, sink size is a reflection of the number of pod bearing branches, number of pods, and number of seeds per pod, while sink activity or seed size is determined by seed-filling rate and length of the effective seed-filling period (SFP; Gbikpi and Crookston 1981; Egli and Leggett 1976; Kaplan and Koller 1974). Sink size is the physical constraint while sink activity is the physiological constraint (Marcelis 1996) for increasing yield potential. The concept of source and sink varies with the stage of plant growth. During the vegetative stage, the priority for assimilates is in the order of roots >young leaves >inflorescence while at the reproductive stage the order changes to fruit >young leaves >flowers >roots (Egli et al. 1989). There is a clear distinction in assimilate partitioning between determinate and indeterminate varieties of soybean. Indeterminate soybeans continue to grow for several weeks after flowering and pod development. During this overlapping vegetative and reproductive growth, the plant has to partition assimilates to both vegetative and reproductive sink tissues. Since the indeterminate soybean begins flowering at the lower nodes of the plant, pods on the bottom of the plant are more mature than the pods on the top portion of the plant. By contrast, the determinate soybeans terminate vegetative growth prior to initiating reproductive development from the middle portion of the canopy, and flowering proceeds both upwards and downwards. Assimilate partitioning in determinate soybean is primarily dedicated towards developing pods after reproductive transition.

Source–sink manipulation studies indicate that the critical and most responsive stage to alteration in source strength is R1–R6 and this has a bearing on the pod number (Board et al. 1995). Within this window, yield at the early reproductive stage (R1 to early R5) is source limited whereas at late reproductive stages (R5 onwards) it is sink limited (Kokubun and Watanabe 1983; Board and Harville 1998). The response of the sink to manipulations (defoliation, shading, pod removal) during seed filling depends on the level of assimilate in the seed and the ability of the seed to respond to change in assimilate supply (Jenner et al. 1991). Carbon and nitrogen assimilates for seed filling are derived from current photosynthates as opposed to storage reserves. Photosynthesis during the SFP provides both the carbon and nitrogen requirement for seed growth. Sucrose forms the major carbon component while nitrogen assimilated via NO_3 contributes to 50 % or more of the nitrogen in the seeds (Layzell and LaRue 1982; Rainbird et al. 1984; Shibles et al. 1987; Harper 1987; Staswick 1989; Wardlaw 1990). A positive correlation exists between sucrose concentrations in the source and seed growth rate (Thompson et al. 1977; Egli et al. 1989). At late reproductive stages when photoassimilation is a limiting factor, starch accumulated in the leaves, stems, and pod walls can also be remobilized to the growing seed. Starch concentration in leaves reduces by 50 % suggesting remobilization of starch to support seed filling (Egli et al. 1980).

Nitrogen requirement for soybean seed growth is fulfilled by nitrate assimilation in the leaves or nitrogen fixation in the nodules. Research has shown that biological nitrogen fixation is the most efficient way to supply the large amount of nitrogen needed by legumes to produce yield. All of the fixed nitrogen is readily available and in the form required for combination with carbohydrates to produce amino acids used for protein synthesis. Since nitrogen fixation in root nodules is directly dependent on the translocation of carbohydrates from leaves, the rate of fixation is fully synchronized with the rate of plant growth. This fine balance between nitrogen supply and demand is another aspect of the high efficiency conferred by biological nitrogen fixation. Nodulation and atmospheric nitrogen fixation is progressively inhibited as the soil nitrate nitrogen level increases, because legumes preferentially use most of the available soil nitrogen before they begin to fix the atmospheric nitrogen by symbiotic association with *Rhizobium* (Udvardi and Poole 2013).

Nitrogen fixation during the vegetative phase is low, but as the plant progresses towards reproductive stages nitrogen fixation increases, reaching a maximum at R5 or early R6 stage (Zapata et al. 1987; Imsande 1988). Nitrogen assimilated in the leaves is transported as asparagine to the growing sinks via phloem (Ohyama and Kawai 1982). Asparagine is abundant in pod walls and stem and is predominant in the embryo, contributing to about 58 % of soluble nitrogen (Rainbird et al. 1984). Soybean plants fix atmospheric nitrogen (N_2) with the help of bacteroids located in the root nodules. For long-distance translocation of fixed nitrogen, ammonia produced during nitrogen fixation is assimilated into glutamine and converted to ureides (allantoin and allantoic acid), which are transported via xylem to the leaves and developing sinks (Layzell and LaRue 1982; Smith and Atkins 2002; Smith et al. 2002; Atkins and Smith 2007). In soybean, the nodule ureide levels can reach concentrations of 94 mM (Streeter and Jeffers 1979). The ratio of allantoin to allantoic

acid translocated may vary from 1:1 to 1:5 depending on the developmental stage of the plant (Rainbird et al. 1984; Gordon et al. 1985). Therefore, ureides are by far the best choice of nitrogen transport in soybean, and 70 % of the fixed nitrogen in soybean is ureides and nearly 95 % of the xylary sap consists of these compounds (Schubert 1981).

Transport of ureides from nodules to the shoot involves ureide transporters. Ureide transporters (UPS—ureide permease) have been identified in *Arabidopsis* (UPS1 to UPS5) and French bean (*Pv* UPS1; Pelissier et al. 2004; Desimone et al. 2002; Schmidt et al. 2004, 2006; Froissard et al. 2006). They transport allantoin but have much higher affinity for purines and pyrimidines. Recently, Collier and Tegeder (2012) characterized two soybean ureide transporters, UPS1-1 and UPS1-2. These transporters are present in the plasma membrane and expressed in nodule cortical cells and vascular endodermis. Unlike the *Arabidopsis* and French bean UPS that transport only allantoin, soybean UPS transports both allantoin and allantoic acid. Repression of UPS in soybean increased ureide accumulation in the nodules by 20–116 %, reducing the concentrations of allantoin and allantoic acid in the xylem sap by 31 % as a result. Suppressed plants had smaller leaves showing nitrogen deficiency symptoms (Collier and Tegeder 2012). Ureides play a central role in nitrogen assimilation and consequently yield potential in soybean.

Water limitation is known to reduce drastically nitrogenase activity and nodule activity, thereby decreasing nitrogen fixation in soybean. The reduction in nitrogen fixation occurs prior to reduction in plant physiological processes. In species that transport nitrogen as amides, nitrogen fixation is less sensitive to water limitation compared to species that transport nitrogen as ureides. Drought-induced ureide accumulation in leaves is also thought to be an inhibitor of nodulation (Sinclair and Serraj 1995). Sinclair et al. (2007) derived soybean lines from a cross between Jackson, a cultivar proven to have N_2 fixation tolerance to drought, and KS4895, a high-yielding line. Using this approach, they identified two lines with potential for higher yields and reduced nitrogen fixation sensitivity to water limitation. Breeding approaches may therefore lead to development of lines with improved yield and reduced nitrogen fixation sensitivity under water stress conditions.

When a balance between the source and sink is reached, another factor comes into play, namely assimilate partitioning efficiency. Partitioning efficiency is the result of a coordinated set of transport and metabolic processes governing the flow of assimilates from source to sink. The flow from source to sink occurs through phloem loading, driven by a solute gradient between the source and the sink (Ho 1979; Wolswinkel 1985; Lang and Thorpe 1986; Patrick 1988; Lang and During 1991). The cross-sectional area of the phloem and the length of the phloem pathway are important determinants of transport. The proximity of source to sink can also play an important role. Studies conducted as early as Pate and coworkers 1977 by Pate showed that, in soybean, pods at a given node receive a major portion of their assimilates from the subtending trifoliate leaf and a very minor contribution came from the leaves situated two nodes above. These findings were corroborated by Fu et al. (1999), using ^{14}C labeling studies. Recent studies on source–sink distance by Liu et al. (2010) indicated that long-distance translocation of assimilates exists in

soybean and mainly supports growth of the later-formed seeds. A number of studies have concluded that partitioning efficiency is regulated more by the sink rather than the source or the transport components (Evans 1975; Gifford and Evans 1981; Farrar 1988; Ho 1988; Verkleij and Challa 1988; Kallarackal and Milburn 1984; Wardlaw 1990).

Strategies to Increase Sink Strength in Soybean

Yield loss in soybean is due to abortion of flowers or young pods (Streeter and Jeffers 1979). Understanding the mechanisms governing abortion could serve as potential targets for enhancing the sink strength by way of flower and pod retention. Soybean has a racemose inflorescence and flowering follows a bimodal and asynchronous pattern (Huff and Dybing 1980; Spollen et al. 1986) resulting in a competition for assimilate; early-formed flowers extract all assimilates leaving very little or no assimilate for the later-formed flowers. Hence, synchronization of flowering pattern could be another avenue for increasing sink strength.

Increasing sink strength can also be attained by creating a physical imbalance in the source sink ratio, or by enhancing the source strength at the transition between R5 and R6 stage when peak nutrient is accumulated in the sink (Henderson and Kamprath 1970). Physical manipulations have been achieved by shade, defoliation, or pod removal (Egli 1998). Shade and defoliation resulted in source-limited yield by reducing seed number and seed size, while pod removal caused sink-limited yield reduction through a decrease in seed number, but increase in seed size.

Shading all side leaflets during pod filling increased significantly the rate of photosynthesis in the unshaded center leaflets, compared to untreated plants. Greater photosynthesis was also reflected in both increased stomatal and mesophyll conductance. Combinations of treatments have also been attempted. Shade applied throughout the SFP reduced seed weight by 37 %. In plants shaded during initial seed filling, seed weight was reduced by 19 %, while shading during the later part of seed filling reduced yield by 28 %. Removal of shade during the later half of the SFP delayed leaf senescence, thereby ensuring functional photosynthesis in the source leaves to supply assimilates to the growing pods (Peet and Kramer 1980). Partial pod removal at the mid-R5 stage reduced pod numbers by 21–29 %. Although seed size increased, it was not enough to compensate for the reduced pod and seed number ultimately impacting yield (Board and Harville 1998). Depodding to increase assimilate supply to the remaining seed usually increases seed size (weight per seed; McAlister and Krober 1958; Hicks and Pendleton 1969; Egli and Leggett 1976; Munier-Jolain et al. 1998), but does not always change individual seed growth rates (SGR; Egli and Leggett 1985; Munier-Jolain et al. 1998).

Studies have shown that increasing the number of sinks or the size of the sink can effectively increase yield (Coombe 1976; Ho 1984; Jenner 1985; Patten et al. 1986). A larger sink was created by increasing the number of isolated nodes fed by a single leaf from one to three. This was achieved by girdling at respective nodal posi-

tions. Increasing sink size did not affect the carbon exchange rate or leaf chlorophyll levels. Increasing the sink size above its normal level does not seem to influence leaf photosynthesis. Although increasing sink number increases yield, the challenge is sink retention on the plants. Soybean is known for its abundance of flowers, but 80% floral/pod abortion is observed (Shibles et al. 1975) resulting in about 30% yield losses.

Biotechnology approaches may, however, provide a solution. Soybean relies predominantly on carbon and nitrogen balance and assimilate import into the developing seeds. Increasing the number of sinks and reducing the rate of flower and pod abscission may also provide an additional approach (Nunes-Nesi et al. 2010; Ainsworth et al. 2012). Enhancing the activity of sucrose transporters for sugar transport has been attempted. Sucrose is transported from the mesophyll tissue to the reproductive sink and is unloaded into the apoplastic space between maternal and filial structures, where it is then taken up by the developing seeds (Thorne 1985; Patrick 1997; Weber et al. 2005; Zhang et al. 2007b). Increasing transport during early stages of pod development is beneficial. Expression of potato sucrose transporter 1 (SUT1) in pea increased sucrose transport to the cotyledons but did not alter yield (Rosche et al. 2002, 2005). It is essential to identify the appropriate sucrose transporter operably linked to a stage-specific promoter to achieve maximum yield. Nitrogen from the leaves is transported to the seeds in the form of amino acids (Rentsch et al. 2007). The importance of amino acid transporters in increasing seed nitrogen concentrations comes from studies on AAP1 and AAP2 (amino acid transporter 1 and amino acid transporter 2; Sanders et al. 2009; Zhang et al. 2010c). To date, about 60 amino acid and nitrogen transporters have been identified in *Arabidopsis*. While these transporters can be potential targets, it is also important to ensure that manipulation of the amino acid transporters results in increased seed protein levels using appropriate expression elements to ultimately increase grain yield.

Independent mechanisms have been discussed for enhancing either the source capacity or sink strength. Multiple approaches such as exposure to high CO_2, overexpression of Calvin cycle enzymes, reducing photorespiration, or continuous light treatment have been used to enhance source capacity. It has been observed that under field situations a higher photosynthetic source capacity could result in a negative feedback mechanism leading to reduced leaf photosynthesis (Okita et al. 2001; Smidansky et al. 2002, 2007) ultimately with little or no impact on yield. A balanced improvement in both source and sink becomes essential to improve productivity. Improving "intrinsic yield" by improving growth under optimal conditions poses a challenge as it is a complex multigenic trait. This opens up another opportunity, that is, addressing abiotic and biotic environmental factors. Given that typical field conditions are suboptimal environments (uncontrolled environments, prone to stochastic environmental change), improving growth and development by manipulating genes (Appendix Tables 4.1 and 4.2) that are identified under controlled laboratory conditions as "stress related" may lead to increased on-farm yield.

Table 4.1 Improving yield opportunity through engineering genes involved in source/sink relationships

Gene name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested/Approach	Reference
Va N-35	Uricase	Moth bean	Ureide biosynthesis	Antisense (CaMV35S (constitutive))	CE	Na-Gyong et al. 1993
Zm AGP	Adenosine diphosphoglucose pyrophosphorylase	Corn	Starch synthesis	Mutant	FT	Giroux et al. 1996
At PHYA	Phytochrome A	Tobacco	Photosynthesis	CaMV35S (constitutive)	CE and FT	Robson et al. 1996
Sc SUC2	Apoplastic invertase	Potato	Sucrose metabolism	Class I patatin (tuber)	CE	Sonnewald et al. 1997
Zm SPS	Sucrose phosphate synthase	Tomato	Starch synthesis	RbcS (leaf)	CE	Murchie et al. 1999
Syn PsaK	Photosystem I subunit K	Synechocystis	Electron transport	Mutant	CE	Nakamoto and Hasegawa 1999
At PhyB	Phytochrome B	Potato	Photosynthesis	CaMV35S (constitutive)	CE	Thiele et al. 1999
At SUT3	Sucrose transporter 3	*Arabidopsis*	Sucrose transport	SUT3 (phloem)	CE	Meyer et al. 2000
At Psa E	Photosystem I subunit E	*Arabidopsis*	Photosystem II efficiency	Mutant	CE	Varatto et al. 2000
Cr Psb Z	Photosystem II subunit Z	Chlamydomonas	Electron transport	Mutant	CE	Swiatek et al. 2001
Nt Psb Z	Photosystem II subunit Z	Tobacco	Electron transport	Mutant	CE	Swiatek et al. 2001
Zm AGP	Adenosine diphosphoglucose pyrophosphorylase	Wheat	Starch synthesis	CaMV35S (constitutive)	CE	Smidansky et al. 2002
At LAS	Lateral suppressor	*Arabidopsis*	Branching	Mutant	CE	Greb et al. 2003

Table 4.1 (continued)

Gene name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested/ Approach	Reference
At ARGOS	Auxin-regulated gene involved in organ Size	*Arabidopsis*	Biomass	CaMV35S (constitutive)	CE	Hu et al. 2003
So SUT	Sucrose transporter	Potato	Sucrose transport	CaMV35S (constitutive)	CE and FT	Leggewie et al. 2003
Zm AGP	Adenosine diphosphoglucose pyrophosphorylase	Rice	Starch synthesis	Sh2 (endosperm)	CE	Smidansky et al. 2003
At MAX4	More axillary 4	*Arabidopsis*	Branching	CaMV35S (constitutive)	CE	Sorefan 2003
At ERECTA	Erecta	*Arabidopsis*	Inflorescence architecture	Native (meristem)	CE	Shpak et al. 2003
Sv PEPC	Phosphoenol pyruvate carboxylase	*Arabidopsis*	Carbon fixation	CaMV35S (constitutive)	CE	Chen et al. 2004
Sc SUC2	Cell wall invertase	*Arabidopsis*	Sucrose metabolism	KnAT (meristem)	CE	Heyer et al. 2004
At PsaD	Photosystem I subunit D	*Arabidopsis*	Photosynthesis	Mutant	CE	Ihnatowicz et al. 2004
Ps Lhcb1–2	Light-harvesting chlorophyll binding 1–2	Tobacco	Photosynthesis	CaMV35S (constitutive)	CE	Labate et al. 2004
At MEX1	Maltose exporter 1	*Arabidopsis*	Starch conversion to sucrose	CaMV35S (constitutive)	CE	Niittyla et al. 2004
Cg PEPC	Phosphoenolpyruvate carboxylase	Purple broad vetch	Carbon fixation	Legumin B4 (seed)	CE	Rolletschek et al. 2004
Ps FBPase	Chloroplastic fructose-1,6-bisphosphatase	*Arabidopsis*	Carbon fixation	CaMV35S (constitutive)	CE	Sahrawy et al. 2004
At SUC5	Sucrose transporter 5	*Arabidopsis*	Sucrose transport	Mutant	CE	Baud et al. 2005

Table 4.1 (continued)

Gene name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested/Approach	Reference
At SBPase	Sedoheptulose-1,7-bisphosphatase	Tobacco	Carbon fixation	CaMV35S (constitutive)	CE	Lefebvre et al. 2005
At MINI3	Miniseed3	*Arabidopsis*	Seed development	Mutant	CE	Luo et al. 2005
Ec PGM	Phosphoglucomutase	Potato	Carbon fixation	B33 patatin (tuber)	CE	Lytovchenko et al. 2005
Ph LIF	Lateral shoot-inducing factor	Petunia	Branching	CaMV35S (constitutive)	CE	Nakagawa et al. 2005
Zm Ra2	Ramosa 2	Corn	Inflorescence architecture; meristem development	Mutant	FT	Bortiri et al. 2006
At ARL	ARGOS like	*Arabidopsis*	Biomass	CaMV35S (constitutive)	CE	Hu et al. 2006
At RAX1	Regulators of axillary meristem 1	*Arabidopsis*	Branching	MYB37 (Shoot tip)	CE	Keller et al. 2006
Ps PPF1	Post floral specific 1	Rice	Delayed senescence	CaMV35S (constitutive)	FT	Li et al. 2006
At AGPase	ADP-glucose pyrophosphorylase	*Arabidopsis*	Starch synthesis	CaMV35S (constitutive)	CE	Obana et al. 2006
At UPS5l	Ureide permease 5 (long isoform)	Yeast	Ureide transport	Not available	CE	Schmidt et al. 2006
At UPS5s	Ureide permease 5 (short isoform)	Yeast	Ureide transport	Not available	CE	Schmidt et al. 2006
At UPS2	Ureide permease 2	Yeast	Ureide transport	Not available	CE	Schmidt et al. 2006
At SUC9	Sucrose transporter 9	*Arabidopsis*	Sucrose transport	AtSUC1 (pollen)	CE	Sivitz et al. 2006

Table 4.1 (continued)

Gene name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested/ Approach	Reference
Cr SBPase	Sedoheptulose-1,7-bi-phosphatase	Tobacco	Photosynthesis	rbcS (leaf)	CE	Tamoi et al. 2006
Gm SBP2	Sucrose-binding protein 2	Tobacco	Sugar transport	Antisense (CaMV35S (constitutive))	CE	Waclawovsky et al. 2006
Os SBPase	Sedoheptulose-1,7-bisphosphatase	Rice	Carbon fixation	Ubiquitin (constitutive)	CE	Feng et al. 2007
So SPS	Sucrose phosphate synthase	Cotton	Sucrose synthesis	CaMV35S (constitutive)	CE	Haigler et al. 2007
Os AGPS2b	ADP-glucose pyrophosphorylase small subunit 2b	Rice	Starch synthesis	Mutant	FT	Lee et al. 2007
Vn GPT1	Glucose-6-phosphate/ phosphate translocator 1	Purple broad vetch	Carbohydrate transport	Legumin B4 (seed)	CE	Rolletschek et al. 2007
Os SUT1	Sucrose transporter 1	Rice	Sugar transport	OsSUT1 (phloem)	CE	Scofield et al. 2007
Os Rcn1	Reduced culm number 1	Rice	Inflorescence architecture	Breeding study	FT	Yasuno et al. 2007
Os Du1	Dull endosperm 1	Rice	Starch synthesis	Mutant	FT	Zeng et al. 2007
Hv AlaAT	Alanine aminotransferase	Canola, Rice	Nitrogen assimilation	Btg26	FT, CE	Good et al. 2007; Shrawat et al. 2008
St SUT4	Sucrose transporter 4	Potato	Sucrose transport	CaMV35S (constitutive)	CE	Chincinska et al. 2008
Zm SPS	Sucrose phosphate synthase	Potato	Sucrose synthesis	Not available	FT	Ishimaru et al. 2008

Table 4.1 (continued)

Gene name	Gene description	Trans-genic host	Mode-of-action classification	Promoter	Environment tested/ Approach	Reference
Os cFBP1	Cytosolic fructose-1,6-bisphos-phatase 1	Rice	Photosyn-thesis	Mutant	Not available	Lee et al. 2008
Zm TB1	Teosinte branched 1	Wheat	Inflo-rescence architecture	Ubiquitin (constitutive)	CE	Lewis et al. 2008
Zm SXD1	Sucrose export defective 1	Corn	Carbo-hydrate transport	Mutant	FT	Ma et al. 2008
At SPS	Sucrose phosphate synthase	Tobacco	Sucrose synthesis	CaMV35S (constitutive)	CE	Park et al. 2008
Os GIF1	Grain incomplete filling 1	Rice	Carbon partitioning	CaMV35S (constitutive) or rice waxy (endosperm)	FT	Wang et al. 2008
Os GIF2	Grain incomplete filling 1	Rice	Carbon partitioning	Native gene (endosperm)	FT	Wang et al. 2008
Os Ghd7	Grain number, plant height, heading date	Rice	Seed devel-opment	Ubiquitin (constitutive)	FT	Xue et al. 2008
At RCA	RuBisCO activase	*Arabi-dopsis*	Photosyn-thesis	CAB3 (leaf)	CE	Kumar et al. 2009
Os HTD2	High tiller-ing dwarf 2	Rice	Inflo-rescence architecture	Mutant	FT	Liu et al. 2009a
At GAPCp1	Glyceral-dehyde-3-phosphate dehydro-genase of plastid 1	*Arabi-dopsis*	Carbon fixation	CaMV35S (constitutive)	CE	Munoz-Ber-tomeu et al. 2009
Os spd6	Small panicle and dwarfness	Rice	Inflo-rescence architecture	Breeding study	FT	Shan et al. 2009
Os RBCS	RuBisCO small subunit	Rice	Carbon fixation	Native (leaf)	CE	Suzuki et al. 2009

Table 4.1 (continued)

Gene name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested/ Approach	Reference
Os ARGOS	Auxin-regulated gene involved in organ size	Rice	Biomass	Not available	CE	Wang et al. 2009a
Os ARGOS	Auxin-regulated gene involved in organ size	*Arabidopsis*	Biomass	Not available	CE	Wang et al. 2009a
Os EP	Erect panicle	Rice	Inflorescence architecture	Breeding study	FT	Wang et al. 2009b
At GPT2	Glucose-6-phosphate/ phosphate translocator 2	*Arabidopsis*	Photosynthesis	Mutant	CE	Kunz et al. 2010
Os PEPC	Phosphoenolpyruvate carboxylase	Rice	Carbon fixation	Knockdown	CE	Masumoto et al. 2010
Cm SPS1	Sucrose phosphate synthase 1	Melon	Sucrose synthesis	CaMV35S (constitutive)	CE	Tian et al. 2010
Le CCD7	Carotenoid cleavage dioxygenase 7	Tomato	Branching	Figwort mosaic virus (constitutive)	CE	Vogel et al. 2010
Hv SUT1	Sucrose transporter 1	Barley	Sucrose transport	Hordein B1 (Endosperm specific)	CE	Weichert et al. 2010
Ptr SUS1	Sucrose synthase 1	*Arabidopsis*	Sucrose metabolism	CaMV35S (constitutive)	CE	Xu and Joshi 2010
Bn SUT1	Sucrose transporter 1	Mustard	Sucrose transport	Breeding study	FT	Li et al. 2011a
Zm AGPase	ADP-glucose pyrophosphorylase	Corn	Starch synthesis	Zein (Endosperm specific)	FT	Li et al. 2011b
At SBPase	Sedoheptulose-1,7-bisphosphatase	Tobacco	Carbon fixation	CaMV35S (constitutive)	FT	Rosenthal et al. 2011

Table 4.1 (continued)

Gene name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested/ Approach	Reference
At SPSA1	Sucrose phosphate synthase A1	*Arabidopsis*	Sucrose synthesis	Mutant	CE	Sun et al. 2011
At TPT &At cFBPase	Triose phosphate/ phosphate translocator (TPT) and cytosolic fructose-1,6-bisphosphatase (cFBPase)	*Arabidopsis*	Sucrose synthesis	CaMV35S (constitutive)	CE	Cho et al. 2012
GmUPS1–1	Ureide permease	Soybean	Ureide transport	RNAi (FMV constitutive)	CE	Collier and Tegeder 2012
GmUPS1–2	Ureide permease	Soybean	Ureide transport	RNAi (FMV constitutive)	CE	Collier and Tegeder 2012
St SUS	Sucrose synthase	Cotton	Sucrose metabolism	Constitutive segment seven promoter (S7)	CE	Shou-Min et al. 2012
At ptAL	Plastidic fructose-1,6-bisphosphate aldolase	Tobacco	Carbon fixation	CaMV35S (constitutive)	CE	Uematsu et al. 2012b
At PGM	Phosphoglucomutase	Tobacco	Starch synthesis	CaMV35S (constitutive)	CE	Uematsu et al. 2012a
Zm Da1–1		Corn	Seed development	Zm ubiquitin (constitutive)	CE	Wang et al. 2012

ADP adenosine diphosphate, *At Arabidopsis thaliana, Bn Brassica napus, CE* controlled environment, *cFBPase* cytosolic fructose-1,6-bisphosphatase, *Cg Corynebacterium glutamicum, Cm Cucumis melo, Cr Chlamydomonas reinhardtii*, *Ec Escherichia coli, FT* field, *FMV* fig mosaic virus, *Gh Gossypium hirsutum, Gm Glycine max, Hv Hordeum vulgare, Le Lycopersicon esculentum, Nt Nicotiana tabacum, Os Oryza sativa, Ph* petunia hybrid, *Ps Pisum sativum, Ptr Populus tremuloides, RNAi* RNA interference, *Sc Saccharomyces cerevisiae, So Spinacia oleracea, St Solanum tuberosum, SUT* sucrose transporter, *Sv Synechococcus vulcanus, Syn* synechocystis, *TPT* triose phosphate/phosphate translocator, *Va Vigna aconitifolia, Vn Vicia narbonensis, Zm Zea mays*

Table 4.2 Improving plant drought tolerance through functional and regulatory genes in conferring drought stress tolerance

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
Va P5CS	Pyrroline-5-carboxylate synthase	Tobacco	Osmolytes	CaMV35S (constitutive)	CE	Kavi-Kishor et al. 1995
Sc TPS1	Trehalose-6-phosphate synthetase	Tobacco	Osmolytes	CaMV35S (constitutive)	CE	Holmstrom et al. 1996
Np SOD	Mn superoxide dismutase	Alfalfa	Detoxification enzymes	CaMV35S (constitutive)	FT	McKersie et al. 1996
Cp IMT1	Myo-inositol o-methyl-transferase (D-ononitol synthesis)	Tobacco	Osmolytes	CaMV35S (constitutive)	CE	Sheveleva et al. 1997
Ec OtsA	Trehalose-6-phosphate synthase (trehalose synthesis)	Tobacco	Osmolytes	CaMV35S (constitutive)	CE	Pilon-Smits et al. 1998
Va P5CS	D1-pyrroline-5-carboxylate synthetase (proline synthesis)	Rice	Osmolytes	Stress inducible (AIPC-ABAinducible)	CE	Zhu et al. 1998
Bs SacB	Fructan	Sugar beet	Osmolytes	CaMV35S (constitutive)	CE	Pilon-Smits et al. 1999
Ms ALR	NADPH-dependent aldose/aldehyde reductase	Tobacco	Detoxification enzymes	CaMV35S (constitutive)	CE	Oberschall et al. 2000
Os CDPK7	CDPK	Rice	Protein kinases	CaMV35S (constitutive)	CE	Saijo et al. 2000
Hv HVA1	LEA-late embryogenesis abundant protein	Wheat	LEA genes	Maize Ubi-1P ubiquitin (constitutive)	CE	Sivamani et al. 2000
Gm BiP	Endoplasmic reticulum-binding protein (BiP)	Tobacco sense and antisense plants	Chaperones	CaMV35S (constitutive)	CE	Alvim et al. 2001

Table 4.2 (continued)

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
As ADC	Arginine decarboxylase	Rice	Osmolytes	ABA inducible	CE	Roy and Wu 2001
Ec OtsA+ OtsB	Trehalose	Rice	Osmolytes	ABA-inducible element ABRC1 coupled with a minimal rice actin 1	CE	Garg et al. 2002
At DREB1B/ CBF1	DREB1/CBF	Tomato	Transcription factors (AP2/ERF family)	CaMV35S (constitutive)	CE	Hsieh et al. 2002
Zm NADP-ME	NADP-malic enzyme which converts malate and NADP to pyruvate, NADPH, and CO_2	Tobacco	Osmolytes	Modified mannopine synthase (guard cell)	CE	Laporte et al. 2002
Hv HVA1	Group 3 LEA protein gene	Oat	LEA genes	CaMV35S (constitutive)	CE	Maqbool et al. 2002
Nt AQP1	PIP1 plasma membrane aquaporin	Tobacco	Water channels, transporters	CaMV35::antisense to PIP1 (constitutive)	CE	Siefritz et al. 2002
Ec TPSP	Bifunctional fusion of the trehalose-6-phosphate (T-6-P) synthase (TPS) and T-6-P phosphatase (TPP) of Escherichia coli	Rice	Osmolytes	Zm Ubi1, ubiquitin (constitutive)	CE	Jang et al. 2003
Ph ZPT2–3	Cys2/ His2-type Zinc–finger	Petunia	Transcription factors (Zinc–finger family)	CaMV35S (constitutive)	CE	Sugano et al. 2003
At APX	Ascorbate peroxidase	Tobacco	Detoxification enzymes	CaMV35S (constitutive)	CE	Badawi et al. 2004
Ds ADC	Polyamine synthesis	Rice	Osmolytes	Zm Ubi1, ubiquitin (constitutive)	CE	Capell et al. 2004

Table 4.2 (continued)

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
At DREB1A/ CBF3	DREB1/CBF	Tobacco	Transcription factors (AP2/ERF family)	rd29a (drought inducible)	CE	Kasuga et al. 2004
Os RWC3	Aquaporin overexpression	Rice	Water channels, transporters	SWPA2 (stress inducible)	CE	Lian et al. 2004
At DREB1A/ CBF3	DREB1/CBF	Wheat	Transcription factors (AP2/ERF family)	rd29a (drought inducible)	CE	Pellegrineschi et al. 2004
Nt NPK1	MAPKKK	Corn	Protein kinases	CaMV35S (constitutive)	CE	Shou et al. 2004
St PPO	Polyphenol oxidases antisense	Tomato	Osmolytes	CaMV35S (constitutive)	CE	Thipyapong et al. 2004
Hv HVA1	Group 3 LEA protein gene	Wheat	LEA genes	CaMV35S (constitutive)	FT	Bahieldin et al. 2005
At DREB1B/ CBF1	DREB1/CBF	Tomato	Transcription factors(AP2/ ERF family)	CaMV35S (constitutive)	CE	Bartels and Sunkar 2005
At lew2	Wilting allele, encodes a subunit of a cellulose synthesis complex	Mutant information: leaf wilting 2–1 and leaf wilting 2–2	Regulatory genes	Mutant	CE	Chen et al. 2005
Sc TPS1	Trehalose-6-phosphate synthase (TPS1)	Tomato	Osmolytes	CaMV35S (constitutive)	CE	Cortina and Culianez-Macia 2005
Gm P5CR	Pyrroline carboxylate reductase (proline accumulation)	Soybean	Osmolytes	heat-shock inducible	CE	Kocsy et al. 2005
At FAD3 (cytosolic) and At FAD8 (chloroplastic)	Increased fatty acid desaturation	Tobacco	Regulatory genes	CaMV35S (constitutive)	CE	Meng et al. 2005

Table 4.2 (continued)

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
At CBF3 AND ABF3	Transcription factor	Rice	Regulatory genes	CaMV35S (constitutive)	CE	Oh et al. 2005
At P5CS, Os P5CS	Pyrroline carboxylate synthase (proline synthesis)	Petunia	Osmolytes	CaMV35S (constitutive)	CE	Yamada et al. 2005
Le TERF1	Ethylene-responsive factor 1	Tobacco	Transcription factor (ERF family)	CaMV35S (constitutive)	CE	Zhang et al. 2005
Ca CAP2	Transcription factor	Tobacco	Transcription factor (ERF family)	CaMV35S (constitutive)	CE	Shukla et al. 2006
Va P5CSF129A	Δ1-pyrroline-5-carboxylate synthetase	Tobacco	Osmolytes		CE	Gubis et al. 2007
Sc TPS1 and Sc TPS2	Trehalose-6-phosphate synthase 1 and 2	Tobacco	Osmolytes	Drought-stress-induced pAtRAB18 and constitutive pAtRBCS1A	CE	Karim et al. 2007
Va P5CS	Δ1-pyrroline-5-carboxylate synthetase	*Triticum aestivum* L. cv. CD200126	Osmolytes	Stress inducible (AIPC-ABA inducible)	CE	Vendruscolo et al. 2007
Ta PP2Ac-1	Catalytic subunit (c) of protein phosphatase 2A		Protein phosphatase	CaMV35S (constitutive)	CE	Xu et al. 2007
WXP1;WXP2	Epicuticular wax accumulation		Osmolytes	CaMV35S (constitutive)	CE	Zhang et al. 2007a
At DREB1A/ CBF3	Dehydration-responsive element-binding protein	*Festuca arundinacea* Schreb	Transcription factor (DREB family)	rd29A promoter (drought inducible)	CE	Zhao et al. 2007
Bs cspB	Cold shock protein	Corn	Chaperones	Os Actin1 (constitutive)	FT	Castiglioni et al. 2008
Ta Ub2	Ubiquitin 2	Tobacco	Protease	CaMV35S (constitutive)	CE	Guo et al. 2008

Table 4.2 (continued)

Gene Name	Gene description	Trans-genic host	Mode-of-action classification	Promoter	Environment tested	Reference
Os iSAP8	Stress-associated protein	Rice/Tobacco	Transcription factor/regulatory protein	Ubiquitin (constitutive)	CE	Kanneganti and Gupta 2008
SodERF3	Ethylene responsive factor 3	Tobacco	Transcription factor (ERF family)	CaMV35S (constitutive)	CE	Trujillo et al. 2008
Bv CMO	Choline monooxygenase	Tobacco	Osmolytes	CaMV35S (constitutive)	CE	Zhang et al. 2008
At AVP1	H+ -PPase	Alfalfa	Osmolytes	CaMV35S (constitutive)	CE	Bao et al. 2009
Gh DREB	AP2/ERF	Wheat	Transcription factor (AP2/ERF)	Zm ubiquitin and At rd29A (drought inducible)	CE	Gao et al. 2009
Os SKIP1	Transcript splicing SKI-interacting protein	Rice	Regulatory genes	CaMV35S (constitutive)	CE	Hou et al. 2009
Os ZFP245	Zinc finger protein	Rice	Transcription factor (zinc finger)	CaMV35S (constitutive)	CE	Huang et al. 2009a
Os DST	DST	Rice	Transcription factor (zinc finger)	RNAi	CE	Huang et al. 2009b
Os DHODH1	Dihydroorotate dehydrogenase	Rice	Osmolytes	CaMV35S (constitutive)	CE	Liu et al. 2009b
Os bZIP72	AREB bZIP	Rice	Transcription factor (AREB bZIP)	CaMV35S (constitutive)	CE	Lu, et al. 2009
Th TsVP	H+ -PPase	Cotton	Osmolytes	CaMV35S (constitutive)	CE	Lv et al. 2009
Os AP37	AP2/ERF	Rice	Transcription factor (AP2/ERF)	Os Cc1 (constitutive)	FT	Oh et al. 2009
Gm BiPD	Bip	Soybean and Tobacco	Chaperones	Duplicated 35S+ alfalfa mosaic virus enhancer	CE	Valente et al. 2009

Table 4.2 (continued)

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
At FTA	Farnesyl-transferase	Canola	ABA sensing	RNAi with AtHPR1 promoter (drought induced in shoot)	FT	Wang et al. 2009c
Os WRKY11	WRKY	Rice	Transcription factor (WRKY)	HSP101 (heat inducible)	CE	Wu et al. 2009
At LOS5	LOS5/ABA3	Rice	ABA biosynthesis	Os HVA22P (stress inducible) and Os Actin1 (constitutive)	FT	Xiao et al. 2009
At CBF3	AP2/ERF	Rice	Transcription factor (AP2/ERF)	Os HVA22P (stress inducible) and Os Actin1 (constitutive)	FT	Xiao et al. 2009
At NPK1	MAP kinase	Rice	Protein kinases	Os HVA22P (stress inducible) and Os Actin1 (constitutive)	FT	Xiao et al. 2009
At NHX1	Na+/H+ antiporter	Rice	Water channels, transporters	Actin1 (constitutive)	FT	Xiao et al. 2009
At SOS2	Ser/Thr kinase	Rice	Protein kinases	Os HVA22P (stress inducible) and Os Actin1 (constitutive)	FT	Xiao et al. 2009
Os GH3	IAA amido synthetase	Rice	Osmolytes	CaMV35S (constitutive)	CE	Zhang et al. 2009
Os NAC45	NAC	Rice	Transcription factor (NAC)	CaMV35S (constitutive)	CE	Zheng et al. 2009
Os DSM2	Beta-carotene hydroxylase	Rice	Osmolytes	CaMV35S (constitutive)	CE	Du et al. 2010
Le SlAREB	AREB bZIP	Tomato	Transcription factor (AREB bZIP)	CaMV35S (constitutive)	CE	Hsieh et al. 2010
Os NAC10	NAC	Rice	Transcription factor (NAC)	RCC3 (root)	FT	Jeong et al. 2010
Gs GST	Glutathione S transferases	Tobacco	Detoxification enzymes	CaMV35S (constitutive)	CE	Ji et al. 2010

Table 4.2 (continued)

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
Os DSG1	E3 ligase	Rice	Protease	RNAi	CE	Park et al. 2010
Le TSRF1	AP2/ERF	Rice	Transcription factor (AP2/ERF)	CaMV35S (constitutive)	CE	Quan et al. 2010
Os NAC5	NAC	Rice	Transcription factor (NAC)	Zm ubiquitin (constitutive)	CE	Takasaki et al. 2010
Ah BADH	Betainealdehyde dehydrogenase	Wheat	Osmolytes	Zm ubiquitin (constitutive)	CE	Wang et al. 2010
Ts CBF1	AP2/ERF	Corn	Transcription factor (AP2/ERF)	Zm ubiquitin (constitutive)	CE	Zhang et al. 2010d
Le JERF1	AP2/ERF	Rice	Transcription factor (AP2/ERF)	ABA induced CaMV35S	CE	Zhang et al. 2010a
Le JEFR3	AP2/ERF	Rice	Transcription factor (AP2/ERF)	CaMV35S (constitutive)	CE	Zhang et al. 2010b
At HARDY	AP2/ER	*Trifolium alexandrinum*	Transcription factor (AP2/ERF)	CaMV35S (constitutive)	FT	Abogadallah et al. 2011
Ca XTH3	Endo-transglucosylase/hydrolase	Tomato	Osmolytes	CaMV35S (constitutive)	CE	Choi et al. 2011
Os SDIR1	E3 ligase	Rice	Protease	RNAi	CE	Gao et al. 2011b
Gm bZIP1	AREB bZIP	Tobacco and wheat	Transcription factor (AREB bZIP)	Tobacco: CaMV35S (constitutive) and rd29A (drought); wheat: ubiquitin (constitutive)	CE	Gao et al. 2011a
Ec betA	Choline dehydrogenase	Wheat	Osmolytes	Zm ubiquitin (constitutive)	CE	He et al. 2011
At DREB1A/ CBF3	AP2/ERF	Lolium perenne	Transcription factor (AP2/ERF)	Zm ubiquitin (constitutive)	CE	Li et al. 2011c

Table 4.2 (continued)

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
Os DREB2A	AP2/ERF	Rice	Transcription factor (AP2/ERF)	rd29a (drought inducible)	CE	Mallikarjuna et al. 2011
Os SQS	Farnesyl-transferase/ squalene synthase	Rice	ABA sensing	RNAi	CE	Manavalan et al. 2012
At DREB1A/ CBF3	AP2/ERF	Wheat and barley	Transcription factor (AP2/ERF)	Double 35S and maize RAB17	CE	Morran et al. 2012
Os DIS1	E3 ligase	Rice	Protease	RNAi	CE	Ning et al. 2011
Ts VP	H+ -PPase	Cotton	Osmolytes	CaMV35S (constitutive)	FT	Pasapula et al. 2011
At IPT	IPT	Rice	Detoxification enzymes	SARK (stress and maturation induced)	CE	Peleg et al. 2011
Os bHLH148	bHLH	Rice	Transcription factor (bHLH)	Os Cc1 (constitutive)	CE	Seo et al. 2011
Os NAC6	NAC	Rice	Transcription factor (NAC)	CaMV35S (constitutive)	CE	Song et al. 2011
Ts BetA and Ts VP	H+ -Ppase+ choline dehydrogenase	Corn	Osmolytes	Zm ubiquitin (constitutive)	CE	Wei et al. 2011
Zm CBF3	AP2/ERF	Rice	Transcription factor (AP2/ERF)	Ubiquitin (constitutive)	CE	Xu et al. 2011
Ta NAC69	NAC	Wheat	Transcription factor (NAC)	Hv Dhn8s (constitutive)/ Hv Dhn4s (drought inducible)	CE	Xue et al. 2011
Sly-miRNA169c	miRNA169	Tomato	ABA sensing	CaMV35S (constitutive)	CE	Zhang et al. 2011b
Xo hrf1	Harpin	Rice	ABA sensing	CaMV35S (constitutive)	CE	Zhang et al. 2011a

Table 4.2 (continued)

Gene Name	Gene description	Transgenic host	Mode-of-action classification	Promoter	Environment tested	Reference
At HB7	Homeodomain-leucine zipper (HD-Zip) transcription factor	Tomato	Transcription factor (HD-Zip)	CaMV35S (constitutive)	CE	Mishra et al. 2012

ABA abscisic acid, *Ah Atriplex hortensis, As Avena sativa, At Arabidopsis thaliana, Bs Bacillus subtilis, Bv Beta vulgaris, Ca Cicer arietinum, Ca Capsicum annuum, CE* controlled environment, *Cp Craterostigma plantagineum, Ds Datura stramonium, Ec Escherichia coli, FT* field, *Gh Gossypium hirsutum, Gm Glycine max, Gs Glycine soja, Hv Hordeum vulgare, Le Lycopersicon esculentum, LEA* late embryogenesis abundant, *miRNA* microRNA, *Ms Medicago sativa, NADPH* reduced form of nicotinamide dinucleotide phosphate, *Np Nicotiana plumbaginifolia, Nt Nicotiana tabacum, Os Oryza sativa, Ph Petunia hybrid, Pv Phaseolus vulgaris, RNAi* RNA interference, *Sc Saccharomyces cerevisiae, St Solanum tuberosum, T-6-P* trehalose-6-phosphate, *Ta Triticum aestivum, Th Thellungiella halophila, TPS* trehalose-6-phosphate synthase, *Ts Triticum sativum, Va Vigna aconitifolia, Xo Xanthomonas oryzae, Zm Zea mays*

Stabilizing Soybean Yield

Grain yield is challenging to dissect as it is determined by a complex network of physiological, genetic, abiotic, and biotic factors. As discussed in the section "Soybean Development and Yield Potential," yield potential is defined as the maximum yield (seed dry matter) of a crop when grown with sufficient water, nutrients, and the absence of unfavorable abiotic and biotic environments (Evans and Fischer 1999). Soybean yield trends in the USA indicate that yield growth rates have not reached a plateau (Fig. 4.2). The average soybean yield in 2012 was 39.6 bu/a, but record yields reported from yield contests in the USA (Iowa, Missouri, and Nebraska 1966–1998) were greater than 67.5 bu/a and in one instance reached 160 bu/a (Specht et al. 1999), demonstrating that future yield growth is possible. Therefore, efforts to improve yield through yield stability (i.e., yield achieved through developing varieties that are less susceptible to variable environmental factors) will be important to increase the speed and ease by which on-farm yield gains are attained in the future.

Yield Stability Under Water-Limiting Conditions

Soybean uses about 450–700 mm of water during the growing season (Dogan et al. 2007; see the section "Global Soybean Production"). Among legumes, soybean is considered a drought-sensitive plant (Clement et al. 2008). Though water limitation affects all stages of soybean growth and development, the most critical stages that

impact yield are flowering and post-flowering (Tran and Mochida 2010; Valliyodan and Nguyen 2006).

Accumulated evidence indicates that soybean yield is more sensitive to water limitation during the early reproductive stage (flowering to early pod expansion; Boyer 1982; Westgate and Peterson 1993), when the rate of pod abortion increases, resulting in fewer pods per plant (Desclaux et al. 2000) ultimately decreasing seed yield (Kokubun et al. 2001). Water limitation between early flowering and early seed fill reduces determinate soybean seed yield primarily by reducing branch vegetative growth resulting in reduced branch seed yield (James et al. 2001). Severe water limitation throughout seed fill reduces seed number at a faster rate than seed mass, germination, or vigor (Dornbos et al. 1989). Soybean plants subject to continuous water deficit from R6 (early seed fill) until maturity lost leaf nitrogen and chlorophyll more rapidly than nonstressed plants (de Souza et al. 1997). The SFP (R7) occurred up to 7 days earlier, resulting in smaller seeds (maximum reduction of 32 %) and up to 44 % less yield.

The major physiological mechanisms adapted by plants to combat water limitation are drought escape, dehydration avoidance, and dehydration tolerance. Plants escape drought by completing their life cycle during periods of sufficient water. The life cycle is shortened and the plants also set seeds. An example of drought escape used in southern USA is the combination of an early soybean planting system with short-season varieties. The short season varieties planted in March or April begin flowering from late April to early May and set pods in late May, thus completing the reproductive cycle before the period of possible drought occurs in July or August (Heatherly and Elmore 2004). Dehydration avoidance is a mechanism where plants use strategies to maintain high water status, which includes efficient water absorption via longer roots or reduced evapotranspiration from the leaf surface by restricting leaf stomatal aperture. On the other hand, dehydration tolerance helps the plants maintain turgor and continue their metabolic activities by protoplasmic tolerance or synthesis of osmoprotectants, osmolytes, or compatible solutes (Turner et al. 2001).

As water limitation is the major abiotic factor affecting soybean yield potential and yield stability across all nonirrigated production areas, identifying traits associated with adaptation to water-limiting conditions and candidate genes governing these traits provide opportunities for increasing yield (Table 4.2).

Physiological Mechanisms Associated with Dehydration Avoidance and Dehydration Tolerance

Grain yield (Y) is a function of three components, viz., the amount of water transpired (T), WUE, and HI; $Y = T \times \mathrm{WUE} \times \mathrm{HI}$ (Turner et al. 2001). The primary strategy to improve yield under water-limiting conditions is maintenance of optimum transpiration, leading to increased WUE. The secondary traits reported to be associated with increasing or maintaining T during water limitation are phenology, photoperiod sensitivity, developmental plasticity, leaf area maintenance, heat tolerance, osmotic adjustment (OA), early vigor, rooting depth and density, and leaf

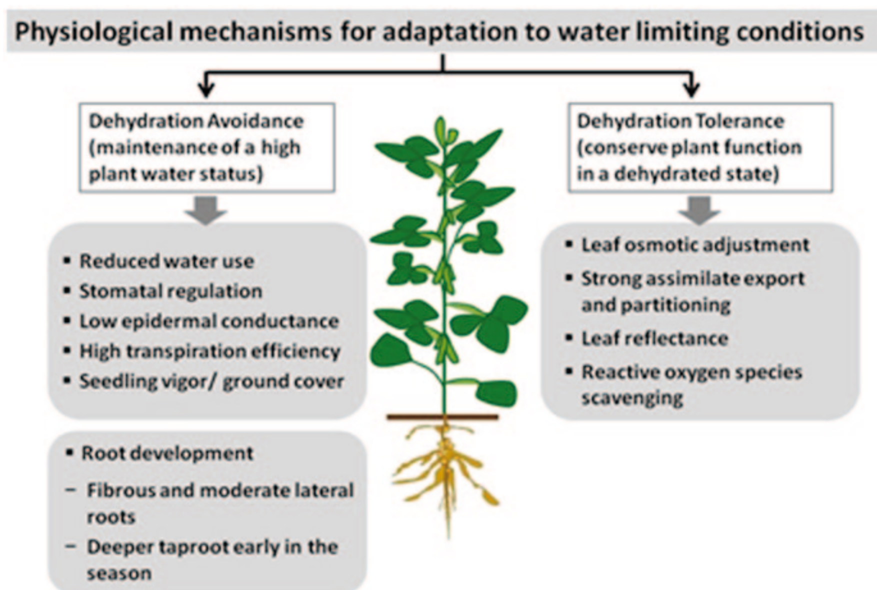

Fig. 4.5 Major physiological traits associated with increased growth and development under field conditions in the context of water availability in soybean

reflectance (Purcell and Specht 2004). These physiological traits act as indicators for resistance to water limitation. Key physiological mechanisms associated with increased growth and development under field conditions during periods of water limitation are shown in Fig. 4.5 and described below.

Reduced Water Use

Plants have evolved developmentally and physiologically to adapt to reduced water use under water limitation. When plant production is defined as a function of water use, it is described as WUE (biomass per unit of water used). WUE for grain yield is not a fixed entity. Genetic variations in WUE have been reported for field crops including soybean (Mian et al. 1996) and are driven mainly by variation in water use rather than by variation in plant production. The positive association between biomass yield and WUE in water-limiting environments suggests that improvement of the WUE of a crop plant should result in superior yield performance via maintenance of higher HI (Wright 1996), and strong assimilate partitioning to the developing sink.

Root Development

Longer taproots help in reaching water available in deeper layers of the soil. In addition, an extensive fibrous root system is useful for tapping subsoil surface moisture

and nutrients such as phosphorus. Water limitation increases biomass partitioning to roots, increasing the root to shoot ratio. An increase in root length was observed in nonirrigated soybeans compared to irrigated plants (Huck et al. 1983). As reported by Hirasawa et al. (1994), soybean plants experiencing water stress early in the growing season had higher seed yields than those exposed to water-limiting conditions after flowering, because the plants developed a larger root system before flowering to overcome the water stress condition. Selecting for traits that contribute to an improved root system would help soybean plants to withstand the intermittent water stress conditions that prevail during reproductive development.

Stomatal Regulation

Water limitation reduces the relative leaf expansion rate, stomatal conductance, and leaf turgor, whereas it increases the abscisic acid (ABA) content in the leaf and xylem (Liu et al. 2003). Decreased stomatal conductance coincided with an increase in xylem ABA and occurred before any significant change in leaf turgor was detected, indicating that chemical signals (root-derived ABA) control stomatal behavior at moderate soil water deficit. Regulation of stomatal aperture/conductance thereby controlling transpiration efficiency (TE) leads to an increase in WUE (Mian et al. 1996) and is a promising physiological trait for soybean grown under typical field conditions.

Osmotic Adjustment

OA, the active accumulation of compatible solutes that occurs in plant tissues in response to an increasing water deficit, helps in maintaining cell turgor, stomatal conductance, and photosynthesis; delays leaf senescence; and reduces flower abortion (Turner et al. 2001). The biosynthesis and accumulation of compatible solutes in response to water-deficit conditions is an important adaptive mechanism to enable restoration of cellular water status by maintaining cellular water potential, stabilizing membrane properties, and by protecting the cellular environment from reactive oxygen species (ROS). The compatible solutes include amines (polyamines, glycine betaine), amino acids (proline), sugars (trehalose, fructan), and sugar alcohols (trehalose, mannitol, galactinol; Rontein et al. 2002). Overproduction of osmoprotectants has been used as one of the approaches to improve abiotic stress tolerance in several target crops.

Epidermal Conductance

Leaf epidermal conductance (ge), the sum of cuticular conductance and any residual stomatal conductance, determines the rate of water loss from leaf tissues under severe water deficit when stomatal closure is maximal. A significant negative

correlation ($r = -0.74$) is reported between ge and WUE under drought in soybean (Hufstetler et al. 2007). Therefore, reduced epidermal conductance is a desirable trait for enhancing drought resistance in soybean.

Leaf Reflectance

Leaf pubescence (presence of hairs on the leaf) increases reflectance from the leaf resulting in lower leaf temperatures under high irradiance, restricted transpiration water loss due to increased leaf boundary layer resistance, and enhanced photosynthesis due to radiation being reflected lower into the canopy (Specht and Williams 1985). Denser pubescence lines are often associated with increased vegetative vigor, greater root density, and a deeper root extension (Garay and Wilhelm 1983). Hence, leaf pubescence density is an important adaptive trait for soybean under field conditions.

ROS Scavenging

ROS are natural by-products of oxygen metabolism and have roles in signaling and homeostasis. However, stress results in accumulation of ROS to toxic levels that negatively impact on plant growth and development. Plants have evolved scavenging systems comprising antioxidant enzymes such as super oxide dismutase (SOD), peroxidase (ascorbate peroxidase, APX), and catalase, and antioxidant compounds such as ascorbate and reduced glutathione. A balance between production and scavenging is essential and ultimately determines plant health (Kar 2011). Altering the activity of antioxidant enzymes by either overexpression or mutation revealed their importance in alleviating damage due to stress (Mittler et al. 2004; Asada 2006). Studies in *Arabidopsis* and *Nicotiana tabacum* plants with mutation in tAPX and Cu–Zn SOD confirm the need for a scavenging mechanism in photosynthesis (Yabuta et al. 2002; Rizhsky et al. 2003; Tarantino et al. 2005). However, the ultimate benefit for improving yield opportunity under field conditions remains unclear.

Signal Transduction Pathways and Genetic Engineering of Candidate Genes to Improve Plant Tolerance to Water-Limiting Conditions

Tolerance or susceptibility to water limitation is a complex phenomenon and may occur at any time during plant development. Under field conditions, multiple stresses simultaneously affect the plant. Therefore, the perception of abiotic stresses and signal transduction to switch on adaptive responses are critical steps in determining the growth and development of plants exposed to challenging environmental

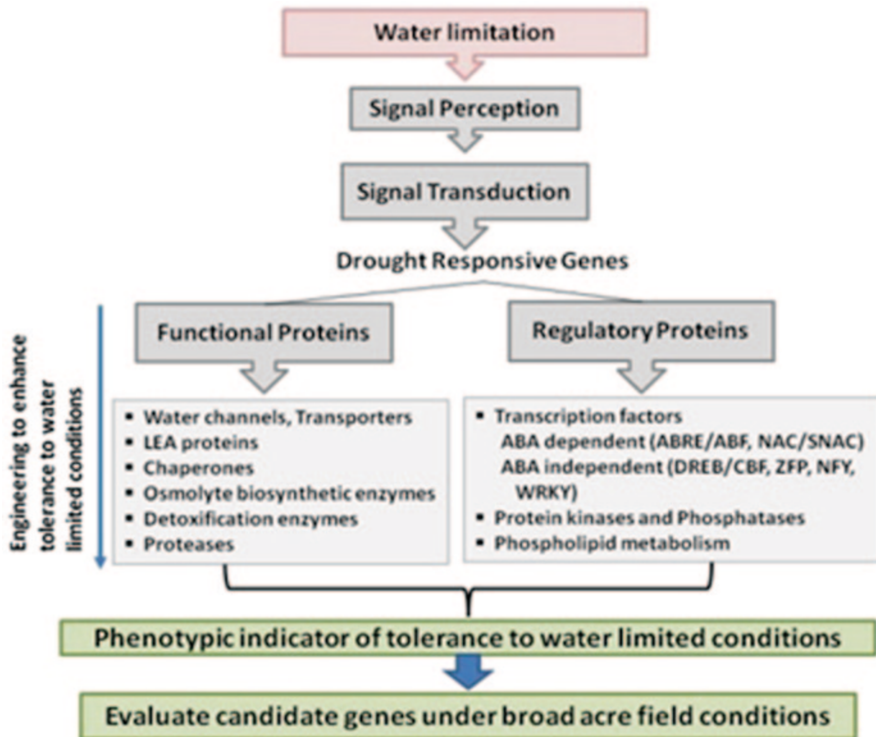

Fig. 4.6. Identification of drought-responsive genes using model system testing under controlled conditions which serve as candidates for improving yield stability in soybean. ABA abscisic acid, DREB dehydration-responsive element binding, LEA late embryogenesis abundant

conditions (Chinnusamy et al. 2004). During the last decade, analysis of model plant species (notably *Arabidopsis*) studied under controlled environmental conditions has led to the discovery of signaling and regulatory pathways that play an important role in response to water deficit (Fig. 4.6). Genes encoding functional and regulatory proteins identified from laboratory screens as impacting growth and development serve as candidates for testing under broad-acre field conditions. Field-grown soybean across all nonirrigated production areas frequently encounters suboptimal conditions (e.g., water limitation) during critical growth periods such as pod formation and seed filling. Thus, there is an opportunity to stabilize yield through managing water relations (drought tolerance or WUE) in the crop plant.

Several drought-responsive genes have been identified, providing the opportunity to modulate their expression in plants and adapt them to water-limiting conditions (Shinozaki and Yamaguchi-Shinozaki 1997). This may be achieved through ectopic expression, downregulation, protein modification, activation, or repression. The candidate genes are classified as:

a. Functional proteins: Genes encoding known enzymatic or structural functions. This includes enzymes for synthesis of osmoprotective compounds, late

embryogenesis abundant (LEA) proteins, chaperones, water channel proteins, ubiquitins, and detoxifying enzymes.

b. Regulatory genes: Genes encoding kinases, phosphatases and transcription factors.

Using transgenic approaches, many of these candidate genes have been expressed in crops of interest and tested under either controlled environment or field conditions. Some studies are discussed below where the transgenic plants have been tested in the field for their ability to survive and maintain yield under water-limiting conditions.

In an effort to identify genes for drought resistance, Xiao and coworkers (2009) expressed CBF3 (DREB1A), LOS5 (ABA3, involved in ABA biosynthesis), NCED2 (ABA biosynthesis), NHX1 (vacuolar Na+/H+ antiporter), SOS2 (CBL-interacting kinase 24/salt overly sensitive 2), ZAT10 (salt tolerance zinc finger, STZ), and NPK1 (MAPKKK) in rice under the control of a constitutive promoter or drought inducible promoter. Field-grown transgenic plants subjected to water limitation at the booting stage showed significantly greater relative yield and relative spikelet fertility compared to wild-type plants under similar conditions.

RNA chaperones are ubiquitous and abundant and are believed to play a role in sustaining active growth by favoring active transcription, translation, and/or ribosome assembly. Constitutive expression of two members of a family of bacterial RNA chaperones, *Escherichia coli* CspA and *Bacillus subtilis* CspB, has shown to confer abiotic stress tolerance in *Arabidopsis,* rice, and maize. Expression of these proteins did not result in any pleiotropic effect in terms of plant size, development, or productivity unlike that observed in other transgenic studies (Kim et al. 2009). Further, their expression was not associated with a yield penalty in high-yielding environments. Consistent with the timing of the water deficit, the positive impact on yield in corn was predominantly via an increase in kernel numbers. Yield was also stable across stress régimes and environments, thus confirming the ability of this family of proteins in delivering broad stress tolerance (Castiglioni et al. 2008).

Although single-gene approaches have yielded positive results, stacking strategies have also been tested to understand the synergistic effect of two or more genes in impacting yield. Wei and coworkers (2011) co-expressed betA (choline dehydrogenase involved in glycine betaine synthesis) from *Escherichia coli* and TsVP (V-H+ -PPase involved in proton pumping) from *Thellungiella halophila* in maize. Glasshouse-grown maize plants at the 10-leaf stage were subjected to water limitation by maintaining soil water content at 15–16% of field capacity. Even under water-limiting conditions, transgenic plants grew vigorously, had greater relative water content (RWC), accumulated more solutes, and had lower cell damage and increased yield compared to the wild-type plants, or plants expressing single genes. This study showed the feasibility of stacking genes that impact different metabolic pathways to enhance tolerance to abiotic stress.

Genetic Engineering of Candidate Genes to Improve Plant Tolerance to Combinatorial Stresses

Under field conditions, plants are often challenged by multiple stresses. Soybean is more sensitive to a combination of drought and heat stress that may occur during the growing season. A survey of all major US weather disasters between 1980 and 2004 (excluding hurricanes, tornadoes, and wildfires) demonstrated that the extent of damage caused by a combination of drought and heat stress is more than individual stresses (Mittler 2006).

Physiological characterization of plants subjected to drought stress, heat stress, or a combination of drought and heat stress reveals that the stress combination has several unique aspects, such as high respiration coupled with low photosynthesis or closed stomata with higher leaf temperatures. For example, during heat stress, plants increase their stomatal conductance in order to cool their leaves by transpiration. However, if heat stress is combined with water limitation, leaf temperatures would be 2–5 °C higher as transpiration is reduced (Rizhsky et al. 2002, 2004). Water-limiting conditions in tobacco and *Arabidopsis* suppress respiration and photosynthesis. Heat shock alone enhances respiration, but does not significantly alter photosynthesis. A combination of both water limitation and heat shock resulted in the suppression of photosynthesis. It has been observed that a combination of drought and heat stress involves the conversion of malate to pyruvate generating NADPH and CO_2, which is possibly recycled into the Calvin–Benson cycle, thereby alleviating the effects of stress on photosynthesis. Energy production in the mitochondria plays a key role in plant metabolism during a combination of drought and heat stress.

The dehydration-responsive element binding (DREB) subfamily of proteins belongs to the larger group of APETALA2/ethylene-responsive element-binding factor (AP2/ERF) proteins. Several stress-inducible DREB genes have been characterized and have been shown to regulate abiotic stress responses in plants. The dehydration-responsive element/C-repeat (DRE/CRT) elements in DREB genes are responsible for inducing stress responses (Mizoi et al. 2012). Class 2 DREB genes comprising eight members in *Arabidopsis* (Sakuma et al. 2002) and five in rice (Matsukura et al. 2010) are induced by dehydration, heat shock, and salinity (Sakuma et al. 2006). DREB2A and DREB2B are the major class 2 DREBs involved in the dehydration response. DREB2A and DREB2C have been shown to mediate a heat stress response through HsfA3 (heat shock factor A3; Yoshida et al. 2008; Chen et al. 2010). In addition to improving tolerance to single stresses, studies have shown that DREB2A functions in both dehydration and heat stress response (Rizhsky et al. 2004). Overexpression of a constitutively active form of DREB2A (rendered by deletion of the negative regulatory domain) induced expression of drought- and heat-stress-responsive genes and the transgenic plants perform better under both water limitation and heat stress (Sakuma et al. 2006).

It is well established that proline accumulates under drought stress and maintains turgor potential of the cells. In addition, studies have shown that proline plays a role in the detoxification of ROS (Floyd and Nagy 1984). Koscy and coworkers (2005) expressed pyrroline-5-carboxylate reductase (P5CR), the final enzyme in proline synthesis in both sense and antisense directions in soybean. Transformants were subjected to simultaneous drought and heat stresses which induced oxidative stress. While the antisense transformants exhibited more injury, enhanced levels of lipid peroxidation, reduced relative water content, and low levels of proline, transformants with enhanced levels of P5CR had higher proline levels, showed reduced injury symptoms, and had lipid peroxidation. This study shows that manipulation of proline levels may help plants tolerate a combination of drought and heat stress.

Microarray studies (Rizhsky et al. 2002, 2004) revealed that the molecular and metabolic response of plants to a combination of drought and heat is unique. Tolerance to a combination of different stresses is likely to be a complex trait involving multiple pathways and cross talk between different sensors and signal transduction pathways. Therefore, combined water and heat stress should be addressed as a new state of abiotic stress and not simply the sum of two different stresses (Mittler and Blumwald 2010). This aspect should be considered when developing transgenic crops.

Candidate genes discussed for drought stress and the combinatorial stresses focus on testing in model plant systems and rice. Studies in soybean are limited and the testing strategy is mostly under controlled environmental conditions. However, candidate genes tested in the model plant systems could serve as potential targets for enhancing drought stress resistance under field conditions. A comprehensive list of genes tested for their ability to impart abiotic stress (water stress and heat stress) tolerance in crops is provided in Table 4.2.

Concluding Remarks

Soybean is the world's most widely grown legume and provides an important source of protein and oil. Soybean production has increased incrementally over the past century through grower adoption of agricultural innovation in the form of agronomic, management practices and genetic improvements. In order to meet the needs of a growing world population without unsustainable expansion of the land area devoted to this crop, new soybean varieties need to have greater intrinsic yield potential and perform well under reduced agronomic inputs.

Soybean breeding programs offer a basis for transgenic yield trait development. Breeders have successfully and steadily made genetic gains in yield wherein the mean performance has increased over time (Specht et al. 1999). Based on findings from several crops, a theme is now emerging that genetic changes that historically have resulted in crop domestication and improvement in conventional varieties have been achieved typically through the selection of plant genes encoding regulatory proteins and modulation of crop physiology (Doebley et al. 2006). Similarly,

advances in plant genomics and systems biology, including the availability of complete genome sequences for both *Arabidopsis* and rice, have identified regulatory genes and networks that control plant physiology. Because regulatory genes naturally modulate cellular processes, they are expected to be candidates for modifying complex traits in crop plants, and play a prominent part of the next generation of biotechnology crops (Century et al. 2008). Thus, the experience of plant breeders in achieving increases in yield over time serves as a valuable guide and resource to molecular biologists attempting to transfer effects observed in controlled environments, to yield on a per-unit basis in field-grown crops such as soybean.

It is predicted that development of new high-yielding soybean varieties will be achieved through targeting yield potential and yield stability. These targets include alteration of source–sink relationships or modulation of responses to abiotic and biotic environmental factors. Transgenes representing a variety of pathways that improve growth and development have been identified from model species testing in controlled environments. Many of these targets have, however, not been tested in soybean or other crops under field conditions. Thus, there is a critical need to bridge the gap between basic science and applied research in the field to enhance yield.

The future growing conditions for soybean will likely be warmer; precipitation is expected to be more variable; and pests, pathogens and weed competition will be altered (Ainsworth et al. 2012). Multiple strategies will therefore be required to stabilize yield under suboptimal conditions encountered across the diversity of environments. Although single-gene approaches have proven successful, the combination of biotechnological traits through "gene stacking" has the potential to further crop improvement (Halpin 2005). Genetically engineered (GE) stacks (also known as stacked or combined events) are produced by combining two or more single transgenic events by conventional breeding and thus offer multiple trait combinations, such as insect control and herbicide tolerance (Que et al. 2010). A system that integrates these next-generation GE stacks with advances in molecular breeding techniques and agronomic practices is predicted to deliver future soybean yield gains.

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Chapter 5
Towards Using Biotechnology to Modify Soybean Seeds as Protein Bioreactors

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Introduction: Changing Seed Protein Composition as a Goal of the Next "Green Revolution"

From a historic perspective, the production of proteins in plant seeds has provided a primary food source and nitrogen input for people and animal feed. Breeding efforts leading to the selection of protein-rich seeds represents a key development in agriculture, creating food surpluses, and making concentrated human populations possible through centralized and distributed food sources. Protein production is ultimately controlled by available nitrogen and the capacity of plants to convert this nitrogen to protein. Among the major crops, few are high-protein plants (Table 5.1), and among these, soybean reigns supreme as the major global protein-source commodity, with almost 300 million t of annual global production (www.soystats.com/2011). Other major crops that produce high concentrations of starch or oil are fundamentally different, as their storage products are the direct end products of photosynthesis containing little nitrogen. The latter is fundamental to human and animal nutrition. While oils, starch, and biomass are basic commodities, it is protein that provides essential nutrients to build muscles and other tissues for animal production and human health. In modern times, proteins have become a much broader and essential commodity beyond food and animal feed. Proteins, in the form of enzymes, are a multibillion-dollar global commodity used in a wide range of activities, from processing foods, cleaning, and converting one type of a material to another. Many of the industrial enzymes are microbial in origin, and

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Table 5.1 Protein content of common crops

Crop plant	Protein (% weight)
Soybean (*Glycine max*)	38
Pea (*Pisum sativum*)	23
Bean (*Phaseolus vulgaris*)	22
Chickpea (*Cicer arietinum*)	20
Broad bean (*Vicia faba*)	23
Lentil (*Lens culinaris*)	25
Maize (*Zea mays*)	5–12
Wheat (*Triticum aestivum*)	10–15
Rice (*Oryza sativa*)	3
Barley (*Hordeum vulgare*)	10
Potato (*Solanum tuberosum*)	8

infrastructure exists for large-scale fermentation of bacterial and fungal cells and subsequent harvesting of the enzymes to varying degrees of purity depending on the intended end use. Other enzymes are derived from plant sources purified directly from organs that are enriched sources of the target protein. Enzymes are but one possible type of heterologous proteins targeted to be produced by plant platforms. Other potentially useful proteins include medically active proteins such as vaccines, now produced routinely in microbial or animal tissue culture systems. The technology to alter seed protein content may also be used to change both seed protein quality and quantity, both useful to enhance animal feed as that is the primary end use of soybean proteins. One of the early goals of plant biotechnology, beginning in the 1980s, was to improve food and feed protein composition by enhancing amino acid balance, digestibility, or by removing antinutritional proteins and allergens. Now 30 years later, the technology and the underlying knowledge of plant biology are on the threshold of bringing these first goals of plant biotechnology to their full potential.

Plant-based protein production systems or plant-source protein bioreactors will become more important as the increasing need for enzymes, medical proteins, and improved protein to meet food or feed challenges continues to rise. Producing proteins on an agricultural scale represents a potential cost–benefit that cannot be matched by the existing fermentation-based production systems. Advantages include increased ease of delivery and scale-up, and decreased risk of contamination with animal and human pathogens. Regulatory acceptance remains a challenge with respect to production and the product. For protein production where the scale remains small, such as vaccines, proteins can be made using quality-controlled systems as required by good manufacturing practices (GMP). For many small-scale production applications, microbes or tissue culture will remain the method of choice (Kusnadi et al. 1997; Alderborn et al. 2010). GMP-certified clean facilities cannot be replicated in either field or glasshouse. However, for many other end uses where there is no need for the tightly controlled production essential for drugs, such as enhanced vegetable proteins or many industrial enzymes that are only partially purified, plant-based protein production can greatly reduce costs and provide scale-up potential that vastly exceeds the capacity and efficiency of industrial biofactory production.

In order to produce economically viable proteins, the input costs and the output efficiency are major engineering design considerations. The value of the target protein is one of the most significant variables; for pharmaceutical proteins that have extremely high value per unit mass, the production cost of these proteins is easily justified, in even the most expensive systems such as animal tissue culture. For industrial enzymes, where the final product is a relatively crude enzyme preparation and the value ranges from US$ 1/kg to US$ 20/kg, the cost of production in microbial systems is often justifiable. In the case of plant production, the economy of scale and cost of production can be a dominant factor for lower value enzymes and/or to competitively undercut the production costs for factory-produced enzymes. Seed-based expression systems provide an attractive solution, and turnkey production/processing equipment can be leveraged from the food/feed industry for enzymes where the product is a crude mixture of proteins. Seed-based production systems offer the additional advantage that a valuable coproduct is often produced, such as seed oil. Assuming that the coproduct can be easily and nondestructively separated from the protein product, the coproduct provides a built-in cost subsidy for protein production, minimizing input costs of field growth and harvest, making the production of the protein product cost-efficient.

Approaches to Alter Seed Protein Content

Early Research to Develop Enabling Technology for Seed Protein Biotechnology The development of biotechnology for production of foreign proteins in seeds represents some of the earliest experiments in plant molecular biology. In the pre-genomics age, gene identification in seeds often relied on random sequencing of inserts representing superabundant messenger RNAs (mRNAs) produced in maturing seeds, or alternatively, complementary DNA (cDNA) expression of fusion proteins coupled with antibody-based selection (Beachy et al. 1978; Goldberg et al. 1981a, b; Turner et al. 1981; Walling et al. 1986). Using these technologies, highly abundant seed storage proteins were among the first plant transcripts to be identified and sequenced (Hill and Breidenbach 1974a, b). Starting with these sequences, subsequent efforts focused on the identification of the upstream promoter and regulatory elements using small insert genomic libraries. The regulatory elements and coding sequences provided the tools to create heterologous genes for plant expression, which in some cases were altered further by site-directed mutagenesis (Hoffman et al. 1987, 1988). Concurrent with the cloning efforts, methodologies were developed to transfer genes into plants, based on either biolistic particle delivery (gene guns) or *Agrobacterium* into plants such as tobacco. Exploiting these tools, laboratories representing academia, government, and industry took the first step toward altering seed composition by expressing foreign genes. These initial forays into biotech seeds were broadly successful, but it was more challenging to produce heterologous proteins in seeds. These positive steps nonetheless created considerable exuberance and excitement for the emerging field of plant biotechnology, touted to be the next "green revolution." These initial experiments also

began to reveal the issues that needed to be resolved for the optimization of protein production in seeds. Although transformation in tobacco was relatively efficient and straightforward, the development of more robust transformation techniques in crops and other plants took years to develop. For example, soybean transformation remains challenging and is restricted to a small self-selected group of laboratories.

From the very inception of seed biotechnology, the emphasis has been on the major seed crops, namely maize, rice, and soybean. Soybean represents the world's largest vegetable protein commodity that is used extensively for nitrogen input for farm animal production, with about 250 million t used annually (www.soystats.com). Soybean protein is deficient in sulfur-containing amino acids so that one of the earliest goals was to develop technology to enhance their content. Initial efforts focused on model systems (Bagga et al. 1995, 1997), and were later extended to soybean (Hagan et al. 2003; Kim and Krishnan 2004; Mainieri et al. 2004). In the 1990s, both *Agrobacterium* and biolistic particle delivery systems were developed for soybean transformation (Trick et al. 1997; Schmidt et al. 2004). However, both are labor intensive and require a high degree of skill and experience for success. Even with these limitations, soybean traits conferring herbicide tolerance have been produced, and Roundup (glyphosate) herbicide-tolerant soybeans have become the dominant global soybean produced (Farre et al. 2010). More recently, soybeans modified to produce low-*trans* fat oils (US 5981781A patent) have been commercialized and may become widely adopted. In contrast, protein modification traits in soybean are largely restricted to the research realm.

Successes and Limitations in Producing Heterologous Proteins in Transgenic Seeds

Many important papers have been published detailing initiating the development of crop plants as protein bioreactors. First, there was the observation that an artificial gene construct comprising a seed-specific promoter-5′ upstream domain, a seed protein open reading frame, and a 5′ terminator sequence can be transferred into a model plant, resulting in seed-specific expression and some accumulation of the transgene-encoded protein product (Hoffman et al. 1987). It was shown further that transgene-encoded heterologous protein is targeted correctly to the appropriate storage site, either to the protein storage vacuole (Levanony et al. 1992), or into endoplasmic reticulum (ER)-derived protein bodies (Herman et al. 1990; Geli et al. 1994; Bagga et al. 1995; Coleman et al. 1996). These primary observations quickly led to additional studies in which compositionally enhanced proteins were expressed to create models for what was expected to become value-added crops of a second "green revolution." Many of the initial experiments focused on sulfur enhancement to correct what is a relative deficiency of methionine in important dicotyledonous crops (Hagan et al. 2003; Kim and Krishnan 2004; Mainieri et al. 2004). In these studies that utilized strong tissue-specific seed storage protein promoters, the fraction of the total seed protein encoded by the transgene was significantly

less than expected from either homologous or heterologous seed storage protein promoters. Typical results for expressing seed storage proteins in transgenic model seed systems yielded less than 1 % of the total seed protein (Hoffman et al. 1987, 1988; Garg et al. 2007; Joensuu et al. 2008; Kim et al. 2004; Moravec et al. 2007; Alvarez et al. 2010), which is far less than would be expected by a proportional allocation of source to produce the seed sink, including the transgene. For instance, if a transgene driven by a storage protein promoter was added in a single copy as an extra gene to a gene family of five endogenous storage protein genes, it would be expected that the transgene-encoded protein should represent roughly one sixth of the total seed-storage protein. Although the transgene may be efficiently expressed, proportionally high concentrations of heterologous protein were not accumulated. As a consequence, the transgene-encoded product made a diminished contribution to the total seed amino acid pool. These results illustrate one of the most persistent impediments to deploying seed biotechnology; it is difficult to substantially alter seed composition due to the poor accumulation of transgenically encoded proteins. While high sulfur proteins, such as zeins, can be produced in transgenic seeds, there are intrinsic aspects of seed physiology that impede production of the transgene product, resulting in only a minor shift in the total seed composition (Bagga et al. 1995, 1997; Coleman et al. 1996; Hoffman et al. 1987; Kim and Krishnan 2004).

Although these early studies of producing heterologous proteins in model seeds did not result immediately in an enabling technology to remodel a seed's composition, other aspects of these studies made important contributions to create the foundation for future biotechnology enhancements. In order for a protein to be produced efficiently in a transgenic platform, it must be targeted to a site where it can be accumulated. In the case of proteins produced by the ER, there are specific targeting and processing mechanisms that facilitate protein accumulation at the correct destination. Targeting sequences for seed and other vacuole proteins were identified based on a combination of deletion and mutation analyses. For vacuole proteins and seed-storage proteins in particular, these sequences have been shown to be peptide sequences that bind to specific targeting receptors located in the *trans* Golgi (Hinz and Herman 2003). Elucidating these targeting sequences is an enabling technology, since in the absence of correct targeting signals, the resulting protein will likely be mistargeted, usually to the cell surface, or alternately remain in the ER where it could be subject to turnover by quality control mechanisms (Enfors 1992; DeWilde et al. 2000; Doran 2006; Drakakaki et al. 2006).

One of the goals of seed protein biotechnology is to produce proteins from nonplant sources. Since nonplant proteins usually do not possess plant-specific targeting sequences, these needs to be added as part of the biotechnology engineering strategy (Herman and Larkins 1999; Bagga et al. 1995; Schmidt and Herman 2008; Alverez et al. 2010). Vacuole-targeting sequences have been shown to encompass several different types, which are located either internally or on the carboxy-terminus of the protein (Robinson et al. 2005). One of the most commonly used vacuole-targeting sequences is the N-terminal propeptide Asn-Pro-Ile-Arg (NPIR) type, which is recognized by members of the BP-80 family of Golgi receptors. The NPIR sequences are usually located near the amino terminus of the protein (Hinz and

Herman 2003). Other significant groups of vacuolar localization sequences include the carboxy-terminal sequence identified in wheat germ agglutinin, as well as a range of internal sequences identified in major storage protein families (Hinz and Herman 2003). Experiments have been conducted based on chimeric genes where targeting sequences, such as the wheat germ agglutinin carboxy-terminal sequence, are fused to heterologous proteins which otherwise lack the targeting sequence, are correctly targeted to the vacuole, mimicking a step needed to enable accumulation of a transgene-encoded protein in seed vacuoles. The results of these studies form one of the key enabling foundations for the design proteins to be expressed in seeds.

Many seed proteins are glycosylated, and in analogy with animal cells, it was initially assumed, and later disproved, that the glycan side chains confer intracellular targeting information. Mutation of N-glycosylation triplets N-S/T of seed glycoproteins showed that the resulting proteins were still correctly targeted and accumulated in seeds. This technology has other applications for the removal of glycosylation sites from heterologous expressed proteins, such as those originating from other eukaryotic species, and to impede the potential for those glycans to undergo additional Golgi processing to add other plant-specific sugars such as galactose, xylose, and arabinose to the core processed N-glycan, that might result in allergenic sensitization (Herman and Burks 2011). The elimination of glycan-processing events removes one potential regulatory impediment to deployment. Conversely, glycans can also be added to specific products to humanize, or animalize, those products (Sturm et al. 1988; Samyn-Petit et al. 2001; Gomord et al. 2004; Karnoup et al. 2005). Experiments have been conducted to coexpress glycan-modifying enzymes in plants that produce human- and animal-specific glycosylation events, resulting in humanized proteins. These products are important in medical applications where there is the potential for the human or animal immune system to react to the protein if it does not possess the correct glycan structure. The capacity to alter or inhibit the attachment glycans has yet to create a commercial product. Nonetheless, glycan modification represents a significant addition to the biotechnologist's tool kit.

Protein Stability and Instability of Foreign Protein Production in Seeds To produce viable protein products in any heterologous system, the proteins must be correctly formed and accumulated in their native conformation. Protein folding occurs in a complex series of events, each of which is dependent on the successful completion of preceding events. Folding and processing occur in a sequential fashion both cotranslationally and posttranslationally, that together lead to the accumulation of a stable functional product. The total permutations of folding events as a protein is produced are immense, but fortunately each step exploits the most energy-efficient state, restricting the almost unlimited number of potential variants that direct the folding in the correct final configuration. The assumption that a protein be correctly folded and processed when produced in a heterologous system assumes that the foreign synthesis environment will faithfully mimic the native environment. Clearly, differences in environments exist between prokaryotes and eukaryotes, as well as between eukaryotes. It is well established that differences in processing

exist between prokaryotic cells and eukaryotic cells. There are numerous examples of eukaryotic proteins produced in prokaryotic cells that are misfolded, producing nonfunctional and often insoluble proteins (Herman 2008).

Protein instability was first observed in plants in experiments designed to modify protein structure by either altering sequence to improve composition, or in deletion, mutants designed to attempt to identify seed protein intracellular targeting information (Hoffman et al. 1988; Enfors 1992; Doran 2006; Benchabane et al. 2008). In other instances, proteins, for example zeins, transferred to transgenic tobacco (Hoffman et al. 1987; Coleman et al. 1996) and later to soybean (Kim and Krishnan 2004) did not accumulate at expected levels for proteins whose transgene transcripts were regulated by strong seed-specific promoters. Pueyo et al. (1995) showed that unstable proteins that possess seed-storage protein vacuolar-targeting sequences are synthesized and translocated to the vacuole and thereafter degraded. This demonstrates that while seed vacuoles are capable of accumulating intrinsic seed storage proteins, they also possess proteolytic enzymes that degrade other proteins. Thus, even when translation and targeting of a foreign protein is successful, accumulation is dependent on the capacity of the protein to persist intact in the vacuolar environment. The susceptibility of foreign proteins to degradation was demonstrated further in experiments designed to produce zeins in tobacco seeds. Zeins in maize accumulate in the ER aided, in part, by complex interactions between the different types of zeins that associate to form an ER-derived protein body separated into two or more domains containing different zeins (Geli et al. 1994). In order to improve accretion and to facilitate protein body formation, different zein genes were coexpressed in tobacco seeds. This approach was shown to improve protein body formation and the initial accumulation of the zein proteins (Bagga et al. 1995, 1997). However, little of the zein remained in mature dry seeds because the protein bodies were sequestered into the seed-storage vacuoles by autophagy where the zeins were apparently degraded (Coleman et al. 1996). This shows what was an apparently successful engineering strategy to produce high sulfur zeins in cytoplasmic-localized protein bodies, mimicking the intrinsic biology of maize, was unsuccessful because of the tobacco seed's intrinsic biology to destroy the protein bodies by autophagy.

There is a large body of literature describing the unfolded protein response (UPR), where misfolded proteins in eukaryotic cells are targeted for destruction. Plants share many, if not all, of the UPR pathways found in diverse animal and fungal cells. From a biotechnology perspective, the UPR is less significant since the objective is to produce correctly formed proteins that should not trigger a UPR response. However, the issue of posttranslational instability of correctly formed proteins remains an underappreciated and an understudied problem that can affect adversely biotechnological engineering strategies. The underlying biology of posttranslational instability of correctly formed proteins requires additional basic research to design future seed protein production platforms, which minimize or eliminate the degradation of correctly folded transgenic protein products.

Seed Protein Bioreactor Applications

Low-Abundance Products Based on the results of early transgenic experiments with tobacco and other model plants, parallel experiments on potential crop models used as seed expression systems typically produced 1 % or less of total protein (Hoffman et al. 1987; Bagga et al. 1995; Karnoup et al. 2005; Piller et al. 2005; Moravec et al. 2007; Powell et al. 2011). From a biotechnological perspective, this places severe constraints on the application and the economic viability of seed protein production platforms. Compositional changes at the 1 % level are too little to alter significantly essential amino acid content. However, a 1 % change can be sufficient to silence allergenic or anti-nutritional proteins, as was shown with the suppression of the immunodominant soybean allergen Gly m Bd 30K or P34 (Herman et al. 2003). A second application for low-abundance products is expression of immunoactive proteins to create vaccines or bioactive proteins, such as growth factors.

The production of functional proteins accumulating at a level of 1 % is sufficient for some applications. A low-abundance transgenic protein product is one that is produced in high enough abundance to be efficacious, but not abundant enough to alter significantly the seed's amino acid composition and overall proteome. Examples of low-abundance proteins include bioactive immunogens used as vaccines, or proteins designed to enhance consumer health or animal production, such as growth hormones. Transgenic expression of low-abundance proteins further offers the potential to mitigate problems, including storage and shipping of seeds, by inhibiting insect feeding. Insects feeding on dried seeds are sensitive to naturally occurring inhibitors, such as amylase inhibitors, which prevent starch metabolism, and production of these has proven effective in laboratory experiments (Morton et al. 2000). The deployment and implementation of this type of strategy could address the large losses that occur in the less developed world during postharvest storage. There are some concerns about using heterologous inhibitors since most, if not all, of these inhibitors have some homology to known human allergens.

For low-abundance proteins, well-established approaches such as inducing accretion in ER-derived protein bodies, or sequestration into protein storage vacuoles, are easily achieved by adding targeting sequences for either ER retention (K/HDEL) or vacuolar targeting. Accumulation of low-abundance proteins could also occur in other cellular compartments. In soybeans, there are two other significant compartments, the oil bodies (van Rooijen and Moloney 1995; Moloney et al. 2008) and proplastids (Daniell et al. 2001, 2005; Garg et al. 2007). Although plastid-based production has been used in leaves, where the plastids are a major compartment, the physical space that the proplastids occupy in seeds is relatively small. Even if plastid-based protein production in seeds were to be relatively successful, the potential physical space available to accumulate proteins will limit the product mass and therefore represent only a small fraction of the total seed protein. To date, there have not been any reported research efforts to extend plastid protein bioreactor technology to seed proplastids. Conversely, oil body protein production is a well-developed technology and could easily be implemented for commercial protein production. The primary strategy for oil body-based protein production is to produce proteins as

a fusion with the major oil body membrane protein oleosin. Oleosins possess several properties which make them well suited to enable transgenic protein production, including the high level of intrinsic protein accumulation in plant seeds, self-targeting and assembling with the oil bodies. Oleosins also tolerate significant additions to the C-terminal domain. An oleosin protein production engineering strategy uses chimeric oleosin fusion proteins from which the fusion proteins are easily purified from seed lysates by centrifugal floatation of the oil bodies. If the fusion protein possesses a cleavable linker, the protein product can be separated efficiently and purified from the oleosin (Boothe et al. 2010). Oleosins are membrane surface proteins that constitute about 1 % of the total seed protein, so as a protein production will be restricted to applications where the goal is a low-abundance protein product. Oil body fusion proteins may be a suitable production platform for some applications, such as immunogen, hormones, and other biomedical product production. Immunogens could be used as bioactive intact fusion proteins, eliminating the need to develop to cleavable linkers between the oleosins and the transgene product. Oil bodies may further serve as effective adjuvants by mimicking the oil emulsion that is often used to enhance the immunoreactivity of proteins.

Vaccine Production Platforms Plant-produced oral vaccines have the potential to immunize people or production animals through food/feed consumption. Plant-based oral vaccines could be produced inexpensively and would not require the intervention of trained personnel for delivery. There have been numerous projects aimed at developing this technology, leading to the demonstration that it is feasible to produce protein antigens in plants that display immunogenic epitopes, eliciting a protective immune response (Ma et al. 2003, 2005; Streatfield et al. 2003; Fischer et al. 2004; Daniell et al. 2001; Boothe et al. 2010). Antigens have been expressed in leaves, fruits, and seeds for oral delivery (Richter et al. 2000; Rigano et al. 2004; Kim et al. 2004; Piller et al. 2005; Obregon et al. 2006; Garg et al. 2007; Moravec et al. 2007; Nochi et al. 2007; Joensuu et al. 2008; Oakes et al. 2009; Joensuu et al. 2009; Alvarez et al. 2010). Plants have also been tested as production systems for vaccines to be purified from plant extracts. The production of antigens by either food delivery or plant production for human is governed by current GMP. These regulations will present compliance challenges since they were not written to encompass the concept of producing medically active proteins in an outdoor or glasshouse environment that cannot be maintained at the same level of cleanliness that is possible in a factory with sterilized incubators and clean production rooms. The compliance and approval issues have hindered deployment of plant-based vaccines for human medical, companion animal, or production animal applications. Nonetheless, this technology remains promising, especially for applications that require inexpensive and easily delivered vaccines in efficiently leveraged production animals and to immunize at-risk wild animal populations. Feed-based vaccines can be stationed as in the field for consumption by wild animal populations and would not require the immediate presence of human intervention. Animal production industries, especially poultry and aquaculture, generate immense numbers at low input costs and profit margins. The economic reality is that the input cost of immunization to be

useful must be kept at very low relative to the average cost of immunization for humans, pets, and work animals. For this reason, oral/feed immunization is likely the most economically viable means of delivery of vaccines for many species of production animals. Experiments with seed-based production in soybean and maize are particularly promising because these are the major global feed sources. Stacking a vaccine trait with other enhanced feed traits would be cost-effective and help meet the global growing needs for the increased animal production.

Food and Feed Additives Another potential soybean platform application is to express low-abundance transgene protein products as feed additives to enhance the digestibility of soybean meal. In addition to their abundant protein and oil content, soybeans contain a smaller amount of starch, oligosaccharides, and phytate, each of which represents one to a few percent of the dry mass. Starch is easily digestible, but the other two reserves, phytate and raffinose-series oligosaccharides, are generally indigestible by production animals, except by ruminants and other animals which harbor bacteria that have the capacity to mobilize these compounds. Phytate, inositol phosphate, provides the post-germination seedling with needed phosphorus reserves. Since it is indigestible by animals, it is the major component of phosphorus waste released into the environment from animal production facilities. Excess phosphorus results in water pollution and promotes harmful algal blooms (Baruah et al. 2004). Phytase produced from fermentors is a widely used additive to animal feed. There have been experiments to directly produce phytase in soybeans (Shi et al. 2007; Bilyeu et al. 2008) to provide a cost-efficient source of the supplemental enzyme to mobilize the phytate, releasing phosphorus in the animal's digestive system that may be assimilated. This strategy has not yet been commercialized due in part to excessive regulatory costs and a competitive supply of inexpensive microbial-produced phytase. Nonetheless, this approach represents a potential value-added trait that could be deployed within the context of larger trait stacks to produce a feed-optimized soybean.

High-Abundance Protein Products

The limitation that seeds generally accumulate only a small fraction of foreign proteins as a part of the total protein is a serious impediment to many biotechnology applications. Without a larger share of the seed's protein being the transgene product, the economics of seed protein reactor platforms even with a valuable co-product, such as oil, is likely to be an economic challenge. There are a few possible ways by which seed protein bioreactor platforms are production limited. Most simply, it is likely that the transcripts encoding the foreign protein are not efficiently translated. Even if the foreign protein's gene expression is regulated by a strong seed-specific promoter, the resulting production/accumulation of foreign proteins still appears to underperform expectation (De Jaeger et al. 2002). It is possible that this underperformance is due to protein instability and turnover of the transgene product

(DeWilde et al. 2000; Benchabane et al. 2008). There is ample documentation that this can be a problem for some engineered proteins that are susceptible to either protein quality control (Vitale and Ceriotti 2004) or vacuolar proteolysis (Muntz 2007; Vierstra 1996). However, the published observations indicate that protein degradation tends to be an all or nothing event, either a protein is stable and accumulates or it is not and does not accumulate, so protein instability does not explain the apparent 1 % common limitation of so many transgene protein products. More likely, transgene product accumulation is limited by the proportional allocation of source nutrient flux into specific components of the accumulating sink. In this context, it is possible that intrinsic proteins have a sink preference, limiting the foreign protein to only a small fraction of the total resulting seed proteome.

Breaking the apparent barrier where seeds, such as soybean, are recalcitrant to produce more than approximately 1 % foreign protein is the key enabling technology to use effectively seeds as protein production platforms. With oil as a coproduct in soybeans and other oil seeds, it should be economically advantageous to produce proteins for end uses that do not require Food and Drug Administration (FDA)-regulated drug quality GMP in these seeds. These applications include the large-scale use of soybeans in animal feed, processed food components, food-processing enzymes. Seed protein production could be a competitive production strategy to fermentor-based industrial enzyme production. Examples of enzymes that would not require GMP-certified production include the growing need for plant cell wall hydrolases used in biofuels, enzymes used in industrial and home cleaning, and enzymes used to process nonfood items, such as processing cotton clothing. Many food-processing enzymes are derived from natural sources and are not produced under GMP circumstances. It is possible that enzymes produced by seed protein bioreactors would similarly be acceptable. The market for industrial enzymes is currently billions of US dollars and increasing, so that even a small fraction of this market could support seed-based enzyme production. The other potential application of producing high-abundance protein in soybean seeds is enhanced animal feed. Although soybean is the vital global commodity for animal feed protein and its protein is relatively high quality, there is ample opportunity to optimize its composition in both general and specialty beans targeted at specific animal production industries, such as aquaculture. If other proteins containing optimized amino acid composition, or increased digestibility by particular species, were to be produced at concentrations rivaling the intrinsic storage proteins, the potential exists to enhance animal production efficiency. If such beans were stacked with other potential traits, such as nutraceuticals and vaccines, an enhanced soybean would contribute significantly to addressing the emerging shortfall projected for global animal feed. To be able to use soybeans to produce industrial proteins or enhanced feed, engineering strategies need to be developed to break the apparent limitations of foreign protein production.

One way to view seeds is as a protein factory programmed to produce a specific set of proteins at predetermined ratios from a genetic determinant program, subject to some alterations as the result of environmental conditions. This concept is supported largely by the observation that transgene protein products do not appear to be major sinks for the seed nutrient pool that is instead allocated to the intrinsic seed storage and ancillary proteins (see prior discussion). Even using strong

promoters, such as those of intrinsic seed storage proteins to drive the transgenic transcript production, still does not result in a larger allocation of nutrients to the transgene protein product. Resolving how to increase the production of transgene protein products is necessary to make seeds a viable protein production platform. For soybeans, one of the first hints at how seeds regulate protein content derived from studies silencing one of the major soybean-storage proteins conglycinin (Kinney et al. 2001). Conglycinin is a member of the large vicilin family of storage proteins that is synthesized from transcripts from a small gene family. In mature soybean seeds, conglycinin constitutes about 20 % of the total seed protein content (Mienke et al. 1981; Nielsen et al. 1989). Cosuppression of conglycinin synthesis resulted in seeds that contained 100 % of the protein content, albeit with a different protein composition or proteome, rather than exhibiting a 20 % shortfall in protein accumulation (Kinney et al. 2001). Proteome analysis shows that in the conglycinin suppressed lines, the other storage protein, glycinin, quantitatively compensated for the conglycinin shortfall. Interestingly, the resulting seeds displayed additional properties that may be leveraged into biotechnology applications. Specifically, a large fraction of the glycinin that exchanges for the conglycinin shortfall remains as proglycinin that is the ER form of the protein, indicating a significant fraction of the exchanged protein does not progress to the vacuole. Electron microscopy of the conglycinin-silenced seeds showed that the proglycinin is sequestered in ER-derived protein bodies that accumulate stably during development and are present in the dry seeds. This result shows soybean's biology of producing and sequestering ER-derived protein bodies is quite different compared to the biology of expressing accreted proteins in other plants, in that protein bodies produced in soybean seeds are not destroyed by autophagy. The capacity of soybeans to produce proglycinin and accumulate it at relatively high concentrations in ER-derived protein bodies, and for proglycinin to persist in the seed through maturation, desiccation, and into germination, indicates that soybeans display a different biology that would make these plants an attractive system to use as a protein bioreactor.

In order to test the capacity of soybeans to store a foreign protein as a stable accretion within protein bodies, green fluorescent protein (GFP) was modified to include both an N-terminal ER signal tag and a C-terminal ER-retention sequence. The resulting construct, when expressed in soybeans under glycinin promoter/terminator control, resulted in the accumulation of GFP to at about 1.5 % of the total protein, with the GFP sequestered within stable ER-derived protein bodies in the dry seeds (Schmidt and Herman 2008). This result indicated that producing ER-directed GFP sequestered in ER bodies mimic the result of conglycinin suppression in that both produce stable proglycinin containing ER bodies which persist into maturation. The proglycinin accumulated in conglycinin suppressed lines was approximately 7 % of the total seed protein content, indicating that soybean seeds can allocate much more of their source to produce a larger sink of novel ER bodies if the protein accumulated is an intrinsic protein. The difference between the 7 % proglycinin accumulations compared to the 1.5 % GFP-HDEL (GFP with carboxy-terminal–HDEL-ER-retention signal) accumulation is fivefold with 7 % close to a nominal 10 % accumulation of transgene product proteins that would make soybeans an economically viable bioreactor. To test whether the capacity for larger

yield of ER-sequestered proteins is a property of intrinsic soybean open reading frames and/or transcripts, the glycinin promoter/terminator regulated, GFP-HDEL line that produced 1.5 % of the total protein was introgressed into the conglycinin suppressor line. The attachment of ER-retention sequences on proteins has been shown previously to result in retention of proteins in the ER lumen (Herman et al. 1990; Pueyo et al. 1995; Torres et al. 2001; Vitale and Pedrazzini 2005; Wandelt et al 1992). This approach been used widely to facilitate protein accretion in the ER, resulting in the formation of ER bodies analogous to ER-derived seed protein bodies (Herman and Schmidt 2004; Herman 2008). The goal of these experiments was to test whether adding an additional glycinin allele, albeit an artificial construct, would be used by the soybean, rebalancing its protein content to favor the GFP glycinin allele mimic. The results showed that by exploiting the proteome rebalancing process, GFP-HDEL accumulation increased from 1.5 to 8 % of the total protein, closely mimicking the result of proglycinin accumulation in conglycinin suppressed seeds (Schmidt and Herman 2008). Thus, the specific open reading frame is not a controlling element but instead the untranslated domain of the transcript does play a role in controlling translational yield. At 8 % of the total protein, this is sufficient yield to enable economically viable industrial enzyme production and is possibly sufficient to mediate changes in seed composition to enhance food and feed.

The success of exchanging conglycinin that constitutes about 20 % of the total protein in soybean seed for other intrinsic and foreign proteins suggests there is potential to enlarge the yield of foreign protein by further exchanges with intrinsic proteins (Fig.5.1). The first step to accomplish this have been taken by producing soybeans in which both conglycinin and glycinin storage proteins are silenced (Schmidt et al. 2011). Although these two proteins constitute about 60 % of the total seed protein, the intrinsic rebalancing process results in seeds that still contain about the same protein content as conventional seed. To compensate for the storage protein shortfall, several other proteins colocalized with the storage proteins in the protein storage vacuole increased in abundance. This suggests a pathway to completely remodel a seed's composition. Soybeans will tolerate high levels of ER bodies sequestering foreign proteins and/or a nearly completely altered vacuolar storage protein composition. Then it should be feasible to suppress a large fraction of intrinsic proteins and reprogram soybeans to produce foreign proteins in exchange. These experiments build on the experience of exchanging β-conglycinin and if successful, will enable developing new feed and protein production seeds.

Assessing the Quality and Safety of Seed Protein Production Platforms to Meet Future Needs

For any new transgenic product, there is a gauntlet of regulatory requirements and issues, both for cultivation and import countries, which will need to be addressed to commercialize any product. In the case of soybean protein bioreactor platforms, these issues may be even more sensitive because soybean is the major vegetable protein in global commerce. This is both a caution and an opportunity, because if

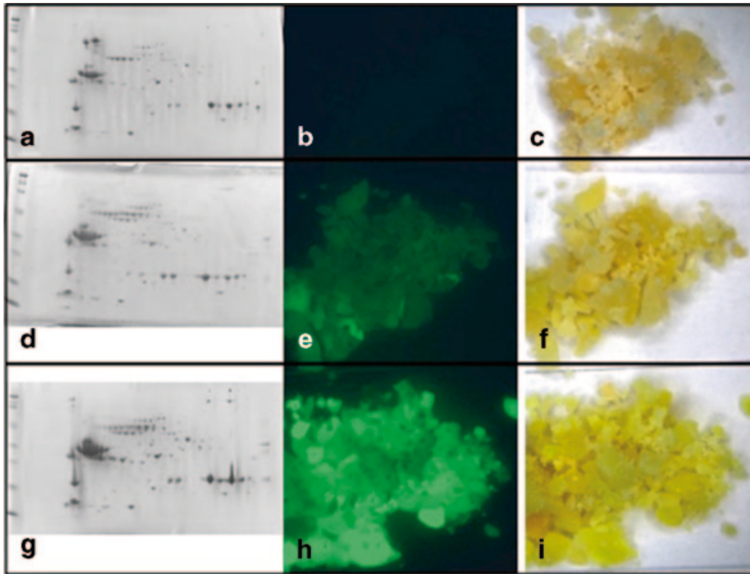

Fig 5.1 **a** A 2-D protein gel of a conventional transgenic soybean seed. **b** Conventional transgenic ground soybean seed under *blue* light. **c** The same conventional transgenic sample from (**b**) under *white* light. **d** A 2-D gel of the proteome isolated from a GFP-ER-targeted transgenic soybean seed. **e** A single-ground GFP-ER-targeted expressing soybean seed ground and viewed under *blue* light. **f** The same GFP-ER-targeted soybean seed seen in (**e**) under *white* light. **g** A 2-D gel of the proteome isolated from a GFP-ER-targeted and β-conglycinin-silenced soybean seed. **h** A single-ground GFP-ER-targeted and β-conglycinin-silenced soybean seed viewed under *blue* light. **i** The same GFP-ER-targeted and β-conglycinin-silenced soybean seed seen in (**h**) under *white* light. *GFP* green fluorescent protein, *ER* endoplasmic reticulum

soybean is used to produce new protein products, its global use and economy of scale makes it an ideal platform to generate new feed or food sources as well as protein biologics. There will be a number of cautions to implementation for any protein to be produced using soybean bioreactor technology. Because soybean is used widely as feed and food, there will always be concerns about the types of proteins produced to avoid a contamination episode such as occurred with the StarLink™ protein in maize (van Putten et al. 2006). For many of the soybean applications, such as improved feed and food, these may not impose any significant difficulties. In other applications like the production of industrial enzymes, many industrial enzymes are already contained within food corresponding to food-processing enzymes which are expected to be present at some level in food products. Examples include papain, cellulases, and other fungal enzymes similar to those in fermented foods. There is less risk associated with potential cross-contamination for enzymes that are already in the food/feed supply. The vast majority of market potential for soybean is in enhanced food/feed where the altered protein composition presents little or no risk. One suggestion is to place a common tag in the DNA that codes the nature of the transgenic industrial product that would make contamination easy to

identify and aid in segregation (Alderborn et al. 2010). Other simple approaches to protect against seed product contamination include visual markers of altered seed coat or cotyledon color, providing a simple visible cue as to whether a harvest being delivered is mixed with other soybeans. Such markers are under development and could, in theory, be color-coded for different end uses such as specific color for aquafeed-optimized soybeans. Because food soybeans are all the same yellow color, there is ample opportunity to introduce color-marking schemes as part of the overall biotechnology engineering strategy. Other potential methods that could have a role in possible cross-contamination resulting from inadvertent growth of seeds are to use chemically inducible promoters to regulate transgene expression, where no transgene product is produced in a field until an inducing chemical is employed. In one experiment testing this strategy, the induction of the transgene in soybean was shown to be effective in maternal cells, but the penetration of the inducer chemical into the pods, and in turn developing seeds was poor indicating that there are technical challenges yet to be resolved to use this approach for seed traits (Semenyuk et al. 2010).

Technology providing rigorous analysis to support regulatory approval is easily obtained with current composition and genomic assessment. It is not widely appreciated, especially among the critics of transgenic technology, the depth of information that can now be obtained for any transgenic or other organism. With the availability of the soybean genome sequence, insertion sites can be determined with precision, whether there are one or more insertions, and whether the insertion is in a genic region, resulting in modification of endogenous genes. With total transcriptome studies, quantifiable changes in gene expression patterns, if any, that are caused directly or collaterally resulting from transgene expression or silencing can be documented, with the alterations of transcripts compared to controls. The availability of modern genomic tools is revolutionizing our understanding of genetic programming and control, and can be translated into strategies to develop new products. More important than genomics and transcriptomics are the state-of-the-art methods to assess proteins, lipids, and metabolites. Mass spectroscopy innovations have made nontargeted total composition analysis of novel organisms routine (Bino et al. 2004). Using techniques such as multidimensional protein identification (MudPit) technology proteomic assessments, a total seed analysis of proteins in transgenics compared to controls are readily accomplished (Miernyk and Hajduch 2011). This allows for precise assessments of any changes in allergens, antinutritional proteins, as well as other more benign proteins to be assessed, and provides a powerful response to the concerns by genetically modified organism (GMO) critics that transgenic modifications will create novel or increased allergens. Similarly, mass spectroscopy using approaches of lipidomics and metabolomics provides a means to assay for alterations in small molecules that may be the goal of the transgenic modification or might be a collateral consequence. The capacity to assay a thousand or more species of small molecules enables assessment of the composition and the quality of a transgenic product at a level that is unprecedented for any other food or feed and can be used to assess the potential food/feed impact. The detailed analysis, if widely available, provides further basic knowledge that will increase

the understanding of the underlying biology and can be leveraged to make further improvements and advancements in transgenic development. The depth of potential analysis is critical, in part, because it provides important regulatory information, but also because it confronts the critics of this technology with overwhelming scientific information as to its safety and effectiveness. Pushing past the blocks created in response to the GMO critics is essential to address the growing global needs for food and feed. It is widely reported and discussed that population increases will certainly happen through this century and will require at least 50 % more food production and at least 100 % more animal feed (Anonymous 2009). This will require new, more efficient, better crops that are capable of growing under less advantageous circumstances. The larger population will need other products, including protein biologics that can be supplied, in part, by plant-based production platforms. The technological developments and underlying basic seed biology have created the framework to implement the next "green revolution."

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Chapter 6
Herbicide Tolerance

Jintai Huang, Christine Ellis, Brian Hauge, Youlin Qi and Marguerite J. Varagona

Introduction

Crops with a genetically engineered (GE) trait conferring herbicide tolerance were among the first biotechnology-derived crops to be commercialized in agriculture. The GE trait conferring tolerance to in-crop application of the herbicide glyphosate was introduced in soy and canola in 1996 and, in cotton in 1997, revolutionizing agricultural practices for these crops. In 1996, biotech corn was introduced that provided tolerance to the herbicide glufosinate. According to the 2012 International Service for the Acquisition of Agri-biotech Applications (ISAAA) report, 100.5 million ha of farmland were planted worldwide with GE crops containing at least one herbicide-tolerance trait (http://www.isaaa.org/). Economic and agricultural advantages to growers have included reduced use of pesticides, increased adoption of reduced or conservation tillage and soil conservation practices, reduced greenhouse gas emissions from agricultural practices, as well as increased yields (Martino-Catt and Sachs 2008). These advantages have economic benefits to farmers, as evident in their choice to invest in these GE crops. Furthermore, the growth in stacked GE traits, i.e., GE crops containing multiple traits combined through conventional

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breeding practices, including insect resistance and/or herbicide tolerance shows that to optimize yield growers prefer to plant crops containing multiple GE traits in combination.

Why have biotech crops with herbicide-tolerance traits been so successful? It is well documented in the literature (Oerke 2006) that weeds are one of the biggest causes of yield loss. Depending on the weed pressure and weed control method, yields can be reduced up to 50% if weeds are not managed well. The methods or systems to control weeds range from hand weeding or hoeing to mechanical tilling and chemical methods. They also include cultural practices, such as the use of cover crops during the fallow months followed by an herbicide treatment, or the in-crop use of selective herbicides that kill the weeds, but are safe to the crop. Agricultural biotechnology has enabled engineering a crop to make it tolerant to one or more broad-spectrum herbicides and therefore allow the in-crop use of those herbicides for simplified but effective weed control. One of the key advantages has been the ease with which growers could practice no-tillage or low-tillage practices by incorporating herbicide-tolerant crops into their farming systems, allowing them to minimize tillage while still controlling emerging weeds through post-planting herbicide application. Additional benefits from combining no-tillage practices with an herbicide-tolerant crop include increased water retention in the soil, less soil disruption, less soil erosion, and increased organic matter in the soil. These agronomic advantages have contributed to the rapid adoption by growers of these simplified farming systems and in turn have delivered important environmental benefits (Cerdeira and Duke 2006).

While the simplicity of the herbicide-tolerant crops is very attractive, their widespread adoption, combined with year-after-year use on the same field, contributed to the selection of weeds that are resistant to the most widely used herbicides (http://www.weedscience.org/summary/home.aspx). James noted in the 2012 ISAAA report that there were 24 weed species known worldwide to be resistant to glyphosate (http://www.isaaa.org/). In some geographies, the presence of herbicide-resistant weeds can drive agricultural practices and strongly influences the choice of inputs by the grower. As for all plant-protection products, agronomic specialists have promoted the use of a diverse weed control system that appropriately uses multiple herbicide modes of action. The coming generations of stacked GE crops containing multiple herbicide-tolerance traits will help simplify effective weed management through the use of multiple herbicide modes of action.

In the past few years, many reviews have been written on different aspects of herbicide traits (Feng et al. 2010a; Green and Castle 2010). This chapter covers the development of the early herbicide-trait systems focusing on the development of the glyphosate-tolerant biotech crop systems and the early glufosinate-tolerant biotech traits. It also discusses the use of trait stacking to fill the immediate needs of farmers facing the challenges of hard-to-control and resistant weeds in their fields and the development of an additional herbicide-trait system for dicamba tolerance.

Glyphosate: A Potent, Nonselective Herbicide

Glyphosate (*N*-phosphonomethyl-glycine) is the most commonly used herbicide in modern agriculture worldwide. Glyphosate was first tested for herbicidal use in 1970 by researchers at Monsanto Company (Duke and Powles 2008) and was commercialized as a nonselective herbicide in 1975. The worldwide adoption of glyphosate-based herbicides stems from its good efficacy against a wide spectrum of weed species, fairly low cost on a per-acre basis, its benign toxicology and environmental safety profile under intended conditions of use (Geisy et al. 2000; Smith and Oehme 1992; Williams et al. 2000). Glyphosate is absorbed readily through plant surfaces (Kirkwood et al. 2000). Once taken up by plant leaves, the unique physicochemical properties of glyphosate allow its systemic translocation via the phloem, in a manner similar to that of the photoassimilate sucrose, to rapidly growing and stronger metabolic sink tissue (CaJacob et al. 2004). Some early work indicated that most crops and weed species lack endogenous mechanisms for catabolism or detoxification of glyphosate, although a number of plant and weed species, mostly legume, are known to have biochemical activities that could convert glyphosate into less toxic compounds such as aminomethylphosphonic acid (AMPA; Reddy et al. 2004, 2008). Rapid uptake, no or limited *in-planta* degradation, systemic translocation to growing points, and a slow mode of action are the primary attributes for the excellent herbicidal efficacy and popularity of glyphosate. The slow mode of action is important since it allows glyphosate to translocate to most parts of the plant before plant injury reaches levels that inhibit translocation. Indeed, glyphosate is regarded as "a once-in-a-century" herbicide (Duke and Powles 2008). For more than 20 years after glyphosate was first registered and commercialized as an herbicide, the utility of glyphosate-based agricultural herbicides remained somewhat restricted from in-crop use due to the nonselective action. Because of this reason, glyphosate-based herbicides were used for controlling weeds prior to crop planting where total vegetation control in the field was needed for seedbed preparation (a practice called burn down), and to certain preharvest applications or in-cropping systems where glyphosate application could be directed to the weeds to avoid contact with crop foliage or other critical vegetation.

Strategies for Engineering Glyphosate Tolerance in Crops

The introduction of a transgenic glyphosate-tolerant (GT) soybean (Roundup Ready® or RR soybean) in 1996 revolutionized agriculture and enabled a new-use pattern for glyphosate-based herbicides. RR soybean is fully tolerant to glyphosate. Thus, glyphosate can be applied "in crop" as a post-emergent herbicide to control weeds without crop injury. The acreage of herbicide-tolerant (HT) crops has increased steadily in the USA since the introduction of RR soybean. Based on United States Department of Agriculture (USDA) survey data, HT soybean acreage

increased from 17% in 1997 to 93% in 2012. HT cotton acreage expanded from about 10% in 1997 to 80% in 2012. HT corn also reached about 73% of US corn acreage in 2012 (http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/). Although several crops with HT traits other than glyphosate tolerance have been marketed over the years, their adoption has been relatively slow (Feng et al. 2010a), and the majority of the HT acreage in the USA has been planted with glyphosate-tolerant traits.

Two basic strategies have been used to engineer GT crops, (a) expression of a transgene encoding a glyphosate-insensitive target enzyme, and (b) expression of transgene-encoding enzymes for glyphosate deactivation or detoxification (CaJacob et al. 2004; Feng et al. 2010a; Pollegioni et al. 2011). The discovery of the chloroplast-localized enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) as the sole target of glyphosate (Steinrücken and Amrhein 1980) offered an opportunity for engineering GT crops through glyphosate-insensitive EPSPSs. EPSPS is present in all plants, bacteria, and fungi, but not in animals. It is a key enzyme in the shikimate pathway involved in biosynthesis of the aromatic amino acids tyrosine, phenylalanine, and tryptophan. Phosphoenol pyruvate (PEP) is the natural substrate of EPSPS, and the product of the reaction is 5-enolpyruvyl shikimate 3-phosphate (EPSP). Because glyphosate competes with PEP for the same catalytic pocket of EPSPS, the challenge in engineering GT crops through insensitive EPSPSs has been to identify EPSPSs with a favorable K_m for PEP but much higher K_i for glyphosate.

EPSPSs from various organisms can be divided into two groups, class I (glyphosate-sensitive) and class II (glyphosate-tolerant) EPSPS, based on their intrinsic sensitivity to glyphosate as well as sequence diversity (Barry et al. 1997, 1992). Class I EPSPS enzymes are present in all plants and in many Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*. Class I EPSPS are inhibited at low micromolar concentrations of glyphosate and do not share significant sequence homologies with the class II enzymes (Barry et al. 1992). Some class I EPSPS variants were found to be insensitive to glyphosate in bacteria (Pollegioni et al. 2011). Plant expression of these glyphosate-insensitive variants did not achieve commercial-level tolerance in transgenic plants. A T102I, P106S double mutant of the maize *epsps* gene (known as the TIPS-EPSPS or 2mEPSPS) was generated by site-directed mutagenesis (Lebrun et al. 2003). The TIPS-EPSPS was found to retain efficient catalytic kinetics but with significantly reduced glyphosate sensitivity (Lebrun et al. 2003). TIPS-EPSPS was used to engineer the first generation of GT corn (event GA21), which was first commercialized in 1998 and is currently marketed by Syngenta.

Class II EPSPSs are found in several naturally occurring glyphosate-resistant microorganisms such as *Agrobacterium* sp. CP4, *Achromobacter* sp. LBAA, *Pseudomonas* sp. PG2982, *Streptococcus pneumonia,* and *Staphylococcus aureus* (Du et al. 2000; Barry et al. 1992; Priestman et al. 2005). Class II EPSPSs are insensitive to glyphosate inhibition and retain catalytic efficiency in the presence of high glyphosate concentrations. The glyphosate-insensitive EPSPS-encoding gene from *Agrobacterium* sp. CP4 (known as the *cp4-epsps* gene) was isolated from the waste

stream of a glyphosate-manufacturing site. CP4-EPSPS is a unique class II enzyme that is highly insensitive to glyphosate, but maintains a normal affinity for PEP (CaJacob et al. 2004; Barry et al. 1992), thereby ensuring continued production of essential aromatic amino acids in GE crops transformed to contain CP4-EPSPS. The first-generation GT soybean, Roundup Ready® soybean (Event 40-3–2), expressed a *cp4-epsps* transgene, and was commercialized in 1996. The *cp4-epsps* gene has been the most commonly used transgene in engineering GT crops by the insensitive-EPSPS approach, due to its unique properties (CaJacob et al. 2004). Other first generation GT crops that were generated by plant expression of CP4-EPSPS include Roundup Ready® cotton (event MON1445), Roundup Ready® canola (event RT73), Roundup Ready® sugar beet (event H7-1), and Roundup Ready® alfalfa (events J101 and J163; CaJacob et al. 2004; Feng et al. 2010a; Green 2012).

An alternative approach in engineering GT crops is by glyphosate deactivation or detoxification. A wide range of soil-borne microbes, including bacteria, actinomycetes, and fungi are known to metabolize glyphosate (Borggaard and Gimsing 2008; Duke 2011). Soil microbes can deactivate glyphosate by two different pathways, namely (a) cleavage of the carbon–phosphorus bond, resulting in the formation of phosphate and sarcosine (the C–P lyase pathway) and (b) oxidative cleavage of the carbon–nitrogen bond by glyphosate oxidoreductase (GOX), resulting in the formation of AMPA and glyoxylate (the AMPA pathway). A glycine oxidase (GO) variant, generated by a rational design approach, together with saturation mutagenesis, was also shown to convert glyphosate into AMPA and glyoxylate, but with a different mechanism (Pedotti et al. 2009). Although some first-generation GT crops, such as Roundup Ready® canola (Event RT73) and sugar beet (Event GTSB77) contain the *GOX* gene, plants transformed with the *GOX* gene alone did not achieve commercial-level tolerance to glyphosate, at least in part, due to the phytotoxicity of AMPA (Reddy et al. 2004, 2008). GT crops developed using the glyphosate-deactivation approach have also been reported. A glyphosate acetyltransferase (GAT) variant having enhanced activity has been used to convert glyphosate into *N*-acteyl glyphosate, which is not herbicidal and does not inhibit EPSPS (Castle et al. 2004; Siehl et al. 2005, 2007).

Development of Second-Generation GT Crops

Improvements to second-generation GT crops were built on knowledge from engineering the first generation. The same properties that make glyphosate an excellent herbicide, such as systemic translocation and lack of *in-planta* degradation, also impose challenges on engineering glyphosate tolerance in transgenic plants. From this point of view, insensitive EPSPSs and glyphosate deactivation each has its own advantages and disadvantages. Because of the systemic translocation and accumulation of glyphosate in the meristem (Pline et al. 2002c), efficient expression of the insensitive EPSPS is required throughout the plant, especially in reproductive meristems and tissues to confer whole-plant tolerance (Chen et al. 2006). For

the glyphosate-deactivation approach, glyphosate is expected to be detoxified once taken up by plants and accumulation would be less in reproductive meristems. However, the speed of deactivation relative to glyphosate translocation and inhibition of EPSPS, especially at higher application rates and/or when sprayed at later plant development stages, is likely to be critical to overall tolerance. It has recently been shown that glyphosate is active against fungi, and the persistence of glyphosate in GT wheat and soybean, expressing an insensitive EPSPS, may help provide fungal disease suppression in these crops (Anderson and Kolmer 2005; Feng et al. 2005, 2008).

In the first-generation GT crops including Roundup Ready® cotton, Roundup Ready® soybean and Roundup Ready® canola, transgene expression was driven by strong native or enhanced viral promoters such as the cauliflower mosaic virus 35S RNA (*CaMV 35S*) or the figwort mosaic virus 34S RNA (*FMV*) promoters. These promoters and their enhanced versions were used because they were shown in early studies to direct strong and constitutive expression during plant growth and development (Odell et al. 1985; Kay et al. 1987). When these strong viral promoters were used to generate GT corn, studies showed that the expression of CP4-EPSPS protein from a construct with the enhanced *CaMV 35S* promoter was high in corn leaf and whole anther, but poor in pollen (cassette 2, Fig. 6.1a). In contrast, the expression of CP4-EPSPS protein from a construct with the *rice actin 1* gene promoter was relatively low in the leaf but high in whole anther and pollen (Heck et al. 2005; Feng et al. 2010a; cassette 1, Fig. 6.1a). These findings are consistent with previous studies showing lack of *CaMV 35S* promoter activity in the tapetum cell layer and microspore mother cells, which are critical for male reproductive functions (Plegt and Bino 1989). These two promoters, therefore, have complementary activities which together generate the desired expression profile for whole-plant tolerance over a wide developmental window. Thus, a double-cassette vector containing each of the two expression cassettes (Fig. 6.1b) with a complementary expression profile was used to engineer the second-generation GT corn (Roundup Ready® Corn 2, event NK603). NK603 has robust vegetative and reproductive tolerance with expanded crop safety (Heck et al. 2005) and has been a popular product since its commercial launch in 2001 (Feng et al. 2010a).

A better understanding of systemic glyphosate translocation and accumulation in meristems in relation to application methods and plant growth stages helped identify strong metabolic sink tissues, especially reproductive tissues, as "at risk" for glyphosate injury (Feng et al. 2010a; Pline et al. 2002c). This knowledge, together with the elucidation of the plant expression profile of the CP4-EPSPS transgene in first-generation Roundup Ready® cotton, greatly facilitated improvement of CP4-EPSPS expression in the second-generation GT cotton (Pline et al. 2002a, b; Chen et al. 2006). The first-generation Roundup Ready® cotton product (event MON1445) was commercialized in 1997 and provided tolerance to in-crop applications of glyphosate through the four-leaf stage (Nida et al. 1996). In event MON1445, the *cp4-epsps* transgene expression is directed by the *FMV* promoter. Research has shown that occasionally a glyphosate application at the four-leaf stage, or very often a late spray at the eight-leaf stage, resulted in boll drop and abnormal

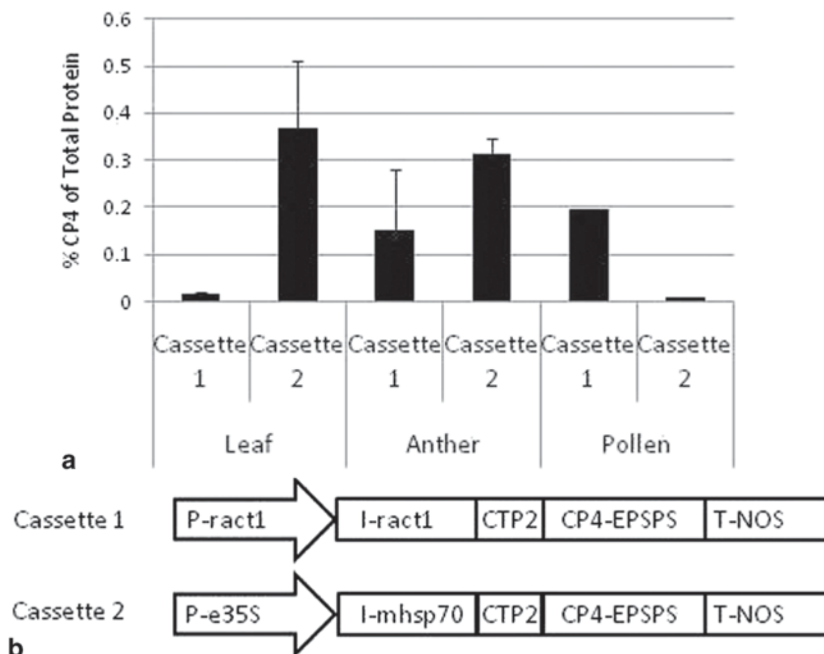

Fig. 6.1 **a** CP4-EPSPS protein levels determined by ELISA analysis in transgenic corn plants transformed with either cassette 1 or cassette 2. Each bar graph shows the concentration of CP4-EPSPS protein in the leaf tissue at development stage V4, anther tissues with most pollen grains removed, or pollen grains as indicated. *Error bars* represent standard deviation. **b** The genetic elements of cassette 1 and cassette 2 used for generating the transgenic events analyzed in **a**. *P-ract1* promoter of the rice actin gene 1, *P-e35S* enhanced CaMV 35S promoter, *I-ract1* first intron of the rice actin gene 1, *I-mhsp70* first intron of the maize heat-shock protein 70 gene; *CTP2* chloroplast transit peptide from *Arabidopsis thaliana* EPSPS (*shkg*), *cp4-epsps* coding sequence for the 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*) gene of *Agrobacterium* sp. strain CP4; *T-NOS* 3′ non-translated region from the nopaline synthase gene of *Agrobacterium*

pollination (Vargas et al. 1998; Jones and Snipes 1999). Gene expression analysis and immunolocalization studies demonstrated reduced accumulation of the CP4-EPSPS protein in the tapetum and developing pollen cells (Pline et al. 2002a, b; Chen et al. 2006), which corroborates early observations that glyphosate spray of the GT cotton event MON1445 can cause pollen degeneration (Vargas et al. 1998; Jones and Snipes 1999). Development of the second-generation GT cotton (Genuity® Roundup Ready® Flex cotton, event MON88913) used a dual-cassette vector, with each cassette containing a *cp4-epsps* gene under the control of a unique chimeric promoter, and where each chimeric promoter consisted of a constitutive plant promoter plus a viral enhancer. Roundup Ready® Flex cotton demonstrated enhanced expression of CP4-EPSPS in the tapetum and developing microspores with improved reproductive and whole-plant tolerance (Chen et al. 2006; Cerny et al. 2010). Roundup Ready® Flex cotton was commercialized in 2006, followed by rapid market adoption (Feng et al. 2010a).

As the studies discussed above indicated, CP4-EPSPS can provide robust glyphosate tolerance over a wide range of expression levels. Therefore, an appropriate expression profile, rather than an excessive level of expression, is more important for whole-plant tolerance because CP4-EPSPS must be present in all critical plant cells where glyphosate accumulates.

The first-generation GT canola, Roundup Ready® canola event RT73, was commercialized in 1996. Event RT73 was generated using a dual-cassette vector with one cassette expressing CP4-EPSPS and one cassette expressing GOX. RT73 provides tolerance to two sequential applications of glyphosate at 0.4 kg a.e. (acid equivalent) ha^{-1} or a single application at 0.6 kg a.e. ha^{-1} up to the six-leaf stage. Using the knowledge gained about expression profile to expand the plant growth stage window of glyphosate application, a second-generation of GT canola, Genuity® TruFlex™ Roundup Ready® canola (event MON88302), was developed. Canola event MON88302 was generated using a single CP4-EPSPS expression cassette with a novel chimeric promoter. Event MON88302 showed enhanced CP4-EPSPS expression in male reproductive tissues compared to the RT73 event, resulting in improved pollen viability following glyphosate spray. During the event selection process, the Roundup Ready 2 canola event MON88302 was selected based, in part, on an expanded glyphosate application rate to 3.6 kg a.e. ha^{-1} and an extended plant growth stage tolerant up to the ten-leaf stage (Feng et al. 2010a). When tested side by side, the RT73 plants showed decreased seed production in response to increased glyphosate rates, whereas seed production of TruFlex™ Roundup Ready® plants (event MON88302) was unaffected by the same glyphosate application rates (Feng et al. 2010a). Pending completion of applicable regulatory approval processes, this second-generation HT canola product will offer growers more flexibility in weed control by having a higher application rate with an expanded application window.

It was determined during the process of evaluation of the first- and second-generation GT crops that the transgene insertion site may have a significant effect on transgene efficacy, and an example of this effect is the development of the Roundup Ready 2 Yield® (RR2Y) soybean. The first-generation GT soybean (event 40-3-2) was generated using a transgene cassette in which an enhanced CaMV35S promoter drives the expression of the *cp4-epsps* gene (Padgette et al. 1995). Yield trials across multiple seasons and environments showed that applications of up to 1.68 kg a.e. ha^{-1} glyphosate to event 40-3-2 (and any of the germplasm lines into which it was introgressed), at any time between early vegetative growth and pod filling, did not adversely affect yield (Delannay et al. 1995; Elmore et al. 2001). However, some independently transformed events generated with the same cassette as 40-3-2 had excellent vegetative tolerance to glyphosate, but showed significant pollen sterility and reduced pod set when treated with glyphosate near the onset of flowering (Delannay et al. 1995). This result suggested that the transgene insertion site may have some effect on the expression profile or efficacy of the transgene used to generate the soybean 40-3-2 event. The same transgene cassette used to generate TruFlex™ Roundup Ready® canola (event MON88302) was used to engineer the second-generation GT soybean. As previously noted, the second-generation GT canola events generated with this cassette exhibited a much improved expression

profile and excellent glyphosate tolerance (Feng et al. 2010a). During the process of selecting the second-generation GT soybean event, molecular breeding technology was applied to analyze and identify transformation events with the transgene inserted at a genomic site or location that could have a positive effect on transgene efficacy. A unique insertion event was identified that was associated with a genomic region or haplotype with beneficial agronomic characteristics. The second-generation GT soybean event selected for commercialization (event MON89788) not only has excellent glyphosate tolerance but also additional agronomic benefits such as increased yield compared to the first-generation RR soybean event 40-3-2 (Feng et al. 2010a). MON89788 was commercialized in North America as Genuity® Roundup Ready 2 Yield® soybean.

Critical Molecular Elements Impacting Plant Expression

As discussed above, the appropriate expression profile of the transgene is essential for whole-plant tolerance over the critical growth stages. This is particularly true for glyphosate tolerance and likely for any potent herbicides that systemically translocate in plants. The improvement to the second-generation GT crops largely relied on using promoters that can drive more appropriate expression (Feng et al. 2010a). However, one of the lessons learned during the development of GT crops was that the promoter may not be the only important regulatory element controlling transgene expression. It has been reported that introns not only enhance gene expression level but also can regulate tissue- and/or cell-specific patterns of gene expression. It was discovered that activation of the floral homeotic gene *Agamous* in *Arabidopsis* required its own first intron (Busch et al. 1999). The first intron of a rice α-tubulin gene, *OsTubA1,* was found to regulate tissue-preferential expression of a transgene (Jeon et al. 2000). Data presented here show that the first intron of the maize heat-shock protein 70 gene (I-*mhsp70*) and the *rice actin 1* gene (I-*ract1*) may regulate tissue- and/or cell-specific transgene expression differently. This differential expression was demonstrated by transformation of corn with one of two separate transgene cassettes, differing only in the introns, and comparing the resulting CP4-EPSPS protein expression pattern, and determining male fertility after glyphosate application (Fig. 6.2). The two cassettes used for these experiments had comparable expression of CP4-EPSPS in vegetative tissues but distinctive expression patterns in the anthers. Cassette 3 showed strong expression in the anther wall, the tapetum cell layer, and the microspore mother cells (Fig. 6.2b.A). In contrast, cassette 4 expressed well in the outer layers of the anther, but had limited or no expression in the tapetum cell layer and microspore mother cells (Fig. 6.2b.B). This expression profile suggested that transgenic plants expressing cassette 3 would be fully tolerant to glyphosate, whereas plants expressing cassette 4 would be sensitive to glyphosate in the anther. Indeed, glyphosate application at the V4 stage, followed by a second glyphosate application at the V10 stage, resulted in complete tassel sterility in transgenic plants harboring cassette 4 (Fig. 6.2c.A), whereas cassette 3 containing

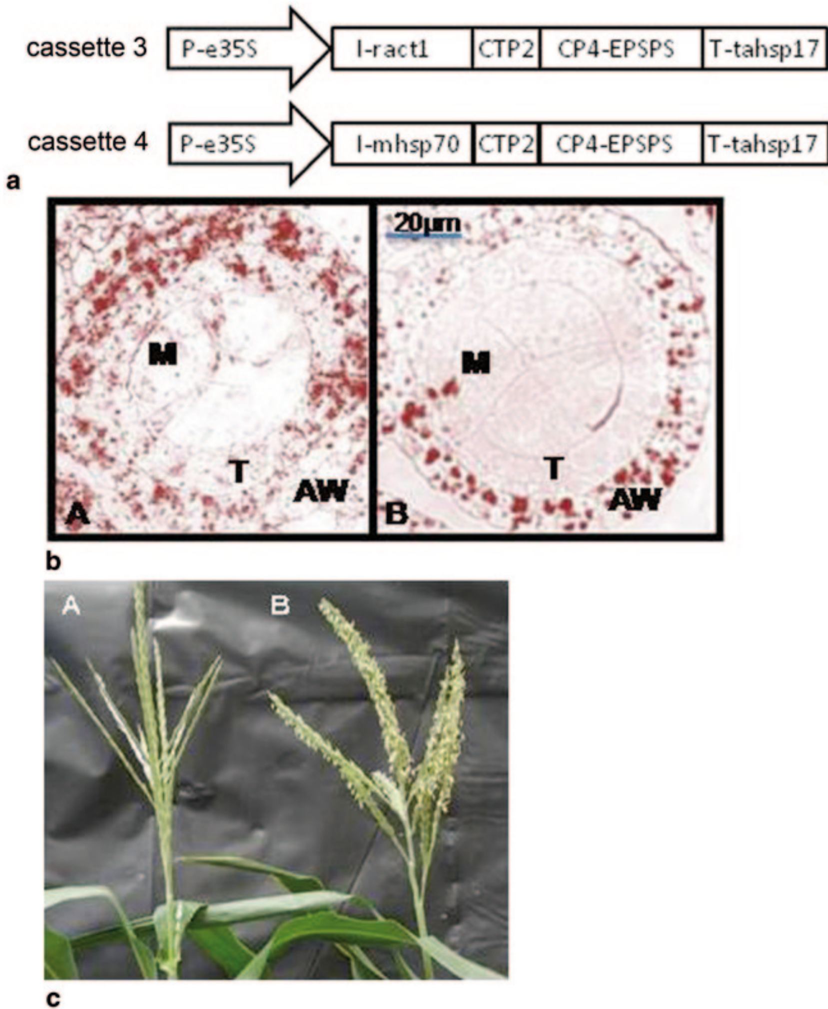

Fig. 6.2 **a** T-DNA cassettes used to generate transgenic corn events. *P-e35S* enhanced CaMV 35S promoter, *I-ract1* first intron of the rice actin gene 1, *I-mhsp70* first intron of the maize heat-shock protein 70 gene, *CTP2* chloroplast transit peptide from *Arabidopsis thaliana* EPSPS (*shkg*), *cp4-epsps* coding sequence for the 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*) gene of *Agrobacterium* sp. strain CP4, *T-tashp17:* 3′ non-translated region of the heat-shock protein 17 gene from wheat, *Triticum aestivum*. **b** Immunolocalization of CP4 EPSPS in developing corn anthers at the microspore mother cell stage of development. *Panel A*: A transverse section through a developing anther from an event containing cassette 3. *Panel B*: A transverse section of an event containing cassette 4. The anther sections were treated with anti-CP4 EPSPS polyclonal antiserum as primary antibody. Positive detection is indicated by development of dark red punctate reaction product. *M* microspore mother cell, *T* tapetum cell layer, *AW* anther wall. Size bar = 20 µm, **c** Transgenic corn plants sprayed with glyphosate at 0.34 kg a.e. ha^{-1} at the V4 stage followed by a second spray at the V10 stage. a: A transgenic event containing cassette 4. b: A transgenic event containing cassette 3

transgenic plants were fully fertile (Fig. 6.2c.B). A vector containing an expression cassette similar to cassette 4 has been used to engineer glyphosate-inducible male sterility for efficient hybrid seed corn production (Feng et al. 2014).

Glufosinate Tolerance

Glufosinate (D, L-phosphinothricin) is the active ingredient in several nonselective herbicides marketed under a number of trade names such as Basta, Challenge, Finale, Ignite, Liberty and Rely. L-phosphinothricin is an amino acid which was identified as the herbicidal component of bialaphos which is a natural extracellular tripeptide produced by *Streptomyces* species. The previously unknown amino acid was discovered independently by two teams; from *Streptomyces viridochromogenes* as reported by Bayer et al. (1972), and from *Streptomyces hygroscopicus* by a team of microbiologists from the Japanese firm Meiji Seika Kaisha (Pline et al. 2002b). Glufosinate is the name given to the chemically synthesized racemic mixture of D-phosphinothricin and L-phosphinothricin. Glufosinate is a fast-acting post-emergence herbicide which displays good efficacy in controlling a wide range of weeds. Glufosinate is absorbed rapidly with greater than 88 % remaining in the treated leaves 72 h after application, while less than 11 % of the absorbed glufosinate reaches the roots (Thompson et al. 1987). It has been demonstrated that transgenic plants transformed with the *pat* gene which confers glufosinate tolerance (see below) metabolize glufosinate differently than non-transformed plants. A single metabolite (acetylated glufosinate) is detected in transgenic plants containing *pat*; while three metabolites (4-methylphosphinico-2-oxo-butanoic acid, 3-methylphosphinico-propanate and 4-methylphosphinico-2-hydroxy-butante) were detected in non-transgenic plants, suggesting that transgene-mediated acetylation of glufosinate competes with endogenous glufosinate metabolism (Dröge et al. 1992; Droge-Laser et al. 1994). Glufosinate was first introduced as an herbicide in Japan in 1984 and was first registered for use as an herbicide in the USA in 1993. The product is now registered for herbicide use in more than 40 countries.

The transgenic Liberty Link® (LL) trait provides tolerance to glufosinate. Glufosinate tolerance is the second most common herbicide-tolerance trait, in part, because it is frequently used as a transformation-selectable marker in the development of other transgenic traits like insect resistance. Liberty Link® corn was commercially available in 1998 and Liberty Link® soybeans were sold for the first time in 2009. Glufosinate-tolerant canola and cotton are also on the market.

Glufosinate tolerance in maize is the result of introducing either the *pat* or *bar* genes which were isolated from *S. viridochromogenes* and *S. hygroscopicus,* respectively. The codons of the *pat* gene were optimized for plant gene expression using synonymous codons which do not alter the amino acid sequence (Murray et al. 1989; Liu and Xue 2005). Events TC1507 and DAS-59122-7 contain a synthetic *pat* gene which was cloned between the CaMV derived 35S promoter and terminator sequences, providing high-level constitutive expression (Odell et al. 1985).

Expression of the *bar* gene in event DAS-06275-8 is regulated by the 35S promoter and enhancer, the alcohol dehydrogenase intron (ADH1) from *Zea mays* and terminator from the *Solanum tuberosum* proteinase inhibitor II.

Glufosinate-tolerant soybean varieties were produced by particle bombardment using a plasmid containing the *pat* gene with the CaMV 35S promoter and terminator. The commercial soybean event A5547-127 also contains partial copies of the bacterial-selectable marker gene *bla* (β-lactamase, encoding ampicillin resistance). One copy of 5′ *bla* sequence and one copy of 3′ *bla* sequence are integrated upstream and downstream of a single copy of the *pat* gene. The *bla* sequences are partial and do not constitute an intact *bla* gene. As expected, they are not expressed.

Glufosinate-tolerant canola (Events HCN92/T45) was developed using the *pat* gene derived from *S. viridochromogenes*—the gene is a synthetic version which was synthesized using synonymous codons preferred by plants. Unlike the previous examples, LL cotton employs the *bar* gene from *S. hygroscopicus*. The expression cassette consists of a codon-optimized *bar* gene flanked by the CaMV promoter and the *nopaline synthase* (*nos*) terminator from *Agrobacterium tumefaciens* (Bevan et al. 1983). Details of the cassettes conferring herbicide-tolerance traits to a variety of GE crops are in Table 6.1.

Table 6.1 Gene expression cassettes conferring herbicide-tolerance traits. (Information is from GM Crop Database which is maintained by The Center for Environmental Risk Assessment (CERA)

Crop	Trait	Transgenic event	Trait cassettes
Canola	Glufosinate tolerance and male sterility	MS8 (DBN230-0028)	P-At-SsuAra/bar/T-G7 P-Nt-Ta29/barnase/T-Nos
Canola	Glufosinate tolerance and sterility restorer	RF3 (DBN212-0005)	P-At-SsuAra/bar/T-G7 P-Nt-Ta29/barstar/T-Nos
Canola	Glufosinate tolerance and selectable marker	HCN92	P-35S/pat/T-35S P-Nos/ntpII/T-octopine synthase
Canola	Glufosinate tolerance	T45(HCN28)	P-35S/pat/T-35S
Canola	Glufosinate tolerance	HCR-1[a]	P-35S/pat/T-35S/T-Nos
Canola	Glyphosate tolerance	GT73 (RT73)	P-FMV/At-CTP2/CP4 epsps/T-Ps-E9 P-FMV/At-CTP1/goxv247/T-Ps-E9
Canola	Oxynil tolerance	OXY-235	P-35S/bxn/T-Nos
Canola	Glyphosate tolerance	MON88302	P-FMV/Tsf1/At-CTP2/CP4 epsps/T-Ps-E9
Canola	Glyphosate tolerance	DP-073496–4	P-At-Ubq10/gat4621/T-St-pinII
Canola	Glufosinate tolerance	HCN10	P-35S/pat/T-35S
Canola	Glyphosate tolerance	GT200	P-FMV/At-CTP2/CP4 epsps/T-Ps-E9 P-FMV/At-CTP1/goxv247/T-Ps-E9
Cotton	Glufosinate tolerance	LLCotton25	P35S/bar/T-Nos

Table 6.1 (continued)

Crop	Trait	Transgenic event	Trait cassettes
Cotton	Glufosinate tolerance and insect control	DAS-24236-5	P-mannopine synthase::4OCS/cry1f/T-ORF25 P-Zm-Ubi1/I-Zm-Ubi/pat/T-ORF25
Cotton	Glufosinate tolerance and insect control	DAS-21023-5	P-ZmUbi1/cry1Ac/T-ORF25 P-mannopine synthase::4OCS/pat/T-ORF25
Cotton	Glyphosate tolerance	MON88913	P-FMV::TSF//At-CTP/CP4 epsps/T-Ps-E9 P-35S::Act8/At-CTP-CP4 epsps/T-Ps-E9
Cotton	Glyphosate and selectable marker	MON1445	P-CMoVb(FMV)/At-CTP2/CP4 epsps/T-Ps-E9 P-35S-nptII/T-nos
Maize	Glufosinate tolerance and insect control	DAS-06275–8	P35S/I-Adh1/bar/T-PinII P-Zm-Ubi1/cry1f/T-PinII
Maize	Glufosinate tolerance and insect control	TC1507	P-Zmubi/cry1F/T-ORF25PolyA P-35S/pat/T-35S
Maize	Glufosinate tolerance and insect control	DAS-59122–7	P-Zm-Ubi/I-Zm-Ubi/cry34Ab1/T-PinII P-Ta-peroxidase/cry35Ab1/T-PinII P-E35S/pat/T-35S
Maize	Glyphosate tolerance and insect control	MON 88017	P-Os-Act/I-Os-act1/CP4 epsps/T-Nos P-E35S/I-Os-act1/Cry3Bb/T-hsp17
Maize	Glyphosate tolerance	NK603	P-Os-act1/CP4 epsps/T-Nos P-E35S/I-Zm-hsp70/CP4 epsps/T-Nos
Maize	Glyphosate tolerance	GA21[b]	P-ract1/I-ract1/CTP-RuBisCo/mZm-epsps/T-Nos
Maize	Glufosinate tolerance and insect control	Bt11	P-35S/I-Zm-Adh/pat/T-nos P-35S/I-Zm-Adh/cry1Ab/T-nos
Soybean	Glufosinate tolerance	GU262	P-35S/pat/T-35S (2 copies)
Soybean	Glufosinate tolerance	A5547-127	P-35S/pat/T-35S
Soybean	Glyphosate tolerance	GTS 40-3-2	P-E35S/Pt-CTP/CP4 epsps/T-Nos
Soybean	Glyphosate tolerance	MON89788	P-FMV::TSF/At-CTP/CP4 epsps/T-Ps-E9

The elements comprising the trait cassettes are ordered 5′ to 3′ with respect to the direction of transcription; *P* Promoter/enhancer, *I* intron, *T* transcription terminator/3′UTR
The cassettes are ordered relative to their respective gene of interest. For traits and gene stacks harboring multiple cassettes, the individual cassettes are present on separate lines. The relative orientation of the cassettes is from top to bottom. For example for event DAS-59122–7 the order of the cassette are (P-Zm-Ubi/I-Zm-Ubi/cry34Ab1/T-PinII)–(P-Ta-peroxidase/cry35Ab1)–(P-E35S/pat/T-PinII). Detailed descriptions of genes of interest and elements can be found in CERA's GM Crop Database (http://cera-gmc.org/index.php?action=gm_crop_database)
[a]Derived from an inter-specific cross with *B. rapa* and the *B. napus* transformation event T45
[b]Single insertion site with three complete copies of modified Zm epsps plus 3 incomplete copies

Biotech Trait Stacks

Over time, a number of weeds have developed resistance to certain herbicides, whether those herbicides were used in a system with conventional crops or in crops enhanced with HT traits derived through modern biotechnology. Since herbicide-resistant weeds cause agronomic issues and can negatively affect yields, overreliance on the use of a single mode-of-action herbicide is not a sustainable option for effective weed control in any agronomic system. The responsible use of products with diverse herbicidal modes of action is an important factor that helps delay the possible onset of weed resistance and provides agronomic options to manage potential issues with resistant or hard-to-control weeds where they exist. Accordingly, HT crops are being developed to have multiple herbicide-tolerance traits through trait stacking, to provide the opportunity for growers to take advantage of the efficacy and spectrum of different chemistries. Most often, such combined trait products ("stacks") are developed using one of two methods—namely, vector stacking and breeding stacking.

For the method referred to as vector stacking, two or more genes are linked physically and the cassette is inserted into the genome as a single unit. Since these stacked traits are integrated at a single genomic/genetic location, they segregate as a single genetic locus, which greatly simplifies the breeding process. Drawbacks to using vector stacks are that the constructs are larger and more complex, and often may contain multiple copies of common gene expression elements such as promoters. Another major challenge of producing efficacious vector stacks is ensuring coordinated expression of each of the individual transgenes in the desired target tissues (Que et al. 2010). While herbicide traits are frequently driven by a constitutive promoter to ensure tolerance in all relevant plant tissues, they are often stacked with traits that provide insect pest control which may require tissue-specific expression. Since enhancer elements, by definition, enhance gene expression independent of orientation and position (Cereghini et al. 1983), they can act on other linked genes in the vector stack which may compromise their desired expression profile. For example, it has been demonstrated that a CaMV 35S promoter/enhancer used to drive expression of the selectable marker affects expression of an adjacent transgene (Yoo et al. 2005). In another case, a 35S enhancer has been shown to affect expression of genes which are 78 kb away (Ren et al. 2004). Strategies have been described to mitigate or eliminate unintended enhancer interactions based on the use of elements such as insulators (Singer et al. 2012).

The second method to produce trait stacks uses traditional plant breeding to combine two or more independently inserted transgenic events, i.e., breeding stacking. The method has been widely used but it becomes increasingly resource intensive for seed production as the number of traits grows. In the context of a commercial breeding program where yield and agronomic improvements need to be maintained, each unlinked transgene doubles the size of the breeding population, limiting the number of loci which can realistically be managed in a breeding program (Hitz 1999; Halpin 2005).

As an example of commercially available stacked product with multiple herbicide-tolerant traits, SmartStax is a branded corn seed trait developed through collaboration between Monsanto Company and Dow AgroSciences LLC. SmartStax hybrids are eight-gene stacks (combination of both vector stacking and breeding stacking) which provide tolerance to two broad spectrum herbicides, glyphosate and glufosinate, and offer multiple modes of action for both above-ground and below-ground insect–pest protection. SmartStax® was developed by conventional breeding of corn lines containing the transformation events MON89034, MON88017, TC1507 and DAS-59122-7. The insecticidal proteins Cry1A.105 and Cry2Ab2 are produced in SmartStax® by one cassette (event MON89034) and the Cry3Bb1 and CP4 EPSPS proteins are produced by a second cassette—(event MON88017). The remaining genes are supplied by event TC1507, encoding the Cry1F protein and event DAS-59122–7 which produces the Cry34Ab1 and Cry35Ab1 proteins. Both cassettes of events TC1507 and DAS-59122-7 also employ the *pat* gene, which served as a selectable marker for plant transformation and provides in-crop glufosinate tolerance in the field.

Stacking traits providing tolerance to glyphosate and glufosinate give growers a choice of two distinct herbicide modes of action for in-crop weed control, as is the case in the example of SmartStax® corn. Both herbicide-tolerance traits have also been stacked in various other combinations with insect protection and other traits in a range of crops (Table 6.2).

Table 6.2 Herbicide-tolerance traits stacks. (Information used in this table is from GM Crop Database which is maintained by The Center for Environmental Risk Assessment (CERA). The details of the trait cassettes conferring herbicide tolerance can be found in Table 6.1. Details of insecticidal and other traits/cassettes not listed in Table 6.2 can be found in CERA's GM Crop Database)

Crop	Product name	Traits	Genes
Canola	InVigor™ Canola	HCN28	*pat*
Canola	InVigor™ Canola	MS1	*bar, barnase*
Canola	InVigor™ Canola	MS1 x RF1	*bar, barnase, barstar*
Canola	InVigor™ Canola	MS1 x RF2	*bar, barnase, barstar*
Canola	InVigor™ Canola	MS1 x RF3	*bar, barnase, barstar*
Canola	InVigor™ Canola	MS8	*bar, barnase*
Canola	InVigor™ Canola	MS8 x RF3	*bar, barnase, barstar*
Canola	InVigor™ Canola	RF1	*bar, barstar*
Canola	InVigor™ Canola	RF2	*bar, barstar*
Canola	InVigor™ Canola	RF3	*bar, barstar*
Canola	Liberty Link™ Independence™	HCN10	*pat*
Canola	Liberty Link™ Innovator™	HCN92	*pat*
Canola	Navigator™ Canola	Oxy235	*bxn*
Canola	Optimum® Gly canola	DP-073496-4	*gat*
Canola	Roundup Ready™ Canola	GT73	*cp4 epsps, gox*
Canola	TruFlex™ Roundup Ready™ Canola	MON88302	*cp4 epsps*

Table 6.2 (continued)

Crop	Product name	Traits	Genes
Cotton	BXN™ Cotton	BNX10211	*bxn*
Cotton	BXN™ Cotton	BNX10215	*bxn*
Cotton	BXN™ Cotton	BNX10222	*bxn*
Cotton	BXN™ Cotton	BNX10224	*bxn*
Cotton	BXN™ Plus Bollgard™ Cotton	31707	*bxn, cry1Ac*
Cotton	BXN™ Plus Bollgard™ Cotton	31803	*bxn, cry1Ac*
Cotton	BXN™ Plus Bollgard™ Cotton	31807	*bxn, cry1Ac*
Cotton	BXN™ Plus Bollgard™ Cotton	31808	*bxn, cry1Ac*
Cotton	BXN™ Plus Bollgard™ Cotton	42317	*bxn, cry1Ac*
Cotton	Fibermax™ Liberty Link™	LLCotton25	*bar*
Cotton	Fibermax™ Liberty Link™ Bollgard II™	LLCotton25 x MON15985	*bar, cry1Ab, cry2A2*
Cotton	GlyTol™	GHB614	*2mepsps*
Cotton	GlyTol™ Liberty Link™	GHB614 x LLCotton25	*2mepsps, bar*
Cotton	Glytol™ x Twinlink™	GHB614 x T304–40 x GHB119	*2mepsps, bar, cry1Ab, cry2Ae*
Cotton	Roundup Ready™ Bollgard II™ Cotton	MON15985 x MON1445	*cp4 epsps, cry1Ac, cry2Ab2*
Cotton	Roundup Ready™ Bollgard™ Cotton	MON531 x MON1445	*cp4 epsps, cry1Ac*
Cotton	Roundup Ready™ Cotton	MON1445	*cp4 epsps*
Cotton	Roundup Ready™ Flex™ Bollgard II™ Cotton	MON88913 x MON15985	*cp4 epsps, cry1Ac, cry2Ab2*
Cotton	Roundup Ready™ Flex™ Cotton	MON88913	*cp4 epsps*
Cotton	TwinLink™ Cotton	T304-40 x GHB119	*bar, cry1Ab, cry2Ae*
Cotton	VIPCOT™ Roundup Ready Flex™ Cotton	COT102 x COT67B x MON88913	*cp4 epsps, cry1Ab, vip3A(a)*
Cotton	WideStrike™ Cotton	281-24-236 x MXB13	*pat, cry1F, cry1Ac*
Cotton	Widestrike™ Roundup Ready Flex™ Cotton	281-24-236 x MXB13 x MON88913	*pat, cry1F, cry1Ac, cp4 epsps*
Cotton	WideStrike™ Roundup Ready™ Cotton	281-24-236 x MXB13 x MON1445	*pat, cry1F, cry1Ac, cp4 epsps*
Maize	Agrisure® 3000GT	BT11 x MIR162 x MIR604	*cry1Ab, mcry3A, pat, vip3Aa20*
Maize	Agrisure® CB/LL	BT11	*cry1Ab, pat*
Maize	Agrisure® CB/LL/RW	BT11 x MIR604	*cry1Ab, mcry3A, pat*
Maize	Agrisure® GT	GA21	*mepsps*
Maize	Agrisure® GT/CB/LL	GA21 x BT11	*cry1Ab, pat,mepsps*
Maize	Agrisure® RW	MIR604	*mcry3A*
Maize	Agrisure® Viptera™ 2100	BT11 x MIR162	*cry1Ab, vip3Aa20, pat*

Table 6.2 (continued)

Crop	Product name	Traits	Genes
Maize	Agrisure® Viptera™ 3110	BT11 x GA21 x MIR162	*cry1Ab, pat, mepsps, vip3Aa20*
Maize	Agrisure® Viptera™ 3111, Agrisure® Viptera™ 4	BT11 x MIR162 x MIR604 x GA21	*cry1Ab, mcry3A, pat, vip3Aa20, mepsps*
Maize	Agrisure™ 3000GT	BT11 x MIR604 x GA21	*cry1Ab, mcry3A, pat, mepsps*
Maize	Agrisure™ GT/RW	MIR604 x GA21	*mcry3A, mepsps*
Maize	Agrisure™ Viptera	MIR162	*vip3Aa20*
Maize	Genuity™ VT Double PRO™	MON89034 x NK603	*cry1A.105, cry2Ab2, p4 epsps*
Maize	Genuity™ VT Triple PRO™	MON89034 x MON88017	*cry1A.105, cry2Ab2, cry3Bb1, cp4 epsps*
Maize	Genuity™SmartStax™	MON89034 x TC1507 x MON88017 x DAS-59122-7	*cry1A.105, cry2Ab2, cry1Fa2, cry3Bb1, cry34Ab1, cry35Ab1, pat, cp4 epsps*
Maize	Herculex® I	TC1507	*cry1Fa2, pat*
Maize	Herculex® I Roundup Ready® 2 Maize	TC1507 x NK603	*cry1Fa2, pat,cp4 epsps*
Maize	Herculex® RW	DAS-59122-7	*cry34Ab1, cry35Ab1, pat*
Maize	Herculex® RW Roundup Ready® 2 Maize	DAS-59122-7 x NK603	*cry34Ab1, cry35Ab1, pat, cp4 epsps*
Maize	Herculex® Xtra	TC1507 x DAS-59122-7	*cry1Fa2, pat, cry34Ab1, cry35Ab1*
Maize	Herculex® Xtra, Roundup® 2 Maize	DAS-59122-7 x TC1507 x NK603	*cry1Fa2, cry35Ab1, cry35Ab1, pat, cp4 epsps*
Maize	Liberty Link™ Yieldgard™ Maize	T25 x MON810	*cry1Ab, pat*
Maize	LibertyLink®	T25	*pat*
Maize	Monsanto Roundup Ready® 2 Maize	NK603	*cp4 epsps*
Maize	YieldGard® Plus	MON863 x MON810	*cry1Ab, cry3Bb1*
Maize	Monsanto YieldGard® Plus with Roundup Ready® 2 Maize	MON863 x MON810 x NK603	*cry1Ab, cry3Bb1, cp4 epsps*
Maize	Optimum™ Intrasect	TC1507 x MON810 x NK603	*cry1Fa2, cry1Ab, pat, cp4 epsps*
Maize	Optimum™ Intrasect XTRA	DAS-59122-7 x TC1507 x NK603 X MON810	*cry1Fa2, cry34Ab1, cry35Ab1, cry1Ab, pat, cp4 epsps*
Maize	Optimum™ Intrasect Xtreme	DAS-59122-7 x TC1507 x NK603 X MIR604 X MON810	*cry1Fa2, mcry3A, cry34Ab1, cry35Ab1, cry1Ab, pat, cp4 epsps*

Table 6.2 (continued)

Crop	Product name	Traits	Genes
Maize	Roundup Ready™ YieldGard™ maize	GA21 x MON810	*cry1Ab, mepsps*
Maize	YieldGard VT™ Rootworm/ RR2®	Mon 88017	*cry3Bb1, cp4 epsps*
Maize	YieldGard VT™ Triple	MON810 x MON88017	*cry1Ab, cry3Bb1, cp4 epsps*
Maize	YieldGard® Corn Borer	MON810	*cry1Ab*
Maize	YieldGard® Rootworm with Roundup Ready® 2 Maize	MON863 x NK603	*cry3Bb1, cp4 epsps*
Maize	YieldGard™ CB + RR	NK603 x MON810	*cry1Ab, cp4 epsps*
Soybean	Cultivance	CV127 (BPS-CV127-9)	*csr1–2*
Soybean	Enlist™ Soybean	DAS68416-4	*pat, aad12*
Soybean	Genuity® Roundup Ready 2 Yield™	MON89788	*cp4 epsps*
Soybean	Genuity® Roundup Ready™ 2 Xtend™	MON87708 x MON89788	*dmo, cp4 epsps*
Soybean	Intacta™ Roundup Ready™ 2 Pro	MON87701 x MON89788	*cp4 epsps, cry1Ac*
Soybean	Liberty Link™ soybean	A2704 (A2704-12)	*pat*
Soybean	Liberty Link™ soybean	A5547 (A5547-127)	*pat*
Soybean	Optimum GAT™	DP-356043-5	*gm-hra, gat*
Soybean	Roundup Ready™ soybean	40-3-2	*cp4 epsps*
Soybean	Vistive Gold™	MON87705	*cp4 epsps, fatb1-A, fad2–1A*

New Traits Conferring New Modes of Action

Dicamba (2-methoxy-3, 6-dichloro benzoic acid) is a member of the synthetic auxin class of herbicides and has been in commercial use since the 1960s. Synthetic auxins can bind to the TIR1 F-box protein that acts as a receptor for natural auxins such as indole-3-acetic acid (Tan et al. 2007). Through this interaction, synthetic auxins may alter gene expression and initiate a cascade of events that ultimately leads to a variety of physiological effects including increased ethylene and abscisic acid production, abnormal cell growth and cell death (Grossmann 2000).

Dicamba is effective against many broad-leaved plants and has been widely used to control these weeds in grass crops such as corn and wheat. Treatment with dicamba results in a variety of symptoms in broad-leaf species including leaf cupping, stem and petiole twisting and rootlength inhibition. Dicamba may also cause some crop injury to grasses depending on germplasm, environmental conditions or developmental timing of application. In corn, symptoms include brace root

Fig. 6.3 Soybean (*top*) sprayed with 1.5 lb/a.c. dicamba in 2005 and corn (*bottom*) plants sprayed with 1 lb/a.c. dicamba in Illinois in 2009

malformation, lodging and twisted whorls. Examples of soybean and corn plants treated with dicamba are shown in Fig. 6.3.

By screening soil and water samples from a dicamba-manufacturing plant, Krueger et al. (1989) isolated several species of bacteria that were capable of using dicamba as a sole carbon source. The enzyme dicamba mono-oxygenase (DMO) was isolated from one of these species, *Stenotrophomonas maltophilia* DI-6 (Chakraborty et al. 2005; Herman et al. 2005). This enzyme is a member of the Rieske non-heme oxygenase family and forms a multicomponent enzyme complex along with ferredoxin and a reductase (Chakraborty et al. 2005; Wang et al. 1997). The latter two proteins form an electron-transfer chain that shuttles electrons from nicotinamide adenine dinucleotide (NADH) to oxygen bound by the DMO enzyme which catalyzes the oxidative demethylation of dicamba (D'Ordine et al. 2009; Dumitru et al. 2009). The resulting breakdown product,

3, 6-dichlorosalicylic acid (DCSA), is non-herbicidal, making DMO a good candidate for engineering dicamba tolerance into crop species (Behrens et al. 2007; Subramanian et al. 1997).

In the absence of ferredoxin and reductase, DMO is not capable of degrading dicamba in vitro (Wang et al. 1997). Because plant ferredoxins resemble those from bacteria, targeting the DMO enzyme to chloroplasts where reduced ferredoxin is abundant, eliminates the need to co-express a bacterial ferredoxin with DMO. Addition of a chloroplast targeting signal to a nuclear-encoded protein or integrating the DMO gene into the chloroplast genome, results in plants that are tolerant to high concentrations of dicamba, indicating that endogenous plant proteins can effectively transfer electrons to DMO (Behrens et al. 2007). Furthermore, targeting DMO to the chloroplast improves dicamba tolerance in corn and soybean as compared to DMO proteins expressed without a targeting signal (Cao et al. 2011; Feng et al. 2010b). These results show that, like EPSPS, effective subcellular targeting is required for optimal activity of DMO.

Expression of DMO in soybean, cotton and corn under the direction of constitutive promoters, has resulted in plants tolerant to dicamba (Cao et al. 2011; Feng et al. 2010b). The first crop engineered for dicamba tolerance is soybean and it is expected to be on the market mid-decade. In this crop, the peanut chlorotic streak virus promoter was used to successfully engineer plants with tolerance to field use rates of dicamba. Plants showed little injury to 0.56 kg ai/ha dicamba (typical field use rate) when treated at preemergence, V3 and R1 growth stages (Brinker et al. 2011; Feng et al. 2010b).

A cotton plant engineered to contain a vector stack, delivering tolerance to both dicamba and glufosinate herbicides, is in commercial development and is also expected to be on the market around the middle of the decade. In this cotton plant, the peanut chlorotic streak virus promoter is used to drive expression of DMO, while the 35S promoter from CaMV is used to drive expression of the *bar* gene to deliver glufosinate tolerance. These herbicide-tolerant cotton plants showed no reduction in yield when treated with 0.5 lb ai/A dicamba at multiple stages from preemergence to 12–15 node stage (Brinker et al. 2012). In addition to soybean and cotton, dicamba tolerance is in development for corn and canola crops.

Summary

Weeds can cause significant yield losses for farmers. For this reason, control of weeds in cultivated fields has been a focus of modern agriculture production and is a persistent challenge. Systems and methods for weed control are many faceted, but introduction of crops having a GE-herbicide-tolerant trait revolutionized management practices in those crops. In the 1990s, the introduction of glyphosate-tolerant and glufosinate-tolerant cropping systems simplified weed control, and led to increased adoption of conservation tillage practices.

Scientists have used the knowledge of the interaction of the herbicide with the crop plant to genetically engineer expression of herbicide-resistance genes in the right cells, at the sufficient level and at the correct time to provide tolerance when the plant is exposed to the herbicide. They have also used the knowledge of the pathways affected to place the enzyme encoded by the gene into the right compartment of the cell to ensure either the detoxification of the herbicide or the mitigation of herbicide action.

With the increasing prevalence of herbicide-resistant weeds, there is a need to develop crops with tolerance to multiple herbicides of different modes of action. These multi-herbicide-tolerant crops are being generated with both breeding and vector stacks of herbicide traits, thereby enabling cropping systems that incorporate the use of herbicides with multiple modes of action to combat weeds and reduce the risk of weeds evolving resistance to any one of the herbicides. Mathematical modeling comparing weed control systems and limited field studies support the effectiveness of the use of herbicides with multiple modes of action for the management of herbicide-resistant weeds (Powles et al. 1997; Diggle et al. 2003; Neve 2008; Lagator et al. 2013; Beckie and Reboud 2009).

A challenge of trait stacking is the ability to co-express multiple genes conferring herbicide tolerance (and any other trait such as insect control) in a single plant with each transgene expressed in a location, level and timing to provide tolerance to all the target herbicides.

Refinement of vector construction will be a focus of future research in the generation of vector-stacked traits. Herbicide-tolerance traits were one of the first uses of biotechnology to agriculture and will likely continue to be one of the areas driving new technologies for crop improvement.

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Part II
Other Economically Important Crops

Chapter 7
Strategies to Increase Heterologous Protein Expression in Rice Grains

Somen Nandi and Gurdev S. Khush

Introduction

Protein synthesis and expression is a tightly regulated process involving many enzymes and cofactors at various steps. Expression of a protein outside of its natural host system is called heterologous protein production, and the product is termed a heterologous protein (Mahmoud 2007; Rai and Padh 2001), or a recombinant protein. In vivo expression of proteins is a very complex process, which involves posttranslational modification of proteins required for stability and biological activity, such as glycosylation, phosphorylation, and correct folding (Desai et al. 2010). There are different types of heterologous proteins, including therapeutic proteins or those used for clinical diagnosis, proteins used as reagents for research and study purposes, and proteins with various industrial applications. Among the above categories, therapeutic proteins constitute a special class, with stringent quality standards, but they usually have high value.

Most of today's therapeutics, drugs, diagnostic molecules, antibodies, and vaccines are made of recombinant proteins. The costs of pharmaceuticals are increasing along with global inflation, and in turn, half of the global population cannot keep up with the cost of medicines and therapeutics. Consequently, there is a clear need to reduce significantly the cost of medicines and make them available to the growing population. Currently, many recombinant molecules are expressed in cell culture systems (e.g., bacterial, mammalian, Chinese hamster ovary cell) that are known

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to be cost-effective, scalable, and scientifically well understood, while allowing for fast, high-level expression of proteins. However, in many cases, microbial systems often fail to deliver correctly folded and functional proteins (Wurm 2004; Oztruk and Hu 2006). In contrast, eukaryotic cells, including plant cells, exhibit a major advantage by allowing the correct assembly and folding of recombinant polypeptides.

The use of plants as bioreactors for the large-scale production of recombinant proteins has emerged as an exciting area of research. The capacity constraints and economic bottlenecks faced with other protein production platforms (microbial, yeast, mammalian) have driven considerable attention towards plant molecular pharming. Utilizing plants for the large-scale production of recombinant proteins is estimated to be 2–10 % the cost of using microbial platforms, and up to 1000-fold more cost-effective than mammalian platforms (Twyman et al. 2003; Sharma and Sharma 2009). Production of heterologous proteins in plant cells is becoming commercially acceptable for human therapeutics (Langer 2010), vaccine antigens (Hefferon 2013), industrial enzymes (Broz et al. 2013), and nutraceuticals (Maxmen 2012). The seed has emerged as one of the most prominent plant organs for the recombinant protein production. In plants, the seed serves as a storage organ that is also required for the establishment of the new generation (Yang et al. 2008). It has been shown that throughout the dormancy and storage periods of the rice seed, its storage proteins remain intact and functional (Tackaberry et al. 2008) and heterologous proteins are also stable in seeds for 2–3 years. This is a considerable advantage over plant cell platforms that accumulate protein in leafy tissue (e.g., tobacco), as they are more prone to hydrolytic inactivation and have much shorter shelf lives. Rice is a self-pollinating crop and has GRAS (generally recognized as safe) designation by the Food and Drug Administration, making it a strong candidate for the large-scale production of heterologous protein for biopharmaceuticals. This chapter evaluates the strategies, needs, and future prospects to increase the heterologous protein expression in rice grains.

Factors that Influence Enhanced Heterologous Protein Expression

The endosperm is the main storage compartment for the rice grain and accounts for more than 80 % of the total seed weight. Thus, it is the target site for protein accumulation. Rice seeds are generally composed of 7–12 % protein and 88–93 % starch. The protein composition of the endosperm is 60–70 % glutelins, 25–30 % prolamins, 5–10 % globulins (Glbs), and 0–5 % other proteins, depending on the rice variety. Protein yields have received considerable attention as they play a key role in the performance of the production platform (Zhang et al. 2010; Broz et al. 2013). The most common strategies to achieve a higher yield of the recombinant protein in rice seeds are by optimizing the promoter region, the untranslated regions, translation efficiency, subcellular localization/targeting of the target protein, codon optimization, knockdown or antisense technology, stable integration of the

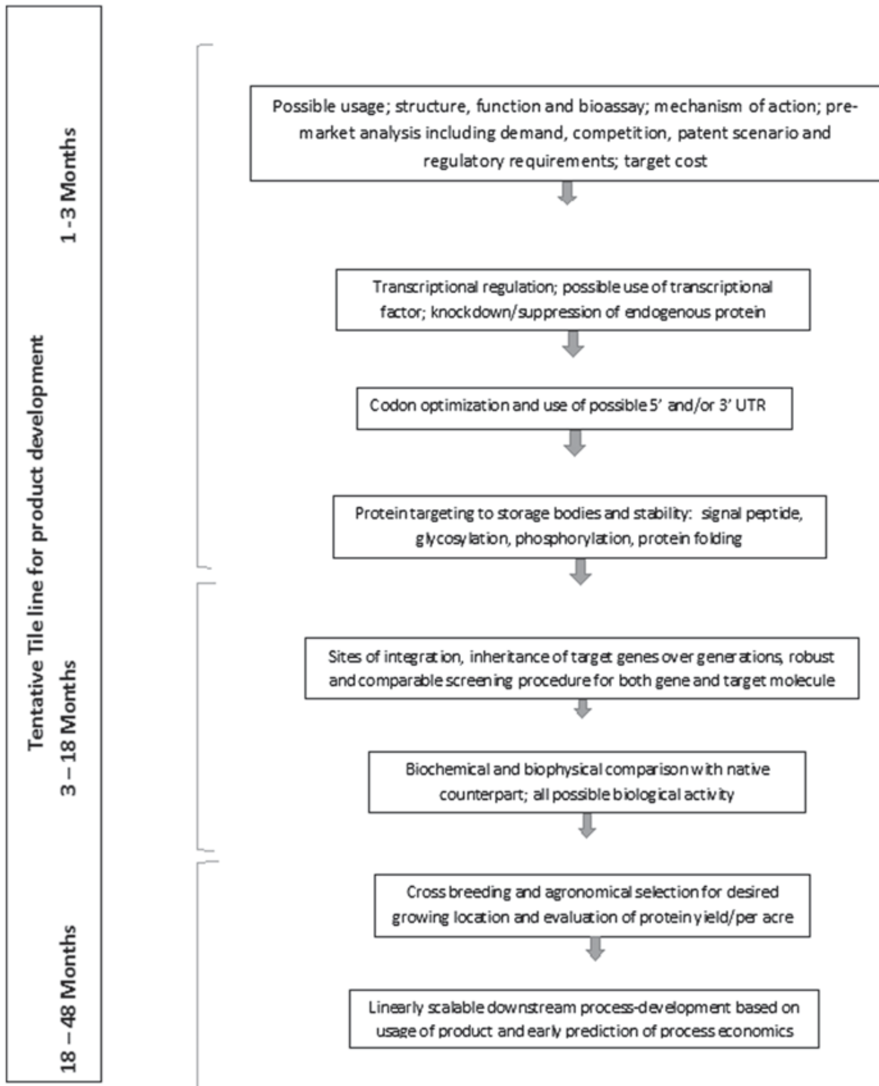

Fig. 7.1 Schematic diagram representing selected fundamental items that need to be addressed for expressing an economically viable heterologous molecule in rice seeds

gene and its copy number, screening, selection, and breeding. A schematic diagram (Fig. 7.1) represents some of the fundamental parameters that should be addressed to express economically viable heterologous molecules in rice seeds. Most of the items are discussed in this chapter, but others, such as selection, characterization, and regulatory need of target molecule and product, will vary on a case-by-case basis and should be addressed before starting the project. Although selection and prior knowledge about the target molecule is one of the key criteria for successful

business, it is not within the scope of the current chapter. The following are the key factors for the enhancement of protein production in rice seeds.

Promoter and Transcriptional Regulation Transcription is controlled by promoter activity and regulated by the cis elements on the promoters. Strong constitutive promoters, such as the cauliflower mosaic virus 35S promoter (Stoger et al. 2000), the rice ubiquitin (Wang et al. 2003), and actin promoters (Huang et al. 2006), have been used to drive the expression in rice seeds. Although these promoters are known to be highly active in plants, they showed low expression in seeds of monocotyledonous (less than 5 % of total seed protein). Constitutive promoters also do not allow for the deposition of the recombinant protein only in seeds. This ultimately reduces the opportunity to develop a cost-effective purification strategy that does not rely on prior art.

Alternatively, seed-specific rice promoters, the ones driving the expression of the major storage proteins (glutelin, prolamin, Glb), have been shown to provide greater seed expression levels than strong constitutive promoters (Qu and Takaiwa 2004). Many rice seed-specific promoters have been characterized for their activity, and among them, the glutelin, Glb, and 10- and 16-kDa prolamin promoters have shown to give the highest expression levels ranging from 6 to 15 % of total seed protein (1–2 % total seed weight; Nandi et al. 2002; Huang et al. 2002; Qu and Takaiwa, 2004; Wakasa et al. 2006a; Wu et al. 1998; Zhang et al. 2010). The use of strong spatiotemporal promoters helps expression of protein at specific times during seed development. In a comparative study of rice seed expression systems, various strong promoters for storage protein genes were evaluated using both transient and transgenic expression systems (Hwang et al. 2001, 2002). β-Glucuronidase (GUS) and human lysozyme genes were used as reporter genes in transient and transgenic analysis, respectively. Rice glutelin 1 (Gt1) promoter and Glb promoters from the respective rice genes showed the strongest promoter activities in the abovementioned study.

Transcription can be enhanced by factors that bind to the cis elements on the promoter. Cis elements are what determine, either independently or synergistically, the spatial and temporal expression of the promoter. The strength of these motifs is dependent on their copy number and location (Rushton et al. 2002). The effects of various transcriptional factors on the recombinant protein expression in rice seeds were studied. These factors include rice endosperm bZIP (REB) binding to the rice Glb promoter, prolamin box-binding factor (PBF) binding, and opaque 2 (O2) regulation of storage protein expression in maize. Rice plants containing the human lysozyme gene were generated both with and without the transcription factors. The results showed a 3.7-fold increase in human lysozyme expression when co-expressing a Glb promoter-specific REB transcriptional factor with the Glb-lysozyme construct (Yang et al. 2001). A significant increase in human lysozyme was observed when co-expressing PBF with the Gt1-lysozyme construct (Yang et al. 2003). Furthermore, transient analysis showed that PBF and O2 can act additively to enhance the expression of the GUS reporter gene in immature rice endosperm (Hwang et al. 2004). Cis elements found in other monocotyledonous species (e.g., coconut, oats)

can be used to direct transgene expression in the rice endosperm, though they have not been proven to be more effective than native seed storage promoter cis-acting elements (Ye et al. 2000). A sequence-specific vacuolar sorting determinant (ss-VSD) has been identified by serial deletions of the rice glutelin gene and its role in the protein-sorting process analyzed by transgenic approaches. Testing site-directed mutagenesis in transient assays showed that the leucine residues in the ssVSD are crucial for protein sorting (Li et al. 2013). Thus, in order to elevate the expression level in transgenic rice seed, the trans-acting factor(s) used should be limited to those native to the plant.

Translational Efficiency and Role of Untranslated Regions The 5′ untranslated region (5′UTR) is located just upstream of the translation initiation site and plays an important role in translation (Kawaguchi and Bailey-Serres 2002). Modifying the 5′UTR of a transgene can increase the expression levels, though it is difficult to precisely evaluate its efficiency, since it works in tandem with many other factors (Mauro and Edelman 2002). The untranslational leader sequence is one of the key elements of translation initiation, a determining factor of the number of peptides produced from each mRNA. Abundant mRNA sets the foundation of effective abundant protein translation. The use of a 5′UTR region from a rice polyubiquitin gene (rubi3) has shown its efficacy in enhancing monocotyledon gene expression (Sivamani et al. 2009). Rubi3 is an abundant protein involved in protein degradation and control of the cell cycle. The 1140-bp 5′UTR of this gene enhances its promoter activity by 20-fold in transient expression assays (Sivamani and Qu 2006; Lu et al. 2008). The highly expressed rice α-tubulin genes (Ostua1, Ostua2, and Ostua3) have been isolated and analyzed for expression. The upstream 5′UTR intron of α-tubulin genes is responsible for their expression (Fiume et al. 2004). The 5′UTR intron in rice β-tubulin gene Ostub16 has also been used to enhance expression levels in rice (Giani et al. 2009). Both transient expression (Hwang et al. 2002) and transgenic analyses (Huang et al. 2002) in rice seeds have showed a high level of target protein expression with the use of a portion of the native 5′UTR sequence of Gt1. The native 5′UTR of a strongly expressed gene is found to be most effective for the recombinant protein expression. Thus, the native 5′ untranslated sequence was often used in expression cassettes.

The 3′ untranslated region (3′UTR), located just downstream of the transcription stop codon, is responsible for pre-mRNA 3′-end formation (cleavage and the addition of poly (A) tail) and helps stabilize the transcript. The poly (A) tail, in particular, plays an important role in determining transcript stability and function (Hunt 2008; Samadder et al. 2008), and a poor 3″UTR can greatly reduce transcript stability (Green 1993). Incorporating 3′UTRs harboring these elements can increase gene expression (Dong et al. 2007). Studies using rice as a host demonstrated that the rice glutelin, Gt1 3′UTR, when used with a constitutive ubiquitin promoter to drive reporter gene expression, caused a 1.8- and 4-fold higher increase in recombinant seed protein levels when compared with a nopaline synthase terminator (Yang et al. 2009). Although very few 3′UTRs have been investigated for their impact on rice transgene expression (Mishra et al. 2006), it is apparent that the choice of a 3′UTR

might contribute to the higher mRNA stability and accumulation of a recombinant protein.

Posttranslational Regulation Compartmentalization of Proteins Posttranslational regulation mainly includes signal peptide cleavage, glycosylation, phosphorylation, and correct protein folding, while the protein is translocated into the endoplasmic reticulum (ER) and transported to the Golgi apparatus. Subsequently, the protein is targeted and transferred to the protein storage vacuole (PSV), ER-derived protein bodies (PB) and other organelles, or secreted into the cytosol (Marty 1999; Neuhaus and Rogers 1998). In these steps, the proteins can be accumulated in large amounts, or turned over rapidly due toprotease activity, depending on the targeting destination of the protein. It has been shown that untargeted heterologous proteins are expressed at low levels in plant cells (Giddings et al. 2000; Larrick et al. 2001; Schillberg et al. 2003) because the proteins are exposed to protease(s) degradation. In rice grain, two PB, PBI, and PBII, are considered the major protein storage systems during endosperm development. Protein bodies provide secure environments for the accumulation and deposition of recombinant proteins, because of limited protease activity. Targeting heterologous protein to PB in rice endosperm cells can be achieved by attaching a signal sequence to a mature peptide of the target molecule, which can guide that molecule through the inner membrane system instead of the cytosol. As soon as the gene is transcribed and processed, mRNA is bound to the sub-domains of the ER, which determines to where the protein is targeted (Choi et al. 2000; Li et al. 1993; Okita and Choi 2002). The synthesized recombinant protein is then targeted to the PB through the protein-trafficking pathway during endosperm development (Vitale and Galili 2001). A comparative study with and without the signal peptide confirms this hypothesis. In the expression of heat stable β-glucanase in barley grain, a signal peptide was used from hordein D, a barley storage protein. The expression level of β-glucanase with the hordein signal peptide is several-fold higher than that of the same construct without the signal peptide (Horvath et al. 2000). A wheat puroindoline b promoter and signal peptide have also been tested. Co-expression of both constructs (Gt1 promoter and its signal peptide with the human lysozyme gene and the puraindoline b promote and its signal peptide with the human lysozyme gene) resulted in an increase in the expression of human lysozyme by 79 % to 8 mg/kg of rice grain flour (Huang and Yang 2005). Electron microscopic studies show that the puroindoline-based construct directed recombinant lysozyme to both PB I and II. Therefore, the use of a particular targeting signal to deposit the recombinant proteins to certain organelles or cell compartments to prevent their degradation (Takagi et al. 2010) is a promising gene expression strategy for heterologous proteins in rice.

Codon-Optimization Translational efficiency is another important element that affects protein synthesis and accumulation. Translational efficiency is highly impacted by codon choice within the gene. Due to genetic codon degeneracy, codon usage has high diversity among different organisms. However, in triple-letter genetic codons, the first and second positions are largely conserved among organisms, usually the third-position codons vary among organisms. For example, the

preferred codons in rice genes at the third position are 100% G or C. This is not always the case for other organisms (e.g., the preferred codons in *Arabidopsis* genes are 15% G or C at the third position of the codons). Therefore, when expressing foreign genes, using the preferred codons of the host can maximize translational efficiency. This has been confirmed by several laboratories (Akashi 2001; Davis 1999; Rouwendel et al. 1997). In the expression of human lysozyme, lactoferrin, and transferrin in rice grain, the codons were optimized for all three synthetic genes (Nandi and Huang 2009; Zhang et al. 2010). For example, in the expression of recombinant human lactoferrin in rice, a total of 92 out of 130 codons were modified resulting in the G+C content being raised from 46 to 68%. The gene was synthesized based on the codon preference of rice genes. Of the 692 codons for the mature peptide of the human lactoferrin gene, 413 codons were changed (Nandi et al. 2002). Further detailed biochemical and biophysical characterization remains consistent with its native counterpart, i.e., human lactoferrin (Nandi et al. 2005). In producing the human blood protein, α-1-antitripsin, in rice culture cells, the expression of the codon-optimized gene was several-fold greater than that of the native gene (Huang et al. 2001; Terashmia et al. 1999). In expressing another protein, subtilisin, the expression of the codon-optimized gene was more than 100-fold greater than that of the native gene. Based on the available data, it is apparent that foreign genes have to be codon-optimized to match the codon preference of the host for the enhanced level of the expression.

Knockdown of Endogenous Protein to Raise Heterologous Protein Expression When rice seeds are potential hosts for the production of heterologous or recombinant proteins, they can compete with endogenous storage proteins. Therefore, attempts have been made to suppress endogenous seed storage proteins to achieve more space for heterologous protein accumulation. It has also been indicated that rice endosperm cells are capable of generating novel storage vesicles for the recombinant protein. When protein body structures of the high-expressing lines of recombinant human lysozyme were evaluated, it was observed that rice endosperm generated novel storage vesicles, or protein body variants, for the recombination protein deposition (Yang et al. 2003). Reduced native storage protein in high lysozyme expressing lines indicates that the recombinant protein can partially compete for ER subdomains with native storage proteins, and chaperones during trafficking. This implies that human lysozyme expression could be increased further by shutting down native storage protein expression, making more space available to recombinant protein deposition. This was tested by reducing the endogenous protein expression via antisense technology. The antisense constructs of glutelin and Glb were introduced using gene stacking into the transgenic lines that expressed high levels of human lysozyme (Huang and Yang 2005). Recombinant human IL-10, a therapeutic treatment candidate for inflammatory allergy and autoimmune diseases, protein yield in rice seed is enhanced by specific suppression of endogenous seed proteins at the same deposit site. This was possible through the selective reduction, via RNA interference (RNAi), of the endogenous seed storage proteins of prolamins or glutelins (Yang et al. 2011). In a similar work, the enhanced production of

human growth hormone was achieved in transgenic rice seeds by co-introduction of RNAi cassettes to suppress the prolamin and glutelin that effectively suppressed endogenous storage protein genes (Shigemitsu et al. 2012). Binding protein (BiP) is the key chaperone involved in folding of secretory proteins, such as seed storage proteins, in the ER lumen. Judicious modification of BiP levels in transgenic rice seeds might provide suitable conditions (Wakasa et al. 2011a) for the production of secretory proteins by alleviating ER stress that ultimately creates more space for heterologous protein.

Stable Integration of Gene, Chromosomal Loci/Position Effects, Gene Copy Number The stable integration of incoming recombinant DNA into cellular DNA is largely a random process, and accordingly, the sites of integration are dispersed over the genome. Thus, high variation has been found in transgenic lines (Nandi et al. 2002, Fig. 6; Huang et al. 2002, Fig. 2) as well as instability in expression levels (Huang and Yang 2005, Fig. 8C). Indeed, in order to meet the requirements for high and stable protein expression, extensive screening is performed to identify those lines that provide optimal protein production (Nandi et al. 2005). Genomic regions can range from highly active (euchromatin) to transcriptionally silenced (heterochromatin) as a result of differential nucleosome arrangements, interactions of nonhistone proteins, and histone modifications and variants (Bernstein and Hake 2006; Ghirlando and Felsenfeld 2008; Mutskov et al. 2007). Euchromatin is often referred to as being in an "open" conformation and possesses irregularly spaced nucleosomes that are highly acetylated and methylated at histone proteins, such as H3K4 and H3K79. The distinct positioning of euchromatin and heterochromatin within the nucleus of eukaryotic cells is thought to correlate with particular environments appointed for chromatin activation and repression, respectively (Heard and Bickmore 2007). The presence of these genomic zones of activity/repression often proves to be a hindrance to plant biotechnology, as chromatin-mediated silencing of the introduced transgene can occur if integration takes place within or near a region of heterochromatin. Once integrated into the cellular DNA, the transgene cassette is affected by neighboring chromosomal elements that modulate the promoter to a considerable extent. Enhancers and silencers directly affect the cis elements of the promoters and may be shielded by insulators.

Procedures have been studied for enhancing the copy number of transgene integration by gene amplification. Transgene copy number can be positively or negatively associated with transgene expression (Hobbs et al. 1993). For stable expression, inheritance of transformed gene(s) over the generations and original copy number might play the key role for overexpressing lines (Nandi and Huang 2009, Figs. 12.3 and 12.4; Broz et al. 2013, Fig. 2B). The transgene integrations are due to genetic rearrangements during gene amplification. These site-dependent chromosomal positions affect trigger significant variability between individual transformants in terms of transgenic expression levels (Singer et al. 2012), as the positioning of transgene insertion is largely a random event in plants. A related phenomenon, known as position effect variation, has been suggested to be the consequence of a stochastic spread or retreat of heterochromatin towards or away from the transgene

(Volfson et al. 2006). Resultant lines become heterogeneous in their expression levels. One possible strategy to counteract this effect in transgenic plants is to flank a transgene with elements that block the spread of heterochromatin, allowing the foreign gene to be expressed appropriately, regardless of its insertion site within the host genome. Barrier insulators, are one such element which have been proposed to play a role in genome organization through the arrangement of chromatin fiber into functional domains, whereby genes in one domain are protected from the regulatory effects of another (Lunyak 2008). Possibly, the most well-studied class of putative barrier elements with potential applications in plant transgenic technology is a 1.3-kb 30 matrix attachment region (MAR) from *Phaseolus vulgaris*. MARs have been suggested to trigger the formation of chromatin loops, thus delimiting the boundaries of discrete chromosomal domains (Bode et al. 2000). For example, the 3′ MAR associated with the tobacco Rb7 gene was found to augment significantly the expression of a flanked transgene when compared to controls lacking this MAR (Allen et al. 1996; Cheng et al. 2001; Ulker et al. 1999) and, in some instances, was also able to decrease variability between transgenic lines through a reduction in transgene silencing (Halweg et al. 2005; Mankin et al. 2003). Similarly, the presence of chicken lysozyme MAR elements (Phi-Van and Stratling 1988) flanking transgenes in tobacco and rice has been found to reduce the variability by reducing silencing effects (Mlynarova et al. 1994; Oh et al. 2005). It has been proposed that these elements will be one of the most important tools for generating transgenic plants with stable expression of foreign genes (Tao et al. 2006). However, despite their promise, results with MAR elements have been somewhat ambiguous and their use in transgenic constructs may not be as straightforward as anticipated initially. Ongoing research in both mammalian (Nehlsen et al. 2009) and plant systems might enable recombinant protein expression by targeting preselected chromosomal loci. Currently, upon random integration, individual cell clones display a highly heterogeneous expression pattern and have to be screened for appropriate, stable expression. Recombinant protein expression in rice is achieved by stable integration of transgenes into the chromosomal DNA of established transgenic lines. The chromosomal surroundings have a strong influence on the expression of transgenes. The exploitation of defined loci by targeting expression constructs with different regulatory elements is an approach to create high-level expression systems. Furthermore, this will allow for the evaluation of the impact of chromosomal surroundings on distinct vector constructs (Nehlsen et al. 2009).

Screening, Selection, and Breeding The integration of transformed recombinant DNA into chromosomes is a random process and individual transformed lines display a highly heterogeneous expression pattern. A robust, sensitive, and stable measurement method for the target molecule is the foundation for screening. It is important to address these issues during very early generations of selection, in order to take advantage of desired level and stable expression over generations, which is always a challenge in a cell culture-mediated platform. The common strategy is to identify multiple high-expressing lines from over hundreds of transformed events, particularly those following a Mendelian segregation ratio (3:1, in the case

of a single dominant gene) in their first generation (Wu et al. 2002). The classical Mendelian ratio permits the assumption that the target gene is in one locus, which can be confirmed later by stable expression, as well as marker-assisted selection (MAS) analysis. The homozygous lines can be identified by second-generation individual seed screening. Finally, a line has to be selected through a combination of desired high expression and stably inherited target gene(s). The selected lines can be crossbreed to agronomically elite and location-specific varieties. Generally, a high-throughput single seed descent (SSD) procedure is followed to expedite this process. Furthermore, it is imperative to perform agronomic selection in the field with the combination of MAS, protein yield, and grain yield. Ultimately, per acre of protein yield has to be derived from yield X (protein+grain). Therefore, careful screening and selection of homozygous lines in early generations, along with the appropriate classical breeding approach (Nandi and Huang 2009), is one of the most powerful strategies to achieve the commercial level expression.

Protein Process Development and Techno-Economics

In the biopharmaceutical industry, a good technology portfolio, strong intellectual property position, and access to capital might not guarantee success. Flexibility, cost-effectiveness, and time to market are the key issues as well. Biopharmaceutical companies are keen to introduce their products to market as quickly as possible to attract a majority of the possible market share. Therefore, the decision for future expansion of any product development process becomes impeded, as this decision must be made quite early, during the product development stage. Such decisions are difficult to change later, primarily due to regulatory constraints. In order to achieve an acceptable return on investment, biopharmaceutical companies focus on reducing the cost of drug or product development and improving the overall time to market. Costs associated with processing for any commercial product are largely dependent on the final product. Thus, the final production cost will be the driving force for commercialization of plant-made recombinant proteins. The cost will be dependent on its intended use, for example, oral or skin care therapeutics, nutraceuticals, pharmaceuticals, or other industrial applications. The product value will be much less for functional food than for a high-purity pharmaceutical product (Wilken and Nikolov 2012). It is very important to keep the integration of process operation in mind during selection and process development of the product. Early analysis of developed processes is pivotal in transforming research and development processes into manufacturing ones (Nandi et al. 2005). This has an immense cost impact, if processes are frozen at the early stages of clinical trials and production (Rathore et al. 2004). The manufacturing cost for plant-produced proteins consists of upstream (biomass production) and downstream recovery and purification costs. The cost of manufacturing in most cases is impacted by protein expression, overall process yield, and production scale.

The downstream processing costs are also affected by the ease of the product recovery, the complexity of clarified plant extracts, protein stability, and required purity (Azzoni et al. 2002). For example, biopharmaceuticals and processing enzymes for cyclic guanosine monophosphate (cGMP) manufacturing may require protein purities as high as 95–99 %, and those for diagnostics about 90 %. Although in both of these cases, the downstream manufacturing processes have to be robust (batch-to-batch repeatability), the main differences would be required documentation and regulatory-related activities. These are often "hidden costs" in the biopharmaceutical manufacturing industry that are not readily available in the published literature and often either unaccounted for or underestimated (Farid 2007). A scientific study using a discrete event modeling (DEM) approach reported that the projected cost of purified recombinant lactoferrin from rice seeds was US$ 5.90 per g (Nandi et al. 2005). It has been reported recently that cost of goods for similar cGMP grade products can be comfortably achieved at US$ 3.75 per g (Broz et al. 2013). This validated the idea that incorporating a linearly scalable protein purification methodology into the manufacturing process will have a major impact later in process economics, as long as the procedure allows for linear scalability of each step. Supporting activities, such as process and cleaning validation, buffer preparation, equipment cleaning, and quality control and quality assurance (QC/QA), can be a substantial fraction of operating costs. For example, the labor cost for validation and QC/QA activities can account easily for more than 50 % of the direct manufacturing labor cost. The breakdown of upstream production and downstream purification costs depend primarily on the end application of biopharmaceutical and industrial proteins being at the opposite ends of the cost and purity spectrum (high to low). In general, the upstream cost for highly purified proteins (90 % and above) from seed crops ranges from 5 to 10 % of the total manufacturing cost, depending on the expression level, purification yield, and annual product output (Evangelista et al. 1998; Mison and Curling 2000; Nandi et al. 2005). An overview of downstream processing steps for bioreactor-, leafy-, and seed-based systems was well presented by Wilken and Nikolov (2012). Representative extraction and purification processes published since previous reviews in 2004 (Menkhaus et al. 2004; Nikolov and Woodard 2004) were summarized in this chapter. The production of human lactoferrin (Nandi et al. 2005), lysozyme (Wilken and Nikolov 2006, 2010), and transferrin (Zhang et al. 2010) produced in rice seeds was shown to be clearly advantageous at least for product concentration (Wilken and Nikolov 2012, Table 1); purity and yield (Wilken and Nikolov 2012, Table 2) were higher over other proteins produced in corn, soybean, and rapeseeds.

Versatility of a Rice Seed-Based Expression System

There are more than 20 plant-derived pharmaceuticals currently in phases I and II of clinical trials and about 10 plant-derived pharmaceuticals that are either in phases III and IV or currently being marketed as a medical device or fine chemical. Plant

Table 7.1 Selected highly expressed recombinant molecules produced in rice seed. The expressed molecules show a wide range of variation in their molecular weight (MW), usage, biological activity, and chemical property.

Target molecule	Approx. MW (kDa)	Description/usage	Reference
Proteins and peptides			
Lactoferrin	80	Prebiotic, anti-inflammatory, toxin binding; pharmaceutical; industrial	Nandi et al. 2002, 2005
Transferrin	76	Iron binding; pharmaceutical; industrial	Zhang et al. 2010
Human serum albumin	67	Carrier protein; medical; industrial	He et al. 2011
T cell epitope peptides Cry j I, Cry j II	55 (varies)	T-cell epitope allergen; clinical	Takagi et al. 2005
Type II collagen	26 (varies)	Cartilaginous tissues; therapeutic use—osteo and rheumatoid arthritis treatment; clinical	Hashizume et al. 2008
Lactostatin	18	Pentapeptide (IIAEK) derived from bovine milk β-lactoglobulin; treat hypercholesterolemia; clinical	Wakasa et al. 2011b
Human IL-10	18	Cytokine receptor, pleiotropic effects in immune regulation and inflammation; clinical; pharmaceutical	Fujiwara et al. 2010
Fibroblast growth factor	17	Stimulate cell proliferation; wound healing; clinical	An et al. 2013
Human IL-7	15	Cytokine receptor; lymphocytes development; clinical; pharmaceutical	Kudo et al. 2013
Mite allergen Der p I	15	Allergen; clinical	Suzuki et al. 2011
Lysozyme	14	Natural antibiotic; pharmaceutical; industrial	Huang et al. 2002
Cholera toxin B subunit	12	Multifunctional protein; immune system; cellular and molecular biology research; clinical research	Oszvald et al. 2008
Human insulin-like growth factor I (IGF-I)	7.5	Mediator of growth hormone; pharmaceutical; industrial	Cheung et al. 2011
Metabolic engineering			
Vitamin A		Essential vitamin, vision; nutraceutical; therapeutic	Ye et al. 2000; and others

Table 7.1 (continued)

Target molecule	Approx. MW (kDa)	Description/usage	Reference
Lignan		Secondary plant metabolites; enhanced matairesinol (antioxidants); nutraceutical	Huang and Yang 2005
Folate		Essential vitamin, biological cofactor DNA repair; nutritional supplements	Storozhenko et al. 2007
Multigene integration			
Multiple transgenes		14 transgenes—5 marker genes and several viral coat protein resistance genes (agronomic)	Chen et al. 1998
Nine transgenes		9 transgenes—5 marker protein and 4 therapeutic proteins	Wu et al. 2002
Nutritional improvement			
Iron		Bound cofactor in heme proteins; nutritional supplements	Goto et al. 1999; and others
Seed specific sulfur-rice-protein		Increased methionine and cysteine; nutritional supplements	Lee et al. 2003
α-Anthranilate synthase		Increased tryptophan; nutritional supplements	Wakasa et al. 2006b
Resveratrol		Polyphenol-type stilbene compound; antioxidant; nutraceutical	Baek et al. 2013

seed, and more specifically rice seed, has emerged as an ideal candidate for the large-scale production of protein therapeutics (Boothe et al. 2010; Broz et al. 2013; Greenham and Altosaar 2013; Kuo et al. 2013; Maxmen 2012) due to its high expression capability, low protease activity, low water content, stable protein storage in ambient conditions, greater biomass, open-field production, and molecular tools available for manipulation. Because of the longer storability of rice seeds, recombinant seed banks could be generated, providing an economically feasible, timely scale-up of therapeutics in response to changing market demands. It is estimated that using achieved yields, enough hepatitis B-antigen to vaccinate all the infants worldwide could be produced on approximately 200 acres of land (Basaran and Rodriguez-Cerezo 2008). There are multiple target molecules that have been successfully produced in rice seeds in academic laboratories and on a commercial scale (http://www.invitria.com). The array of molecules and the molecular weight varies significantly. These molecules include recombinant proteins, peptides, monoclonal antibodies, multi-subunit proteins, vaccine antigens, fusion proteins, enzymes, and nutritional enrichment (Bhullar and Gruissem 2013). Selected, highly expressed recombinant molecules produced in rice seed are listed in Table 7.1. The expressed

molecules have a wide range of variation in their usage and in biological activity and chemical properties.

Future Perspectives: Glycan Modulation of Glycoproteins by In Vitro Enzymatic Approaches

Most proteins are glycosylated, and these glycosylations involving many branched or linear chains, exhibiting particular O- or N-linkages (Delehedde et al. 2006; Ohtsubo and Marth 2006), have consequently made recombinant proteins more complex products to engineer than initially thought. Lately, there have been many examples of therapeutic products that have failed in clinics because they were not bearing the appropriate, if any, glycosylation (Harcum 2006; Zucca et al. 2006). Posttranslational modifications are critical, and are usually required for biological activity (Wurm 2004; Kiss et al. 2010). Experiments with sialylated proteins have demonstrated an ability to improve protein half-life in animal models. Studies involving recombinant human erythropoietins (rhEPO), where a sialylated version of the target protein continued to accumulate 9 days after infiltration when compared with a non-sialylated version, showed a gradual decrease in rhEPO over the same period of time (Jez et al. 2013). Another study in mice, involving recombinant butyrylcholinesterase (rBuChE), demonstrated that the polysialylated version of the protein had up to a sixfold increase in pharmacokinetic properties over the non-sialylated rBuChE, while providing a protection level virtually equal to that of the native version of the BuChE protein (Ilyushin et al. 2013).

The production of therapeutically important proteins in plant cells has attracted increasing attention and initiated scientific investigation (Langer 2010). Nevertheless, a barrier for producing human glycoproteins in plant cells is a significant difference in their N-glycan structures. Both high-mannose type and complex-type N-glycans are common in plant glycoproteins (Kiss et al. 2010). Different from the complex N-glycan structures in human glycoproteins, which are present with or without an α1–6-linked core fucose, plant complex N-glycan structures may have an α1–3-linked core fucose. In addition, instead of a bisecting β1–4-linked N-acetylglucosamine (GlcNAc) in human complex N-glycans, plant complex N-glycans may have a bisecting β1–2-xylose. Furthermore, unlike common β1–4-linked N-acetylgalactosamine (Gal-NAc) structures with or without an additional β1–3-linked fucose (e.g. Lewis x-type structures) in human complex N-glycans, β1–3-linked Gal1-3GlcNAc structures with or without an additional α1–3-linked fucose (e.g., Lacto-N-biose or Lewis x-type structures; Yu et al. 2010) exist in plant glycans. Lastly, plants do not have a biosynthetic pathway for adding terminal sialic acid residues, which are common in human glycoproteins. The presence of α1–3-linked core fucose and bisecting α1–2-xylose in plant glycoproteins has caused unwanted immunogenicity and stimulated the production of human-like glycoproteins as therapeutics by in vivo metabolic engineering of N-glycan biosynthetic pathways (Sugiarto et al. 2011; Yu et al. 2006), in vitro glycan remodeling using the combination of glycosidases

and glycosyltransferases, or a combination of both methods. In recent years, most attempts to sialylate proteins that resemble native glycan structures have been carried out using plants and bacteria, with chemical and enzymatic modification systems using both in vitro and in vivo methods. The in vitro chemical modifications have primarily been achieved through PEGylation. In the process of PEGylation, a polyethylene glycol (PEG) chain is attached to a protein or peptide (Harris and Chess 2003). Several studies have demonstrated the effectiveness of PEGylation in improving protein half-life in various animal models. PEGylated recombinant interleukin-11 (rhIL11) retention increased by about 60-fold over non-PEGylated rhIL11 in mice (Takagi et al. 2007). In another study with nephrectomized rats (Zamboni 2003), the rate of filgrastim cleared by the body decreased from 44.5 ml/hour/kg in the non-PEGylated protein to 9.4/ml/hour/kg in the PEGylated protein. Two recent studies showed site-specific enzymatic polysialylation of a theraputic protein is possible (Lindhout et al. 2011; Sohn et al. 2013). Sometimes, simple sialylation reactions are not enough to increase the sialic acid content. A combined reaction using galactosyltransferase, sialyltrasferase, and their sugar substrates at the same time is needed along with the reduced incubation time to retain the activity while increasing sialylation (Sohn et al. 2013). Recently, at the University of California, Davis, and in other few laboratories have been developing several efficient one-pot multienzyme systems for adding GlcNAc or GalNAc, β1–3-linked galactose (Yu et al. 2010), β1–4-linked galactose (Chen et al. 2010), α1–3-linked fucose (Sugiarto et al. 2011), as well as terminal α2–3- or α2–6-linked sialic acid (Sugiarto et al. 2011; Thon et al. 2011a, b; Yu et al. 2005) to glycans and glycoconjugates. We have developed (Nandi et al. unpublished data) several efficient one-pot multienzyme systems for adding GlcNAc, β1–4-linked galactose, and α2–3/6-linked sialic acid to glycans and glycoconjugates. These in vitro enzymatic systems have been successful with mannosidases to modify N-glycans of plant-produced rBuChE. Mass spectrometry-based site-specific glycan analysis of this plant-produced therapeutic glycoprotein has also been developed, but needs further improvement. Purification and in vitro glycan modification have been achieved, but are currently inefficient in terms of the amount of the glycoprotein produced and the glycoforms that are ideal for in vitro glycan modification. Improved understanding of the reaction kinetics of each of the enzyme catalyzing steps under different reaction conditions, and enzyme and product stability under various reaction conditions, is also needed to allow more strategic process design, optimization, and yield improvement of active sialylated glycoproteins. Therefore, it is expected that in vitro enzymatic glycan modification should be achievable commercially for recombinant proteins in the near future.

Conclusion

Plant molecular pharming has undergone considerable advancement in recombinant protein production and is recognized as a promising opportunity to meet the future demands for biopharmaceuticals. The drastic shortage of protein-based thera-

peutics has pushed collaboration between industries, molecular pharming start-up companies and academia. The proof-of-concept trials have shown that the large-scale and effective production is possible, using plant system as hosts (Basaran and Rodriguez-Cerezo 2008). While the transgenic plant platform continues to mature, research and development interests will likely shift from upstream to downstream processing to improve the overall productivity, which has been the case for more established biotechnology industries (Huang 2004; Gottschalk 2008). Depending on the product type and application, downstream processing can account for a significant portion of the total operating costs for product manufacture (Wilken and Nikolov 2012). Thus, the development of efficient and selective extraction and purification processes as early as possible is essential for favorable economics. Molecular pharming as an industry will see considerable growth and attention over the next decade with the increasing demand for protein therapeutics and the era of follow-on biologics. In order to fully utilize the advantages of seeds, careful selection of target molecule, advances in expression technology, and downstream purification will require the most consideration as they are the key factors that determine the economic performance of the product. Finally, processes for modifying glycans on glycoproteins are becoming another field of research as the effects of glycan structures on the stability, immunogenicity, and efficacy of therapeutic glycoproteins often influence the efficacy of the product.

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Chapter 8
Wheat Biotechnology: Current Status and Future Prospects

Huw D. Jones

Wheat as a Global Commodity Crop

In 2010, wheat was grown on nearly 217 million ha worldwide (more than any other food crop) and a total of 651 million t of grain was harvested, making it the third most-cultivated cereal after maize and rice (www.faostat.fao.org). It is one of the main staple food crops providing approximately 20 % of our daily needs of protein and calories. Wheat is traded on global commodity markets which have become accustomed to a good annual harvest and large reserves. However, global demand, estimated at 666 million metric tons (MMT) in 2010, is predicted to increase in line with population growth and reach 880 MMT (a 40 % increase) by 2050 (Weigand 2011). Significant action will be required to maintain this positive situation.

Further complicating the supply/demand balance are extreme weather events, unpredictable longer-term climate patterns and changing regional preferences in crop choice/suitability. Recent localised severe weather and poor harvests have resulted in price volatility with notable spikes in 2008 and 2012 (Fig. 8.1). The volatility in the wheat market was exacerbated by the Russian export ban in 2010 resulting from an extreme heatwave in the grain producing regions of southwestern Russia. The highest July temperatures in 130 years brought severe drought, causing the annual harvest of the biggest wheat producing region (Volga) to be reduced by more than 70 % on that of the previous year (Wegren 2011; Welton 2011). The US wheat supplies for 2013/2014 are projected at 2917 million bushels, down 7 % from 2012/2013 (Vocke and Liefert 2014) and there is evidence that growers in some US states are moving away from wheat in favour of biotech soya and corn. Quoting statistics from the US Department of Agriculture, Rookhuyzen (2012) stated that wheat acreage for South Dakota declined by 1.2 million acres, or 28 %, between 1981 and 2011 and the acres planted with spring wheat in 2012 were the lowest in

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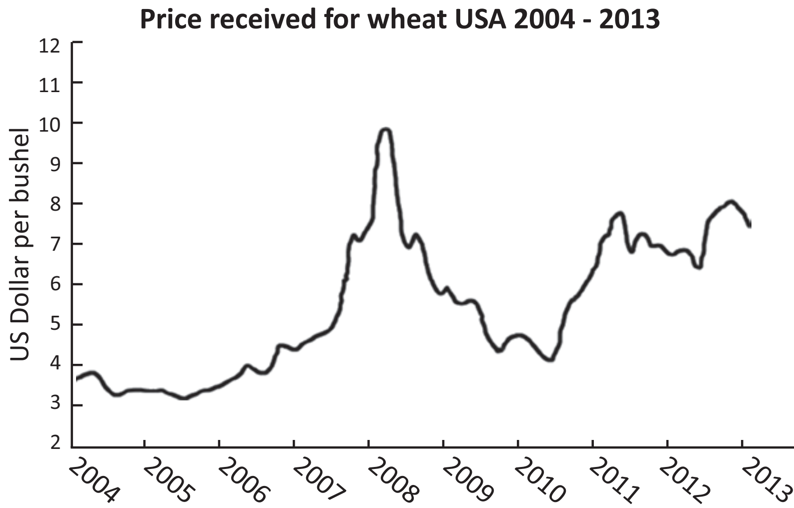

Fig. 8.1 Fluctuations in US wheat price 2004–2013. Peaks in 2008 and 2012 were due mainly to severe weather in some growing regions

the state history. In comparison, corn acreage increased from 3.4 to 5.2 million and soya bean acreage went from 780,000 to 4.1 million during the same period of time. This author also stated that the total US wheat acres plummeted from 88.3 million to 53.6 million, or 39 %, in the last three decades, while corn increased by 4 million acres and soya beans by 10 million acres during that time.

Despite these localised losses in wheat growing areas, there was a record high in global production in 2013, with latest figures indicating a 7.6 % rise to 710 million t (FAO, 2013). To further elevate these production levels in a sustainable manner to keep pace with the increasing global demands for wheat, will need considerable innovation in agriculture and calls particularly for research and development in wheat breeding and biotechnology.

Why Is Wheat a Biotech Late-Starter?

Despite its global importance as a global food and feed commodity, wheat is not yet marketed to growers as a biotech crop. However, this is not due to fundamental gaps in technology or scientific understanding, nor because there are no good targets for genetic improvement. Progress in wheat tissue culture and genetic manipulation does not significantly lag behind crops such as maize, soya, cotton and canola, all of which are major biotech crops and collectively were planted on 170.3 million ha in 2012 (James 2012). Robust gene discovery, transformation and phenotyping

platforms for wheat exist in the major crop biotechnology companies and, maybe with a lower capacity, in several publicly funded organisations.

Glyphosate-resistant (Roundup Ready; RR) wheat was close to commercial release in 2004 before substantial resistance from influential sectors of the USA and Canadian wheat growers contributed to a decision by Monsanto Corporation to discontinue its efforts to win regulatory approval as discussed by Blechl and Jones (2009). The event (MON 71800) possessed a gene from a soil bacterium that encoded the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which protects the plant from the harmful effects of glyphosate. It appeared that North American growers were concerned they would lose export markets in Europe and Asia (Berwald et al. 2006). There was also some discussion about the relatively low demand for weed control in the US spring wheat (Stokstad 2004). However, BASF is successfully marketing non-genetically modified (non-GM) herbicide-tolerant 'Clearfield' wheat (made by mutation breeding) in the USA, Canada and Australia, implying at least some growers approve of the weed control offered by these Imidazolinone-resistant varieties.

Since the early uncertainties over RR wheat, the US National Association of Wheat Growers (NAWG) and the US Wheat Associates (USW), which represent thousands of growers across the USA, now publically support GM wheat and are encouraging biotech companies to pursue novel GM traits that will benefit the wheat industry. In contrast, the Canadian Wheat Board is less positive and currently appears to have no consensus or clear position on GM wheat. These views are influenced and compounded by the special importance that wheat and bread have in our society. Wheat is viewed differently from other commodity crops and has a unique cultural and religious significance as a food. Because wheat is such an important human food crop, iconography including the spike (ear) or the sheaf (garb) can be found in paintings, carvings and heraldic coats of arms throughout history. Wheat has an 'image' associated with a good supply of wholesome food and many well-respected food-related organisations (e.g. FAO, United Nations, and the US Department of Agriculture) have incorporated it into their logos. Its historic significance is reinforced, by the quote from the classical Greek philosopher Socrates (469–399 BC) who said, 'Nobody is qualified to become a statesman who is entirely ignorant of the problems of wheat'.

Despite the issues outlined above, the pressure for higher yields and more efficient and sustainable use of farmland will drive biotechnology research and development activity in many traits and it is likely that biotech or genome-editied wheat will be cultivated commercially within a decade.

Sequencing the Wheat Genome

Many excellent genomic resources exist for cereals. Rice, maize, barley and *Brachypodium* are frequently used as model species for cereal genomics and the sequence of these species were published between 2002 and 2012 (Goff et al. 2002;

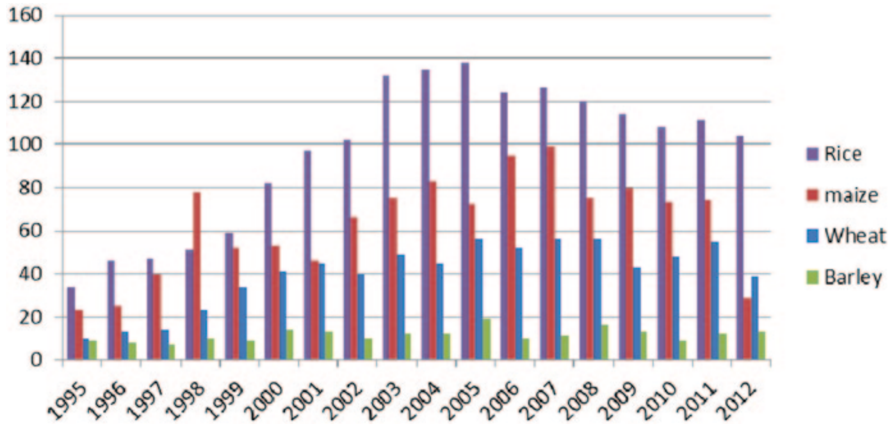

Fig. 8.2 Numbers of research publications that focus on genetic modification of the major cereals found in a survey of the scientific literature from 1995 to 2012. For each of the crops included, the Boolean terms used to search 'Thomson Web of Science' are listed below. For all searches, the time span was set to 'All Years' and the search language was 'English'.Title=(wheat) AND Title=(transform* OR *Agrobacterium* OR transgen* OR biolistic*) NOT Title=(tobacco OR *Arabidopsis* OR fourier OR energy OR wave* OR transgeneration* OR soil OR economic* OR mice OR cow*) Title=(rice) AND Title=(transform* OR *Agrobacterium* OR transgen* OR biolistic*) NOT Title=(tobacco OR *Arabidopsis* OR fourier OR energy OR wave* OR transgeneration* OR soil OR economic* OR mice OR cow*) Title=(barley) AND Title=(transform* OR *Agrobacterium* OR transgen* OR biolistic*) NOT Title=(tobacco OR *Arabidopsis* OR fourier OR energy OR wave* OR transgeneration* OR soil OR economic* OR mice OR cow*) Title=(maize OR corn) AND Title=(transform* OR *Agrobacterium* OR transgen* OR biolistic*) NOT Title=(tobacco OR *Arabidopsis* OR fourier OR energy OR wave* OR transgeneration* OR soil OR economic* OR mice OR cow*)

Mayer et al. 2012; Schnable et al. 2009; Vogel et al. 2010). The full sequence of *Sorghum* was also published in 2013 (Mace et al. 2013). Many T-DNA tagged or otherwise mutated populations exist, particularly in rice and maize, although for wheat functional genomics, barley or *Brachypodium* are often more useful models and good resources also exist for these plants. A survey of the primary research literature on genetic modification of the major cereals shows that the number of publications per year increased from 1995 to the mid-2000s and then declined slightly (Fig. 8.2). As expected, there were more papers focussing on rice and maize transgenics compared to wheat and barley. However, the number of papers describing wheat transformation experiments has remained relatively consistent since the early 2000s at about 50 per year. Wheat genomic translational research recently received a major boost with the publication of a draft wheat genome sequence (Brenchley et al. 2012). The wheat variety Chinese Spring (CS42) was selected for sequencing because of its wide use in the past genome studies. The DNA was sequenced using Roche 454 pyrosequencing technology to generate 85 Gb of sequence (220 million reads), corresponding to approximately a fivefold coverage on the basis of an

estimated genome size of 17 Gb (5× larger than the human genome). The sequence can be searched using 'BLAST' software at the CerealsDB website:http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/Documents/DOC_CerealsDB.php.

In addition to this, a consortium of scientists (IWGSC) http://www.wheatgenome.org/ is making good progress towards establishing a high-quality reference sequence of the wheat genome. They aim to construct chromosome-based physical maps anchored to the genetic maps of Chinese Spring, and to sequence the complete genome. The survey sequence is complete and the announcement in January 2014 that Bayer CropScience committed approximately € 1 million to the project should ensure the timely completion of physical maps for all 21 chromosomes. A different approach was taken by a group of Chinese laboratories who produced draft genome sequences of two progenitor species of hexaploid wheat, the D-genome donor *Aegilops tauschii* (Jia et al. 2013) and the A-genome donor *Triticum urartu* (Ling et al. 2013). Together, these genome sequences will give valuable insight into the structure and function of the complicated polyploid genome of bread wheat and provide candidate gene sequences for functional genomics and reverse genetics.

Transformation as a Tool for Translational Genomics Research

Transformation is a powerful research tool for gene discovery and functions to investigate genetically controlled traits, and the majority of papers on wheat transformation included in Fig. 8.2 describe some aspects of functional genomics research where a genetic manipulation is used to investigate the function of a predetermined nucleotide sequence. This is a valuable process in its own right, but also forms a step in the applied research pipeline leading to commercialisation of a new GM variety. It provides key underpinning knowledge to inform and short-cut conventional breeding strategies. There is already significant information on the wheat genome sequence and there are very large volumes of additional 'next generation' RNA and DNA sequence data that are constantly deposited into Sequence Read Archive (SRA) databases, forming raw materials for functional genomics research.

Methods for Wheat Transformation

The first fertile transgenic wheat plants were made using particle bombardment and were reported 20 years ago (Becker et al. 1994; Nehra et al. 1994; Vasil et al. 1993; Weeks et al. 1993). The target tissue for transformation was the immature seed embryo which can be induced by media containing growth regulators and sugars to form regenerable callus. Although successful transformation has been reported from other explants such as immature inflorescences (Barcelo et al. 1994; He and Lazzeri 1998; RascoGaunt and Barcelo 1999), regenerable embryogenic callus

(Vasil et al. 1992) and mature seeds (Miroshnichenko et al. 2011), there has been no viable and sustained alternative to the scutellum tissue of the immature embryo for reliable and efficient wheat transformation. The optimal age of caryopses is 11–16 days postanthesis (Pastori et al. 2001). At this developmental stage, the grains are at early–medium milk stage and contain translucent embryos each 1–3 mm in length, which are optimal for transformation (Jones 2005). The generation of transgenic wheat lines through tissue culture is labour- and materials-intensive and time-consuming, and can increase the chance of genetic instability due to unpredictable somaclonal variation. This has served as a driver to investigate various *in planta* (germ line) methods for wheat transformation. Such methods, targeting the germ line cells in seeds (Supartana et al. 2006) or developing inflorescences in a similar way to the floral-dip process available for *Arabidopsis* (Zale and Steber 2006), have now been reported for wheat, but retain a 'niche' position compared to conventional methods.

The biolistics method is still used in many laboratories because it delivers DNA over a large area of target tissue, is reasonably efficient and appears less genotype-dependent than other methods (Altpeter et al. 2005; Ingram et al. 2001; Rasco-Gaunt et al. 1999; Sparks and Jones 2009; Vasil and Vasil 1999; Vasil et al. 1991, 1992; Weeks et al. 1994). However, to maximise DNA delivery and minimise cell damage, variables such as the micro-carrier material, the mass of DNA precipitated onto the surface of the micro-carriers, propellant force and target distance must all be optimised for specific genotypes and explant types (Altpeter et al. 1996; Harwood et al. 2000; Ingram et al. 1999; Rasco-Gaunt et al. 1999). Biolistics can also be utilised to deliver DNA into the genomes of mitochondria and chloroplasts. Perceived advantages, particularly in terms of lower copy number have driven significant research into using *Agrobacterium tumefaciens* (synonym *Rhizobium radiobacter*) for the DNA transfer process, and many laboratories now report successful wheat transformation using *Agrobacterium* (Binka et al. 2012; Campa et al. 2005; Cheng et al.1997; Cheng et al. 2003; Ding et al. 2009; Guo et al. 1998; Haliloglu and Baenziger 2003; Hamid et al. 2012; He et al. 2010; Jones et al. 2005; Khanna and Daggard 2003; Mahalakshmi and Khurana 1995; Marks et al. 1989; McCormac et al. 1998; Mitic et al. 2004; Mooney et al. 1991; Murin et al. 2011; Parrott et al. 2002; Patnaik et al. 2006; Pérez-Piñeiro et al. 2012; Przetakiewicz et al. 2004; Pukhalskii et al. 1996; Rashid et al. 2012; Shi et al. 2011; Song et al. 2012; Tamas-Nyitrai et al. 2012; Trifonova et al. 2001; Wu et al. 2003, 2008; Xia et al. 1999). One particular unpublished method developed by the Japan Tobacco company (PureWheat Technology) has been licensed to specific laboratories who report wheat transformation efficiencies of more than 50%.

Genotype Dependency

The precise response of wheat explant tissues to a regime of tissue culture is highly genotype dependent and extensive variation has been reported (Barro et al. 1999; Carman et al. 1988; Fennell et al. 1996; Machii et al. 1998; Maddock et al. 1983;

Redway et al. 1990; Sears and Deckard 1982; Shimada 1978; Viertel et al. 1998). Some wheat cultivars are more responsive and regenerable than others. Several reports have demonstrated better transformation and regeneration from specific lines of the spring wheat 'Bobwhite' (Fellers et al. 1995; He and Lazzeri 1998) and this has become known as a transformation model for wheat (Janakiraman et al. 2002). However, 'Bobwhite' is not an isogenic variety, but actually a heterogeneous set of lines originally derived from the cross 'Aurora'//'Kalyan'/'Bluebird 3'/'Woodpecker'. Transformation efficiency of 129 sister lines all generically called 'Bobwhite' were compared and eight demonstrated transformation efficiencies of more than 60 %. One of these, 'Bobwhite SH 98 26' was identified as a super-transformable wheat line (Pellegrineschi et al. 2002).

The limitations of a lack of highly transformable elite commercial lines have been discussed previously by Varshney and Altpeter (2001) who compared the culture response of 38 European winter wheat varieties and breeding lines. From the genotypes studied, these authors identified nine with transformation efficiencies between 0.2 and 2 %. Pérez-Piñeiro et al. (2012) listed in excess of 50 wheat genotypes, including durum, emmer, spring and winter types that they have tested for their transformability.

Control of Transgene Expression

The transcription and translation of genes is regulated at different levels and with a range of mechanisms. The core promoter together with other *cis*-acting regulatory sequences includes response elements, 3′ and 5′ untranslated regions. Introns and polyadenylation signals are non-coding sequences that form part of this control. It is routine in research and commercial applications to generate a chimeric DNA construction where the promoter/enhancer elements, the coding region and the terminator originate from different genes, and often from different species. The promoter from the 35S gene of the cauliflower mosaic virus (Odell et al. 1985) gives strong and generally constitutive expression, and is commonly used in dicotyledonous plants. However, for wheat and other cereals, a wide range of other promoters have been validated. Two monocotyledon promoters commonly used in preference to viral sequences for wheat are the maize ubiquitin-1 promoter, usually with its first intron (Christensen and Quail 1996; Christensen et al. 1992), or the rice actin promoter and first intron (McElroy et al. 1991). The expression patterns from 22 different promoters used in transgenic wheat have been reviewed by Jones (2005); Jones and Sparks (2009) and references therein. Many are constitutive, but to supply the research into the end-use qualities of wheat grain, there are a significant number of seed- and other tissue-specific promoters that have been validated using reporter gene expression and utilised in research projects (Table 8.1) (Al-Saady et al. 2004; Chrimes et al. 2005; Pistón et al. 2008a, b; Somleva and Blechl 2005; Stoger et al. 1999b; Wiley et al. 2007). It is obvious that a high-level expression of a novel protein in transgenic plants could divert energy away from normal life-cycle

Table 8.1 Promoter sequences analysed for their expression and tissue-specificity or inducibility in wheat with indicative references

Promoter common name	Plasmid name (Ref.) and promoter/reporter gene/terminator cassette	Species: mode of transformation	Expression reported	Ref.
Promoters with generally constitutive expression patterns				
CaMV35S	CaMV35S::Gus	Wheat cv. Bobwhite; stable transformation via *Agrobacterium* strain C58 (ABI) into immature embryos and embryogenic calli	GUS expression was detected in all of the tissues tested	(Cheng et al. 1997)
	CaMV35S:: sgfpS65T (Chiu et al. 1996)	Wheat (cv. Fielder) stable transgenics using *Agrobacterium* (AGL0) transformation of pre-cultured immature scutella	GFP expression in the roots, immature seed coat and germinating immature embryo	(Weir et al. 2001)
	CaMV35S::Gus	Three Indian wheat varieties transformed with *Agrobacterium* LBA4404	GUS expression in callus and leaf tissue	(Patnaik et al. 2006)
Adh1	pBargus (Fromm et al. 1990). Maize Adh1 plus Adh1intron::uidA (Gus):	Wheat (cv. Parvon & RH770019); stable transgenics using bombardment of regenerable callus	GUS expression reported in Phosphinothricin (PPT) PPT-resistant calli, root tips and seeds	(Vasil et al. 1992)
Ubi1	pAHC25 (Christensen and Quail 1996; Christensen et al. 1992). Maize Ubi1 plus Ubi1 intron:: uidA (Gus)	Wheat (NILs L88-6 and L8831); stable transgenics using bombardment of immature scutella	Constitutive histochemical GUS expression reported, generally strong in young, metabolically active tissues and in pollen grains	(Rooke et al. 2000)
	Ubi1 intron:: sgfp (S65T)	Winter wheat var. Certo; *Agrobacterium* transformation with LBA4404	GFP expression in embryogenic callus and developing shoots	(Hensel et al. 2009)

Table 8.1 (continued)

Promoter common name	Plasmid name (Ref.) and promoter/reporter gene/terminator cassette	Species: mode of transformation	Expression reported	Ref.
Act1	pDB1 (Becker et al. 1994). Rice Actin plus 1st in/ex::uidA (Gus)	Wheat (var. Florida) stable transgenics using bombardment of immature scutella)	Histochemical GUS activity reported in leaves	(Becker et al. 1994)
	pAct1sGFP-1 (Cho et al. 2000) Rice Actin 1 plus 1st in/ex::sgfp(S65T)	Wheat (cv. Fielder) stable transgenics using bombardment of immature scutella	GFP expression reported in regenerable callus, leaves, developing shoots and embryos	(Jordan 2000)
	pRC-62 (Datla et al. 1991; McElroy et al. 1990) Rice Actin1D plus 1st in/ex:::uidA(Gus)/*npt*II	Wheat (cv. Fielder) stable transgenics using bombardment of immature scutella)	Histochemical GUS activity reported in leaves, ovary, stigma, anthers and pollen	(Nehra et al. 1994)
H2B	pHP12679 Maize histone H2B plus Ubi1 intron 1:: uidA(Gus)::pinII	Wheat (var. Cadenza); stable transgenics using bombardment of immature scutella	Constitutive. Gus expression reported in all tissues tested	(Rasco-Gaunt et al. 2003)
Promoters with seed-specific expression patterns				
HMWG	pHMW-GUS Wheat high molecular weight glutenin subunit. Glu-1D-1:: uidA (Gus). (-1191 to+58)	Wheat (durum var. Ofanto); stable transgenics using bombardment of immature inflorescences	Endosperm specific 10–12 dpa	(Lamacchia et al. 2001)
LMWG	pLMWG1D1-326 pLMWG1D1-938. Wheat low molecular weight glutenin subunit LMWG1D1 (two lengths−326 to+30, and−938 to+30)	Wheat (cv. Bobwhite); stable transgenics using bombardment of immature scutella	Endosperm specific, on ~14 dpa, longer promoter stronger	(Stoger et al. 1999b)

Table 8.1 (continued)

Promoter common name	Plasmid name (Ref.) and promoter/reporter gene/terminator cassette	Species: mode of transformation	Expression reported	Ref.
B- and D-hordein	pHorB- and pHorD-Gus. Barley B- and D-type hordeins (1043 + 834 bp respectively:: uidA (Gus)	Wheat (cv. Bobwhite); stable transgenics using bombardment of immature scutella	Histochemical GUS activity reported in endosperm and absent in leaves, roots and flowers	(Piston et al. 2008b)
ETC (endosperm transfer cell-specific) promoter	pTdPR60::gus	Wheat cv. Bobwhite was transformed using bombardment	The strongest GUS expression in wheat was found in the endosperm transfer cells	(Kovalchuk et al. 2009)
LTP (lipid transfer protein)	pTaPR61::gus	Wheat cv. Bobwhite was transformed using biolistic bombardment	The strongest GUS expression in wheat was found in the endosperm transfer cells	(Kovalchuk et al. 2012)
Pin A and B	Pina::uidA Pinb::uidA (Digeon et al. 1999) Wheat puroindoline a and b::uidA (Gus)::nos 3’	Wheat (var. Cadenza and durum var. Ofanto); stable transgenics using bombardment of immature inflorescences and scutella	GUS expression reported in starchy endosperm cells only	(Wiley et al. 2007)
α_Gliadin	592 bp α-gliadin (van Herpen et al. 2006) promoter fragment used to drive GUS	Wheat (var. Cadenza); stable transgenics using bombardment of immature scutella	Starchy endosperm, sub-aleurone and aleurone 11–8 days after flowering until maturity	(Van Herpen et al. 2008)
αamy1 and 2	pα1GT/pα2GT (Huttly and Baulcombe 1989). Wheat alpha amylase 1 or 2:: uidA (Gus)	Wheat (var. Cadenza); stable transgenics using bombardment of immature scutella	Histochemical GUS reported in scutellum and embryonic axis of seeds at 4 days post germination	(Stone 2003)
Promoters with other expression patterns				
Lem1	pBSD5sGFP Barley Lem1:: sgfp(S65T)::nos 3’	Wheat (cv. Bobwhite); stable transgenics using bombardment of immature scutella	Tissue-specific expression of GFP reported in outer floret organs at anthesis	(Somleva and Blechl 2005)

Table 8.1 (continued)

Promoter common name	Plasmid name (Ref.) and promoter/reporter gene/terminator cassette	Species: mode of transformation	Expression reported	Ref.
AGPL1	pAGP2::GUS (Thorneycroft et al. 2003) wheat ADP-glucose pyrophosphorylase large subunit:: uidA (Gus)	Wheat (cv. Chinese Spring) stable transgenics using bombardment of embryo-derived calli	Tissue-specific expression of GUS reported in endosperm, pollen and carpel	(Chrimes et al. 2005)
		Wheat (cv. Chinese Spring) stable transgenics using bombardment of embryo-derived calli	Tissue-specific expression of GUS reported in endosperm and aleurone	(Thorneycroft et al. 2003)
End1	pPsEND1gusA (Gomez et al. 2004) pea END1(2731 bp):: uidA (Gus)	Wheat (cv. Bobwhite); stable transgenics using bombardment of immature scutella	GUS expression localised to pollen; microspores binucleate and pollen tube stages	(Pistón et al. 2008a)
HSP	Barley pHvhsp17::gus (Marmiroli et al. 1993)	Wheat var. Cadenza transformed using biolistics	GUS expressed in response to 40C heat shock for 1–2 h in all tissues tested	(Freeman et al. 2011)
Abiotic Stress-induced	Wheat TaAIDFa promoter (2.7 kb upstream of ATG) cloned with GUS	Transient GUS expression analysed in bombarded wheat (variety not identified)	GUS expressed under stress treatments, including mock drought (2 % PEG), salinity, low temperature and exogenous ABA	(Xu et al. 2008)
	LEA-like rd29A gene promoter from *Arabidopsis thaliana,* cloned with GUS	Transient GUS expression analysed in bombarded wheat immature embryogenic calluses	GUS gene was induced by polyethylene glycol treatment	(Gao et al. 2005)

GFP green fluorescent protein, *ABA* abscisic acid, *ADP* adenosine-5′-diphosphate, *PEG* polyethylene glycol

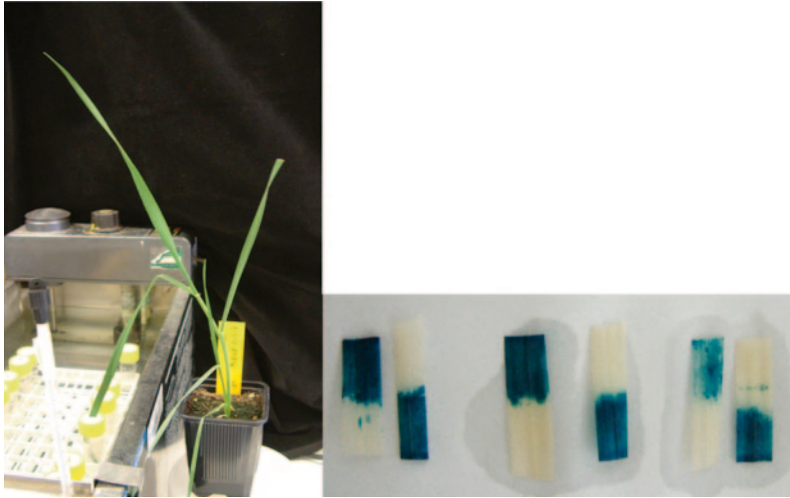

Fig. 8.3 Induction of GUS expression in leaves of wheat lines possessing a GUS gene under the control of the HSP17 heat-shock promoter. Only those parts of the leaf that were exposed to 38–40 °C for 1–2 h showed expression of the reporter gene

processes and from the final harvestable products. Thus, where appropriate, it is better to switch on the expression of a transgene only where and when it is required. In transgenic wheat, only three promoters inducible by abiotic stresses have been described previously. An abscisic acid (ABA)-responsive element from the barley HVA22 gene fused to a rice actin minimal promoter was induced by drought in transgenic wheat plants (Vendruscolo et al. 2007). The rd29A promoter from *Arabidopsis thaliana*(which acts through an ABA-independent pathway) was also demonstrated to be induced by drought (Pellegrineschi et al. 2004). The inducibility by a short 40 °C heat shock and resulting expression pattern of the uidA/GUS gene driven by the barley heat-shock promoter Hvhsp17 was characterised in transgenic wheat (Freeman et al. 2011; Fig. 8.3).

Target Traits for Wheat Biotechnology

Input Traits

Good bread wheat has a requirement for high nutrient inputs and is host to a broad spectrum of pathogens, insect pests, nematodes and herbivores that would decimate yield if not effectively controlled. In high-yielding, modern commercial farming environments, these nutrient requirements, pests and diseases together with weeds are managed by good agronomic practice, including appropriate crop rotations and chemical sprays. The expense of research, development and registration of new

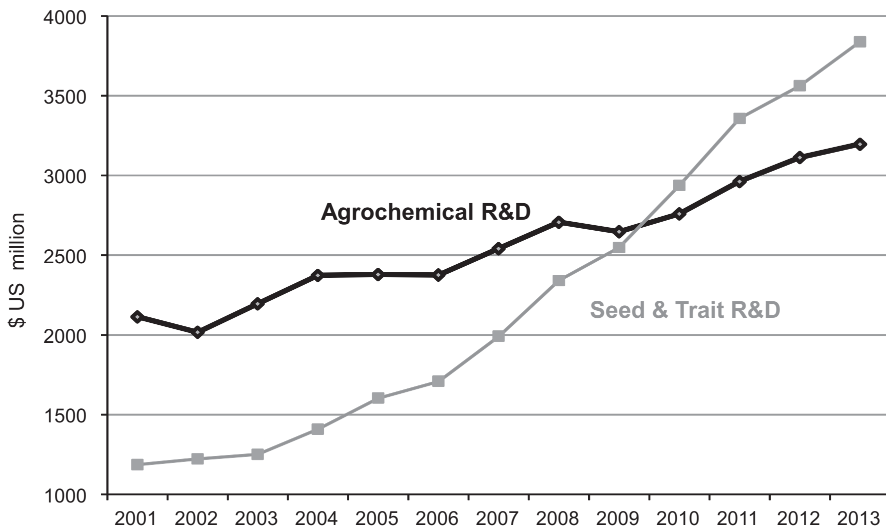

Fig. 8.4 From a low base in 2000, increase in investment in biotechnology research and development (R&D) has now overtaken agrochemical R&D. Source: The Global Agrochemical and Seed Markets Industry Developments. AgriFutura Newsletter No. 168. Oct 2013. Phillips McDougall Ltd., info@phillipsmcdougall.com

active ingredients for agrochemicals, particularly pesticides, is prohibitively costly. This combined with the increasingly stringent safety requirements and withdrawal from the market of existing formulations means that the age of 'peak agrochemical' may have passed already. In a related observation, work done by Phillips McDougall Ltd, consultants to the agribusiness industry, indicates that since 2010 there is more investment in biotechnology, seed and trait R&D compared with agrochemical R&D, although both are still increasing (Fig. 8.4).

Tolerance to Abiotic Stresses

Wheat yields are particularly vulnerable to extreme abiotic stresses and, as the pressure for land and water increases, the availability of varieties that can withstand extremes of drought, salinity and temperature is likely to become increasingly important. The effect of drought on cereal production can be large enough to affect the economy of the whole wheat-producing regions. For example, serious drought combined with high temperatures in Victoria, SE Australia, reduced the wheat supplied by this region by 70 % in 2007/2008. As discussed earlier, similar severe conditions forced the Russian government to ban temporarily all wheat exports during the same period. The genetic basis for tolerance to abiotic stresses

is complex and likely to require controlling the expression profiles of several genes via transcription factors. However, some biotech approaches have shown promising results. For example, plants sometimes react to stress by producing osmoprotectants such as sugars. Trehalose, a non-reducing disaccharide of glucose, is one of the most effective osmoprotectants. Several strategies leading to its accumulation have been envisaged in both model and crop plants using genes of bacterial, yeast and, more recently, plant origin. Significant levels of trehalose accumulation have been shown to provide protection against abiotic stresses in transgenic plants (Almeida et al. 2007; Benaroudj et al. 2001; Garg et al. 2010; Jang et al. 2003).

In another approach, International Maize and Wheat Improvement Centre (CIMMYT) focussed on the *dreb1A* gene from *Arabidopsis thaliana* that shows enhanced tolerance to drought, low temperature and salinity. Dehydration responsive element binding (DREB) proteins constitute a large family of transcription factors that induce the expression of a large number of functional genes and impart stress endurance to plants (Dubouzet et al. 2003; Kasuga et al. 1999; Taji et al. 2002). CIMMYT scientists engineered this gene into wheat plants under the control of the rd29A stress-inducible promoter (Pellegrineschi et al. 2004). Almost a decade ago, in March 2004, they took a significant and historic step when they used these lines in the first field trials of genetically modified wheat in Mexico, which were repeated in 2012 (Centro Internacional de Mejoramiento de Maiz y Trigo 2012).

Work in Australia at the Department of Environment and Primary Industries and Commonwealth Scientific and Industrial Research Organisation (CSIRO) has led to several field trials to test genes that regulate biochemical pathways to promote normal growth under reduced availability of water. These include the genes encoding NAC, C2H2 (ZFP), AP2 and heat-shock transcription factors. Combined in these trials are also genes for altered amylose content (silencing starch branching enzymes), improvement of nitrogen use efficiency (alanine aminotransferase) and altered grain composition (silencing glucan water dikinase) (Australian Government 2012). However, even if the trials are successful, the GM traits will undoubtedly require further testing and are unlikely to be integrated into commercial breeding lines before 2020.

Bread-Making Quality

The importance of wheat as a source of protein and calories in the human diet has not surprisingly led research to understand and improve its end-use qualities. The ability to make bread and the wide range of other processed foods from wheat flour is determined by the unique properties of the grain storage proteins. Much research has targeted gluten, the protein network that underlies wheat dough properties and allows the making of leavened wheat products (Shewry et al. 2003). In order for wheat dough to make leavened bread, the gluten must have a balance of

elasticity and extensibility. Variation in one group of proteins, the high molecular weight glutenin (HMWG) subunits, is particularly important in determining dough strength in different wheat varieties (Branlard and Dardevet 1985; Gupta and Macritchie 1994; Payne et al. 1987; Popineau et al. 1994). In the European cultivars tested, HMW-GS composition accounted for 45–70 % of the variation in dough strength (Shewry et al. 2003) and led to a series of experiments to alter the number and type of HMWG subunits in transgenic wheat. Genes encoding subunits Ax1, Dx5, Dy10 and a hybrid between Dx5 and Dy10, each under control of its native HMW-GS promoter, were added to various wheat backgrounds. Expression of a 1Ax1 subunit in lines lacking the native gene resulted in increased dough strength and improved bread-making quality (Alvarez et al. 2000; Barro et al. 1997; Darlington et al. 2003; Mao et al. 2013; Popineau et al. 2001; Vasil et al. 2001). Similarly, a substantial increase in dough strength was reported in transgenic lines expressing both the 1Dx5 and 1Dy10 proteins (Anderson and Blechl 2000). When the levels of 1Dy10 alone were elevated, dough properties as determined by farinograph quality were reported to be improved in both undiluted and blended flours (Graybosch et al. 2013). In contrast, overexpression of the 1Dx5 gene in bread wheat or in durum wheat resulted in doughs that were difficult to hydrate and lines possessing high expression levels could not be mixed in a 2-g mixograph without blending (Alvarez et al. 2001; Blechl et al. 2007; Popineau et al. 2001; Rakszegi et al. 2005; Rooke et al. 1999).

Three HMW-GS transgenes, encoding HMW-GS 1Ax1, 1Dx5 and 1Dy10 L, were combined in various ways by conventional crossing of individual transgenic plants (Leon et al. 2010). All lines with transgenic subunits showed greater levels of glutenin proteins compared to the control variety ‘Anza’, but these increases were compensated by lower amounts of gliadins. Some combinations had significantly superior dough, and demonstrated that stacking HMW-GS transgenes by conventional crossing is a valid strategy for the improvement of wheat quality.

Gluten is a complex polymer and many variables that determine dough quality remain unquantified. Nevertheless, the results achieved by expressing the 1Ax1 subunit show that genetic manipulation can be used to develop cultivars with increased dough strength. Summarising almost 20 years of research using transgenic approaches to study the genetic basis of bread-making quality, one of the pioneers and leading authorities, Peter Shewry (Shewry 2009) commented that ‘It is perhaps not surprising that the results have been “mixed”, but some conclusions can be drawn’. Firstly, expression of an additional HMW subunit gene can lead to increased dough strength, even when a modern good quality wheat cultivar is used as the recipient (Field et al. 2008; Rakszegi et al. 2008). However, the effect depends on the precise HMW subunit gene which is used and on the expression level, with the transgenes resulting in over-strong (too elastic) gluten properties in some cases. Thus, although transgenesis is a realistic strategy to increase dough strength in wheat, it is also necessary to have an understanding of the underlying mechanisms in order to optimise the experimental design”.

Strategies for Insect Resistance

Many insect species cause significant yield losses in cereal production systems and our ability to control these pests using conventional chemical means is becoming limited through increasingly strict legislation on human and animal health, pollution and other environmental safety issues. Thus, one obvious route to deliver future insect control is by new breeding approaches, including introgression of genes from ancestral or other alien species (Harper et al. 2011), along with genetic modification, which is now being seen as much more valuable in the landscape context (Lu et al. 2012; Pickett et al. 2014). Over the past two decades, a range of biotechnology solutions have been proposed or investigated. Transgenic approaches to control insects have relied classically on a toxic mode of action such as cry proteins from *Bacillus thuringiensis*, plant lectins or protease inhibitors (Gatehouse et al. 2011). However, novel pest control ideas are also emerging involving cross-kingdom gene silencing using RNAi (Burand and Hunter 2013) or methods for altering insect feeding or alarm behaviour to deter such pests from crop plants (Pickett et al. 2014).

Cry toxins are powerful and effective agents against lepidopteran and coleopteran pests and commercial GM varieties of maize and cotton are available that control lepidopteran insects by production of Cry1Ab, Cry1Ac, Cry1F and Cry2Ab2 proteins. Cry3 toxins, such as Cry3Bb1, Cry34Ab1 and Cry35Ab1 with activity against coleopterans are also being used commercially, particularly in maize to protect against rootworms. Recently, a non-Cry Bt protein (Vip3) was introduced into maize (Gatehouse et al. 2011). However, Bt toxins do not affect hemipteran pests such as aphids, and other strategies are needed for this order of economically important insects. Plant lectins, particularly those of the *Galanthus nivalis* agglutinin (GNA) class, have been used experimentally for aphid-resistance in cereals. For example, improved resistance to corn aphid was observed in field trials of transgenic maize plants with GNA expression controlled by a phloem-specific promoter (Wang et al. 2005). Transgenic wheat plants expressing GNA at levels greater than 0.04 % of total soluble protein decreased the fecundity of grain aphids, but had no effect on their survival (Stoger et al. 1999a). However, there are reports of the risk of unintended cross-species agglutination and significant off-target effects of transgenic lectins. For instance, adverse effects on predatory ladybirds (*Adalia bipunctata*) and parasitoids (*Aphidius ervi*) via aphids in the food chain have been reported (Birch et al. 1999; Hogervorst et al. 2009). In addition, other sublethal impacts on parasitoids have been recorded, such as reduced longevity, reduced fecundity and extended development times (Romeis et al. 2003; Tomov et al. 2003; Wakefield et al. 2010). At the time of writing, lectin-based GM strategies have not been commercialised.

Many insects, particularly Lepidoptera, depend on serine proteases as their primary digestive enzymes and there are many reports of the potential of inhibitors of these proteases to protect crops from herbivory or pathogenic infection. Serine protease inhibitors have been readily identified as potential candidates for

the development of insect-resistant transgenic crops (Schlueter et al. 2010), and their expression in rice to reduce insect pests in the field has been documented (Huang et al. 2005; Qiu 2008). Expression of a maize proteinase inhibitor gene in elite japonica rice varieties resulted in enhanced resistance to the striped stem borer, and was considered as a promising strategy to protect rice plants against this pest (Vila et al. 2005). In China, cotton cultivars expressing a modified cowpea trypsin inhibitor (CpTI) along with Cry1Ac, were released commercially in 2000 (He et al. 2009).

Expression in transgenic plants of double-stranded RNA (dsRNA) designed against insect target genes has been shown to give protection against pests through RNA interference (RNAi),opening the way for a new generation of insect-resistant crops (Baum et al. 2007; Gordon and Waterhouse 2007; Mao et al. 2011; Price and Gatehouse 2008). The theory is simple and compelling. The crop plant is engineered to produce a double-stranded RNA molecule with a high level of sequence specificity to a key insect gene. Both the injection and feeding of dsRNA have proven to be effective for a range of insect species. After uptake of the dsRNA, gene expression of the target sequence is silenced. Many target proteins could be envisaged including those involved in basic metabolism, digestion and fecundity. It has been demonstrated that a range of insect cells possess RNAi activity (Roether and Meister 2011; Terenius et al. 2011). With the purpose of identifying target genes for silencing in aphid gut, Zhang et al. (2013) performed RNA sequencing on the alimentary canals of grain aphids before and after feeding on wheat plants. They identified 16 genes that were significantly up or down-regulated upon feeding and suggested these were good targets for RNAi insect control strategies. However, this RNAi approach is still in infancy and requires further investigation. Since it is thought that dsRNA itself cannot replicate in the insects, relatively large amount of dsRNA is needed to block effectively the expression of the targeted gene.

In a GM approach first suggested by Pickett (1985), it was proposed that aphid pests could be repelled and their parasitoids attracted by expressing in plants, genes for the biosynthesis of the aphid alarm pheromone. The same author argued that long-term strategies for pest control will utilise natural metabolites that, acting by non-toxic modes of action, affect in more sophisticated ways than current pesticides, behavioural and developmental processes in the pest organisms (Pickett et al. 2014). An example of such a metabolite is the aphid alarm pheromone comprising the sesquiterpene (E)-β-farnesene (EBF). When *Arabidopsis* was engineered genetically to produce this compound, it repelled the peach–potato aphid, *Myzus persicae*, and caused increased foraging by the parasitoid wasp *Diaeretiella rapae* (Beale et al. 2006). GM wheat engineered to emit EBF gave a strong alarm response in the cereal aphids *Sitobion avenae, Metopolophium dirhodum* and *Rhopalosiphum padi* in laboratory assays. Also, parasitoid wasps, *Aphidius ervi,* spent longer foraging on the GM wheat plants under field simulation (Pickett et al. 2014).

Concluding Remarks

Wheat is already grown on more land than any other crop and the global demand is predicted to increase by a further 50% by 2050. Despite this, the application and commercialisation of biotechnology approaches in wheat lag behind those of maize, soya, cotton and canola. There has been significant progress in sequencing the genomes of bread wheat and its progenitors, along with research to associate genes or genetic markers to specific traits. Flexible vector construction platforms and robust and efficient protocols also exist for wheat transformation. Thus, the tools and technologies are largely in place and it is likely that wheat varieties developed using some type of molecular breeding technique will be grown commercially within a decade.

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Chapter 9
Sorghum Transformation: Achievements, Challenges, and Perspectives

Phat T. Do and Zhanyuan J. Zhang

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a drought-tolerant crop which can grow in marginal land areas where the growth of other cereals is limited. It is the fifth most important cereal after wheat, rice, maize, and barley (Food and Agricultural Organization of the United Nations 2013). Sorghum can be used as a source of food for humans and animals, as well as raw materials for the production of alcoholic beverages and bioenergy (Dahlberg et al. 2011). The gluten-free flour of sorghum makes it suitable for celiac patients. In addition, sorghum consumption can improve human health due to its high antioxidant phenolics and low cholesterol content (Taylor et al. 2006; Dahlberg et al. 2011). Sorghum is a dietary staple for about 500 million people in more than 30 countries of the semi-arid tropics, especially in Africa and Asia (Dahlberg et al. 2011). In 2011, an excess of 55 million tons of sorghum was harvested from about 35 million ha grown worldwide, with an average yield of 1.5 t/ha. Of these, the USA dedicated about 1.6 million ha and produced more than 5.4 million tons with an average yield of 3.4 t/ha (Food and Agricultural Organization of the United Nations 2013). Recently, ethanol production has become one of the fastest growing segments in the US sorghum industry and has led to the single largest value-added market for grain sorghum producers in America. Currently, about 15–20 % of the US domestic sorghum production is used for manufacturing of ethanol and its coproducts (Dahlberg et al. 2011).

Both natural and man-made interventions affect sorghum production. Natural factors include fungal diseases (Little et al. 2012; Tesso et al. 2012), insects (Guo et al. 2011), abiotic stress (Tari et al. 2012), and the parasitic weed like *Striga* (Khan

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et al. 2000). Biofuel conversion not only cuts into food-based yields but also presents new problems on how to gain the most efficiency from sorghum plants for the ethanol process. Therefore, efforts have been made to improve sorghum varieties to reduce the impacts of these limiting factors on sorghum agronomical performance. To date, most sorghum varietal improvements have been achieved through conventional breeding (Grootboom et al. 2010). However, traditional breeding for crop improvement has several limitations, including its inability to sustain yield and productivity indefinitely (Vasil 1994). In recent years, plant biotechnology, including molecular genetics and genomics as well as plant transformation, has provided a powerful means to supplement traditional breeding approaches. Plant transformation has a unique role in varietal improvement and offers a much faster approach to accomplish genetic gains for various traits (Gurel et al. 2009; Grootboom et al. 2010). These gains will contribute to both food and biofuel industries as they relate to sorghum production.

Despite the difficulties in sorghum tissue culture and transformation progresses have been made (Zhu et al. 1998; O'Kennedy et al. 2006), twenty years after the first transgenic sorghum was developed (Casas et al. 1993), several successes in sorghum transformation have been reported which employ different transformation methods such as *Agrobacterium*-mediated transformation, particle bombardment, electroporation, and pollen-mediated transformation. More recently, transformation studies have focused primarily on using marker genes to establish, develop, and improve transformation and regeneration processes (Nguyen et al. 2007). The production of transgenic sorghum with agronomic traits such as nutrient improvement, pest resistance, disease, and stress tolerance has been reported (Zhao and Tomes 2003; Gao et al. 2005b; Maheswari et al. 2010; Arulselvi et al. 2011). Low transformation frequency and transgene silencing are limiting factors for sorghum varietal improvement by genetic engineering. As a result, more attempts have been made to overcome these obstacles in order to meet the requirements of sorghum consumption and biofuel production.

This review discusses the contributions of genetic transformation to sorghum improvements with emphasis on transformation methods, sources of explant tissues, promoters, and various candidate genes. In addition, challenges and possible strategic solutions to sorghum transformation are also discussed.

Transformation Methods Employing Different Types of Explants

Although a tissue culture system for sorghum was reported about four decades ago (Gamborg et al. 1977), less progress has been made in sorghum transformation than in other cereals (Nguyen et al. 2007). Microprojectile- and *Agrobacterium*-mediated transformation methods are two main approaches that have been developed and applied for sorghum transformation. Other methods such as electroporation and pollen-mediated transformation have also been reported.

Microprojectile Transformation

Due to the host limitations by *Agrobacterium tumefaciens,* early studies on sorghum transformation focused on direct DNA delivery methods. The first two reports on sorghum transformation described the use of protoplasts and cell suspension cultures combined with electroporation, but without success in obtaining stable transgenic sorghum plants (Battraw and Hall 1991; Hagio et al. 1991). Fertile transgenic sorghum plants were first obtained by microprojectile bombardment of immature embryos of sorghum genotype P898012 (Casas et al. 1993). This method was later applied to transformation of immature inflorescences and other explants, such as leaf tissues and calli, with constructs carrying reporter, selectable marker, and target genes (Kononowicz et al. 1995; Casas et al. 1997; Zhu et al. 1998). The transformation efficiency of the above bombardment method was very low, around 0.08–1 %, despite some modifications (Casas et al. 1997; Able et al. 2001; Emani et al. 2002). The transformation efficiency was improved to 1.3 % by the optimization of transformation conditions, including bombardment parameters such as acceleration pressure, target distance, and gap width, as well as experimentation with different types of explants (Tadesse et al. 2003). Although immature and mature embryos, shoot tips, and embryogenic calli were used in this study, transgenic sorghum plants were obtained only from immature embryos and shoot tips. Using shoot apices as explants for bombardment reduced the time for transgenic sorghum regeneration, but could cause transgene instability in transgenic plants (Girijashankar et al. 2005). Consequently, immature embryos were used thereafter as favored explants for microprojectile bombardment. Recently, many studies aiming at introducing different genes of interest have employed alternative explant tissues, which included inflorescences, shoot tips, or calli derived from immature embryos for sorghum transformation (Grootboom et al. 2010; Maheswari et al. 2010; Raghuwanshi and Birch 2010; Kosambo-Ayoo et al. 2011; Brandao et al. 2012). However, these studies showed low transformation efficiencies from 0.3 to 1.3 %.

Most recently, Liu and Godwin (2012) reported a substantial improvement in particle bombardment-mediated sorghum transformation with a frequency of 20.7 %; furthermore, more than 90 % of transgenic plants exhibited normal growth and fertility under glasshouse condition. High frequencies of callus induction and shoot regeneration were achieved by using genotype Tx430 and an increase or addition of $CuSO_4$, KH_2PO_4, L-proline, and L-asparagine in the culture medium. DNA delivery conditions were also optimized with 0.6 μm gold particles, 18.5 cm flying distance, and 1000 psi helium pressure.

Agrobacterium-Mediated Transformation

Agrobacterium-mediated transformation has been used in many sorghum transformation studies. However, as with other cereal plants, this method is still subject to certain limitations that hinder sorghum transformation progress and reduce

transformation efficiency. In 2000, Zhao and his colleagues first reported the production of stable transgenic plants obtained using *Agrobacterium*-mediated transformation. In this study, immature embryos were used as explants and the transformation frequency ranged from 0.95 to 2.34 %, greater than the frequency of the bombardment method used at that time. Later studies showed further improvement of *Agrobacterium*-mediated transformation. Carvalho et al. (2004) increased the transformation to 3.5 % by optimization of the infection, cocultivation, and selection conditions. By using mannose and kanamycin instead of herbicidal agents, the transformation rate was achieved at 3.3–4.5 % (Gao et al. 2005a; Howe et al. 2006). Transgenic plant recovery further reached 5 % as some factors related to callus induction, inducible treatments (e.g., cold-pretreatment of immature seeds, reduction of phenolic compounds, and tissue culture microenvironment), were considered and optimized (Nguyen et al. 2007). Gurel et al. (2009) reported an 8.3 % transformation frequency by utilizing the heat treatment of immature embryos before inoculation. Other attempts have been made to optimize parameters related to cocultivation and regeneration media, but further improvements have not been reported (Jambagi et al. 2010; Kimatu et al. 2011). Recently, the frequency of sorghum transformation via *Agrobacterium*-mediated delivery was improved dramatically by 33 % (Wu et al. 2013). This was achieved by modifications of media and the utilizing of supper binary vectors. In general, all previous results demonstrated that immature embryos were the most efficient explants for sorghum transformation by *Agrobacterium*-mediated method.

Other Transformation Methods

Electroporation was first utilized by combining with protoplast culture for sorghum transformation (Ou-Lee et al. 1986; Battraw and Hall 1991). Nevertheless, this method could not be further developed and applied widely because of the lack of a protoplast-to-plant regeneration system. The electroporation of protoplasts for transformation utilizes high-voltage electric pulses applied either directly or indirectly to a solution containing plasmid DNA and protoplasts (Ou-Lee et al. 1986). To date, as is the case with most plant species, electroporation of sorghum protoplasts has been reported only for transient transgene expression and no transgenic plant has ever been obtained using this method.

Pollen-mediated transformation was another approach in sorghum transformation, inspired by previous success in several plant species including maize (Wang et al. 2001). Pollen was subjected to ultrasonication in a sucrose solution containing plasmid, and then the treated pollen was used to pollinate stigmas of the male sterile plants. In the case of sorghum transformation, the integration and inheritance of the introduced gene were confirmed in T0 plants using Southern-blot hybridization and antibiotic resistance in the T1 generation (Wang et al. 2007). The disadvantages of this method include low transformation frequency and difficulties in seed production due to damage of pollen after ultrasonication. Furthermore, as is the case with other direct transformation methods, a large number of transgene copies inserted

into the sorghum genome were observed as the target for gene silencing. Table 9.1 summarizes key studies in sorghum transformation.

Promoters

Promoters have drastic effects on the success of plant transformation. Using suitable promoters is essential to improve the transgenic frequency and transgene expression and, therefore, it gains considerable attention from many laboratories. It is desirable to identify strong promoters that not only provide a high expression level of the introduced genes but also avoid transgene-induced gene silencing in the target cells.

In most early studies of sorghum transformation, the cauliflower mosaic virus (CaMV35S) promoter was used in both bombardment and *Agrobacterium*-mediated delivery methods. Despite the lower efficiency in dicotyledonous cells, this promoter has been used extensively for transformation of sorghum and other monocotyledons. The strength of the CaMV35S promoter was determined by the expression levels of transgenes in T0 and T1 plants (Casas et al. 1993, 1997; Carvalho et al. 2004). To improve the expression of transgenes in sorghum and other cereals, an intron sequence (i.e., *il* sequence of maize) was inserted in the 5′ untranslated region (5′ UTR) behind the 35S promoter (Gallie and Young 1994; Vain et al. 1996; Tadesse et al. 2003).

Monocotyledonous promoters were utilized as a potential way to enhance sorghum transformation. The *uidA* and *hpt* genes controlled by the maize alcohol dehydrogenase promoter (*adh1*) were transferred into sorghum via bombardment in the earliest study (Hagio et al. 1991). Although stable transformation was reported using sorghum cell suspension cultures, the efficiency was very low. The maize ubiquitin 1 promoter (*ubi1*) was first used for transgenic sorghum through *Agrobacterium*-mediated transformation (Zhao et al. 2000). Mendelian segregation in the T1 generation was confirmed by screening for herbicide resistance. Furthermore, by using the *ubi1* promoter and a good source of embryos, a higher frequency of stable transformation was reported than in previous studies. Able et al. (2001) evaluated the influence of three promoters involving *actin1,* CaMV35S, and *ubi1* on sorghum transformation by expressing two reporter genes, *uidA* and *gfp*. This study indicated that the transient expression of *uidA* gene controlled by *ubi1* was significantly higher than with the other promoters.

In separate efforts to improve transformation efficiency, various promoters including *actin1, adh1,* CaMV35S, HBT is a chimeric promoter with the 35S enhancer (Hind III-35S400bp-Hind III) fragment fused to the basal promoter (that includes the TATA box, transcription initiation site, and 5 untranslated re-gion) of C4-pyruvate orthophosphate dikinase gene (C4PPDK) (Jeoung et al., 2002). The strength of these promoters was explained by the order *ubi1*>CaMV 35S>*HBT* for green fluorescent protein (GFP) expression in calli of Tx430 genotype and *ubi1*>CaMV35S>*act1*>*adh1* for β-glucuronidase (GUS) constructs. The activities of these heterologous promoters *adh1, act1,* CaMV35S, and *ubi1* were compared by using

Table 9.1 Information about transgenes, promoters, and DNA delivery methods in sorghum transformation

Features	Transgenes	Promoters	DNA-delivery methods
Reporter	*gus* (*uidA*)	*CaMV35S*; *adh1*; *act1*; *ubi1*	Bombardment; *Agrobacterium*-mediated; electroporation; pollen-mediated transformation
	Gfp, Sgfp65T (improved *gfp*)	*CaMV35S*; *act1*; *ubi1*; *α-kaf*	Bombardment; *Agrobacterium*-mediated
	luc⁺ (luciferase)	*ubi1*	Bombardment
	R and Cl maize anthocyanin regulatory elements	*CaMV35S*	Bombardment
Selectable	*bar*	*CaMV35S*; *act1*; *ubi1*	Bombardment; *Agrobacterium*-mediated
	pmi	*ubi1*	*Agrobacterium*-mediated
	htp	*CaMV35S; ubi1*	Bombardment, *Agrobacterium*-mediated
	nptII	*act1*; *CaMV35S*; *ubi1*	Bombardment; *Agrobacterium*-mediated; PEG-mediated transformation
	CAT gene	*CaMV35S*	Electroporation
Stress tolerance	*CryIAb*	*ubi1*	Bombardment; *Agrobacterium*-mediated
	CryIAc	*mpiC1*; *ubi1*	Bombardment
	harchi (chitinase) and *harcho* (chitosanase)	*ubi1*	Bombardment
	Chi11 (rice chitinase)	*ubi1*	*Agrobacterium*-mediated
	mtlD gene encoding for mannitol-1-phosphate dehydrogenase	*CaMV35S*	Bombardment
	tlp (encoding TLP)	*ubi1*	*Agrobacterium*-mediated
	OsCDPK-7	*ubi1*	*Agrobacterium*-mediated
Nutrient improvement	*dhdps-rl*	–	Bombardment
	lysine-rich HT12	–	*Agrobacterium*-mediated
	sorghum lys1 tRNA synthase elements (TC2 or SKRS)	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	sorghum gamma-kafirin-1	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	sorghum gamma-kafirin-2	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	sorghum delta-kafirin-2	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	lysine alpha-ketogluterate reductase	*maize zein CZ19 B1*	*Agrobacterium*-mediated
	CrtI	*sorghum beta-kafirin promoter*	*Agrobacterium*-mediated

act actin, *adh* alcohol dehydrogenase, *CMV35S* cauliflower mosaic virus, *CAT* chloramphenicol acetyltransferase, *CDPK* calcium-dependent protein kinases, *kaf*kafirin, *PEG* polyethylene glycol, *TLP* thaumatin-like protein, *ubi* ubiquitin

the *uiA* gene in an effort to optimize transformation conditions (Tadesse et al. 2003). The histochemical staining and enzymatic activity assay of the *gusA* gene in samples demonstrated that *ubi1* was the strongest promoter followed by *actin1, adh1,* and *CaMV35S*. The *ubi1* promoter was also used with different target genes, such as *manA* and *tlp,* for sorghum transformation (Gao et al. 2005b; Gurel et al. 2009). To date, *ubi1* is still considered to be the most efficient promoter for transgene expression in sorghum and is used predominantly in sorghum studies (Grootboom et al. 2010; Kosambo-Ayoo et al. 2011; Jambagi et al. 2010; Raghuwanshi and Birch 2010; Liu and Godwin 2012)

Several promoters of plant genes were also exploited successfully in sorghum genetic engineering in some individual studies. In a maize study (applicable to sorghum), the protease inhibitor gene *mpiC1* was induced in response to mechanical wounding and insect feeding. In an attempt to increase insect resistance, Girijashankar et al. (2005) used the maize *mpiC1* promoter to drive *CryIAc* and introduce the transgene into sorghum via shoot apices-based transformation. These authors observed a stronger expression of the *CryI*Ac gene under the control of the *mpiC1* promoter than the maize *polyubiquitin1* promoter. Recently, the kafirin promoter (*α or β kaf*) was used in sorghum transformation (Ahmad et al. 2012; Wu et al. 2013). This promoter contained endosperm specificity-determining motifs, a prolamin-box, the O2-box 1, CATC, and TATA boxes required for *α-kafirin* gene expression. This report showed that *ubi1*-GFP expression was detected throughout the plant, while the *α-kafirin*-GFP was expressed only in seeds. This success suggested a new venue for studying sorghum grain quality by using the *α-kaf* seed-specific promoter through genetic transformation.

Selectable Marker and Reporter Genes

Selectable Marker Genes

An efficient selection system can be seen as the key for successful transformation. Monocotyledons are known to have a more narrow range of available marker genes than dicotyledons due to a natural endogenous resistance to some selective agents (Tadesse et al. 2003). However, various selectable marker genes have been utilized in sorghum transformation. These maker genes could be divided into three main groups, including antibiotic resistance (*hpt*, *npt*II), herbicide resistance (*bar*), and nutrient assimilation (*man A*).

The stable integration of neomycin phosphotransferase II (*npt*II) *gene* in transgenic sorghum was first reported by Tadesse et al. (2003). In this study, geneticin selection was used to avoid the release of phenolic substances. Mendelian inheritance of *npt*II in T1 generation was confirmed by using geneticin resistance analysis of T1 seedlings. Later studies also verified that *npt*II was an efficient antibiotic marker for transgenic selection (Howe et al. 2006; Mall et al. 2011; Liu and Godwin 2012). Likewise, the hygromycin phosphotransferase gene (*hpt*) conferring hygromycin

resistance was also used as a good selectable marker for sorghum transformation (Hagio et al. 1991; Carvalho et al. 2004; Nguyen et al. 2007; Raghuwanshi and Birch 2010). However, as is the case with other plants, the disadvantage of using antibiotic-resistance selectable markers for sorghum is the possible migration of these genes to infectious bacteria (Balter 1997).

The bialaphos resistance gene, *bar,* encodes *p*hosphinothricin *a*cetyl *t*ransferase (PAT) conferring herbicide resistance and is one of the most efficient selectable markers for sorghum transformation. Some glufosinate ammonium-based herbicides, such as phosphinothricin (PPT), Basta, and bialaphos, could be used as selection agents in experiments that utilize the *bar* gene. Different concentrations of these herbicides have been used to select transgenic plants based on the types of explants and different stages during the regeneration process. For example, a 0.6% aqueous solution of Ignite/Basta (glufosinate 200 mg/mL) was used for leaf painting (Casas et al. 1993); up to 10 mg/L PPT was supplemented to callus-induction medium, while lower concentrations of PPT from 1 to 5 mg/L were applied in different stages of callus development and shoot regeneration (Zhao et al. 2000; Emani et al. 2002; Tadesse et al. 2003; Lu et al. 2009). Basta was used for the selection of embryogenic calli and somatic embryos at concentrations from 1 to 2.5 mg/L (Girijashankar et al. 2005; Arulselvi et al. 2010; Grootboom et al. 2010). The advantage of using the *bar* gene is to produce herbicide-resistant plants. Nevertheless, *bar* selection seems to be a leaky system resulting in many escapes in sorghum. In addition, there was concern about transmission of the *bar* gene via pollen to wild relatives of sorghum (Gao et al. 2005a).

The phosphomannose isomerase (*pmi*) gene, isolated from *Escherichia coli,* has been used as a positive selectable marker gene to eliminate the risk of herbicide and antibiotic resistance genes in other monocotyledons such as maize, rice, and wheat (Wright et al. 2001; Lucca et al. 2001). The *pmi* enzyme converts mannose-6-phosphate into fructose-6-phosphate, which can be used as a carbon source for plant cells. The mannose selection system was used for sorghum transformation initially by Gao et al. (2005a). In this study, medium containing 1–2% mannose was applied for embryogenic callus selection; the integration and expression of the *pmi* gene in progeny were confirmed by Southern and western blots, respectively. The high transformation efficiency was indicated to be 2.88% for Pioneer 8505 and 3.30% for C401 genotypes. Afterwards, other independent reports again indicated the efficiency of mannose selection in sorghum transformation (Gurel et al. 2009; Grootboom et al. 2010). Until now, the highest frequency of *Agrobacterium*-mediated sorghum transformation was obtained by using the *mpi* selection system (Gurel et al. 2009; Wu et al. 2013).

Reporter Genes

Among the various reporter genes, *uidA* and *gfp* are used extensively for transformation of most plant species. The *uidA* gene coding for GUS has been utilized in

many sorghum transformation studies employing all transfer methods (Casas et al. 1993, 1997; Lu et al. 2009; Arulselvi et al. 2010; Grootboom et al. 2010; Brandao et al. 2012). The chief advantage of *uidA* is its simple detection system when compared to other reporter genes because the transient and stable expression of GUS in tissue is easily visualized without specific equipment. However, the *uidA* detection system is limited by the loss of tissue samples to the destructive assay, X-Gluc staining.

The green fluorescent protein (GFP) gene, isolated from jellyfish (*Aequorea victoria*), can be used as a reporter gene to monitor stable expression and avoid destructive assays. GFP has been found to be superior to other markers in many cases because of some favorable properties such as no need for exogenous substrates and easy visualization (Able et al. 2001; Hraska et al. 2006). In many previous studies, the marker gene, *gfp,* was transferred into sorghum alone or together with other target genes by different methods (Jeoung et al. 2002; Gao et al. 2005b; Gurel et al. 2009; Jambagi et al. 2010; Ahmad et al. 2012; Liu and Godwin 2012). Using the *gfp* gene to detect transgenic materials for plant transformation has two advantages because it is highly sensitive and nondestructive. Conversely, *gfp* detection requires expensive equipment, which is a disadvantage of *gfp* as a reporter gene. Another disadvantage is that high concentrations of *gfp* could adversely affect organogenesis, which in turn can cause sterility (Jeoung et al. 2002). The reduced regeneration efficiency by *gfp* accumulation in the cell organelles was also reported in some plant species (Haseloff and Amos 1995; Able et al. 2001).

In some studies, other reporter genes have been introduced into sorghum. Casas et al. (1993) reported that the stable expression of R and C1 maize anthocyanin regulatory elements was obtained in transgenic sorghum plants under control of the CaMV35S promoter. In this study, anthocyanin accumulation could be seen in order to initially evaluate the efficiency of the sorghum transformation system. In addition, the *luc+* gene coding for firefly luciferase was transferred into both grain sorghum (Kononowicz et al. 1995) and sweet sorghum (Raghuwanshi and Birch 2010). The integration and expression of this gene in transformed sorghum plants was confirmed by genomic Southern blot analysis and the luciferase assay. Recently, DsRed-encoded 28-kDa red fluorescent protein was overexpressed in sorghum genotype Tx430 and the expression of this protein was observed in different organs such as roots, leaves, shoots, and seeds (Wu et al. 2013).

Stress Tolerance Genes

Pest Tolerance

In order to reduce the damage on sorghum development and yields caused by many insect species, *Bacillus thuringiensis* (Bt) toxin genes have been transferred into this crop. Girijashankar et al. (2005) introduced different constructs involving *ubi-*

cry1Ab, ubi-cry1Ac, and *mpiC1-cry1Ac* into sorghum by particle bombardment. The expression and inheritance of the *Bt* genes were confirmed in T1 plants by partial tolerance against first instar larvae of the spotted stem borer (*Chilo partellus Swinhoe*). However, *Bt* protein accumulated at very low contents of 1–8 ng/g of fresh tissue of mechanically wounded leaves. In a recent report, Zhang et al. (2009) utilized *Agrobacterium*-mediated transformation to transfer the *CryIAb* gene into three sorghum cultivars, 115, ICS21B, and 5–27, with an average transformation efficiency of 1.9 %. Different expression levels of *Bt* protein in transgenic plants were detected by Western blotting and enzyme-linked immunosorbent assay (ELISA) assays. Furthermore, transgenic plants with a high content of *Bt* protein displayed a tolerance to pink rice borer (*Sesamia inferens*). The barrier for utilization of *Cry* family genes is the very low content of *Bt* protein obtained in transgenic sorghum plants. These contents are far below the lethal dose required to give complete protection against some major insect species (Girijashankar et al. 2005).

Fungi Tolerance

The rice chitinase gene (*Chi11*), which may have a protective role against fungal pathogens, is known as the first potentially agronomically useful gene introduced into sorghum. The presence of *Chi11* in transgenic sorghum was confirmed by Southern blotting, and the expression was indicated by the improvement of resistance to disease incited by fungus (Zhu et al. 1998; Krishnaveni et al. 2001; Arulselvi et al. 2011). Both chitinase (*harchit*) and chitosanase (*harcho*) genes, isolated from *Trichoderma harzianum,* were introduced into sorghum in attempts to improve resistance to fungal diseases such as anthracnose caused by *Colletotrichum sublineolum* (Kosambo-Ayoo et al. 2011). The transgenic plants displayed greater tolerance to anthracnose as compare to the parent wild types in both *in planta* and *ex planta* infection assays with *C. sublineolum*. Similarly, the *tlp* gene, i.e., encoding thaumatin-like protein (TLP), enhanced resistance to fungal diseases and drought and was transferred into sorghum with the *gfp* gene (Gao et al. 2005b). The result showed a 100 % correlation between *gfp* expression and the presence of the *tlp* gene in transgenic plants. In addition, the strong expression of TLP was indicated by western blot analysis.

Abiotic Stress Tolerance

Although the *tlp* gene, which has a function of enhancing drought tolerance, was introduced into sorghum, the presence of this transgene was verified in T0 and T1 generations. However, the response of transgenic plants to fungus or drought was not shown (Gao et al. 2005b). To enhance the tolerance to water deficit and NaCl stress, the *mtlD* gene encoding for mannitol-1-phosphate dehydrogenase from *E. coli* was used for sorghum transformation (Maheswari et al. 2010). The improved

drought tolerance of transgenic sorghum was illustrated by the increased retention of leaf water. Moreover, there was a significantly improved maintenance in root and shoot growth of transformed plants under NaCl stress (200 mM).

Calcium-dependent protein kinases (CDPKs) are known as key players in the responses of plants to environmental attacks. Therefore, the CDPK-7 gene isolated from rice (genotype Nipponbare) was transferred into sorghum to enhance abiotic stress tolerance (Mall et al. 2011). The presence and expression of this gene was confirmed in transformed sorghum by molecular analysis. However, improvement in the tolerance to cold and salt stress was not observed under tested conditions. Instead, the result showed a lesion mimic phenotype and upregulation of a number of pathogen-related proteins along with transcripts linked to photosynthesis.

Nutrient Modifications

Despite the use of sorghum as a human and animal food source, it has a low nutritional quality, e.g., being relatively poor in protein and lipid. Overproduction of the essential, but limiting amino acid, lysine, is known as a good strategy to improve sorghum grain quality. The first study on genetic engineering to improve sorghum grain quality was accomplished by Yohannes et al. (1999). In this investigation, a mutated *dhdps-rl* gene, encoding a feedback-insensitive dihydro-picolinate synthetase enzyme leading to increased lysine accumulation, was introduced into sorghum by bombardment. Later, Zhao and Tomes (2003) used the high-lysine protein gene (HT12) for sorghum transformation via *Agrobacterium*-mediated transformation. The reported transformation rate was 2.1% and expression of HT12 in transgenic plants led to a 50% increase in total grain lysine. Sorghum lys1 tRNA synthase elements (TC2 or SKRS), together with the *bar* gene in a 2 T-DNA system, were introduced into sorghum (Lu et al. 2009). The average transformation frequency was 0.7%, the presence of the target gene was confirmed in T1 generation plants, and marker-free transgenic sorghum plants were obtained. However, the expression of this gene and the change in lysine content were not described. Recently, Wu et al. (2013) used a super binary vector, PHP166, for sorghum transformation with the aim to improve the concentration of pro-vitamin A, mineral bioavailability, protein quality, and protein digestibility in seeds. The multiple- and single-copy intact integrations of the T-DNA were verified in transgenic plants, but transgene expression was not reported.

Challenges in Sorghum Transformation

Clearly, transformation plays a unique role in sorghum genetic improvement and biological studies and has gained significant attention from scientists around the world. However, the transformation efficiency, even two decades after the first

production of fertile transgenic sorghum, remains too low to satisfy the requirements of sorghum genetic engineering. This is in sharp contrast with some other cereal crops, whose transformation protocols have been improved considerably. Progress in sorghum transformation has been hampered by many difficulties associated with tissue culture, the transformation process itself, and transgene silencing.

Tissue Culture Barrier

Reproducible generation of transgenic plants depends on an efficient tissue culture system. However, sorghum is considered to be the most recalcitrant crop among the cereals for its *in vitro* response (Gao et al. 2005a; Pola and Mani 2006; Girijashankar et al. 2007; Arulselvi and Krishnaveni 2009; Sadia et al. 2010). Accumulation of phenolic compounds and a high degree of genotype dependence are known as the major barriers for sorghum tissue culture.

The release of phenolics into the medium was a well-known problem for tissue culture due to strong negative effects on cell differentiation, somatic development, and plant regeneration (Zhao et al. 2000; Tadesse et al. 2003; Gao et al. 2005a; Howe et al. 2006). These compounds not only decreased the frequency of sorghum regeneration but also were toxic to *Agrobacterium* cells in transformation experiments (Nguyen et al. 2007). More phenolic substances observed in red sorghum, hybrid sorghum, and some public varieties hinder the use of these genotypes for regeneration and transformation (Gao et al. 2005a; Nguyen et al. 2007). A number of culture manipulations have been developed to alleviate the effects of phenolic compounds in tissue culture such as reducing the sub-culturing intervals, the addition of polyvinylpolypyrrolidone (PVPP) to the medium (Zhao et al. 2000; Gao et al. 2005a; Lu et al. 2009), and the use of activated charcoal and cold pretreatment (Nguyen et al. 2007). However, short subculture intervals require more labor and materials, which raises the cost of the culture process. PVPP and activated charcoal reduce the effective concentration of certain growth regulators and therefore affect the in vitro response of the tissue (Howe et al. 2006).

To date, the successful recovery of transgenic plants through *Agrobacterium*-mediated or particle bombardment was achieved mainly using immature embryos, in spite of various explants utilized, which include immature embryos, inflorescences, or shoot tips. Nevertheless, the frequency of callus induction and plant regeneration from immature embryos varies widely and depends especially on plant genotype. Consequently, different genotypes have different transformation efficiencies even though the same culture and transformation conditions are employed (Casas et al. 1993,1997; Zhao et al. 2000; Able et al. 2001; Gao et al. 2005a; Howe et al. 2006; Raghuwanshi and Birch 2010; Kosambo-Ayoo et al. 2011). Casas et al. (1993) reported that after DNA delivery, only three of eight genotypes produced embryogenic calli on selection medium, and only genotype P898012 regenerated plants under bialaphos selection. Genotype dependence was again demonstrated as the drawback for tissue culture in recent reports on sorghum regeneration (Maheswari et al. 2010;

Jogeswar et al. 2007; Arulselvi and Krishnaveni 2009). Sorghum genotypes such as Tx430 and P898012 have been considered to be appropriate materials for regeneration and transformation, regardless of the fact that many sorghum genotypes have been screened and used in studies with this plant. Therefore, it is imperative to compare these genotypes alongside experiments to identify highly regenerable genotypes (Kumar et al. 2011; Gurel et al. 2009; Howe et al. 2006), and to establish further an optimal protocol for tissue culture and transformation.

Transformation Conditions

Agrobacterium-mediated sorghum transformation is known to have advantages over other methods, especially for generating a high proportion of plants with single copy of transgenes and reduced chances of gene silencing and instability (Zhao et al. 2000; Gao et al. 2005a, b; Howe et al. 2006; Nguyen et al. 2007; Lu et al. 2009). However, similar to some other cereals, sorghum has been recalcitrant to *Agrobacterium*-mediated transformation. The interaction between bacterial cells and sorghum tissue could be improved by preinduction of *Agrobacterium* with acetosyringone, using tissues that have actively dividing cells, and heat–cold pretreatment of explants (Verma et al. 2008; Gurel et al. 2009). Other ways to increase transformation include the use of greater concentrations of *Agrobacterium* or longer cocultivation time (Zhao et al. 2000). Nevertheless, the above treatment conditions could be plant species- or genotype-dependent and, therefore, may not necessarily promote high transformation efficiency and could even cause negative effects on transgenic plant recovery. Zhao et al. (2000) reported that too high concentration of bacteria caused serious damage of explant tissues during the *Agrobacterium* inoculation period, and the overgrowth of bacteria interfered with callus growth on the medium. This observed when high concentrations of bacteria were used, contributing to the failure in transgenic regeneration (Gao et al. 2005b). Moreover, *Agrobacterium* is a plant pathogen which is capable of inducing plant necrosis; it also reduces regeneration and transformation efficiency (Hansen 2000). In fact, this problem has been reported in several sorghum transformation studies (Gao et al. 2005b; Nguyen et al. 2007). Additionally, immature embryos proved to be sensitive to *Agrobacterium* infection and embryo death after cocultivation was the limiting factor in improving transformation efficiency (Carvalho et al. 2004).

Likewise, the low frequency of sorghum transformation via microparticle bombardment was known to be associated with the difficulty of DNA delivery and tissue damage (Able et al. 2001). Increasing particle flow by using a higher acceleration pressure could improve DNA delivery, but at the same time, it could cause more extensive tissue damage which is detrimental to callus induction, cell differentiation, and plant recovery. For example, at a high pressure of particle flow (1800 psi), more than 90 % of bombarded tissues became necrotic; regenerable calli and somatic embryos did not develop (Tadesse et al. 2003). Similarly, in a separate study, 10 % of the shoot apices were killed when high helium gas pressure was employed

for bombardment (Girijashankar et al. 2005). Although several parameters such as the microprojectile size, DNA coating of the microprojectiles, distance to the target tissue, and the velocity of gas flow were evaluated and optimized, the efficiency of sorghum transformation via bombardment was still less than those of other crops (Able et al. 2001; Tadesse et al. 2003; Liu and Godwin 2012).

Finally, selection pressures influence cell differentiation and reproduction of transgenic tissue. Negative selective agents, such as antibiotics or herbicides, have been known to cause detrimental effects on plant tissue culture and hinder the regeneration process (Zhao et al. 2000; Gao et al. 2005b). Untransformed cells subjected to stress by selection substrates release phenolic compounds that are toxic for transformed cells. For example, the release of phenolic substances from herbicide-treated explants during the regeneration process was a key reason for failure in the production of transgenic sorghum plants via phosphinothricin selection (Tadesse et al. 2003; Lu et al. 2009). In some cases, the selection pressure on sorghum tissue could be reduced by using a low concentration of selection agents in combination with rapid selection to regenerate plants (Lu et al. 2009) or by using visual maker genes such as *gfp* without using antibiotics or herbicides as the selection agents (Gao et al. 2005b). However, these approaches would allow generating more "escapes" (i.e., nontransgenic events), decrease the efficiency of selection process, and increase the time and resources necessary for the analysis of transformed plants.

Transgene Silencing

Transgene silencing has been observed in both dicotyledons (Matzke and Matzke 1995) and monocotyledons (Iyer et al. 2000). Methylation of the introduced DNA and homology-dependent ectopic pairing were known as the major pathways leading to transgene inactivation (Demeke et al. 1999; Iyer et al. 2000; Fagard and Vaucheret 2000). In sorghum transformation, transgene silencing appears to be a problem because it is not attributed to variation in copy number or the method of transformation. For example, the GUS gene has been widely used in sorghum transformation. However, the silencing of this gene was indicated in many reports. Early studies showed that GUS-transformed cells did not display blue staining upon incubation with the histochemical substrate X-Gluc, or they showed a very low level of GUS activity (Hagio et al. 1991; Battraw and Hall 1991). Casas et al. (1993) observed that the GUS gene was not expressed after sustained periods of culture although the presence of this gene was confirmed by Southern analysis. They suggested that the expression of transgenes was inactivated by DNA methylation in the transformed sorghum cells. In 1997, Casas and his colleagues also observed that GUS activity could not be detected in T1 plants containing the GUS gene. Zhu et al. (1998) also found that both *bar* and rice chitinase genes were present, but silenced at certain developmental stages in a few primary transgenic plants (T0) as confirmed by Southern and western blots, respectively. Emani et al. (2002) confirmed that multiple copies of the *bar* as well as the *gus* genes had integrated into

the sorghum genome. The expression of the *bar* gene was observed in T0, T1, and T2 generations. However, GUS expression was not found in all tissues tested from regenerated T0 plants. Moreover, by using reactivation agents and different promoters, these workers demonstrated that methylation-based transgene silencing was the reason for the suppression and inactivation of transgenes.

Future Perspectives

Over the last two decades, since the production of the first transgenic sorghum plants, many sorghum transformation studies with various DNA delivery methods have been reported. Not only various marker genes have been used to establish, confirm, and optimize sorghum transformation protocols but also some agronomical important genes such as genes for pest, disease and abiotic tolerance have been transferred into sorghum. Future sorghum transformation research efforts will continue to focus on enhancing the value of sorghum for food consumption and biofuel production.

Improvement of Grain Quality

Grain sorghum is a major staple for millions of people in Africa and Asia, and a major livestock feed in developing countries. Nevertheless, the low nutritional content is limiting its value as food and feed. Attempts to improve the lysine content of sorghum grain using transformation were reported in early studies (Yohannes et al. 1999; Zhao and Tomes 2003), and the need for such an improvement has gained more attention recently from scientists around the world. As discussed earlier, Ahmad et al. (2012) studied the endosperm-specific expression of the α-kafirin promoter that was isolated from sorghum using the *gfp* gene as a reporter. This result implied that the identification of a sorghum grain-specific promoter could open up the opportunity to express ectopically candidate genes in endosperm for grain quality improvement.

Sorghum grains are known to have relatively poor digestibility in comparison to those of other cereal grains. Kafirins, the main sorghum proteins resistant to digestion, account for more than 80 % of the protein in the endosperm of the sorghum grain (Hamaker et al. 1995). These proteins are cotranslationally translocated to the endoplasmic reticulum (ER) and assembled into discrete protein bodies which tend to be poorly digestible in food and feed applications (Kumar et al. 2012). Therefore, using genetic engineering techniques to reduce the expression of different kafirin subclasses is a promising approach to improve sorghum grain quality (Da Silva et al. 2011a, b; Kumar et al. 2012).

In the attempt to improve the staple food for about 300 million people in Africa, the Africa Biofortified Sorghum (ABS) project was established by the collaboration

of 13 organizations with two main phases. It was initiated by 2005 and scheduled for completion in 2015. Achieving increased beta-carotene concentration and stabilization, increasing iron and zinc bioavailability, and improvement in protein digestibility are targeted traits that have been the main focus in this project. The progress of ABS updated on September 2012 showed that hundreds of transgenic events have been produced and analyzed for enhanced beta-carotene. The next steps of the ABS are to determine and optimize the final transgenic constructs for the β-carotene gene and Fe and Zn bioavailability gene. Moreover, transgenic sorghum should be evaluated by using animal model systems (The Africa Biofortified Sorghum 2012).

Increase Biofuel Conversion

Due to the multiple uses of sorghum, there are now several research programs being developed that emphasize the development of grain, particularly sweet and cellulosic sorghums, for biofuel production (Rooney et al. 2007). Sorghum starch and sugar are now being used for biofuel production. Modifications in starch deposition, digestibility, and sugar content would strongly influence ethanol production from sorghum grain (Rooney et al. 2007). Thus, the improvement of starch and sugar contents of sorghum grain using genetic engineering is predicted to gain more effort from researchers globally. In addition, a large and sustainable supply of biomass must be made for profitable biofuel production from lignocellulose. This will require the development of specialty crops for bioenergy production (Rooney et al. 2007). However, high biomass but low saccharification potential would waste energy and labor for harvesting, storing, transporting, and biofuel production. Hence, increasing biomass as well as saccharification yield will maximize biofuel yield. As a consequence, this could be another area in which sorghum transformation could play a role to accelerate energy production. Wang et al. (2011) identified two markers on sorghum chromosomes which are associated with saccharification yield. They found that these markers are physically close to genes which encode plant cell wall synthesis enzymes. They further proposed to evaluate the impact of these candidate genes on saccharification in sorghum through genetic transformation.

For the second-generation biofuel (cellulose ethanol), lignin is known to impede conversion of lignocellulose into ethanol. Cellulosic biomass is always more difficult than starch to be broken down into sugars due to the presence of lignin and the complex structure of cell walls. Modifying the chemical structures of lignin components and/or reducing plant lignin could decrease pretreatment costs in bioethanol production from cellulosic biomass (Ragauskas et al. 2006). Using genetic engineering to reduce lignin content has been attempted for some plant species such as hybrid poplar (Hu et al. 1999) and switchgrass (Fu et al. 2011; Xu et al. 2011). Recently, Dien et al. (2009) indicated that some *brown midrib (bmr)* mutations in forage sorghum not only reduced lignin content significantly but also improved glucose yields of sorghum biomass. Therefore, changing lignin components and content by genetic engineering would be important strategies to increase the potential of sorghum as a biofuel feedstock.

Exploitation of Sorghum Genomes

The sorghum genome has been sequenced by the whole-genome shotgun (WGS) method and approximately 98 % of the total predicted genes (34,496) have been placed in their chromosomal context (Paterson et al. 2009). These genomics resources offer great potential to improve sorghum genetically. Using genetic transformation to introduce, express, and modulate genes in transgenic plants represents a very powerful tool to examine directly gene functions, and also provides a means to broaden the sorghum germplasm for genetic improvement. Verma et al. (2011) induced and generated stable *Ds*-tagged mutants in sorghum via *Agrobacterium*-mediated transformation. The *Ds*-tagged mutants are used commonly for mutagenesis and functional genomics. Thus, this result could be seen as a good example for the utilization of sorghum transformation to study genome functions. Most recently, precise genome editing technologies have emerged and advanced rapidly. These technologies, particularly CRISPR/Cas9 [Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR Associated (Cas) 9] as a simple and powerful approach (Gaj et al., 2013; Li et al., 2013; Shan et al., 2013), deems to enhance sorghum genome exploitation, benefiting sorghum genetic studies and transgene-free variety development.

Conclusion

Sorghum is one of the most important crops in the world due to its food value and potential for bioenergy production. Genetic engineering is capable of supplementing traditional methods of improving sorghum as a food and feedstock. Among the DNA-delivery methods that have been utilized for sorghum transformation, the bombardment and *Agrobacterium*-mediated methods are the most efficient. Some agronomical traits such as nutrient improvement, pest resistance, disease tolerance, and stress tolerance have been achieved through sorghum genetic engineering. Several factors are known to play an important role in sorghum genetic engineering. Promoters have great impact on the success of sorghum genetic engineering because they directly influence the expressions of transgenes in sorghum. *Ubi1,* a maize ubiquitin 1 promoter, was indicated as the strongest promoter for sorghum transformation and was used in recent studies with both marker genes and genes of interest. Furthermore, the use of *mpiC1* and *α-kafirin* promoters through transgenic approaches has excellent potential for sorghum genetic improvement. Herbicide and antibiotic selection systems have been used widely in sorghum transformation. However, the high pressure of these negative selective agents on cell differentiation and development reduces regeneration and transformation efficiency. Moreover, there is a concern about possible migration of *bar* and antibiotic genes to wild relatives of sorghum, or to infectious bacteria. Using mannose selection as a positive selection system has overcome the side effect of the negative selective agents and

has indeed increased sorghum transformation efficiency. Sorghum has been known to be the most recalcitrant crop for genetic engineering. Nevertheless, to date, sorghum engineering frequency has increased significantly due to improvements in tissue culture and transformation conditions. In addition, genome sequencing, together with discovery of candidate genes and promoters, will continue to be very useful for sorghum genetic engineering. These new genetic resources provide opportunities to develop sorghum varieties with important traits required for food consumption and bioenergy production. New emerging transgene technologies especially precise genome editing technology including CRISPR/Cas9 should revolutionize sorghum genetic improvements and biology studies.

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Chapter 10
Biotechnology for Insect Pest Management in Vegetable Crops

Rohini Sreevathsa, Amolkumar U. Solanke and P. Ananda Kumar

Introduction

Vegetables are essential for a well-balanced diet, supplying many essential nutrients not found in staple starch crops such as rice, wheat, and corn. Additionally, there is evidence that diets rich in vegetables can lower the risk of heart disease, strokes, and several forms of cancer, as well as improve gastrointestinal health and vision. Long-term studies have shown that plant-based diets provide increased longevity, and that vegetables fight the "hidden hunger" of malnutrition. Vegetable cultivation is a significant part of the agricultural economy, especially in the developing world. In countries like India where the population is predominantly vegetarian, vegetables form a vital constituent of the diet. China is the world's largest producer of vegetables followed by India. These two countries have 61 % of the world's vegetable cultivated area and contribute to 71 % of the world's vegetable production (Table 10.1).

Besides providing benefits to consumers, farmers involved in vegetable production usually earn much higher incomes compared with cereal producers, with per capita farm income up to fivefold greater. Worldwide, the area of arable land devoted to vegetables is expanding faster than other crops, at 2.8 % per annum (Shelton 2012). Vegetables are high-value commodities, but they also have high cosmetic standards. Insect damage can impact on their appearance and, consequently, the desirability to consumers. The main method of insect control has been the frequent use of conventional pesticides. Although statistics for insecticide use worldwide are combined for vegetables and fruit (45 % of total insecticide value), if vegetables

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Table 10.1 Major vegetable-producing countries of the world. (Source: FAO)

	Country	Production (2007)	Production (2004)
1	China	146,902,838 m/t	423,369,004 m/t
2	India	29,117,400 m/t	80,528,500 m/t
3	Vietnam	6,600,000 m/t	6,450,000 m/t
4	Philippines	4,400,000 m/t	3,800,000 m/t
5	Nigeria	4,285,000 m/t	4,845,000 m/t

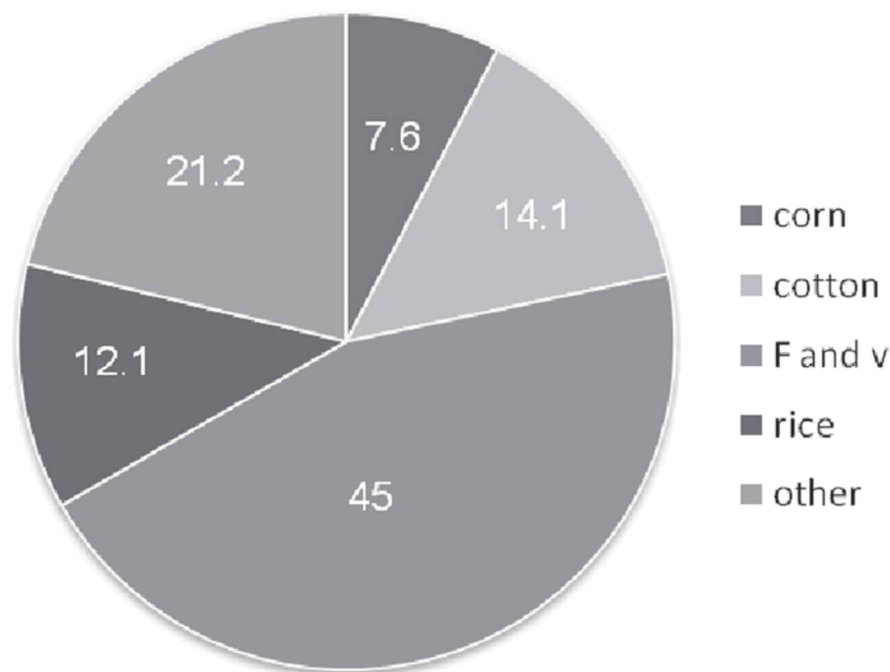

Fig. 10.1 Worldwide insecticide use in major crops (Shelton 2012)

were conservatively estimated to equal half of this total (22.5 %), the insecticide use for vegetables would exceed that for corn (7.6 %) plus cotton (14.1 %; Fig. 10.1). The heavy dependence on insecticides in vegetables can increase the residues which can affect human health and the environment (Shelton 2012).

In addition to losses due to biotic and abiotic stresses, improper storage and lack of timely access to processing facilities all contribute to lower yields and loss before the consumer market. The advances in plant tissue culture, transformation, and molecular biology tools have greatly benefited crop improvement programmes. The widespread use of transgenic cotton and maize with insect control traits demonstrates the utility of this technology. Transgenic vegetables that are resistant to insect pests have also been produced. This chapter is a compendium of the biotechnological approaches used in vegetable crops for the development of insect-resistant plants.

Table 10.2 Major insect pests of vegetable crops in India

Crop species	Insect pest
Cabbage/ cauliflower	Diamondback moth (*Plutella xylostella*) Webworm (*Hellula undalis*) Hairy caterpillar (*Spilosoma obliqua*)
Tomato	Fruit borer (*Helicoverpa armigera*) Epilachna beetle (*Epilachna* sps.) Tobacco caterpillar (*Spodoptera litura*)
Brinjal	Shoot and fruit borer (*Leucinodes orbonalis*) Epilachna beetle (*Epilachna* sps.) Lacewing bug (*Urentius echinus*) Jassids
Chilli	Thrips Aphids (*Aphis gossypii*)
Potato	Aphid (*Myzus persicae*) Tube moth (*Phthorimaea operculella*)
Pea	Pea aphid (*Macrosiphum pisi*) Pod borer (*Helicoverpa armigera*) Pea weevil (*Bruchus pisorum*)
Onion	Thrips (*Thrips tabaci*) Head borer (*Helicoverpa armigera*)
Okra	Spotted bollworm (*Earias* sps.) Jassid
Spinach	Aphids
Cucurbits	Red pumpkin beetle (*Aulacophora* sps.) Fruitfly (*Dacus cucurbitae*) Aphids

Insect Pests of Vegetable Crops

India's climate is predominantly tropical and subtropical. Although good for agriculture, this environment results in strong and diverse insect pressure on crops. The most damaging insect pests are in the orders; Lepidoptera, Coleoptera, and Diptera. Some of the major insect pests of vegetable crops are summarized in Table 10.2 confirming that insect pest management in vegetables is of paramount importance. The development of insect-resistant plants will both combat the insect pests and reduce pesticide usage. A durable solution would be to genetically transform elite genotypes and breeding lines of vegetable crops with traits that confer resistance to key insect pests. Research experience gives an encouraging view of the potential to improve vegetable crops.

Promoters for Transgene Expression for Pest Management

In order to generate effective transgenic plants for insect pest management, a high level of expression of the transgene in specific tissues, specific organs, and/or under specific conditions is essential. The induction of insecticidal genes and linking their

expression to the times of insect or pathogen attack is as important as the selection of effective insecticidal genes. High-level expression of a gene of interest can be attained by the use of key regulatory elements called "promoter(s)" which drive transcription. Promoters offer a fundamental control in gene expression, and there is a considerable interest in isolating and studying plant promoters. The choice of a promoter can result in constitutive, exogenously controlled, temporally controlled, or spatially controlled expression. The promoter is the key *cis*-acting regulatory region on the genome that controls the transcription of the adjacent coding gene body into messenger RNA (mRNA; Buchanan et al. 2000). Promoter sequences are located in the 5′ flanking or upstream region of the transcribed gene. mRNA is further translated into peptides or proteins. The transcription of mRNA is carried out by an enzyme, RNA polymerase II, with other transcription factors that recognize signals and elements present in its promoter region. These regulatory elements in the promoter region vary from gene to gene and are responsible for differential expression patterns of respective genes. The most common motif present in promoters is the TATA element where the TATA-binding protein (TBP) binds. This protein is part of a complex of polypeptides that recruit the RNA polymerase II to begin transcription. The other motifs are the transcription start site and the CCAAT consensus sequence. A core promoter or minimal promoter sequence includes only a TATA box and a transcription start site. The variability in gene expression is observed when other diverse, semi-conserved sequence elements are present within regulatory regions of the genes. These are generally present upstream or 5′ of the RNA polymerase binding site. Protein factors responsible for controlling the level and pattern of the gene expression bind to these elements. In short, promoters are a set of transcription control modules clustered around the initiation site of RNA polymerase II. Promoters can be grouped into several categories based on their ability to regulate temporal and spatial expression of genes.

Constitutive Promoters

Constitutive promoters induce the expression of gene of interest irrespective of the developmental stage, tissue, or the environmental conditions. These promoters are generally used to express insecticidal proteins at high concentration in all tissues of the plant.

The first few constitutive promoters used for the expression of transgenes in plants were isolated from plant pathogens, including the opine and cauliflower mosaic virus *35S* (CaMV35S) promoters. The CaMV35S has been used extensively as a constitutive promoter (Odell et al. 1985). It is more successful in dicotyledons than monocotyledons, likely due to different regulatory factors. Another class of extensively used constitutive promoters are from ubiquitin genes isolated from various plants like *Arabidopsis* (Callis et al. 1990), potato (Garbarino and Belknap 1994), tobacco (Genschik et al. 1994), and rice (Wang and Oard 2003). A polyubiquitin promoter from soybean has also confirmed to have a strong constitutive expression

(Hernandez-Garcia et al. 2009). Other constitutive promoters used for production in transgenic potato plants include the *mannopine synthase* gene promoter, tobacco cryptic constitutive promoter, Mac promoter, a hybrid of the mannopine synthase promoter and CaMV*35S* promoter enhancer region in potato, rice actin promoter, and maize alcohol dehydrogenase 1 promoter (Sharma and Sharma 2009). Various other constitutive promoters that are characterized and are available for use in plants include the banana actin promoter (Hermann et al. 2001), C1 promoter of cotton leaf curl Multan virus (Xie et al. 2003), cassava vein mosaic virus promoter (Verdaguer et al. 1996), and the nopaline synthase promoter (Stefanov et al. 1991). Since all these promoters are expressed constitutively across species, these promoters can also be exploited for constitutive insecticidal protein expression in vegetable crops.

Tissue-Specific Promoters

Tissue-specific promoters are preferred for use in vegetable biotechnology to express insecticidal genes when and where they are needed. These promoters control gene expression in specific tissues and at certain stages of development. They are very helpful to accumulate the insecticidal proteins in specific tissue types such as tubers, roots, vegetative organs or seeds, and reproductive organs like fruits and pods, limiting any possible negative effects on plant growth and development. In addition, the restriction can be beneficial of an insecticidal transgene product to tissue besieged by insect pests instead of harvestable material. Thus, targeted expression is important for the future development of vegetable crops because of public acceptance of less intrusive transgene expression. Often, homologous promoters can deliver tissue-specific expression of any gene because they have all the DNA motifs that interact with native transcriptional regulators. This is one of the main reasons for identification of tissue-specific promoters from plants and tissues. Expression of the mannose-specific snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) in transgenic rice plants using constitutive or phloem-specific promoters resulted in plants that were partially resistant to rice brown plant hopper (*Nilaparvata lugens*) and other hemipteran pests. Reductions of up to 50 % in survival were observed, with reduced feeding, development, and fertility of survivors (Rao et al. 1998; Foissac et al. 2000). The tomato pz7 and pz130 gene promoters for expression in ovary, tobacco RD2 gene promoter for root-specific expression, banana thioredoxin (TRX) promoter, and melon actin promoter for fruit-specific expression are other examples of tissue-specific promoters.

Fruit-Specific Promoters Fruit-specific promoters control the expression of genes in the mature ovary tissue of a fruit. Many fruit-specific promoters have been isolated from tomato, like the E8 promoter (Deikman and Fischer 1988), 2A11 promoter (Pear et al. 1989), polygalacturonase promoter (Fraser et al. 2002), and from apple such as the 1-aminocyclopropane-1-carboxylate (ACC) oxidase promoter (Atkinson et al. 1998). Schaart et al. (2002) confirmed the floral- and fruit-tissue-

specific activity of petunia FBP7 (floral binding protein 7) promoter by expressing the beta-glucuronidase reporter gene in transgenic strawberry plants.

Tuber/Storage-Organ-Specific Promoters Tuber/storage-organ-specific promoters that enhance or suppress the expression in root cells can help combat soil pests. The known tuber- or storage-organ-specific promoters include the potato patatin B33 gene (Liu et al. 1991), potato patatin PAT 21 gene (Jefferson et al. 1990), potato GBSS (granule-bound starch synthase gene; Visser et al. 1991), and sweet potato sporamin gene (Maeo et al. 2001).

Seed/Seed-Coat-Specific Promoters Several promoters have been characterized which restrict expression to the seeds. A few examples of seed-specific promoters are the bean beta-phaseolin gene promoter (Bustos et al. 1989) and wheat gbss1 gene promoter (Kluth et al. 2002). The seed-coat-specific promoter from pea, PsGNS2, is used to express insecticidal proteins only in the seed coat and not in cotyledons (Buchner et al. 2002). The bean (Phaseolus vulgaris) alpha-amylase inhibitor gene was expressed in seeds of transgenic garden pea (Pisum sativum) and other grain legumes, using a strong seed-specific promoter (Shade et al. 1994). The resulting seeds contained up to 3% of the alpha-amylase inhibitor protein and seeds were resistant to stored product pests, such as larvae of bruchid beetles, and field pests, including larvae of the pea weevil Bruchus pisorum (Morton et al. 2000).

Green-Tissue-Specific Promoters Green tissue, especially foliage, is the main component of some vegetable crops. Many green-tissue-specific promoters have been isolated. These include the rbsS 3A promoter from pea (Gilmartin and Chua 1990), CAB2 promoter from Arabidopsis (Carre and Kay 1995), and RAc promoter from alfalfa (Potenza et al. 2004). These promoters can be used for concentrating expression in green tissues. A green-tissue-specific light-inducible C4-PEPC promoter has been used to express cry1Ab gene in transgenic potato plants for the control of the tuber moth (Phthorimaea operculella; Hagh et al. 2009).

Root/Nodule-Specific Promoters Root- and nodule-specific promoters can help to protect vegetable crops from soil borne insects and nematodes. A root-specific plant promoter TobRB7 has been isolated and characterized in tobacco by Yamamoto et al. (1991). A rolD promoter from A. rhizogenes (Leach and Aoyagi 1991) and domain A of CaMV35S (Benfey and Chua 1989) are other examples of root-specific promoters. Other root-nodule-specific promoters are the VfEnod12 promoter from Vicia faba (Fruhling et al. 2000), Nvp30 promoter from bean (Carsolio et al. 1994) and leghemoglobin promoter from Sesbania rostrata (Szabados et al. 1990).

Inducible Promoters

Tissue-specific promoters are useful to control expression in particular tissues but their expression pattern always depends on endogenous *trans*-activating factors. In this case, inducible promoters are a very powerful tool in genetic engineering be-

cause the expression of genes under the control of these promoters can be regulated to function at certain stages of development of an organism or a particular tissue. These inducible (sometimes repressible) promoter systems are quite useful for regulation, because their performance is not dependent only on endogenous factors but external triggers can control expression of genes. These promoters are of two types, chemically regulated and physically regulated. The promoters modulated by abiotic stress factors such as light, oxygen levels, heat, cold, and wounding are physically regulated. Promoters which are inducible by wounds or stress are potentially useful for the engineering of insecticidal proteins that are turned on only at the time of attack, whereas a global or systemic induction system can protect the whole plant from insect attack. The best examples of wound inducible promoters are potato *wun1* and proteinase inhibitor II (*pin2*), which direct extensive wound and pathogen inducible expression but have very little or no expression without stimulus (Logemann et al. 1989; Xu et al. 1993). Promoters that respond to chemical compounds like antibiotics, copper, alcohol, tetracycline, steroids, and herbicides, which are not found naturally in the organism of interest, are chemically inducible promoters and allow the induction of gene activity upon application of the stimulus. These are independent of biotic or abiotic triggers. Such inducers should be inexpensive, easy to apply, and nontoxic for commercialization. The use of chemical-inducible promoters in combination with the chemical-responsive transcription factor can further restrict the target transgene expression to specific organs, tissues, or even cell types (Zuo and Chua 2000). These regulated gene expression systems may be useful to many valuable biotechnological applications such as conditional expression of Bt *cry* genes, targeted expression of herbicide resistance genes, synchronous flowering, and ripening in vegetable crops.

Synthetic or Artificial Promoters

The construction of synthetic promoters required a basic knowledge of modular arrangement of *cis*-acting elements that include the TATA box, necessary for recruiting the RNA polymerase II, a transcription start site and the CCAAT consensus sequence, which are required for an active eukaryotic promoter along with the enhancer regions. Promoters also have a diverse range of elements, which either upregulate or downregulate the activity of genes. Artificial promoters have been constructed in the past by engineering *cis*-elements which include enhancers, activators, or repressors, upstream to the core promoter. Many studies suggested that the strength of promoters depend upon the motif copy number and the spacing between these motifs (Gurr and Rushton 2005). In order to increase the strength of the CaMV*35S* promoter, multiple copies have been used of its tobacco mosaic virus (TMV) omega enhancer element. It was shown that it also enhances translation of the transgene in both eukaryotes and prokaryotes with no alteration in organ specificity (Holtorf et al. 1995). A hybrid promoter, *mac,* was constructed by incorporating part of the *mas* promoter and the enhancer region of the CaMV*35S* promoter

to express a thermostable cellulase E2 or E3 from *Thermonospora fusca* in alfalfa, potato, and tobacco. This promoter increased expression 10–15 times in hypocotyls as well as in roots (Ziegelhoffer et al. 1999). The combinations of regulatory sequences from octopine synthase and mannopine synthase gene promoters were used to construct a hybrid promoter *(Aocs)3AmasPmas* by combining a triple repeat of the *ocs* activator sequence, *mas* activator element, and *mas* promoter (Ni et al. 1995). Zhu et al. (2008) developed a tuber-specific and cold-inducible chimeric promoter to express in potato by using different combinations of the low-temperature-responsive elements (LTRE) from the *Arabidopsis* cor15a promoter and the tuber-specific and sucrose-responsive sequence (TSSR) from potato class I patatin promoter. Based on the successful use of synthetic promoters, they potentially play a large role in the future of transgenic expression and thus biotechnology.

Transgenics for Insect Pest Management in Vegetable Crops

Genetic engineering has helped revolutionize agriculture. Together with genomics, the contribution of transgenic technology has been immense in crop improvement programmes of important crops, including vegetables. There has been a considerable progress in the development of insect pest resistance in vegetables. A durable and eco-friendly alternative for pest management is therefore to genetically transform elite genotypes and breeding lines of vegetable crops using genes that encode insecticidal proteins. As with Bt cotton, the development of insect-resistant plants in vegetables demonstrate several advantages such as decreased pesticide usage, environmental friendly footprint, and decreased input cost to the farmer. There are several genes belonging to different classes of bacterial-, plant- and animal-derived proteins which have been shown to be insecticidal towards a range of economically important insect pests from different orders. These include delta endotoxins of *Bacillus thuringiensis,* protease inhibitors, alpha amylase inhibitors, lectins, and chitinases. Various strategies that have been used and others that have future potential in the development of insect-resistant vegetable crops are discussed in this chapter.

Insecticidal Proteins of *Bacillus Thuringiensis*

Bacillus thuringiensis is a gram-positive soil bacterium, which produces proteinaceous crystalline inclusion bodies during sporulation. There are many subspecies and serotypes of *Bt* with a range of well-characterized insecticidal proteins or *Bt* toxins. At present, it has been estimated that more than 60,000 isolates of *Bt* are being maintained in culture collections worldwide. Known *Bt* toxins kill insects belonging to the orders Lepidoptera, Coleoptera, Diptera (Hofte and Whiteley 1989) and also nematodes (Feitelson et al. 1992). Insecticidal δ-endotoxins of *Bt* have acquired significance in recent years because of their specificity to target insects,

toxicity at very low concentrations, and environment friendly nature (Kumar et al. 1998). The Bt genes are one of the thoroughly characterized genes for insect resistance. Primarily, *Bt* toxins are classified based on homology of toxin gene sequences and the spectrum of insecticidal activity (Hofte and Whiteley 1989). The crystalline protoxins are inactive, until they are solubilized by gut proteases (Tojo and Aizawa 1983; Milne and Kaplan 1993). The protoxins are activated in the alkaline midgut by trypsin-like proteases to toxins. In general, 500 amino acids from the C terminus of 130 kDa protoxins and 28 amino acids from the N terminus are cleaved, leaving a 55–65-kDa protease-resistant toxic active core comprising the N terminal half of the protoxin (Hofte and Whiteley 1989). The active toxin consists of three distinct structural domains. Domain I (seven α-helices) determines toxicity and mediates pore formation. Domain II (three β-sheets) determines receptor binding and specificity, whereas domain III (two β-sheets) is involved in receptor binding and protein processing (Schnepf et al. 1998). The active toxin binds to specific receptors located on the apical brush border membrane of the columnar cells in the midgut of the target insect, the α–helices penetrate the membrane and lead to the formation of pores (ion channels). The toxicity of *Bt* lies in the organization of α-helices derived from domain I. The toxin-induced pores form in the columnar cells and allow rapid fluxes of ions leading to swelling of the cells and osmotic lysis. The disruption of gut integrity leads to death of the insect through starvation or septicaemia (Sneh and Schuster 1981; Salama and Sharaby 1985). A number of putative receptors have been identified and include aminopeptidase N proteins and cadherin-like proteins. Transgenic plants expressing Bt toxins were first reported in 1987, and following this initial study, numerous crop species have been transformed with genes encoding a range of different Cry proteins targeted towards different pest species. Since bacterial *cry* genes (genes encoding Bt toxins) are rich in A/T content compared to plant genes, both the full-length and truncated versions of these *cry* genes have had to undergo considerable modification of codon usage and removal of polyadenylation sites before successful expression in plants.

Vegetable Crops Engineered with Bt cry Genes

There has been considerable success in the development of transgenics vegetables expressing *cry* genes; several crop species have been transformed with cry genes to target their respective pests. Table 10.3 lists the various biotech vegetable crops developed using *Bt* genes. Some of these efforts are described below.

Tomato Expression of Bt genes in tomato was one of the first examples of genetically modified plants against insects (Fischhoff et al. 1987). The major focus in tomato has been the use of *cry1Ab* genes for resistance against the fruit borer, which is the major pest (Kumar and Kumar 2004). Tomato has also been engineered with *cry*1Ac to protect it from *H. armigera* (Mandaokar 2000). Transgenic tomato with Bt genes were also developed by Monsanto in 1989 (Delannay et al. 1989) with significant protection being observed against tobacco hornworm (*Manduca sexta*), tomato

Table 10.3 Transgenic vegetable crops developed for insect pest resistance

Genes	Crop	Effective against	Reference
Bacillus thuringiensis cry genes			
cry1Ab	Tomato	Lepidopteran pests	Delannay et al. 1989
cry1Ac		*H. armigera*	Mandaokar et al. 2000
		Tobacco hornworm *Manduca sexta*	Fischhoff et al. 1987
cry3	Potato	Colorado potato beetle	Jansens et al. 1995; Perlak et al. 1993
cry3A		Colorado potato beetle	Arpaia et al. 1997
cry3B		Potato tuber moth *Phthorimaea operculella*	Arpaia et al. 2000; Stewart et al. 1999
cry1Ab		Potato tuber moth	Mohammed et al. 2000; Canedo et al. 1999
cry1Ac9		Potato tuber moth	Davidson et al. 2002
cry5			Douches et al. 2002
			Li et al. 1999
cry1Ac		Potato tuber moth	Ebora et al. 1994
cry1Ab + vip3Aa	Sweet corn	*Helicoverpa zea*	Burkness et al. 2010
cry1C	Broccoli	Diamond back moth, Cabbage looper, Cabbage butterfly	Cao et al. 1999
cry1Ab	Cabbage	Diamond back moth	Xiang et al. 2002; Bhattacharya et al. 2002
cry1Ac	Broccoli Cabbage	Diamond back moth	Metz et al. 1995a; Metz et al. 1995b
cry3Aa *cry3B*	Brinjal (Eggplant)	Colorado potato beetle	Arpaia et al. 1997; Hamilton et al. 1997; Iannacone et al. 1997; Jelenkovic et al. 1998; Chen et al. 1995; Arencibia et al. 1997
cry1Ab		Fruit and shoot borer	Kumar et al. 1998
Protease inhibitor genes			
Cowpea trypsin inhibitor	Cabbage	Small cabbage white *P. rapae*	Fang et al. 1997
	Tomato	Spodoptera litura	Mandal et al. 2002
	Potato	Tomato moth, *Lacanobia oleracea*	Gatehouse et al. 1997
Sweet potato trypsin inhibitor	Taiwan cauliflower	*Plutella xylostella*	Ding et al. 1998
Oryzacystatin	Potato	Colorado potato beetle	Lecardonnel et al. 1999; Cloutier et al. 2000
Potato trypsin inhibitor-II	Tomato	*Heliothis obsoleta Liriomyza trifolii*	Abdeen et al. 2005

Table 10.3 (continued)

Genes	Crop	Effective against	Reference
Alpha-amylase inhibitors			
α-amylase inhibitor gene from Phaseolus vulgaris	Pea	Pea weevil (*Bruchus pisorum*)	Shade et al. 1994
α-amylase inhibitors (alpha AI-1 and AI-2)			Morton et al. 2000; Schroeder et al. 1995
α-amylase inhibitor	Adzuki bean	Pea bruchid	Ishimoto et al. 1996
Plant lectins			
Snowdrop lectin	Potato	Potato aphid	Down et al. 1996
			Gatehouse et al. 1996
		Tomato moth	Gatehouse et al. 1997
	Tomato	Tomato moth	Fitches et al. 1997
Other novel strategies			
Isopentenyl transferase	Tomato	Tobacco hornworm	Smigocki 1997
Vegetative insecticidal protein	Sweet corn	*Helicoverpa zea*	Burkness et al. 2010
Cholesterol oxidase			Corbin et al. 2001
Anionic peroxidase	Tomato	Potato peach aphid	Dowd and Lagrimini 1997
Tryptophan decarboxylase			Schuler et al. 1998
Novel insecticidal proteins from *Photorhabdus luminescens Serratia Xenorhabdus*			Liu et al. 2003; Gatehouse 2008; Pardo-Lopez et al. 2013
Engineering volatile communication compounds			Gatehouse 2008
RNAi strategy			Mao et al. 2007; Baum et al. 2007; Kumar et al. 2009; Zha et al. 2011

fruit worm (*Helicoverpa zea*), and tomato pinworm (*Keiferia lycopersicella*). Field trials were also carried out by Monsanto, Novartis, and Mycogen with tomatoes carrying the *cry1Ab* gene (Krattiger 1997). However, no transgenic event has been commercialized to date.

Potato Potato is another important vegetable infested by insects like the Colorado potato beetle (CPB) and the potato tuber moth. Transgenics have been developed in potato to combat these insect pests with the help of various *cry* genes. Earlier, transgenic plants were developed in potato with *cry1Ac* gene against potato tuber moth (Ebora et al. 1994). Potato cultivars expressing the *cry3A* toxin against CPB were the first transgenic vegetables developed for human consumption (Jansens et al. 1995). In 1997, a potato product called "NewLeaf" which combined the Bt-*cry3A* and virus resistance was commercialized. However, the product was later withdrawn in 2001 because of various issues related to public acceptance. Davidson et al. (2002) developed transgenic potato with the *cry1Ac9* gene, which induced more than 40 % mortality in the potato tuber moth. Several other transgenic potatoes were developed by other laboratories targeting the two pests of potato with other *cry* genes, such as *cry3a, cry5,* and *cry3b* (Arparia et al. 1997; Innacone et al. 1995; Stewart et al. 1999).

Sweet Corn Presently, the only commercial transgenic vegetable crop harbouring the Bt gene is sweet corn. The viable Bt event carried the *cry1Ab* gene against the European corn borer, producing 100 % clean ears. Though the product was effective and was given wide publicity in 1998, it saw a decline by 1999 due to adverse reaction from the antagonists of GM crops. In order to increase the toxicity of the transgenic crops against European corn borer, researchers pyramided the *cry1Ab* event with another insecticidal protein, *vip3A* (Burkness et al. 2010). Trials in the areas heavily infested with *H. zea* demonstrated superior performance of the stacked event. In 2010–2011, Monsanto developed another pyramided event in sweet corn harbouring *cry1A.105* and *cry2Ab2*. The transgenic plants showed >99 % clean ears in high-pressure areas with *H. zea.* Although growers are rapidly adopting this product, an emerging noctuid lepidopteran pest, western bean cutworm may pose problems because of its resistance to *cry1A* or *cry2A* (Shelton 2010). Therefore, pyramiding the existing varieties with genes like *cry1F* could help the corn industry mitigate resistant issues.

Crucifers Another group of prominent vegetables that are a target for genetic engineering are the crucifers, such as cauliflower, cabbage, and broccoli. The major insect that attacks these vegetables is the diamond back moth (DBM; *Plutella xylostella*). Several *Bt* genes have been introduced for conferring resistance to DBM and other Lepidoptera (Earle et al. 2004; Paul et al. 2005). Synthetic *cry1C* was transferred to broccoli against *Plutella* (Cao et al. 1999) and later pyramided with *cry1A* gene (Cao et al. 2002). Unfortunately, DBM had developed resistance to *cry1A* gene (Mittal et al. 2007). This led the scientific community to choose other genes that were more effective against DBM. However, there were several other transgenic events developed in broccoli and cabbage using Bt genes such as *cry1Ab* (Bhattacharya et al. 2002) and *cry1Ab* or *cry1Ac* (Xiang et al. 2000). Transgenic cauliflower plants were also developed using the gene *cry9Aa* with high levels of activity against DBM. Transgenic cabbage was developed with *cry1C* genes against the cabbage butterfly, *Pieris rapae*. It was thought that the development of transgenics in the crucifers against DBM would be of significant utility to society. This led to the formation of a public private partnership program involving Nunhems, a major

vegetable breeding company and public partners like Asian Vegetable Research and Development Centre (AVRDC, Taiwan), the Centre for Environmental Stress and Adaptation Research at the University of Melbourne, Cornell University, USA, and the National Resources Institute, UK. The main aim was to tackle successful development of transgenics in crucifers with the effective genes to which the insect has not developed resistance. This has led various groups working towards viable transgenic events with commercialization potential.

Eggplant Eggplant is a popular vegetable crop grown in the tropics and subtropics and commonly known as “brinjal” in India and Bangladesh. One of the major pests attacking eggplant in Europe and North America is the CPB. The Bt gene *cry3B* was used to combat this pest (Chen et al. 1995). At first, the protein expressed in the plant was not sufficient enough to kill the insect. Subsequently, transgenic events were developed using a mutagenized version of *cry3B* (Iannacone et al. 1995; Arpaia et al. 1997). These new events demonstrated better activity against the insect. Further, events with a fully synthesised version of the gene showed improved resistance as demonstrated by the mortality of both the neonates and adult CPB. This represents an environmentally safe way of pest control.

The major insect pest attacking eggplant resulting in large crop losses in India and parts of South Asia is the fruit and shoot borer (FSB; *Leucinodes orbonalis*). At least 60 sprays are given by the farmers during the entire crop season to protect the plant against this pest. Therefore, development of resistance to this pest as well as using the transgenic technology would be highly beneficial. To achieve this, transgenic plants were developed first at the Indian Agricultural Research Institute (IARI), New Delhi (Kumar et al. 1998) using a codon-modified *cry1Ab* gene. Subsequently, a private company, Mahyco, developed FSB-resistant brinjal using the *cry1Ac* gene, which showed good control of the pest in glasshouse and field trials. Not only did field trials demonstrate the superior performance of the transgenic plants but analysis also revealed that the product would give yield increase, economic benefit, and health benefit in terms of reduced pesticide usage. Several tests showed the safety of the product. The latter was recommended for environmental release by the Genetic Engineering Approval Committee (GEAC) of the Government of India, but in 2010, a moratorium was imposed on its commercial release. India awaits the lift of the moratorium, while Bangladesh approved the release of transgenic brinjal in October 2013.

Increasing the Efficiency of Bt Toxins by Novel Approaches

Various strategies have been developed to increase the efficiency of the toxins as well as for resistance management (Gatehouse 2008; Pardo-Lopez et al. 2013). These include:

1. Using multiple cry toxins to generate transgenic crops, i.e., pyramiding toxins.
2. Combining domains from different cry toxins (domain swap) and developing chimeric cry proteins with novel specificities.

3. Mutagenesis of three-domain cry toxins to increase toxicity towards target pests.
4. Development of fusion proteins, that is, a gene construct containing a single translationally fused coding sequence encoding two cry proteins.

Transgenic Plants Expressing Inhibitors of Insect Digestive Enzymes

The concept of employing genes encoding Bt toxins to produce insect-resistant transgenic plants arises from the successful use of Bt-based biopesticides. A number of other strategies for protecting crops from insect pests actually exploit endogenous resistance mechanisms. Genes encoding such defensive proteins were obvious candidates for enhancing crop resistance to insect pests. Interfering with digestion, and thus affecting the nutritional status of the insect, is a strategy widely employed by plants for defence, and has been investigated extensively as a means of producing insect-resistant crops.

Numerous studies since the 1970s have confirmed the insecticidal properties of a broad range of protease inhibitors from both plant and animal sources. Plant protease/proteinase inhibitors are polypeptides or proteins that occur widely and naturally in plants and are a part of the plant defence machinery against herbivory. Proteinases in insects include serine, cystine, aspartic acid, and metalloproteases that catalyse the release of amino acids from dietary protein. Serine and cysteine proteinase inhibitors have been reported to inhibit the growth and development of a wide range of insects, mainly lepidopteran and coleopteran species (Gatehouse et al. 1993). The antimetabolic mode of action of these inhibitors is not fully understood. Direct inhibition of digestive enzymes is not considered as the main effect, but could be complemented with the hypersecretion of digestive enzymes caused by the presence of inhibitors leading to the depletion of amino acids. It has also been observed that the proteinase inhibitors not only affect gut digestive enzymes but also water balance, moulting, and enzyme regulation in insects (Boulter 1993). Proof of concept for exploiting such molecules for crop protection was first demonstrated with expression of a serine protease inhibitor from cowpea (CpTi), which was shown to significantly reduce insect growth and survival. Experiments with transgenic plants and artificial diets have shown that CpTi affects a wide range of lepidopteran and coleopteran species (Gatehouse and Hilder 1994). CpTi has been used in the development of transgenic plants in various crop species against these types of insects (Table 10.3). Transgenic cabbage plants with the *CpTi* gene have shown resistance to *P. rapae* (small white butterfly; Fang et al. 1997), while a trypsin inhibitor gene from sweet potato expressed in Taiwan cauliflower conferring resistance to insects (Ding et al. 1998). A CpTi from *B. Juncea* was introduced into tomato and demonstrated resistance to *Spodoptera litura* (Mandal et al. 2002).

Since many economically important coleopteran pests predominantly utilize cysteine proteases for protein digestion, inhibitors for this class of enzyme (cystatins)

have been investigated as a means for controlling pests from this order. Oryzacystatin, a cysteine protease inhibitor isolated from rice seeds, is effective towards both coleopteran insects and nematodes when expressed in transgenic plants. Transgenic potatoes expressing oryzacystatin encoding the oci gene resulted in sufficient mortality of the CPB (Lecardonnel et al. 1999; Cloutier et al. 2000). Similarly, the cysteine/aspartic protease inhibitor equistatin, from sea anemone, is also toxic to several economically important coleopteran pests, including the CPB.

More recent studies have included the stacking of different families of inhibitors to increase the spectrum of activity. A major limitation, however, to this strategy for control of insect pests arises from the ability of some lepidopteran and coleopteran species to respond and adapt to ingestion of protease inhibitors by either overexpressing native gut proteases or producing novel proteases that are insensitive to inhibition. Thus, detailed knowledge about the enzyme–inhibitor interactions, both at the molecular and biochemical levels, together with detailed knowledge on the response of insects to exposure to such proteins is essential to exploit this strategy effectively.

Transgenic Vegetable Crops with α-Amylase Inhibitors

Another type of plant insecticidal enzyme inhibitors that are produced in response to herbivory are the α-amylase inhibitors. Six different types of α-amylase inhibitors, i.e. lectin-like, knotton-like, cereal-type, Kunitz-like, gamma-purothionin-like, and thaumatin-like, can be used in pest control (Franco et al. 2002). These show tremendous diversity, modes of action and different specificities against diverse α-amylases. However, the introduced gene should not affect the plant's own alpha amylases and the nutritional value of the crop. These inhibitors are attractive candidates for the control of seed weevils as these insects are highly dependent on starch as their energy source.

The α-amylase inhibitors from some legume seeds, which are similar to legume lectins in sequence, have been shown to be effective towards coleopteran seed weevils (Table 10.3). The bean (*Phaseolus vulgaris*) α-amylase inhibitor gene was transferred to garden pea (*Pisum sativum*) using a strong seed-specific promoter (Shade et al. 1994; De Sousa-Majer et al. 2007). The resulting seeds contained up to 3 % of the foreign protein and were resistant to storage pests, such as larvae of bruchid beetles, and field pests, such as larvae of the pea weevil *Bruchus pisorum* (Morton et al. 2000). This strategy is basically directed toward coleopteran seed herbivores, with a neutral or acidic gut pH, so the inhibitor is not inactivated. Despite these results, commercialization of transgenic crops expressing this α-amylase inhibitor gene has not taken place. Safety concerns have arisen as a result of immunological reactions in mice fed peas expressing the α-amylase inhibitor protein (Prescott et al. 2005). However, in a recent study (Lee et al. 2013), it was demonstrated that the mice fed with transgenic plants material harbouring the α-amylase

inhibitor gene as well as non-transgenic beans and peas demonstrated the same kind of allergic response. This study demonstrated the vitality in the analysis of allergenic responses in mice upon consumption of plant products.

Transgenic Vegetable Crops Expressing Lectins

Lectins are a heterogeneous group of carbohydrate-binding proteins which have a protective function against a range of organisms. They are particularly effective against insects, viz., homopteran, coleopteran, lepidopteran, and dipteral. Although there are some lectins that are toxic to mammals and cannot be used in crop improvement programmes, there are some non-toxic lectins that can be used specifically towards the homopterans. This finding is of considerable interest as the Bt genes are not found to be effective against homopterans. Research through bioassays and artificial diets has proven the efficacy of lectins against insects (Powell 2001; Powell et al. 1995; Sauvion et al. 1996; Bandyopadhyay et al. 2001; Banerjee et al. 2004). Research has been conducted with lectins including snowdrop agglutinin (GNA; *Galanthus nivalis*), *Allium sativum* leaf agglutinin (ASAL), and ConA (Concavalin A). The mode of action of lectins is still not clear. However, some of them have been shown to bind to midgut epithelial cells (Gatehouse and Hilder 1994). GNA has been shown to not only bind to the epithelial cells but also accumulate in the fat bodies, ovarioles, and haemolymph suggesting its passage into the circulatory system resulting in the systemic effect. One of the receptors for GNA in brown plant hopper gut is a subunit of ferritin, indicating that GNA may be interfering with metal homeostasis within the insect.

Among lectins, GNA has shown to be very effective against aphids and rice brown plant hopper (Hilder et al. 1995; Rao et al. 1998; Ramesh et al. 2004) and a 25-kDa homodimeric allium sativum leaf lectin (ASAL; Dutta et al. 2005a, b; Sadeghi et al. 2007; Saha et al. 2006). Among the vegetable crops, potato and tomato have been engineered with the snowdrop lectin (Table 10.3). The bioefficacy analysis of potato engineered with snowdrop lectin showed that the fecundity of the potato aphid was reduced considerably (Down et al. 1996). Similarly, potato lectin transgenic plants against the potato peach aphid also showed not only reduced fecundity but also reduced establishment (Gatehouse et al. 1996). Snowdrop lectin also enhanced the resistance of potato to the larvae of tomato moth, demonstrating that the effect of snowdrop lectin was anti-feedant rather than insecticidal (Gatehouse et al. 1997).

Various studies demonstrate that the levels of protection conferred by expression of lectins in transgenic plants are generally not high enough to be considered commercially viable. However, the absence of genes with proven high insecticidal activity against homopteran pests may well mean that transgenic crops with partial resistance may still find acceptance in agriculture. Moreover, pyramiding lectins with other insecticidal genes could also be beneficial.

Other Novel Approaches (New Proteins) for Insect Resistance

There are several other strategies that could be used to combat insect pests (Table 10.3). They are:

Isopentenyl Transferase

The key enzyme in cytokinin synthesis isopentyl transferase was isolated from *Agrobacterium tumefaciens* and transformed into tomato using a wound-inducible promoter (Smigocki 1993). The transformed plants showed resistance to tobacco hornworm (*Manduca sexta*) and also potato peach aphid (*Myzus persicae*).

Vegetative Insecticidal Proteins

Unlike the Bt toxins which are inclusion bodies in sporulating *Bacillus thuringiensis,* vegetative insecticidal proteins (VIPs) are secreted proteins from the same bacillus. The VIPs have shown efficacy against a wide range of lepidopteran and coleopteran pests (Estruch et al. 1996). The VIP proteins also act on the gut epithelial cells where they bind to the cells and bring about progressive degradation inducing gut paralysis and death (Yu et al. 1997). Several transgenic events have been developed using *vip3a* in cotton and corn (Christou et al. 2006). Transgenic sweet corn has been developed, stacking *vip3a* and *cry1Ab* for resistance to *Helicoverpa zea.* VIP, along with Bt toxins, can form an effective way to control insect pests.

Cholesterol Oxidase

Another option for the development of insect-resistant plants can be the enzyme cholesterol oxidase from bacteria, which is thought to promote membrane destabilization. Expression constructs containing part or all of the coding sequence of the protein, or the coding sequence fused to a chloroplast-targeting peptide, resulted in production of active enzyme in transgenic tobacco (Corbin et al. 2001). However, phenotypic abnormalities were observed in transgenic plants unless the enzyme was localized in chloroplasts, possibly as a result of interference with steroidal signalling pathways. Leaf tissue from all transgenic plants was toxic to boll weevil larvae. The cholesterol oxidase gene appears to be an obvious candidate for introduction into the chloroplast genome rather than the plant nuclear genome, which would avoid potential problems caused by enzyme activity in the cytoplasm.

Anionic Peroxidase

Anionic peroxidases can be used as an alternate candidate for insect protection. A tobacco anionic peroxidase was cloned and expressed in tomato (Dowd and Lagrimini 1997). The transgenic plants showed significant levels of resistance to several lepidopterans, coleopterans, and also the potato peach aphid. The mode of action of peroxidises is highly complex, and this insecticidal activity can be linked to inactivation of digestive enzymes or production of highly reactive toxic enzyme products.

Tryptophan Decarboxylase

Another contender that acts as an anti-oviposition agent, anti-feedant, or as an inhibitor of larval/pupal development is tryptophan decarboxylase (TDC). This enzyme converts tryptophan to the indole-alkaloid tryptamine which brings about control of insects. This was observed when TDC from periwinkle was expressed in tobacco, which brought about control of the whitefly, *Bemicia tabaci* by reduction in reproduction by 90 %.

Novel Insecticidal Proteins from Other Bacteria

Studies have revealed the presence of insecticidal proteins in several other bacteria which could be incorporated into plants (Gatehouse 2008). Nematodes of the family *Heterorhabditidae* species harbour bioluminescent gram-negative enterobacteria named *Photorhabdus luminescens.* When nematodes enter an insect host, bacterial cells from the nematode gut are released into the insect circulatory system. Toxins secreted by the bacteria cause cell death in the insect host, leading to lethal septicaemia. Research demonstrated that the bacteria contained a large number of potentially insecticidal components encoded by toxin complex loci *tca, tcb, tcc,* and *tcd*. One of the orally toxic components, toxin A, was selected for further study. The encoding gene *tcdA* was cloned and assembled into expression constructs, containing 5′ and 3′ untranslated region sequences from a tobacco osmotin gene to improve expression levels of mRNA and protein in transgenic plants. It was observed that expression of toxin A at levels 0.07 % of total soluble protein in leaves of transgenic *Arabidopsis* plants gave almost complete protection against larvae of the lepidopteran tobacco hornworm (*Manduca sexta*; Liu et al. 2003). Leaf extracts from these plants were also toxic to corn rootworm, showing cross-species protection.

In addition, novel insecticidal proteins produced by other bacteria such as *Serratia* and *Xenorhabdus* spp. could be used potentially in insect control programmes in combination with Cry toxins from Bt (Bravo et al. 2011). These genes offer an attractive alternative to *Bt* genes for deployment in transgenic plants. Further, pyramiding of both the *Pht* and *Bt* genes will also be a viable alternative, and commercial development of this technique is likely.

Secondary Metabolites as Candidates for Insect Resistance

Secondary metabolites such as alkaloids, steroids, foliar phenolic esters, terpenoids, saponins, flavonoids, and non-protein amino acids act as potent protective chemicals.

Engineering Secondary Metabolism of Volatile Communication Compounds

Plants produce several volatile compounds which act as plant protectants. Engineering these volatiles is a novel strategy for insect pest resistance. There have been studies in tobacco and *Arabidopsis* where volatile composition has been altered by RNAi. The research involved suppression of cytP450 oxidase gene expressed in trichomes and constitutive overexpression of a plastid dual linalool/nerolidol synthase (Wang et al. 2001; Aharoni et al. 2003), respectively. Although the transgenic plants demonstrated resistance to aphid colonization, they were not completely resistant. Another strategy was to overexpress genes that produce volatiles which can be used as attractants for natural enemies of pests. *Arabidopsis* plants transformed with the maize terpene synthase gene TPS10 emitted several sesquiterpene volatiles normally produced in maize. These compounds when produced by the transformed *Arabidopsis* attracted parasitoid wasps that attack maize pests (Schnee et al. 2006). Yet another strategy that can be exploited is producing volatiles used by the insects to communicate with each other. The sesquiterpene (E)-β-farnesene is an alarm pheromone in aphids that attracts aphid predators and parasitoids. When *Arabidopsis* was transformed with the (E)-β-farnesene synthase gene from mint, the transgenic plants showed significant levels of resistance to aphid because they attracted the aphid parasitoid, *Diaeretiella rapae.*

RNAi Strategy for Insect Control

Conferring insect pest resistance through host-derived RNAi can be another strategy (Pattanayak et al. 2012). Disrupting gene function by the use of RNAi has been a well-established technique in insect genetics based on delivery by injection into insect cells or tissues. The observation that RNAi could also be effective in reducing gene expression, measured by mRNA level, when fed to insects (Turner et al. 2006) has led to extrapolation of the strategy to insect control. Transgenic maize producing double-stranded RNA (dsRNA) directed against V-type adenosine triphosphatase (ATPase) of corn rootworm showed suppression of mRNA in the insect and reduction in feeding damage compared to controls (Baum et al. 2007). Similarly, RNAi was used to reduce the level of a detoxification enzyme (Cyt P450 gene CYP6AE14) for gossypol to protect tobacco and *Arabidopsis* against cotton bollworm. The RNAi resulted in *H. armigera* to become more sensitive to gossypol

in its diet (Mao et al. 2007). Studies on target genes for the control of *H. armigera* identified acetylcholine esterase gene. In vitro RNAi against this gene caused mortality, growth inhibition, reduction in pupal weight, malformation, and fecundity, showing the potential of this approach to control bollworm (Kumar et al. 2009). Similar in vitro RNAi studies have demonstrated the utility of five putative genes for the control of sap-sucking insects (Borgio 2010). Recent work on host-delivered RNAi has also been demonstrated in transgenic rice against brown plant hopper, which is a sap-sucking insect (Zha et al. 2011). However, similar results have yet to be translated for producing insect-resistant plants in vegetable crops.

Resistance Management

The fundamental purpose of the deployment of resistance genes in transgenic plants is to manage the insect pest population and to prevent the development of resistance in insects. Resistance management strategies try to prevent or diminish the selection of rare individuals carrying resistance genes and hence to keep the frequency of resistance genes sufficiently low for insect control. Strategy development generally relies on theoretical assumptions and computer models simulating insect population growth under various conditions (Tabashnik 1994). The proposed insect management strategies include the use of multiple toxins, crop rotation, and high or ultrahigh doses paired with spatial or temporal refugia. The most promising of all the strategies are refugia. The strategy would reduce the possibility of resistant insects from mating with other resistant insects, thereby preventing the creation of resistant population. This is achieved by ensuring that there are always plenty of susceptible insects nearby which mate with the few resistant ones. The basic principle of high-dose strategy is to deploy plants with high levels of expression of the toxin with the expectation that it would take a long time for insects to overcome the toxin. It assumes that most or all the resistance is recessive and that most resistance carriers would be heterozygous. A viable complementary strategy that is best adopted with the above two strategies is the deployment of multiple resistance or stacking of resistance genes. The strategy requires more than one resistance gene with different modes of action/receptors. It could be achieved by using more than one *cry* genes with different *cry* genes or *cry* and *vip* genes, or a combinatorial construct with many genes. The targeted expression can also be taken as a strategy to improve resistance management. In this case, a toxin gene is expressed only specifically in a certain tissue or part of the plant, or only at a critical period of crop growth. This would allow plenty of susceptible insects to breed normally, thus increasing their predator and parasitic populations, while at the same time being prevented from causing damage at critical times during plant development.

One of the most important tools for resistance management is to apply integrated pest management (IPM) strategies in transgenic crop cultivation. The use of biological control methods (viz., predators, viruses, and fungi), botanical pesticides (neem and pyrethrum), crop rotation and sanitation, traditional methods coupled

with minimum application of chemical insecticides, would improve synergistically the performance of transgenic crops.

Conclusions and Future Perspectives

The first Bt vegetable was developed by Fischhoff et al. (1987), who engineered tomato plants resistant to tobacco hornworm (*Manduca sexta)* and the tomato fruit worm (*Heliothis virescens*). Since that time, many other Bt vegetable crops have been developed, but only potatoes and sweet corn have been commercialized, and only sweet corn remains in the market. Meanwhile, the area planted to Bt field crops (cotton and corn) continues to increase. Vegetable crops suffer from a variety of insect pests. Research has shown that biotechnological approaches can be implemented for the development of vegetable crops resistant to insect pests. This requires the identification of a large and varied number of *cry* genes, along with the already validated ones to be deployed into vegetables. Strategies involving multiple genes for effective resistance management are imperative. The transgenic technology coupled with effective IPM would create a sustainable approach that can greatly benefit farmers.

In India, there was hope that Bt eggplant (brinjal) would have a much smoother path to commercialization and that it would become the first Bt food crop. The need for the crop is undeniable because of its high pesticide load, the direct financial benefit to growers, and the increased safety to consumers and farm workers. With the recent approval for commercialization given by the Bangladesh Government, Bt brinjal would certainly provide tangible benefits of biotechnology to the farmers and consumers, as well as a durable means for crop improvement.

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Chapter 11
Enhancement of Sugar Yield by Introducing a Metabolic Sink in Sugarcane

Luguang Wu

Introduction

Sucrose is currently the major product from sugarcane worldwide. Increasing sucrose yield can be accomplished in one of two ways by either increasing sugarcane biomass while maintaining the same concentration of sucrose, or increasing the sucrose content of the cane, but the correlation between these two traits is low. There is an increased profitability of the latter approach because of reduced costs related to harvesting, transport and milling compared with increased biomass. Sugarcane is currently well below the theoretical physiological limits of sugar accumulation (Waclawovsky et al. 2010). However, in recent years, it has proven very difficult to achieve incremental improvements in this trait through conventional breeding and selection (Jackson 2005), even though considerable progress has been made in biomass production to improve sugar yield per unit land area by sugarcane breeding. Even through molecular approaches (Grof and Campbell 2001), endogenous gene manipulations in sugarcane metabolism have not achieved improvement in whole-plant sugar accumulation to date (Botha et al. 2001; Vickers et al. 2005; Groenewald and Botha 2008).

There has been a long-lasting dispute as to whether current sugarcane varieties have reached the peak of sugar accumulation (Inman-Bamber et al. 2011). This argument appears to be answered by the experimental results that new sinks have been generated by the heterologous sucrose-modifying enzymes in sugarcane transformants (Nell 2007; Wu and Birch 2007). Specifically targeting a highly efficient bacterial enzyme, sucrose isomerase, to the vacuole allowed for the conversion of sucrose into isomaltulose. This resulted in a doubling of sugar content, including isomaltulose accumulation, without reduction in sucrose (Fig. 11.1). This remarkable enhancement in total sugar concentration was termed SugarBooster, along with

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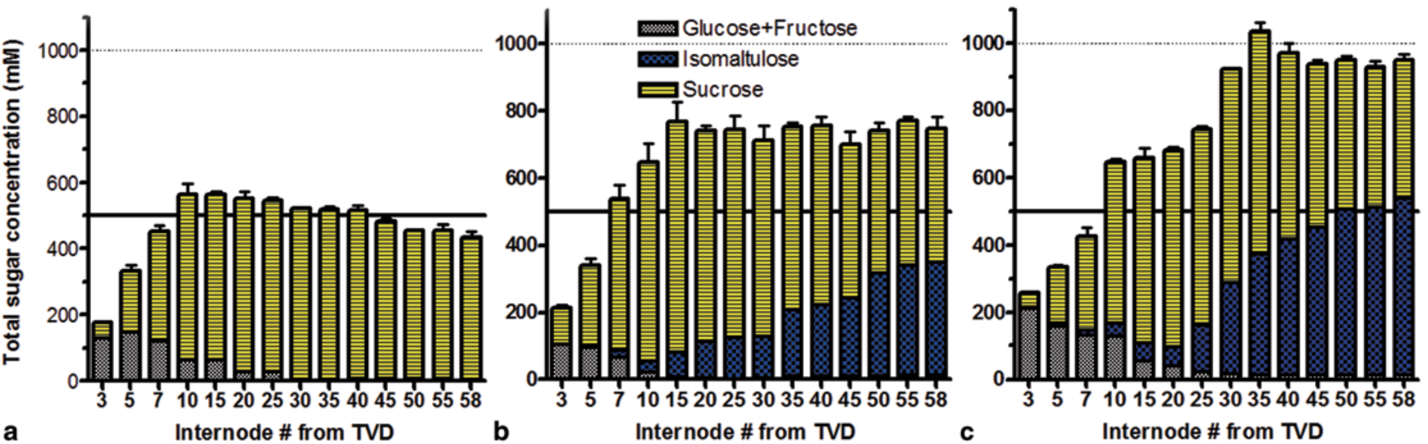

Fig. 11.1 Sugar accumulation profiles of sugarcane cultivar Q117 (**a**) and transgenic lines N3.2 (**b**) and N3.2H (**c**) showing substantial isomaltulose accumulation and enhanced total sugar concentration in juice. The plants were 16 months old with 59 internodes in the third vegetative generation. Results are means of three replicates, with standard errors shown for sucrose results. Analysis of variance (ANOVA) with Bonferroni post-tests showed significant differences ($P < 0.001$) in total sugar concentration between Q117 (**a**) and N3.2 (**b**) or N3.2H (**c**) in juice from internodes older than #10. These developmental profiles of sugar accumulation were stable over three tested vegetative generations. *TVD* top visual dewlap (Wu and Birch 2007)

no major reduction in carbon partitioning to cell wall components, as well as no significant influence on plant growth and development under glasshouse conditions. Another example of SugarBooster effects was demonstrated by Nell (2007) showing improved sugar content in sugarcane stem by up-regulating a fructosyltransferase gene from the plant, *Cynara scolymus*. This gene transformation in sugarcane led to 78 % of stem sucrose being converted to fructants-like 1-ketose and inulin, resulting in a 63 % increase in total soluble sugar content compared to the parent controls. The remarkable increases in sugar concentration by manipulating foreign genes like sucrose isomerase and fructosyl transferase surpass the former ceiling in stored sugar content, indicating that the addition of a new vacuolar-compartmentalized metabolic sink for sucrose deregulated prior constraining processes on sugar accumulation. Multiple biochemical processes were shown to be altered through analyses of SugarBooster transgenic plants to partition the carbon flux to sugar (Nell 2007; Wu and Birch 2007, 2010).

In order to increase sugar content in sugarcane stalks, four rate-limiting steps were identified in an early review (Grof and Campbell 2001) consisting of (1) leaf reactions, (2) rate of phloem loading, (3) sucrose transport to the site of storage and (4) the loss of sucrose to support vegetative growth. In the process of sugar accumulation, each component of the above-mentioned steps sends and receives signals from other components, along with plant growth and development. In SugarBooster plants, for example, it is hypothesized that the isomaltulose production takes part in the signalling processes, which results in metabolic changes (Fig. 11.2). However, sugarcane stalks (culms) are a challenging system for experiments to understand the physiological mechanisms. Historically, some important insights have been gained through experiments on sugarcane tissue slices and cell cultures (Moore 1995; Bindon and Botha 2001). The transgenic sugarcane line engineered to express a vacuole-targeted sucrose isomerase, which shows SugarBooster effects, was also found to accumulate sucrose to twice the concentration of the background genotype Q117 in heterotrophic cell cultures, without adverse effects on cell growth (Wu and Birch 2010). Key metabolites and activities of enzymes related to sucrose metabolism were analysed systemically in the suspension cell lines to understand the SugarBooster effects (Wu and Birch 2010).

This chapter outlines the progress and prospects of SugarBooster effects from both the SugarBooster plants and their suspension cell cultures. With supporting data from other plants, these are presented in the context of how new understandings in physiology and biochemistry will impact on the further targets for gene manipulation to enhance sucrose accumulation in sugarcane.

Photosynthesis

SugarBooster lines allow new insights into the mechanisms by which plants regulate sugar accumulation, a pivotal question in plant biology (Rolland et al. 2002; Koch 2004; Fernie et al. 2005). SugarBooster lines increased photosynthetic

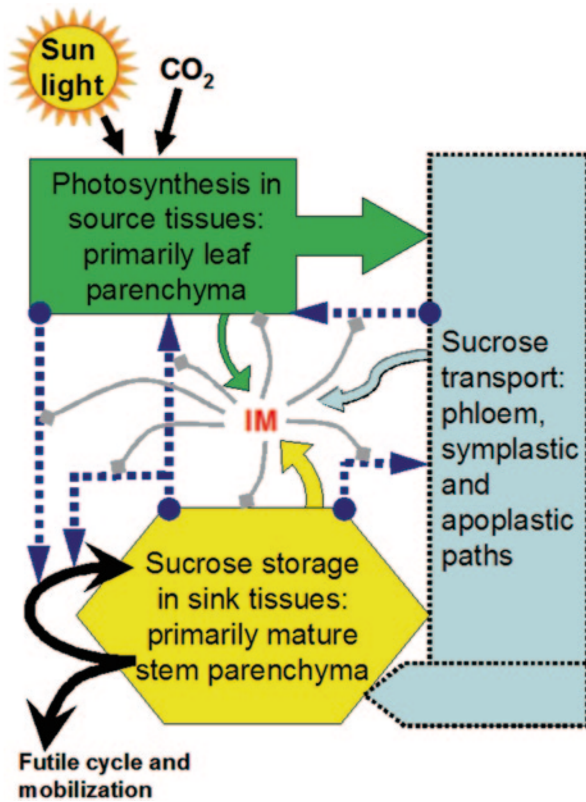

Fig. 11.2 Isomaltulose (IM) or other sucrose isomerase product interferes with signalling between the source, transport and sink tissues, resulting in enhanced sugar accumulation. IM is generated from different parts shown by *green*, *yellow* and *light blue arrows*. Signalling pathways are shown by *broken arrows*

activity (Wu and Birch 2007). Their high total sugar phenotype is also accompanied by delayed leaf senescence, and enhanced sucrose loading rates in source tissues (Wu and Birch 2007). Each of these activities can contribute to the observed high-sugar yields. Direct induction or suppression on expression of genes related to photosynthesis by isomaltulose has not been reported. There were no reports indicating isomaltulose being a co-factor to enhance activities of enzymes related to light harvesting or dark reactions in carbon assimilation. The improvement of photosynthesis in SugarBooster plants might be a secondary effect, which is consistent with the hypothesis that sink capacity regulates photosynthesis and overall carbon supply from the source, which has been proven by experiments using physiological perturbations of sink–source relations, by either leaf removal or shading, or cold girdling of the sugarcane stem (McCormick et al. 2008b, c, a; McCormick et al. 2009). Sink size and activity control photosynthesis by feedback responses, including sugar-regulated expression of photosynthetic genes (McCormick et al. 2008c, a; Urban et al. 2008). As an adaptation mechanism, sink stimulation of photosynthesis as a function of strong sinks in the plants (i.e., fruits, storage organs and seeds) has also been detailed in other plant species (Kaschuk et al. 2009; Herold 1980; Paul and Foyer 2001).

Sugar Transport

Sucrose is the principal product of photosynthesis used for the distribution of assimilated carbon in plants. Transport mechanisms and efficiency influence the photosynthetic productivity by relieving product inhibition and contribute to plant vigour by controlling source/sink relationships and biomass partitioning (Ayre 2011; Patrick et al. 2013). Sucrose is synthesized in the cytoplasm in the source leaves and may, through plasmodesmata, move from cell to cell or may cross membranes to be stored or exported to the apoplasm for uptake into adjoining cells (Patrick et al. 2013). Sucrose requires essential surface proteins to facilitate efficient membrane transport. Transport across the tonoplast by facilitated diffusion, antiport with protons and symport with protons have been proposed (Ayre 2011). Bush developed experimental tools of isolated membrane vesicles to reveal the biochemical features of sucrose transport across plant membranes, including $K_{0.5}$ for both H^+ and sucrose, electrogenicity, pH dependence, stoichiometry and specificity (Bush 1990, 1993). The use of these experimental tools on SugarBooster lines revealed enhanced sucrose loading rates in source tissues (Wu and Birch 2007). Consistently, suspension culture cells of the SugarBooster line have also shown improved sucrose uptake (Wu and Birch 2010).

In other crops, assimilate transport has also been improved when sink capacity was increased. From studies on competition between two wheat ears containing different numbers of grains, but receiving assimilate from a common source leaf equidistant from the two sinks (Cook and Evans 1978), it was concluded that a stronger sink generates a steeper gradient in sieve-tube assimilate concentration leading to flow from more distant sources than does a weak competing sink. The rate of assimilate accumulation into sink tissues reflected sink strength in tomato fruit (Ho 1996). Cell turgor is an important regulator of sucrose uptake in this tissue and, thus, may be an important determinant of sink strength in tissues that store sucrose (Wyse et al. 1986). Sucrose transporters, which have an indispensable role in the regulation and sucrose transport, are highly regulated (Aoki et al. 2003; Williams et al. 2000; Afoufa-Bastien et al. 2010). A model was proposed (Vaughn et al. 2002) that sink strength would change the rate of sucrose unloading and influence sucrose content in the phloem, which, in turn, regulates sucrose transporter expression. If sink strength is weak, sucrose concentrations increase throughout the phloem symplasm and repress sucrose transporter expression in companion cells. Repressed sucrose transporter expression in companion cells results in reduced uptake from the apoplasm, and finally increased carbohydrate in mesophyll cells and the feedback inhibition on photosynthesis (Stitt et al. 2010). Phosphorylation cascades are involved in the sucrose-mediated regulation of sucrose transporter expression (Ransom-Hodgkins et al. 2003).

Carbon Partitioning

Control and improvement in distribution and storage of photosynthetic assimilates are important aspects in yield. Photosynthesis and translocation in relation to crop yield have been reviewed previously (Loomis et al. 1979; Nasyrov 1978; Gifford and Evans 1981; Veneklaas et al. 2012). In SugarBooster lines, there was correspondingly more sugar per unit fresh weight, with no significant change in the insoluble (fibre) content of 9–10 % fresh weight in mature internode tissues (Wu and Birch 2007). This indicates increased photosynthate storage as sugar, rather than altered partitioning between sugar and fibre. In high-sugar cultivars of sugarcane, the water content typically decreases down the stalk with increased sucrose content, to a minimum of about 70 % moisture in mature internodes (Bull and Glasziou 1963). In SugarBooster lines, there was about 60 % moisture in the oldest internodes (Wu and Birch 2007).

In suspension cell cultures, when sugar concentrations in the medium were similar at early stages of subculture, the SugarBooster line showed greater incorporation from a pulse-labelled fructose into all cellular metabolite pools and into respired carbon dioxide. The proportion of metabolised label converted into sugars increased at the expense of fibre (Wu and Birch 2010).

Sugar Futile Cycle in Storage Tissues

Sucrose is an unusual storage compound because it is soluble and readily metabolised. Cycling of carbon between sucrose and hexoses, as a result of concurrent synthesis and degradation of sucrose, evidently occurs in all sugarcane sucrose-storing cells and is believed to be primarily responsible for regulating sucrose accumulation. This phenomenon was first described in young sugarcane internodal tissue (Sacher et al. 1963; Hatch et al. 1963), and subsequently in young and older culm tissue (Batta and Singh 1986), as well as in cell suspension cultures (Dancer et al. 1990; Veith and Komor 1993). It has been estimated that 22 % of stored sucrose is digested and re-synthesized by 'futile cycling' (Uys et al. 2007). Isomaltulose, previously described as a non-metabolizable sugar (Sinha et al. 2002; Fernie et al. 2001), can also be digested in plant cells, even though its degradation is much slower than that of sucrose (Wu and Birch 2011). In order to improve accumulated sugar concentrations, sugarcane breeders have been trying to either decelerate the degradation of sucrose or accelerate the resyntheses of the disaccharides (Fig. 11.3).

Sucrose, an assimilate unloaded from the phloem, may move across and distribute into three cellular compartments, namely the cell wall and intercellular spaces (the apoplastic compartment), cytoplasm (the metabolic compartment) and vacuole (the storage compartment). Different invertase isoforms are associated with each of these compartments, these being neutral invertase (NI) in the cytoplast, soluble acid invertase (SAI) in the vacuoles and cell wall invertase (CWI) in the apoplast. Also, sucrose can be degraded by SuSy in the cytoplasm.

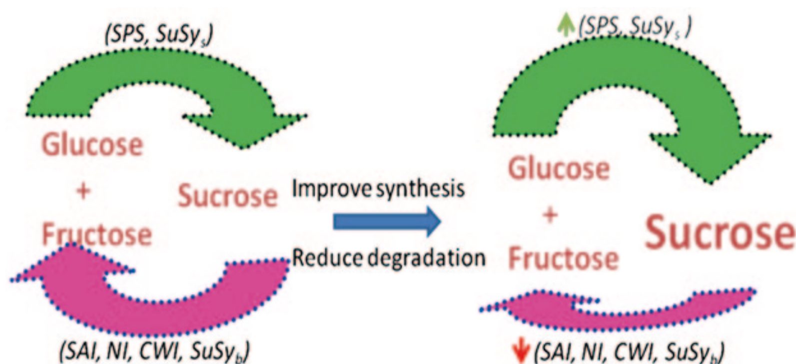

Fig. 11.3 A model of enhancement of sucrose accumulation by manipulating enzyme performance to accelerate sucrose synthesis or to decelerate sucrose degradation in the futile cycle. *CWI* cell wall invertase, *NI* neutral invertase, *SAI* soluble acid invertase, *SPS* sucrose phosphate synthase, $SuSy_b$ sucrose synthase in sucrose breakage direction, $SuSy_s$ sucrose synthase in sucrose synthesis direction

Invertases have long been considered as principal regulators of sugarcane growth and more specifically, sucrose accumulation (Gayler and Glasziou 1972). Relative to the control, CWI activity in a SugarBooster cell suspension line was reduced by 50–75 %, accompanied by much slower hydrolysis of sucrose in the culture medium to hexoses during the period of rapid sucrose accumulation in the cells (Wu and Birch 2010). Nevertheless, sugar uptake by the SugarBooster line was faster, which would result in different osmotic environments for the suspension cells between the SugarBooster and the control. In contrast, CWI activities were 50–80 % greater in the central storage parenchyma-rich zone of the mature stems of SugarBooster plants than in Q117 controls, but not in the peripheral fibre- and vascular-rich zone (Wu and Birch 2007). Higher CWI activities in the sucrose storage tissues increase the gradient from vascular bundles and reflect the improved sink strength in the matured stem of SugarBooster plants. Suspension cells during the sucrose accumulation phase appear physiologically closer to storage parenchyma cells in the transition between the stages of most active growth and most active sucrose accumulation, rather than mature stem storage parenchyma cells (Wu and Birch 2010). The hydrolysis of sucrose by CWI and the subsequent import of hexoses into target cells appear to be crucial for appropriate metabolism, growth and differentiation in plants (Roitsch 1999; Sherson et al. 2003). Strong evidence from transgenic carrots indicated important roles played by CWI in plant growth and development (Tang et al. 1999).

Intracellular enzymes for sucrose cleavage in the vacuole (SAI) and cytoplasm (NI and SuSy cleavage direction) were all significantly less in the SugarBooster line. In contrast, sucrose biosynthesis activities (sucrose phosphate synthase (SPS) and SuSy synthesis direction) were unaltered under the standard reaction conditions for extracted enzymes (Wu and Birch 2010).

Studies on relationships among different genotypes of sugarcanes indicate that major differences in sucrose accumulation among the population could be attributed to the difference between activities of SAI and SPS, provided SAI is below the critical threshold concentration (Zhu et al. 1997b; Zhu et al. 1997a). Antisense suppression on SAI expression in the vacuole of sugarcane cells in liquid culture increased the sucrose concentration by twofold (Ma et al. 2000). However, up to a 70 % reduction of SAI activity in the immature internodes of transgenic sugarcane plants did not have a significant impact on sucrose concentration (Botha et al. 2001).

A kinetic model of sucrose accumulation (Rohwer and Botha 2001) pointed out cytosolic NI as one of the three promising targets that may lead to higher sucrose concentrations if manipulated. Antisense suppression on NI led to increased sucrose concentrations, with a demonstrable reduction in sucrose cycling in cell suspensions (Rossouw et al. 2007). This kind of antisense suppression on NI reduced the activity by 40 % compared to the non-transgenic controls, resulting in a sucrose content increase of 25 and 14 % in the immature and mature culms of transgenic sugarcane plants, respectively (Rossouw et al. 2010). A SuSy increase in the culms of transgenic plants compensated for reduced NI (Rossouw et al. 2010), which made the metabolic effects from NI suppression in transgenic plants weaker than that observed in suspension cells (Rossouw et al. 2007).

The thermodynamics of sucrose hydrolysis by invertases is irreversible (Avigad 1982; Kruger 1997). However, the reaction catalysed by SuSy is freely reversible, with a theoretical equilibrium constant (K_{eq}) in the direction of sucrose degradation ([UDP-glucose] [fructose]/[UDP] [sucrose]) of 0.15–0.56 (UDP, uridine diphosphate; Morell and Copeland 1985; Avigad 1964; Delmer 1972) and with reported ΔG values of -1.4 to 4.7 kJ mol^{-1} for the sucrose synthesis direction (Geigenberger and Stitt 1993). The significance of SuSy cannot be ignored in sugarcane, as it plays multiple roles such as sucrose unloading in the sink tissues, cell wall synthesis, carbon storage as sucrose or starch accumulation, internode elongation in sugarcane and respiration in all plant species (Koch et al. 1996; Martin et al. 1993; Moore 1995; Lingle and Smith 1991; Chourey et al. 1998; Sturm et al. 1999; Botha and Black 2000; Lingle and Dyer 2001; Schrader and Sauter 2002; Bieniawska et al. 2007). Though SuSy catalyses a reversible reaction, it is believed that it mainly reacts in the direction of degradation in mature sugarcane stem tissues (Claussen et al. 1985; Schafer et al. 2004). In sucrose isomerase transformed sugarcane suspension cell lines, SuSy activity in cleavage direction showed the most consistent and strongest down-regulation among all sucrose-hydrolysing enzymes, along with highly accumulated sucrose content (Wu and Birch 2010). Antisense expression of a SnRK1 in potato resulted in decreased expression of SuSy in tubers and the loss of sucrose inducibility of SuSy transcripts in leaves (Purcell et al. 1998). Along with reduced SnRK1 activity in the SugarBooster suspension cell lines compared to the controls detailed in the following sections, SuSy transcripts were reduced (unpublished data).

SuSy is encoded by multiple genes in plant species, playing individual roles, but having expression patterns overlapping in numerous plants such as corn, *Arabidopsis,* pea and rice (Carlson et al. 2002; Chourey et al. 1998; Barratt et al. 2001;

Bieniawska et al. 2007). In polyploid sugarcane, it is believed that SuSy may be encoded by multiple genes. Three forms of SuSy proteins were partially purified from sugarcane tissues (Schafer et al. 2005). Full-length SuSy genes have been cloned (Lingle and Dyer 2001). A negative association was found between the SuSy expressed sequence tag (EST) marker and sucrose content (da Silva and Bressiani 2005). Pinto et al. (2010) also found that two restriction fragment length polymorphisms (RFLP) markers derived from ESTs encoding SuSy enzymes were correlated negatively with both cane yield and sugar yield across plants and ratoons. A positive correlation was found between transcripts of a specific SuSy member and SnRK1 activity in suspension cells and mature stem tissues of SugarBooster lines, but a negative association between levels of transcripts of the specific SuSy member and accumulated sucrose contents (unpublished data). Further work on EST characterization, gene cloning and manipulation will help understand the complex relationships between SuSy isoforms and sucrose accumulation within the stem parenchyma tissues.

Considering that the glycolytic pathway in the cytosol would use hexoses from sucrose degradation, Scheepers (2005) down-regulated by more than 90% the gene expression of aldolase, an important glycolytic enzyme. Unfortunately, this strategy did not perturb glycolytic carbon flux, carbon partitioning or sucrose accumulation in transgenic sugarcane plants. In contrast, down-regulation of the expression of pyrophosphate-fructose 6-phosphate 1-phosphotransferase (PFP) gene in transgenic sugarcane plants has demonstrated a developmental stage-specific role in sugar accumulation (Groenewald and Botha 2008; van der Merwe et al. 2010). PFP, with ATP-dependent phosphofructokinase, represents control points for rerouting hexose phosphates to respiratory pathways and is likely involved in hexose utilization in cytoplasm after sucrose cleavage. Analyses on metabolites of these transgenic plants further demonstrated the regulatory roles for PFP in sucrose futile cycle in young culms and gluconeogenesis in older ones (van der Merwe et al. 2010). However, whole transgenic plant stalks with down-regulated PFP had decreased total sugar concentration and increased fibre content (Groenewald and Botha 2008).

Sucrose Synthesis and Sucrose-Related Metabolites with Potential Allosteric and Regulatory Effects

Sucrose can be re-synthesized by SPS or SuSy in the cytosol. SPS catalyses the production of sucrose phosphate from UDP-glucose (UDPG) and fructose 6-phosphate (F6P). Sucrose-phosphate phosphatase (SPP) is highly active, converting sucrose phosphate to sucrose. Thus, physiological concentrations of sucrose phosphate are extremely low, resulting in SPS irreversibility (Krause and Stitt 1992). Tight correlation was demonstrated between stem SPS activity and sucrose concentration in different sugarcane varieties and segregating populations (Botha and Black 2000; Grof et al. 2007). Therefore, SPS was considered as a primary target for metabolic

manipulation to increase sucrose accumulation. Even though successful over-expression of maize SPS in tomato, resulting in greater dry weight, number of fruit and higher sucrose concentration, was first reported in 1991 (Worrell et al. 1991; Laporte et al. 1997), so far, the over-expression of this enzyme activity in transgenic sugarcane plants has not led to altered sugar yields. Attempts to over-express a spinach SPS gene in sugarcane suggested that this gene was highly prone to transgene silencing (Grof et al. 1996). There was no enhancement in sucrose content compared to non-transgenic controls in sugarcane field trials with an SPS over-expression construct (Vickers et al. 2005). There are several isoforms of SPS present in sugarcane (McIntyre et al. 2006) with tissue-specific expression patterns (Grof et al. 2006). With increasing knowledge of sugarcane genome structure, EST collections and functional analysis, up-regulation of SPS may also be worth pursuing by employing a specific member and optimizing developmental patterns. As described in detail in the following examples, the enhancement of SPS capacity by improving its physiological environment could also stimulate sucrose accumulation.

The key enzymes in plant sucrose metabolism are all regulated at multiple levels, including reversible effects of phosphorylation, allosteric modulators, substrate/product concentrations at the subcellular location(s) and employing various enzyme isoforms (Winter and Huber 2000; Lunn and MacRae 2003; Koch 2004; Rolland et al. 2006; Vargas and Salerno 2010). Several of these metabolites, such as trehalose-6-phophate (T6P), glucose-6-phophate (G6P), glucose-1-phophate (G1P), fructose-6-phophate (F6P) and UDPG, as well as SnRK1 enzyme activity, were analysed in SugarBooster suspension cell lines, for further insight into the mechanism of enhanced sucrose accumulation (Wu and Birch 2010).

T6P is a low-abundance molecule in plants that responds to sucrose or hexose phosphate pool concentrations, and putatively coordinates metabolism with development in response to carbon availability and stress. Evidence for a direct role of trehalose as a signal molecule in plants is less compelling (Paul et al. 2008a; Paul et al. 2008b). T6P concentration was steady in the control cells, whereas the concentration doubled during the period of enhanced sucrose accumulation in the SugarBooster suspension cells (Wu and Birch 2010).

In plants, SnRK1 activity regulates many functions at transcriptional and metabolic levels in response to carbon and energy status. The effects vary depending on interacting regulatory factors in different tissues. For example, SnRK1 typically activates photosynthesis and degradation processes (including SuSy breakage activity), while down-regulating biosynthetic processes (including SPS and TPS;T6P synthase) during a ‘starvation response’ in growing leaves (Baena-Gonzalez et al. 2007), whereas it enhances starch storage in potato (*Solanum tuberosum* L.) tubers and cereal endosperm (Halford and Hey 2009). Recently, it has been shown that T6P and G6P at micromolar concentrations strongly inhibit SnRK1 activity in cell extracts from the sugarcane suspensions (Wu and Birch 2010). Consistently, T6P was found to reduce SnRK1 activity from actively growing *Arabidopsis thaliana* tissues, but not in mature leaves, which lack an unknown essential cofactor (Zhang et al. 2009).

UDPG is a key activated intermediate used in the synthesis of sucrose (via SPS, SPP or SuSy), T6P (via trehalose phosphate synthase (TPS)), cell wall components (via cellulose synthase and UDPG dehydrogenase, UDPG-DH), and potentially for starch biosynthesis or catabolism (via the reversible actions of SuSy and UDP sugar-pyrophosphatase; Kotake et al. 2004; Baroja-Fernandez et al. 2009). UDPG concentration was doubled, along with the decreased UDPG-DH, in the suspension cells of SugarBooster lines relative to the controls (Wu and Birch 2010). Compared to controls, enhanced sucrose content and increased SPS activity have been reported in transgenic sugarcane plants with suppressed UDPG-DH activity (Bekker 2007). Interestingly, cell wall synthesis in the UDPG-DH-silenced transgenic plants was compensated partly via activation of an alternative pathway, the myoinositol oxygenation pathway for cell wall precursor synthesis (Bekker 2007).

High UDPG concentration may also be from the high content of G6P in the suspension cells of SugarBooster lines compared to the controls (Wu and Birch 2010), since G6P can be isomerised to G1P by phosphoglucose mutase. With G1P, UTP is lysed to produce UDPG and pyrophosphate. UDPG is then used to produce sucrose. Although it sounds complex, it is a very energy-efficient process, ultimately requiring fewer molecules of triphosphate per molecule of sucrose to store. Recent experiments (unpublished data) showed G1P also doubled in the suspension cells of the SugarBooster line compared to the control.

An excess of pyrophosphate, which is produced as a by-product of UDPG biosynthesis, is known to inhibit sucrose synthesis in a feedback loop (Neuhaus and Stitt 1991). In an innovative approach by expression of a yeast-derived pyrophosphatase gene driven by a leaf-specific promoter, both stem and leaf sucrose content increased by 25 and 43 %, respectively, in transgenic sugarcane (Wang and Zhang 2011).

High concentrations of substrates of UDPG and F6P in sucrose synthesis might be one of the high-sucrose mechanisms in SugarBooster lines. G6P and F6P are generally in equilibrium through the action of phosphoglucose isomerase. Both were present in the SugarBooster cells at about twice the concentrations in the control, even before the sucrose concentrations in these cell lines diverged (Wu and Birch 2010).

Elevations of other sugar phosphates, such as T6P, G1P and G6P, have the potential to affect both direct and indirect enhancement of sucrose synthesis via SPS. Keeping SPS levels constant, apparent SPS activity can be improved by reducing the ratio of inactive (phosphorylated) to active (dephosphorylated) through phosphorylation by SnRK1 (Halford and Hey 2009; Toroser et al. 2000). The putative regulatory molecule T6P varied significantly between the cell lines in the range of 4 nmol g^{-1} FW in Q117 to10 nmol g^{-1} FW in SugarBooster (Wu and Birch 2010). The T6P in this cellular concentration range (7.5 M) strongly inhibits (50 %) SnRK1 activity from sugarcane sink cells (Wu and Birch 2010), which is consistent with effects in *A. thaliana* seedlings (Zhang et al. 2009; Nunes et al. 2013). Also, G1P, which doubled its content in the cells of SugarBooster lines (unpublished data), could strongly inhibit SnRK1 activity and showed synergistic effects with T6P (Nunes et al. 2013). Moreover, G6P (200–400 nmol g^{-1} FW) was far more abundant

than T6P in sugarcane cells. It was also elevated by ~80 % in the SugarBooster line relative to its control (Wu and Birch 2010). This G6P concentration caused a highly significant (17 %) reduction in SnRK1 activity in sugarcane cell extracts (Wu and Birch 2010), which is consistent with the results from *A. thaliana* seedlings (Zhang et al. 2009; Nunes et al. 2013). The G6P inhibition on SnRK1 is cumulative with T6P and G1P (Nunes et al. 2013). Kinetic models show that both T6P and G1P follow the same partial non-competitive mechanism, but each has distinct binding sites and regulation; G6P follows a hyperbolic mixed-type mechanism which affects both the binding of ATP and the formation of product (Nunes et al. 2013). In addition, G6P at 0.6 mM (approximately the concentration in the SugarBooster cell extracts) increased SPS activity in sugarcane cell extract more than threefold (Wu and Birch 2010), which is consistent with its known role as an allosteric activator (Winter and Huber 2000; Lunn and MacRae 2003). Under routine assay conditions, there was no evidence for enhanced SPS activity as described earlier, whereas assays conducted at measured cellular G6P concentrations showed a highly significant (twofold) increase in sucrose synthesis from the SugarBooster line (Wu and Birch 2010). The regulation on metabolism at multiple levels is depicted graphically in Fig. 11.4.

Sucrose isomers are evidently sensed by plant cells (Figs. 11.2, 11.4), with effects that differ from sucrose (Loreti et al. 2000; Fernie et al. 2001; Sinha et al. 2002; Atanassova et al. 2003). Therefore, it is plausible that exceptional SugarBooster phenotypes among sucrose isomerase transgenic lines reflect the sensitivity of sugar signalling to isomaltulose produced in different patterns through integration position effects (Birch and Wu 2004). However, diverse 'sugar phenotypes' were obtained in the case of sugarcane engineered to express a vacuole-targeted sucrose isomerase (Birch and Wu 2004). Constitutive expression of cytosolic sucrose isomerase is disruptive (Bornke et al. 2002; Wu and Birch 2007). Therefore, diverse phenotypes may stem from multiple effects of sucrose isomers as signal and storage compounds when accumulated in various developmental and compartmental patterns following different transgene integration events (Figs. 11.2, 11.4). In preliminary experiments, the SugarBooster line was remarkable for enhanced sucrose accumulation in cell cultures. Not all cell lines engineered with the same sucrose isomerise construct showed enhanced sucrose accumulation, even though it was highly reproducible in this SugarBooster line. Plants appear to lack efficient transporters for the sucrose isomer-isomaltulose (Loreti et al. 2000; Fernie et al. 2001; Sinha et al. 2002; Atanassova et al. 2003), so exogenous application is unlikely to mimic intracellular conversion. It is not surprising to find that exogenous isomaltulose did not convert Q117 to high-level sucrose accumulation. In addition, isomaltulose production in the SugarBooster suspension cells declined over the course of subcultures, whereas enhanced sucrose accumulation was highly reproducible. Southern analysis indicated the SugarBooster had several copies of transferred genes integrated at separate locations (unpublished data), so collateral genetic changes cannot be excluded. Furthermore, field evaluation of SugarBooster lines of diverse sugarcane genotypes demonstrated that even though isomaltulose

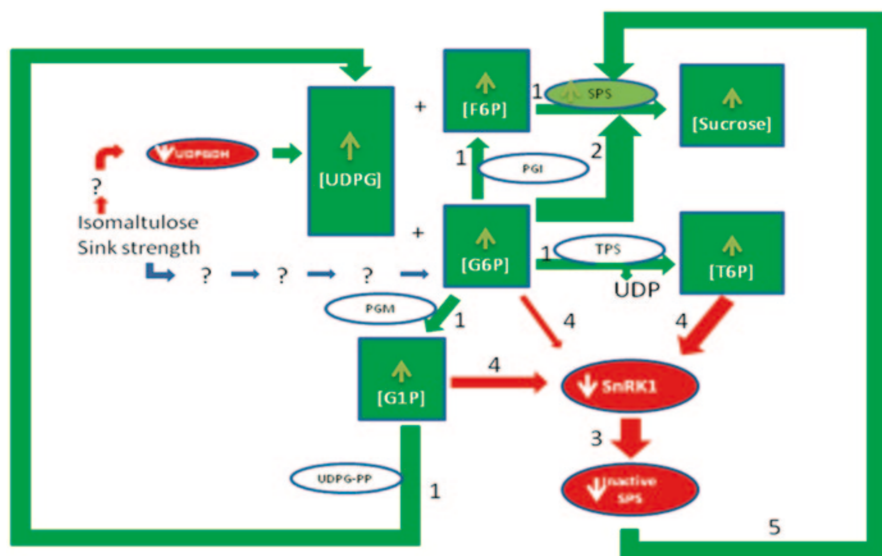

Fig. 11.4 Potential regulations in SugarBooster lines. Metabolites are represented by *green squares* with up-regulated contents (shown by *green up-arrow heads*) compared to the controls. *Ovals* represent various enzymes: *light green ovals* show they are stimulated (shown by *up-arrow heads*); *red* ones indicate they are inhibited by the metabolites (shown by *white down-arrow heads*); *grey* ones indicate the enzymes have not been tested in the related articles. *Green arrows* show the positive effects on the reaction and *red arrows* indicate inhibition on enzyme activities. *Question marks* indicate unknown signal transduction pathways. *Numbers* represent mechanisms of metabolic regulations: 1 substrate concentrations, 2 allosteric effects, 3 reversible phosphorylation, 4 direct inhibition, 5 active enzyme ratio. *F6P* fructose-6-phosphate, *G1P* glucose-1-phosphate, *G6P* glucose-6-phosphate, *PGI* phophoglucose isomerase, *PGM* phophoglucose mutase, *SnRK1* sucrose non-fermenting-1-related protein kinase, *SPS* sucrose phosphate synthase, *T6P* trehalose-6-phosphate, *TPS* trehalose phosphate synthase, *UDP* uridine diphosphate, *UDPG* uridine diphosphate glucose, *UDPGDH* uridine diphosphate glucose dehydrogenase, *UDPG-PP* uridine diphosphate glucose pyrophosphorylase

production could be maintained, the high isomer production was accompanied by a reduction of sucrose production, resulting in no increase or decrease in total sugar content (Basnayake et al. 2012). This leaves the role of sucrose isomers in triggering enhanced sucrose accumulation in the SugarBooster lines open to discussion.

It is fascinating that multiple processes in the SugarBooster line were all altered in a direction consistent with enhanced sucrose accumulation. To date, the manipulation of individual endogenous genes has not surmounted the ceiling in sucrose accumulation accomplished by conventional sugarcane breeding (Botha et al. 2001; Vickers et al. 2005; Groenewald and Botha 2008). It may be that development requires parallel activation and/or repression of multiple enzymes with shared flux control, which could be achievable through alterations to regulate entire pathways rather than single 'rate-limiting' enzymes (Morandini 2009).

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Part III
Enabling Technologies

Chapter 12
Zinc Finger Nuclease-Mediated Gene Targeting in Plants

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DNA Double-Strand Breaks (DSBs)

The creation and repair of DNA DSBs is of central importance to the recombination between DNA sequences (Xu and Price 2011). Pioneering studies in yeast have highlighted the importance of DSBs in both meiotic (Keeney 2001) and mitotic (Lisby and Rothstein 2007) DNA recombination. The induction of genomic DSBs and their repair via various homologous and nonhomologous processes is well established (Haber 2007). Many of the genes involved in DSB repair have been elucidated and found to be conserved across a broad range of life-forms (Li et al. 2011), although the contributions of each to the DNA repair process have dramatically changed during evolution (Sonoda et al. 2006). These studies have highlighted the dual role of DSB formation and resolution as a means of both promoting genetic diversity by facilitating DNA sequence exchange and conserving genomic integrity via DNA repair.

DSBs can be repaired using homologous sequences, i.e., from a sister chromatid or other related template DNA, via pathways involving a collection of proteins which facilitate strand resection, invasion, annealing, and synthesis reactions resulting in an intact DNA sequence (Rajesh et al. 2011). Alternative pathways of DSB repair involve nonhomologous end joining (NHEJ) of DNA sequences whereby cleaved ends are religated without regard for homology, often resulting in deletions or insertions at the cleavage site (Wu et al. 2012). These complexes of apparently competing processes effectively repair DSBs with varying degrees of fidelity (Shibata et al. 2011).

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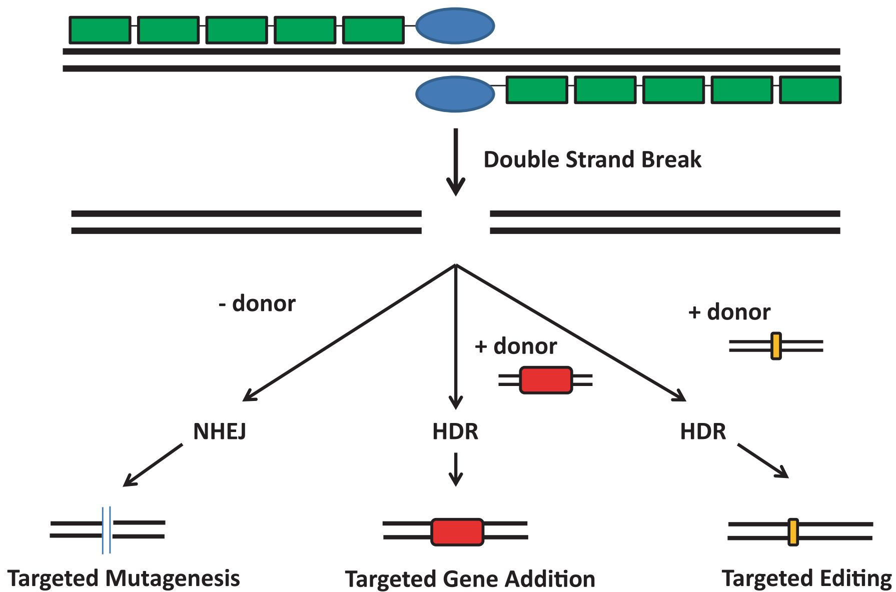

Fig. 12.1 ZFNs facilitate targeted genome modifications. ZFNs can be designed to specific genomic sequences to enable targeted gene addition, gene editing, and targeted mutagenesis. Delivery of ZFNs into cells results in targeted double-strand DNA breaks that are repaired by cellular repair mechanisms such as NHEJ and HDR. Repair of the double-strand DNA break by NHEJ leads to introduction of indels (insertions/deletions) at the cut site and targeted mutagenesis in the genome. Repair in the presence of an exogenous DNA molecule carrying a gene of interest (donor) with homology to the break site leads to targeted gene insertion. Presence of specific mutations in the donor result in edits at desired locations in the genomic sequence. *DSB* double-strand break, *NHEJ* nonhomologous end joining, *HDR* homology-directed repair, *indels* insertions/deletions. Zinc finger DNA-binding domains are represented by *green rectangles* and the *blue circle* represents the Fok1 nuclease domain

In higher plants, it appears as if DSBs are most typically repaired via NHEJ where sequence-independent repair often results in deletions, insertions, and/or rearrangements at the break site (Gorbunova and Levy 1999; Puchta 2005). Although not completely understood, it appears as if several NHEJ pathways in plants operate to repair DSBs (Charbonnel et al. 2011). If homologous sequences are in close proximity to the DSB, high-fidelity, homology-directed repair has been observed to occur in plant cells (Roth et al. 2012; Siebert and Puchta 2002).

The ability to generate DSBs, thereby stimulating the cell's DNA repair processes, represents a means of facilitating genetic modification (Fig. 12.1). The error-prone nature of NHEJ repair makes induction of DSBs a method for inducing mutations (Carroll 2011). Intervening sequence elimination following the formation and repair of concurrent DSBs is a means of generating various sorts of gene deletions (Lee et al. 2010). Homology-directed repair of DSBs enables transgene integration (Lombardo et al. 2011) and genome editing (McMahon et al. 2012).

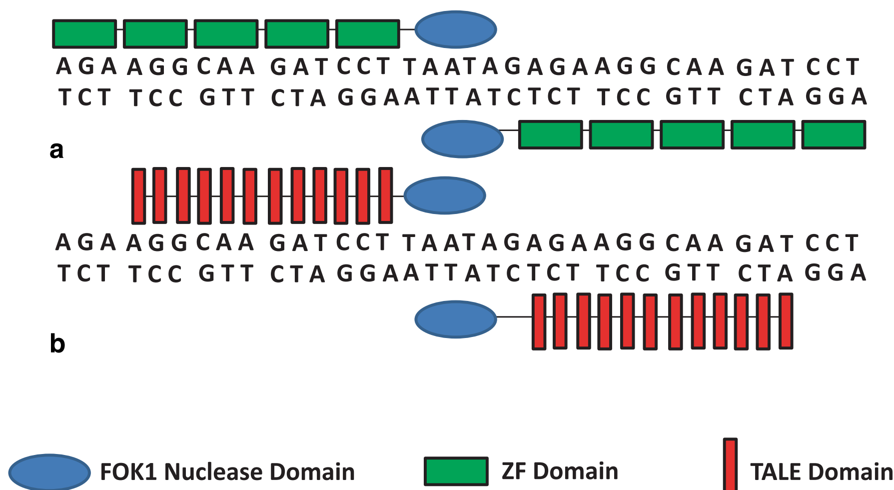

Fig. 12.2 Double-strand DNA binding by site-specific nucleases. Schematic representation of a ZFN (a) ZFN and a TALEN (**b**) bound to DNA. ZFNs recognize and bind DNA through the zinc finger domains (*green*, **a**) and each finger binds a nucleotide triplet. DNA binding by TALENs is mediated by TALE effector (*red*, **b**) domains with single nucleotide specificity. The nonspecific *Fok-1* nuclease domain is shown in *blue*

Designed Zinc Finger Nucleases (ZFNs)

To take advantage of DSB repair for controlled genome modification, a method is required for targeted DNA cleavage (Puchta and Fauser 2013). Previously, targeted DSBs could only be made in plant genomes following pre-integration of restriction enzyme cleavage sites and expression of genes encoding the corresponding restriction enzyme (Salomon and Puchta 1998). Recently, ZFNs have been described that allow for DSB formation at endogenous plant loci (de Pater et al. 2013; Shukla et al. 2009; Townsend et al. 2009; Zhang et al. 2010). ZFNs are engineered restriction enzymes consisting of a nonspecific cleavage domain and sequence-specific DNA-binding domains designed to create site-specific DSBs (Porteus and Carroll 2005). In this way, DSBs can be targeted to investigator-specified sites by engineering and delivering novel sequence-specific restriction enzymes capable of binding and cleaving endogenous genomic DNA (Tzfira et al. 2012).

Zinc finger protein domains consist of ~30 amino acids which, upon chelating a zinc atom, fold into ββαstructures capable of binding specific DNA triplets (Pabo et al. 2001). Key amino acid residues in the α helix dictate sequence-specific binding, while the remaining amino acids maintain a consensus backbone structure with a modular architecture (Durai et al. 2005). Linking such modular structures together allows for the creation of DNA-binding domains capable of recognizing predetermined stretches of sequence (Fig. 12.2a). The development of designed ZFNs that cleave DNA at predetermined sites depends on the reliable creation of zinc finger

Table 12.1 Use of ZFNs for targeted genome modification in plants

Species	Description of the study	Reference
Targeted mutagenesis		
Arabidopsis thaliana	Stably integrated a ZFN cleavage site along with a ZFN gene. Mutated target sequence	Lloyd et al. (2005)
Arabidopsis thaliana	Stably integrated a *GUS* reporter gene disabled with a stop codon in a ZFN cleavage site. Mutated the stop codon following retransformation with a ZFN gene	Tovkach et al. (2009)
Arabidopsis thaliana	Stably integrated a ZFN cleavage siteRetransformed with ZFN to generate targeted mutations	de Pater et al. (2009)
Arabidopsis thaliana	Transformed with a gene encoding a ZFN designed to cleave, *ADH1* and *TT4*	Zhang et al. (2010)
Arabidopsis thaliana	Transformed with a gene encoding a ZFN designed to cleave *ABI4*	Osakabe et al. (2010)
Glycine max	Transformed with a gene encoding a ZFN designed to cleave *DCLa* and *DCLb*	Curtin et al. (2011)
Nicotiana tabacum	Transformed with a gene encoding a ZFN designed to cleave *SuRA*	Maeder et al. (2008); Townsend et al. (2009)
Zea mays	Transformed with a gene encoding a ZFN designed to cleave *IPK1*	Shukla et al. (2009)
Gene deletion		
Nicotiana tabacum	Stably integrated a ZFN cleavage site-flanked *GUS* reporter gene in one plant and a corresponding ZFN in another. Deleted reporter gene in hybrid and progenies	Petolino et al. (2010)
Site-specific transgene integration		
Arabidopsis thaliana	Stably integrated a ZFN cleavage site Retransformed with ZFN and homologous donor for targeted transgene integration	de Pater et al. (2009)
Nicotiana tabacum	Co-delivered ZFN and homologous donor DNA to repair a nonfunctional *GUS*/*NPTII* fusion gene	Wright et al. (2005)
Nicotiana tabacum	Gene addition into a pre-integrated partial *PAT* gene flanked by ZFN cleavage sites via co-delivery of homologous donor DNA and ZFN gene	Cai et al. (2009)
Nicotiana tabacum	Targeted *PAT* gene integration into *CHN50* using a designed ZFN	Cai et al. (2009)
Nicotiana tabacum	Stably integrated a ZFN cleavage site-flanked *GFP* gene and replaced with an incoming ZFN cleavage site-flanked *HPT* gene co-delivered with a ZFN gene	Weinthal et al. (2013)

Table 12.1 (continued)

Species	Description of the study	Reference
Zea mays	Targeted PAT gene integration into *IPK1* with autonomous and nonautonomous homologous donor DNA and designed ZFNs	Shukla et al. (2009)
Zea mays	Stably integrated an *AAD1* gene flanked by ZFN cleavage site and targeted a *PAT* gene into the locus	Ainley et al. (2013)
Genome editing		
Arabidopsis thaliana	Modified an endogenous *PPO* gene using homologous donor DNA with 2 mutations and a designed ZFN	de Pater et al. (2013)
Nicotiana tabacum	Generated specific mutations of *SuRA* and *SuRB* using homologous donor DNA and designed ZFNs	Townsend et al. (2009)

ZFN zinc finger nuclease

protein domains that can specifically recognize the chosen target site within a genome. The design, assembly, and validation of such DNA-binding proteins based on modular zinc fingers are becoming more routine (Hurt et al. 2003; Isalan et al. 2001; Maeder et al. 2008; Mandell and Barbas 2006). ZFN design services are commercially available (e.g., ComposZr® from Sigma-Aldrich).

DNA cleavage is facilitated by a sequence-independent nuclease domain from the bacterial type IIS restriction endonuclease *Fok*I(Kim et al. 1996). To cut DNA and generate a DSB, the *Fok*I nuclease domain needs to dimerize at the cleavage site (Bitinaite et al. 1998). A ZFN is created by linking the *Fok*I cleavage domain to the C-terminus of a tethered series of zinc finger protein domains designed to bind a specific DNA sequence. Upon binding of two adjacent ZFN pairs to sequences flanking the intended cleavage site in a precise orientation and spacing relative to each other, the *Fok*I domains dimerize thereby facilitating DSB formation (Fig. 12.2a). ZFNs have been used to create targeted DSBs and enable genome modification in a broad spectrum of genomes, including human (Lombardo et al. 2007; Moehle et al. 2007; Perez et al. 2008; Porteus and Baltimore 2003; Provasi et al. 2012; Sebastiano et al. 2011; Urnov et al. 2005; Wilen et al. 2011), hamster (Santiago et al. 2008), mouse (Osiak et al. 2011), pig (Hauschild et al. 2011), frog (Young et al. 2011), zebra fish (Doyon et al. 2008), insect (Beumer et al. 2006; Bibikova et al. 2002), roundworm (Morton et al. 2006), and *Plasmodium* (Straimer et al. 2012). The present chapter reviews the use of designed ZFNs for inducing targeted DSBs and facilitating genome modification in plants (Table 12.1).

Targeted Mutagenesis

The ability to modify specific gene sequences is an indispensable tool for systematic analysis of plant gene function (Perry et al. 2003). Since DSB repair in plants appears to be primarily via NHEJ (Gorbunova and Levy 1999; Puchta 2005) and, since NHEJ in plants tends to be rather error-prone (Britt 1999), targeted DSB formation is a path toward targeted mutagenesis (Lyznik et al. 2012). Designed ZFNs appear to be ideally suited for such an application.

The first proof-of-concept study demonstrating ZFN-mediated targeted mutagenesis in plants involved the mutation of an introduced construct comprising a ZFN cleavage site and a corresponding ZFN under the control of a heat shock promoter (Lloyd et al. 2005). The experimental system involved an *Eco*R1 restriction sequence within the ZFN cleavage site which could be lost upon mutation, due to NHEJ-induced deletion or insertion, thereby allowing mutations to be identified. TOPO-cloning of polymerase chain reaction (PCR) products amplified from genomic DNA from heat-treated T_1 *Arabidopsis* seedlings with single copy integration of the construct revealed mutation frequencies across multiple independent transgenic events, measured by lost *Eco*R1 restriction sites, to be in the range of 1.7–19.6 % based on a random sampling of clones. Sequencing of the *Eco*R1-minus clones illustrated the types of mutations resulting from DSB repair. Most of the mutations (78 %) were simple deletions of 1–52 bp. Simple insertions (1–4 bp) and combinations of insertions and deletions were also observed at lower frequency. These frequencies should be considered to represent an underestimate of the actual mutation frequency. Based on the design of the targeting construct, larger deletions (> 62 bp) which removed the PCR primer binding sites would not have been observed in this study. In fact, NHEJ-mediated deletions of 0.2–2.0 kb have been commonly observed and 50 % of all such deletions were found to be > 100 bp (Gorbunova and Levy 1999). Nonetheless, this study clearly demonstrated that ZFN-mediated DSB formation can lead to targeted mutations.

In a similar study, *Arabidopsis* plants, stably transformed with a target construct comprising an *Eco*R1-containing ZFN cleavage site, were retransformed with corresponding ZFN-expressing constructs driven by various promoters (de Pater et al. 2009). Most *Eco*R1-resistant DNA fragments amplified from transgenic plants contained deletions ranging from 1 to 80 bp. Small insertions (1–14 bp) and larger deletions (up to 200 bp) were also observed. Mutation frequency was estimated to be about 2 % based on a random sampling of cloned PCR fragments. Reverse transcription polymerase chain reaction (RT-PCR) was used to estimate relative ZFN expression. Driving the ZFN gene with a stronger promoter appeared to be more effective at generating mutations.

Additional examples of the ability of ZFN expression to mediate targeted genome modification via NHEJ DSB repair involved the mutation of a disabled reporter gene (Cai et al. 2009; Tovkach et al. 2009). In one study (Tovkach et al. 2009), a *GUS* gene, engineered to carry a TGA stop codon within a ZFN cleavage site—and thereby rendered nonfunctional, was stably transformed into tobacco. As expected,

transgenic tissue did not express the *GUS* reporter gene. Cocultivation of transgenic tissue with an *Agrobacterium* strain harboring a construct containing a corresponding ZFN expression cassette resulted in small sectors of positive GUS staining. Similarly, *Arabidopsis* plants stably transformed with the nonfunctional *GUS* gene and a ZFN under the control of a heat shock promoter expressed GUS following high-temperature induction. Sequence analysis of the target site following PCR amplification identified several single nucleotide deletions and substitutions resulting in an open reading frame expected to encode an active *GUS* gene. This mutation was also facilitated using viral delivery of a ZFN (Vainstein et al. 2011). In another study (Cai et al. 2009), a reporter construct carrying a *GFP* gene disabled by the insertion of a 2.8-kb stretch of heterologous DNA containing a ZFN-binding site was stably integrated into tobacco cell cultures. A tandem repeat of 540 bp in the two *GFP* gene fragments served as a substrate for intrachromosomal repair. Upon retransformation with a ZFN gene, fluorescent foci were visible and PCR analysis confirmed homology-directed repair of the targeted DSB.

Mutations at endogenous gene loci have also been demonstrated following expression of designed ZFNs (Maeder et al. 2008; Shukla et al. 2009; Townsend et al. 2009). Tobacco protoplasts were transformed with a ZFN designed to cleave a specific site within the *SuRA* gene. Among 66 transgenic plants regenerated, three displayed single base mutations in the SuRA gene (Maeder et al. 2008). Similarly, ZFNs designed to cleave *SuRA* and *SuRB* genes displayed varying degrees of specificity relative to creating site-specific mutations (Townsend et al. 2009). A ZFN designed to cleave within the maize *IPK1* gene was transiently expressed in cultured maize cells after which multiple deletions and insertions were observed following deep sequencing of PCR amplified products (Shukla et al. 2009).

Genes encoding ZFNs designed to recognize *Arabidopsis ADH1* and *TT4* driven by an estrogen-inducible promoter resulted in somatic mutation frequencies of 7 and 16 %, respectively (Zhang et al. 2010). The mutations were typically 1–142 bp insertions or deletions localized at the ZFN cleavage site and were often found to be biallelic, i.e., homozygous. A ZFN gene, designed to recognize the *Arabidopsis ABI4* gene sequence, driven by a heat shock promoter, upon induction, resulted in up to 3 % mutagenesis of the binding site and the appearance of expected phenotypes, i.e., abscisic acid (ABA) and glucose insensitivity, in homozygous progeny (Osakabe et al. 2010). In a similar study, independent mutations in the paralogous *DCLa* and *DCLb* soybean genes involved in RNA silencing were generated using designed ZFNs (Curtin et al. 2011). Taken together, these results suggest the general utility for basic and applied studies of making site-specific mutations by expressing ZFNs designed to create targeted DSBs and induce NHEJ repair.

Mutation breeding in plants has resulted in numerous commercially relevant varieties in a broad spectrum of crop species (Maluszynski 2001). Conventional methods of mutagenesis used to generate genetically-modified crops typically involve random perturbations in the DNA sequence, using treatment with chemicals such as ethyl methanesulfonate (Watanabe et al. 2007), physical methods such as fast neutron radiation (Li et al. 2001) or naturally occurring genetic mechanisms such as transposable elements (Mathieu et al. 2009) combined most recently with

sequence-specific screening (McCallum et al. 2000). Such approaches have serious limitations, such as the lack of observable phenotypes, in highly duplicated genomes such as those found in modern domesticated crop species (Pham et al. 2010). More targeted transgenic approaches, such as RNAi-based gene silencing, have been fraught with unanticipated phenotypic consequences presumably due to lack of specificity and potential off-target effects (Duxbury and Whang 2004). The ability to modify single or multiple gene copies in duplicated genomes of crop species would represent a powerful means of generating new genetic variants. Targeted mutagenesis via sequence-specific DSB formation and repair using designed ZFNs enables such a capability.

Gene Deletion

As complete plant genomic sequences become elucidated, the need to assign functions to unknown genes becomes increasingly important. This is most effectively approached via reverse genetics and the analysis of gene disruptions, including silencing (Baulcombe 1999), insertional mutants (Feldmann 1991), and deletions (Koornneeff et al. 1982). Conventional methods of creating plant gene deletions, such as exposure to fast neutron emission, combined with molecular analysis of pooled arrays of mutant DNA, have resulted in the assembly of large deletion libraries covering most known genes in *Arabidopsis* and rice (Li et al. 2001). The ability to generate investigator-specified deletions by creating targeted DSBs, followed by subsequent intervening sequence removal via DNA repair, represents an increasingly powerful refinement for genome modification. In human cell cultures, predetermined genomic DNA segments up to 15 mega-bp were deleted following expression of ZFNs designed to cleave at specific loci (Lee et al. 2010). Targeted deletions of promoter or exon sequences by generating DSBs in intergenic regions or introns could result in targeted gene knockouts, including multigene disruption. By virtue of the polyploid nature of most crop species, agronomically relevant genes exist as multiple copies such that single gene disruptions may not result in discernable phenotypes (Pham et al. 2010). The ability to knockout multiple homologous genes simultaneously with carefully designed ZFNs might be particularly useful for crop improvement.

Proof of concept for ZFN-mediated gene deletion was obtained in a recent study involving the removal of a ZFN cleavage site-flanked reporter gene from a stably transformed plant by crossing it with a second plant expressing a corresponding ZFN gene (Petolino et al. 2010). A target construct, containing a *GUS* reporter gene flanked by ZFN cleavage sites, was used to generate transgenic tobacco target events. A second construct, containing a ZFN gene driven by a strong constitutive promoter, was used to generate separate transgenic ZFN events. Homozygous T_1 target plants, which expressed the *GUS* reporter gene, were crossed with homozygous T_1 ZFN plants, which expressed the ZFN gene. Numerous *GUS*-negative hybrid plants were observed (up to 35 % in one cross). Evidence for complete deletion of a 4.3-kb sequence between the ZFN cleavage sites was obtained and sequence

verified in hybrid plants and progenies. Since ZFNs can be designed to cleave a wide range of DNA sequences, the results from this study constitute a general strategy for creating targeted deletions.

Site-Specific Transgene Integration

The ability to introduce exogenous DNA into a predetermined location within the plant genome would greatly enhance the precision and predictability of transgenic technology. The potential mutagenic effects of random DNA integration and the unpredictable consequences of position effect on transgene behavior could be circumvented by targeting transgenes to specific genomic locations.

Early attempts at targeted transgene integration used a combination of integrated, nonfunctional selectable marker genes and exogenous DNA homologous and complementary to the integrated target (Offringa et al. 1990; Paszkowski et al. 1988). Transgene integration into the target site was achieved under selective conditions following correction of the nonfunctional selectable marker gene at very low frequency, i.e., estimated to be in the range of 10^{-4}–10^{-5}. In similar approaches, nonfunctional *ALS* gene fragments, carrying mutations that specified resistance to various herbicides, were used to target the endogenous gene loci in tobacco (Lee et al. 1990) and rice (Endo et al. 2007). Using herbicide selection, transgenic events were obtained that suggested that homologous recombination between the exogenous DNA and the endogenous gene had occurred at estimated frequencies in the range of 10^{-4}–10^{-5}. "Brute force" attempts at generating transgenic events via homologous recombination without direct selection corroborated the extremely low frequency of targeted transgene integration (Miao and Lam 1995). Some success was reported using a combination of positive and negative selection to enrich for targeted events, whereby a targeting construct containing an antibiotic resistance gene within and a cytosine deaminase gene outside sequences homologous to an endogenous locus allow for selection against random integration in the presence of fluorocytocine (Xiaohui Wang et al. 2001). Subsequently, rice *Waxy* and *adh2* genes were successfully targeted using a similar approach whereby a diphtheria toxin gene was used as a negative selectable marker (Terada et al. 2007; Terada et al. 2002). Attempts to enhance targeted transgene integration by modifying DNA repair pathways, such as co-expressing recombinase genes (Reiss et al. 2000; Shaked et al. 2005; Shalev et al. 1999), or knocking out genes associated with NHEJ (Jia et al. 2012), have met with limited success. Clearly, homology-directed repair does occur in plants and can facilitate targeted transgene integration; however, the frequency of targeted versus random integration appears to be too low for practical use with conventional transformation technology.

The yeast mitochondrial endonuclease, *I-sceI,* which has an 18-bp recognition sequence, has been used to demonstrate the importance of homology-directed repair of DSBs for targeted transgene integration (Puchta et al. 1996). A target construct containing an *I-sceI* restriction site flanking a partially deleted antibiotic resistance

gene was transformed stably into tobacco. Retransformation with a repair construct containing sequences homologous to the target construct and complementary to the deleted antibiotic resistance gene together with an *I-sceI* expression construct resulted in targeted transgene integration at the *I-sceI* cleavage site. Using different ratios of *Agrobacterium* strains harboring the repair versus the *I-secI* construct, it appeared as if the induction of DSBs by the *I-sceI* was rate limiting, i.e., the best targeting frequency (18.8×10^{-3}) was achieved using a 1:9 ratio of repair: *I-sceI* strain. Thus, the induction of DSB formation and its repair via homology-directed processes are a key to targeted transgene integration.

Using analogous strategies, targeted transgene integration into transgenic reporter loci via homology-directed repair has also been demonstrated after ZFN-mediated DSB formation in tobacco (Cai et al. 2009; Wright et al. 2005). Following stable integration of a defective *GUS/NPT*II reporter gene containing a 600-bp deletion and a ZFN cleavage site, transgenic protoplasts were electroporated with DNA encoding the corresponding ZFN and donor DNA homologous to the target and capable of correcting the deletion. Homology-directed repair of the reporter gene occurred in more than 10% of the protoplasts across multiple transgenic events, i.e., target chromosomal positions (Wright et al. 2005). In a similar study, a pre-integrated reporter construct containing a 3′ partial herbicide resistance gene fragment flanked by ZFN binding sites allowed for in vitro selection following targeted integration of a complementary 5′ sequence from an incoming donor DNA co-transformed with a ZFN-expressing construct (Cai et al. 2009). Approximately 6 kb of target sequence between two ZFN cleavage sites was excised and replaced by 1.9 kb of donor DNA sequence using 1.2 and 1.7 kb of homology directly flanking each of two induced DSBs. These studies clearly illustrate the efficacy of ZFN-mediated DSB induction and the ability to effectively target exogenous DNA using homology-directed repair. NHEJ-mediated repair of DSBs has also been used to integrate DNA sequences in a targeted manner (Weinthal et al. 2013). ZFN-mediated cassette exchange was facilitated between an incoming promoter-less *hpt* gene and a pre-integrated GFP reporter gene both flanked with the same ZFN cleavage sites.

The ability to design ZFNs to cleave virtually any DNA sequence and thereby create investigator-modified, site-specific DSBs has allowed for targeted transgene integration into endogenous gene loci. Using a yeast-based system for screening ZFN efficacy (Doyon et al. 2008), ZFNs were designed against native gene sequences including, tobacco endochitinase (Cai et al. 2009) and maize *IPK1* (Shukla et al. 2009). An herbicide resistance gene driven by a constitutive promoter flanked on each side by 750 bp of endochitinase, *CHN50*, gene sequence was co-delivered with a ZFN expression cassette via *Agrobacterium* (Cai et al. 2009). Although the majority of the resulting transgenic events were the result of random integration, 5–10% of the events appeared to be targeted to the *CHN50* locus. Four different ZFN pairs targeting exon 2 of the maize *IPK1* gene were independently co-delivered with donor constructs containing a herbicide resistance gene cassette flanked by 815 bp of sequence homologous to IPK1 (Shukla et al. 2009). Two different donor constructs were used for targeted integration into the maize *IPK1* gene locus. One carried an autonomous herbicide resistance gene with its own promoter, whereas a second comprised a nonautonomous, i.e., promoter-less, gene that relied on precise trapping of

the endogenous *IPK1* promoter for expression and herbicide resistance. All four ZFN pairs drove targeted gene addition into their respective target sites, albeit with different efficiencies. In addition, site-specific transgene integration was successful using either donor construct with frequencies ranging from 3.4–22.3 % and 16.7–100 % for autonomous and nonautonomous constructs, respectively. Moreover, both monoallelic and biallelic insertions into the *IPK1* locus were observed. These exciting results with designed ZFNs not only extend transgenic technology to targeted transgene integration into endogenous genomic loci but also to include important crop species.

Genome Editing

The ability to make specific modifications to plant genome sequences in order to truly edit genes in a precise and predicable fashion would not only enhance basic understanding of plant biology but also ultimately result in genetically enhanced crops with new traits and improved performance. A recent study suggests that this capability might not be too far from reality (Townsend et al. 2009). Specific mutations in *SuR* genes in tobacco result in resistance to different imidazolinone herbicides. ZFNs were designed to cleave a specific sequences within the tobacco *SuRA* and *SuRB* genes. Electroporation of protoplasts with DNA encoding these engineered ZFNs along with donor DNA templates containing specific mutations resulted in herbicide resistance resulting from homology-directed processes. A surprising outcome was that mutation frequencies in the range of 2 % were observed with up to 1.3 kb removed from the DSB. Although this study relied on herbicide resistance for identifying edited events, the frequencies observed were high enough for screening via high-throughput DNA analysis. A ZFN designed to recognize the *Arabidopsis PPO* gene was co-delivered with a truncated *PPO* gene containing two mutations resulting in tolerance to the herbicide butafenacil using *Agrobacterium* floral dip transformation (de Pater et al. 2013). Targeted PPO modification was observed at a frequency of 3.1×10^{-3}. The combination of sequence-specific DNA cleavage by designed ZFNs and homology-directed DSB repair at investigator-specified break sites makes precise genome modification a reality. This capability, in combination with rapid advances in genome sequencing and bioinformatics, bodes well for the future of plant functional genomics and crop improvement.

Alternative Nuclease Technologies

Although ZFNs have become the most well-established tools for precise genome engineering, alternative nucleases are also available, such as those based on DNA binding domains from transcription activator-like effector (TALE) proteins (Boch and Bonas 2010) or “meganucleases” encoded by mobile introns (Arnould et al. 2011). TALEs are a family of proteins, first discovered in the plant pathogen *Xanthomonas* sp., that contain variable N- and C-terminal domains and a conserved central domain

for DNA binding (Boch et al. 2009). The DNA-binding domain consists of a variable number of tandem 34 amino acid repeats (Fig. 12.2b), whereby binding specificity is determined by the repeat-variant di-residues (RVDs) at positions 12 and 13, which specifically recognize a single nucleotide (Bogdanove and Voytas 2011; Deng et al. 2012; Moscou and Bogdanove 2009). A one-to-one correspondence of the RVDs to a single nucleotide enables TALE designs for any target DNA sequence of interest with a high degree of specificity, though the RVD binding is not completely independent of its neighbor in TALE derivatives (Streubel et al. 2012). TALE-*Fok1* nuclease (TALENs) fusions have been shown to facilitate genome modifications in several species, including human (Hockemeyer et al. 2011), rat (Tesson et al. 2011), zebra fish (Sander et al. 2011), worms (Wood et al. 2011), and plants (Cermak et al. 2011).

In contrast, designing ZFNs is more complex as each finger can only recognize a nucleotide triplet and there are multiple zinc finger designs for a given triplet of base pairs, with complex contextual interactions. Detailed knowledge of DNA binding of individual zinc fingers as well as the influence of various combinations of zinc fingers on binding specificity and affinity is required. Ease of design, high degree of specificity, minimal documented off-target effect, and low cost make TALENs an attractive alternative to ZFNs. Indeed, several recent reports of successful targeted mutagenesis following expression of designed TALENs suggest that this type of nuclease may represent a powerful addition to the arsenal of tools for plant genome modification (Li et al. 2012; Zhang et al. 2013). However, the larger size of TALENs (~3×) might limit their activity in plant cells primarily by effecting their expression negatively. Also, due to their pathogenic origins, TALENs might have a higher regulatory hurdle to cross for product development. Well controlled, comparative studies of ZFNs, and TALENs in plants will be critical for understanding their relative merits for precision genome engineering.

"Meganucleases" are naturally occurring gene-targeting proteins that function as homodimers comprising two identical subunits each 160–200 amino acid residues in size, but also active as a single peptide of two tandem repeat monomers joined together by a linker sequence (Stoddard 2011). Meganucleases typically bind to 20–30 bp DNA target sites which provide remarkable specificity, a primary reason for pursuing these proteins as for genome modification. In contrast to ZFNs and TALENs, the cleavage and DNA-binding domains of meganucleases are not clearly separated. Attempts to reengineer DNA contact points of the endonuclease can be challenging and often compromise nuclease activity (Taylor et al. 2012). Because of these engineering challenges, only a handful of academic groups and companies routinely engineer meganucleases that target novel DNA sites.

Most recently, RNA-guided nucleases from bacteria and archaea, referred to as "clustered regulatory interspaced short palindromic repeats" or CRISPRs have been adapted for genome modification whereby short segments of DNA are transcribed into RNAs which direct sequence-specific cleavage by Cas proteins (Wiedenheft et al. 2012). Using this system, targeted mutations were made in *Arabidopsis* BRI1, JAZ1 and GAI, and in rice ROC5 (Feng et al. 2013).

One of the main challenges associated with the routine deployment of designed nuclease technology for crop improvement is the relative inefficiencies of transgen-

ic event production in all but a few plant species. Recently, *in planta* gene targeting was demonstrated using the meganuclease *I-SceI* (Fauser et al. 2012). In this study, three constructs were transformed independently into *Arabidopsis*: (i) a target with a broken reporter gene and nuclease cleavage sites, (ii) a donor with sequences complementary to the broken reporter, nuclease cleavage sites, and sequences homologous to the target, and (iii) the meganuclease which cuts in both the target and donor. Single copy, homozygous plants for each construct were generated and intermated in the following manner, [(target × donor) × nuclease]. The target contained a 3′ partial *GUS* reporter gene sequence and two *I-SceI* nuclease cleavage sites. The donor contained a 5′ partial *GUS* reporter gene, two *I-SceI* nuclease cleavage sites, sequences homologous to the target and two flanking identical sequences for single strand annealing repair following excision. Nuclease cleavage at the donor locus released the 5′ *GUS* gene fragment and the homologous sequences which provided a template for repair of the target. Observed targeting frequencies were as high as ~1 % on a progeny seed basis. This approach was corroborated in maize whereby inducible expression of I-SceI, combined with in vitro selection on kanamycin, allowed for the detection of the somatic repair of an *NPT*II gene (Ayar et al. 2013).

Future Prospects

The availability of custom targeting reagents such as designed ZFNs, together with the development of high-resolution molecular methods and bioinformatics for trait characterization, is likely to rapidly advance precision genome engineering in plants to enable product development in the near future. It is anticipated that targeted mutagenesis, gene excision, and genome editing will be routinely deployed for functional genomics and trait discovery. Some of these applications of precision genome engineering are likely to be regulated differently, i.e., as non-transgenic (Waltz 2011) and, as such, resulting changes in regulatory policies may have positive economic and social consequences. Similarly, current transgenic product development methods involve the random integration of transgenes into the plant genome, such that generating events and screening them for a trait of interest is time and cost intensive. The ability to target transgene integration into a predetermined genomic site should result in events whereby undesired side effects would be minimized and cycle times associated with product development reduced as event-specific analysis and characterization is simplified. Moreover, additional routes to product development are also likely through retargeting of transgenic loci leading to transgene stacking (Ainley et al. 2013; D’Halluin et al. 2013). In addition, from a trait discovery standpoint, targeting experimental constructs to specific genomic loci effectively removes variability associated with position effect thereby providing a uniform background against which genes and gene constructs can be screened to find lead candidates for new traits. Clearly, the enhanced precision relative to DNA manipulation, made possible by designed ZFNs, opens up some intriguing possibilities for both basic and applied research.

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Chapter 13
Engineered Minichromosome Technology in Plants

James A. Birchler

Introduction

Molecular breeding and transgenic approaches to improving crop plants have revolutionized agricultural practices during the past few decades. Other technologies also hold promise for manipulating the genomes of crop plants, further modifying the genome sequence and incorporating aspects of synthetic biology into crop development programs in the future. One such technology might be engineered minichromosomes or artificial chromosomes. Such entities provide the potential to design a chromosome to specification in attempts to improve crops in various ways.

The potential of plant artificial chromosomes is that multiple genes could be added to plants as a single entity without linkage to other chromosomes. This approach to transformation would also avoid the potentially mutagenic effects of random insertion in the genome, and generate a platform to which one could continue to add more genes or otherwise rearrange the contents (Gaeta et al. 2012). Such chromosomes bring synthetic biology approaches to applications in biotechnology and agriculture as well as basic studies. There is the potential to stack multiple genes and assemble different transgenes that confer desirable traits for plants, to add whole biochemical pathways that confer new properties to them, or to use plants as factories for the mass production of specific proteins or metabolites.

Artificial chromosomes were first generated in yeast by assembling centromeres, a selectable marker, an origin of replication, and capped by telomeres on the ends as an in vitro construct (Murray and Szostak 1983). When this assembly was introduced back into yeast cells, it functioned as an independent chromosome. Subsequently, artificial chromosomes were produced in cultured human cells (Harrington et al. 1997). In this case, centromere sequences were introduced and conglomerates of the input DNA were made by the cell to produce an artificial chromosome. In another approach, the ends of chromosomes, or telomeres, were transformed and

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were found to cleave the chromosome at the sites of potential integration (Farr et al. 1992, 1995; Heller et al. 1996). Using this approach, the chromosome arms were removed leaving only the centromeric region, and then the remaining sequences were engineered using homologous recombination.

The "buildup" method first demonstrated in yeast apparently holds a certain intrigue and was attempted in plants, but it is not presently clear if this approach has worked (Gaeta et al. 2012). It has now become known that there is an epigenetic component to centromere function in plants (and other species) in that the presence of the canonical centromere repeats will not necessarily condition the assembly of a kinetochore (Birchler and Han 2009). In plants, it became known that centromeres could become inactive and several examples have been documented in maize (Han et al. 2006; Gao et al. 2011). It is also the case that centromeres can arise at positions that have no canonical centromere repeats (Nasuda et al. 2005; Fu et al. 2013). Further, transformation of large sections of the centromere arrays will produce stable integrations (Phan et al. 2007). The size of endogenous centromeres for normal function is currently beyond the size of DNA fragments that can be introduced into plant cells (Gaeta et al. 2012). Two reports claiming success via the buildup method have been published (Carlson et al. 2007; Ananiev et al. 2009). A detailed critique of them has been published (Gaeta et al. 2012) and will not be reiterated here other than to note that it is questionable whether the observed entities were in fact as claimed.

Despite the above consideration, it is theoretically possible that the buildup method could work. The fact that reactivation of inactive centromeres occurs (Han et al. 2009) might imply that the centromere arrays can, at some low frequency, organize a kinetochore where a preexisting one did not exist. However, the basis of the reactivation is still unknown and the fidelity of these cases is uncertain. Indeed, in a case in which a fragment was recovered that had had an inactive centromere, the site of the new active centromere had actually shifted (Zhang et al. 2013), again illustrating a disconnect between centromere repeats and activity. Also, attempts to target the centromere-specific histone H3 to synthetic DNA arrays were successful in the targeting aspect, but not in producing a functional centromere (Teo et al. 2013). Thus, further research is needed to understand whether the buildup method of producing artificial chromosomes will work in plants.

The "top down" method of telomere-mediated truncation to remove chromosome arms in order to use an endogenous centromere in the engineered construct has proven successful in plants (Yu et al. 2006, 2007). This approach overcomes the epigenetic aspect of centromere specification by using the endogenous centromere and appending added genes to the truncated chromosome. While the truncation events are presumably random in the genome, the frequency is sufficiently high that very small engineered minichromosomes can be recovered by this method (Yu et al. 2007; Xu et al. 2012; Gaeta et al. 2013).

When plant telomere arrays are placed inside the right border of an *Agrobacterium* T-DNA, a fraction of the potential integrations results in chromosomal fracture at that site and the transgene is appended to the end of the chromosome (Yu et al. 2006). Although the mechanism is not known, presumably, the left border is ligated

to the chromosomal DNA break and the telomere capping apparatus is attracted to the right border, which prevents ligation into the chromosome at this side of the T-DNA. Truncation has been found to occur via *Agrobacterium* and biolistic transformation (Yu et al. 2007). This technique is effective on both normal A and supernumerary B chromosomes. Telomere-mediated truncation in plants was first demonstrated in maize (Yu et al. 2006) but has also been shown to occur in *Arabidopsis* (Nelson et al. 2011; Teo et al. 2011), barley (Kapusi et al. 2012), and rice (Xu et al. 2012).

Coincident with telomere-mediated truncation in plants, the lack of efficient homologous recombination systems for placing genes on truncated chromosomes was overcome by including the transgenes of interest in the truncating constructs. In this way, the minichromosomes generated do not need to be modified subsequently to carry the genes of interest.

Nevertheless, the ability to add to minichromosomes is a desired feature. As a proof of concept, a site-specific recombination cassette of the Cre-lox system was included in the original cases of telomere-mediated truncation. In the presence of Cre recombinase, it was demonstrated that lox sites at the end of the chromosome would participate in interchromosomal recombination events (Yu et al. 2007).

The original finding of telomere-mediated chromosomal truncation used the placement of telomere arrays within the introduced transgene. However, based upon the fact that cotransformations of different sequences often result in cointegration (De Neve et al. 1997; Radchuk et al. 2005), attempts were made to cause truncation via cobombardment of genes and free telomeres. The principle involved is that if telomeres are ligated to the desired genes in the correct orientation, then when incorporated into the chromosome, truncation would occur at the side where the telomere resides and the genes of interest would ligate to the broken end of the chromosome. Other orientations might truncate chromosomes, but if the selectable marker is not transferred to the plant chromosome, such events would not survive selection. This approach has been demonstrated in rice (Xu et al. 2012) and maize (Gaeta et al. 2013).

Truncation of normal chromosomes will produce a monosomic state for the region of the genome that is lost. Thus, these broken chromosomes are unlikely to survive through the haploid gametophyte generations if the deficiency produced eliminates vital genes. Nevertheless, minichromosomes have been recovered containing basically the centromere of a normal chromosome (Gaeta et al. 2013). However, there are a few procedures that can be used to circumvent this issue. First, tetraploids can be used as the target of truncation. This was first found to occur in maize (Yu et al. 2007) and then was used intentionally in *Arabidopsis* (Nelson et al. 2011; Teo et al. 2011) and barley (Kapusi et al. 2012). In this case, a truncated chromosome can be recovered because in a tetraploid plant, other copies of the homologous chromosome will be present in the gametophytes and will supply the gene functions otherwise missing in the truncated chromosome.

Another way in which this issue can be overcome is to target supernumerary B chromosomes for truncation. These chromosomes are basically inert and are not needed in the genome. Thus, deletions of them are not detrimental. In maize, the

frequency of truncations of B chromosomes via biolistic transformation exceeded that of A chromosomes (Yu et al. 2007), suggesting that their recovery was much more efficient. B chromosomes have been studied extensively in maize (Carlson 1986) and rye (Jones and Houben 2003). The latter B chromosome has been transferred to wheat, making B chromosomes available as a target for truncation in this cereal as well.

Another method is to use telotrisomics as the starting material (Xu et al. 2012). These stocks have an extra chromosome arm present in their genomes, but because of their mechanism of formation, only one chromosome arm is present. An engineered minichromosome will be formed if the truncation occurs near the centromere of the telotrisomic. This method has been demonstrated in rice.

Engineered B chromosomes have the property that the dosage can be manipulated either to study dosage effects of the added genes or to amplify the output of the genic cargo. With the maize B chromosome, truncation of the distal tip of the long arm will eliminate the nondisjunction property (Ward 1973; Roman 1947) and transmission will behave as a normal A chromosome. However, because the nondisjunction function is *trans*-acting, the addition of normal B chromosomes back into the genotype will restore the nondisjunction activity. Using this approach, as many as 19 engineered B chromosomes could be accumulated in a selfing and selection scheme (Masonbrink and Birchler 2012a).

Small chromosomes in maize behave in ways that are distinct from normal-sized chromosomes (Han et al. 2007; Masonbrink and Birchler 2012b) and this behavior should be taken into account with regard to engineered minichromosomes. First, pairs of small chromosomes seldom will find their homologue in early prophase when other members of the karyotype are undergoing homologue pairing. The consequence of this fact is that from a pair of chromosomes, the two will independently assort rather than segregate from each other. Thus, they will not be transmitted to all of the progeny. The second property of note is that sister chromatids of small chromosomes in maize will separate in meiosis I, in contrast to full-sized chromosomes. The normal homologue pairs separate with sister chromatid cohesion intact in meiosis I. However, there appears to be a size threshold under which the sister cohesion dissolves even in the event that a pair of small chromosomes does show homologous pairing (Han et al. 2007). Because of this behavior, the sisters separate in meiosis I and randomly progress to one pole or the other in meiosis II.

The randomized distribution of small chromosomes creates a situation in which a parent plant with one chromosome will be transmitted to somewhat less than half of the progeny, and the presence of two will not generate a progeny with 100 % representation. Some progeny will be missing the minichromosome while some might have multiple copies. In circumstances in which a high fidelity of transmission is desirable, it might be possible to place pollen selection on a single copy of the minichromosome. Thus, only pollen grains carrying the minichromosome would function, and when used as a male parent, transmission of the minichromosome should be present in all of the progeny. Alternatively, truncated B chromosomes that retain substantial portions of the chromosome will pair in meiosis and exhibit sister cohesion (Han et al. 2007; Masonbrink and Birchler 2012b), so such

chromosomes with added transgenes will show normal chromosomal transmission. Also, such adjustments are not necessary for species that have a sexual breeding program followed by vegetation propagation in the field. The mitotic stability of truncated chromosomes is very high, so breeding with a truncated chromosome to add it to desired genotypes followed by vegetative propagation could proceed at the present state of the art.

The potential of engineered minichromosomes would be enhanced with the development of procedures to modify them in vivo and to add new sequences. A proof of concept of in vivo modification involved the removal of the selection marker from a maize minichromosome (Gaeta et al. 2013). Using flanking direct repeats of lox sites around the selection marker, plants with the minichromosome were crossed to a line with constitutive expression of the Cre recombinase. Cre catalyzed recombination between the lox sites (Dale and Ow 1990), forming a circular molecule that was excised and, in doing so, removed the selectable marker (see Srivastava et al. 1999; Zubco et al. 2000; Wang et al. 2005; Kerbach et al. 2005) while leaving behind a single lox site. Future constructs could be designed in such a manner that the selectable marker could be removed, and then the remaining lox site following a promoter utilized to select for additions to the minichromosome. Such procedures have been demonstrated for chromosomal inserts (Albert et al. 1995; Srivastava et al. 2004; Yau et al. 2011; Ow 2011) and there is no reason to believe that they cannot be applied to minichromosomes.

The development of engineered minichromosomes is still in its infancy as a field of research. However, the groundwork has been laid for the generation of the basic platforms and their modification in vivo. Future developments that provide a means to add greater numbers of genes at the time of truncation, or in subsequent recombination events, would result in minichromosomes with increased numbers of genes. Such additions to an artificial chromosome will address basic questions, such as how different compositions will affect the behavior of an artificial chromosome in a cell, and practical questions of how to stack multiple genes for the extensive range of properties one might want to introduce into a plant.

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Chapter 14
In Planta Transient Expression Systems for Monocots

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Introduction

Advances in genome and transcriptome sequencing technologies have led to ever-increasing volumes of data being generated, with a corresponding explosion in the numbers of candidate genes of interest being identified from plants and plant-associating organisms. Identification of the functions of these genes poses a major challenge. The more traditional approaches to determining gene function have included abolishing or highly reducing gene expression using chemical or irradiation mutation, or transfer DNA (T-DNA) insertion mutation. Alternatively, the more recent approach has been to generate stable transgenic plants that over-express a fragment of a target gene in order to trigger silencing of the corresponding endogenous plant gene, using the process of post-transcriptional gene silencing (also known as RNA interference, RNAi). Stable transformation of plants to overexpress genes of interest to elucidate their function is also common, particularly in model organisms.

In non-model plants and, in particular in many monocotyledonous species, the often large genome sizes, low transformation efficiency and long life-cycles may mean that these stable technologies may be at best, laborious and time-consuming, and at worst, non-applicable. Even in monocots such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), for which stable transformation has become a relatively routine technique, transformation protocols have been optimized for only a handful of cultivars in each species.

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As a consequence of these drawbacks, there has been increasing interest in transient *in planta* expression systems that allow expression of double-stranded RNAs (dsRNAs) that trigger silencing of endogenous plant genes and, hence, reduce the expression of proteins of interest. These systems allow relatively rapid prescreening of candidate genes for specific traits or cellular responses. This chapter focuses mainly on viral-based transient gene silencing and protein expression systems available for monocots and recent advances therein. However, some common nonviral-based transient systems are also discussed for comparison along with their associated advantages and limitations.

RNAi-Based Downregulation of Endogenous Plant Genes

Virus-Induced Gene Silencing (VIGS)

Virus-induced gene silencing (VIGS) has been used during the past two decades to study the function of plant genes, first in model dicotyledonous species, but now increasingly in commercially important monocotyledonous species. The technique exploits a plant antiviral defence mechanism called post-transcriptional gene silencing (Waterhouse et al. 2001) in which viral RNA is targeted for degradation in a sequence homology-based manner. The genome of a viral vector is engineered to express a short fragment of a transcribed sequence of a plant gene of interest. When the modified virus infects and spreads throughout inoculated test plants, it triggers silencing both of itself and of the endogenous gene corresponding to the plant gene sequence inserted into the vector. This results in the reduction of target gene transcript levels and, subsequently, target gene activity (Lee et al. 2012).

VIGS is particularly useful for prescreening large numbers of candidate genes, and for studying essential genes whose silencing would have a embryonic lethal phenotype. Whilst nearly 30 viruses have been adapted for VIGS in numerous dicot species (Huang et al. 2012), to date there have only been 5 monocot-infecting viruses reported to have been adapted for VIGS: *Barley stripe mosaic Virus* (BSMV), *Brome mosaic virus* (BMV), *Rice tungro bacilliform virus* (RTBV), *Cymbidium mosaic virus* (CymMV) and, most recently, *Bamboo mosaic virus* (BaMV) and its associated satellite RNA satBaMV. Details of the VIGS systems reported to date are summarized in Table 14.1. A brief description of each is given below.

BSMV-Based VIGS Systems

BSMV, the type member of the *Hordeiviridae,* was the first monocot-infecting virus to be adapted for VIGS. It has a tripartite genome which comprises the positive-sense RNAα, RNAβ and RNAγ. The first described BSMV-based VIGS system

Table 14.1 Virus-induced gene-silencing (VIGS) systems available for gene silencing in monocots

Viral source of vectors	Reference	Promoter	Cloning site for insert sequences	Other modifications to viral genome	Inoculation method
BSMV	Holzberg et al. (2002)	T7	*Pac*I and *Not*I sites downstream of stop codon introduced at 3′ end of γb ORF	Majority of βa (*CP*) gene deleted	Rub-inoculation with *in vitro* RNA transcripts
	Tai et al. (2005)	T7	*Bam*HI site created by modifying start codon of γb ORF	Start codon of βa ORF mutated, blocking translation of CP	Rub-inoculation with *in vitro* RNA transcripts
	Bruun-Rasmussen et al. (2007)	T7	*Sma*I, *Pac*I and *Bam*HI sites introduced immediately downstream of γb ORF	(βa ORF retained)	Rub-inoculation with *in vitro* RNA transcripts
	Zhou et al. (2007)	T7	*Nhe*I site downstream of γb ORF	–	Rub-inoculation with *in vitro* RNA transcripts
	Meng et al. (2009)	35S	*Pac*I and *Not*I sites downstream of γb ORF	HDV ribozyme sequence introduced at 3′-end of each viral cDNA sequence	Biolistic transformation with DNA plasmids
	Pacak et al. (2010a)	T7	LIC site downstream of γb ORF	–	Rub-inoculation with *in vitro* RNA transcripts
	Yuan et al. (2011)	Double 35S	LIC site downstream of γb ORF	TRSV satellite RNA ribozyme sequence introduced at 3′-end of each viral cDNA sequence	Agroinfiltration into *Nicotiana benthamiana* intermediate host, then sap inoculation onto cereal plants
	Kawalek et al. (2012)	T7	Introduced *Bam*HI site at the end of βc ORF	Used in combination with modified RNAγ clone created by Bruun-Rasmussen et al. 2007[a]	Rub-inoculation with *in vitro* RNA transcripts
BMV	Ding et al. (2006)	T3	*Hind*III site within 3′-untranslated region of RNA3	Intergenic region of F-BMV RNA3 replaced with that of R-BMV RNA3, generating a chimeric genome into which foreign sequences are cloned; used in combination with F-BMV RNA1 and RNA2	Rub-inoculation with *in vitro* RNA transcripts

Table 14.1 (continued)

Viral source of vectors	Reference	Promoter	Cloning site for insert sequences	Other modifications to viral genome	Inoculation method
	Sun et al. (2013)	Double 35S	*Nco*I and *Avr*II sites immediately downstream of *CP* ORF in chimeric RNA3 clone	HDV ribozyme sequence introduced at 3′-end of each viral cDNA sequence	*Agrobacterium*-mediated vacuum infiltration into rice leaves
	Pacak et al. (2010b)	T7	*Spe*I and *Bam*HI sites separated by a 337-nt spacer sequence inserted downstream of *CP* ORF on RNA3	Cloning site on RNA3 is followed by 295 nt from 3′-end of R-BMV RNA1	Rub-inoculation with *in vitro* RNA transcripts
RTBV	Purkayastha et al. (2010)	Maize ubiquitin	*Pac*I and *Mlu*I sites downstream of truncated ORF IV	ORF I, II and part of ORF IV deleted, leaving only ORF III and a truncated ORF IV; a tRNA-binding site and the Kozak sequences introduced upstream of ORF III	Agroinfiltration into rice (*Oryza sativa*) plants
CymMV	Lu et al. (2007)	T3	*Sma*I site between a duplicated fragment of the CP subgenomic promoter and the CymMV *CP* gene	–	Rub-inoculation with *in vitro* RNA transcripts
BaMV + satBaMV	Liou et al. (2013)	35S	In the BaMV genome, *Eco*RV, *Nco*I and *Not*I sites have been introduced between a duplicate CP subgenomic promoter and the BaMV *CP* gene *Eco*NI site towards the 3′-end of the *P20* ORF in satBaMV	–	Mechanical inoculation of DNA constructs onto leaves of *Chenopodium quinoa* intermediate host, then sap inoculation onto *Brachypodium distachyon* plants

[a] Allows fragments to be inserted into two regions of the BSMV genome (each on a different genomic RNA)

ORF open reading frame, *LIC* ligation-independent cloning, *CP* coat protein, *BSMV Barley stripe mosaic virus*, *BMV Brome mosaic virus*, *RTBV Rice tungro bacilliform virus*, *CymMV Cymbidium mosaic virus*, BaMV *Bamboo mosaic virus*, *HDV Hepatitis delta virus*, *TRSV Tobacco ringspot virus*

(Holzberg et al. 2002) was based on complementary DNA (cDNA) clones of the ND18 strain of BSMV under the control of the bacteriophage T7 promoter (Petty et al. 1989). Holzberg et al. (2002) added *Pac*I and *Not*I sites downstream of the *γb* gene in the plasmid representing BSMV RNAγ, for the insertion of foreign sequences. A stop codon was introduced between the *γb* open reading frame (ORF) and the *Pac*I site to prevent translation of the inserted sequences. In addition, the majority of the *βa* gene, which encodes the viral coat protein (CP), was deleted from the genomic RNAβ. Deletion of the CP appeared to enhance silencing efficiency, although it also had the less desirable effect of increasing viral-induced symptom severity.

There are now a number of variant BSMV–VIGS vectors available, the main details of which are summarized in Table 14.1. In most of these systems, foreign gene fragments are introduced into RNAγ using cloning sites downstream of the *γb* ORF, as in the original BSMV–VIGS vector developed by Holzberg et al. (2002). An exception is the variant system developed by Tai et al. (2005), in which the start codon for the *γb* gene sequence was modified to create a *Bam*HI site for the insertion of a foreign sequence. This modification also blocked translation of the BSMV *γb* silencing suppressor. Kawalek et al. (2012) introduced a cloning site for foreign sequences at the end of the *βc* ORF in the plasmid representing BSMV RNAβ. When combined with wild-type RNAα and the RNAγ VIGS vector developed by Bruun-Rasmussen et al. (2007; see also Table 14.1 for details) this facilitated simultaneous silencing of two unrelated genes when fragments targeting different genes were inserted into each of the cloning sites on RNAβ and RNAγ. However, delivery of a foreign gene fragment from RNAβ appeared to induce weaker silencing than when gene fragments were inserted into RNAγ (Kawalek et al. 2012).

Other modifications to the BSMV–VIGS vectors have been made in attempts to increase the throughput and ease of use of this research tool. A DNA-based BSMV–VIGS system developed by Meng et al. (2009) eliminated the costly *in vitro* transcription step by cloning the BSMV cDNA sequences from the vectors described by Holzberg et al. (2002) into separate binary vectors (whilst retaining the *βa* gene in the RNAβ). The T7 promoter was replaced with the 35S promoter from *Cauliflower mosaic virus* and a ribozyme sequence added downstream of each viral cDNA sequence to generate the correct 3′-end after transcription. Particle bombardment could then be used to introduce the plasmids containing the BSMV genomes into barley seedlings (Meng et al. 2009). Another variant retained the T7 promoter but replaced the original cloning site downstream of the CP ORF with a ligation-independent cloning (LIC) site, to facilitate efficient insertion of foreign gene sequences (Pacak et al. 2010a). Yuan et al. (2011) combined these two approaches by cloning the BSMV genomes into binary vectors under the control of a double 35S promoter and introducing a LIC site downstream of the *γb* ORF. These plasmids were then delivered via agroinfiltration into the leaves of an intermediate host, *Nicotiana benthamiana,* and the sap from these plants used to rub-inoculate leaves of a large number of monocot plants (Yuan et al. 2011).

BMV-Based VIGS Systems

BMV is a positive-strand RNA virus of the genus *Bromovirus,* with a tripartite genome comprising RNA1, RNA2 and RNA3. Ding et al. (2006) created and modified BMV clones to produce a hybrid BMV strain (H-BMV)-based VIGS vector. RNA1 and RNA2 from the rice-infecting fescue strain of BMV (F-BMV) were used in combination with RNA3 of the Russian strain of BMV (R-BMV). This allowed foreign gene sequences to be inserted downstream of the CP ORF using a unique *Hin*dIII site in the cDNA clone of R-BMV RNA3 (the F-BMV cDNA clone contained two *Hin*dIII sites). Successful silencing of the *phytoene desaturase* (*PDS*) gene using the H-BMV vector was demonstrated in barley, rice (*Oryza sativa*) and maize (*Zea mays*). However, in order to reduce the severity of visual symptoms induced by the virus itself, Ding et al. (2006) proceeded to modify the cDNA clone representing F-BMV RNA3 by replacing a portion of the intergenic sequence between the movement and CP genes with the corresponding fragment from the R-BMV RNA3 cDNA clone. This intergenic region from R-BMV RNA3 had been shown previously to be responsible for a higher accumulation of R-BMV RNA3 and subgenomic RNA4 during plant infection (Hema and Kao 2004). The resulting BMV vector containing the chimeric RNA3, and RNA1 and RNA2 from F-BMV accumulated to higher levels than the parental F-BMV strain and induced fewer disease symptoms than R-BMV or H-BMV in infected rice plants (Ding et al. 2006).

The chimeric BMV VIGS system has been modified further more recently by transferring each of the BMV cDNA clones into a binary vector between a double 35S promoter and a ribozyme sequence (Ding et al. 2010; Sun et al. 2013; Table 14.1). In addition, a new multiple cloning site has been added at the 3′-end of the CP ORF to allow directional cloning of foreign gene fragments into the chimeric RNA3 clone. This DNA-based BMV vector can be introduced into rice plants via *Agrobacterium*-mediated vacuum infiltration (Ding et al. 2010).

Pacak et al. (2010b) used a different R-BMV-based vector for VIGS studies in rice. In this vector, which was originally developed for studying viral RNA recombination (Alejska et al. 2005), foreign gene sequences are also inserted at a cloning site downstream of the CP ORF. This cloning site comprises *Spe*I and *Bam*HI sites separated by a 337-nt spacer sequence which allows expression of either separated inverted repeats or a single-gene fragment.

RTBV-Based VIGS System

RTBV is a member of the genus *Pararetrovirus,* with a monopartite double-stranded DNA genome that contains four ORFs. ORF III encodes a polyprotein which is post-translationally processed into the CP, an aspartate protease and a reverse transcriptase enzyme with ribonuclease H activity. It is possible to remove ORF I, ORF

II and part of ORF IV (leaving only ORF III and the remainder of ORF IV) from the virus without removing its ability to replicate and spread systemically in host plants (Purkayastha et al. 2010). These authors generated a RTBV-derived VIGS vector by cloning a 6.1-kb fragment, representing ORF III and truncated ORF IV from an Indian isolate of RTBV, as a partial dimer into the T-DNA of a binary vector. This fragment also incorporated the constitutively expressed maize ubiquitin promoter, a transfer RNA (tRNA)-binding site and the Kozak sequences at the 5′-end, whilst a multiple-cloning site for the insertion of foreign gene fragments was introduced at the 3′-end of the fragment. VIGS of the marker gene *PDS* using this RTBV vector was demonstrated in rice (Purkayastha et al. 2010).

CymMV-Based VIGS System

The potexvirus CymMV, which infects many species within the *Orchidaceae,* is the first virus to have been adapted specifically for VIGS in non-grass monocots (Lu et al. 2007). CymMV has a monopartite positive-sense single-strand RNA genome of approximately 6200 nt, encoding an RNA-dependent RNA polymerase; triple gene block movement proteins 1, 2 and 3; and CP. A symptomless strain of CymMV was modified to function as a VIGS vector by inserting a duplicated fragment (60-nt long) of the subgenomic promoter of the *CP* gene immediately upstream to the original CP subgenomic promoter. Foreign gene fragments are inserted downstream of this duplicated promoter which subsequently drives transcription of the foreign sequence during viral infection. VIGS of the marker gene *PDS* and the orchid floral organ identity gene *PeMADS6* were demonstrated in the native orchid species *Phalaenopsis amabilis* (moon orchid) and in a commercial cultivar *Phalaenopsis* Sogo Musadium (Fig. 14.1).

BaMV-Based VIGS System

BaMV, another member of the *Potexvirus* genus, also has a monopartite positive-sense single-strand RNA genome that contains five conserved ORFs (Lin et al. 1994; Yang et al. 1997). It is the most recent monocot-infecting virus to have been modified to function as a VIGS vector and the only one for which a satellite RNA associated with the virus has also been modified for VIGS application (Liou et al. 2013). The modified BaMV VIGS vector was generated by the duplication of the subgenomic promoter for the BaMV *CP* gene, which was inserted upstream of the original CP subgenomic promoter. A multiple cloning site was introduced downstream of the duplicated promoter to allow insertion of foreign gene fragments (Table 14.1).

Fig. 14.1 *Cymbidium mosaic virus* (CymMV)-mediated VIGS of the floral organ identity gene *PeMADS6* in floral tissue of *Phalaenopsis* spp. (reproduced with permission from Lu et al. 2007). Plants were inoculated with buffer (**a**, **d** and **g**), an empty virus control construct (**b**, **e** and **h**) or with a CymMV VIGS construct targeting *PeMADS6* for silencing (**c**, **f** and **i**). **a**–**c** and **g**–**i**, *Phalaenopsis* Sogo Musadium. **d**–**f**, *Phalaenopsis amabilis* var. *formosa*. Green streaks developed on the sepals, petals and lips of flowers of plants in which *PeMADS6* had been silenced (indicated by *black arrows*), but not on flowers of buffer treated or virus control-infected plants. VIGS virus-induced gene silencing

The BaMV-based VIGS system differs from other VIGS systems for monocots in that the satellite RNA associated with BaMV, satBaMV, can be modified to express a second gene fragment during virus infection, such that two unrelated genes can then be silenced simultaneously when fragments from different genes are inserted into the BaMV and satBaMV genomic sequences (Liou et al. 2013; see also Sect. 2.2.3). Satellite RNAs are dependent on their associated (helper) virus for replication, encapsidation and cell-to-cell movement (Roossinck et al. 1992). Therefore, satBaMV replicates and induces silencing only when it is co-inoculated onto a host plant together with BaMV (Liou et al. 2013).

satBaMV is a linear RNA molecule of 836 nucleotides, encoding a 20-kDa protein (P20) which is flanked by 5′- and 3′-untranslated regions (Lin and Hsu 1994). Foreign gene fragments can be inserted into satBaMV at an *Eco*NI cloning site present towards the 3′-end of the P20 ORF. Silencing of single-gene targets using either the BaMV-based vector or a modified satBaMV with an unmodified (i.e. not carrying a target gene fragment) BaMV helper virus has been demonstrated in two model species, namely *N. benthamiana* and *Brachypodium distachyon* (Liou et al. 2013). However, simultaneous silencing of two target genes by expressing a fragment of one gene from BaMV, and a fragment of the second gene from satBaMV has only been demonstrated in *N. benthamiana* (Liou et al. 2013).

Comparison of the Infection Biology of the Viral Vectors Currently Adapted for VIGS

One of the first considerations when assessing the applicability of a VIGS vector is the viral vector host range (see Table 14.2). However, of equally high importance is the biology of the host–virus interaction. For example, BSMV can be transmitted through the seed and pollen of infected plants in many species, and BSMV-mediated VIGS has been demonstrated in wheat ears, pollen and developing grain and even in the progeny of infected plants (Bennypaul et al. 2012; Lee et al. 2012; Ma et al. 2012b). BSMV-mediated VIGS has also been reported in wheat roots (Bennypaul et al. 2012) and wheat stems (Fig. 14.2), as well as in the leaves of many monocot species (Holzberg et al. 2002; Scofield et al. 2005; Renner et al. 2009; Demircan and Akkaya 2010; Wang et al. 2010; Pacak et al. 2010a; Martin et al. 2013). By contrast, BMV, CymMV, RBTV and BaMV have not been reported to be seed or pollen transmitted (Description of Plant Viruses database, www.dpvweb.net), and it is unlikely that these viruses would be able to mediate VIGS in pollen or developing grain. Nonetheless, the CymMV-based VIGS system has been used to silence gene expression in orchid floral tissue (Lu et al. 2007; Fig. 14.1). Currently, there are little data available on the applicability of BMV-, RBTV- or BaMV-mediated VIGS to plant tissues other than leaves.

The Potential for Improving Existing VIGS Systems

VIGS was first deployed as a reverse genetics tool in monocot species for the functional analysis of host genes involved in plant–fungal pathogen interactions (Hein et al. 2005; Scofield et al. 2005). In recent years, there has been an increased application of VIGS to disciplines beyond fungal plant pathology, such as those relating to the study of plant cell wall biosynthesis (Oikawa et al. 2007; Held et al. 2008), insect–wheat interactions (van Eck et al. 2010), nutrient uptake and translocation (Pacak et al. 2010a; Li et al. 2014), plant–virus interactions (Shi et al. 2011; Cao et al. 2012), abiotic stress tolerance (Liang et al. 2012; Rong et al. 2014), leaf development (Wang et al. 2011; Ma et al. 2012a), grain development (Bennypaul et al. 2012), and flower development (Lu et al. 2007), amongst others. As the application of VIGS in monocots has increased, naturally so has interest in developing and modifying existing VIGS systems for enhanced deployment. In particular, there have been significant advances in improving BSMV- and BMV-based VIGS. However, it is evident that there are still many limitations associated with these systems. The approaches that have been used to reduce or overcome these limitations are discussed later, together with potential methods for further improving existing VIGS systems in monocots. As the aim of this chapter is to provide an overview of existing transient expression systems in monocotyledonous species, the potential of modifying other viruses as VIGS vectors is not discussed here.

Table 14.2 Potential for exploiting existing VIGS vectors in other monocotyledonous species

Virus adapted for VIGS	Virus host range[a]	Monocotyledonous species targeted by VIGS	References[b]
BSMV	Infection under experimental conditions demonstrated for more than 240 members of the *Poaceae*, some members of the *Chenopodiaceae* (in which BSMV forms local lesions) and one member each of the *Solanaceae*, *Amaranthaceae* and *Primulaceae*; also infects *Nicotiana benthamiana*	Barley (*Hordeum vulgare*)	Holzberg et al. (2002)
		Wheat (*Triticum aestivum*)	Scofield et al. (2005)
		Brachypodium distachyon	Demircan and Akkaya (2010)
		Culinary ginger (*Zingiber officinale*)	Renner et al. (2009)
		Haynaldia villosa	Wang et al. (2010)
		Oat (*Avena sativa*)[c] Oat (*Avena strigosa*)[c]	Pacak et al. (2010a)
		Ryegrass (*Lolium temulentum*)	Martin et al. (2013)
BMV	Infects many members of the *Poaceae*; also infects several *Chenopodium* species (in which BMV forms local lesions) and *N. benthamiana*	Barley (*Hordeum vulgare*)	Ding et al. (2006)
		Rice (*Oryza sativa*)	
		Maize (*Zea mays*)	
		Sorghum (*Sorghum bicolor*)	Martin et al. (2011)
RTBV	Infects a limited range of species within the *Poaceae* and *Cyperaceae*	Rice (*Oryza sativa*)	Purkayastha et al. (2010)
CymMV	Infects mainly *Orchidaceae*, but also species in a few other plant families	*Phalaenopsis* spp.	Lu et al. (2007)
BaMV	Infects bamboo species from at least seven genera; also reported to infect *N. benthamiana*, *Brachypodium distachyon* and *Hordeum vulgare*, but not *Oryza sativa* or *Zea mays*	*Brachypodium distachyon*	Liou et al. (2013)

[a] Virus host range information as described on the Description of Plant Viruses database (www.dpvweb.net) or on the Virus Identification Data Exchange database (http://jpkc.jluhp.edu.cn/zwkx/zwbl/improve/ref/vide/refs.htm)

[b] References given are the first papers describing VIGS in the relevant virus–host combinations

[c] Only weak silencing observed

VIGS virus-induced gene silencing, *BSMV Barley stripe mosaic virus*, *BMV Brome mosaic virus*, *RTBV Rice tungro bacilliform virus*, CymMV *Cymbidium mosaic virus*, BaMV *Bamboo mosaic virus*

Fig. 14.2 *Barley stripe mosaic virus*-mediated VIGS of *phytoene desaturase* (*PDS*) gene in wheat leaves, leaf sheaths and ears. Silencing of *PDS* results in a photobleached phenotype in aerial tissues of BSMV:*asPDS*-infected wheat plants (**a**). Upper uninoculated leaves from control (BSMV:*asGFP*-infected; *top*) or BSMV:*asPDS*-infected (*bottom*) plants. *B–C*. Leaf sheaths of **b** BSMV:*asGFP*-infected and **c** BSMV:*asPDS*-infected wheat plants. *D–E*. Ears of **d** BSMV:*asGFP*-infected or **e** BSMV:*asPDS*-infected plants. VIGS virus-induced gene silencing

Assessing and Reducing Interference from Virus-Induced Symptoms

An important consideration when utilizing VIGS to investigate plant gene function is that infection of host plants by the virus vector itself may influence host developmental and cellular processes. Varying degrees of chlorosis and stunting are common symptoms induced by viral infections, and may make the phenotype due to silencing of target genes difficult to analyze when symptoms are severe. It is therefore important to include appropriate controls during VIGS experiments, to determine the effect or influence of virus infection itself on the phenotype under investigation. This is particularly true when studying responses to abiotic or biotic stresses, as virus infection may activate certain host defence signalling pathways that could

affect plant responses to other pathogens or stresses. For example, infection of wheat plants with BSMV was reported to enhance resistance to the blast fungus *Magnaporthe oryzae,* although the interaction with *Blumeria graminis,* the causal agent of powdery mildew disease, did not appear to be affected (Tufan et al. 2011).

The severity of symptoms induced by BSMV- and BMV-silencing vectors has been shown to be variable between different varieties or genotypes within the same species (reviewed by Ramanna et al. 2013). Careful selection of plant varieties for VIGS experimentation can help to mitigate the problem of severe VIGS vector-induced symptoms that may obscure or confuse gene function analyses. However, silencing efficiency is also variable and the choice of host genotype may therefore require a compromise between varietal differences in silencing efficiency and severity of symptoms induced by the VIGS vector (see the section 'Silencing Stability and Efficiency').

Another possible strategy to minimize undesirable side effects of infection by the viral vector on host development and metabolic processes is to develop a VIGS vector that induces mild or no visible symptoms. The CymMV-based VIGS vector was developed using a specially selected symptomless strain of the virus (Lu et al. 2007). The RTBV-based VIGS vector also infects rice plants without inducing visible systems (Purkayastha et al. 2010). However, in the RTBV-VIGS system, this was suggested to be due to the replacement of the original RTBV promoter by the maize ubiquitin promoter, as it is thought that the symptoms normally associated with RTBV infection in rice are related to the sequestration of two host transcription factors via interactions with a Box II element in the viral promoter (Dai et al. 2008). Thus, it may be possible to minimize symptom severity by utilizing different viral strains, or by modifying or removing elements associated with symptom induction (if these are known) in the viral vector. It must be noted though that the absence of visible virus-induced symptoms does not indicate that there are no effects on host metabolism and signalling at the cellular level. A complete lack of virus symptoms on experimental plants could also mean that plant infection would have to be verified by enzyme-linked immunosorbent assay (ELISA)- or reverse transcription polymerase chain reaction (RT-PCR)-aided detection of the virus vector, rather than by monitoring the appearance of viral symptoms.

Silencing Stability and Efficiency

A range of factors affects the stability and efficiency of silencing that can be achieved in a VIGS experiment. A problem associated particularly with VIGS is the question of foreign gene fragment insert stability in the virus vector. In small grain cereal species in particular, VIGS phenotypes are generally observed only in two or three systemically infected leaves and in the intermediate stem tissue when young vegetative stage plants are inoculated (Holzberg et al. 2002; Scofield et al. 2005; Ding et al. 2006). Usually, the silencing phenotype in newer leaves becomes increasingly patchy and incomplete until it appears to fade entirely. This tends to be associated

with the loss of the foreign gene insert from the virus vector through recombination (Bruun-Rasmussen et al. 2007). Both the size and possibly the actual sequence of the insert appear to affect insert stability and, therefore the efficiency of silencing. Studies carried out with fragments of different lengths inserted into the BSMV vector indicated that fragments larger than 500 bp are often unstable, whilst fragments smaller than 120 bp may not efficiently induce silencing (Scofield et al. 2005; Bruun-Rasmussen et al. 2007; Yuan et al. 2011).

The orientation and design of foreign gene fragments in the vector may also have a significant effect on silencing efficiency. Fragments inserted into the BSMV or BMV vectors in antisense orientation generally induce more efficient silencing (and never less efficient silencing) when compared to fragments inserted in sense orientation (Lacomme et al. 2003; Pacak et al. 2010b). The expression of a short direct-inverted repeat from a BSMV vector was suggested to induce stronger and more stable silencing than expression of an antisense fragment for the same target gene (Lacomme et al. 2003). However, later studies showed that short inverted repeats were in some cases highly unstable and less efficient than antisense fragments inserted into BSMV and BMV VIGS vectors (Pacak et al. 2010a; b). Therefore, it may be that the stability of short inverted repeats is dependent on their specific sequence.

VIGS construct design can be aided by the use of predictive tools such as the siRNA-Finder (si-Fi) software developed at IPK-Gatersleben in Germany (http://labtools.ipk-gatersleben.de/). This software allows the researcher to pinpoint regions of a target gene sequence that are most likely to generate a large number of small interfering RNAs (siRNAs) efficient in inducing gene silencing. si-Fi can also be used to help design constructs for stable RNAi or VIGS with no or minimal off-target silencing effect (provided the complete genome or the transcriptome sequences from the host plant species are available). Whereas the *in silico* predictions are not a substitute for *in planta* validation of the efficiency of VIGS constructs, si-Fi can be very useful when designing VIGS constructs (Lee et al. 2014).

The choice of host genotype is another important factor that influences silencing efficiency. Several studies have shown that there can be a substantial variation in the level and persistence of silencing induced by the same VIGS construct in different varieties or genotypes within the same host species (Hein et al. 2005; Meng et al. 2009; Cakir and Tör 2010; Bennypaul et al. 2012). In some wheat genotypes inoculated at the two-leaf stage, BSMV-induced silencing of *PDS* may persist throughout the developing plant into the flag leaf and spike, and may even be inherited in a percentage of the progeny of these plants, whilst in others the photobleaching phenotype associated with *PDS* silencing is no longer visible in the sixth leaf (Bruun-Rasmussen et al. 2007; Bennypaul et al. 2012; Lee et al. 2012).

An additional consideration related to VIGS is that of the ambient temperature. Generally, daytime temperatures of 20–24 °C appear to yield better BSMV-induced gene silencing in wheat and barley than temperatures of 16–18 °C or 26–28 °C (Scofield et al. 2005; Bruun-Rasmussen et al. 2007; Cakir and Tör 2010). Similarly, Purkayastha et al. (2010) observed RTBV-mediated silencing of *PDS* in the majority (around 80 %) of inoculated rice plants when the plants were kept at 27 °C, but

not in plants in glasshouses maintained at 30 °C, although there were also other differences in the growth conditions that may have affected silencing efficiency. In addition to the effect of temperature, it has been found that light intensities greater than 300 μmol m^{-2} s^{-1} appear to induce more severe symptoms in wheat cultivars in response to BSMV infection in specific growth room conditions (i.e. 23 °C day/20° C night, 16 h light), perhaps because the higher light levels compound the stress induced by virus infection (Lee WS, Kanyuka K, unpublished).

Silencing of Multiple Genes

As VIGS, like all other RNAi-based techniques, involves silencing of target genes based on their sequence homology to the silencing inducer dsRNA expressed inside plant cells, it is possible using one VIGS construct to knock-down expression of related or even families of genes by targeting sequences conserved between these genes. What is more difficult is to silence two unrelated genes in the same plant cell. Limited success has been achieved by inserting fragments of two individual genes joined *in cis* into BSMV RNAγ (Cakir and Scofield 2008; Cakir and Tör 2010). This enabled simultaneous silencing of the two target genes although the degree of silencing for each gene was highly variable between replicate experiments, and was always less efficient than silencing induced by constructs targeting only one plant gene. Interestingly, the order the fragments were inserted into the virus vector appeared to affect the degree of silencing observed for each gene (Cakir and Tör 2010). Mixed infections in which BSMV RNAα and RNAβ were combined with two BSMV RNAγ sequences, each carrying a fragment of a different target gene, also induced limited silencing of both genes in the same area of leaf tissue. However, silencing efficiency again was highly variable, and it was not possible to confirm whether both genes were silenced in the same plant cell (Cakir and Scofield 2008; Cakir and Tör 2010). In this respect, the BaMV/satBaMV combined VIGS system described in Sect. 2.1.5 differs because the helper virus and satellite RNA replicate together in the same host cell. Hence, when BaMV and satBMV have been modified to carry different silencing constructs, any observed silencing of target genes is likely to have been activated in the same plant cell as discussed further later.

More success was observed in a recent study in which two individual fragments targeting different genes were inserted into BSMV RNAβ and RNAγ, respectively (Kawalek et al. 2012). Simultaneous silencing of the two target genes was observed consistently, although silencing from RNAγ was always stronger than from RNAβ, probably because BSMV RNAγ accumulates to much higher levels than that of RNAβ during plant infection. However, foreign inserts in BSMV RNAβ were more stable than sequences inserted into RNAγ (Kawalek et al. 2012). Interestingly, when fragments targeting the same gene were inserted into both RNAβ and RNAγ, the overall level of silencing was much better than when fragments were expressed from one genomic RNA component alone, suggesting that this could be an attractive strategy for inducing more efficient and possibly more stable silencing of a single target gene or gene family (Kawalek et al. 2012).

In a different approach, simultaneous silencing of two genes in the same plant cell using BaMV and its associate satellite RNA satBaMV was reported in *N. benthamiana* by Liou et al. (2013). In this study, *green fluorescent protein gene* (*GFP*)-transgenic *N. benthamiana* plants were co-infected with BaMV modified to express a fragment of the *sulphur* gene and satBaMV carrying a fragment of *GFP*. Although simultaneous silencing of two genes has not yet been demonstrated in a monocot species with this system, successful silencing of single-gene targets by expressing a gene fragment from either BaMV or satBaMV has been reported in *B. distachyon* (Liou et al. 2013), indicating that this may be a viable system for multiple gene silencing in monocots. However, recombinant BaMV and satBaMV from which gene sequence inserts had been partially or fully lost (see the section 'Silencing Stability and Efficiency') could be detected already in both the directly inoculated and systemically infected leaves of *B. distachyon* plants by 16 days post inoculation (Liou et al. 2013). Further studies and investigation into the stability and duration of silencing induced by this BaMV-/satBaMV-based system are required to assess applicability of the system to other monocotyledonous species.

Expanding the Host Range of Existing VIGS Systems

To date, most monocot VIGS studies have been carried out predominantly in the crop species barley (*Hordeum vulgare*), hexaploid wheat (*Triticum aestivum*) and, to a lesser extent, maize (*Zea mays*) and rice (*Oryza sativa*). However, the successful application of VIGS has been reported for a growing number of other, mostly small grain cereal crops (Table 14.2), although in many of these only silencing of the visual marker *PDS* has been demonstrated (Renner et al. 2009; Demircan and Akkaya 2010; Pacak et al. 2010a; Martin et al. 2013). Both BSMV and BMV have a broad experimental host range, particularly within the *Poaceae* (Table 14.2), and there is considerable potential for the application of VIGS to other grass and non-grass species using the vectors currently available. For example, in a recent publication (Ramanna et al. 2013), there was mention of an unpublished but successful use of the BMV binary VIGS vector for gene silencing in foxtail millet (*Setaria italica*) and switchgrass (*Panicum virgatum*). Apparently, this was done by first delivering the BMV plasmids into *N. benthamiana* leaves via agroinfiltration and using the sap to rub-inoculate leaves of monocots. Similarly, CymMV has a broad host range within the *Orchidaceae,* one of the two largest families of angiosperms. Therefore, future applications for this VIGS vector may be expected to extend beyond the orchid species within the genus *Phalaenopsis*.

When testing the applicability of existing VIGS systems in other monocotyledonous species, multiple varieties or accessions should be tested in order to identify genotypes in which efficient silencing is coupled with moderate or mild virus-induced symptoms (see sections 'Assessing and Reducing Interference from Virus-Induced Symptoms' and 'Silencing Stability and Efficiency'). In addition, certain genotypes may be resistant to the VIGS vector, whilst other accessions of

the same species may be susceptible. For example, of 150 barley accessions and landraces tested at Rothamsted Research with the ND18 wild-type strain of BSMV, 89 were potentially suitable hosts for further VIGS studies, 32 developed overly severe symptoms and there was poor or no infection in 29 genotypes (Lee WS, Ruiz O, Kanyuka K, unpublished).

The currently available BMV-VIGS vectors are based on either the R-BMV strain or a hybrid virus comprising sequences from the two different strains, R-BMV and F-BMV (see the section 'BMV-Based VIGS Systems'). Similarly, almost all of the BSMV-VIGS vectors described to date are based on the ND18 strain of BSMV. The one exception is a vector system described by Pacak et al. (2010a), comprising RNAα and RNAβ from the oat-infecting strain CV42 in combination with RNAγ modified for insertion of foreign sequences from the ND18 strain. The ND18 strain does not infect oat. Using this hybrid BSMV, Pacak et al. (2010a) were able to observe limited photobleaching due to silencing of *PDS* in diploid oat (*Avena strigosa*) and hexaploid oat (*Avena sativa*) cultivars. Whilst the silencing phenotype observed was not very robust in this study, these results demonstrate the potential for utilizing hybrid or alternative viral strains to expand the VIGS target host range.

Nonviral Transient-Induced Gene-Silencing Systems

There are a number of nonviral transient gene-silencing systems that utilize the innate plant defence mechanism of post-transcriptional gene silencing. As with VIGS, these systems involve the delivery of dsRNA fragments to plant cells that activate the host RNA silencing machinery, leading to sequence-specific degradation of a target RNA. Two of these systems, namely microprojectile bombardment and *Agrobacterium*-mediated delivery, have been applied for gene function studies in monocots. The advantages and disadvantages associated with each system are discussed below.

Microprojectile/Biolistic-Bombardment-Based Expression

Microprojectile bombardment, otherwise known as biolistic bombardment, has been used extensively for single-cell transient overexpression of DNA in tissues of monocot plants since the early 1990s (see section 'Microprojectile/Biolistic Bombardment'). In 2000, microprojectile bombardment of dsRNA into leaf epidermal cells was used to trigger transient-induced gene silencing (TIGS) of target genes in maize, barley and wheat (Schweizer et al. 2000). Microprojectile bombardment-mediated TIGS has also been demonstrated in rice protoplasts and leaf cells (Miki and Shimamoto 2004). Since then, this technique in monocots has been applied most extensively to the identification and study of wheat and barley genes involved in resistance or susceptibility to different *formae speciales* of the powdery mildew

fungus *Blumeria graminis* (Azevedo et al. 2002; Christensen et al. 2004; Douchkov et al. 2005; Dong et al. 2006; Zimmermann et al. 2006). Powdery mildews only attack the outermost epidermal cell layer, thus making this single-cell TIGS system highly amenable to the study of cereal–powdery mildew interactions. As TIGS is triggered within 2–3 days after the target tissues have been bombarded with tungsten or gold microparticles (of approximately 0.4- or 2-μm diameter, respectively) coated with dsRNA (Schweizer et al. 2000) this technique has the advantage of being relatively rapid. Furthermore, the development of high-throughput cloning methods for the generation of inverted-repeat RNAi constructs for microprojectile bombardment, such as one based on the Gateway cloning system and reported by Douchkov et al. (2005), means that libraries of gene sequences can be generated and screened in a high-throughput manner.

Another advantage of microprojectile bombardment-mediated TIGS is that plasmids carrying silencing constructs can be co-bombarded with plasmids containing reporter gene constructs. As co-bombarded plasmids have a high co-expression rate, this aids the identification of dsRNA construct transformed cells as reporter gene expression can be used as an indicator of successful transformation (Schweizer et al. 1999; see also section 'Microprojectile/Biolistic Bombardment'). Indeed, microprojectile bombardment can be used to deliver multiple plasmid vectors into the same cell, as the gold or tungsten particles can be coated with plasmid DNA comprising a mixture of different vectors. This was demonstrated by Marzin and colleagues (2008), who co-bombarded barley leaf epidermal tissue with three plasmids; two encoding GFP and *Discosoma* sp. red fluorescent protein (DsRed), respectively, and one carrying an RNAi construct in order to trigger TIGS against a candidate gene of interest. This combination was used to test candidate barley genes for their involvement in cell-autonomous responses to dehydration stress by monitoring the effect of TIGS of target genes on DsRed fluorescence. The fluorescence of DsRed is reduced under denaturing conditions, such as those imposed by drought stress (Marzin et al. 2008). Although TIGS of multiple gene targets using co-bombardment of two or more dsRNA constructs has yet to be reported in monocots, this is a distinct possibility.

A notable disadvantage of microprojectile bombardment-mediated TIGS is that silencing is only triggered in directly bombarded cells. Thus, although this technique is useful for studying genes involved in cell-autonomous processes or single-cell interactions in leaf or potentially root epidermal cell layers (see the section 'Microprojectile/Biolistic Bombardment'), it is not suitable for studying genes involved in developmental processes or plant–pathogen interactions which involve multiple cells or whole tissues (Marzin et al. 2008). The set-up costs of establishing a microprojectile bombardment system can also be expensive.

Agrobacterium-Mediated Delivery

Agrobacterium tumefaciens-mediated transient gene-silencing assays have been used increasingly to study gene function in a number of dicotyledonous species (Kapila et al. 1997; Johansen and Carrington 2001; Wroblewski et al. 2005;

Bhaskar et al. 2009). *A. tumefaciens* delivers RNAi constructs into plant cells where they trigger TIGS. Unlike microprojectile bombardment, *Agrobacterium*-mediated TIGS does not require specialized equipment, and silencing of target genes is induced throughout agrobacteria-infiltrated tissue, allowing the study of genes involved in multicellular processes. However, many monocotyledonous species are considered to be either a non-host or a poor host for *A. tumefaciens*. It is for this reason that *Agrobacterium*-mediated transformation of monocotyledonous cereal plants is relatively inefficient. As a consequence, cereal transformation groups as well as specialist laboratories tend to work with only 1–2 cereal genotypes and 1–2 compatible *A. tumefaciens* strains capable of transferring the T-DNA into the host plant's nuclear DNA (Sparks et al. 2014). Similarly, transient *Agrobacterium*-mediated expression in non-embryogenic tissues, such as leaves, is especially problematic in cereal species. This is because the standard pressure infiltration of *Agrobacterium* suspensions into these tissues using a needleless syringe is very difficult due to several intrinsic structural features, such as extensive epidermal cuticular waxes, considerable silica content and the low volume of intercellular space. In various dicotyledonous species, this procedure, known as 'agroinfiltration', is used routinely to explore gene function (Vaghchhipawala et al. 2011).

In spite of the difficulty of agroinfiltration into cereal leaves, a method for *Agrobacterium*-mediated TIGS in leaves through transient gene expression in rice (*O. sativa*) has recently been reported (Andrieu et al. 2012). Leaves of *japonica* and *indica* rice plants were mechanically wounded using 600-μm diameter needles mounted onto a custom-made apparatus. The wounded leaves were then incubated for 30–60 min in a suspension of *Agrobacterium* ($OD_{600\,nm}$ of 0.5–0.8) containing the surfactant Silwet L-77. Andrieu et al. (2012) were able to introduce hairpin RNAi constructs targeting the *phytoene desaturase* (*OsPDS*) and *SLENDER 1* (*OsSLR1*) genes into rice leaves, and demonstrated siRNA accumulation in the agroinfiltrated leaf area for both constructs. This was associated with a reduction of the targeted rice mRNA concentrations in both the agroinfiltrated and adjacent tissues, indicating that the gene-silencing mechanism had been activated. The same procedure was also used to overexpress transiently the *β-glucuronidase* (*gus*) reporter gene in rice leaves (Andrieu et al. 2012; see also the section '*Agrobacterium*-Mediated Expression'). It remains to be determined whether other rice genotypes and, indeed, other cereal species such as wheat and barley are equally amenable to transient transformation via this new procedure.

Transient Expression Assays

Microprojectile/Biolistic Bombardment

The initial concept of transient expression was first developed using electroporation of isolated plant protoplasts (Fromm et al. 1985). Microprojectile bombardment for DNA delivery directly into live plant cells was developed soon after (Klein et al. 1987).

This method remains one of the most frequently used techniques for stable genetic transformation of various monocotyledonous cereals (Sparks and Jones 2014). In microprojectile bombardment-mediated transformation, the construct designed for *in planta* protein expression typically contains a complete gene or just the coding sequence (CDS) of a gene of interest flanked by an appropriate promoter on its 5′-end, with an appropriate terminator sequence on its 3′-end. The gene construct is coated onto gold or tungsten microparticles (approximately 0.4–2 µm in size), which are then bombarded using high pressure from a helium gun into cells that exhibit embryogenic competence, such as immature embryos, scutella, immature inflorescences, and shoot tips. Finally, genetically transformed plants are regenerated from these bombarded tissues following labour-intensive tissue culture procedures.

Due to its relative simplicity, speed and ability to deliver DNA into various plant organs, tissue and cell types, microprojectile bombardment is also often used for transient gene expression. For instance, this method is particularly useful for the identification of novel promoters and promoter elements and for analyses of their activity, e.g. level and timing of expression or tissue and organ specificity. Most frequently, the constructs used in these analyses have novel promoter sequences upstream of genes that encode reporter proteins, such as GFP, β-glucuronidase (GUS) or luciferase, expression of which can be visualized easily by microscopy-based techniques and can be quantified (Fig. 14.3).

Microparticle bombardment has also proved to be useful for analyzing the localization of proteins to particular cellular compartments, such as the nucleus, vacuole, plasma membrane, endoplasmic reticulum (ER) and Golgi bodies, amongst others. Expression constructs used in these studies typically contain a gene of interest fused in frame to GFP or another fluorescent protein reporter, e.g. yellow fluorescent protein (YFP), DsRed, mCherry fluorescent protein or monomeric red fluorescent protein (mRFP) and placed under the control of a strong constitutively active promoter such as that found in the rice actin (*Act-1*) or maize ubiquitin (*Ubi-1*) genes. Subcellular localization of the fluorescent protein tag can be analyzed using confocal laser scanning microscopy. Peels of onion epidermal cells and wheat leaf sheath epidermal cells are frequently used for these studies, as these cells are relatively large, translucent and lack chloroplasts (Fig. 14.4).

In monocots, genes for anthocyanin biosynthesis are commonly used reporters for transient expression assays, and have been exploited to demonstrate the applicability of microprojectile bombardment-mediated gene expression in maize aleurone and tassel primordial tissues (Klein et al. 1989; Dupuis and Pace 1993), wheat inflorescences (Leduc et al. 1994) and barley coleoptile and leaf epidermal cells (Nelson and Bushnell 1997). GUS reporter protein fusions have also been used to demonstrate the viability of this transient expression technology in the leaves, coleoptiles and roots of rice, barley and perennial ryegrass (*Lolium perenne*; Hensgens et al. 1993), and in co-bombardment studies as a marker of transformed cells in many monocots. Since the late 1990s to early 2000s, transient expression and/or gene silencing of candidate genes using particle bombardment of leaf cells in monocots has also been applied in the field of molecular plant pathology. This technology has revolutionized research on the cell biology and

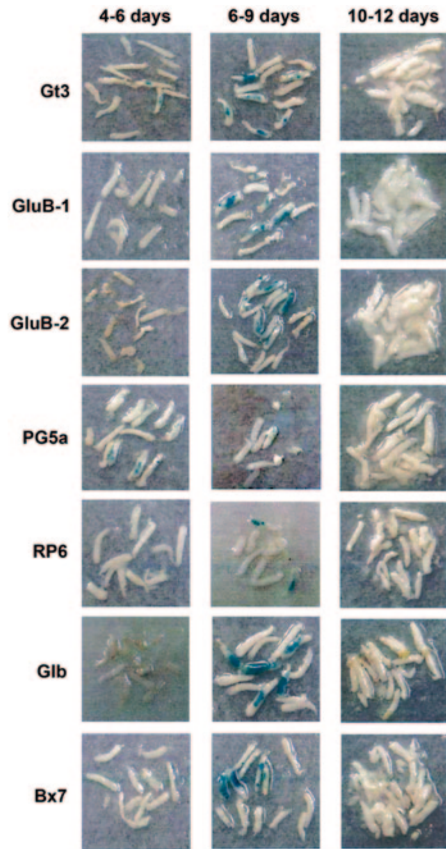

Fig. 14.3 Effects of developmental age of caryopses on transient expression of the *GUS* chimeric constructs under the control of cereal seed storage protein gene promoters (reproduced with permission from Hwang et al. 2001). Promoters: rice glutelin gene *Gt3,* rice glutelin gene *GluB-1,* rice glutelin gene *GluB-2,* rice prolamin gene *PG5a*, rice prolamin gene *RP6*, rice globulin gene *Glb,* and wheat glutenin gene *B×7*. Rice immature endosperm was isolated from three different stages of caryopses and used as a target material for biolistic bombardment. Histochemical analysis of bombarded endosperm was carried out following 24-h incubation at 25 °C

molecular analyses of cereal–powdery mildew (*Blumeria graminis* f. sp. *tritici* and *B. graminis* f. sp. *hordei*) interactions. These obligate biotrophic fungal plant pathogens, mainly known to infect wheat and barley, invade exclusively epidermal leaf cells, which are ideal for bioimaging studies. In addition, defences mounted by the plant in response to powdery mildew appear to operate cell autonomously, and can be dissected using a biolistic single-cell transformation approach. The short life cycle of powdery mildew (3–5 days from the initial spore germination to the production of the next generation of asexual spores) also permits the use of detached leaves, and the cutting out of 'windowpanes' of only epidermal cells, which simplifies the pathoassays. In this approach (Panstruga 2004; Hückelhoven and Panstruga 2011), detached leaves are bombarded with microprojectiles carrying at least two different constructs, one for overexpression or silencing of a candidate gene, and another for expression of a reporter protein, e.g. GUS or GFP, and then inoculated with fungal conidiospores. Co-bombardment is known to result in a high frequency of construct co-integration in plant cell nuclear DNA, such that

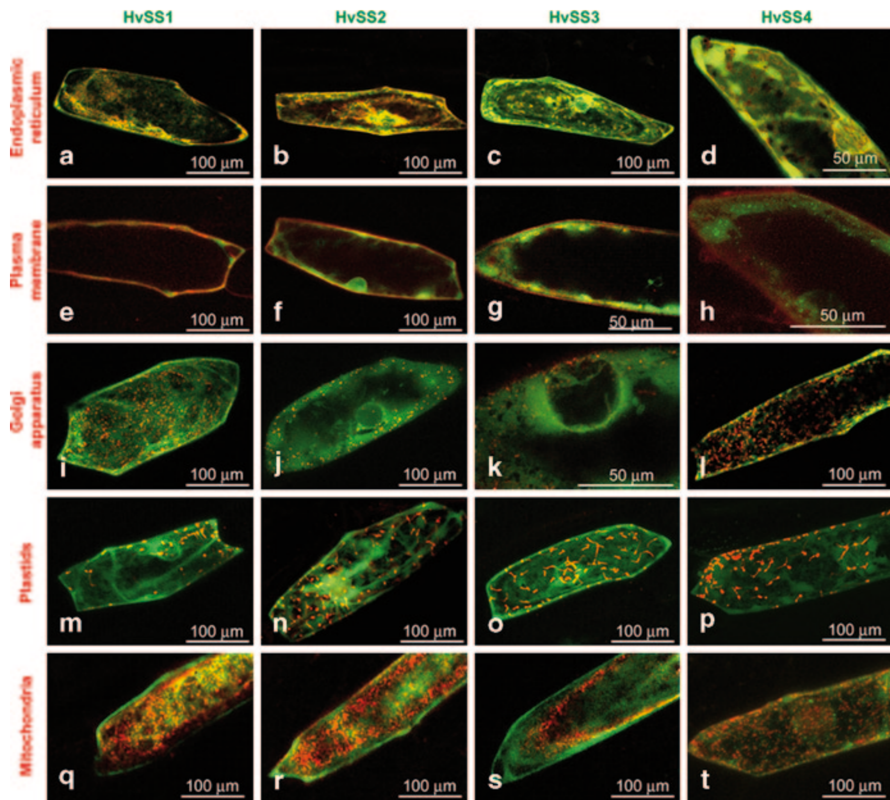

Fig. 14.4 The overlay projections of confocal stacks spanning onion epidermal cells simultaneously transformed with the GFP-tagged barley sucrose synthase genes and the mCherry-tagged subcellular markers (reproduced with permission from Barrero-Sicilia et al. 2011). Barley sucrose synthases: *HvSS1*: **a**, **e**, **i**, **m**, **q**; *HvSS2*: **b**, **f**, **j**, **n**, **r**; *HvSS3*: **c**, **g**, **k**, **o**, **s**; *HvSS4*: **d**, **h**, **l**, **p**, **t**. Five different organelle markers specific for (**a**–**d**) endoplasmic reticulum, (**e**–**h**) plasma membrane, (**i**–**l**) Golgi apparatus, (**m**–**p**) plastids and (**q**–**t**) mitochondria. *GFP* green fluorescent protein

the majority of single-leaf epidermal cells that express the reporter protein is also likely to be expressing a test gene construct. It is therefore possible to determine whether the introduced test construct has had any impact on the outcome of the particular cereal genotype–powdery mildew isolate interaction by assessing the success of infection or monitoring fungal development throughout the infection time course in the reporter protein-expressing plant cells (Fig. 14.5). This type of experimentation can also be supplemented by using various compounds that stain specifically fungal structures and/or certain cellular compartments, providing additional valuable insights and better understanding of cell biology of fungal–plant interactions (Weis et al. 2013). This remains a very intensive area of research, which continues to provide exciting novel discoveries.

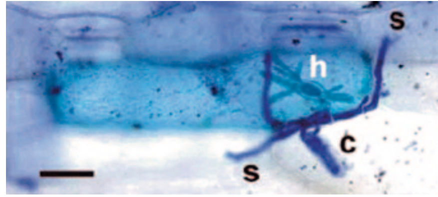

Fig. 14.5 Micrograph of successful fungal penetration on a biolistically transformed barley epidermal cell (reproduced with permission from Panstruga 2004). A barley leaf was bombarded with a *GUS* reporter construct. Subsequently, the leaf was inoculated with powdery mildew conidia and, at 48 h post inoculation, stained for GUS activity. Fungal structures were highlighted by Coomassie Brilliant Blue for microscopic evaluation. Successful penetration is indicated by the presence of a haustorium and elongating secondary hyphae. *c,* conidiospore; *h,* haustorium; *s,* secondary hyphae. Scale bar = 20 µm

Agrobacterium-Mediated Expression

A. tumefaciens-mediated transfer and transient expression of binary Ti plasmid-based vector constructs in leaf cells has been used extensively for testing gene function in a number of dicotyledonous species. In contrast to the microprojectile bombardment-mediated method used to introduce constructs into plant tissues, which allows heterologous protein expression or gene silencing in single cells, *A. tumefaciens* is able to deliver a binary T-DNA vector to the genome of most of the plant cells in the infiltration zone of the leaf, and also allows experiments to be carried out using non-sterile glasshouse-raised plants.

Unfortunately, the standard methods of agroinfiltration developed for dicots do not work well in many monocotyledonous species (see discussion in the section '*Agrobacterium*-Mediated Delivery'). Therefore, several strategies have been used to improve this procedure for monocots, including mechanical wounding of tissues prior to or during agroinfiltration (e.g. by sonication, mixing by vortex with carborundum, or wounding of plant tissue with a needle), and the incorporation of additives to the agroinfiltration medium. Surfactants, such as Li700 and Silwet-L77, and/or various thiol compounds including L-cysteine and dithiothreitol, are thought to inhibit wound- and pathogen-induced responses (Chen et al. 2010).

In a typical example, Requesens et al. (2010) developed an efficient and reliable transient *A. tumefaciens*-mediated assay for testing the functionality of endosperm-preferred promoters in maize. In this method, kernels were isolated from maize ears by cutting the kernels at the base with a scalpel, and dissecting out and discarding the embryos. The pericarp was peeled away exposing approximately three quarters of the endosperm surface, thus increasing the area available for contact with the agrobacteria. The remaining developing endosperm was co-cultivated with *Agrobacterium* suspensions for 3–5 days following vigorous mixing by vortexing and subsequent sonication for 30 s.

Wounding of plants in these bioassays may interfere with functional analyses of stress-related genes. Vacuum infiltration has been used as an alternative method

for introducing *Agrobacterium* into otherwise unwounded leaves in harvested switchgrass (*Panicum virgatum*; VanderGheynst et al. 2008). Detached leaves were vortexed with a suspension of *A. tumefaciens* carrying a GUS reporter construct and non-ionic surfactant added before the application of a low-pressure vacuum (25 kPa). The infiltrated leaves were incubated on moistened sterile filter paper at 22 °C in the dark for 3–6 days. Although GUS expression could be detected in the switchgrass leaf cells after 3 days incubation, leaf decay was observed by 6 days of incubation (VanderGheynst et al. 2008). In a more recent report, a method was described for *Agrobacterium*-mediated transient expression in several cereals (namely rice, barley, maize, oats, rye, sorghum and wheat), which allows functional analyses of genes involved in various abiotic stress responses and which eliminates the need for wounding of plants (Dhadi et al. 2012). In this procedure, young 12–15-day--old plantlets were uprooted, cleaned, co-cultivated with *Agrobacterium* harbouring the expression construct in half strength Murashige and Skoog (0.5 MS) basal salt medium supplemented with acetosyringone, a natural phenolic wound response product, at 28 °C for 15 h on a rotating platform. After co-cultivation, the plantlets were incubated for 8–12 h with 0.5 MS medium supplemented with the antibiotic carbenicillin to prevent bacterial contamination.

In almost all the published work to date, *A. tumefaciens*-mediated protein expression has been shown to be maintained only transiently, usually during the first 5–7 days after agroinfiltration or cocultivation. However, a new method involving cocultivation of mature seeds with agrobacteria appears to enable a longer period of transient expression (Fursova et al. 2012). The seeds were trimmed with scissors to remove about the uppermost one third of each seed, leaving the intact embryo and a 'sufficient' portion of the cotyledon. Trimmed seeds were incubated with *A. tumefaciens* in the presence of acetosyringone, Silwett-L77 and plant extracts from *Nicotiana tabacum* leaves (these are known to contain metabolites inducing Agrobacteria to initiate gene transfer) for 30 h at 21 °C (Fursova et al. 2012). Seed trimming appeared to aid *Agrobacterium* penetration through the intracellular spaces of the seed tissues and allowed efficient transformation of the embryonic cells. On average, 10 % of the cocultivated seeds were able to germinate and the resulting plantlets maintained active expression of proteins for up to 12 weeks. This allowed analyses of the effects of gene expression at different stages throughout plant development. Currently, this method has been described only for the model grass species *B. distachyon*. However, this method may prove to be very useful in functional genomics studies if it can be applied to other monocots, including cereal crops.

Bacterial Type III Secretion System-Based Expression

As discussed earlier, *Agrobacterium*-mediated transient expression is not very efficient in many monocots, especially in wheat and barley, whereas microprojectile bombardment-mediated gene expression is technically challenging, with expensive set-up costs. This technology also only permits protein expression in individual

cells scattered among many untransformed cells. These drawbacks of the 'conventional' technologies necessitate development of novel transient expression systems for cereals that allow higher throughput analyses and expression in all or most cells in the target tissue or region.

Many pathogenic bacteria use the type III secretion system (T3SS), a complex protein assembly that is said to resemble a syringe with a needle, to inject small secreted proteins (effector proteins) into the cytoplasm of targeted plant cells to initiate and aid infection (Block et al. 2008). During the past decade, T3SS of various plant pathogenic bacteria have been utilized for protein delivery into cells of dicots, mainly *Arabidopsis thaliana* and *Nicotiana benthamiana*. In this system, a heterologous protein is fused to the N-terminus of one of the well-characterized bacterial effector proteins, such as AvrRps4 or AvrRpm1, that contain signal peptides for T3SS-mediated delivery. The main focus of many of these studies has been functional analyses of cytoplasmic effector proteins predicted in the genome sequences of various bacterial, oomycete and fungal pathogens of plants (Sohn et al. 2007; Whisson et al. 2007; Rentel et al. 2008; Fabro et al. 2011; Goritschnig et al. 2012).

Very recently, a similar approach has been applied for delivery of pathogen effector proteins into leaf cells of monocots. The bacterial pathogen of rice, *Burkholderia glumae,* was used in a rice leaf sheath inoculation assay for *in planta* expression, and subcellular localization analyses of effector proteins of the rice blast fungus *Magnaporthe oryzae* (Sharma et al. 2013). In the same study, the *B. glumae* system was shown to deliver fluorescently labelled fungal effectors to wheat and barley leaf sheath cells and, therefore, this system may be applied for effector discovery. However, the pathogenic nature of this bacterium may restrict its use.

Another system utilizes the soil-dwelling bacterium *Pseudomonas fluorescens*. This benign species is non-pathogenic on plants and currently seems to have greater potential to become used widely for studies on the detailed characterization of effectors from different plant-pathogenic organisms. The *P. fluorescens* strain Pf0–1 naturally lacks an endogenous T3SS-encoding region as well as candidate type-III effector genes. Thomas et al. (2009) integrated the entire T3SS-encoding region from a plant pathogenic bacterium, *Pseudomonas syringae* pv. *syringae* strain 61, into the genome of *P. fluorescens* Pf0–1. This engineered strain, known as EtHAn (*E*ffector-*t*o-*H*ost *An*alyzer), is capable of delivering individual bacterial type III effector proteins, or effector proteins of a nonbacterial origin (as C-terminal fusions to the T3SS signal), directly into the cells of different dicotyledonous plants. The EtHAn-mediated effector delivery system has been shown to work moderately well in wheat (Yin and Hulbert 2011). A modified and improved version of this system has been used recently in a relatively high-throughput screen of stem rust fungus *Puccinia graminis* f. sp. *tritici* effectors in wheat. This resulted in identification of a candidate avirulence effector capable of inducing genotype-specific hypersensitive response that depended on the presence of the *Sr22* resistance gene (Upadhyaya et al. 2014).

Virus-Mediated Overexpression

Transient expression using plant virus vectors is known as VOX, *v*irus-mediated *o*vere*x*pression, and can provide rapid and high-level production of a recombinant protein. Since the mid-1990s, many plant viruses (mainly those with positive-sense single-stranded RNA genomes) have been cloned and modified to express foreign peptides and proteins *in planta*. Full-length virus vectors can spread systemically within a plant to colonize the majority of the phloem sink tissue, and are easily transmitted to new plants when scale-up is required. Therefore, this technology proved to be very useful, especially for moderate-to-large scale production of a variety of biopharmaceutical proteins (Hefferon 2012). Other important uses of plant virus vectors include the investigation and manipulation of metabolic pathways, monitoring virus trafficking and defining function of virus-encoded proteins (through expression of reporter proteins such as GFP or GUS), functional characterization of host disease resistance genes and pathogen effector proteins, and cellular protein localization studies.

For cereals, only two plant RNA virus vectors have been described, namely BSMV and *Wheat streak mosaic virus* (WSMV), to be capable of systemic expression of heterologous proteins (Tatineni et al. 2011; Lee et al. 2012). The most widely used in wheat and barley is the BSMV vector system, initially developed for investigations of viral cell-to-cell and long distance movement (Haupt et al. 2001; Lawrence and Jackson 2001). The most commonly used BSMV vector variants are those designed to express recombinant proteins as fusions to the C-terminus of the small (17 kDa) viral γb protein. Using this type of vector, for example, Manning et al. (2010) achieved expression of a functionally active fungal (*Pyrenophora tritici-repentis*) ToxA effector protein in wheat. However, for many applications, it is advantageous to be able to produce a free heterologous protein without a viral γb protein, which may have a negative impact on protein activity, function or its intracellular localization. This can be achieved, for example, by introducing an autoproteolytic 2A peptide of picornaviruses between the fused sequences providing co-translational cleavage and release of free proteins. Using this approach, Lee et al. (2012) successfully expressed a number of relatively small proteins, including iLOV, a flavin-based fluorescent reporter protein and the necrosis-inducing secreted effector protein Nip1 from the fungal pathogen *Rhynchosporium commune,* in barley and wheat. In these experiments, Nip1 elicited systemic necrosis specifically in barley genotypes carrying the cognate *Rrs1* resistance gene (Fig. 14.6). In another BSMV vector design, GFP and 42 rice coding gene sequences (CDS), between 200- and 1800-nt in size, were expressed as N-terminal fusions to the haemagglutinin epitope (HA) tag linked to the 2A self-cleaved peptide sequence followed by BSMV γb (Pogue and Holzberg 2012). Western blot analyses of systemically infected barley tissue using anti-HA antibodies confirmed expression of 38 out of 43 recombinant protein products (Fig. 14.7). Reassuringly, 2A cleavage rates of 60–95 % were observed. Apparently, this vector was also capable of expressing proteins requiring maturation

Fig. 14.6 BSMV::*RcNip1* induces necrosis (*arrows*) specifically in barley 'Atlas 46' carrying the cognate resistance gene *Rrs1* photographs taken at 8 days after virus inoculation. *BSMV Barley stripe mosaic virus*

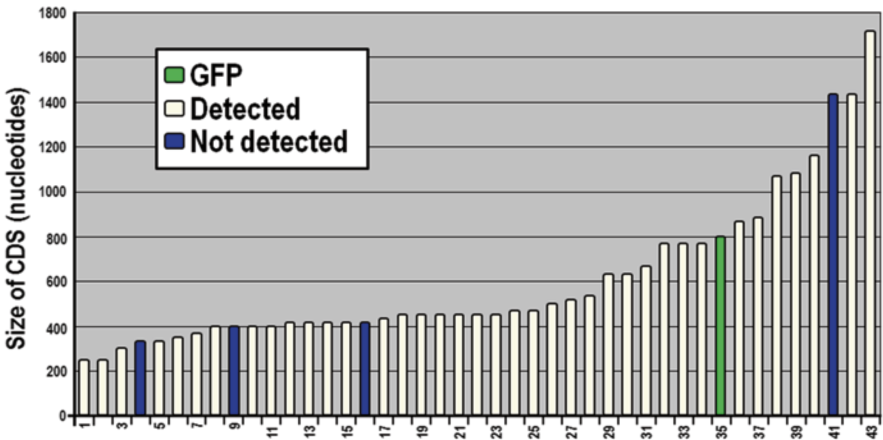

Fig. 14.7 BSMV-mediated expression of free GFP or heterologous proteins (reproduced with permission from Pogue and Holzberg 2012). Rice coding gene sequences (CDS) are arranged according to size. CDS whose predicted proteins were detected by Western blot analysis are *unshaded*, while *blue shaded bars* indicate the lack of detectable HA-tagged protein. *Green bar* represents the HA-tagged GFP control. *BSMV Barley stripe mosaic virus*, *GFP* green fluorescent protein

through the plant secretory pathway, which demonstrates the ability of the 2A cleavage system to deliver functionally active proteins to distinct subcellular fates. Moreover, the expression was shown to be relatively stable and at least in the case of GFP, its expression was observed regularly in leaves 1–4 above the inoculated leaf and maintained for up to 18 days post inoculation (Pogue and Holzberg 2012).

Concluding Remarks

The difficulty of obtaining stable transformants of most monocotyledonous species has contributed to the lag in gene function analyses in comparison to dicotyledonous species. However, as discussed in this chapter, there have been considerable advances in recent years in both the range and reliability of transient *in planta* expression systems available for both RNAi-mediated downregulation and overexpression of target genes of interest in monocots. In combination with the ongoing explosion of genomic and transcriptomic data being generated for many monocotyledonous species, major advances are foreseen in understanding gene function in these plants, and processes involved in interactions between monocots and associated organisms will continue to accelerate. In future, the existing technologies described in this chapter are likely to be improved further, thereby lowering costs whilst simultaneously increasing efficiency.

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Chapter 15
Recent Advances in *In Planta* Transient Expression and Silencing Systems for Soybean Using Viral Vectors

Steven A. Whitham, Alan L. Eggenberger, Chunquan Zhang, R. V. Chowda-Reddy, Kathleen M. Martin and John H. Hill

Introduction

Transient methods for overexpressing and silencing plant genes provide platforms for rapid analysis of gene function and regulation. Several methods have been used widely in plants to express or silence genes that avoid the generally more time- and labor-intensive generation of stable transgenic plants. Transient methods allow experiments to be performed on the order of hours, days, or weeks versus months or years for stable transgenes, if they are possible at all. In this post-genomic era, the sequences of many crop genomes, including soybean (Schmutz et al. 2010), are now available. Transient expression and silencing methods, combined with the genome information provide powerful approaches for high-throughput screens to associate genes with traits and to further dissect the functions of proteins and regulatory sequences.

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Transient gene expression and silencing studies require the introduction of nucleic acids, typically DNA, into cells in the appropriate tissue of intact plants, cultured cells, or protoplasts. The nucleic acid may be introduced by biological, mechanical, or chemical techniques that are feasible in a given plant species. Biological techniques employ *Agrobacterium* spp. such as *A. tumefaciens* and *A. rhizogenes* that transfer DNA segments defined by specific border sequences into plant cells. The transferred DNA is then imported into the nucleus where it is expressed. Mechanical techniques involve biolistic bombardment of DNA-coated particles into plant cells. Micron- or submicron-sized gold or tungsten particles are propelled rapidly into plant cells by high-pressure gas. The particles release their nucleic acid payload inside the cell for uptake into the nucleus where expression of the genes of interest occurs. The use of chemicals to transform protoplasts involves $CaCl_2$ and polyethylene glycol, which promote uptake of DNA contained in the solution bathing the protoplasts. All of these techniques enable overexpression and silencing of genes in the cells that directly receive the nucleic acid. While these methods can greatly accelerate gene function analyses, the expression or silencing is limited to a few cells or patches of cells.

Viral vectors provide powerful platforms for transient gene expression and silencing throughout the plant. As an alternative to stable transformation, gene function studies using plant viral vectors in crop plants are rapid, flexible, of higher throughput, effective in a wider range of genotypes, and less expensive. In the cases of viruses possessing RNA genomes, the viral genome is cloned as a complementary DNA (cDNA) and its transcription is placed under control of prokaryotic or eukaryotic promoters. Prokaryotic promoters, such as T7, direct the T7 polymerase to initiate synthesis of plant virus RNA at the first base of the genome. This process is known as in vitro transcription, because the RNA synthesis is performed outside of the cell. The RNA transcripts are infectious and can be introduced into plants by rub-inoculation or particle bombardment to initiate new systemic infections. If the viral genome is placed under control of a promoter that functions in plant cells, such as the *Cauliflower mosaic virus* 35S promoter (P35S), then P35S will direct the synthesis of the plant virus RNA at the first base of the genome in the inoculated cells. Thus, the viral RNA transcripts are produced in vivo. DNA-based infectious clones can be introduced into plant cells by rub-inoculation, particle bombardment, or *Agrobacterium* infiltration. In viral vectors, the viral genomes have been modified to enable the insertion of sequences that are foreign to the virus. These foreign sequences may be of plant origin or from other organisms, and they may be used for purposes of protein expression or gene silencing. Viruses with DNA genomes, such as the geminivirus *Tomato yellow leaf curl virus* (TYLCV), have also been engineered to accept foreign sequences, and they show promise as vectors for gene expression and silencing in a broad range of plants as well (Peretz et al. 2007). Several viruses have been investigated for these uses in soybean and some have been used more widely than others.

This chapter focuses on seven viral vector systems that have been used in soybean for overexpression and/or virus-induced gene silencing (VIGS) applications. We discuss a functional analysis pipeline that utilizes a *Bean pod mottle virus*

(BPMV)-based vector that has been used for investigating soybean defense gene networks on a relatively high-throughput scale. We conclude by discussing potential future applications of viral vectors in soybean. For additional information on viral vectors that emphasize gene silencing, we refer the reader to these recent, excellent reviews for soybean and other legumes (Kasai and Kanazawa 2012; Pflieger et al. 2013) or plants in general (Lange et al. 2013). Detailed protocols for VIGS in several dicotyledons and monocotyledons are available in Becker (2013).

Viral Vectors that Have Been Used in Soybean

Approximately 70 viruses have been shown to infect soybean (Saghai Maroof et al. 2008; Zhou et al. 2011; Han et al. 2012). At least seven of these viruses have been used with some measure of success as vectors for gene expression and/or VIGS in soybean. For comparative purposes, features of these viruses are summarized in Table 15.1. In this section, we introduce each of these viruses with a discussion of the viral properties, infectious clones, capabilities, and limitations. The varied features and properties of the viruses affect strategies that must be used to modify their genomes for protein expression and VIGS. Plant viral gene expression and VIGS systems are by nature dependent on the viral life cycle as well as on plant–virus interactions. Therefore, we conclude this section with a brief discussion of biosafety considerations, because most recombinant viral clones will retain some degree of pathogenicity.

Potyviruses Potyviruses are used primarily as expression vectors since one of the potyvirus proteins, helper component-proteinase (HC-Pro), is a potent suppres-

Table 15.1 Properties of viruses used as vectors for gene expression and/or silencing in soybean

Virus	Genus	Morphology	Genome segments	Genome strategy	Application		Silencing suppressor[a]
					Expression	VIGS	
ALSV	*Cheravirus*	Sphere	2	Polyprotein	Yes	Yes	?
BPMV	*Comovirus*	Sphere	2	Polyprotein	Yes	Yes	?CP
CLYVV	*Potyvirus*	Rod	1	Polyprotein	Yes	No	HC-Pro
CMV	*Cucumovirus*	Sphere	3	Subgenomic	No	Yes	2b
SMV	*Potyvirus*	Rod	1	Polyprotein	Yes	No	HC-Pro
TRV	*Tobravirus*	Rod	2	Subgenomic	Yes	Yes	MP, P1b
TSV	*Ilarvirus*	Sphere	3	Subgenomic	No	Yes	?2b

ALSV *Apple latent spherical virus*, BPMV *Bean pod mottle virus*, ClYVV *Clover yellow vein virus*, CMV *Cucumber mosaic virus*, SMV *Soybean mosaic virus*, TSV *Tobacco streak virus*, TRV *Tobacco rattle virus*

[a] "?" indicates that the silencing suppressor is unknown or that the protein has been identified as a silencing suppressor in another virus species within the genus but not confirmed in this species

sor of VIGS. Potyviruses are characterized by having an ~10-kilobase (kb) RNA genome that is translated into a single, large polyprotein that is processed by virus-encoded proteinases to yield mature viral proteins (Fig. 15.1; Shukla et al. 1994; Urcuqui-Inchima et al. 2001). Recently, an additional open reading frame (ORF) was discovered in potyviruses that is produced as a fusion to the N terminus of the P3 protein (Fig. 15.1; Chung et al. 2008). Expression of foreign genes is achieved by their insertion into the virus genome so that the virus ORF is maintained, and the foreign protein is cleaved from the polyprotein by the action of one or more of the viral proteinases. Proteinase cleavage sites are generally designed so that the amino acid sequences of the flanking viral proteins are unaffected. For existing potyvirus vectors, this requires that the foreign protein has at least a carboxyl-terminal (C-terminal) addition, and often an amino-terminal (N-terminal) addition for correct processing from the viral polyprotein.

Soybean mosaic virus (SMV) has been developed for expressing foreign genes by introduction of a cloning site and nuclear inclusion a (NIa) proteinase cleavage site between the P1 and HC-Pro cistrons of the virus genome (Wang et al. 2006; Fig. 15.1). The foreign protein is processed from the polyprotein at its N-terminus by the proteolytic activity of the P1 proteinase, which cleaves a tyrosine/serine bond at its C-terminus. This requires addition of four amino acids to the N-terminus of the foreign protein. Alternatively, where maintenance of a native N-terminus is important, a sequence encoding an NIa proteinase cleavage sequence can be added to the 5′ end of the foreign gene during cloning which results in the addition of 0–1 amino acids to the N-terminus. The foreign protein is cleaved from the polyprotein at its C-terminus by the action of the NIa proteinase on an introduced heptapeptide cleavage sequence which adds nine amino acids to the introduced protein. The polyprotein expression strategy results in all proteins being made in equimolar amounts, with relative yields of the foreign proteins determined largely by their stability. Since the SMV virion is a filamentous particle, there is no strict encapsidation limit for the virus RNA and thus no strict limit on the size of the introduced gene. In practice, however, larger genes are less stable in the SMV vector. Though it has not been explored extensively, approximately 2 kb may be the upper size limit for inserts in this vector.

Clover yellow vein virus (ClYVV) has also been used as a potyvirus expression vector in soybean (Masuta et al. 2000). Similar to SMV, a cloning site between P1 and HC-Pro has been used for expression of foreign sequences of up to 1.8 kb (Fig. 15.1). In addition, a second position between nuclear inclusion b (NIb) and coat protein (CP) has also been used (Fig. 15.1; Wang et al. 2003), with greater stability of foreign inserts compared to the P1/HC-Pro site, though there are no reports that this version has been used in soybean. Independent attempts to use this NIb/CP site in SMV were not successful (A.L.E. and R.V.C.R., unpublished), possibly due to disruption of RNA sequences required for the virus life cycle.

Bean pod mottle virus (BPMV) BPMV is a member of the genus *Comovirus* within the subfamily *Comoviridae* (family *Secoviridae*; (Lomonossoff and Ghabrial 2001; Table 15.1). BPMV has a bipartite positive-strand RNA genome consisting of

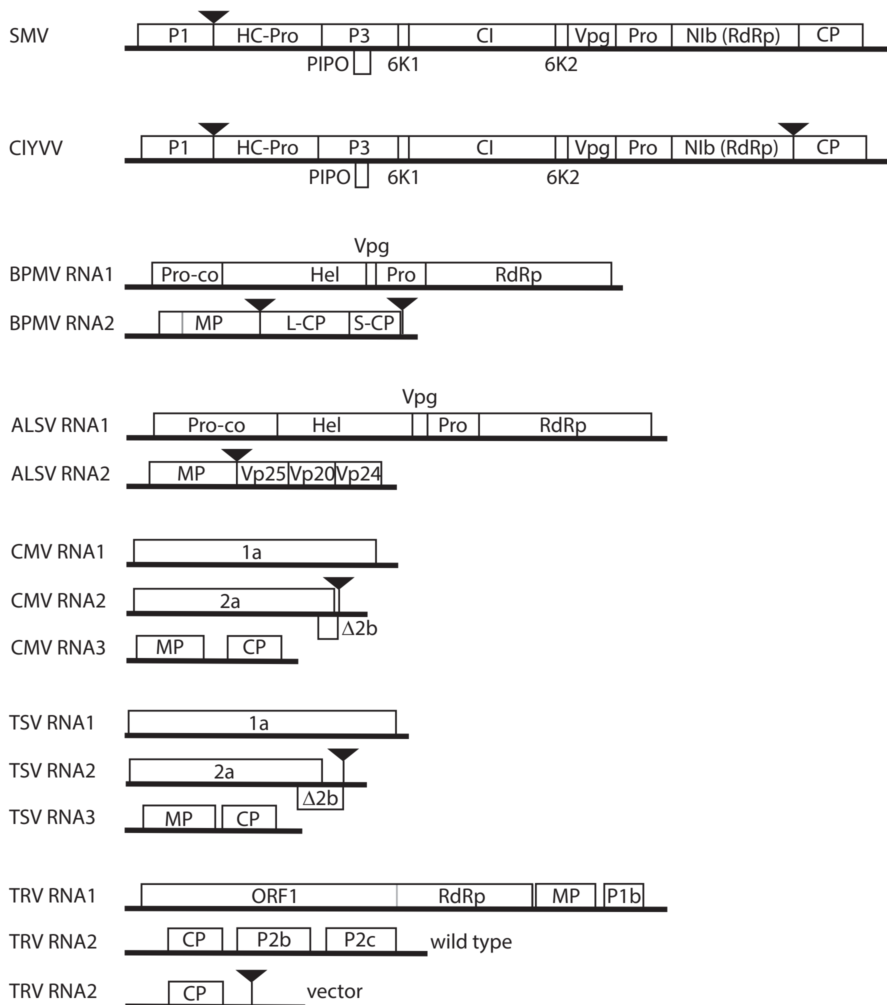

Fig. 15.1 Genome structures of viruses used as vectors in soybean. The *black triangles* indicate positions of cloning sites for foreign inserts in the viral vectors. The *gray line* separating ORF1 from RdRp in TRV represents the position of a leaky stop codon that results in production of a fusion protein. SMV *Soybean mosaic virus*, ClYVV *Clover yellow vein virus*, BPMV *Bean pod mottle virus*, ALSV *Apple latent spherical virus*, CMV *Cucumber mosaic virus*, TSV *Tobacco streak virus*, TRV *Tobacco rattle virus*. *Δ2b* truncated 2b protein, *MP* movement protein, *CP* capsid protein, *HC-Pro* helper component-proteinase, *VPg* viral protein genome-linked, *RdRp* RNA-dependent RNA polymerase, *Hel* helicase, *Pro* proteinase, *Pro-co* proteinase cofactor, *CI* cylindrical inclusion, *PIPO* pretty interesting potyviral open reading frame, *6K1* 6 kilodalton protein 1, *6K2* 6 kilodalton protein 2, *NIa* nuclear inclusion a, *NIb* nuclear inclusion b

RNA1 (approximately 6.0 kb) and RNA2 (approximately 3.6 kb) that are individually encapsidated in isometric particles with a diameter of 28 nm. There are two distinct subgroups of BPMV strains: subgroups I and II. In addition, inter-subgroup reassortants and recombinants have also been previously isolated and extensively characterized (Gu and Ghabrial 2005; Zhang and Ghabrial 2006; Zhang et al. 2010; Bradshaw et al. 2011). Members of all subgroups of various geographic isolates have been used in the development of three generations of BPMV vectors (Zhang and Ghabrial 2006; Zhang et al. 2009b, 2010).

BPMV uses a polyprotein synthesis and cleavage strategy for the expression of proteins encoded by RNA1 and RNA2. BPMV RNA1 contains a large ORF encoding a single 200-kilodalton (K) polyprotein precursor, which is processed subsequently into five mature gene products designated as proteinase cofactor (Co-pro, 32 K), helicase (58 K), VPg (viral protein genome-linked), proteinase (Pro, 24 K), and RNA-dependent RNA polymerase (Pol or RdRp, 87 K; Fig. 15.1). In general, these proteins are required both for replication of viral genomic RNAs and for processing the polyproteins encoded by them (Lomonossoff et al. 1985; Eggen and van Kammen 1988; Dessens and Lomonossoff 1991; Peters et al. 1992; Carette et al. 2002; Pouwels et al. 2002; Gu and Ghabrial 2005; Zhang et al. 2010). BPMV RNA2 has two start codons and thus can be translated into either a 105- or 95-K polyprotein. The 105-K polyprotein is processed to 58-K cofactor of RNA2 replication (CR), large coat protein (LCP), and small coat protein (SCP), while the 95-K polyprotein is processed to the 48-K movement protein (MP), LCP, and SCP. The 48-K MP protein shares its C-terminal region with the 58-K CR protein which is required for RNA2 replication (Van Bokhoven et al. 1993). A suppressor of RNA silencing has not yet been identified for BPMV, although the SCP of the related *Cowpea mosaic virus* has this function (Canizares et al. 2004).

BPMV RNA2 has been engineered to carry foreign inserts in two different places (Fig. 15.1). The first position lies at the junction of the MP and LCP (Zhang and Ghabrial 2006; Zhang et al. 2009b, 2010; Fig. 15.1). At this site, the foreign insert must be cloned in frame with the viral polyprotein so that it will be properly translated. The foreign peptide is cleaved from the viral polyprotein at natural and engineered cleavage sites flanking the insertion. This function is performed by the viral proteinase carried on RNA1. A second strategy to introduce foreign sequences into BPMV RNA2 involves the placement of a cloning site immediately after the stop codon for the viral polyprotein (Zhang et al. 2010). By using this position, it is not necessary to clone foreign inserts in frame with the viral polyprotein. This is advantageous for targeting untranslated regions of messenger RNAs (mRNAs) or regulatory regions such as promoters for silencing, and it is possible to easily insert foreign sequences in either the sense or antisense orientation without concern for the ORF. A vector with its cloning site after the stop codon cannot be used for protein expression.

RNA- and DNA-based BPMV vector systems have been developed and used successfully, and detailed protocols have been published for both (Kachroo and Ghabrial 2012; Zhang et al. 2013). The systems utilize infectious DNA clones of the viral genomic RNAs that have been placed under control of the T7 (RNA-based)

or P35S (DNA-based) promoters. To inoculate plants with the clones of vectors under control of the T7 promoter, RNA transcripts are made in vitro and then rub-inoculated on soybean leaves. Inoculation of the DNA-based vectors utilizes biolistic delivery of the cDNA clones of RNA1 and RNA2 into soybean leaf cells from which infectious RNA copies of the viral genomes are produced in vivo. The BPMV vectors cause symptoms in soybean, and the severity can be modulated by selecting different RNA1 strains that vary from mild, moderate, to severe symptoms (Zhang et al. 2010). Selection of the appropriate RNA1 allows gene expression and silencing to be optimized in conjunction with viral pathogenicity, with more severe symptoms making it easier to identify BPMV-infected plants but possibly masking effects of gene silencing. BPMV readily infects leaves, stems, roots, and flowers (Juvale et al. 2012), but it is rarely seed transmitted, although it can be found in the seed coat (Krell et al. 2003). Therefore, the vector may have limited utility for investigating seed and embryonic traits, although this has not been thoroughly tested.

Apple Latent Spherical Virus (ALSV) ALSV is a member of the genus *Cheravirus*, also in the *Comoviridae* (Le Gall et al. 2007; Table 15.1). ALSV has a bipartite genome with RNA1 (6813 nucleotides) encoding viral replication and proteinase functions and a smaller RNA2 (3385 nucleotides) encoding a movement protein and three CPs (Li et al. 2000). Each RNA genome contains a single large ORF encoding polyproteins that are cleaved into the mature viral proteins by the protease carried on RNA1. ALSV virions encapsidate a single genomic RNA, and therefore, plants must be inoculated with two particles, one carrying RNA1 and the other carrying RNA2, to initiate systemic infection. The strategy of engineering ALSV to be a vector for gene expression and VIGS is similar to that of BPMV described above. RNA2 was modified to carry additional foreign sequences and remain below the packaging limit of the spherical virus particles. The cloning site has been engineered between the MP and first subunit of the CP (Li et al. 2004; Yaegashi et al. 2007; Fig. 15.1), therefore any sequence carried by this virus must be expressed in frame with the RNA2 polyprotein. Duplication of the proteinase cleavage site between the MP and CP allows inserted protein sequences to be cleaved away from the viral polyprotein. ALSV-based vectors have been used to express marker genes and plant genes as well as silence plant genes (Li et al. 2004; Yaegashi et al. 2007).

Interestingly, ALSV has a wide host range that encompasses plant species across the rosid clade including soybean, apple, cucurbits, petunia, *Nicotiana* sp., and *Chenopodium* sp. (Igarashi et al. 2009). Therefore, this virus is useful as a vector in a wide variety of important crop plants for which stable transformation is challenging. Soybean plants inoculated with ALSV at the cotyledon stage initially develop mosaic symptoms on the unifoliolate and first and second trifoliolate leaves, but symptoms do not appear on subsequent leaves (Yamagishi and Yoshikawa 2009). The ALSV-infected plants continue to develop normally with respect to flowering and seed set. The lack of symptoms reduces the possibility that the effects of the virus itself will interfere with the phenotype of experimental plants in which genes are being silenced or expressed.

In the ALSV vector system, DNA copies of the viral genomic RNAs are cloned into plasmids under control of the P35S and the nopaline synthase terminator (Tnos; Li et al. 2004). The RNA1 and RNA2 plasmids are co-inoculated onto *Chenopodium quinoa* leaves to initiate systemic infections. To inoculate soybean plants, total RNA extracted from the systemically infected *C. quinoa* plants is coated onto gold particles, which are then bombarded biolistically into soybean cotyledons or leaves. A detailed protocol for using the ALSV vector in soybean was recently published (Yamagishi and Yoshikawa 2013).

Cucumber Mosaic Virus (CMV) CMV is a member of the *Bromoviridae* in the genus *Cucumovirus* (Table 15.1). CMV has a tripartite genome that is packaged in spherical viral capsids. RNA1 encodes the replicase, RNA2 encodes the helicase and 2b silencing suppressor, and RNA3 encodes the MP and CP (Fig. 15.1). The 2b and CP are in different reading frames than the helicase and MP, respectively, and are expressed from subgenomic mRNAs. CMV virions encapsidate a single genomic RNA, and therefore plants must be inoculated with a mixture of three particles carrying RNA1, RNA2, and RNA3 to initiate systemic infection. RNA2 was modified for insertion of foreign sequences after the stop codon of a truncated 2b coding sequence (Otagaki et al. 2006; Fig. 15.1). Because the cloning site is after the stop codon, the CMV-based vectors can be used for VIGS but not for expressing foreign proteins.

Collectively, CMV strains have a wide host range of more than 1200 plant species (Scholthof et al. 2011) and some of its strains systemically infect soybean. In the CMV vector system developed by Otagaki et al. (2006), DNA copies of the viral genomic RNAs were cloned into plasmids under control of the prokaryotic T7 promoter, which is recognized by T7 polymerase to produce full-length transcripts of the viral RNAs in vitro. The in vitro transcripts of RNA1, RNA2, and RNA3 are mixed together and then co-inoculated onto *N. benthamiana* leaves to initiate systemic infections. Sap from infected *N. benthamiana* plants is used subsequently to rub-inoculate soybean plants. The original CMV vector is derived from the Y strain of CMV, which does not infect soybean. To overcome this obstacle, a pseudo-recombinant is used that has a mixture of RNA1 and RNA2 derived from CMV-Y and RNA3 derived from the soybean strain of CMV (CMV-Sj; Nagamatsu et al. 2007). Plants inoculated with this pseudo-recombinant at the unifoliolate leaf stage did not develop obvious symptoms of infection. The CMV-infected plants continue to develop normally with respect to flowering and seed set. The lack of symptoms reduces the possibility that the effects of the virus itself will interfere with the phenotypes of experimental plants in which genes are being silenced.

Tobacco Streak Virus (TSV) TSV is a member of the *Bromoviridae* in the genus *Ilarvirus* (Table 15.1). TSV has a tripartite genome with a genome organization and gene expression strategy similar to CMV. However, it packages a fourth RNA (RNA4), which is the mRNA for its CP, and it is also required to initiate viral infection. Positions in each of the three genomic RNAs were investigated as potential cloning sites for foreign inserts (Jossey 2012). Foreign inserts were tolerated in

RNA2 without debilitating the virus, and the inserts were sufficiently stable to elicit VIGS of target genes. Similar to the CMV cloning strategy that limits the vector to VIGS applications, the TSV cloning site is located after the stop codon of a truncated 2b protein (Fig. 15.1). The TSV vector can carry foreign inserts of up to 175 nucleotides that can be maintained during seed transmission. However, a larger 317 nucleotide insert debilitated the virus and was rapidly deleted as the virus replicated and spread throughout the plant.

DNA copies of the TSV genomic RNAs 1–4 were cloned into a plasmid to place them under control of P35S and Tnos (Jossey 2012). In addition, this plasmid includes a *Hepatitis delta virus* ribozyme (HDVr) fused to the 3′ end of the viral genomic cDNAs, which cleaves the transcribed RNA to produce authentic 3′ termini. Soybean plants are inoculated biolistically with a mixture of the four DNA clones. Infected plants develop symptoms of mosaic, leaf curling, and necrosis, but they recover and new leaves are symptom-free. The recovered plants continue to develop normally to flowering and seed set.

Tobacco Rattle Virus (TRV) Vectors based on TRV, type member of the genus *Tobravirus,* have been used extensively for VIGS in a number of dicotyledons (Lange et al. 2013), including one report of their use for VIGS in soybean (Jeong et al. 2005). TRV has a bipartite genome, with the 6.8-kb RNA1 encoding proteins for replication, movement, and silencing suppressor activity, while the 3.9-kb RNA2 encodes the TRV CP. RNA2 also encodes two proteins that are not essential for infection (2b and 2c) but are involved in nematode transmission. Viral proteins are translated only from the 5′ ORFs of genomic RNAs or subgenomic RNAs. TRV silencing vectors have been made by replacing nonessential genes of RNA2 with sites that enable cloning through the use of restriction enzymes, Gateway recombination, or ligation-independent cloning (Ratcliff et al. 2001; Burch-Smith et al. 2004; Caplan and Dinesh-Kumar 2006; Dong et al. 2007; Bachan and Dinesh-Kumar 2012). Foreign genes are expressed from RNAs transcribed from subgenomic promoters. Thus, modifications are not required to the foreign proteins.

Biosafety The viruses mentioned earlier are potentially pathogenic on soybean and other host plants, and the vectors derived from them are regulated as recombinant viral plant pathogens. From a containment point of view, it is best if the virus cannot be transmitted by its normal vector, especially in areas where soybean or alternate hosts are found. For the potyvirus group, mutations in either HC-Pro or CP can abolish aphid transmission (Granier et al. 1993; Huet et al. 1994; Atreya et al. 1995; Blanc et al. 1997; Peng et al. 1998; Llave et al. 2002) and, in fact, these mutations tend to occur rapidly in mechanically transmitted virus populations. Passage by aphids is necessary to maintain aphid transmission. BPMV is transmitted by bean leaf beetle and other beetles, but the specific motifs that mediate this transmission are not characterized. A beetle-free environment is sufficient to contain BPMV to the experimental plants.

Other examples of ways to enhance biosafety of viral vectors include deletion of an essential virus gene and complementation with the gene expressed in a transgenic plant. Bedoya et al. (2010) constructed a *Tobacco etch potyvirus* vector

with the NIb replicase gene deleted. This vector could only infect transgenic tobacco expressing the NIb gene. For tobravirus-based expression or VIGS vectors, deletion of the 2b protein, which is required for nematode transmission but is not essential for virus infection or movement, makes it harder for the engineered virus to escape.

Viruses as Vectors for Gene Expression in Soybean

Use of viral vectors to transiently express foreign genes in soybean has some advantages over stable transformation approaches. Viral expression is quick and cost-effective with no plant regeneration required; thus, by increasing throughput, it can be used for any plant within the host range of the virus, making it possible to use in a range of genetic backgrounds, and the viruses are not incorporated into the plant genome allowing for separation of virus from the plant in future generations (Diaz-Camino et al. 2011). There are also potential limitations that must be kept in mind in the experimental designs, such as symptoms and other plant responses to the virus that can interfere with phenotypes, variability from plant-to-plant and experiment-to-experiment, and the ability of the virus to infect relevant cell types and tissues. The first two limitations can be dealt with primarily by using appropriate controls and multiple, independent biological replications of experiments. The latter is a function of the biology of the virus, and if the virus does not infect cell types or tissues of interest, then it may not be possible to use a viral vector-based strategy.

Four of the seven viruses described above have been used for expression of foreign genes in soybean (Table 15.1). Whether or not a virus can be used to express foreign genes is a function of the cloning strategy for inserts and how well the viral genome tolerates the addition of foreign sequences. As discussed earlier, the cloning sites in both CMV and TSV are after the stop codon of their truncated 2b proteins. Therefore, it is not possible to express proteins from these viruses, and, moreover, their genomes do not seem to tolerate inserts of more than a few hundred nucleotides, which is less than the size of most ORFs. The capacity of the virus genome for foreign RNA is an issue, because ORFs can extend to 3 kb or more. Due to capsid-volume constraints, capacity for foreign genes is less for icosahedral viruses, e.g., BPMV, than it is for rod-shaped viruses like SMV, which can encapsidate more RNA through the addition of more CP subunits. The ability to express foreign sequences may also be affected by other factors, such as insert sequence. A range of proteins has been expressed in soybean using SMV, ClYVV, BPMV, and ALSV vectors as presented below. For purposes of discussion, we divided these proteins into the following functional groups, namely marker, pathogen, and plant proteins.

Marker Proteins The marker proteins that have been expressed in soybean by viral vectors include visible markers such as β-glucuronidase (GUS), green fluorescent protein (GFP), and *Discosoma* sp. red fluorescent protein (DsRed) and herbicide-selectable markers such as bialaphos resistance (BAR). SMV expression vec-

tors based on strains G2 (isolate N, SMV-N) and G7 (SMV-G7) were first tagged with GUS and GFP in studies of bacterial avirulence genes (Wang et al. 2006). The smaller GFP gene was very stable in systemically infected plants, whereas the GUS coding sequence suffered some deletion in upper leaves of transfected plants when analyzed by reverse transcription polymerase chain reaction (RT-PCR). GUS expression was, however, still readily apparent in the eighth trifoliolate of infected plants (Wang et al. 2006). SMV GUS, DsRed, and yellow fluorescent protein (YFP) have also been used to track virus infection and movement in studies of *Rsv1*- and *Rsv3*-mediated resistance to SMV (Zhang et al. 2009a; Hajimorad et al. 2011; Wen et al. 2013; R.V.C.R., unpublished). Another strain of SMV, G7H, was modified in similar fashion to express reporter and other genes (Seo et al. 2009).

The potyvirus ClYVV was used to express GFP in soybean (Masuta et al. 2000) at the P1 and HC-Pro junction with the addition of an NIa cleavage site. The use of other positions in the viral genome as cloning sites was investigated, and the junction of NIb/CP could also be used to express foreign sequences. For proteins that cannot be stably expressed at the P1/HC-Pro junction, it may be possible to express them from the junction of NIb/CP and vice versa (Wang et al. 2003). Thus, context within the viral genome can affect the expression of foreign proteins. For some potyviruses, such as *Turnip mosaic virus,* total capacity can be increased if foreign genes are inserted at multiple sites (Beauchemin et al. 2005). In this example, simultaneous expression of GFP and GUS was possible in *Brassica perviridis* when these genes were inserted between the P1/HC-Pro and NIb/CP junctions. It may not be possible to utilize multiple insert locations in all the potyviruses, because attempts to use the NIb/CP junction as a cloning site in SMV have been unsuccessful (A.L.E., R.V.C.R., unpublished). However, the use of other sites may be possible based on successes in *Potato virus A* and *Turnip mosaic virus* (Chen et al. 2007; Kelloniemi et al. 2008).

BPMV has been used to express several foreign proteins ranging in size up to approximately 1.4 kb (Zhang and Ghabrial 2006; Zhang et al. 2009a, 2010). These inserts were stable in BPMV after multiple passages in soybean, and quantification of GFP protein showed that expression levels of nearly 1% of total soybean leaf protein is possible (Zhang and Ghabrial 2006). Furthermore, Zhang and Ghabrial (2006) indicated that GFP fluorescence could be detected in seed coats suggesting that viral gene expression could continue until late into plant development. Attempts to express larger proteins such as GUS (1.8 kb) have been unsuccessful probably due to size limitations imposed by the icosahedral particles of this virus (C.Z., unpublished). While it has not been determined precisely, the size limit for foreign inserts in BPMV is apparently somewhere between 1.4 and 1.8 kb. Co-expression of two relatively small genes was demonstrated by expressing GFP and BAR from a version of BPMV RNA2 (pBPMV-IA-V5) that can carry two ORFs with an intervening proteinase cleavage site (Zhang et al. 2010). This proteinase releases the two proteins from one another allowing them to function independently within cells. The proteins are functional in soybean allowing visualization of GFP fluorescence under ultraviolet light and conferring resistance to glufosinate herbicides. The total size of GFP, BAR, and the intervening proteinase is approximately 1.4 kb, which is within the size limit of the BPMV vector.

Pathogen Genes SMV-N has also been used to express two effector genes from *Pseudomonas syringae, avrB* and *avrPto* (Wang et al. 2006). The AvrB and AvrPto proteins are post-translationally modified by covalent attachment of a myristic acid moiety to the N-termini of the proteins, which serves as a membrane anchor essential for their proper localization and function (Nimchuk et al. 2000; Shan et al. 2000). Thus, it was necessary for the NIa proteinase recognition sequence to be placed immediately adjacent to the AvrB and AvrPto sequence to preserve the native N-termini of these proteins required for the myristoylation. Expression of *avrB* from SMV resulted in avirulence in cultivars with the cognate *Rpg1-b* resistance gene, and expression of both *avrB* and *avrPto* enhanced virulence of SMV in susceptible cultivars. These data show that AvrB and AvrPto functioned as expected when expressed from SMV-N, and they strongly indicate that myristoylation occurred correctly after processing by the NIa proteinase.

The P19 protein of *Tomato bushy stunt virus,* CP of *Turnip crinkle virus,* and HC-Pro protein of *Tobacco etch virus* were expressed from BPMV. These three proteins are well characterized viral suppressors of RNA silencing (Zhang and Ghabrial 2006). A major function of these proteins is to promote viral infection by interfering with antiviral RNA silencing. A common observation when two viruses with silencing suppressors that function by different mechanisms coinfect a plant is that the plant becomes more diseased than if infected by either virus alone. This is known as synergism, which can also be observed when a virus expresses a silencing suppressor from another unrelated virus (Vance et al. 1995; Pruss et al. 1997). Indeed, when these silencing suppressors were expressed from BPMV, the plants exhibited enhanced symptoms reminiscent of synergism (Zhang and Ghabrial 2006).

Plant Genes Yamagishi and Yoshikawa (2011a) explored the potential of flowering locus T (FT) to promote precocious flowering in soybean when expressed from ALSV. The *Arabidopsis thaliana* FT coding sequence was cloned into ALSV RNA2, and this recombinant ALSV-FT clone was used to infect soybean plants of different maturity groups and growth habits. The *A. thaliana* FT ORF shares 71 % nucleotide identity with the most similar soybean ORF, which is below the threshold of nucleotide identity required for VIGS. ALSV-FT-infected plants flowered at the four to seven node stage regardless of maturity group or growth habit. Furthermore, expression of *A. thaliana* FT halted vegetative stem growth in indeterminant soybean cultivars and promoted early maturation in all cultivars tested. The results demonstrated that FT has a positive role in promoting soybean flowering and maturation, and they suggested that ectopic expression of *A. thaliana* FT could be used to hasten the generation time of soybean irrespective of maturity group or growth habit, which could be beneficial in plant breeding applications (Yamagishi and Yoshikawa 2011b, 2011a).

BPMV has been used recently to overexpress the soybean calmodulin 4 protein (*GmCaM4*) in soybean (Rao et al. 2014). Overexpression of *GmCaM4* resulted in enhanced resistance to salt stress and infection by the oomycete pathogen *Phytophthora sojae* and the necrotrophic fungal pathogens *Alternaria tenuissima* and *Phomopsis longicolla*. Enhanced resistance was associated with increased accumulation

of the phytohormone jasmonic acid and upregulation of pathogenesis-related genes *PR-1a, PR-2, PR-3,* and *PR-4,* which are markers for induced plant defenses. Western blot analysis of *GmCaM4* demonstrated that the protein was indeed expressed at higher levels by the BPMV vector. In contrast, plants infected by a BPMV construct designed to silence *GmCaM4* were more sensitive to salt stress, accumulated less jasmonic acid, and had reduced levels of pathogenesis-related gene expression. The overexpression construct carried the full ORF of *GmCaM4,* while the silencing construct carried a 198 nucleotide fragment corresponding to the 5′ untranslated region of the *GmCaM4* mRNA. This study demonstrates that it is possible to use BPMV to overexpress soybean genes in soybean by expressing the full ORF even though the virus is an effective vector for silencing soybean genes as discussed in the next section. However, additional work may be necessary to determine if constructs that initially overexpress genes of interest in plants eventually silence the genes as infection time proceeds.

Viruses as Vectors for VIGS in Soybean

Genes are often associated with traits through loss of function phenotypes. Loss of gene function can be achieved through point mutations, deletions, insertions, and RNA silencing. VIGS is a form of RNA silencing that has been used extensively to silence transiently the expression of a wide variety of plant genes to assess their roles in diverse aspects of plant biology. VIGS exploits antiviral RNA silencing mechanisms that normally protect plants from viral infections. During VIGS, the fragment of a plant gene carried by the virus programs the RNA silencing system to degrade the mRNA corresponding to the target gene. Double-stranded RNA (dsRNA), produced during viral replication, that is part of the natural structure of viral genomic or subgenomic RNAs, is targeted by the RNA silencing system for degradation. RNA silencing is initiated when Dicer-like (DCL) enzymes cleave dsRNA into 21–25 nt fragments referred to as small RNAs (sRNAs). One strand of these sRNAs becomes integrated into the RNA-induced silencing complex (RISC) where it serves as a guide to direct the Argonaute (AGO) protein to cleave any complementary RNA sequence within the cell. Subsequently, RNAs cleaved by RISC are degraded further, or they can serve as templates for RNA-dependent RNA polymerases to produce additional dsRNA to amplify the silencing signal.

While this pathway is typically induced and directed toward viruses, it can be manipulated to silence the expression of plant genes. In VIGS, viruses are engineered to carry a fragment of a plant target gene. During replication of the recombinant virus, dsRNA of both viral and plant origin are produced, resulting in the accumulation of sRNAs derived from both the virus and the plant target gene. RISC programmed with the sRNAs of plant origin targets and cleaves both the recombinant viral genome and the mRNA produced by the target gene and any homologs with sufficient complementarity. Degradation of the mRNA results in decreased expression or silencing of the plant target gene. Because the RNA degradation initi-

ated by RISC is determined by the guide sRNA sequence, VIGS is programmable and sequence specific. Furthermore, any mRNA in the cell that has sufficient identity to the target sequence can be degraded as well. This means that VIGS can be readily directed to knock down the expression of duplicated genes and gene family members that can frequently have redundant functions. Knocking down expression of related genes can be a drawback of VIGS, because if duplicated genes are nearly identical in sequence, then it is difficult to design a VIGS strategy to silence specifically only one member of the family.

VIGS has the advantage over other approaches in that it can be done rapidly, usually taking a few weeks to induce discernable phenotypes, and it does not require a stable transformed plant. VIGS can be used to allow comparison of silencing in different genetic backgrounds of the same species or even different plant species. An important concern when using VIGS is that the virus itself must successfully infect the plant in order to generate a silencing phenotype. Viruses elicit responses at the molecular and macroscopic levels as they invade their hosts (Whitham and Wang 2004; Whitham et al. 2006; Mandadi and Scholthof 2013). Frequently, the virus is capable of producing symptoms of infection on the host that can potentially interfere with or complicate phenotypic analyses. The systemic infection and symptoms must be accounted for in order to determine the effect of the silencing of the gene of interest, and thus, control experiments are crucial that utilize viral vectors that lack inserts, or that carry inert inserts.

Five of the seven viruses listed in Table 15.1 have been tested for VIGS in soybean, these being BPMV, ALSV, CMV, TSV, and TRV. The potyviruses SMV and ClYVV have not proven to be useful for VIGS, probably due to the activity of their HC-Pro silencing suppressors. This section discusses the use of each of these viruses for VIGS and provides examples of their efficacy in studies of soybean gene function. The utility and limitations of these vectors for studying different aspects of plant biology are also considered.

BPMV VIGS BPMV vectors have been used most widely for VIGS of soybean genes, and VIGS has been used successfully in large-scale screens. Here, some general results and observations related to BPMV VIGS are discussed, with additional details on large-scale screens being presented in a later section. VIGS studies have included marker genes such as *phytoene desaturase* (*Pds*; endogenous gene) and *GFP* (transgene), genes associated with plant defense to pathogens and abiotic stress, genes associated with plant growth and development, and house-keeping genes. The first example of BPMV VIGS in soybean was the silencing of *Pds* resulting in a photobleaching phenotype that correlated with reduced mRNA transcript levels (Zhang and Ghabrial 2006). More detailed analysis of *Pds* silencing demonstrated that the most extensive photobleaching phenotype and reduced mRNA expression could be achieved by targeting the 3′ end of the *Pds* ORF cloned in the antisense orientation into BPMV (Zhang et al. 2010). This was made possible by the development of a new version of the vector containing the cloning site just after the stop codon of BPMV RNA2, eliminating the need for inserts to be cloned in frame with the viral polyprotein. Interestingly, two other studies noted that the 3′ end of

the ORF was most effective in silencing *GFP* and *GmNPR1* expression (Pandey et al. 2011; Juvale et al. 2012). These three examples suggest that constructs targeting the 3′ ends of transcripts may be the most effective for silencing, but work with additional genes is needed to determine the generality of this observation. The versions of the BPMV vector that allow for antisense target sequences may suppress gene expression by up to 95 % or more, which is more effective than vectors containing inserts in the sense orientation that generally reduce mRNA expression in the 50–75 % range (Zhang et al. 2010; Juvale et al. 2012).

A major challenge in studying functions of genes in soybean and other crop plants results from genome duplication events in their evolutionary histories (Grant et al. 2000; Schlueter et al. 2006, 2007; Schmutz et al. 2010). For many genes, there are two homologous pairs that share high identity within their coding sequences. Simultaneous silencing of these homologs is desirable to overcome the likely problem of functional redundancy achieved by targeting conserved sequences in the coding region. However, it can be useful to silence individual family members or perhaps one homeologous pair of genes. This is often not possible when targeting the coding sequences within the ORFs that tend to be highly conserved. However, targeting the 5′ or 3′ untranslated regions that tend to be less conserved can be used to silence individual homologs or a homeologous pair of genes. Rao et al. (2014) reported that targeting 198 nucleotides from the 5′ untranslated region of *GmCaM4* silenced specifically this gene and resulted in approximately 50 % reduction in transcript levels. To target this sequence, these authors used a BPMV vector that required a single nucleotide change in the cloned sequence in order to change a stop codon into an amino acid-encoding codon. This was necessary because the insert had to be expressed in frame with the viral polyprotein. Alternatively, it is possible to use versions of BPMV vectors that possess the cloning site after the stop codon in RNA2 to target 5′ and 3′ untranslated regions for silencing (Zhang et al. 2010). In these vectors, it is not necessary to eliminate stop codons from the target sequence.

The BPMV vector is useful for gene silencing in a range of tissues throughout plant development. This was analyzed systematically by using it to silence a GFP transgene constitutively expressed under control of the soybean ubiquitin promoter, which allowed silencing to be nondestructively evaluated (Juvale et al. 2012). GFP was silenced in leaves, petioles, stems, flowers, and roots over a 45-day time course. Pods and seeds were not tested. Although the virus is often found in the seed coat, it is rarely transmitted through seed to the next generation. Because of this, it is expected that silencing will not be effective in seeds, but this remains to be evaluated. Silencing reached a maximum in the third and fourth leaves by 21 days post inoculation, and then declined. In roots, the silencing was more effective in the upper part of the root system compared to near the root tips. Silencing was not observed in all flowers, but silencing was very effective in those in which it was observed. In general, BPMV VIGS correlates well with the accumulation of the viral vector in various tissues (Zhang et al. 2010; Juvale et al. 2012).

ALSV VIGS ALSV has been used to silence *Pds* in proof-of-concept experiments and for genes involved in flowering and secondary metabolism. Effective silencing

of *Pds* was observed by photobleaching under high light conditions within 10–14 days after inoculation (Igarashi et al. 2009). Patchy photobleaching was observed on the third trifoliolate leaves, and uniform photobleaching was observed on the fourth and fifth trifoliolate leaves, which persisted through at least 50 days after inoculation. Silencing of a myb transcription factor, *GmMYB-G20–1,* that controls flower color in soybean, resulted in flowers that had irregular pigmentation and demonstrated that the vector is also effective in flowers (Takahashi et al. 2013). Igarashi et al. (2009) also explored the impact of *Pds* fragment size on photobleaching phenotype and insert stability in tobacco. They found that foreign inserts in the range of 100–200 nucleotides effectively induced *Pds* silencing, and they were more stable than inserts of 300 and 408 nucleotides.

ALSV has been used to silence *Pds* and *isoflavone synthase 2* (IFS2) genes in seed and has been transmitted through the seed where it causes silencing in progeny plants (Yamagishi and Yoshikawa 2009). However, the seed transmissibility of VIGS was dependent on the cultivar. Of the six cultivars tested, silencing in the seed and progeny plants was most frequent in "Enrei". VIGS of *Pds* was observed in 33 % of seedlings from "Enrei" plants showing photobleaching. Soybean *IFS2* was silenced in 36 % of cotyledons from seed randomly harvested from "Enrei" plants that were infected with the soyIFS2-ALSV construct, demonstrating that the silencing was effective in the embryo. The seed transmissibility of ALSV, coupled with its ability to induce VIGS in seed and progeny plants, makes it possible to use this vector to target genes involved in seed traits and early plant development.

CMV VIGS CMV VIGS has been used to investigate functions of genes involved in flavonoid biosynthesis. Silencing of *chalcone synthase 7* (*CHS7*) in a brown-seeded cultivar resulted in yellow seed coats and 70–80 % reduction in the accumulation of isoflavones derived from flavonoid precursors that correlated well with reduced *CHS7* mRNA levels in the 55–90 % range (Nagamatsu et al. 2007). Silencing of the flavonoid 3′-hydroxylase gene (*sf3'h1*) reduced transcript levels by about 70 % and led to decreased levels of the flavonoids kaempferol and quercetin in leaves (Nagamatsu et al. 2007). Nagamatsu et al. (2009) further explored the silencing of *sf3'h1,* because pigmentation of pubescence was not reduced in their earlier study as would have been expected. Previous experiments had been performed in a glasshouse, and when experiments were moved to a more tightly controlled growth chamber environment, loss of pigmentation in pubescence was observed in leaves and seed pods. The reduced pigmentation in pubescence was associated with a much lower quercetin/kaempferol ratio in plants in the growth chamber. Their results suggested a threshold level of silencing that had to be achieved to observe colorless pubescence. In addition, growth conditions of plants used in VIGS experiments can affect dramatically the outcomes of experiments, even when degradation of target transcripts is improved by just a few percentage points.

CMV VIGS has also helped to confirm identification of the *Determinate 1* (*Dt1*) gene by a candidate gene and genetic mapping approach (Liu et al. 2010). The dominant *Dt1* allele is present in cultivars in which vegetative growth continues through most of the growing season, whereas cultivars carrying the recessive *dt1*

allele cease vegetative growth upon flowering. Soybean homologs of the *Terminal Flower 1* (*TFL1*) gene were investigated based on the function of this gene in other species in controlling the transition from vegetative growth to flowering. The *GmTFL1b* homolog mapped to a genetic interval containing *Dt1* and it co-segregated with stem growth habit. CMV VIGS targeting a 139 nucleotide fragment from exon 4 of *GmTFL1b* resulted in plants that terminated vegetative growth and formed terminal flowers earlier than controls. The VIGS data combined with complementation of the *dt1* allele by transformation with *GmTFL1b* from an indeterminant (*Dt1*/*Dt1*) cultivar, and sequence analysis of cultivars with different stem growth habits, clarified soybean *Dt1* as an ortholog of *TFL1*.

TSV VIGS TSV was used to silence *Pds* and *magnesium chelatase* genes, but silencing of these genes was inconsistent and occurred at a low frequency in infected plants (Jossey 2012). Interestingly, the silencing could persist and be seed transmitted at rates of 3–8 % depending on the construct. These results suggest that TSV is promising as a viral vector for silencing genes in seed and in the next generation, but additional development and optimization are required of the system.

TRV VIGS Despite its wide success for VIGS in many dicotyledons (Senthil-Kumar and Mysore 2011a; Lange et al. 2013), TRV has only been used to silence soybean *Pds* in the cultivar "Somyung" (Jeong et al. 2005). Three different inoculation methods were tested, but only an *Agrobacterium* drench (agrodrench) at the crown of the plant yielded infected plants. No infected plants were obtained after *Agrobacterium* infiltration of the leaves or spraying plants with an *Agrobacterium* suspension. The *Pds* silencing in the agrodrench-inoculated plants caused them to become chlorotic, but the silencing phenotypes presented did not appear to be as robust as with other viral vectors that cause plants to become pale yellow to white. It is possible that an extensive screen of soybean germplasm, followed by optimization, might identify genotypes that can better support TRV VIGS. For example, such a strategy was used recently to establish TRV VIGS in *Gerbera hybrida* in which a screen of 21 cultivars and subsequent optimization steps led to identification of a single cultivar that consistently supported TRV VIGS (Deng et al. 2012).

Large-Scale BPMV VIGS Screens to Establish Soybean Defense Gene Networks

The DNA-based BPMV vector has been used for large-scale studies to identify genes participating in the signaling networks regulating general defense and specific resistance to pathogens. At this time, it is impractical to pursue screening on a genome-wide scale. Therefore, our approach has been to select candidate genes associated with traits of interest, engineer recombinant BPMV clones to target these genes, and then test if VIGS alters defense phenotypes. Candidate gene lists were generated from multiple, complementary sources of data. The three main sources of candidate genes that we have used are (i) genes from gene expression profiling data,

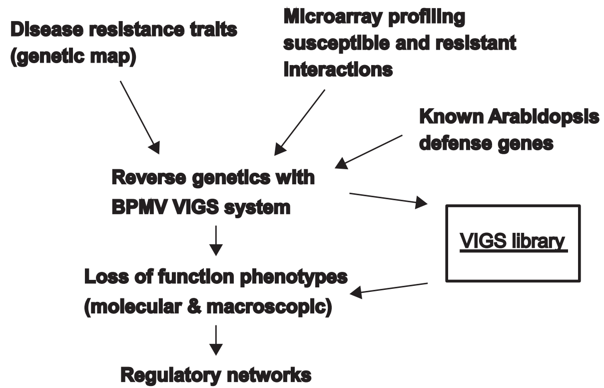

Fig. 15.2 General strategy for large-scale virus-induced gene silencing (VIGS) screens using the *Bean pod mottle virus* (BPMV) viral vector to identify genes involved in soybean defense gene regulatory networks

(ii) soybean homologs of genes with known functions in model plants such as *A. thaliana,* and (iii) genes mapping to genetic intervals containing known resistance genes (Fig. 15.2).

1. Candidates from expression profiling: We and others have generated extensive gene expression profiling data on the responses of soybeans and other plants to pathogens, abiotic stresses, and insect pests. These data provide lists of genes that are expressed differentially in response to these environmental stimuli. Hundreds or thousands of upregulated and downregulated genes can be identified from these experiments using rigorous statistical criteria. Even with techniques such as VIGS, it is not practical to test experimentally the role of each of these genes in a given soybean trait. Therefore, we have prioritized the genes based on functional annotation such as whether the gene is likely to have a regulatory function, possibly controlling the expression of many other genes. For example, differentially expressed transcription factors and kinases are thought to be excellent targets since they frequently control the activities and/or expression of many other genes. These genes represent regulatory hubs whose loss of function can have significant effects on the trait of interest. We also utilize information from the literature to identify genes in key biochemical pathways that are also predicted to have important roles in defense.
2. Homologs of genes with defense functions in model plants: Model plants such as *A. thaliana* have been utilized extensively to understand key genetic and molecular circuitry of plant immunity. Most of the genes regulating immunity in *A. thaliana* have homologs in the soybean genome that can be identified readily by searching the soybean gene predictions. These genes may or may not be differentially expressed during defense responses, so this approach of transferring research from a model system to a crop plant provides a way to identify potentially important regulatory genes independent of their expression profiles.
3. Genes that map to genetic intervals containing traits of interest: This is a powerful approach that capitalizes on genome sequence, gene prediction, genetic mapping, VIGS, and the knowledge that many disease resistance traits are conferred

by genes encoding nucleotide binding site leucine-rich repeat (NLR) proteins. NLRs that map to these genetic intervals can be targeted by VIGS to determine if their silencing results in loss-of-resistance phenotypes. However, NLRs are frequently found in tandemly repeated clusters of genes that share high nucleotide identity, and thus, it might not be feasible to use VIGS to determine exactly which of the genes is the actual resistance gene in question. In cases where the genetic interval containing a resistance gene does not include NLRs, VIGS can be used to target multiple genes in the interval in conjunction with other genetic data to pinpoint the gene conferring resistance.

Using these approaches, we have begun accumulating a library of BPMV VIGS clones for silencing soybean genes. Because this library has been developed mostly to investigate genes associated with the soybean immune system, it is most useful for investigating soybean defense against a variety of pathogens and environmental stresses. A list of the clones, their target sequences, primers, and images of plants is available at the webpage http://www.soybase.org/SoyVIGS/Welcome.html. Interested parties may browse through the clones or conduct a search to see if a VIGS clone targeting a specific gene is available. The clones are archived as plasmid DNA, and for most, we maintain a stock of infected tissue that is used to inoculate experimental plants.

After the BPMV VIGS clones are made, they are inoculated onto soybean by DNA particle bombardment for amplification. The infected tissue derived from each viral clone is lyophilized and stored at −20 °C for use in future experiments. These tissues can be used to inoculate experimental plants for phenotypic analyses. The use of infectious tissue improves the efficiency and uniformity of infection, and it is much simpler and less expensive than particle bombardment. We and our collaborators have used this analysis pipeline in large-scale screens to identify genes that participate in the regulatory networks controlling resistance to *Phakopsora pachyrhizi* (soybean rust), SMV, and *Heterodera glycines* (soybean cyst nematode, SCN; (Meyer et al. 2009; Liu et al. 2011; Pandey et al. 2011; Liu et al. 2012; Zhang et al. 2012; Liu and Whitham 2013). In most cases, these genes have been identified through loss-of-function phenotypes characterized by increased susceptibility in resistant genotypes. Genes resulting in loss-of-resistance when silenced encode the actual resistance genes specifying pathogen recognition, or genes that function in the signaling networks downstream of the resistance genes. We have also observed that silencing of *GmMAPK4,* from a screen of 30 mitogen-activated protein kinase (MAPK) genes, results in gain-of-resistance phenotypes because it negatively regulates aspects of the soybean immune system (Liu et al. 2011).

Soybean Rust All three approaches have been used to select candidate genes for soybean rust resistance mediated by *Rpp4* and *Rpp2*. The *Rpp4* resistance gene was mapped to a genetic interval on chromosome 18 (Silva et al. 2008), and following sequencing of Williams 82 BAC clones spanning this interval, three genes encoding NLR proteins were identified among 15 predicted genes (Meyer et al. 2009). These three NLRs are most similar to lettuce *RGC2,* which confers resistance to downy mildew. The three NLRs share 87–95 % nucleotide identity in their

coding sequences. Williams 82 lacks any known resistance genes to soybean rust, and therefore, primers were designed to PCR amplify homologous sequences from a resistant parent of the *Rpp4* genotype. This analysis showed that the *Rpp4* parent carries additional copies of the NLR sequence, suggesting a more complex locus than in susceptible Williams 82. The homologs were all related by at least 92% nucleotide identity, which is sufficient for simultaneous silencing by VIGS. VIGS constructs were designed to target the nucleotide binding domain and a portion of the leucine-rich repeat region. Both VIGS clones effectively silenced expression of the NLR genes at the *Rpp4* locus, and they resulted in loss-of-resistance phenotypes. The BPMV empty vector did not affect expression of these genes or the *Rpp4* resistance phenotype. These results demonstrated that *Rpp4* is encoded by an NLR gene related to lettuce *RGC2.* However, the sequence identity shared among the NLR genes prevented VIGS from being used to identify the specific *Rpp4* gene. An important implication of this work was the demonstration that the combined resources of soybean map position, genome sequence, and VIGS could be utilized to identify genes associated with specific traits in soybean (Schmutz et al. 2010).

A large-scale VIGS screen of 140 unique constructs was used to identify 11 genes in the *Rpp2* signaling network using information from model systems and results from gene expression profiling (Pandey et al. 2011). A subset of the constructs was designed to silence soybean homologs of *A. thaliana* genes that function downstream of resistance genes or in basal resistance networks that require the phytohormones salicylic acid, jasmonic acid, and/or ethylene. Results from VIGS experiments showed that, indeed, some of these conserved genes are required for *Rpp2* resistance (Pandey et al. 2011). The majority of constructs were designed based on results from an extensive microarray profiling time course of the *Rpp2*-mediated resistance response. Bioinformatic analyses of the differentially expressed genes indicated that regulatory genes such as WRKY and MYB transcription factors and genes encoding enzymes in the phenylpropanoid pathway and lignin biosynthesis were important. VIGS experiments demonstrated that these classes of genes were indeed required for *Rpp2* function (Pandey et al. 2011).

MAPKs A screen of 30 constructs targeting MAPKs revealed that silencing of soybean MPK4 (*GmMPK4*) resulted in gain-of-resistance phenotypes that were very similar to those observed in *A. thaliana* (Liu et al. 2011). Phenotypic changes include spontaneous cell death, intense pigmentation, and increased expression of pathogenesis-related genes, salicylic acid, and hydrogen peroxide. Concomitant with these changes, GmMPK4-silenced plants are also more resistant to viral and oomycete pathogens. Microarray profiling of the *GmMPK4*-silenced plants showed that most of the genes identified by VIGS as required for *Rpp2* function became strongly upregulated. These results indicate that one of the functions of GmMPK4 is to negatively regulate the *Rpp2* resistance network, and they demonstrated the utility of combining BPMV VIGS with gene expression profiling analyses in soybean.

SMV A screen of 82 BPMV VIGS constructs was conducted to identify genes involved in the *Resistance to Soybean mosaic virus 1* (*Rsv1*) signaling network (Zhang et al. 2012). This screen mostly utilized constructs designed as described

above for the soybean rust studies. The one exception was a construct designed to silence *Rsv1* candidate genes. *Rsv1* was mapped to a genetic interval on chromosome 13 that contains multiple highly homologous NLR genes (Hayes et al. 2004). These genes were targeted simultaneously with a single VIGS clone, which caused a loss-of-resistance phenotype, confirming that at least one member of this family of NLRs encodes *Rsv1*. As with *Rpp4,* it is not possible to design a VIGS clone that targets specifically individual members of the *Rsv1* family. This study, combined with results from others, identified nine additional genes that are also required for *Rsv1* function. These include *EDS1* and *PAD4,* which are shared with the *Rpp2* study and likely function early in resistance signaling, and two WRKY transcription factors that are distinct from WRKY transcription factors required in *Rpp2* resistance. These results suggest that *Rsv1* and *Rpp2* networks share some common upstream features, but may bifurcate to involve different downstream components.

SCN The soybean rust and SMV examples show how VIGS can be used to silence genes in pathogens infecting leaves. BPMV VIGS has also been effective in screens of genes required for resistance to SCN in roots (Liu et al. 2012; Kandoth et al. 2013). The SCN resistance gene *Rhg4* was mapped to a genetic interval containing two genes that were not similar to previously identified plant disease resistance genes (Liu et al. 2012). Based on extensive evidence, including VIGS of genes present in the interval, *Rhg4* was determined to encode a methyl transferase protein involved in one-carbon metabolism. VIGS of this gene resulted in reproducible loss-of-resistance phenotypes in which significantly more cysts were found on roots of *Rhg4*-silenced plants versus the empty vector control. A very important consideration for these studies was the establishment of appropriate conditions under which nematode loss-of-resistance phenotypes could be identified reproducibly. In light of this, specific protocols were developed for VIGS of genes involved in soybean–SCN interactions in roots (Kandoth et al. 2013).

Conclusions and Future Prospects

We have highlighted many successes of using viral vectors for gene expression and silencing, but we may only be scratching the surface of the possible uses of these vectors (Senthil-Kumar and Mysore 2011a), and there is room for improvements to viral vectors and the protocols for inoculation. One of the limiting steps in using the vectors is inoculation. TRV and *Potato virus X* (PVX) vectors have been used widely in the plant research community by virologists and nonvirologists, in no small part due to the ease of *Agrobacterium* inoculation procedures. Such inoculation procedures have not been developed in soybean for the viruses presented here, because soybean is generally very difficult to infiltrate with suspensions and the *Agrobacterium* transformation is not efficient. Recently, a method was developed for transient gene expression by *Agrobacterium* infiltration (Azhakanandam and Su 2012), which could enable *Agrobacterium* inoculation directly in soybean. An-

other possibility is developing *Agrobacterium* inoculation of surrogate hosts that are highly susceptible to *Agrobacterium* and viral vectors. It would then be easy to generate large amounts of infectious tissues that could be archived and used to inoculate experimental soybean plants for replicated experiments.

Another exciting possibility is heritable silencing of which there are at least two possible forms as discussed in detail by Senthil-Kumar and Mysore (2011a). The first is nonintegration-based transmissible posttranscriptional gene silencing (PTGS). Initially, VIGS was considered to be transient and not passed to the next generation, because there is no integration of RNA virus genomes into host genomes. However, VIGS has been shown to be transmissible to progeny in barley—*Barley stripe mosaic virus* (BSMV; Bruun-Rasmussen et al. 2007), soybean—ALSV (Yamagishi and Yoshikawa 2009), *N. benthamiana*—TRV (Senthil-Kumar and Mysore 2011b), and tomato—TRV (Senthil-Kumar and Mysore 2011b). In these examples, continued silencing was correlated with transmission of the recombinant viral vector to first-generation progeny at percentages ranging from 11 to 48 % for barley—BSMV depending on the cultivar, 20–30 % for soybean—ALSV in the cultivar "Enrei", and 1 % for *N. benthamiana*—TRV. The frequency of transmission can increase in subsequent plant generations, reaching 90–100 % by the third generation in the barley—BSMV system and 10 % in the second-generation progeny in the *N. benthamiana*—TRV system (Senthil-Kumar and Mysore 2011b; Bennypaul et al. 2012). Interestingly, by the third generation, barley plants became asymptomatic even though BSMV was present, so that viral symptoms no longer interfered with assessing VIGS phenotypes.

The second form of heritable silencing is transcriptional gene silencing (TGS), which is initially triggered by sRNAs produced by the recombinant virus that direct methylation of target DNA in the host. This DNA methylation is an epigenetic change that is heritable and leads to decreased transcription of the target gene due to modified histone methylation that alters chromatin structure (Paskowski and Whitham 2001). TRV and PVX can induce heritable methylation and TGS of coding and promoter sequences of transgenes in plant genomes (Jones et al. 1999; Sonoda and Nishiguchi 2000; Jones et al. 2001; Otagaki et al. 2006). More recently, two endogenous genes have been silenced by TGS using a CMV vector. The petunia gene, *Chalcone synthase A* (*CHS-A*) was silenced using a CMV vector that carried a 222 nucleotide fragment targeting the bases −224 to −2 relative to the *CHS-A* transcription start site, and the tomato colorless nonripening gene (*CNR*) was silenced using a 286 nucleotide fragment from 2.4 kb upstream of the coding sequence (Kanazawa et al. 2011). TGS of *CHS-A* resulted in reduced or aberrant flower pigmentation in inoculated plants and in progeny plants, and TGS of *CNR*-inhibited fruit ripening. The silencing and DNA methylation persisted in progeny plants in the absence of the CMV vector, demonstrating that the virus is not required to maintain TGS of these genes. Because the virus is not required for maintenance of TGS, phenotypes can be assayed independent of viral symptoms. It will be interesting to test if CMV, ALSV, or BPMV can induce heritable silencing in soybean either by PTGS or TGS. Success may depend on establishing optimal environmental conditions or identifying a soybean line that is amenable to these forms of heritable silencing.

The nonintegration-based transmissible PTGS and TGS can alter phenotypes across multiple generations, but they do not result in heritable, stable changes in DNA sequence. Technologies being developed for precise editing of plant genomes include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs) (Liu et al. 2013). These technologies are built on different protein platforms for recognizing specific DNA sequences in genomes and modifying DNA sequences at or near the recognition sites. The ZFN and TALEN technologies rely solely on protein-mediated recognition of DNA that is engineered by manipulating the amino acid sequences in positions that specify recognition of DNA bases. The CRISPR technology requires coexpression of a Cas9 protein and a guide RNA that directs the protein to act at a specific site in the genome that has sequence complementarity to the guide RNA. One can envision scenarios in which it is possible to express components of these DNA modification systems in viral vectors to modify plant genomes without integration of a transgene. If a viral vector is able to express the protein in meiotic cells or early in embryo development, it may be possible to achieve site-specific DNA modification without transformation.

MicroRNAs (miRNAs) are regulatory sRNAs that are encoded in plant genomes. They regulate gene expression by directing cleavage of mRNAs that are complementary in sequence or by inhibiting translation of such mRNAs. miRNA mimics are complementary in sequence to miRNAs, and they sequester miRNAs by preventing binding to the endogenous target sequence (Franco-Zorrilla et al. 2007; Todesco et al. 2010). Viral vectors can be used to investigate miRNA function by expressing miRNA mimics, which would lead to misexpression of the endogenous target mRNAs. Thus, it might be possible to use viral vectors to assess rapidly miRNA function in soybean, as has been shown in *N. benthamiana* (Sha et al. 2014).

RNA silencing mediated by viral vectors directs degradation of endogenous and transgene mRNAs. However, it may be possible to silence genes of other organisms that colonize or feed on soybean plants by expressing fragments of genes from these organisms from a viral vector. This process of silencing genes in a pathogen or pest by inducing silencing against one or more of their genes within the host plant is known as host-induced gene silencing (HIGS; Nunes and Dean 2012). HIGS can be used to assess the functions of genes in pathogens and pests, or it may be used to develop novel management strategies to control them. Silencing of *Puccinia striiformis* (wheat stripe rust) genes was induced using the BSMV vector, which demonstrates that a viral vector can be used for this purpose (Yin et al. 2011). Soybean is attacked by a variety of pathogens and pests and development of HIGS using viral vectors could be a valuable approach to assessing gene functions in these organisms or selecting potential pathogen or pest genes to be exploited for HIGS-based disease management strategies.

The last point that we explore is that of the numerous traits involved in domestication of soybean and other legumes that are likely to have resulted from loss-of-function mutations, but which have not yet been introgressed into some important landrace groups or allied agronomic species. Traits involved in legume domestication include nonshattering characteristics, increases in seedling vigor, rapid germi-

nation, larger inflorescences, larger seeds, thicker stems and more upright plants with loss of vinyness, earliness, and loss of photoperiod dependency (Koinange et al. 1996; Liu et al. 2007). VIGS may provide a valuable approach needed to help to identify the genes responsible for these traits when combined with map-based cloning, association mapping, or candidate gene strategies as discussed earlier for *Rpp4, Rhg4,* and *Dt1*.

In conclusion, viral vectors have proven useful for expression and silencing of genes in soybean, and there appear to be many new and exciting developments and applications that are possible that will ultimately aid in soybean improvement. Viral vectors have been used in directed studies and large-scale screens to investigate and identify genes involved in a variety of traits including immunity, response to abiotic stress, development, pigmentation, and domestication. Collectively, the vectors are effective in several different tissues such as leaves, stems, petioles, flowers, seeds, and roots, and they can be used to silence genes as diverse as resistance genes, transcription factors, kinases, and biosynthetic enzymes. The viral vector systems that have been developed vary in their ability to be used for gene expression, VIGS, or both. In addition, they likely differ in their effectiveness in different tissues although this remains to be thoroughly investigated for all of the vector systems. Use of a particular viral vector must be carefully considered depending on the purpose (expression or VIGS) and the target tissue.

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